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Propriedades e Secreção das enzimas digestivas e caracterização das membranas microvilares intestinais de insetos.

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ABREVIATURAS

AlaβNA: L-alanina - β - Naftilamida

ArgβNA: L-arginina - β - Naftilamida

AspβNA: L-Aspartico β - Naftilamida

GluβNA: L-Glutâmico - β - Naftilamida

LeuβNA: L-Leucina - β - Naftilamida

LpNA : L-Leucina - p - Nitroanilida

MetβNA: L-Metionina - β - Naftilamida

ProβNA: L-Prolina - β - Naftilamida

1. Introdução

1.1. Considerações iniciais

Insetos são organismos bem sucedidos, que adaptaram-se aos mais variados nichos ecológicos. O número de espécies de insetos existentes supera de longe o número de espécies de todos os outros animais reunidos. Estima-se que haja cerca de 900.000 espécies diferentes de insetos e entre essas, 90.000 são pragas (Gallo et. al., 1988).

Os insetos são vetores de várias doenças e já provocaram grandes desgraças na humanidade, tais como a peste negra no século XIV. Na agricultura, além de sugar a seiva e ingerir fragmentos de plantas, disseminam doenças, principalmente causadas por vírus, que podem aniquilar uma lavoura inteira. No Brasil, prejuízos na produção agrícola vão de 7 a 79%, dependendo da cultura considarada (Pfadt, 1978; Gallo et. al., 1988).

A capacidade que os insetos tem de viver em praticamente qualquer local da Terra e de adaptar-se a variados tipos de alimento, torna-os objetos de estudos extremamente interessantes. Estudos sobre esses animais são também realizados na tentativa de encontrar estratégias para combatê-los. Embora a luta do homem contra os insetos seja antiga, a descoberta do DTT por volta de 1930 fez com que os recursos para pesquisa sobre insetos fossem diminuídos drásticamente. A pesquisa só recomeçou nas décadas de 50 e 60, após verificar-se o fenômeno da resistência aos inseticidas.

A descoberta em plantas, de proteínas inibidoras de enzimas digestivas de insetos chamou atenção para a possibilidade de usar essas enzimas como alvo para desenvolver novas técnicas de controle de insetos (Ryan, 1990). Uma abordagem de sucesso é produzir plantas transgênicas que expressam, por exemplo, inibidor de trypsina (Hilder et. al., 1987). Essa abordagem vai se beneficiar do conhecimento

adquirido sobre as propriedades das enzimas digestivas (que diferem entre vários grupos) e sobre a distribuição das enzimas no canal alimentar (o que determina se as enzimas alvo são ou não acessíveis aos inibidores).

Outras substâncias usadas para controle de insetos são as δ-endotoninas, uma classe de proteínas tóxicas produzida por Bacillus thuringiensis. Essas toxinas formam um canal na membrana plasmática das células intestinais causando lise e morte celular (English & Slatin, 1992; Knouls & Dow, 1993). As toxinas só formam canais em insetos suscetíveis, embora o papel dos receptores na formação do poro seja controverso. Um melhor conhecimento da bioquímica das microvilosidades das células intestinais é um passo necessário para se entender melhor o mecanismo de ação das δ-endotoninas. Desse modo, métodos mais específicos e eficientes de usá-las podem ser encontrados.

A observação de que os insetos adquirem resistência também às δ-endotoninas, reforçou a idéia de que técnicas diversificadas de ataque as pragas devem ser utilizadas (Mc Gaughey & Whalon, 1992)

Outros métodos de controle podem ser vizualizados usando o conhecimento crescente sobre digestão em insetos. Assim, enzimas digestivas purificadas até a homogeneidade podem ser usadas para formar anti-corpos a serem expressos em plantas transgênicas. Técnica similar pode ser usada com proteínas purificadas das microvilosidades celulares, certificando-se antes que os anticorpos sejam capazes de cruzar a membrana peritrófica do inseto alvo.

Desde a minha iniciação científica, tenho trabalhado com vários aspectos relacionados com a digestão nos insetos. Nessa tese enfocarei alguns desses tópicos, nos quais a minha contribuição foi maior. Esses tópicos são: a) Propriedades das aminopeptidases e dipeptidases de insetos; b) Caracterização das membranas plasmáticas que revestem as células do intestino médio e c) Secreção de enzimas digestivas em insetos.

Essa introdução pretende dar um panorama sobre o que existe na literatura a respeito dos tópicos abordados nessa tese. Ela pretende também comentar o que estamos fazendo atualmente e o que pretendemos fazer no futuro sobre esses temas.

1.2 As aminopeptidases intestinais de insetos

Aminopeptidases (EC 3.4.11) hidrolisam um aminoácido a partir da porção N-terminal da cadeia polipeptídica e são classificadas com base na sua dependência de íons metálicos (normalmente Zn^{++} ou Mn^{++}) e na sua especificidade com relação ao substrato.

Nos mamíferos, as aminopeptidases digestivas não são encontradas no lúmen intestinal, tendo sua atividade restrita aos enterocitos. A aminopeptidase N(EC 3.4.11.2) é uma enzima inserida na membrana microvilar por um peptídeo ancorador hidrofóbico. Ela hidrolisa principalmente substratos contendo alanina na proção N-terminal, podendo agir também em outros, principalmente os contendo leucina. Ela não atua quando esse aminoácido é a prolina (ver Norén et. al., 1986; Sanderink et. al., 1988).

Outra aminopeptidase que ocorre como proteína integrante da microvilosidade do enterócito é a aminopeptidase A (EC 3.4.11.7). Essa enzima cliva principalmente substratos contendo aspartato ou glutamato na posição amino-terminal. Possui fraca atividade quando esse aminoácido é arginina ou histidina e não hidrolisa LeuβNA. É caracteristicamente ativada por Ca^{++} (Norén et. al., 1986; Maroux et. al., 1988; Sanderink et. al., 1988).

No citossol do enterocito é encontrada a leucina aminopeptidase (EC 3.4.11.1), a aminopeptidase B (EC 3.4.11.6) e a tripeptídeo aminopeptidase (EC 3.4.11.4).

A leucina aminopeptidase possui ampla especificadade, mas hidrolisa

preferencialmente ligações peptídicas adjacentes a um resíduo de leucina amino terminal. leucinamida é considerado seu substrato específico e ela hidrolisa também Leu-Gly e Pro-Gly. Sua atividade sobre LpNa e LeuβNA é muito pequena (Sjostrom & Norén, 1986; Sanderink et. al., 1988). Estudos recentes mostraram que a prolil-aminopeptidase (EC 3.4.11.5) é na verdade idêntica à leucina aminopeptidase (Matsushima et. al., 1991).

Aminopeptidase B atua principalmente sobre substratos que contém lisina ou arginina na posição N-terminal, clivando também dipeptídeos com essa estrutura. Não atua sobre LeuβNA ou AlaβNA (Sjostrom & Norén, 1986; Sanderink et. al., 1988).

A tripeptídeo aminopeptidase é responsável por quase toda a atividade solúvel sobre tripeptídeos. É ativa sobre vários tripeptídeos, inclusive com prolina N-terminal. Não são hidrolisados substratos que contém um aminoácido carregado na posição N-terminal ou uma prolina na segunda posição (Sjostrom & Norén, 1986; Sanderink et. al., 1988).

Em insetos, do ponto de vista enzimológico, as proteinases são as peptídeo hidrolases melhor conhecidas, enquanto poucos dados existem sobre aminopeptidases, carboxipeptidases e dipeptidases (Law et. al., 1977; Vonk & Western, 1984; Applebaum, 1985). Nesses animais, as aminopeptidases devem desempenhar um papel importante na digestão de proteínas, uma vez que essas enzimas geralmente são mais ativas e mais numerosas que as carboxipeptidases.

A maioria dos trabalhos envolvendo a caracterização de aminopeptidases de insetos realizados até o início da década de 80, utilizaram como fonte de enzima a fração solúvel de homogeneizados do intestino médio (Ward, 1975a, b e c; Gooding & Rolseth, 1976; Baker & Woo, 1981, Van der Westhuizen et. al., 1981). Embora esse procedimento possa produzir dados moleculares relevantes, ele não é adequado para esclarecer o significado fisiológico dessas enzimas e, além disso, são

desprezadas as aminopeptidases que possam ocorrer ligadas às membranas plasmáticas.

Estudando a larva de Tineola bisselliella (Lepidoptera), Ward (1975b e c) obteve a separação das aminopeptidases por eletroforese em gel de poliacrilamida em dois grandes grupos. Um deles com migração intermediária e outro com alta mobilidade eletroforética. As enzimas desses dois grupos foram parcialmente purificadas e caracterizadas, revelando propriedades muito semelhantes dentro de cada grupo, mas muito diferentes entre os grupos. Embora seja possível que essas aminopeptidases sejam produtos de vários genes diferentes (como foi observado em Drosophila melanogaster, ver Kylsten et. al., 1992), é mais provável que as aminopeptidases de cada grupo derivem de um só precursor proteico, através de proteólise limitada. A aminopeptidase de T. bisseliella que apresenta maior migração eletroforética, hidrolisa LeuβNA > ArgβNA > MetβNA > ProβNA, não hidrolisa significativamente GluβNA e age em di- e tripeptídeos. A aminopeptidase de migração eletroforética intermediária é muito ativa sobre MetβNA, além de hidrolisar bem LeuβNA e ArgβNA. Especificidade muito semelhante a essa última é encontrada na aminopeptidase intestinal do Coleoptera Attagenus megatoma (Baker & Woo, 1981).

Na larva de Rhynchosciara americana (Diptera), nós tínhamos encontrado aminopeptidases no fluído ectoperitrófico (fluído contido no espaço luminal do intestino, entre a membrana peritrófica e o epitélio, ver Terra et. al., 1979), na fração solúvel das células e integrando as membranas plasmáticas celulares do intestino (Ferreira & Terra, 1980, 1982).

Nós decidimos isolar e caracterizar as várias aminopeptidases presentes em R. americana, com o objetivo de compreender o papel fisiológico de cada uma delas e de contribuir para o conhecimento das propriedades dessas enzimas nos insetos.

No fluído ectoperitrófico de R. americana, encontrou-se duas

aminopeptidases bastante ativas sobre substratos contendo leucina na posição amino terminal. Após sua semi-purificação, verificou-se que elas são idênticas às encontradas na fração solúvel celular (Ferreira & Terra, 1984). Essas enzimas recuperadas na fração solúvel correspondem na realidade a moléculas adsorvidas ao glicocálix celular, (ver Ferreira et. al., 1990; Klinkowstrom et. al., 1994a). Essas duas aminopeptidases apresentam algumas propriedades em comum tais como Km para substratos contendo leucina e arginina e K_i para alguns inibidores. Elas diferem no pI e na capacidade de hidrolisar alguns substratos (Ferreira & Terra, 1984; Klinkowstrom et. al., 1994a). Essas enzimas possuem especificidade ampla em relação ao amino ácido amino terminal, e hidrolisam melhor tetrapeptídeos que tri- e dipeptídeos (Ferreira & Terra, 1984). Outra aminopeptidase solúvel detectada nas larvas de R. americana ocorre majoritariamente associada ao glicocálix das células dos cecos gástricos. Essa enzima é semelhante a Aminopeptidase A de mamíferos (EC 3.4.11.7) apresentando alta atividade sobre AspβNA e hidrolisando pouco LpNA e ProβNA. Tanto quanto sabemos, essa é a primeira aminopeptidase A descrita em insetos. Essa enzima é inibida competitivamente por hidroxamato de aspartato (K_i=0,1 mM) e, ao contrário da enzima de mamíferos, ela não é ativada por Ca^{++} (Klinkowstrom et. al., 1994a).

Na membrana plasmática das células dos cecos de R. americana, demonstrou-se a existência de duas aminopeptidases. Uma responsável por cerca de 80% e outra por 20% da atividade microvilar (Ferreira & Terra, 1985). Essas duas enzimas apresentam uma especificidade em relação a substratos sintéticos semelhante aquela da enzima solúvel. Com relação aos peptídeos, a enzima majoritária prefere tripeptídeos, enquanto a minoritária prefere tetrapeptídeos (Ferreira & Terra, 1985, 1986a e b). Alguns parâmetros cinéticos foram determinados na aminopeptidase majoritária solubilizada por Triton X-100 ou liberada para a solução por ação da papaína. Valores muito semelhantes foram

encontrados para as duas formas da enzima (Ferreira & Terra, 1986b).

Todas as aminopeptidases de insetos que tiveram sua especificidade estudada, são semelhantes à aminopeptidase N de mamíferos (EC 3.4.11.2). A única exceção é a aminopeptidase presente no glicocálix das células dos cecos de R. americana que, conforme comentado anteriormente, assemelha-se à aminopeptidase A de mamíferos (EC 3.4.11.7).

Embora aminopeptidases sejam usualmente metaloenzimas, poucos trabalhos tentaram verificar o papel do metal na atividade dessas enzimas de insetos. Nós mostramos que a aminopeptidase majoritária presente nas membranas plasmáticas do intestino de R. americana possui um íon metálico envolvido em catálise. Realizamos também experimentos de inibição múltipla e de proteção da inativação por EDTA conferida por inibidores competitivos. Pudemos assim propor a existência de um sub-sítio hidrofóbico e um sub-sítio hidrofílico no sítio ativo da enzima e que a ocupação do sub-sítio hidrofóbico aumenta a exposição do metal (Ferreira & Terra, 1986a). Fenantrolina inibe as aminopeptidases de R. americana de modo diferente do EDTA. Ela forma um complexo ternário com a enzima e o metal (Ferreira & Terra, 1984) ou liga nas vizinhanças do sítio ativo mudando o grau de exposição desse íon (Ferreira & Terra, 1986a).

Os dados obtidos sobre a especificidade das aminopeptidases de R. americana permitiram propor o seguinte esquema para a digestão de proteínas: Oligômeros formados dentro da membrana peritrófica (membrana acelular quitino-proteica, que envolve o alimento) passam para o fluído ectoperitrófico. Aí eles são atacados pelas aminopeptidases que preferem peptídeos longos. A hidrólise desses oligômeros prossegue no glicocálix das células dos cecos por ação dessas mesmas enzimas e pela aminopeptidase semelhante à aminopeptidase A de mamíferos. Na membrana das microvilosidades dos cecos, oligopeptídeos são atacados por aminopeptidases semelhantes à aminopeptidase N de mamíferos. A aminopeptidase

minoritária (20% da atividade microvilar), que tem especificidade semelhante a das enzimas presentes no fluído ectoperitrófico, termina a digestão intermediária de oligopeptídeos. Desse modo, ela fornece substrato para a aminopeptidase majoritária presente na membrana. Dipeptídeos resultantes da ação da aminopeptidase microvilar majoritária sobre pequenos peptídeos (usualmente tripeptídeos), são hidrolisados por dipeptidases.

Aminopeptidases diferentes, presentes em compartimentos intestinais distintos, foram também encontradas por nós em Rhodnius prolixus (Hemiptera). Esse inseto possui três aminopeptidases ativas sobre substratos contendo leucina na posição amino terminal. Uma delas é encontrada no conteúdo luminal (Mr=61.000) e é bem ativa também sobre Leu-Gly-Gly. As outras duas (Mr=61.000 e Mr=240.000) ocorrem aprisionadas entre as membranas interna e externa das microvilosidades. A aminopeptidase de alto Mr possui alta atividade sobre Leu-Gly-Gly, enquanto a de baixo Mr praticamente não é ativa sobre esse substrato (Ferreira et. al., 1988). É possível que a atividade das aminopeptidases presentes nesses dois compartimentos seja diferente, dependendo do tamanho da cadeia oligopeptídica, como foi comentado para as aminopeptidases de Rhynchosciara americana. Nós não prosseguimos no estudo das aminopeptidase de Rhodnius prolixus, devido a dificuldade na obtenção dos animais e da pouca estabilidade dessas enzimas a alguns métodos de separação de proteínas.

Desde que nós iniciamos o estudo das aminopeptidase de Rhynchosciara americana, somente dois trabalhos foram publicados tratando especificamente de aminopeptidases de insetos (Billingsley, 1990; Takezue et. al., 1992). Outros trabalhos reportavam algumas propriedades de aminopeptidases de insetos, embora mais preocupados com aspectos mais gerais da digestão (ver Terra & Ferreira, 1994).

Aminopeptidases foram detectadas nas membranas microvilares intestinais

de várias ordens de insetos (ver Terra & Ferreira, 1994). A maioria das aminopeptidases parecem estar inseridas na membrana por intermédio de um peptídeo ancorador hidrofóbico. Essa inferência baseia-se em uma boa solubilização das enzimas por Triton X-100 e uma liberação pelo menos parcial das moléculas por ação da papaína. Esse é o caso das aminopeptidases encontradas por nós em R. americana (Ferreira & Terra, 1985, 1986b) e das aminopeptidases de Trichosia pubescens (Espinoza-Fuentes & Terra, 1986), Musca domestica (Terra et. al., 1988), Anophelis stephensi (Billingsley, 1990), Erinnyis ello (Santos & Terra, 1986) e Spodoptera frugiperda (Ferreira et. al., 1994a). Somente a aminopeptidase de Bombyx mori parece inserir-se na membrana plasmática através de uma âncora de glicosil-fosfatidil inositol (Takesue et. al., 1992).

No futuro, pretendemos estudar o efeito de fenantrolinas não quelantes (como a 1,7- e a 4,7-fenantrolina) e de substâncias semelhantes a fenantrolina (como o fenantrol e a fenantridina) nas aminopeptidases de R. americana. Os resultados obtidos devem permitir esclarecer se a ligação dessas moléculas com a aminopeptidases envolve ou não interação com o íon metálico presente no sítio ativo. Estudo semelhante deverá ser feito com aminopeptidases de outras fontes biológicas.

1.3. Dipeptidases intestinais de insetos

No enterocito de mamíferos, todas as dipeptidases são solúveis. A principal delas é a dipeptideo hidrolase (EC 3.4.13.11), muitas vezes chamada de Gly-Leu hidrolase porque esse substrato é de regra o mais hidrolisado por essa enzima. Ela exibe ampla especificidade, podendo hidrolisar muitos substratos. Gly-Gly e peptídeos contendo prolina e histidina na posição carboxi terminal são maus substratos ou são resistentes a ação dessas enzimas (ver Das & Radhakshisman, 1972; Norén et. al., 1973). A prolina dipeptidase (ou prolidase, EC 3.4.13.9)

hidrolisa dipeptídeos do tipo X-Pro, com pouquíssima atividade sobre alguns outros (Sjostrom & Norén, 1986).

Carnosinase (EC 3.4.13.3) e prolil dipeptidase (ou prolinase, EC 3.4.13.8) foram isoladas de alguns órgãos de mamíferos mas, tanto quanto temos conhecimento, nenhuma delas foi purificada a partir de intestino. Estudos mais recentes sugerem que essas duas atividades são devidas a uma só enzima (Lenney, 1990).

Em insetos, as dipeptidases são as peptídeo hidrolases menos estudadas. Há poucos trabalhos em que essa enzima foi ensaiada, e uma quantidade ainda menor de trabalhos tentou caracterizá-la.

Em Locusta migratoria e Dysdercus fasciatus, Khan (1962) detectou atividade sobre Ala-Gly no lúmen e nas células do tubo digestivo. Em Pheropsophus aequinoctialis atividade hidrolítica de Gly-Leu foi encontrada no tubo digestivo na forma solúvel (cerca de 40%) e ligada a membrana (cerca de 60%). Provavelmente a enzima ligada a membrana está associada às microvilosidades (Ferreira & Terra, 1989). Em Drosophila melanogaster, homogeneizados do tubo digestivo hidrolisam Leu-Pro e Phe-Leu (Hall, 1988). Embora esses resultados surgiram a ocorrência de dipeptidases nesses insetos, não é possível descartar que pelo menos em parte essa atividade resulte da ação de aminopeptidases.

A ocorrência de uma dipeptidase verdadeira foi mostrada em Rhodnius prolixus, embora sua especificidade em relação ao substrato não tenha sido estudada (Garcia & Guimarães, 1979). Atividade dipeptidásica distinta da aminopeptidase também foi evidenciada no tubo digestivo da larva de Musca domestica (Jordão & Terra, 1989) e de Spodoptera frugiperda (Ferreira et. al., 1994a e b), utilizando-se Gly-Leu como substrato. Em M. domestica há uma só dipeptidase nas membranas microvilares das células intestinais. Já S. frugiperda possui duas dipeptidases: uma solúvel associada ao glicocálix e outra ligada às

membranas microvilares das células do intestino médio.

Levando em consideração as dipeptidases encontradas em mamíferos, os resultados descritos em insetos estão refletindo a atividade da Gly-Leu dipeptidase. Somente em D. melanogaster parece ocorrer também uma prolidase (Hall, 1988).

Nas larvas de R. americana foi detectada atividade sobre Gly-Leu solúvel e ligada as membranas microvilares intestinais (Ferreira & Terra, 1980). Nesse animal, amplo estudo foi feito sobre digestão terminal de proteínas, descrevendo-se as propriedades das aminopeptidases (ver ítem anterior). Em vista disso, decidimos iniciar um estudo mais detalhado das dipeptidases digestivas de insetos, usando as larvas de R. americana.

No intestino desse animal nós encontramos duas dipeptidases solúveis, que são capazes de hidrolisar Gly-Leu e Pro-Gly, porém com eficiência diferente. Elas diferem também na sua sensibilidade a EDTA e no grau de inibição causado por 1,10-fenantrolina e aminoacil hidroxamatos. A inibição causada por fenantrolina parece não ser devida a remoção do metal, uma vez que é revertida por diálise. Nas membranas microvilares intestinais de R. americana, detectou-se uma dipeptidase que também hidrolisa Gly-Leu e Pro-Gly. Essa enzima foi solubilizada por Triton X-100 e liberada para a solução por ação da papaína (Klinkowstrom et. al., 1994b). Essas três dipeptidases são semelhantes a dipeptídeo hidrolase de mamíferos (EC 3.4.13.11), embora sejam muito mais ativas sobre Pro-Gly que a enzima dos vertebrados (Norén et. al., 1973). Detectou-se também, nas larvas de R. americana, atividade sobre β-alanil-histidina (Carnosina). Essa atividade não é devida as enzimas que clivam Gly-Leu e Pro-Gly. Nenhuma atividade sobre Gly-Pro foi encontrada (Klinkowstrom et. al., 1994b).

Uma vez que as dipeptidases são tão pouco conhecidas em insetos, nós pretendemos no futuro detectar e caracterizar os vários tipos de dipeptidases em diferentes ordens de insetos.

1.4. Membranas plasmáticas das células intestinais de insetos.

A primeira preparação de microvilosidades de células intestinais de mamíferos foi feita em 1961 (Miller & Crane, 1961). Nesses animais, é bem conhecida a ocorrência de um grande número de hidrolases integrantes da membrana microvilar que participam dos estágios finais da digestão de proteínas e carboidratos, como os complexos sacarase-isomaltase, lactase-florizina hidrolase e maltase-glicoamilase, aminopeptidase A e aminopeptidase N (Norén et. al., 1986). A presença de glicoproteínas e a composição de carboidratos nas membranas microvilares foram determinadas por diversos autores, como Kim & Perdomo (1974), Chritchley et. al., (1975) e Cooper & Kent (1978). Posteriormente, foi demonstrado que a maior parte das glicoproteínas presentes consiste de hidrolases digestivas, como revisado por Matter & Hauri (1991). A composição lipídice dessas membranas também tem sido extensivamente estudada e foi recentemente revisada por Proulx (1991).

Em insetos, as membranas plasmáticas microvilares intestinais apenas começaram a ser estudadas. Mesmo preparações totais de microvilosidades foram feitas apenas a partir de alguns animais. Provavelmente isso se deve ao fato do tamanho reduzido desses animais e de não haver enzimas marcadoras das membranas das microvilosidades intestinais que sejam universais em insetos.

A primeira preparação de microvilosidades intestinais de insetos foi feita utilizando cecos gástricos da larva de R. americana em 1980 (Ferreira & Terra, 1980). Essa preparação foi realizada utilizando-se a técnica de precipitação diferencial por cátions divalentes (Ca^{++} ou Mg^{++}), desenvolvida para mamíferos por Schmitz et. al., (1973). Essa mesma técnica foi usada por Hanozet et. al., (1980), para obtenção de microvilosidades das células colunares do Lepidoptera Phisolamia cynthia. Porém, somente em 1986 mostrou-se que esse tipo de preparação não

estava contaminado pelas microvilosidades das células calciformes presentes nos Lepidoptera (Santos et. al., 1986).

Um método diferente de preparar microvilosidades das células colunares de Lepidoptera foi introduzido por Cioffi e Wolfersberger (1983). Esse método baseia-se no rompimento sequencial do tecido por ultrasom. A preparação resultante é pouco contaminada, mas o rendimento é menor que o obtido usando-se precipitação diferencial por cátion divalente (Santos et. al., 1986; Eisen et. al., 1989).

Desde 1980, quando a primeira preparação de microvilosidades foi obtido, essas técnicas foram aplicadas para cerca de dez insetos diferentes e várias hidrolases foram detectadas nas microvilosidades (ver Terra & Ferreira, 1994). Mostrou-se também que as microvilosidades intestinais dos Lepidoptera, possuem diferentes transportadores de aminoácidos (Giordana et. al., 1989; Hennigan et. al., 1993).

Trabalhos visando determinar a composição química das membranas microvilares intestinais utilizaram preparações de microvilosidades totais. Nessas preparações, mediu-se a relação fósforo/proteína (Wolfersberger et. al., 1987, usando Lepidoptera) e determinou-se a composição polipeptídica após separação do material por eletroforese em gel de poliacrilamida (Wolfersberger et. al., 1987, usando Lepidoptera e Houk et. al., 1986 usando Diptera).

Essas preparações de microvilosidades estão contaminadas, principalmente por elementos do citoesqueleto. Desse modo, medidas químicas realizadas nessas preparações não podem ser comparadas com dados obtidos na literatura sobre membranas purificadas.

Conforme foi comentado anteriormente, as membranas plasmáticas intestinais são uma importante região de contato entre o organismo e o meio ambiente, e desempenham funções fisiológicas importantes. Um melhor

conhecimento sobre essas estruturas pode, eventualmente, levar ao desenvolvimento de novas técnicas de controle de insetos ou a aprimorar as técnicas já existentes.

Devido ao exposto acima e para aprimorar o conhecimento existente sobre essas organelas, nós nos propusemos a isolar e caracterizar as membranas plasmáticas intestinais de insetos de várias ordens.

O primeiro inseto que nós utilizamos para o estudo das membranas plasmáticas intestinais foi Rhodnius prolixus, que pertence a ordem Hemiptera.

Na maioria dos insetos, a membrana plasmática que está em contato direto com o lúmen do tubo digestivo é a membrana das microvilosidades das células colunares. Os Hemiptera, entretanto, apresentam uma membrana lipo-proteica que reveste as microvilosidades e formam expansões para o lúmen do intestino. Estudos utilizando criofratura mostraram que essas membranas possuem poucas proteínas (Lane & Harrison, 1979; Andries & Torpier, 1982). Alguns autores achavam que elas poderiam ter função semelhante a do glicocálix (Marshall & Cheung, 1970), enquanto outros propuseram que elas poderiam estar substituíndo a membrana peritrófica (Lane & Harrison, 1979; Billingsley & Dorene, 1983) que está ausente nesses animais. Essas hpóteses foram aventadas sem que houvesse qualquer evidência bioquímica.

Trabalhando com Rhodnius prolixus, nós verificamos que a α-manosidase é marcadora das membranas microvilares, e α-glicosidase marcadora das outras membranas, que nós denominamos perimicrovilares. Uma aminopeptidase foi encontrada no espaço perimicrovilar. Essa compartimentação das enzimas vai a favor da hipótese dessas membranas possuírem uma função semelhante á da membrana peritrófica. Nós conseguimos, pela primeira vez, separar as membranas microvilares e perimicrovilares e mostramos que as últimas possuem pequena densidade, como era de se esperar pelos dados de criofratura. (Ferreira et. al.,

1988). A caracterização dessas membranas não foi continuada devido a pequena quantidade de material biológico disponível e a dificuldade existente em separar as duas membranas.

Os outros insetos que nós utilizamos para caracterizar as membranas plasmáticas intestinais foram o Coleoptera Tenebrio molitor e os Diptera Rhynchosciara americana e Musca domestica, que pertencem a famílias distintas. Conforme comentado acima, nessas ordens a membrana plasmática em contato com o lúmem intestinal é a membrana plasmática microvilar. Os métodos rotineiramente aplicados em mamíferos para separar membranas microvilares, a partir de uma preparação de microvilosidades, não puderam ser usados em insetos. Entretanto, conseguimos adaptar com sucesso um procedimento que não leva a boas preparações em mamíferos. Preparamos membranas microvilares de diferentes regiões do intestino médio dos insetos e determinamos proteína, lipídeos, carboidratos, colesterol, fósforo e densidade. Os resultados mostraram que, quanto mais evoluído é o inseto, maior a concentração de proteínas presente nas membranas microvilares intestinais. A mesma distribuição é observada analisando-se as razões lipídeo/proteína, colesterol/proteína e as densidades. A concentração de proteína também varia dependendo da região do tubo digestivo e acompanha a distribuição encontrada para as enzimas digestivas ligadas a membrana (Jordão et. al., 1994).

Comparando-se a razão lipídeo/proteína obtida por nós nos insetos com as descritas para vertebrados, verificamos que as membranas microvilares de T. molitor são as mais pobres em proteínas. As membranas microvilares intestinais de R. americana e da região anterior do intestino de M. domestica tem aproximadamente a mesma concentração de proteínas que as membranas microvilares de vertebrados. Já a região posterior do intestino de M. domestica apresenta as membranas microvilares mais ricas em proteínas (ver Jordão et. al.,

1994; Proulx, 1991).

Pretendemos expandir esse estudo para várias ordens de insetos (Membranas microvilares intestinais de Lepidoptera já estão sendo preparadas). Pretendemos também verificar quais proteínas presentes nas membranas microvilares são molecularmente semelhantes entre as várias ordens. Para isso, anti-soros serão preparados contra uma mistura de proteínas purificadas das membranas microvilares de determinado inseto. Esses anti-soros serão usados para detectar proteínas semelhantes em outras ordens, através de Western-Blots. O objetivo desse tipo de estudo é, entre outros, encontrar uma ou mais proteínas que desempenhem uma função fisiológica importante (por exemplo, transporte de íons) e que seja comum a vários insetos nocivos. Conforme comentado anteriormente, esse tipo de enfoque pode orientar o desenvolvimento de novas técnicas de controle de insetos.

1.5. Secreção de enzimas digestivas nos insetos.

Um dos aspectos mais polêmicos da digestão em insetos é o processo pelo qual as enzimas são secretadas pelo intestino médio (Chapman, 1985).

De um modo geral são postulados três tipos de secreção. Umas delas é a exocitose, onde haveria a formação de um grânulo de secreção a partir de vesículas do complexo de Golgi, que posteriormente se fundiram com a membrana plasmática. Os outros tipos são a secreção holócrina, onde as células desintegram-se completamente no processo de secreção; e a secreção apócrina, onde há extrusões citoplasmáticas para a região luminal. Um variante do processo secretor do tipo apócrino que parece ocorrer em alguns insetos é o chamado microapócino (ou microvesículação microvilar), onde pequenas vesículas brotam a partir das membranas apicais das células colunares, levando junto uma pequena parcela de citoplasma e de membranas plasmática.

Certos autores consideram que a desintegração total das células e a

ocorrência de grande extrusões citoplasmáticas não representariam processos de secreção por células digestivas, mas apenas eventos associados a morte de células e sua eliminação para o lúmen intestinal. Algumas evidências apoiam esse ponto de vista. As referidas células apresentam, por exemplo, várias alterações que seguramente indicam que estão em processo de degeneração fisiológica dentro dos mecanismos usuais de renovação celular (ver Andries, 1983). Além disso, essas células são encontradas em ordens de insetos que possuem um ciclo de vida longo, e portanto necessitam de uma grande renovação das células epiteliais velhas (Khan & Ford, 1962; Wigglesworth, 1984). Também foi demonstrado, em Hemiptera, que o número dessas células aumenta quando o animal é submetido a jejum e volta a seu número normal logo após o animal ser realimentado (Khan & Ford, 1962), o que não seria compatível com um envolvimento em secreção de enzimas digestivas.

É interessante notar que, em mamíferos, os enterocitos velhos são liberados para o lúmen intestinal para serem digeridos e as enzimas digestivas ligadas a membrana são recicladas por brotamento de fragmentos da membrana para o lúmen (Haffen et. al., 1986). Esses eventos seriam morfológicamente semelhantes àqueles atribuídos a uma secreção holócrina ou apócrina.

Do ponto de vista bioquímico, há poucos dados que sugerem um ou outro processo de secreção em insetos. Khan & Ford (1962) não encontraram relação entre o aparecimento de α-glicosidase no lúmen e a presença de extrusões citoplasmáticas das células do tubo digestivo de um Hemiptera e Espinoza-Fuentes et. al., (1987), mostraram a ocorrência de algumas enzimas digestivas de Musça domestiça em grânulos de secreção. Graf et. al., (1986), utilizando imunocitoquímica, demonstraram que a secreção de tripsina pelo intestino médio de Aedes aegypti é feita pelo processo exocítico clássico. Esses autores foram capazes de demonstrar a presença da enzima no complexo de Golgi, logo após o animal ter se alimentado e seguiram seu caminho no grânulo de secreção até ela ser

liberada para o lúmen por exocitose. O mesmo processo parece ocorrer na secreção de tripsina por Stomoxys calcitrans (Lehane, 1989; Moffatt & Lehane, 1990).

A secreção microapócrina já foi observada em algumas ordens de insetos (ver Terra & Ferreira, 1994). No Lepidoptera E. ello, foi proposto um engenhoso modelo tipo microapócrino para a secreção de amilase e tripsina (Santos et. al., 1983 e 1986). Observou-se que grande parte da atividade dessas enzimas são ligadas a membrana quando se faz fracionamento sub-celular (Santos et. al., 1983 e 1986). Essas enzimas são secretadas e encontradas em grandes quantidades no lúmen do tubo digestivo, em contato com o alimento. Verificou-se também que o epitélio ventricular apresenta vesículas que se destacam lateralmente das microvilosidades e vesículas que fundem-se entre si e com o topo da microvilosidade, brotando em direção ao espaço ectoperitrófico. Essas vesículas aparentemente não representam um artefato produzido pela fixação do tecido porque quando o fluído ectoperitrófico é coletado e centrifugado, elas estão presentes no sedimento resultante. Nessas vesículas encontra-se amilase e tripsina ligadas a membrana e com alta atividade específica. Baseados nesses e em outros resultados os autores propuseram o modelo comentado a seguir: amilase e tripsina estariam inicialmente ligadas a membrana de vesículas presentes no citoplasma celular. Essas vesículas iriam para a microvilosidade de onde se destacariam lateralmente (saindo uma vesícula com dupla membrana) ou iriam até o topo da microvilosidade, fundindo-se entre si e com a membrana microvilar. Durante esse trajeto, parte das enzimas seriam solubilizadas e parte continuaria presa a membrana. No lúmen as membranas seriam dissociadas pelo alto pH reinante (cerca de 9.5) ou seriam incorporadas à membrana peritrófica.

A tripsina presente no lúmen intestinal de larvas de Musca domestica pode ser secretada por um mecanismo semelhante ao proposto para E. ello. Duas linhas de evidências levaram a essa hipótese: a) em adição à tripsina solúvel luminal, uma

atividade tríptica também é observada ligada a membranas celulares do ventrículo, o que levou Espinoza-Fuentes et. al., (1987) a proporem que a enzima solúvel seria à derivada da forma ligada à membrana e b) Terra et. al., (1988) observaram a ocorrência de brotamentos laterais de pequenas vesículas nas microvilosidades do epitélio, ao analisarem micrografias eletrônicas do intestino médio de larvas de M. domestica.

Tentando contribuir para o estudo da secreção de enzimas digestivas nos insetos, utilizamos dois Coleoptera: Pheropsophus aequinoctialis e Tenebrio molitor. Esses animais possuem algumas de suas células intestinais com extrusões citoplasmáticas. Nós encontramos evidências de que a secreção de enzimas em ambos insetos ocorre por exocitose, indicando que as extrusões citoplasmáticas não devem estar envolvidas nesse processo (Ferreira & Terra, 1989; Ferrerira et. al., 1990). No futuro pretendemos separar as células com extrusões citoplasmáticas presentes no intestino de Tenebrio molitor utilizando técnicas desenvolvidas para remoção de células velhas no intestino de mamíferos (Weiser, 1973; Raul et. al., 1977). Após tratamento, o tecido será inspecionado por microscopia eletrônica e submetido a um fracionamento sub-celular. Os resultados serão comparados com aqueles obtidos a partir de células não tratadas.

Para confirmar o modelo de secreção microapócrina (que foi proposto inicialmente para E. ello, ver acima), purificamos a tripsina solúvel presente na larva de Musca domestica (Diptera). Essa enzima purificada foi utilizada na formação de anti-corpos em coelho. O soro obtido está sendo usado para localizar imunocitoquimicamente a enzima. Os resultados obtidos até o momento reforçam o modelo microapócrino de secreção. O mesmo tipo de estudo deverá ser realizado em breve com a amilase do Lepidoptera Spodoptera frugiperda.

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SOLUBLE AMINOPEPTIDASES FROM CYTOSOL AND LUMINAL CONTENTS OF *RHYNCHOSCIARA AMERICANA* MIDGUT CAECA

PROPERTIES AND PHENANTHROLINE INHIBITION

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Abstract—*Rhynchosciara americana* midgut caecal cells contain two major cytosolic aminopeptidases which are resolved by electrophoresis but which have the same M_r value (115,700), as determined by gradient ultracentrifugation, and have pI values of 8.7 and 7.8. Electrophoretic migration of the two aminopeptidases in polyacrylamide gels of different concentration suggests they differ only in net charge. Thermal inactivation of both cytosolic aminopeptidases follow apparent first-order kinetics with identical half-lives. The two cytosolic aminopeptidases have the same K_m values when either leucine-*p*-nitroanilide or arginine-*p*-nitroanilide are substrates. Midgut luminal fluid displays two major aminopeptidases resolved by electrophoresis which have the same properties as the two cytosolic aminopeptidases. The cytosolic aminopeptidases purified by electrophoresis have the same pH optimum of 8.0 and Tris (K_i 107 mM) and 1,10-phenanthroline (K_i 14 μM) both act as simple linear competitive inhibitors. The enzymes are true aminopeptidases with a broad specificity towards aminoacyl-β-naphthylamides and are more active on tetra and tripeptides than on dipeptides. The data support the assumption that the cytosolic aminopeptidases from caecal cells, which are similar to those in luminal fluid, are enzymes en route to their being secreted and that they differ only in net charge. Furthermore, the properties of the aminopeptidases are in accordance to their proposed role of oligopeptide digestion in the ectoperitrophic fluid.

Key Word Index: *Rhynchosciara americana*, cytosolic aminopeptidases, luminal aminopeptidases, midgut aminopeptidase, aminopeptidase specificity, protein digestion, terminal digestion

INTRODUCTION

Based on the distribution of peptide hydrolases among different midgut regions, it was proposed that digestion of proteins occurs, in the larvae of *Rhynchosciara americana*, in three spatially organized steps (Terra *et al.*, 1979; Ferreira and Terra, 1980, 1982a). The first occurs in the endoperitrophic space under the action of a trypsin-like proteinase, resulting in oligopeptides which diffuse through the peritrophic membrane. The second phase of digestion occurs in the ectoperitrophic space (mainly in the caeca) and it consists of the hydrolysis of oligopeptides mainly by aminopeptidases (carboxypeptidases are much less active than aminopeptidases in all midgut regions in *R. americana*, cf. Ferreira and Terra, 1982b). The major part of the terminal digestion occurs in the cells of the midgut caeca and a minor part in the cells of the posterior ventriculus, by the action of aminopeptidases bound in the plasma membrane covering the cell microvilli and probably also by intracellular aminopeptidases (Ferreira and Terra, 1980).

The determination of hydrolase activities (using different aminoacyl-β-naphthylamides as substrates) in subcellular fractions of midgut caecal cells confirmed previous work (Ferreira and Terra, 1980) showing that aminopeptidases occur only in the microvilli and cytosol of caecal cells (Ferreira and Terra, 1982a). Furthermore, the authors showed that the soluble and microvillar aminopeptidases have different properties and, based mainly in electrophoretical data, they proposed that the majority of the soluble aminopeptidases from the caecal cells are enzymes en route to their being secreted.

In this paper we report the purification and characterization of the soluble aminopeptidases from the midgut caecal cells and lumen. The data showed that the enzymes have similar properties and that they are more active on tetra and tripeptides than on dipeptides, in accordance to their proposed role of oligopeptide digestion in the ectoperitrophic fluid.

MATERIALS AND METHODS

Materials

Acrylamide, L-arginine-β-naphthylamide (ArgβNA), L-arginine-*p*-nitroanilide (ArgpNA), ethylenediaminetetracetic acid (EDTA), *N*-γ-L-glutamyl-β-naphthylamide (GluβNA), L-leucine-*p*-nitroanilide (LpNA), L-leucine-β-naphthylamide (LeuβNA), DL-methionine-β-naphthylamide (MetβNA), L-proline-β-naphthylamide (ProβNA), bisacrylamide, peptides and M_r standards were purchased from Sigma Chemical Co. (St Louis, Missouri, U.S.A.). Ampholytes were from Serva Fine Chemicals (Heidelberg, Germany). All the other reagents were of analytical grade from E. Merck (Darmstadt, Germany) and J. T. Baker (Phillipsburg, New Jersey, U.S.A.). The solutions were prepared in glass-double-distilled water.

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Animals

R. americana (Diptera: Sciaridae) were reared as described by Lara *et al.* (1965). We used only mature feeding larvae at the end of the second period of the fourth instar (Terra *et al.*, 1973).

Preparation of samples

Larvae were dissected in ice-cold 0.1 M NaCl. The gut was removed, rinsed with 0.1 M NaCl and after it was transferred to a dry glass slide, the luminal fluid was collected from the two large caeca with the aid of a capillary. The caeca were removed from the midguts and after they have been rinsed thoroughly with 0.1 M NaCl, they were homogenized in pH 7.0 isotonic KCl solution (in a sufficient volume to contain 2.5 mg protein/ml) with the aid of an Omni-mixer (Sorvall) at 15,000 rpm for 20 sec at 4°C. The homogenates, after being filtered through a piece of nylon mesh of 45 μm pore size, were centrifuged at 100,000 g for 1 hr at 4°C. The supernatants could be stored for at least one year at −20°C without noticeable change in activity of the aminopeptidase.

Hydrolase assays and protein determination

Hydrolase assays were conducted, unless otherwise specified, in 0.1 M sodium phosphate buffer pH 8.0 at 30°C. Naphthylamine liberated from aminoacyl-β-naphthylamides, nitroaniline from aminoacyl-p-nitroanilides and phenylalanine and leucine from the different peptides were determined by the methods of Hopsu *et al.* (1966), Erlanger *et al.* (1961) and Nicholson and Kim (1975), respectively. In each determination, incubations were continued for at least four different periods of time and the initial rates were calculated. All assays were performed so that the measured activity was proportional to protein and to time. Protein was determined as described previously (Terra *et al.*, 1979).

Inhibition studies

The enzymes were incubated in 0.1 M sodium phosphate buffer pH 8.0 at 30°C with four (or five) different concentrations of the tested inhibitor in each of five different concentrations of the substrate. The substrate (LpNA) concentrations used were: 0.2, 0.4, 0.6, 0.8 and 1.0 mM. The inhibitor concentrations used were: Tris, 10, 50, 100, 250, 500 mM; 1,10-phenanthroline, 0.01, 0.02, 0.03, 0.05, 0.1 mM. In the experiment with Tris as an inhibitor, the ionic strength in assay tubes was maintained constant (600 mM) by the addition of suitable amounts of NaCl. The K_i values were determined from replots of slopes and intercepts of Lineweaver–Burk plots against inhibitor concentration (cf. Segel, 1975).

Polyacrylamide gel electrophoresis

Samples were applied to gels of different polyacrylamide gel concentrations prepared as described by Hedrick and Smith (1968) in glass tubes of 5 mm i.d. and 100 mm length. The electrophoretic separation, the fractionation of gels in a gel fractionator and the collection of gel fractions with a fraction collector were performed as described by Terra and Ferreira (1983). The M_r values of the enzymes assayed in the fractions were calculated by the method of Hedrick and Smith (1968), using the migration rates (in the different gels) of myoglobin (M_r 17,800), ovalbumin (M_r 43,000), catalase (M_r 232,000) and ferritin (M_r 450,000) as reference standards. The recoveries of the activities applied to the gels were approx. 70%.

Density gradient centrifugation

Samples (0.2 ml) of aminopeptidase preparations, containing 1.5 mg of bovine haemoglobin and 50 μg of bovine liver catalase, were layered on top of 4.6 ml linear glycerol gradients (5–30, w/v) made up in 50 mM-sodium phosphate buffer, pH 6.2. The centrifugations and the collection of fractions were performed as described previously (Terra and Ferreira, 1983). The M_r values of the enzymes assayed in the fractions were calculated by the method of Martin and Ames, (1961), using the sedimentation rates of bovine haemoglobin (M_r 64,500) and bovine liver catalase (M_r 232,000) as reference standards. The recoveries of the activities applied to the gradients were approx. 95%.

Isoelectric focusing in polyacrylamide gels

Isoelectric focusing was performed as described by Terra *et al.* (1978), in columns of 7.5% polyacrylamide gel containing 1% ampholytes pH 2–11, after pre-focusing for 30 min at 31 V/cm. The recoveries of the activities applied to the gels were approx. 25%.

Thin-layer chromatography of amino acids and peptides

Reaction media, reference peptides and amino acids were spotted on to thin layers of silica gel G (250 μm thick). Chromatograms were developed with n-butanol–acetic acid–water (80:20:20 by vol) and the compounds were detected with ninhydrin (Brenner *et al.*, 1969).

RESULTS

Electrophoresis, isoelectric focusing and density-gradient centrifugation of the soluble aminopeptidases

There are two major aminopeptidases from midgut caecal cytosol which are resolved by polyacrylamide gel electrophoresis (Fig. 1A). In addition to the major aminopeptidases, it is possible to resolve minor aminopeptidases (less than 10% of total aminopeptidase activity) from some cytosol preparations (results not shown). The aminopeptidase of lower migration rate (AP_1) has pI 8.7 and the one of higher migration rate (AP_2) has pI 7.8 (Fig. 1C). Both aminopeptidases sediment in density-gradients as a protein with M_r 115,700 ± 9800 (Fig. 1B). Results similar to those in Fig. 1 were obtained using the luminal fluid from midgut caeca as a source of enzymes.

The migration rates of AP_1 and AP_2 from caecal cytosol and luminal fluid were determined in electrophoretical runs accomplished in polyacrylamide gels of different concentrations (Fig. 2). The fact that the lines of Fig. 2 are approximately parallel suggests that all the aminopeptidase molecules have the same M_r value (122,200 ± 24,400 average ± SD, $n = 4$), whereas the different intercepts indicate the molecules have different pI values (cf. Hedrick and Smith, 1968).

Kinetic similarity among soluble aminopeptidases

AP_1 and AP_2 purified by electrophoresis from caecal cytosol and luminal fluid display similar K_m values in relation to LpNA and ArgpNA (Table 1). This suggest that the aminopeptidases in the four samples are identical from a kinetical point of view.

Thermal inactivation of soluble aminopeptidases

Thermal inactivation of both AP_1 and AP_2 (purified by electrophoresis) from caecal cytosol follows an apparent first-order kinetics with similar half-lives (58 and 57 min respectively), for a period of time of at least three half-lives (Fig. 3). This suggests that there is only one aminopeptidase in each preparation and that the aminopeptidases in both preparations are similar to each other. Results similar to those in Fig. 3 were obtained using AP_1 and AP_2 purified from the luminal fluid of midgut caeca.

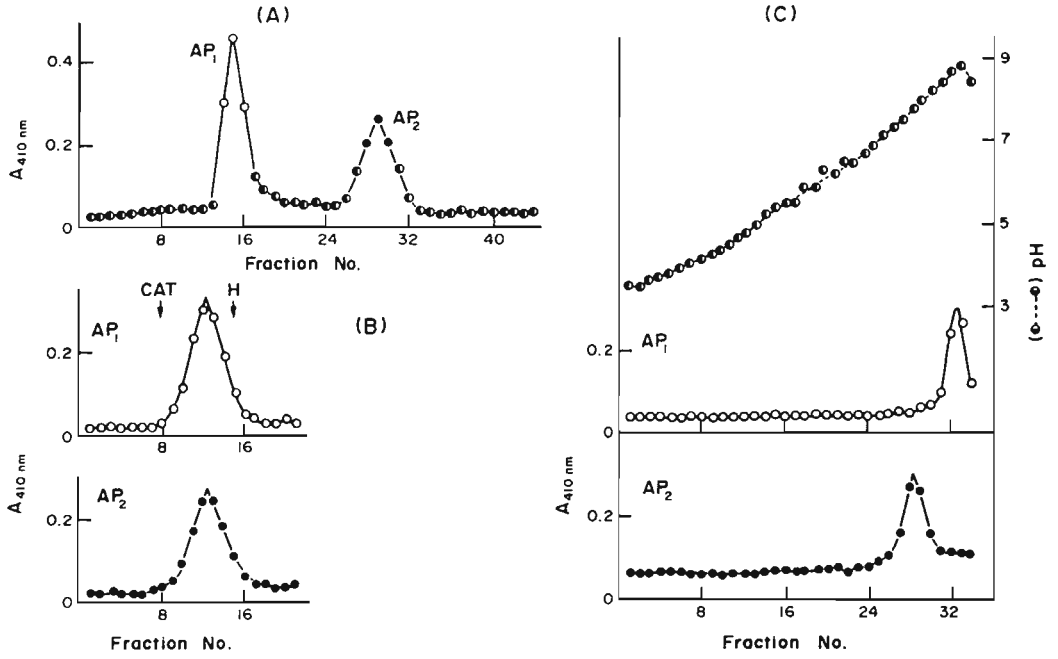

Fig. 1. Physical properties of the caecal cytosol aminopeptidases from *R. americana*. (A) Electrophoretic separation in 7% polyacrylamide gel columns. The three most active fractions (represented by open (○) or solid (●) circles) corresponding to each peak (AP_1 and AP_2 respectively) were pooled for later use. (B) Sedimentation profiles of aminopeptidases in a linear glycerol gradient. Samples were pooled fractions corresponding to either AP_1 (○) or AP_2 (●) from the experiment described in (A). Fractions were collected from the bottom of the tube. CAT, bovine liver catalase (M_r 232,000); H, bovine haemoglobin (M_r 64,500). (C) Isoelectric focusing of aminopeptidases. Samples were pooled fractions corresponding to either AP_1 (○) or AP_2 (●) from the experiment described in (A). Profiles from several other preparations are similar to those shown. Assays were accomplished with 1.0 mM LpNA as substrate. Details are given in Materials and Methods.

Effects of pH and temperature on the caecal cytosol aminopeptidase

The effect of pH on the caecal cytosol aminopeptidase suggests the existence of two prototropic groups in the active site of the free enzyme (pK_a 6.9 and 7.9) and enzyme–substrate complex pK_a (6.5 and pK_a 8.5) (Fig. 4). The pK_a values were determined according to Dixon's rules and they can be only considered to be approximate, due to the closeness of the values (see Discussion in Segel, 1975). The enzyme has an apparent optimum pH of approx. 8.0 (0.1 M sodium phosphate buffer) (Fig. 4).

The energy of activation of the caecal cytosol aminopeptidase determined in saturating conditions

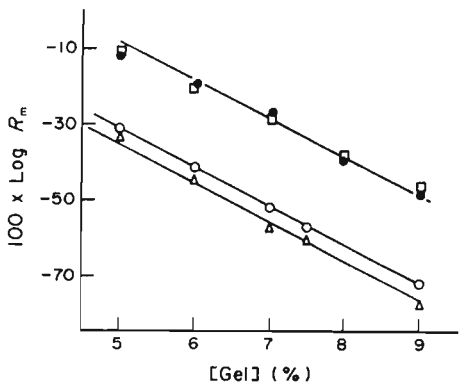

Fig. 2. Effect of different polyacrylamide gel concentrations on the electrophoretical migration of soluble caecal aminopeptidases from *R. americana*. Caecal cell cytosol: ○, AP_1; ●, AP_2. Caecal luminal fluid: △, AP_1; □, AP_2. R_m, electrophoretic migration of the enzyme in relation to the tracking dye. Each data point represent a single determination. Other details as in legend to Fig. 1.

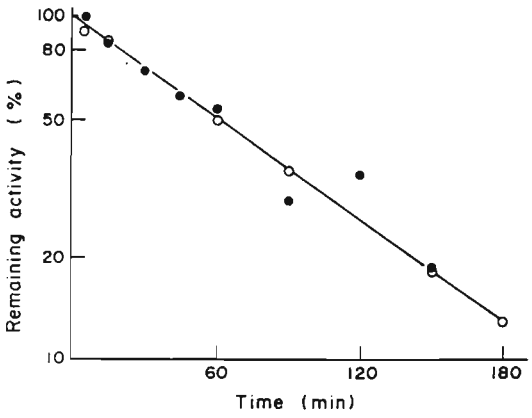

Fig. 3. Thermal inactivation, at 50°C, of the purified caecal cytosol aminopeptidases from *R. americana* midgut caecal cells. ○, AP_1; ●, AP_2. The enzyme sources were the pooled fractions described in the legend to Fig. 1. Each data point represent a single determination. Other details as in the legend to Fig. 1.

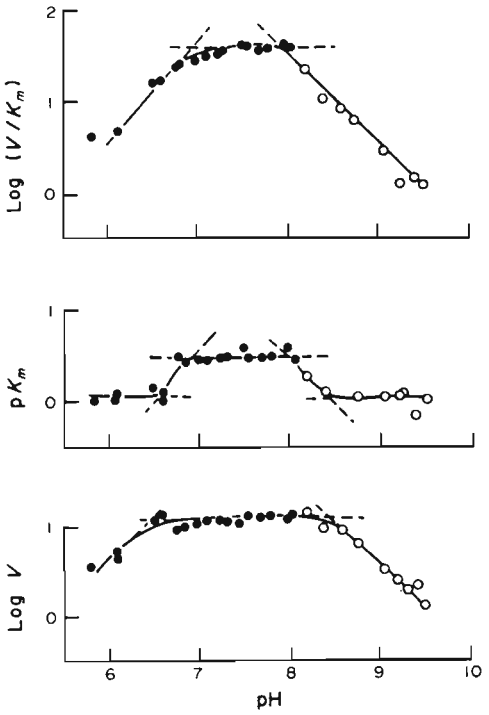

Fig. 4. Effect of pH on the stability and on some kinetic parameters of the caecal cytosol aminopeptidase from *R. americana*. The enzyme samples were incubated at the different pH values at several LpNA concentrations, and apparent V and apparent K_m values were calculated as described in the legend to Table 1. The assays performed at 30°C in 100 mM sodium phosphate buffer (●) and 100 mM sodium borate buffer (○). For the determination of the pH-stability, the enzyme was left for 2 hr at 30°C at different pH values, before being diluted 10-fold by the addition of 0.1 M sodium phosphate buffer, pH 8.0, followed by assays in these conditions. The enzyme is stable in all pH values displayed. Units: V, μM sec^{-1}; K_m, mM; V/K_m, 10^3 sec^{-1}.
Each data point represent a single determination.

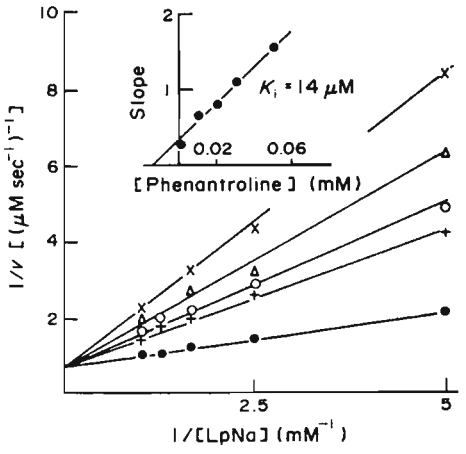

Fig. 5. Inhibition of the purified caecal cytosol aminopeptidase from *R. americana* by 1,10-phenanthroline at pH 8.0. Lineweaver–Burk plots for different concentrations of phenanthroline; inset, replot of slopes calculated from Lineweaver–Burk plots against the concentration of phenanthroline. The enzyme source was the combined fractions AP_1 and AP_2 purified as described in the legend to Fig. 1.

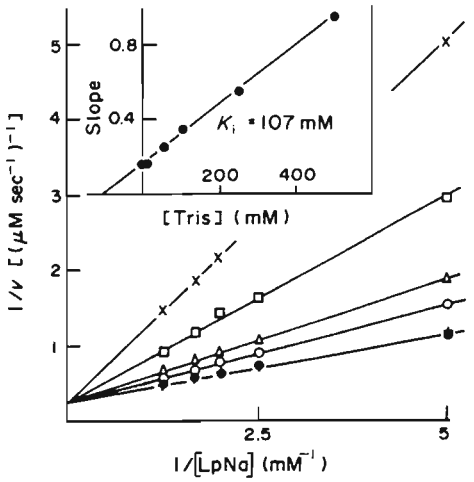

Fig. 6. Inhibition of the purified caecal cytosol aminopeptidase from *R. americana* by Tris at pH 8.0. Lineweaver–Burk plots for different concentrations of Tris; inset, replot of slopes calculated from Lineweaver–Burk plots against the concentration of Tris. The enzyme source was the combined fractions of AP_1 and AP_2 purified as described in the legend to Fig. 1.

between 20 and 50°C (six different temperatures were used) is 33.8 kJ/mol (8.06 kcal/mol). The enzyme is completely stable for at least 2 hr at 40°C in 0.1 M sodium phosphate pH 8.0.

Inhibitors and substrate specificity of the purified caecal cytosol aminopeptidase

EDTA (0.17 mM, final concentration) does not inhibit, whereas 1,10-phenanthroline (0.17 mM, final concentration) causes a 85% inhibition in the purified aminopeptidase. This inhibition is completely reversed by dialysis against buffer. 1,10-Phenanthroline (K_i 14 μM, Fig. 5) and Tris (K_i 107 mM, Fig. 6) are simple linear competitive inhibitors of the aminopeptidase.

Table 1. K_m values for LpNA and ArgpNA corresponding to the aminopeptidases purified by electrophoresis from *R. americana* midgut caecal cytosol and luminal fluid*

Enzyme	Substrate	K_m (mM)
Cytosol, AP_1	LpNA	0.51 ± 0.07
Cytosol, AP_2	LpNA	0.46 ± 0.10
Fluid, AP_1	LpNA	0.47 ± 0.06
Fluid, AP_2	LpNA	0.55 ± 0.03
Cytosol, AP_1	ArgpNA	0.10 ± 0.02
Cytosol, AP_2	ArgpNA	0.11 ± 0.01
Fluid, AP_1	ArgpNA	0.12 ± 0.01
Fluid, AP_2	ArgpNA	0.10 ± 0.01

*Cytosol AP_1 and AP_2 correspond to fractions pooled as described in Fig. 1, whereas luminal fluid AP_1 and AP_2 correspond to fractions analogous to those from cytosol samples. The enzymes purified by electrophoresis were incubated with five different concentrations (range 0.2–1.0 mM) of substrates in 0.1 M sodium phosphate buffer pH 8.0 at 30°C. Kinetic parameters (means ± SD; $n = 5$) were determined by a weighted linear regression by the procedure of Wilkinson (1961) with the aid of a programmable pocket calculator (Texas Instruments TI 59).

Table 2. Substrate specificity of the purified soluble *R. americana* midgut caecal aminopeptidase

Substrate	K_m (mM)	V (nM sec^{-1})	$10^4 \times V/K_m$ (sec^{-1})
LeuβNA	0.064 ± 0.011	792 ± 5	125 ± 22
ArgβNA	0.046 ± 0.007	368 ± 18	79 ± 16
MetβNA	0.161 ± 0.029	527 ± 26	32.7 ± 7.5
ProβNA	0.56 ± 0.03	228 ± 1	4.09 ± 0.23
Gly-Phe	29 ± 9	163 ± 45	0.056 ± 0.018
Phe-Gly	4.68 ± 0.27	273 ± 9	0.583 ± 0.053
Leu-Gly	1.67 ± 0.29	54.2 ± 9.5	0.33 ± 0.11
Phe-Gly-Gly	2.69 ± 0.28	1073 ± 38	3.99 ± 0.56
Leu-Gly-Gly	2.68 ± 0.48	823 ± 68	3.07 ± 0.80
Phe-Gly-Gly-Phe	1.07 ± 0.12	718 ± 25	6.71 ± 0.99

Purified aminopeptidase (Ap_1 and Ap_2 were combined, details in the legend to Fig. 1) was incubated with five different concentrations of each of the listed substrates, in 0.1 M sodium phosphate buffer pH 8.0 at 30°C. Kinetic parameters (means ± SD; $n = 5$) were determined by a weighted linear regression. GluβNA was also tested, but was found not to be hydrolyzed by the enzyme. Details are given in the legend to Table 1 and in Materials and Methods.

Thin-layer chromatography of the products of the action of the purified caecal cytosol aminopeptidase on peptides (those listed in Table 2) demonstrate that the enzyme is a true aminopeptidase which hydrolyses the N-terminal peptide bonds in tripeptides and tetrapeptides. The purified aminopeptidase shows a broad specificity in relation to the N-terminal aminoacyl-residue (Table 2) and hydrolyses tetra and tripeptides much more efficiently than dipeptides. Activity of the enzyme toward Gly-Phe (Table 2) is very weak, whereas no activity was found in relation to GluβNA.

DISCUSSION

The role of midgut caeca soluble aminopeptidases

The two major soluble aminopeptidases found in the midgut caeca cell cytosol seem to differ only in net charge as do those in midgut caecal lumen. These assertions are based on the following: (a) the aminopeptidases (from caecal cytosol or lumen) have the same M_r value, as determined by density-gradient centrifugation and by electrophoresis, and display different pI values, as judged by isoelectric focusing and electrophoresis; (b) the aminopeptidases display identical K_m values for LpNA and ArgpNA and the thermal inactivation kinetics of the enzymes are similar.

Comparison of the electrophoretic migration of aminopeptidases present in the soluble fraction of *R. americana* midgut caecal cells and in the midgut luminal contents led Ferreira and Terra (1982a) to describe a minor aminopeptidase restricted to the cytosol, which may have an intracellular function. Nevertheless, the results discussed above suggest the presence of only one enzyme with aminopeptidase activity (identical in cells and in luminal fluid) displaying different charges as a result (presumably) of differential glycosilations. Such differential glycosilations are not infrequent among secretory enzymes, amylases for example (cf Kauffman *et al.*, 1970). Thus, it is highly probable that the aminopeptidases found in midgut cell cytosol are aminopeptidases en route to their being secreted and are probably without an intracellular function, as has been suggested previously for the majority of the aminopeptidase activity of the cytosol (Ferreira and Terra, 1982a). The minor aminopeptidase activities described previously (Ferreira and Terra, 1982a) and which are observed in some (but not all) preparations might be other charge variants of single aminopeptidase protein.

The marked preference of the soluble aminopeptidase for tetra and tripeptides in relation to dipeptides lends support to the assumption that this enzyme is involved in the luminal intermediary digestion of proteins. Its role is apparently important, since it is known that carboxypeptidases are not very active in *R. americana* midgut lumen (Terra *et al.*, 1979).

Properties of the midgut caeca soluble aminopeptidases

The *R. americana* midgut caeca soluble aminopeptidases resemble those from other insect sources in their broad specificity towards aminoacyl-β-naphthylamides, in their higher activity on tripeptides rather than on dipeptides, in their inhibition by Tris and phenanthroline and in their low sensitivity towards EDTA (Ward, 1975a, b; Baker and Woo, 1981). Furthermore, the soluble aminopeptidases from *R. americana*, like those of *Tineola bisselliella*, occur as families of charge variants of what seem to be the same enzyme protein (Ward, 1975a, b).

Aminopeptidases are usually metallo-enzymes which become inactivated by extensive dialysis against metal chelators such as EDTA (Baker and Woo, 1981). Otherwise, 1,10-phenanthroline (a chelator weaker than EDTA, cf. Sillén and Martell, 1964) inhibition of aminopeptidases has been described in conditions in which efficient chelation is not supposed to occur (e.g. when EDTA does not inhibit the enzyme) (Ward, 1975a, b). In the present paper, we showed that 1,10-phenanthroline is a strong (K_i 14 μM) simple linear competitive inhibitor of the midgut soluble aminopeptidase. Thus, its effect should not depend on the removal of metal ions from the enzyme (which should result in an irreversible inhibition), although it is possible that phenanthroline binds reversibly to the active site through some metal ion in the region of the active site. Nevertheless, it is possible that phenanthroline binding to the aminopeptidase depends more on the ring system of phenanthroline than in its chelator properties.

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MINOR AMINOPEPTIDASES PURIFIED FROM THE PLASMA MEMBRANE OF MIDGUT CAECA CELLS OF AN INSECT (*RHYNCHOSCIARA AMERICANA*) LARVA

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Abstract—*Rhynchosciara americana* midgut caecal cells display in their plasma membranes, as judged by electrophoresis, a major (T_1) and three minor (T_2, T_3 and T_4) aminopeptidases which are solubilized by Triton X-100, and a major (P_1) and two minor (P_2 and P_3) aminopeptidases which are released by papain treatment. Previous work showed that T_1 corresponds to P_1. Aminopeptidases T_1 and T_3 have the same M_r value as determined by density-gradient centrifugation and by electrophoresis, and display different pI values, as judged by isoelectric focusing and electrophoresis. Furthermore, T_1 and T_3 purified by electrophoresis display identical K_m values for arginine *p*-nitroanilide and leucine *p*-nitroanilide and the same K_i values for leucine hydroxamate, hydroxyl amine and isoamyl alcohol. The data suggest that T_1 and T_3 differ only in net charge, implying that T_2 and T_4 should be related to P_2 and P_3. P_2 and P_3 purified by electrophoresis display identical K_i values for arginine hydroxamate, leucine hydroxamate, hydroxylamine and isoamyl alcohol and also identical substrate specificities. P_2 and P_3 show a broad specificity in relation to the N-terminal aminoacyl residue and hydrolyses tetrapeptides more efficiently than tripeptides and much more efficiently than dipeptides. The data suggest that T_2 and T_4 as well as P_2 and P_3 are respectively different aggregation states of a minor native membrane bound aminopeptidase solubilized by detergent and released by papain. The native aminopeptidase is probably a tetramer of identical (or similar) subunits, which, due to its substrate specificities, produces short oligopeptides (mainly tripeptides) which are the preferred substrates for the major membrane-bound aminopeptidase.

Key Word Index: Membrane-bound aminopeptidase, midgut aminopeptidase, aminopeptidase specificity, protein digestion, terminal digestion

INTRODUCTION

The intermediary and final digestion of proteins in larvae of *Rhynchosciara americana* are supposed to occur through the action of a luminal aminopeptidase and an aminopeptidase bound in the plasma membrane covering the midgut caecal cell microvilli (Terra *et al.*, 1979; Terra and Ferreira, 1981; Ferreira and Terra, 1980, 1982).

The *R. americana* luminal aminopeptidase was purified and was shown to display a broad specificity towards the N-terminal aminoacyl-residue and to hydrolyze tetra and tripeptides much more efficiently than dipeptides (Ferreira and Terra, 1984). These properties lend support to the assumption that this enzyme is involved in the luminal intermediary digestion of proteins. Recently, the *R. americana* major membrane-bound aminopeptidase was purified and characterized (Ferreira and Terra, 1985). The data showed that it has a broad specificity in relation to the N-terminal aminoacyl-residue and that it hydrolyzes tripeptides more efficiently than tetra and dipeptides. This agrees with the proposal that terminal digestion of proteins occurs mainly on the surface of midgut cells.

In this paper we describe the partial purification and characterization of the minor aminopeptidases present in the plasma membrane covering the microvilli of the *R. americana* midgut caeca cells. The data suggest that the minor aminopeptidase is a tetramer, which may dissociate in different conditions, and which is more active on tetrapeptides than on tripeptides.

MATERIALS AND METHODS

Materials

Acrylamide, L-arginine-β-naphthylamide (ArgβNA), L-arginine-*p*-nitroanilide (ArgpNA), ethylenediaminetetracetic acid (EDTA), L-leucine-*p*-nitroanilide (LpNA), L-leucine-β-naphthylamide (LeuβNA), DL-methionine-β-naphthylamide (MetβNA), L-proline-β-naphthylamide (ProβNA), bisacrylamide, peptides, 1,10-phenanthroline, and M_r standards were purchased from Sigma Chemical Co. (St Louis, Missouri, U.S.A.). Ampholytes were from Serva Fine Chemicals (Heidelberg, FRG). All the other reagents were of analytical grade from E. Merck (Darmstadt, FRG) and J. T. Baker (Phillipsburg, New Jersey, U.S.A.). The solutions were prepared in glass-double distilled water.

Animals

Rhynchosciara americana (Diptera: Sciaridae) were reared as described by Lara *et al.* (1965). We used only mature feeding female larvae at the end of the second period of the fourth instar (Terra *et al.*, 1973).

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Solubilization of membrane aminopeptidases in Triton X-100 and papain

The preparation of midgut caecal plasma membranes, the solubilization of membrane aminopeptidases in Triton X-100 (10 mg Triton X-100/mg of protein) and in papain (1 mg papain/10 mg of protein) were performed as previously described (Ferreira and Terra, 1983).

Hydrolase assays and protein determination

Hydrolase assays were conducted, unless otherwise specified, in 50 mM Tris–HCl buffer pH 7.2 at 30°C. Naphthylamine liberated from aminoacyl-β-naphthylamides, nitroaniline from aminoacyl-*p*-nitroanilides and phenylanine and leucine from the different peptides were determined by the methods of Hopsu *et al.* (1966), Erlanger *et al.* (1961) and Nicholson and Kim (1975), respectively. Other details as in Ferreira and Terra (1984). Protein was determined as described previously (Terra *et al.*, 1979).

Inhibition studies

The enzymes were incubated in 50 mM Tris–HCl buffer pH 7.2 at 30°C with four (or five) different concentrations (in the range of 0.1 to 2 K_i) of the tested inhibitor in each of five different concentrations of LpNA (in the range of 0.2 to 1 mM). The K_i values were determined from replots of slopes and intercepts of Lineweaver–Burk plots against inhibitor concentration (cf. Segel, 1975).

Polyacrylamide gel electrophoresis

The electrophoretic separation, the fractionation of gels in a gel fractionator and the collection of gel fractions with a fraction collector were performed as described by Terra and Ferreira (1983). The electrophoretical determination of M_r values followed Ferreira and Terra (1984). When the material submitted to electrophoresis was in detergent, the gels and the fractionation buffer contained 0.1% Triton X-100. The recoveries of the activities applied to the gels were approx. 80%. M_r values are displayed as means ± range for two determinations.

Isoelectric focusing in polyacrylamide gels

Isoelectric focusing was performed as described by Terra *et al.* (1978), in columns of 7.5% polyacrylamide gel containing 1% ampholytes pH 2–11, after pre-focusing for 30 min at 31 V/cm. The recoveries of the activities applied to the gels were approx. 27%.

Density-gradient centrifugation

The preparation of the glycerol gradients, the centrifugations and the collection of fractions were performed as described previously (Terra and Ferreira, 1983). The M_r values of the enzymes assayed in the fractions were calculated by the method of Martin and Ames (1961), using the sedimentation rates of bovine haemoglobin (M_r 64,500) and bovine liver catalase (M_r 232,000) as reference standards. The recoveries of the activities applied to the gradients were approx. 90%. M_r values are displayed as means ± range for two determinations.

Thin-layer chromatography of amino acids and peptides

Reaction media, reference peptides and amino acids were spotted onto thin layers of silica gel G (250 μm thick). Chromatograms were developed with *n*-butanol–acetic acid–water (80:20:20 by volume) and the compounds were detected with ninhydrin (Brenner *et al.*, 1969).

RESULTS

Solubilization of the midgut aminopeptidases by detergent and papain

About 80% of the activity originally present in the midgut caecal membranes of *R. americana* is solubilized in Triton X-100, and about 50% of the original activity is released into solution by 15 to 30 min of treatment with papain. In each case, the solubilized and the non-solubilized activity add up to the activity found originally in the preparation of membranes.

Detergent forms of the midgut aminopeptidases

There are four aminopeptidases (T_1, T_2, T_3 and T_4) solubilized by Triton X-100 which are resolved by polyacrylamide gel electrophoresis (Fig. 1A). T_2 sediments in density gradients (pH 6.2) as two proteins with M_r 104,000 ± 7000 and 210,000 ± 10,000 (Fig. 1B) with pI values of 6.6 and 7.9 (Fig. 1C). In alkaline density gradients (pH 8.0), T_2 displays a single peak sedimenting as a protein with M_r 166,000 ± 3000. Electrophoresis accomplished in polyacrylamide gels of 5 different concentrations provided data for calculating the T_2 M_r value as 220,000 ± 25,000. T_4 has an M_r of 107,000 ± 7000 (Fig. 1A) and the pI is 6.8 (Fig. 1C). The small amounts of T_2 and T_4 which may be recovered from the gel columns precluded further comparison between them.

T_3 has an M_r of 160,000 ± 10,000 and the pI is 7.0 (Fig. 1), whereas the M_r of T_1 is 169,000 ± 2000 and the pI is 7.8 (Ferreira and Terra, 1985). The migration rates of T_1 and T_3 were determined by electrophoresis with polyacrylamide gels of different concentrations (Fig. 2). The fact that the lines of Fig. 2 are approximately parallel suggests that the two aminopeptidases have the same M_r value (170,000 ± 2000), whereas the different intercepts indicate that they have different pI values (see Hedrick and Smith, 1968).

T_1 and T_3 purified by electrophoresis display similar K_m and K_i values in relation to several compounds (Table 1). Both enzymes are stable from pH 6.4 to 9.5 at 30°C for at least 4 hr and their pH optimum is 7.2 in Tris–HCl buffer.

Papain forms of the midgut aminopeptidases

There are three aminopeptidases (P_1, P_2 and P_3) released by papain which are resolved by polyacrylamide gel electrophoresis (Fig. 3A). According to Ferreira and Terra (1985) P_1 has an M_r of 207,000 and a pI of 7.8. The M_r of P_2 is 174,000 ± 3000 (in the absence of detergent) (Fig. 3B) or 180,000 ± 20,000 (in the presence of detergent) and the pI is 7.1 (Fig. 3C). P_3 has an M_r of 106,000 ± 2000 (in the absence of detergent) (Fig. 3B) or 112,000 ± 9000 (in the presence of detergent) and a pI of 6.6 (Fig. 3C).

The migration rates of P_2 and P_3 were determined by electrophoresis in polyacrylamide gels of 5 different concentrations. The results confirmed the existence of only one molecular species of aminopeptidase in each of the preparations. The M_r values calculated from these data were: P_2, 170,000 ± 6000; P_3, 100,000 ± 12,000.

P_2 and P_3 purified by electrophoresis display similar K_m and K_i values in relation to several compounds (Table 1). Both enzymes are stable from pH 6.3 to 9.6 at 30°C for at least 4 hr and their pH optimum is 7.7 in Tris–HCl buffer. Treatment of P_2 with Triton X-100, followed by electrophoresis, results in the appearance of a small amount of an aminopeptidase

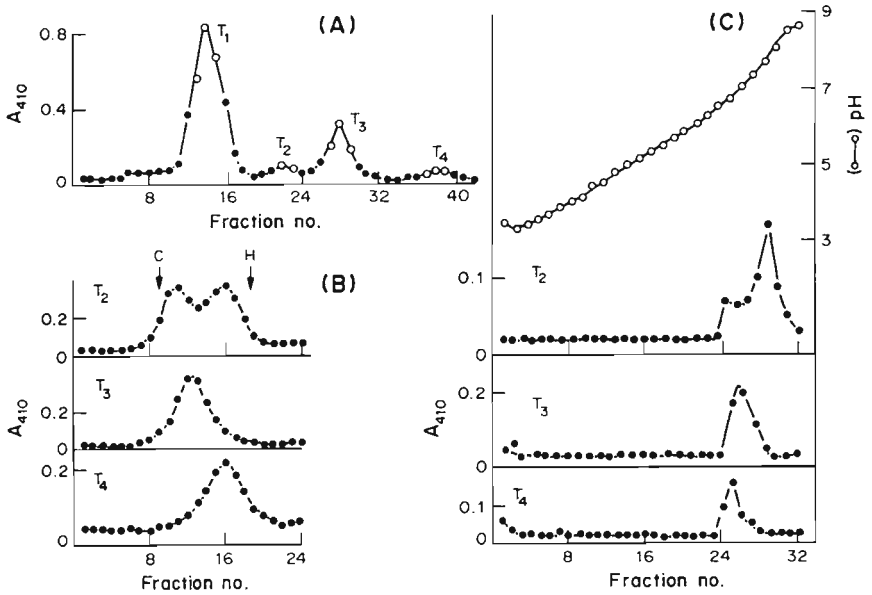

Fig. 1. Physical properties of the Triton X-100-solubilized midgut aminopeptidase from *R. americana*. (A) Electrophoretic separation in 5% polyacrylamide gel column. The most active fractions (represented by open circles) corresponding to each peak from several gel columns were pooled for later use and named hereafter T_1, T_2, T_3 and T_4, respectively. (B) Sedimentation profiles of the aminopeptidases purified as described above. M_r markers: C, bovine liver catalase (M_r 232,000); H, bovine haemoglobin (M_r 64,500). (C) Isoelectric focusing of the aminopeptidases purified as described above. Profiles obtained from several other preparations are similar to those shown. Assays were accomplished with 1.0 mM LpNA as substrate. Details are given in Materials and Methods.

with a migration rate similar to P_3. Otherwise, P_3 after a similar procedure remains unchanged.

1,10-Phenanthroline (1 mM final concentration in reaction media) causes complete inhibition of P_2 and P_3. This inhibition is completely reversed by dialysis against Tris buffer. In contrast to phenanthroline, 2 mM EDTA does not affect the activity of either P_2 or P_3.

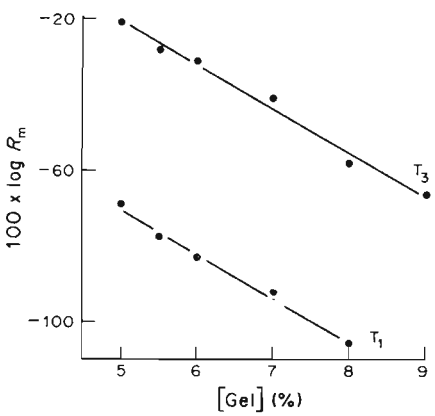

Fig. 2. Effect of different polyacrylamide gel concentrations on the electrophoretic migration of the aminopeptidases T_1 and T_3 from *R. americana* midguts. R_m, electrophoretic migration of the enzyme in relation to the tracking dye. Each data point represent a single determination. Other details as in legend to Fig. 1.

Substrate specificity of the aminopeptidase P_2 and P_3

The aminopeptidase P_3, which was purified in 6% polyacrylamide gel columns (see legend to Fig. 3), was subjected to electrophoresis using 8% polyacrylamide gel columns and gel fractions were assayed with all the substrates listed in Table 2. Only one peak of activity, with identical migration, was found for all the substrates tested. Thin-layer chromatography of the products of the action of P_3 on peptides (those listed in Table 2) demonstrated that the enzyme is a true aminopeptidase which hydrolyzes the N-terminal amino acid in tripeptides and tetrapeptides. P_3 shows a broad specificity in relation to the N-terminal aminoacyl-residue (Table 2) and hydrolyzes tetra and tripeptides much more efficiently than dipeptides. Activity of the enzyme upon Gly-Phe, Gly-Leu and ProβNA is very weak.

Results obtained with the aminopeptidase P_2 were similar to those obtained with P_3 in relation to all substrates. Dipeptides could not be studied due to a contamination of P_2 with a dipeptidase.

Aminopeptidases in the insoluble residue after papain treatment

The insoluble residue remaining after papain treatment of the membrane fraction of *R. americana* midgut cells contains only one major aminopeptidase (TP, Fig. 4A), with an M_r of 104,000 ± 7000 (Fig. 4B), which is resolved into three aminopeptidases by isoelectric focusing (Fig. 4C). Since there is only one major peak of aminopeptidase activity migrating

Table 1. K_m and K_i values for several ligands to midgut aminopeptidases purified by electrophoresis*

Ligand	K_i or K_m (mM)				
	T_1†	T_3	P_2	P_3	TP
Substrates					
ArgpNa	1.5	1.6 ± 0.1	3.3 ± 0.3	3.6 ± 0.4	0.32 ± 0.02
LpNA	1.4	1.8 ± 0.1	1.8 ± 0.2	1.8 ± 0.2	0.58 ± 0.05
Inhibitors					
L-Arginine hydroxamate	0.24	—	0.15 ± 0.02	0.16 ± 0	0.23 ± 0.02
L-Leucine hydroxamate	0.027	0.023 ± 0.003	0.033 ± 0.003	0.027 ± 0.005	0.024 ± 0.02
Hydroxyl amine	5.4	5.9 ± 0.1	3.5 ± 0.2	3.57 ± 0.04	6.3 ± 0.8
Isoamyl alcohol	62	56 ± 3	300 ± 4	310 ± 10	70 ± 6

*T_1 and T_3 correspond to aminopeptidases purified as described in Fig. 1, P_2 and P_3 as in Fig. 3 and TP as in Fig. 4. K_m values (means ± SEM, $n = 10$) were determined by a weighted linear regression by the procedure of Wilkinson (1961) with the aid of a programmable pocket calculator (Texas Instruments T159). The K_i values of the inhibitors (mean ± range, two determinations), which were all simple intersecting linear competitive inhibitors (cf. Segel, 1975), were determined as described in Materials and Methods. †Taken from Ferreira and Terra (1985).

($M_r = 107{,}000 \pm 3000$) in polyacrylamide gels of five different concentrations, the widely spaced peaks of activity observed during isoelectric focusing are probably due to some kind of dissociation related to the pH and ionic strength of the gels. TP and T_1 present similar K_i values and different K_m values for several compounds (Table 1). Although heat denaturated TP is more active than heat denaturated T_1, their initial rates of denaturation are identical (Fig. 5).

DISCUSSION

Properties of membrane-bound midgut aminopeptidases

The two major detergent-solubilized midgut aminopeptidases (T_1 and T_3 in Fig. 1A) seem to differ only in net charge. These assertions are based on the following: (a) the aminopeptidases have the same M_r value as determined by density gradient centrifugation and by electrophoresis, although they display different pI values as judged by isoelectric focusing and electrophoresis; (b) the aminopeptidases display identical K_m values for LpNA and ArgpNA and identical K_i values for leucine hydroxamate, hydroxyl amine and isoamyl alcohol.

Since it is reasonable to suppose that T_1 and T_3 are one and the same enzyme (see above), and T_1 was shown to correspond to the major papain released midgut aminopeptidase (P_1 in Fig. 3A; Ferreira and Terra, 1985), the remaining Triton-solubilized aminopeptidases T_2 and T_4 are probably related to the minor papain released aminopeptidases P_2 and P_3. The finding that 15–20% of the aminopeptidase activity initially present in midgut cell membranes is accounted for by $T_2 + T_4$ or $P_2 + P_3$ supports this assertion. These figures were calculated from the areas under the profiles in Fig. 1A and Fig. 3A (and others similar to them) and taking into consideration the relative amounts of aminopeptidase put into solution by Triton X-100 and papain. T_2 displays M_r values of 104,000, 166,000 or 210,000 depending on

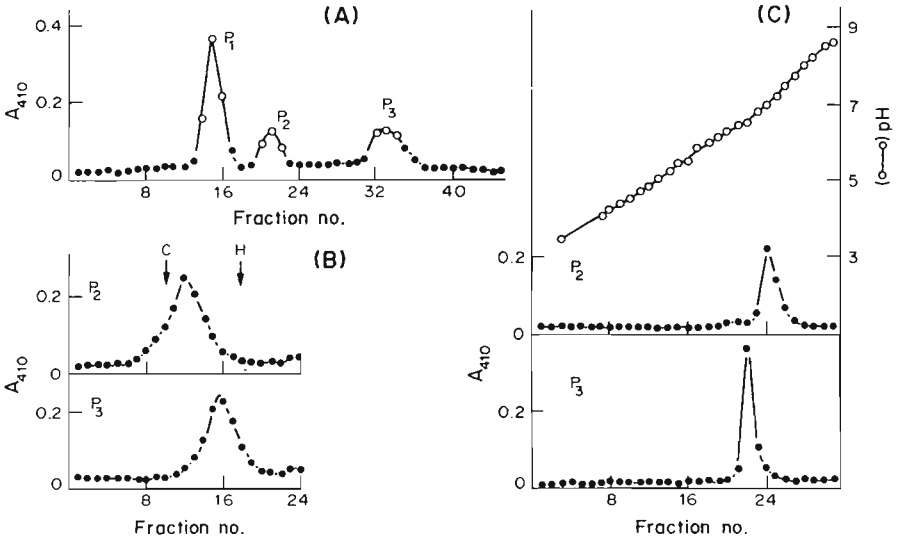

Fig. 3. Physical properties of the papain-released midgut aminopeptidases from *R. americana*. (A) Electrophoretic separation in 6% polyacrylamide gel column. The most active fractions (represented by open circles) corresponding to each peak from several gel columns were pooled for later use and named hereafter P_1, P_2 and P_3 respectively. (B) Sedimentation profiles of the aminopeptidases purified as described above. (C) Isoelectric focusing of the aminopeptidases purified as described above. Profiles obtained from several other preparations are similar to those shown. Other details as in the legend to Fig. 1.

Table 2. Substrate specificity of P_3 purified from *R. americana* midgut caeca*

Substrate	K_m (mM)	V (mM sec^{-1})	$10^4 \times V/K_m$ (sec^{-1})
LeuβNA	0.28 ± 0.03	175 ± 8	6.3 ± 0.9
ArgβNA	0.64 ± 0.07	83 ± 6	1.3 ± 0.2
MetβNA	0.32 ± 0.02	81 ± 2	2.5 ± 0.2
ArgpNA	3.6 ± 0.4	84 ± 6	0.23 ± 0.04
LpNA	1.8 ± 0.2	222 ± 1	1.2 ± 0.3
Phe-Gly	1.2 ± 0.1	16.6 ± 0.5	0.14 ± 0.02
Leu-Gly	1.30 ± 0.03	18.9 ± 0.1	0.140 ± 0.004
Leu-Gly-Gly	0.37 ± 0.02	64 ± 1	1.7 ± 0.1
Phe-Gly-Gly	0.65 ± 0.05	81 ± 2	1.3 ± 0.1
Phe-Gly-Gly-Phe	0.55 ± 0.03	106 ± 1	1.9 ± 0.1

*Purified P_3 (see the legend to Fig. 3) was incubated with five different concentrations of each of the listed substrates, in 50 mM Tris–HCl buffer, pH 7.2 at 30°C. Kinetic parameters (mean ± SEM, n = 15) were determined by a weighted linear regression by the procedure of Wilkinson (1961) with the aid of a programmable pocket calculator (Texas Instruments T159). Gly-Leu, Gly-Phe and ProβNA are hydrolyzed slowly by the enzyme.

the pH conditions in which the determination is accomplished. This variation suggests that T_2 is a tetramer made up of similar subunits (M_r value of about 50,000), which dissociates into a trimer or dimer depending on the pH of the medium. T_4 resembles closely the "dimer" form of T_2, both in M_r value and pI (compare T_4 data with T_2 data in Fig. 1). Otherwise, P_2 and P_3 display similar kinetic parameters (Tables 1 and 2), which agrees with the assumption they are different assemblies of the same (or similar) monomers. Furthermore, treatment of P_2 with Triton X-100 followed by electrophoresis, results in the appearance of an aminopeptidase with a migration similar to P_3.

According to Ferreira and Terra (1985), P_1 corresponds to a dimer of a fragment (M_r 97,000) formed by the action of papain upon the native major (M_r = 169,000) aminopeptidase. The aminopeptidase (TP, M_r = 104,000) solubilized in detergent from the residue remaining after the papain treatment displays an initial heat denaturation rate and K_i values similar to T_1, although its K_m values and activity in the heat denaturated state are different. It is possible that TP is a fragment (or a family of fragments to account for the pI values measured, Fig. 4C) of the major aminopeptidase (M_r = 169,000), different from that which dimerizes into P_1 and displaying different kinetics and solubility properties.

The role of midgut aminopeptidases in protein digestion

Midgut membrane-bound aminopeptidases have been found in insects other than *R. americana*, such as *Drosophila melanogaster* (Walker *et al.*, 1980), *Bombyx mori* (Sumida and Eguchi, 1983), *Erinnyis ello* (Santos and Terra, 1984) and *Tipula abdominalis* (Sharma *et al.*, 1984). Nevertheless, the substrate specificities of those aminopeptidases are unknown, which makes difficult any speculation on their precise role in the terminal digestion of proteins.

The data discussed in this paper support the assertion that there are only two aminopeptidases in the *R. americana* midgut caeca cell membranes: a major

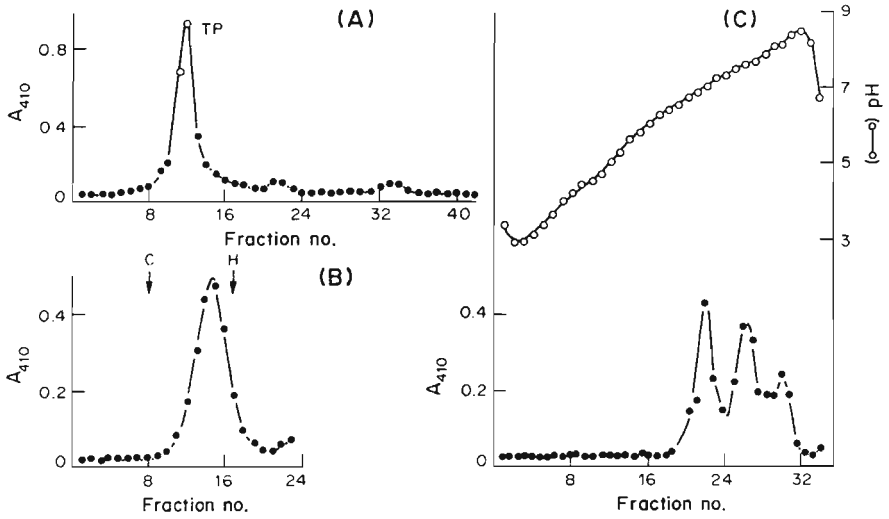

Fig. 4. Physical properties of the aminopeptidases solubilized by Triton X-100 from the insoluble material remaining after papain treatment of *R. americana* midgut cell membranes. (A) Electrophoretic separation in 5% polyacrylamide gel column. The most active fractions (represented by open circles) from several gel columns were pooled for later use and named hereafter TP. (B) Sedimentation profile and (C) isoelectric focusing of the aminopeptidases purified as described above. Profiles obtained from several other preparations are similar to those shown. Other details as in legend to Fig. 1.

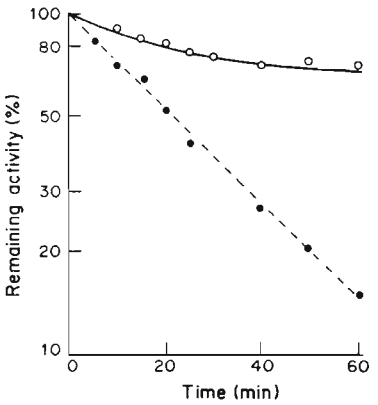

Fig. 5. Thermal inactivation, at 50°C of the purified membrane-bound aminopeptidases from *R. americana* midgut caecal cells. (○) TP; (●) T_1. The enzyme sources were the pooled fractions described in the legends to Figs 1 and 4, respectively. Each data point represents a single determination. The curves are theoretical. The solid line was calculated assuming $k = 7.4 \times 10^{-4}\,sec^{-1}$ and the denaturated enzyme display 65% of the activity of the native enzyme. The interrupted line was calculated assuming $k = 5.4 \times 10^{-4}\,sec^{-1}$.

enzyme accounting for about 80% of the total aminopeptidase activity and a minor portion accounting for 20% of that activity. The major aminopeptidase displays two charged isomers (T_1 and T_3) and, according to Ferreira and Terra (1985), displays broad specificity towards the N-terminal aminoacyl residue, prefers tripeptides to tetrapeptides and slowly hydrolyzes all tested dipeptides. The minor aminopeptidase seems to be a tetramer of similar subunits which may dissociate for a different extent depending on the environmental conditions. It has a broad specificity towards the N-terminal aminoacyl residue and, in contrast to the major aminopeptidase, is more active on tetrapeptides than on tripeptides.

The minor membrane-bound aminopeptidase, which has substrate specificities similar to those of the luminal aminopeptidase, is postulated to finish the intermediary digestion of oligopeptides (started by the luminal aminopeptidase), thus forming substrates for the major membrane-bound aminopeptidase. Dipeptides resulting from the action of the major membrane-bound aminopeptidase on small oligopeptides are probably hydrolyzed by dipeptidases which, based on preliminary results, are present mainly in the plasma membrane and cytosol of midgut caecal cells.

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Substrate Specificity and Binding Loci for Inhibitors in an Aminopeptidase Purified from the Plasma Membrane of Midgut Cells of an Insect (*Rhynchosciara americana*) Larva

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Plasma membrane-bound aminopeptidases (EC 3.4.11.2) are found in the midgut cells from *Rhynchosciara americana* larvae, and are recovered in soluble form after papain treatment. The major papain-released aminopeptidase (M_r 207,000 and p*I* 7.8) was shown to be a true aminopeptidase with a broad specificity toward aminoacyl-β-naphthylamides and to be more active on tetra and tripeptides than on dipeptides. The purified aminopeptidase is inactivated by EDTA according to a kinetics which is half order in relation to EDTA. Leucine hydroxamate (K_i 27 μM) and hydroxylamine (K_i 5.4 mM) completely protect the enzyme from inactivation by EDTA, whereas isoamyl alcohol (K_i 62 mM) increases the inactivation rate. There are 2.3 binding sites in the enzyme for phenanthroline, which makes the binding of the substrate in the enzyme difficult, changes the enzyme-substrate into a more productive complex, and increases the inactivation rate of the enzyme by EDTA by 87-fold. The data support the proposal that the enzyme has a metal ion which is catalytically active and that the enzyme displays two subsites in its active center: a hydrophobic subsite, to which isoamyl alcohol binds exposing the metal ion, and a polar subsite, to which hydroxylamine binds.

Digestion of proteins in the larvae of the fly *Rhynchosciara americana* occurs in three spatially organized steps (1-3). The first takes place inside the peritrophic membrane under the action of a trypsin-like proteinase. The second phase of digestion occurs outside the peritrophic membrane (largely in the caeca) and it consists of the hydrolysis of oligopeptides mainly by luminal aminopeptidases [carboxypeptidases are much less active, see Ref. (4)]. The final digestion of peptides occurs in the cells of the midgut caeca and to a minor extent in the cells of the posterior ventriculus, by the action of aminopeptidases bound in the plasma membrane covering the cell microvilli (5).

Midgut membrane-bound aminopeptidases have been found in insects other than *R. americana*, such as *Drosophila melanogaster* (6), *Bombyx mori* (7), *Erinnyis ello* (8), and *Tipula abdominalis* (9). Nevertheless, the characterization of gut plasma-membrane-bound aminopeptidases has been attempted almost exclusively in mammals [see review in Ref. (10)]. As far as we know, there has hitherto been no comprehensive study of insect gut membrane-bound aminopeptidases.

In the present paper we describe some of the physical and kinetic properties of the major plasma-membrane-bound aminopeptidase (EC 3.4.11.2) from midgut caecal cells of *R. americana*.

MATERIALS AND METHODS

Materials

Acrylamide, L-arginine-β-naphthylamide (Arg-βNA), L-arginine-*p*-nitroanilide (Arg*p*NA), L-aspartic-

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acid α-(β-naphthylamide) (AspβNA), ethylenediamine-tetracetic acid (EDTA), L-leucine-*p*-nitroanilide (LpNA), L-leucine-β-naphthylamide (LeuβNA), DL-methionine-β-naphthylamide (MetβNA), L-proline-β-naphthylamide (ProβNA), bisacrylamide, peptides, 1,10-phenanthroline, and relative molecular weight (M_r) standards were purchased from Sigma Chemical Company (St. Louis, Mo.). Ampholytes were from Serva Fine Chemicals (Heidelberg, Germany). All the other reagents were of analytical grade from E. Merck (Darmstadt, Germany) and J. T. Baker (Phillipsburg, N. J.). The solutions were prepared in glass-double-distilled water.

Animals

R. americana (Diptera: Sciaridae) were reared as described by Lara *et al.* (11). We used only mature feeding female larvae at the end of the second period of the fourth instar (12).

Release of Membrane Aminopeptidases by Papain

The preparation of midgut caecal plasma membranes and the solubilization of membrane aminopeptidases by papain (1 mg papain/10 mg of protein) were performed as previously described (13).

Hydrolase Assays and Protein Determination

Hydrolase assays were conducted, unless otherwise specified, in 50 mM Tris/HCl buffer, pH 7.2, at 30°C. Naphthylamine liberated from aminoacyl-β-naphthylamides, nitroaniline from aminoacyl-*p*-nitroanilides, and phenylalanine and leucine from the different peptides were determined by the methods of Hopsu *et al.* (14), Erlanger *et al.* (15), and Nicholson and Kim (16), respectively. In each determination, incubations were continued for at least four different periods of time (usually 30, 60, 90, and 120 min) and the initial rates were calculated. All assays were performed so that the concentration of product released increased linearly with time. These initial rates were proportional to protein concentration. Protein was determined as described previously (1).

Polyacrylamide Gel Electrophoresis

Samples were applied to gels of different polyacrylamide gel concentrations prepared as described by Hedrick and Smith (17) in glass tubes of 5 mm i.d. and 100 mm length. The electrophoretic separation, the fractionation of gels in a gel fractionator, and the collection of gel fractions with a fraction collector were performed as described by Terra and Ferreira (3). The apparent M_r values of the enzymes assayed in the fractions were calculated by the method of Hedrick and Smith (17), using the migration rates (in the different gels) of myoglobin (M_r 17,800), ovalbumin (M_r 43,000), catalase (M_r 232,000), and ferritin (M_r 450,000) as reference standards. The recoveries of the activities applied to the gels were approximately 80%.

Isoelectric Focusing in Polyacrylamide Gels

Isoelectric focusing was performed as described by Terra *et al.* (18), in columns of 7.5% polyacrylamide gel containing 1% ampholytes, pH 2–11, after prefocusing for 30 min at 31 V/cm. The recoveries of the activities applied to the gels were approximately 25%.

Density-Gradient Centrifugation

Samples (0.2 ml) of membrane proteins released by papain, containing 1.5 mg of bovine hemoglobin and 50 μg of bovine liver catalase, were layered on the top of 4.6 ml linear glycerol gradients (5–30%, w/v) made up in 50 mM sodium phosphate buffer, pH 6.2. The centrifugations and the collection of fractions were performed as described previously (3). The apparent M_r values of the enzymes assayed in the fractions were calculated by the method of Martin and Ames (19), using as reference standards the sedimentation rates of bovine hemoglobin (M_r 64,500) and bovine liver catalase (M_r 232,000). The recoveries of the activities applied to the gradients were approximately 100%.

Thin-Layer Chromatography of Amino Acids and Peptides

Reaction media, reference peptides, and amino acids were spotted on to thin layers of silica gel G (250 μm thick). Chromatograms were developed with *n*-butanol:acetic acid:water (80:20:20 by volume) and the compounds were detected with ninhydrin (20).

RESULTS

Papain Forms of the Midgut Aminopeptidases

About 50% of the activity originally present in the midgut caecal membranes of *R. americana* is released into solution after treatment with papain for 15 to 30 min. The solubilized and the nonsolubilized activity add up to the activity found originally in the preparation of membranes. There are three papain-released aminopeptidases which are resolved by electrophoresis in polyacrylamide gels (Fig. 1A). The major aminopeptidase was purified by

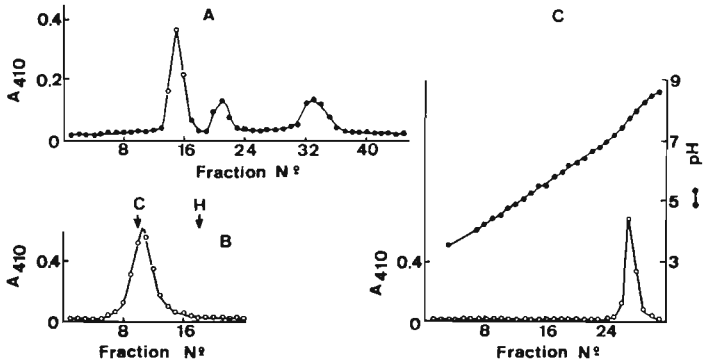

FIG. 1. Physical properties of the papain-released midgut aminopeptidases (A) Electrophoretic separation in a 6% polyacrylamide gel column. The most active fractions (represented by open (O) circles) corresponding to the major peak from several gel columns were pooled for later use and named hereafter "purified midgut aminopeptidase." (B) Sedimentation profile in a linear glycerol gradient of the major aminopeptidase purified as described above. M_r markers: C, bovine liver catalase (M_r 232,000); H, bovine hemoglobin (M_r 64,000). (C) Isoelectric focusing of the major aminopeptidase purified as described above. Profiles obtained from several other preparations are similar to those shown. Assays were accomplished with 1.0 mM LpNA as substrate.

electrophoresis as detailed in the legend to Fig. 1A. The migration rates of the purified aminopeptidase were determined by electrophoretic analyses performed in polyacrylamide gels of five different concentrations. The results showed the existence of only one molecular species of aminopeptidase in purified preparations from papain-treated membranes. The purified aminopeptidase has an apparent relative molecular mass of 207,000 ± 4000 (n = 3) as determined in density gradients (Fig. 1B), which agrees well with the value calculated from electrophoretic data (202,000). The enzyme is stable from pH 6.4 to pH 9.5 at 30°C for at least 4 h. It has a pH optimum of 7.2 in Tris/HCl buffer, and a pI of 7.8 (Fig. 1C).

Substrate Specificity of the Purified Midgut Aminopeptidase

Thin-layer chromatography of the products of the action of the purified aminopeptidase on peptides (those listed in Table I) demonstrated that the enzyme is a true aminopeptidase which hydrolyzes the N-terminal amino acid in tripeptides and tetrapeptides. The purified aminopeptidase shows a broad specificity in relation to the N-terminal aminoacyl residue (Table I).

Inhibitors of the Purified Midgut Aminopeptidase

L-Leucine hydroxamate (K_i 27 μM) is a strong, L-arginine hydroxamate (K_i 0.24 mM), an intermediate, and hydroxylamine (K_i 5.4 mM) and isoamyl alcohol (K_i 62 mM) are weak competitive inhibitors of the purified aminopeptidase. The results shown in Fig. 2 indicate that leucine hydroxamate and hydroxylamine bind at the same site, since the interaction factor (α) of the hydroxamate and hydroxylamine in the enzyme-inhibitor complex must be ∞ for the plot to result in a family of parallel straight lines and the Yagi-Ozawa plot (interrupted line in Fig. 2) to be linear (23). A similar conclusion can be drawn from multiple inhibition data using leucine hydroxamate and isoamyl alcohol as inhibitors. Otherwise, the interaction factor (α) between hydroxylamine and isoamyl alcohol is 2.2. (Fig. 3). This indicates that hydroxylamine and isoamyl alcohol bind at different although close sites in the aminopeptidase active center.

Effect of Phenanthroline and EDTA on the Purified Midgut Aminopeptidase

1,10-Phenanthroline (1 mM, final concentration in reaction media) causes com-

TABLE I

SUBSTRATE SPECIFICITY OF THE PURIFIED MIDGUT CAECAL AMINOPEPTIDASE

Substrate	K_m (mM)	V (nM·s^{-1})	$10^4 \times V/K_m$ (s^{-1})
LeuβNA	0.28 ± 0.03	1320 ± 80	47 ± 8
ArgβNA	0.23 ± 0.03	360 ± 30	16 ± 4
MetβNA	0.38 ± 0.02	580 ± 10	15 ± 1
Arg*p*NA	1.5 ± 0.2	430 ± 30	2.9 ± 0.5
LpNA	1.4 ± 0.1	1600 ± 90	11 ± 1
Phe-Gly	1.34 ± 0.07	74 ± 1	0.55 ± 0.04
Leu-Gly	1.7 ± 0.3	120 ± 10	0.7 ± 0.2
Leu-Gly-Gly	0.72 ± 0.04	770 ± 10	10.7 ± 0.7
Phe-Gly-Gly	1.20 ± 0.05	780 ± 10	7 ± 1
Phe-Gly-Gly-Phe	1.5 ± 0.2	710 ± 30	5 ± 1

Note. Purified aminopeptidase (see the legend to Fig. 1) was incubated with five different concentrations of each of the listed substrates, in 50 mM Tris/HCl buffer, pH 7.2, at 30°C. Kinetic parameters (means ± SE) were determined by a weighted linear regression by the procedure of Wilkinson (21) using a program written in Basic (22). Gly-Leu, Gly-Phe, AspβNA, and ProβNA are hydrolyzed slowly by the enzyme. Purified aminopeptidase displays only one peak of activity, with identical electrophoretical migration in 8% polyacrylamide gel columns, for all the substrates tested.

plete inhibition of the purified midgut aminopeptidase. This inhibition is completely reversed by dialysis (24 h) against Tris buffer. The enzyme has 59% of control activity in the presence of 0.34 mM phenanthroline and 100% in the presence of 0.15 mM phenanthroline. When the enzyme inhibited by 0.34 mM phenanthroline is diluted so that the final phenanthroline concentration is 0.15 mM, 100% activity ensues. Changes in enzyme concentration alone do not affect this inhibition. Furthermore, the degree of inhibition observed is not enhanced by preincubation of phenanthroline with the midgut aminopeptidase. Thus, the inhibition of the purified midgut aminopeptidase by phenanthroline is instantaneous and freely reversible. Fig. 4A shows that phenanthroline increases the V_{max} of the aminopeptidase by sevenfold, even in phenanthroline concentrations as low as 0.2 mM. Thus, the phenanthroline bound at

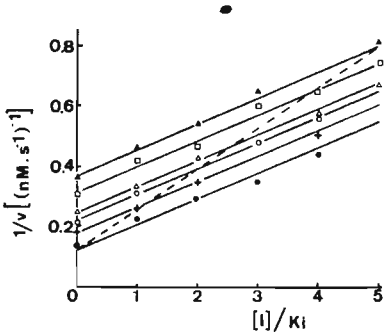

FIG. 2. Multiple inhibition of purified midgut aminopeptidase by hydroxylamine (I) and (●) 0, (+) 30, (○) 60, (△) 90, (□) 120, and (▲) 150 μM, L-leucine hydroxamate; Yonetani and Theorell (23) plot.

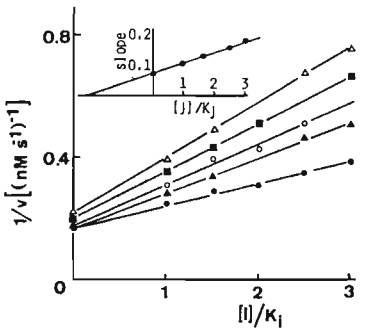

FIG. 3. Multiple inhibition of purified midgut aminopeptidase by hydroxylamine (I) and isoamyl alcohol (j); Yonetani and Theorell (23) plot. The inset shows a replot of the slopes from the previous plot against the relative concentration of isoamyl alcohol. α, the intercept in the $[j]/K_j$ axis, is the interaction factor between the inhibitors in the enzyme-inhibitor complex.

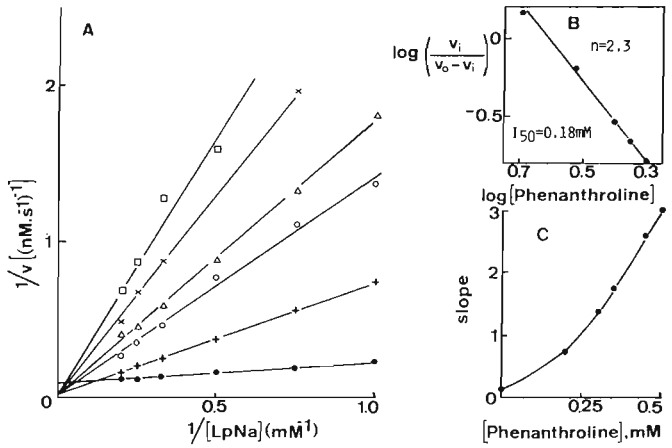

FIG. 4. Inhibition of purified midgut aminopeptidase by 1,10-phenanthroline. (A) Lineweaver-Burk plots for different concentrations (mM) for phenanthroline. (●, 0; +, 0.2; ○, 0.3; △, 0.35; ×, 0.45; □, 0.5). (B) Hill plot of phenanthroline inhibition data. Initial velocities were determined in the absence (v_0) or in the presence (v_i) of different concentrations of phenanthroline at constant LpNA concentration (4 mM). n, the slope of the plot, estimates the number of inhibitor binding sites in the enzyme (24). I_{50} is the concentration of phenanthroline which inhibits 50% of the activity of the enzyme. I_{50} changes from 0.15 mM, corresponding to 1 mM LpNA to 0.20 mM, corresponding to 5.0 mM LpNA. Hill plots for six different LpNA concentrations results in the same value for n(2.3). (C) Replot of slopes from Lineweaver-Burk plots against the concentration of phenanthroline. If slopes are plotted against phenanthroline concentration raised to the 2.3 power, the curve become rectilinear.

the activating site must dissociate from the enzyme with a K_D much lower than 0.2 mM. In addition to an activating effect, phenanthroline displays an inhibitory effect which seems to be of the competitive type. This follows from the observation that increasing concentrations of phenanthroline change the slopes but not the intercepts of Lineweaver-Burk plots, if we discount the activating effect of phenanthroline (Fig. 4A). A replot of slopes from the Lineweaver-Burk plots against concentration of phenanthroline is parabolic (Fig. 4C), suggesting that more than one molecule of inhibitor is binding at each active site (24). This is confirmed by the Hill plot of phenanthroline inhibition data (Fig. 4B), which indicates the existence of 2.3 binding sites for phenanthroline in each aminopeptidase active site.

Inhibition of the midgut aminopeptidase activity by 0.34 mM phenanthroline is reversed (there is even an activation) in the presence of Zn^{2+} ions (Fig. 5). Zn^{2+} ions alone inhibit the enzyme. Mg^{2+} does not affect the enzyme inhibited by phenanthroline, whereas Mn^{2+} enhances the observed inhibition (Fig. 5).

The purified aminopeptidase which was inactivated 75% by EDTA did not recover the initial activity after dialysis against 50 mM Tris/HCl buffer, pH 7.2, for 24 h. The apparent K_m value of the EDTA-inactivated enzyme, however, did not differ from the values of the native enzyme. It seems likely that the measurable activity of the

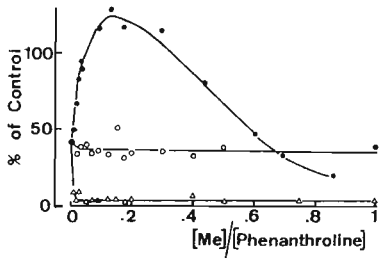

FIG. 5. Effect of metal ions in the reversal of phenanthroline inhibition. Phenanthroline concentration: 0.34 mM. [Me], metal ion molar concentration: ●, Zn^{2+}; ○, Mg^{2+}; △, Mn^{2+}. Control: activity in the absence of phenanthroline and metal ions.

partially inactivated enzyme represents residual unmodified enzyme rather than altered enzyme with distinct kinetic properties. Attempts to reactivate the enzyme by preincubation with 1 nM, 1 μM, 1 mM, and 5 mM $ZnCl_2$ or 1 and 10 mM $MgSO_4$ in 50 mM Tris/HCl buffer, pH 7.2, at 37°C for up to 4 h were unsuccessful.

The inactivation of the purified aminopeptidase by EDTA in 50 mM Tris/HCl buffer, pH 7.2, at 37°C follows pseudo first-order kinetics for at least three half-lives. The reaction order with respect to EDTA is 0.5 ($k = 4.77 \times 10^{-4}$ $M^{0.5}$ s^{-1}) (Fig. 6). Since EDTA has two metal binding sites, the data support the conclusion that the removal of only one metal ion is sufficient to inactivate the enzyme.

L-Leucine hydroxamate protects the aminopeptidase from inactivation by EDTA (Fig. 7). The concentration of the inhibitor for half-maximal protection (29 μM) is in good agreement with the K_i determined from inhibition of activity. Extrapolation to infinite inhibitor concentration indicates complete protection of the enzyme. Similar protection was found with hydroxylamine, which confers half-maximal protection of the enzyme at 6.6 mM.

Isoamyl alcohol (Fig. 8) and phenanthroline (Fig. 9) increase (in saturating concentration) the inactivation rate of the aminopeptidase by EDTA by a factor of about 2 and 87, respectively. Apparent half-maximum increases are observed with 60 mM isoamyl alcohol (which agrees with its K_i) and with 0.11 mM phenanthroline.

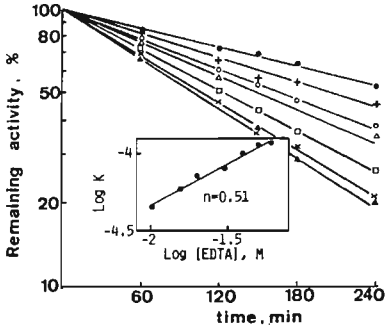

FIG. 6. Effect of the concentration of EDTA on the inactivation rate of the purified midgut aminopeptidase at 37°C. EDTA concentrations (mM): ●, 10; +, 15; ○, 20; △, 30; □, 40; ×, 50; ▲, 60. The inset shows a plot of log k (k is the observed first-order rate constant for enzyme inactivation) against log [EDTA]. n, the slope of the plot, estimates the number of molecules of EDTA inactivating each active site of the enzyme (25).

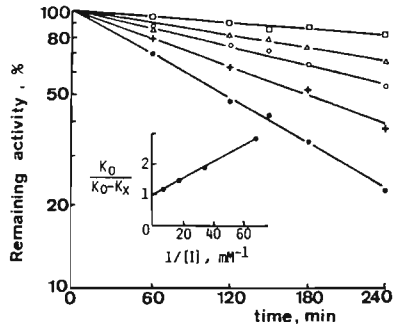

FIG. 7. Inactivation of the purified midgut aminopeptidase by EDTA (60 mM, 37°C) in the presence of 0 (●), 15 (+), 30 (○), 60 (△), 150 (□) μM L-leucine hydroxamate (I). The inset shows a plot of $k_0/(k_0 - k_x)$, where k_0 and k_x are the inactivation rate constants in the absence and presence, respectively, of L-leucine hydroxamate versus the reciprocal of L-leucine hydroxamate concentration.

DISCUSSION

Substrate Specificity and the Essential Role of a Metal Ion in Catalysis by the Midgut Membrane-Bound Aminopeptidase

An aminopeptidase activity was shown, by several criteria, to be bound to the plasma membrane covering the microvilli of *R. americana* midgut caecal cells (4, 5). This activity is released into solution by papain treatment as three proteins with different electrophoretic migration (Fig. 1). The major aminopeptidase was purified and shown to prefer, in contrast to the caecal luminal aminopeptidases from *R. americana* (26), tripeptides rather than tetrapeptides. This lends support to the proposal (1, 4, 5, 26) that intermediary digestion of protein takes place in the lumen of the midgut caeca while terminal digestion occurs at the surface of the cellular microvilli, both through the action of aminopeptidases. Carboxypeptidases are not very active in *R. americana* midgut (1).

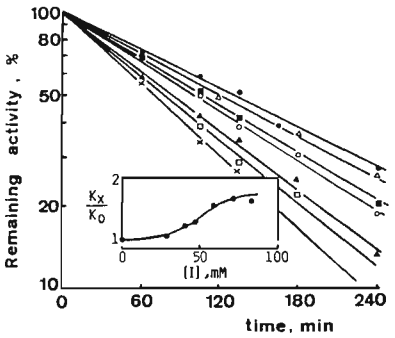

FIG. 8. Inactivation of the purified midgut aminopeptidase by EDTA (60 mM, 37°C) in the presence of 0 (●), 30 (△), 42 (■), 48 (○), 60 (▲), 72 (□), 84 (×) mM isoamyl alcohol. The inset shows a plot of k_x/k_0, where k_0 and k_x are the inactivation rate constants in the absence and presence, respectively, of isoamyl alcohol versus the isoamyl alcohol concentration. The curve showing data plotted as in the inset of Fig. 7 is not rectilinear.

A metal ion is involved in catalysis considering the following: (a) the action of EDTA upon the enzyme results in a completely inactive enzyme; (b) the order of reaction in relation to EDTA is 0.5, which indicates that the removal of only one metal ion is sufficient to inactivate the enzyme; (c) the presence of a bulky competitive inhibitor in the active site of the enzyme confers total protection against inactivation by EDTA; (d) Zn^{2+} not only restores full activity to a phenanthroline-inhibited midgut aminopeptidase, but it also raises the activity of the enzyme to above the control level.

Binding Loci for Inhibitors in the Midgut Membrane-Bound Aminopeptidase

Hydroxamates are linear competitive inhibitors of the midgut aminopeptidase and their strength of binding seems to depend to a great extent on the hydrophobicity of the side chain. Because hydroxamates are supposed to bind in a bidentate manner to metal ions in the active site of several proteases (27), it is conceivable that the active site of the midgut aminopeptidase contains a hydrophobic subsite and a polar subsite, the latter one close to the metal ion. Hydroxylamine probably binds at the polar subsite, since it is excluded from the active site by leucine hydroxamate and it completely protects the enzyme from inactivation by EDTA. Isoamyl alcohol probably binds at the hydrophobic subsite, since it is excluded from the active site by leucine hydroxamate, but it does not protect the enzyme from inactivation by EDTA. Indeed, isoamyl alcohol increases the inactivation rate of the enzyme by EDTA. The increase in the reactivity of the metal ion caused by isoamyl alcohol is probably a consequence of a conformational change in the enzyme which leaves the metal ion more exposed. The polar and hydrophobic subsites are probably close, since multiple inhibition analysis showed that some interference exists with simultaneous binding of hydroxylamine and isoamyl alcohol. In a similar study, Baker *et al.* (28) were able to show that subsites are about a methylene group diameter apart in the active center of a microbial aminopeptidase.

The inhibition of the midgut aminopeptidase by phenanthroline is instantaneous and reversible upon dilution. Preincubation does not enhance the degree of inhibition, and the effect of phenanthroline depends only upon its concentration in the reaction mixture. In this respect the midgut aminopeptidase is similar to horse liver alcohol

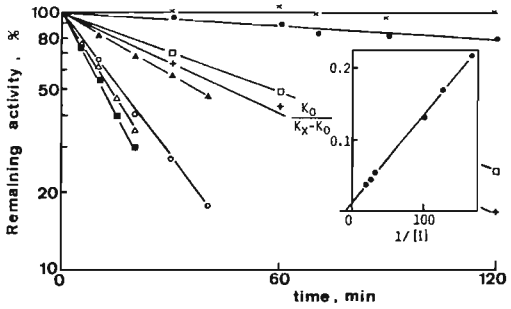

FIG. 9. Inactivation of the purified midgut aminopeptidase by EDTA (10 mM, 37°C) in the presence of 0 (●), 24 (□), 37 (+), 40 (▲), 80 (○), 120 (△), and 200 (■) μM phenanthroline. The inset shows a plot of $k_0/(k_x - k_0)$, where k_0 and k_x are the inactivation constants in the absence and presence, respectively, of phenanthroline versus the reciprocal of phenanthroline concentration. The enzyme in the presence of phenanthroline (200 μM) alone (×) are completely stable for at least 120 min.

dehydrogenase (29) and to leucine aminopeptidase (30). Nevertheless, it differs from both enzymes in the nature of the effect caused by phenanthroline. Whereas phenanthroline is a linear competitive inhibitor of the alcohol dehydrogenase and leucine aminopeptidase, phenanthroline affects the midgut aminopeptidase in a complex way.

Chelating agents are usually considered to inhibit metalloenzymes by removing metal ions from the active site or by forming an inactive ternary complex with the metal and the enzyme (31). The action of EDTA on the midgut aminopeptidase is probably of the first type, whereas the action of phenanthroline is probably of a third type. It is possible that phenanthroline molecules (2.3 per active site) bind in the neighborhood of (or at) the active site preventing the binding of substrate molecules. Otherwise, the observed increase in V_{max} caused by phenanthroline should depend on the binding of the phenanthroline in the neighborhood of the hydrophobic subsite leading to changes in the degree of exposure of the active site metal ion, in a similar way as proposed for isoamyl alcohol. This proposal is further supported by the finding that phenanthroline binding increases the (irreversible) inactivation rate of the enzyme by EDTA, whereas it alone inhibits but does not irreversibly inactivate the enzyme at a measurable rate.

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THE DETERGENT FORM OF THE MAJOR AMINOPEPTIDASE FROM THE PLASMA MEMBRANE OF MIDGUT CAECA CELLS OF *RHYNCHOSCIARA AMERICANA* (DIPTERA) LARVA

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Abstract—1. Plasma membrane-bound aminopeptidases from *Rhynchosciara americana* midgut caeca cells may be solubilized by Triton X-100 or may be released by papain treatment.

2. The major detergent solubilized aminopeptidase was purified by electrophoresis and was shown to have M_r 169,000 and pI 7.8.

3. K_m values for two substrates (L-arginine-*p*-nitroanilide and L-leucine-*p*-nitroanilide) and K_i values for four linear inhibitors (arginine hydroxamate, leucine hydroxamate, hydroxyl amine and isoamyl alcohol) corresponding to the major detergent-solubilized aminopeptidase are identical to those of the major papain released aminopeptidase, which was previously purified and characterized.

4. Both aminopeptidase forms display the same pH optimum (7.2 in Tris–HCl buffer) and follow the same thermal inactivation kinetics (half-lives of 23 min at 50°C).

5. Treatment of the major detergent form of the aminopeptidase with papain results in the appearance of a M_r 97,000 aminopeptidase.

6. The data support the proposal that the papain-form of the aminopeptidase (M_r 207,000) results from the dimerization of the products of the papain action upon the major native (M_r 169,000) aminopeptidase.

INTRODUCTION

Rhynchosciara americana midgut caecal cells display in their plasma membranes, as judged by electrophoresis, a major (T_1) and three minor (T_2, T_3, T_4) aminopeptidases which are solubilized by Triton X-100, and a major (P_1) and two minor (P_2 and P_3) aminopeptidases which are released by papain treatment (Ferreira and Terra, 1985). The data suggested that T_2 and T_4 as well as P_2 and P_3 are respectively different aggregation states of a minor native membrane bound aminopeptidase solubilized by detergent and released by papain. This minor aminopeptidase seems to be a tetramer, which may dissociate in different conditions, and is more active on tetrapeptides than on tripeptides (Ferreira and Terra, 1985).

The major papain released aminopeptidase (P_1) was purified and shown to prefer, in contrast to the minor aminopeptidase, tripeptides rather than tetrapeptides (Ferreira and Terra, 1986). Since there is evidence that T_1 and T_3 differ only in net charge (Ferreira and Terra, 1985), it is possible that T_1 corresponds to P_1.

In this paper we describe the partial purification and characterization of the major detergent solubilized aminopeptidase (T_1) and show that it corresponds to the major papain released aminopeptidase (P_1) from *R. americana* midgut cell membranes. This lends support to the proposal (Ferreira and Terra, 1985) that the minor membrane-bound aminopeptidase, which has substrate specificities similar to those of the luminal aminopeptidase, finishes the intermediary digestion of oligopeptides (started by the luminal aminopeptidase), thus forming substrates for the major membrane-bound aminopeptidase.

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MATERIALS AND METHODS

Materials

Acrylamide, L-arginine hydroxamate, L-arginine-*p*-nitroanilide (ArgpNA), bisacrylamide, L-leucine hydroxamate, L-leucine-*p*-nitroanilide (LpNA), peptides and M_r standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ampholytes were from Serva Fine Chemicals (Heidelberg, Germany). All the other reagents were of analytical grade from E. Merck (Darmstadt, Germany) and J. T. Baker (Phillipsburg, NJ, USA). The solutions were prepared in glass–double-distilled water.

Animals

Rhynchosciara americana (Diptera: Sciaridade) were reared as described by Lara *et al.* (1965). We used only mature feeding female larvae at the end of the second period of the fourth instar (Terra *et al.*, 1973).

Solubilization of membrane aminopeptidases in Triton X-100

The preparation of midgut caecal plasma membranes, the solubilization of membrane aminopeptidases in Triton X-100 (10 mg Triton X-100/mg of protein) were performed as previously described (Ferreira and Terra, 1983).

Hydrolase assays and protein determination

Hydrolase assays were conducted, unless otherwise specified, in 50 mM Tris–HCl buffer pH 7.2 at 30°C. Nitroaniline liberated from aminoacyl-*p*-nitroanilides were determined by the method of Erlanger *et al.* (1961). In each determination, incubations were continued for at least four different periods of time and the initial rates were calculated.

All assays were performed so that the measured activity was proportional to protein and to time. Protein was determined as described previously (Terra *et al.*, 1979).

Inhibition studies

The enzymes were incubated in 50 mM Tris–HCl buffer pH 7.2 at 30°C with four (or five) different concentrations (in the range of 0.1 to 2 K_i) of the tested inhibitor in each of five different concentrations of LpNA (in the range of 0.2 to 1 mM). The K_i values were determined from replots of slopes and intercepts of Lineweaver–Burk plots against inhibitor concentration (cf. Segel, 1975).

Polyacrylamide gel electrophoresis

Samples were applied to gels of different polyacrylamide gel concentrations prepared as described by Hedrick and Smith (1968) in glass tubes of 5 mm int. dia and 100 mm length. The electrophoretic separation, the fractionation of gels in a gel fractionator and the collection of gel fractions with a fraction collector were performed as described by Terra and Ferreira (1983). The gels and the fractionation buffer contained 0.1% Triton X-100 and a pigment prepared from *R. americana* midguts was replaced, as a substitute for bromophenol blue as a tracking dye. The pigment, although chemically unknown, was proved to be a good tracking dye in conditions where bromophenol blue associates with detergent micelles, thus resulting in false determinations of migration rates (R_m) (unpublished results). The M_r values of the enzymes assayed in the fractions were calculated by the method of Hedrick and Smith (1968), using the migration rates (in the different gels) of myoglobin (M_r 17,800), ovalbumin (M_r 43,000), catalase (M_r 232,000) and ferritin (M_r 450,000) as reference standards. The recoveries of the activities applied to the gels were approximately 80%.

Isoelectric focusing in polyacrylamide gels

Isoelectric focusing was performed as described by Terra *et al.* (1978), in columns of 7.5% polyacrylamide gel containing 1% ampholytes pH 2–11 and 0.17% Triton X-100, after pre-focusing for 30 min at 31 V/cm. The recoveries of the activities applied to the gels were approximately 25%.

Density-gradient centrifugation

Samples (0.2 ml) of membrane proteins solubilized with Triton X-100, containing 1.5 mg of bovine liver catalase, were layered on the top of 4.6 ml linear glycerol gradients (5–30%, w/v) made up in 50 mM sodium phosphate buffer, pH 6.2. The centrifugations and the collection of fractions were performed as described previously (Terra and Ferreira, 1983). The M_r values of the enzymes assayed in the fractions were calculated by the method of Martin and Ames (1961), with as reference standards the sedimentation rates of bovine haemoglobin (M_r 64,500) and bovine liver catalase (M_r 232,000). The recoveries of the activities applied to the gradients were approximately 100%.

RESULTS

About 80% of the activity originally present in the midgut caecal membranes of *R. americana* is solubilized in Triton X-100. The solubilized and the non-solubilized activity add up to the activity found originally in the preparation of membranes.

There are four aminopeptidases (T_1, T_2, T_3 and T_4) solubilized by Triton X-100 which are resolved by polyacrylamide gel electrophoresis (Fig. 1A). The major detergent-solubilized form of the aminopeptidase has M_r 169,000 ± 1600 (Fig. 1B) and pI 7.8 (Fig. 1C), whereas the major papain-released form has M_r 207,000 ± 4000 (in the absence of detergent) or 217,000 ± 18,000 (in the presence of detergent) and pI 7.8 (Ferreira and Terra, 1986).

The migration rates of the major detergent and papain forms of the midgut aminopeptidase were determined in electrophoretic runs accomplished in polyacrylamide gels of 5 different concentrations

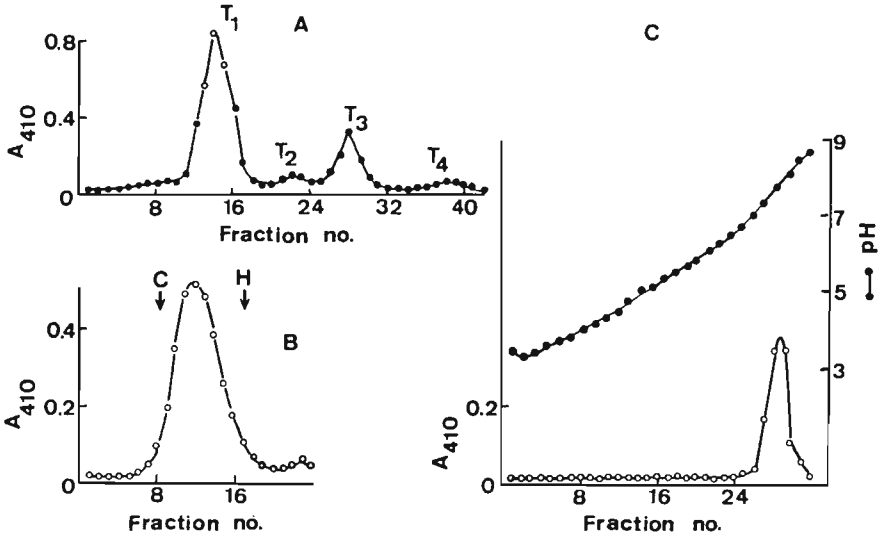

Fig. 1. Physical properties of the Triton X-100-solubilized midgut aminopeptidase. (A) Electrophoretic separation in 5% polyacrylamide gel column. The most active fractions (represented by open circles) corresponding to the major peak from several gel columns were pooled for later use and named hereafter "purified detergent form of the major midgut aminopeptidase". (B) Sedimentation profile of the major aminopeptidase purified as described above. M_r markers: C, bovine liver catalase (M_r 232,000); H, bovine haemoglobin (M_r 64,500). (C) Isoelectric focusing of the major aminopeptidase purified as described above. Profiles obtained from several other preparations are similar to those shown. Assays were accomplished with 1.0 mM LpNA as substrate. Details are given in Materials and Methods.

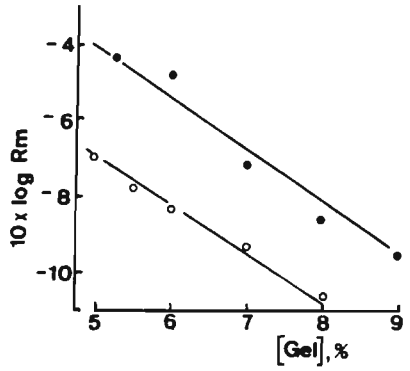

Fig. 2. Effect of different polyacrylamide gel concentrations on the electrophoretic migration of the major aminopeptidases from *R. americana* midguts. R_m, electrophoretic migration of enzyme in relation to the tracking dye. Each data point represent a single determination, D, (○) purified detergent form of the major midgut aminopeptidase (details in Fig. 1A); P, (●) purified papain form of the major midgut aminopeptidase (details in Ferreira and Terra, 1986).

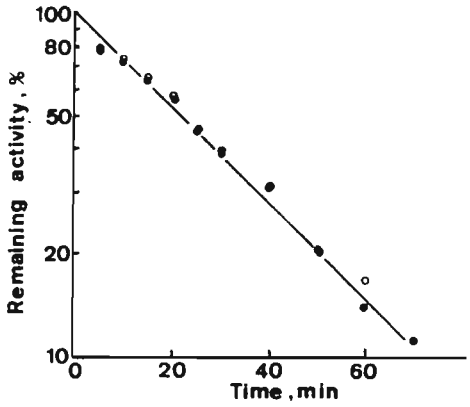

Fig. 3. Thermal inactivation, at 50°C, of the major aminopeptidases from *R. americana* midguts. Each data point represents a single determination. D, (○); P (●). Other details as in legend to Fig. 2.

(Fig. 2). The results confirmed the existence of only one molecular species of aminopeptidase in purified preparations from both papain-treated and detergent-treated membranes. The M_r values calculated from electrophoretic data were: detergent-form, 170,000; papain-form, 202,000.

Treatment of the purified detergent form of the aminopeptidase with papain (1 mg papain/10 mg protein) results in the appearance of a M_r 97,000 ± 1900 aminopeptidase. Otherwise, pre-incubation of the purified papain form of the aminopeptidase with 0.1% Triton X-100 for 30 min at 30°C does not change its M_r value.

The purified detergent and papain forms of the aminopeptidases are stable from pH 6.4 to pH 9.5 at 30°C for at least 4 hr and their pH optimum is 7.2 in Tris–HCl buffer. The detergent form of the aminopeptidase displays K_m values for substrates and K_i values for inhibitors similar to those of the papain form of the aminopeptidase (Table 1). Thermal inactivation, at 50°C, of both the purified detergent and papain forms of the aminopeptidase follows apparent first-order kinetics with similar half-lives (23 min and 22 min, respectively), for a period of at least 3 half-lives (Fig. 3).

Table 1. K_m and K_i values for several ligands to the major midgut aminopeptidase purified by electrophoresis*

	K_i or K_m (mM)	
	Detergent form	Papain form†
Substrates		
Arg pNa	1.4 ± 0.1	1.5
LpNA	1.3 ± 0.1	1.4
Inhibitors		
L-Arginine hydroxamate	0.25 ± 0.01	0.24
L-Leucine hydroxamate	0.027 ± 0.007	0.027
Hydroxylamine	4.7 ± 0.5	5.4
Isoamyl alcohol	60 ± 10	62

*The detergent form was purified as described in Fig. 1, and the papain described in Ferreira and Terra (1986). K_m values (means ± SEM, n = 10) were determined by a weighted linear regression by the procedure of Wilkinson (1961) using a program written in Basic (Oestreicher and Pinto, 1983). The K_i values of the inhibitors (means ± range, two determinations), which are all simple intersecting linear competitive inhibitors (see Segel, 1985), were determined as described in Materials and Methods.

†Taken from Ferreira and Terra (1986).

DISCUSSION

An aminopeptidase activity was shown, by several criteria, to be bound to the plasma membrane covering the microvilli of *R. americana* midgut caecal cells (Ferreira and Terra, 1980, 1982). The major detergent-solubilized form of the membrane-bound *R. americana* aminopeptidase (M_r 169,000) is the only aminopeptidase which occurs in sufficient amount to correspond to the major papain-released form of the aminopeptidase (M_r 207,000). Furthermore, catalytic parameters, K_i values and pH and thermal stabilities are identical whichever enzyme form is studied. Treatment of the purified detergent form of the aminopeptidase with papain results in the appearance of a M_r 97,000 aminopeptidase. Thus, it is possible that the major papain-form of the aminopeptidase results from the dimerization of the products of the papain action upon the M_r 169,000 aminopeptidase.

The major papain-form of the aminopeptidase is a true aminopeptidase with a broad specificity toward the N-terminal aminoacyl residues, and displays a weak activity upon dipeptides (Ferreira and Terra, 1986). This enzyme, in contrast to the caecal luminal aminopeptidase from *R. americana* (Ferreira and Terra, 1984), prefers tripeptides rather than tetrapeptides. This lends support to the proposal (Terra *et al.*, 1979; Ferreira and Terra, 1980, 1982, 1984, 1985) that intermediary digestion of proteins in *R. americana* larvae takes place in the lumen of the midgut caeca and terminal digestion in the surface of the cellular microvilli, both through the action of aminopeptidases. Carboxypeptidases are not very active in *R. americana* midgut (Terra *et al.*, 1979).

Midgut membrane-bound aminopeptidases have been found in insects other than *R. americana*, such as *Drosophila melanogaster* (Walker *et al.*, 1980), *Bombyx mori* (Sumida and Eguchi, 1983), *Erinnyis ello* (Santos and Terra, 1984) and *Tipula abdominalis* (Sharma *et al.*, 1984). Nevertheless, the characterization of those enzymes has not yet progressed

far enough to permit a detailed comparison with the *R. americana* midgut membrane-bound aminopeptidases.

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DIGESTIVE ENZYMES TRAPPED BETWEEN AND ASSOCIATED WITH THE DOUBLE PLASMA MEMBRANES OF *RHODNIUS PROLIXUS* POSTERIOR MIDGUT CELLS

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Abstract—Subcellular fractions of the cells of the posterior midgut of *Rhodnius prolixus* nymphs were obtained by conventional homogenization, under isotonic or hypotonic conditions, followed by differential centrifugation. Alkaline phosphatase and membrane-bound α-mannosidase are more abundant in fractions in which vesicles displaying brush-borders predominate. α-Glucosidase is associated with large membranous structures, although its subcellular distribution is different from that of alkaline phosphatase and α-mannosidase. α-Mannosidase-carrying membranes were resolved from α-glucosidase-carrying membranes in sucrose gradients, supporting the hypothesis that these enzymes are part of respectively, protein-rich inner and protein-poor outer microvillar membranes. To account for the soluble enzyme activities that sediment with vesicles displaying brush borders, major amounts of aminopeptidases are assumed to be trapped in the space between outer and inner microvillar membranes, from where they are set free by homogenization and (or) freezing and thawing. There are at least three different aminopeptidases, based on their activities toward several substrates and on sedimentation data. The results favor the view that oligomers derived from partial digestion of polymeric food are hydrolyzed down to monomers by enzymes trapped between microvillar membranes or on the surface of midgut cells. The use of microvillar membranes as a peritrophic membrane by *R. prolixus* is thought to be a derived character evolved from a putative phloem feeder Hemiptera ancestor.

Key Word Index: *Rhodnius prolixus*, outer microvillar membranes, inner microvillar membranes, double plasma membranes, resolution of microvillar membranes, microvillar enzymes

INTRODUCTION

The surface of midgut cells of all Hemiptera, except the xylem-feeders, is covered by a system of membranes. These, in the more detailed papers (Reger, 1971; Lane and Harrison, 1979; Andries and Torpier, 1982; Baerwald and Delcarpio, 1983), are described as being membranes which ensheath the midgut microvilli, forming an outer microvillar membrane which maintains a constant distance from the inner (or true) microvillar membrane, and which may extend far into the midgut lumen. Freeze-fracture replicas showed that the outer membranes, except for those directly ensheathing the microvilli, are almost free from intramembranous particles, thus resembling myelin sheets (Lane and Harrison, 1979; Andries and Torpier, 1982). Apparently the membranes ensheathing the microvilli are set in position by columns obliquely disposed between the outer and inner microvillar membranes (Lane and Harrison, 1979).

The function of the outer microvillar membranes have been described as a glycocalyx (Marshall and Cheung, 1970) or a kind of peritrophic membrane (Burgos and Gutierrez, 1976; Lane and Harrison, 1979; Baerwald and Delcarpio, 1983; Billingsley and Downe, 1983). The outer microvillar membrane is completely different from the so-called cell glycocalyx, which corresponds to the carbohydrate moieties of the integral proteins. Thus, its function should be different. Although the outer microvillar membrane may function as a kind of peritrophic membrane in Hemiptera displaying midgut luminal digestion, such as blood- and seed-suckers, biochemical support for this hypothesis is lacking. This hypothesis, up to the present, relies only on ultrastructural grounds (Burgos and Gutierrez, 1976; Lane and Harrison, 1979; Baerwald and Delcarpio, 1983) and on suggestive cytochemical data (Billingsley and Downe, 1985). Furthermore, there is no hypothesis to explain why those insects use microvillar outer membranes as a peritrophic membrane instead of a true (chitinous) peritrophic membrane.

Recently, *Rhodnius prolixus* nymphs have been subjected to a detailed study on the properties and

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midgut distribution of their digestive enzymes (Terra *et al.*, 1988). The results showed that aminopeptidase is a soluble enzyme enveloped by cell membranes and that there is a major membrane-bound α-glucosidase and a major membrane-bound α-mannosidase, in addition to minor soluble counterparts.

In the present paper, data are presented which suggest that α-mannosidase and α-glucosidase are integral proteins of respectively microvillar and outer microvillar membranes, and that aminopeptidase is trapped between these membranes. Furthermore, we describe the resolution of the outer from the inner microvillar membrane, taking advantage of the fact that they have different amounts of intramembranous particles (and hence buoyant densities) and discuss the origin and role of these membranes in Hemiptera.

MATERIALS AND METHODS

Animals

R. prolixus (Hemiptera: Reduviidae) were reared in the laboratory at 50–60% r.h. at 28 ± 1°C. The insects were fed on citrated sheep blood every 25–30 days through a special feeding apparatus (Garcia *et al.*, 1975). The animals used in this study were fifth-instar nymphs 7 days after being fed with blood.

Preparation of samples

Nymphs were dissected in cold saline (206 mM NaCl) and posterior midgut free from contents and contaminating tissues were isolated. Posterior midgut luminal fluids were recovered as the fluid leaking out from the dissected organ every time it was flushed with saline. Differential centrifugation of midgut homogenates were accomplished as follows. Posterior midgut tissue was homogenized using a Omni-mixer (Sorvall) at 5000 rpm for 2 × 15 s in an isotonic (206 mM KCl, pH 7.0) or in a hypotonic (2 mM Tris/HCl buffer, pH 7.0, containing 50 mM mannitol) medium. The homogenates were filtered through a piece of nylon mesh of 45 μm pore size, then were adjusted to contain material from 10 animals/ml and centrifuged at 4°C. The following fractions were collected: P_1, pellet resulting from centrifuging at 600 *g* for 10 min; P_2, pellet from 3300 *g* for 10 min; and P_3, pellet from 25,000 *g* for 10 min. No sediment was visible after centrifugation of this supernatant at 100,000 *g* for 60 min. This supernatant was considered the soluble fraction of the cells. After differential centrifugation the fractions were collected and homogenized, and following three freezing–thawing cycles they were centrifuged at 100,000 *g* for 60 min. The supernatants and pellets corresponding to each fraction were assayed for several enzymes. The recovery of each hydrolase activity in subcellular fractions were between 75 and 105% of the homogenate activity.

Membrane fractions for sucrose gradient-ultracentrifugation were prepared as follows. Total membranes were obtained by centrifuging midgut homogenates (prepared in distilled water) at 25,000 *g* for 30 min. The pellets after being homogenized in water were submitted to three freezing–thawing cycles and to another centrifugation at 25,000 *g* for 30 min. The resulting pellets were resuspended in water and stored. The membranes present in the posterior midgut luminal fluid were prepared by centrifuging fluid (collected as described above) diluted 5-fold with water at 25,000 *g* for 30 min. The pellets were resuspended in water, and after three freezing–thawing cycles they were again centrifuged at 25,000 *g* for 30 min. The pellets were resuspended in water and stored.

Prior to sucrose gradient-ultracentrifugation, the membrane preparations were centrifuged at 600 *g* for 2 min, and the pellets discarded. This is necessary to decrease the amount of pellet which is formed during ultracentrifugation. The samples (0.2 ml) were applied on the top of 4.6 ml linear gradients of sucrose (4.5–45%, w/v) prepared in 50 mM sodium acetate buffer pH 5.5 containing 1 mg/ml bovine serum albumin. The presence of albumin in the gradients is necessary to avoid enzyme inactivation. The centrifugations of the gradients were carried out at 96,000 *g* for 15 h at 4°C. Fractions of 0.2 ml were collected starting close to the bottom of the tubes (about 0.1 ml of the gradients were left behind) in order to avoid collecting the pellet formed during the ultracentrifugation. The densities of the gradient fractions were determined from their refractive indexes measured with the aid of a refractometer.

Glycerol-gradient centrifugations

Samples (0.2 ml) of preparations, containing 1.5 mg of bovine hemoglobin and 50 μg of bovine liver catalase were layered on the top of 4.6 ml linear glycerol gradients (5–30%, w/v) made up in 50 mM sodium phosphate buffer, pH 6.2. Centrifugations and collection of fractions were performed as described previously (Terra and Ferreira, 1983). M_r values of enzymes assayed in the fractions were calculated by the method of Martin and Ames (1961), using sedimentation rates of bovine hemoglobin (M_r 64,500) and bovine liver catalase (M_r 232,000) as reference standards. The recoveries of the activities applied to the gradients were 60–80%.

Electron microscopy

Tissue or subcellular fractions were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 h at 4°C. After being rinsed with 0.2 M sucrose in the same cacodylate buffer, the fractions were postfixed in 1% osmium tetroxide in cacodylate buffer for 1 h at 4°C and washed in 0.1 M NaCl. En-bloc staining was performed in aqueous 1% uranyl acetate for 16–18 h. After dehydration in graded ethanol at room temperature, the material was embedded in Polylite 8001 polyester resin (Resana S. A., Brazil; Coiro *et al.*, 1972). Ultrathin sections were cut using a Porter–Blum MT II ultramicrotome, stained with lead citrate (Reynolds, 1963), and examined in a Siemens Elmiskop IA electron microscope operated at 60 kV.

Hydrolase assays

Protein was determined according to Bradford (1976) using ovalbumin as a standard. Succinate dehydrogenase and lactate dehydrogenase were assayed according to Ackrell *et al.* (1978) and Bergmeyer and Bernt (1974), respectively, as detailed elsewhere (Santos and Terra, 1984).

Other enzymes assayed and substrates used were (enzyme, substrate): acid phosphatase, *p*-nitrophenyl phosphate; alkaline phosphatase, *p*-nitrophenyl phosphate; aminopeptidase, L-leucine-*p*-nitroanilide (LpNA); α-glucosidase, *p*-nitrophenyl α-D-glucoside; α-mannosidase, *p*-nitrophenyl α-D-mannoside. The assays were described previously (Terra *et al.*, 1988). Aminopeptidase was also assayed with L-leucine β-naphthylamide (Leu β NA) and Leuglygly as substrates according to Hopsu *et al.* (1966) and Nicholson and Kim (1975), respectively.

In each determination of all the enzyme activities, incubations were carried out at 30°C for at least four different periods of time and the initial rates were calculated. A unit of enzyme is defined as the amount required to catalyze the cleavage of μmol of substrate/min.

RESULTS

Differential centrifugation of midgut homogenates

The subcellular distribution of enzymes in *R. prolixus* posterior midgut cells is displayed in Figs 1 and 2 and the electron micrographs of the fractions,

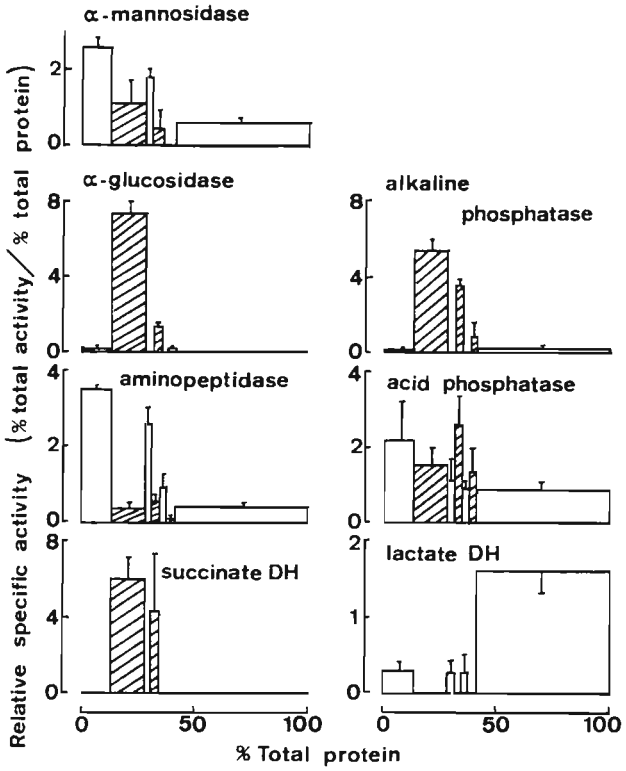

Fig. 1. Distribution of hydrolases among the subcellular fractions of *R. prolixus* posterior midgut in isotonic conditions. Homogenizing medium: 206 mM KCl, pH 7.0. The following fractions were collected: P_1, pellet resulting from centrifuging at 600 *g* for 10 min; P_2, pellet from 3300 *g* for 10 min; P_3, pellet from 25,000 *g* for 10 min; S, final supernatant. After differential centrifugation, all fractions were homogenized, and after several freezing–thawing cycles they were centrifuged at 100,000 *g* for 1 h. The supernatants and pellets corresponding to each fraction were assayed. Clear areas correspond to supernatants and shaded ones to pellets. The data are means and SEM based on determinations carried out in three independent preparations obtained from 150 nymphs each. Fractions (supernatants and pellets) are displayed from left to right in the order they were obtained (P_1, P_2, P_3, and S, respectively). The length of horizontal bars (with or without histogram bars) is proportional to the percentage of total protein. The absence of a histogram means that no activity was found in the corresponding sample.

including of particulate material in luminal contents, are shown in Fig. 3.

Alkaline phosphatase is found mainly in fraction P_1, when tissue is homogenized in isotonic conditions (Fig. 1), and is more evenly distributed in the several fractions when tissue is homogenized in hypotonic conditions (Fig. 2). In both conditions, the activity found in the final supernatant is very small. This suggests that *R. prolixus* posterior midgut alkaline phosphatase is plasma membrane bound, as described for Diptera (Ferreira and Terra, 1980) and Lepidoptera (see Santos *et al.*, 1986 for references). The fact that fraction P_1 (isotonic) displays numerous microvillar structures (Fig. 3B), whereas these structures are rarely visible in fraction P_1 (hypotonic) (Fig. 3C), supports this hypothesis. The intracellular distribution of membrane bound-α-mannosidase (shaded areas in Figs 1 and 2) is similar to that of alkaline phosphatase.

α-Glucosidase is membrane-bound and occurs mainly in fraction P_1, regardless of the homogenizing condition used (Figs 1 and 2). This suggests that the enzyme is associated with large membranous structures which are distinct from the plasma membranes, where alkaline phosphatase is bound. Inspection of Fig. 3A leads to the suggestion that α-glucosidase may be associated to the outer microvillar membranes, in contrast to alkaline phosphatase which may be associated to the inner (or true) microvillar membrane.

Aminopeptidase is a soluble enzyme which is more enriched in fraction P_1 (isotonic). The low aminopeptidase activities found associated to membranes (shaded areas in Fig. 1) may be due to unspecific adsorption of the soluble enzymes to membranes. Nevertheless, it is not possible to discard the occurrence of a minor membrane-bound aminopeptidase. The intracellular distribution of soluble α-mannosidase and soluble acid phosphatase is similar to that of aminopeptidase (Figs 1 and 2).

Sucrose-gradient sedimentation of midgut homogenates and midgut fractions

The membranes recovered from total posterior midguts (Fig. 4), as well as from luminal contents (Fig. 5a), which are rich in membranes (Fig. 3E), and fraction P_1 (hypotonic) (Fig. 5b) and fraction P_3 (hypotonic) (Fig. 5c), are resolved into low density

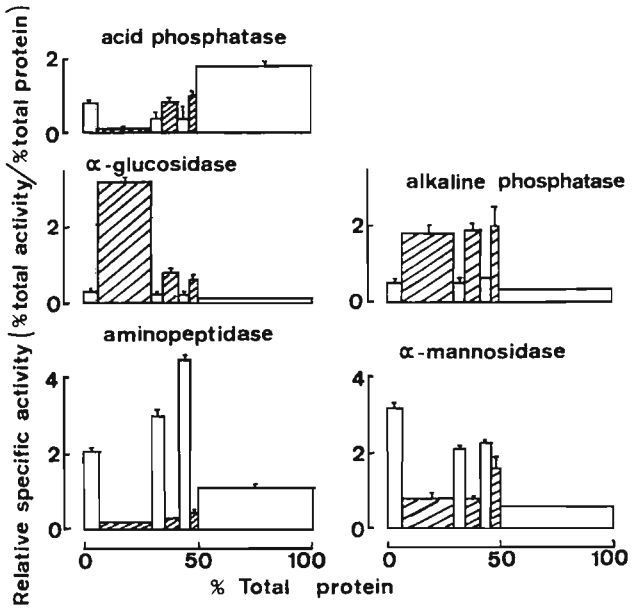

Fig. 2. Distribution of hydrolases among the subcellular fractions of *R. prolixus* posterior midgut in hypotonic conditions. Homogenizing medium: 2 mM Tris/HCl buffer, pH 7.0, containing 50 mM mannitol. The data are means and SEM based on determinations carried out three independent preparations obtained from 60 nymphs each. Other details as in the legend to Fig. 1.

α-glucosidase-carrying membranes and high density α-mannosidase-carrying membranes, with apparent buoyant densities of 1.068 ± 0.003 g/cm^3, and 1.086 ± 0.002 g/cm^3, respectively.

Properties of aminopeptidases isolated from different preparations

There is a major low (M_r 61,000) molecular weight and a minor high (M_r 240,000) molecular weight aminopeptidase hydrolyzing LpNA in cell fractions (S and T in Fig. 6). The low molecular weight aminopetidase hydrolyzes Leu β NA rapidly and Leuglygly poorly, whereas the reverse is true for the high molecular weight aminopeptidase. Luminal contents display only a low molecular weight (M_r 61,000) aminopeptidase which is, nevertheless, different from the cellular enzyme, since it hydrolyzes both Leu β NA and Leuglygly well.

DISCUSSION

Subcellular distribution of hydrolases and terminal digestion in R. prolixus posterior midguts

Alkaline phosphatase is probably associated to the inner (true) microvillar membranes of posterior midgut cells of *R. prolixus* based on the following evidence. (1) It is membrane-bound, and (2) it occurs in major amounts in the fractions in which vesicles displaying brush-borders predominate (fraction P_1, isotonic, Fig. 3B). Since membrane-bound-α-mannosidase follows alkaline phosphatase in cell fractions obtained by differential centrifugation in isotonic and hypotonic conditions (Figs 1 and 2), it is possible that α-mannosidase is also bound to the true microvillar membranes.

α-Glucosidase seem to be associated with large membranous structures, taking into account that it is membrane-bound and it is recovered in major amounts in fraction P_1, whichever homogenizing condition had been used (Figs 1 and 2). Since its distribution among cell fractions is distinct from that of alkaline phosphatase (and membrane-bound-α-mannosidase) (see Figs 1 and 2), it is possible that

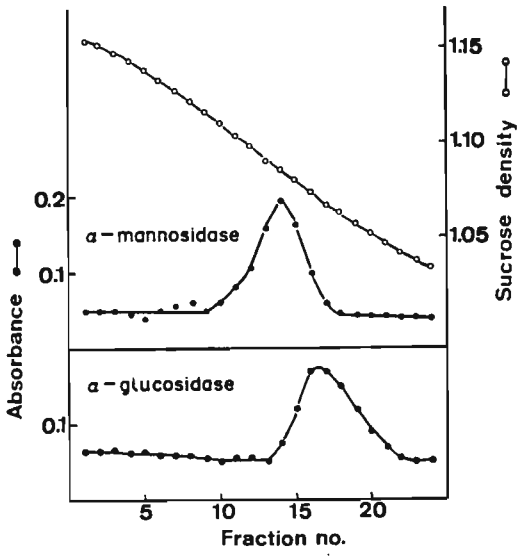

Fig. 4. Typical resolution of posterior midgut membranes in sucrose gradients. Gradient fractions were assayed for α-glucosidase and α-mannosidase and their densities were determined with the aid of a refractometer. The sedimentation profiles obtained from other preparations were similar to these. Details in Materials and Methods.

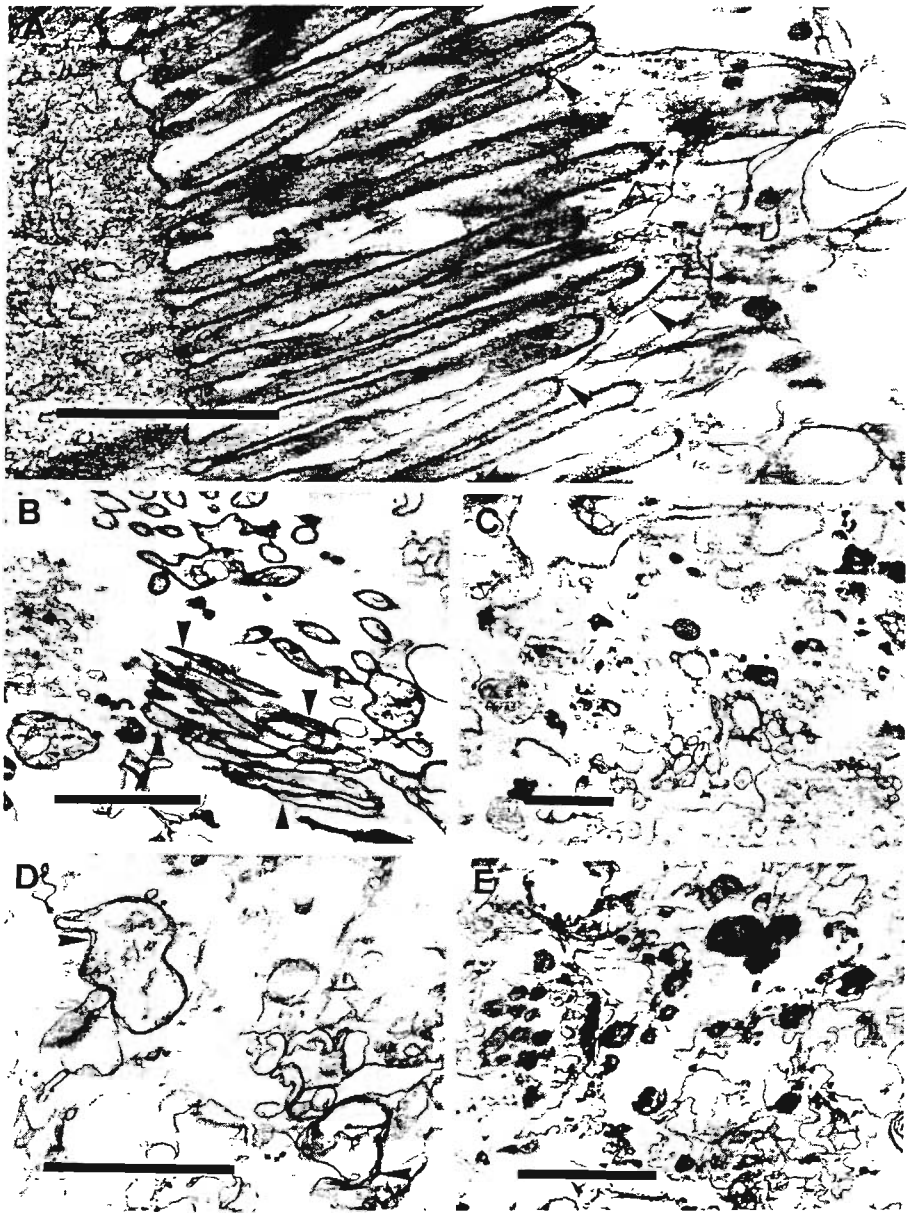

Fig. 3. (A) Detail of the apex of posterior midgut cell, and the extension of the outer membrane into the midgut lumen (arrowheads). (B) Fraction P_1 (isotonic), as detailed in Fig. 1; note microvilli (arrows). (C) Fraction P_1 (hypotonic) as detailed in Fig. 2; note absence of microvilli. (D) Fraction P_3 (isotonic) as detailed in Fig. 1; note double membranes (arrows). (E) Membranes recovered from luminal contents. Scale bar = 1 μm.

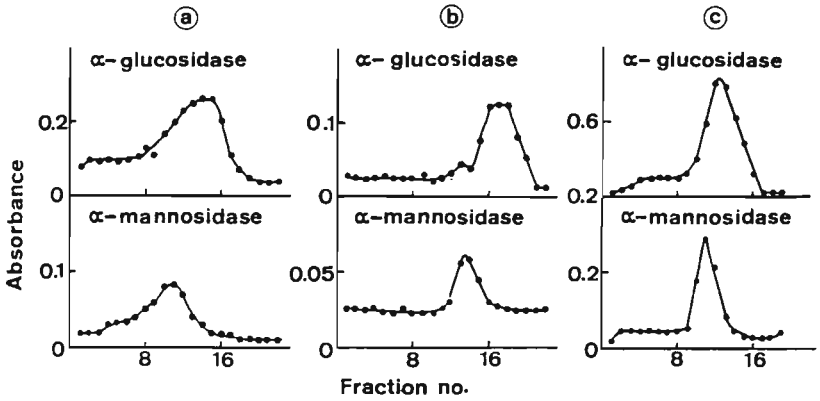

Fig. 5. Typical resolution in sucrose gradients of membranes present in different preparations. (a) Posterior midgut lumen; (b) pellet corresponding to fraction P_1 of Fig. 2; (c) pellet corresponding to fraction P_3 of Fig. 2. Other details as in the legend to Fig. 4.

α-glucosidase is associated to the outer microvillar membranes. According to Lane and Harrison (1979), the outer microvillar membranes are almost devoid of intramembranous particles, thus resembling myelin sheets. Therefore, the outer microvillar membranes must display a lower buoyant density than the inner microvillar membranes. In agreement with this hypothesis, it was possible to resolve α-glucosidase-carrying membranes from α-mannosidase-carrying membranes in sucrose gradients (Figs 4 and 5a–c). The apparent buoyant density of the α-glucosidase-carrying membrane was found to be 1.068 $\pm$ 0.003 g/cm^3, which agrees with the densities determined for plasma membranes almost lacking proteins (see Evans, 1978). The apparent buoyant density of the α-mannosidase-carrying membrane was found to be 1.086 $\pm$ 0.002 g/cm^3. This density is lower than the apparent buoyant density (1.095 $\pm$ 0.002 g/cm^3, unpublished results) of microvillar membranes prepared from *Musca domestica* larval midguts (Espinoza-Fuentes *et al.*, 1987) and determined under conditions similar to those of *R. prolixus*. This discrepancy suggests that the inner microvillar membranes are still associated with fragments of outer membranes, and that these latter fragments are devoid of α-glucosidase activity. The finding of large amounts of double membrane-vesicles in fraction P_3 (isotonic) (Fig. 3D) gives support to the above-mentioned hypothesis. The maintenance of the association of the inner and outer microvillar membranes in cell fractions is not unexpected. According to Lane and Harrison (1979), the membranes directly ensheathing the microvilli are maintained together by proteins obliquely disposed between the outer and inner microvillar membranes.

Membrane-bound-α-glucosidase (Terra *et al.*, 1988) and membrane-bound-α-mannosidase (Ferreira *et al.*, 1988) were solubilized in Triton X-100 and have had several of their properties determined.

In spite of the possibility of the occurrence of a minor membrane-bound aminopeptidase, the majority of this enzyme is soluble. Nevertheless, if aminopeptidase is really soluble, the major amounts of it that are recovered in fraction P_1 (isotonic) must be explained. It is possible that the aminopeptidases in this fraction are cytosolic enzymes bound into cell fragments, but this has no support from intracellular distribution data of lactate dehydrogenase, a cytosolic marker (Fig. 1). It is more likely that these activities in fraction P_1 result from enzymes trapped inside vesicles formed from outer microvillar membranes or trapped in the space between outer and inner microvillar membranes, since electron microscopic examination of fraction P_1 reveals large vesicles possessing brush-borders with double membranes (Fig. 3B). After freezing and thawing, these vesicles probably change in structure, the enzyme formerly trapped being released. The activity of aminopeptidase found in the soluble fraction of cells may correspond to molecules inside the space enveloped by the outer microvillar membranes, and which are rendered free on tissue homogenization. The increase of aminopeptidase activity in this fraction, when the tissue is homogenized in hypotonic medium (which may lead to swelling and rupture of the vesicles) agrees with this hypothesis. Sedimentation data (Fig. 6) suggest that there are at least three different aminopeptidases in *R. prolixus* posterior midgut and that they may be compartmentalized. One aminopeptidase being found in luminal contents and at least two trapped between the outer and inner microvillar membranes. It is important to stress that the majority of the aminopeptidase activity is cellular (Terra *et al.*, 1988). It is possible that the activity of these aminopeptidase differ in relation to the length of the oligopeptide chain, as shown to be the case for *R. americana* midgut aminopeptidases (Ferreira and Terra, 1984, 1985, 1986).

The intracellular distribution of soluble α-mannosidase and soluble acid phosphatase is similar to that of aminopeptidase. Nevertheless, these enzymes are, in contrast to aminopeptidase, also found in significant amounts in posterior luminal contents (Terra *et al.*, 1988). It is interesting to add that the properties of *R. prolixus* soluble acid phosphatase is similar to those of the best known lysosomal acid phosphatases (Terra *et al.*, 1988).

Combining all the data discussed in this paper, it is possible to advance a model on the terminal digestion occurring in *R. prolixus* posterior midgut

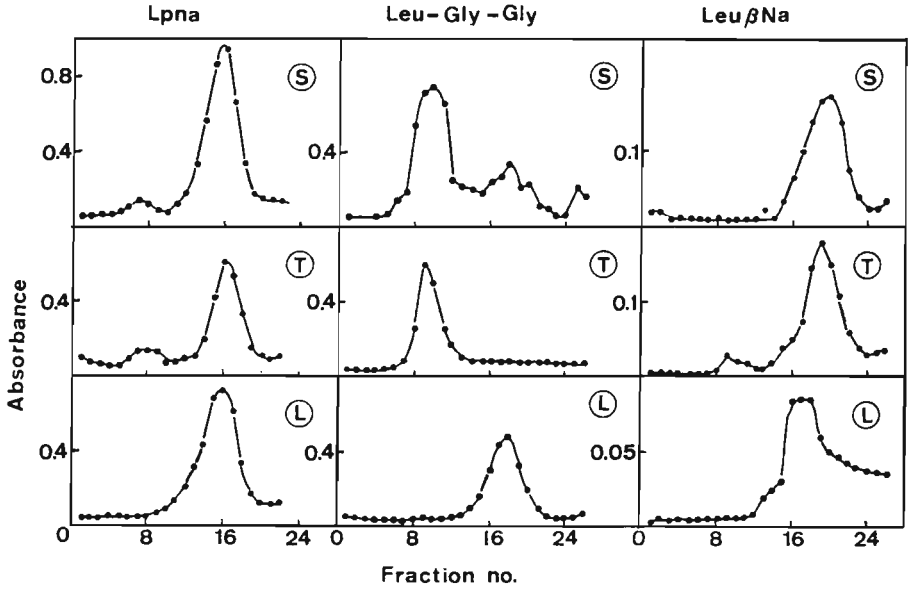

Fig. 6. Resolution in glycerol gradients of posterior midgut aminopeptidases active on LPNA, Leuglygly and Leu βNA. *L*, soluble luminal contents; S, final supernatant of differential centrifugation (fraction s, Fig. 1); T, trapped enzymes in fraction P_1 (supernatant corresponding to fraction P_1, Fig. 2). The sedimentation profiles obtained from other preparations were similar to these. Details in Materials and Methods.

(Fig. 7). This model is also supported by cytochemical data which showed that aminopeptidase (active on Leu β NA) is somehow associated with the cell microvilli and outer microvillar membranes (Billingsley and Downe, 1985).

Evolutionary origin and the physiological role of the outer microvillar membranes in Hemiptera

The ancestor of the Hemiptera is supposed to have been a sap sucker similar to present day Homoptera (Goodchild, 1966). Sap-suckers may feed on phloem, xylem or cambium fluid. The major constituents of phloem fluid is sucrose (5–25%, w/v), whereas free amino acids (0.03–0.13% w/v), potassium and some organic acids are also present in significant amounts (Mittler, 1958; Peel and Weatherley, 1959). Xylem fluid contains 0.1–0.2% (w/v) dry-matter, with potassium ions accounting for about 50% of its osmolarity, and with amino acids and sucrose present in trace amounts (Cheung and Marshall, 1973a). Cambium fluid contains solutes from xylem and phloem (Marshall and Cheung, 1975). The lack of a true peritrophic membrane in all Hemiptera (early reports describing a peritrophic membrane in Hemiptera were not supported by further research, see Terra, 1988, for a review) is probably related to the absence of polymeric nutrients in sap. In xylem-feeders, a midgut structure (the so-called filter chamber) concentrated the sap many times before its nutrients were absorbed (Cheung and Marshall, 1973a,b; Marshall and Cheung, 1974). Since phloem sap cannot be much concentrated, mechanisms which do not rely in a concentration step are necessary to assure the absorption by phloem-feeders of organic compounds (mainly amino acids), present in minute concentrations in phloem sap. According to Terra (1988), the midgut outer microvillar membranes provide such a mechanism. The inner and outer microvillar membranes limit a closed compartment, the outer membrane-enveloped compartment (OMC). If the inner microvillar membranes actively transport potassium ions (the most important ion in sap, see above) from the OMC into the midgut cells, there will be a K^+ concentration gradient between the gut luminal sap and the OMC. This concentration gradient may be used as a driving force for the active absorption of organic compounds through appropriate protein carriers present in the outer microvillar membranes. Organic compounds, once in the OMC, may diffuse through the OMC fluid up to carriers in the microvillar surface. This movement is probably enhanced by a transfer of water from midgut lumen to midgut cells, following (as solvatation water) the trans-membrane transport of compounds and ions through the putative carriers.

Predators, seed-suckers, and hematophagous bugs are supposed to have evolved from phloem sap-sucking bugs, which lost the peritrophic membrane and acquired the outer microvillar membranes for efficient absorption from dilute diets. They probably retained the outer microvillar membranes as a substitute for the usual (chitinous) peritrophic membrane in the compartmentalization of digestion. This compartmentalization may have in *R. prolixus* the following functions (1) to prevent unspecific binding of undigested material onto inner (true) microvillar membrane hydrolases and/or transporting proteins; (2) to let the monomers be produced (from food oligomers) only close to the surface of the inner microvillar membranes, in the neighbourhood of membrane carriers. Prevention of binding of undigested material onto the surface of the microvillar

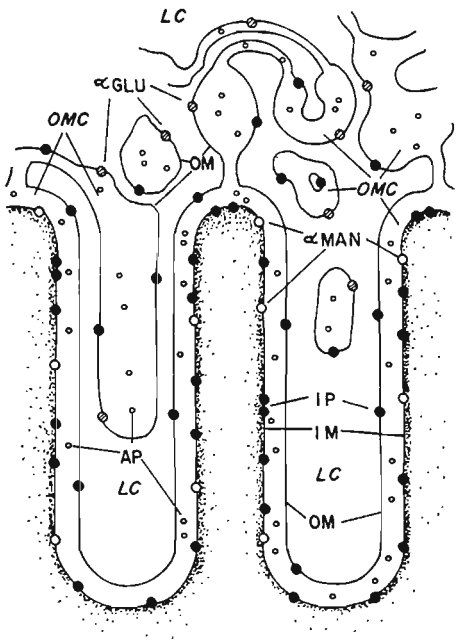

Fig. 7. Model for the structure and physiological role of the microvillar border of the *R. prolixus* posterior midgut. The inner (true) microvillar membrane (IM) is ensheathed by an outer microvillar membrane (OM), which extends toward the luminal compartment (LC) with a dead end. The inner and outer microvillar membranes limit a closed compartment, the outer membrane-enveloped compartment (OMC). The inner is rich and the outer microvillar membrane is poor in integral proteins (IP). Oligopeptides formed in the luminal compartment through the action of cathepsins on hemoglobin, after being shortened by a minor luminal aminopeptidase, were transported into OMC to fuel a major aminopeptidase (AP), which products were further digested by dipeptidases and then absorbed. The role of carboxypeptidases in this process remains to be revealed. The OM-bound α-glucosidase (α-GLU) and the IM-bound α-mannosidase (α-MAN) probably digest the carbohydrate moieties of peptides resulting from the digestion of blood glycoproteins.

membranes is important to assure efficient final digestion and absorption. This prevention is accomplished in the insects other than Hemiptera by the peritrophic membrane, and in mammals, by the gastrointestinal mucus (see Terra, 1988, for a review).

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SPATIAL ORGANIZATION OF DIGESTION, SECRETORY MECHANISMS AND DIGESTIVE ENZYME PROPERTIES IN *PHEROPSOPHUS AEQUINOCTIALIS* (COLEOPTERA: CARABIDAE)

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Abstract—Aminopeptidase (soluble form M_r 110,000), carboxypeptidase A (soluble form M_r 47,000), maltase (a dimer composed of two identical M_r 60,000 subunits) and trypsin (two charge isomers with M_r 34,000) are found in major amounts in the crop and midgut tissue, whereas amylase (a trimer of three identical M_r 18,000 subunits) and cellobiase (a trimer of three identical M_r 27,000 subunits) occur mainly in the crop and midgut contents. Subcellular fractions of midgut cells were obtained by conventional homogenization, followed by differential centrifugation or differential calcium precipitation. The results suggest that part of the aminopeptidase and carboxypeptidase A activity is bound to microvilli, that major amounts of trypsin and maltase are trapped in the cell glycocalyx and finally that soluble aminopeptidase, amylase and cellobiase occur in intracellular vesicles. The data support the hypothesis that most protein and carbohydrate digestion takes place in the crop under the action of enzymes passed forward from the midgut, after being secreted by exocytosis. Nevertheless, part of the intermediate and final digestion occurs at the surface of the midgut cells. The peculiar features of the digestion of *P. aequinoctialis* beetles, including their partly fluid peritrophic membranes, are thought to be derived from putative Coleoptera ancestors.

Key Word Index: crop digestion, Carabidae peritrophic membrane, carabid carbohydrases, carabid proteases, digestion and phylogeny, digestive enzyme secretion

INTRODUCTION

The digestive process occurs over three phases: initial, intermediate and final digestion. The initial digestion consists of a decrease in the molecular weight of the food molecules through the action of polymer hydrolases such as amylase and trypsin. Intermediate digestion is the hydrolysis by oligomer hydrolases (exemplified by aminopeptidases and carboxypeptidases) of oligomeric food molecules to dimer and/or small oligomers, which during the final digestion are split into monomers by dimer hydrolases, such as disaccharidases and dipeptidases. Depending on the phylogenetic position of the insect these phases may occur in different gut or midgut compartments (see review by Terra, 1988).

Digestive enzymes are passed forward from the midgut into the foregut both in lower (Adephaga: see Cheeseman and Pritchard, 1984a) and in higher (Polyphaga; see Terra *et al.*, 1985) Coleoptera. Thus, the role of the crop in beetle digestion is a function of its size in relation to the midgut and of the nature of the digestive enzymes found in the midgut luminal contents. The crop is generally absent or very slightly developed in beetle larvae and in Polyphaga adults, whereas it is usually present in adult Adephaga (Crowson, 1981). The predaceous larvae of *Pyrearinus termitilluminans* (Polyphaga: Elateridae) regurgitate onto their prey their midgut contents (they have no crops) which accomplishes initial digestion. Preliquefied material is then ingested by larvae and the intermediate and final digestion take place on the surface of the midgut cells (Colepicolo—Neto *et al.*, 1986).

The majority of the gut proteinase activity is found in the crop of predaceous Carabidae (Adephaga) adults (Cheung and Gooding, 1970; Cheeseman and Pritchard, 1984a; Cheeseman and Gillott, 1987). Although such studies suggest that initial digestion occurs in the crop of the beetles, they do not provide information on intermediate and final digestion. Furthermore, except for a few studies on proteinases (Gooding and Huang, 1969; Cheung and Gooding, 1970), the molecular properties of Adephaga digestive enzymes are unknown.

In this paper we describe the distribution and properties of several hydrolases occurring in different gut regions of *Pheropsophus aequinoctialis* (Coleoptera: Carabidae) adults. The results suggest that the crop is the main site of digestion, although at least part of the intermediate and final digestion occurs at the surface of the midgut cells. Furthermore, we propose from the data that the peculiar digestive features displayed by Carabidae beetles, including their partly fluid peritrophic membranes, are derived from putative Coleoptera ancestors.

MATERIALS AND METHODS

Animals

Adults of the predaceous species *Pheropsophus aequinoctialis* (Coleoptera, Adephaga, Carabidae, subfamily Brachininae, tribe Brachinini) are found mainly in north and northeast Brazil. These bombardier beetles were collected in the field by Dr K. Zinner and were maintained in our laboratory at a relative humidity of 50–60% at 24 ± 2°C. The insects were fed daily on oats and twice a week on *Musca domestica* larvae. Insects of both sexes with digestive tracts having ample food were used in the determinations.

pH of gut contents

Adults were dissected in cold 0.3 M NaCl after which the guts were transferred to a dry glass slide, and divided into a foregut, anterior midgut, posterior midgut and hindgut. To the contents of each section was added 20 μl of a 10-fold dilution of a universal pH indicator (Merck, Darmstadt; pH 4–10). The resulting colored solutions were compared with suitable standards.

Preparation of samples of gut sections

Beetles were immobilized by placing them in ice, after which they were dissected in cold 0.3 M NaCl. After the removal of the whole gut, the foregut, the midgut tissue, the peritrophic membrane with contents and the hindgut were pulled apart. Cephalic segments, Malpighian tubules, foreguts, midgut tissue and hindguts, after being rinsed thoroughly with 0.3 M NaCl, were homogenized in double distilled water using a Potter–Elvehjem homogenizer. All the above preparations were then passed through a nylon mesh of 100 μm pore size. Peritrophic membranes and contents were homogenized like gut preparations, without previously rinsing with saline, and centrifuged at 10,000 *g* for 10 min at 4°C. All samples were stored at −20°C until use.

Differential centrifugation of midgut homogenates was accomplished as follows. Midgut tissue was homogenized with the aid of a Potter–Elvehjem homogenizer (15 strokes) in 565 mM mannitol, 5 mM EDTA, 5 mM Tris–HCl buffer, pH 7.0. The homogenates, after being filtered through a piece of nylon mesh of 45 μm pore size, were adjusted to contain material from 7 animals/ml and centrifuged at 4°C. The following fractions were collected: P_1, pellet resulting from centrifugation at 600 *g* for 10 min; P_2, pellet from 3300 *g*, 10 min; P_3, pellet from 25,000 *g*, 10 min; P_4, pellet from 100,000 *g*, 60 min; S, supernatant from 100,000 *g*, 60 min. After differential centrifugation the fractions were collected and homogenized and, following three freezing and thawing cycles, they were centrifuged at 100,000 *g* for 60 min. The supernatants and pellets corresponding to each fraction were assayed for several enzymes. The recovery of each hydrolase activity in subcellular fractions was 80–110% of the homogenate activity.

Differential calcium precipitation for the purification of microvilli was performed according to Ferreira and Terra (1980) by the procedure of Schmitz *et al.* (1973). The recovery of each hydrolase activity in subcellular fractions was 80–110% of the homogenate activity.

Polyacrylamide gel electrophoresis

Samples were applied to gels of different polyacrylamide gel concentrations prepared as described by Hedrick and Smith (1968) in glass tubes of 5 mm i.d. and 100 mm length. The electrophoretic separation, the immersion of gels in buffer to decrease their concentration of Tris, the fractionation of gels in a gel fractionator and the collection of fractions with a fraction collector are detailed in Terra and Ferreira (1983). Recoveries of the activities applied to the gels were 5–10% for carboxypeptidase A, 30–50% for cellobiase, maltase and trypsin and 80–120% for aminopeptidase and amylase.

Isoelectric focusing in polyacrylamide gels

Isoelectric focusing was performed as described by Terra *et al.* (1978), in columns of 7.0% polyacrylamide gel containing 1% ampholytes pH 3–10. Midgut samples were added before acrylamide polymerization or were applied after polymerization and pre-focusing (30 min at 31 V/cm) on the top of the alkaline side of the gel. Samples added before polymerization were used in the assay of aminopeptidase and cellobiase, whereas those added after polymerization were used in the assays of amylase, carboxypeptidase A and trypsin. For maltase assays, both procedures are reported. Recoveries of the activities applied to gels were 2–15% for amylase and carboxypeptidase A and 70–110% for the other enzymes.

Density-gradient centrifugation

Samples (0.2 ml) of preparations, containing 1.5 mg of bovine hemoglobin and 50 μg of bovine liver catalase were layered on the top of 4.6 ml linear glycerol gradients (5–30%, w/v) made up in 50 mM sodium phosphate buffer, pH 6.2. In some experiments, the gradients were prepared in 50 mM glycine–NaOH buffer, pH 9.0. Centrifugations and collection of fractions were performed as described previously (Terra and Ferreira, 1983). M_r values of enzymes assayed in the fractions were calculated by the method of Martin and Ames (1961), using sedimentation rates of bovine hemoglobin (M_r 64,500) and bovine liver catalase (M_r 232,000) as reference standards. Recoveries of the

Table 1. Assay conditions and methods used in the determination of enzymes in *P. aequinoctialis* adults*

Enzyme	Substrate	Concentration	pH	Substance or group determined	Reference
Alkaline phosphatase	NPP	4 mM	10.4	Nitrophenolate	Terra *et al.* (1979)
Aminopeptidase	LpNa	1 mM	8.0	Nitroaniline	Erlanger *et al.* (1961)
Amylase	Starch	0.5%	5.5	Reducing groups	Noelting and Bernfeld (1948)
Carboxypeptidase A	ZGlyPhe	15.8 mM	8.0	Phenylalanine	Nicholson and Kim (1975)
Cellobiase	Cellobiose	7 mM	5.0	Glucose	Dahlqvist (1968)
Dipeptidase	GlyLeu	5 mM	8.0	Leucine	Nicholson and Kim (1975)
Lactate DH	Lactate	50 mM	7.4	Dye reduction	Bergmeyer and Bernt (1974)
Maltase	Maltose	7 mM	5.5	Glucose	Dahlqvist (1968)
Succinate DH	Succinate	20 mM	7.5	Dye reduction	Santos and Terra (1984)
Trypsin	BAPA	0.83 mM	8.5	Nitroaniline	Erlanger *et al.* (1961)

*All assays were performed in 30°C in media at the indicated pH values. The buffers (50 mM) used were: citrate–sodium phosphate (pH 3–7), Tris–HCl (pH 7–9), Gly–NaOH (pH 10.4) and, in carboxypeptidase A assays, boric acid–NaOH. The reaction medium with starch contained, unless otherwise specified, 10 mM NaCl besides buffer; with ZGlyPhe, 50 mM NaCl; and with NPP, 2.5 mM $ZnCl_2$. Incubations have been carried out for at least four different periods of time and the initial rates of hydrolysis calculated. All assays were performed under conditions such that activity was proportional to protein concentration and to time. A unit (U) of enzyme is defined as the amount that catalyzes the cleavage of 1 μmol of substrate (or bond)/min.

Abbreviations: BAPA, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide; DH, dehydrogenase; LpNA, L-leucine-*p*-nitroanilide; NPP, *p*-nitrophenyl phosphate; ZGlyPhe, *N*-carbobenzoxy-glycyl-L-phenylalanine.

activities applied to the gradients were 2–3% for carboxypeptidase A and 85–110% for the other enzymes.

Hydrolase assays and protein determination

Protein was determined according to Bradford (1976) using ovalbumin as a standard. Enzymatic assays were accomplished as described in Table 1.

Inhibition studies

Enzymes were incubated with different concentrations of inhibitor in each of six different concentrations of the substrate. Ionic strength in assay tubes with Tris was maintained constant by addition of suitable amounts of NaCl. K_i values were determined from replots of slopes and intercepts of Lineweaver–Burk plots against inhibitor concentration (Segel, 1975).

RESULTS

Gut pH and the presence of peritrophic membrane

Gut luminal contents are more acid in the crop than elsewhere. The pH of gut contents increases slightly along the midgut and decreases again in the hindgut (Fig. 1). However, the differences found are small.

The presence of the peritrophic membrane in the midgut was detected by dissection. For this, the midgut was divided into an anterior and a posterior region and the contents of each region were carefully removed and added to a drop of saline. Lumen contents in the posterior midguts were always found enclosed in a membrane. In the anterior midgut, lumen contents were occasionally found in a membrane similar to that in the posterior midgut. Nevertheless, in most instances, anterior luminal contents were only surrounded by a viscous material and, in a few cases, contents were free. There seems to be a relationship between development of the peritrophic membrane and the amount of food in the midgut.

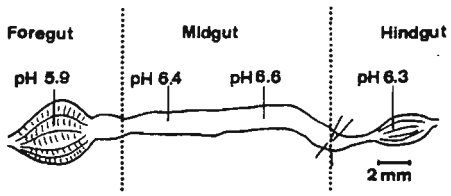

Fig. 1. pH of gut contents at different sites in the adult of *P. aequinoctialis*. The data are averages of at least 11 determinations which were reproducible within 0.1 pH units.

Luminal contents were found free in the anterior midgut only in beetles whose midguts were almost empty. Optical and electron micrographs (to be published elsewhere) show that the peritrophic membrane occurs in both midgut regions.

Distribution of hydrolases in gut regions

Digestive enzymes are found in major amounts in the *P. aequinoctialis* foregut and midgut, whereas only minor amounts are recovered from hindguts (Table 2). Head homogenates display negligible amounts of digestive enzymes (<2% of the activities found in foregut and midgut) and, except for aminopeptidase, the same is true for Malpighian tubules. Aminopeptidase in Malpighian tubules amounts to about $40 \pm 15\%$ (mean and SEM, $n = 3$) of the sum of the activities found in foregut and midgut with a specific activity of 90 ± 30 mU/mg protein (mean and SEM, $n = 3$). Thus, although Malpighian tubules certainly present an aminopeptidase activity, the other activities found in them, and in head homogenates as well, should be contaminants. This suggests that the digestive enzymes found in the foregut and midgut are synthesized and secreted in the midgut and passed forward into the foregut. The foregut is covered with a cuticle and is not secretory (Chapman, 1985).

The specific activity of hydrolases is constant in cells and in contents along the midgut (Table 2) and is higher in the latter than in the former (Table 2). Nevertheless, in terms of percentage of total activity, aminopeptidase, carboxypeptidase A, maltase and trypsin are found in larger amounts in midgut cells than in midgut contents (Table 2).

Subcellular distribution of hydrolases in midgut cells

Midgut tissue was homogenized in water with the aid of a Potter–Elvehjem homogenizer and, after centrifuging the homogenates at 100,000 *g* for 60 min at 4°C, the resulting supernatant and pellet were assayed for several enzymes. The following activities (% total activity) were found in the supernatant (the remaining activities were recovered in the pellet) (mean and SEM, $n = 3$): alkaline phosphatase, 91 ± 2; aminopeptidase, 48 ± 5; amylase, 96 ± 1; carboxypeptidase A, 55 ± 4; cellobiase, 94 ± 1; dipeptidase, 38 ± 5; maltase, 98 ± 1; trypsin, 97 ± 2. The results suggest that part of the aminopeptidase, carboxypeptidase A and dipeptidase activity is membrane bound and part is soluble, whereas the activity

Table 2. Hydrolases present in different regions of the gut of *P. aequinoctialis* adults*

Enzyme	Foregut (crop)	Midgut cells		Midgut contents		Hindgut
		Anterior	Posterior	Anterior	Posterior	
Aminopeptidase	35 (24)	21 (32)	16 (38)	12 (65)	16 (60)	5 (37)
Amylase	50 (2000)	9 (900)	8 (1000)	14 (5000)	19 (5000)	3 (1200)
Carboxypeptidase A	58 (550)	14 (280)	10 (350)	6 (460)	12 (600)	1 (70)
Cellobiase	60 (6)	9 (4)	5 (5)	10 (19)	16 (13)	1 (2)
Maltase	39 (500)	22 (700)	15 (700)	9 (1000)	15 (1100)	1 (124)
Trypsin	29 (24)	28 (44)	17 (41)	11 (60)	15 (70)	2 (7)

*Results are relative activities displayed as percentage of the sum of activities found in foregut and midgut and specific activities (in parentheses) displayed as mUnits/mg protein. Figures are means based on determinations carried out in four different preparations obtained from 25 adults each. SEM were found to be 5–30% of the means. The amount of protein found in each region (μg/animal, mean ± SEM, $n = 4$) was: foregut, 600 ± 150; anterior midgut cells, 290 ± 30; posterior midgut cells, 180 ± 30; anterior midgut contents, 96 ± 7; posterior midgut contents, 110 ± 10; hindgut, 100 ± 30; Malpighian tubules, 180 ± 60.

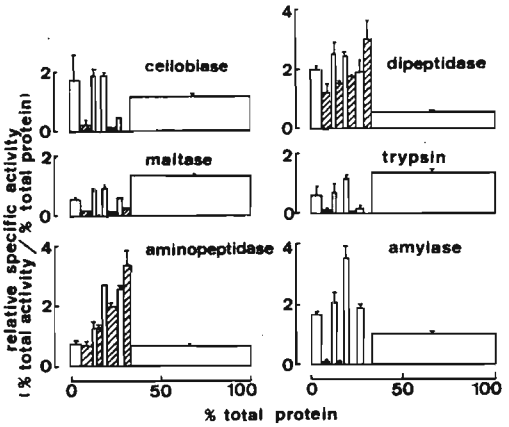

Fig. 2. Distribution of hydrolases among the subcellular fractions of *P. aequinoctialis* midgut. Homogenizing medium: 5 mM EDTA, 565 mM mannitol, 5 mM Tris–HCl, pH 7.0. The following fractions were collected: P_1, pellet resulting from centrifuging at 600 *g* for 10 min; P_2, pellet from 3300 *g* for 10 min; P_3, pellet from 25,000 *g* for 10 min; P_4, pellet from 100,000 *g* for 60 min; S, final supernatant. After differential centrifugation, all fractions were homogenized, and after several freezing–thawing cycles they were centrifuged at 100,000 *g* for 1 h. The supernatants and pellets corresponding to each fraction were assayed. Clear areas correspond to supernatants and shaded ones to pellets. The data are means and range based on determinations carried out in two independent preparations obtained from 25 adults each. Fractions (supernatants and pellets) are displayed from left to right in the order they were obtained (P_1, P_2, P_3, P_4 and S, respectively). The length of horizontal bars (with or without histogram bars) is proportional to the percentage of total protein. The absence of a histogram means that no activity was found in the corresponding sample.

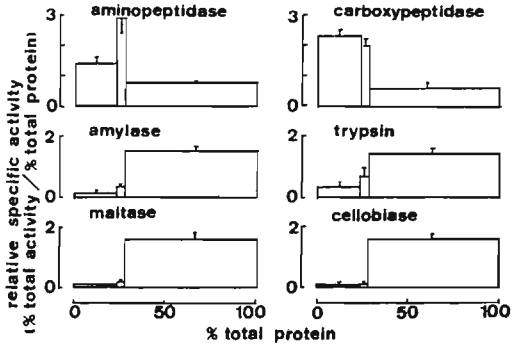

Fig. 3. Differential calcium precipitation of *P. aequinoctialis* midgut homogenates. Midgut cells were homogenized with an Omni-mixer (Sorvall) at 5000 rpm for 2 × 15 s in 50 mM mannitol, 2 mM Tris, pH 7.1 and passed through a 45 μm pore nylon net. The filtrate was made up to 10 mM in $CaCl_2$ and was centrifuged after 10 min. The following fractions were collected: C_1, pellet 3300 *g* for 10 min; C_2, pellet 20,000 *g* for 15 min; C_3, final supernatant. The data are means and range based on determinations carried out in two independent preparations obtained from 30 animals each. Fractions are displayed from left to right in the order they were obtained (C_1, C_2 and C_3, respectively).

of the other enzymes is entirely soluble in the cells. This is in agreement with data displayed in Fig. 2, which shows that only aminopeptidase and dipeptidase occur in significant quantity in membranes (shaded areas in Fig. 2). Carboxypeptidase A was not assayed in subcellular fractions because it is completely inactivated by EDTA present in the homogenizing medium. Due to this fact, dipeptidase assays were included to have peptide hydrolase data other than aminopeptidase data. Lactate dehydrogenase and succinate dehydrogenase were assayed in subcellular fractions to see whether they could be used as enzymic markers of cytosol and mitochondria, respectively. However, <30% of their homogenate activities were recovered in subcellular fractions. Alkaline phosphatase, a plasma membrane marker for several insects (e.g. Diptera: Ferreira and Terra, 1980; Lepidoptera: Wolfersberger, 1984; Santos *et al.*, 1984; Hemiptera: Ferreira *et al.*, 1988) is a soluble enzyme in *P. aequinoctialis* midgut tissue (see above). Therefore, the subcellular distribution of enzymes is based on sedimentation data alone. The high specific activity of aminopeptidase and dipeptidase in membranes from all subcellular fractions (except P_1) (Fig. 2) suggests that they are microvillar enzymes (see Ferreira and Terra, 1980 for references). The highest specific activities of soluble (clear areas in Fig. 2) aminopeptidase, amylase, cellobiase and dipeptidase are found in fractions P_2 or P_3. This suggests that these enzymes are present inside vesicles, from which they are set free on freezing and thawing. Amylase and lysozyme with similar behaviors on subcellular fractionation were shown to be contained inside secretory vesicles in *Musca domestica* larval midguts (Espinoza-Fuentes *et al.*, 1987). Data in Fig. 2 suggest that maltase and trypsin are present mainly in the soluble fraction of the cells. These enzymes may be cytosolic or, more likely, enzymes trapped in the cell glycocalyx, from which they are set free upon homogenizing (Santos *et al.*, 1986).

Figure 3 shows that only aminopeptidase and carboxypeptidase A are enriched (relative specific activity higher than one, the relative specific activity of the homogenate) in fraction C_2. This agrees with the results discussed previously, since according to research done in several insect orders (review: Terra, 1988) fraction C_2 contains mainly cell microvilli.

Properties of midgut hydrolases

P. aequinoctialis midgut maltase activities are resolved by polyacrylamide gel electrophoresis into three peaks [Fig. 4(A)]. Migration data [Fig. 4(B) and (C)] suggest that peak 3 corresponds to a molecule with M_r 54,000, whereas peaks 1 and 2 correspond to molecules with identical M_r values (120,000), but with different pI values [straight lines with identical slopes and with different intercepts, Fig. 4(B)]. Maltase sediments at pH 6.2 and 9.0 as a single molecule with M_rs 125,000 and 54,000, respectively [Fig. 5(A) and (B) and Table 3]. The data suggest that maltase is composed of a peptide chain with an M_r value of about 60,000, which may occur singly or as dimers, depending mainly on the medium pH. This assertion is supported by the different pI values found for maltases depending on the experimental conditions employed [Fig. 5(C) and (D)]. Tris is a strong competitive inhibitor of maltase [Fig. 6(A)]. Other properties

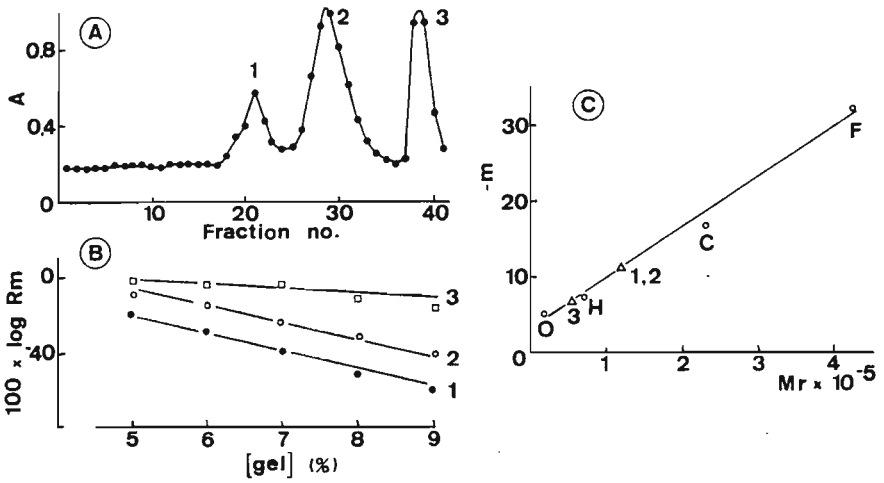

Fig. 4. Electrophoretic properties of the midgut maltases from *P. aequinoctialis* adults. (A) Electrophoretic separation of midgut maltases in 6% polyacrylamide gel. (B) Effect of different polyacrylamide gel concentrations on the electrophoretical migration of midgut maltases. R_m, electrophoretic migration of the enzyme in relation to the tracking dye. The straight lines 1, 2 and 3 correspond to the peaks 1, 2 and 3 displayed in Fig. 4(A). (C) Determination of the M_r of midgut maltases by electrophoresis in different concentrations of polyacrylamide gel by the method of Hedrick and Smith (1968). The slopes of the plots displayed in Fig. 4(B) are *m* values. M_r markers: O, ovalbumin (M_r 43,000); H, bovine hemoglobin (M_r 64,500); C, catalase (M_r 232,000); and F, ferritin (M_r 450,000).

of the midgut maltase are shown in Table 3. Taking into account the *P. aequinoctialis* midgut pH, it is likely that the maltase occurs *in vivo* as a dimer.

The techniques used in the study of other *P. aequinoctialis* digestive enzymes were similar to those described for maltase. Due to this fact, only brief descriptions of the properties of those enzymes will be presented.

P. aequinoctialis midgut amylase migrates in electric fields in polyacrylamide gels and sediments in glycerol gradients (pH 6.2 or 9.0) as a single molecule with M_rs 18,000 and 63,000, respectively. In the same conditions, *P. aequinoctialis* midgut cellobiase behaves as a molecule with M_r 27,000 and 70,000, respectively. Both enzymes display only one peak of activity after isoelectric focusing. The data suggest that in the midgut the amylase and the cellobiase are composed of three identical subunits. Amylase isolated from glycerol gradients made up in sodium phosphate buffer, pH 6.2, were diluted to become only 2.5 mM in sodium phosphate. Samples of this preparation were assayed after the additon of (a) starch; (b) starch and 5 mM calcium acetate; and (c) starch and 10 mM sodium chloride. Since the assays gave identical results, it is likely that *P. aequinoctialis* amylase is not activated by calcium or chloride. Cellobiase is inhibited by Tris [Fig. 5(B)]. Other properties of amylase and cellobiase are shown in Table 3.

Electrophoretic data suggest that there are, in *P. aequinoctialis* midguts, two trypsins with M_r 34,000 and different pI values, whereas sedimentation data reveal only one trypsin with M_r 34,000. Since isoelectric focusing resolves only one trypsin activity, it is probable that one of the activities observed in electrophoresis denatures during focusing. Other properties of *P. aequinoctialis* trypsin are displayed in Table 3. The trypsin from another carabid was partially characterized (Gooding and Huang, 1969). Its pH optimum is similar to that of the *P. aequinoctialis* enzyme, although its M_r value is smaller (16,600).

There is only one molecular species of soluble aminopeptidase and carboxypeptidase A in midguts

Table 3. Physical and kinetic properties of some soluble midgut hydrolases from *P. aequinoctialis* adults*

Enzyme	pH optimum	Activity in gut pH (% of maximum) Crop	Midgut	K_m	Tris K_i (mM)	pI	M_r Centrifugation	Electrophoresis
Aminopeptidase	8.0	77	91	0.15 ± 0.01 mM	—	6.2	110,000	110,000
Amylase	5.5	88	75	0.061 ± 0.005%	—	6.3	63,000	18,000
Carboxypeptidase A	8.0	32	45	0.57 ± 0.05 mM	—	4.7	47,000	59,000
Cellobiase	5.0	85	64	1.5 ± 0.1 mM	3.0	6.8	71,000	27,000
Maltase	5.5	86	63	3.0 ± 0.1 mM	0.61	see Fig. 5(B) and (C)	125,000; 66,000†	54,000; 120,000
Trypsin	8.5	31	52	0.13 ± 0.02 mM	—	4.3	34,000	34,000

*pH optimum and pI values were determined as described in Material and Methods. K_m values (means and SEM, $n = 10$) were determined by a weighted linear regression by the procedure of Wilkinson (1961) using a program written in BASIC by Oestreicher and Pinto (1983). Since there seems to be only one type of catalytic site for all the enzymes, K_m values are reliable. Tris K_i were taken from Fig. 6(A) and (B). M_r values (relative molecular weights) were calculated from ultracentrifugation data according to Martin and Ames (1961) or from electrophoresis data similar to those displayed in Fig. 4(A) and (C) according to Hedrick and Smith (1968).

†Ultracentrifugation performed at pH 9.0.

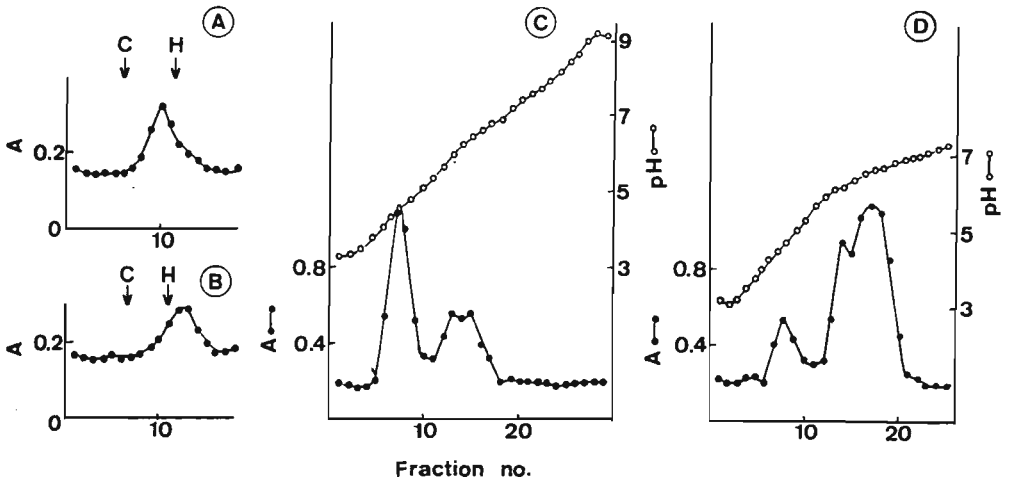

Fig. 5. Ultracentrifugation and isoelectric focusing of midgut maltases from *P. aequinoctialis* adults. (A) and (B) are sedimentation profiles of midgut maltases in a linear glycerol gradient prepared at pH 6.2 (A) or pH 9.0 (B). M_r markers: C, bovine liver catalase; and H, bovine hemoglobin. (C) and (D) are isoelectric focusing profiles of midgut maltases. Isoelectric focusing was performed with midgut samples added to acrylamide before polymerization (C) or applied to the gels from the alkaline side after the polymerization (D).

of *P. aequinoctialis*, as revealed by polyacrylamide gel elecrophoresis, isoelectric focusing and density-gradient centrifugation. The properties of these enzymes are described in Table 3.

In order to detect soluble enzymes involved in final digestion which might be restricted to the midgut, the following experiment was performed. Supernatants (100,000 *g*, 60 min, 4°C) corresponding to homogenates of midgut tissue and of crop with contents were submitted to ultracentrifugation in glycerol gradients. After assaying for aminopeptidase and maltase, M_r values were calculated. The results showed that the enzymes from both sources are identical.

DISCUSSION

Spatial organization of digestion and secretory mechanisms in P. aequinoctialis adults

The peritrophic membrane in *P. aequinoctialis* adult midguts is always well defined in the posterior region, whereas in the anterior region it may be lacking (starving insects), be fluid (the majority of cases) or it may be condensed in a few cases.

After secretion, peritrophic membrane precursors form a fluid material. This material may remain as such, but in the majority of the insects it condenses into the definitive membrane (Richards and

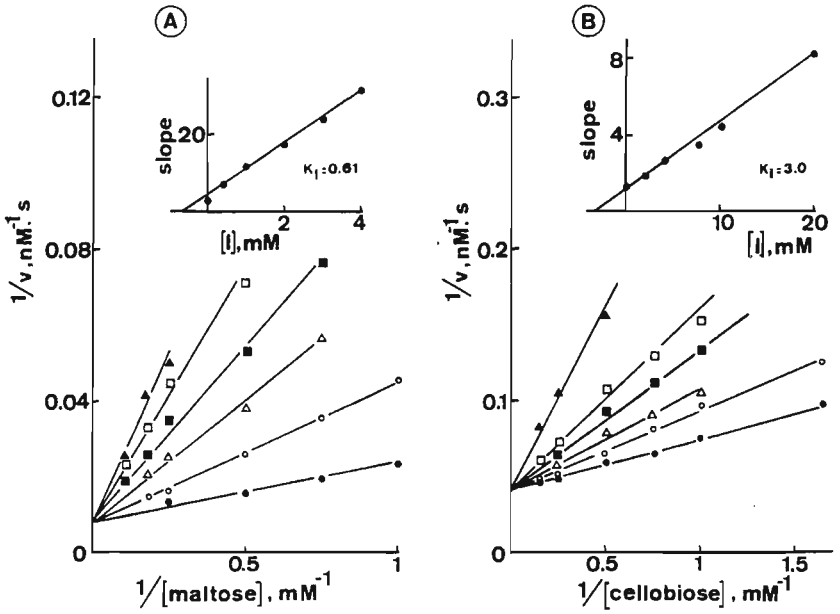

Fig. 6. Inhibition of midgut maltase (A) and cellobiase (B) from *P. aequinoctialis* adults by Tris. Lineweaver–Burk plots for different concentrations of Tris; inset, replot of slopes of Lineweaver–Burk plots against the concentration of Tris (I).

Richards, 1977). Since the fluid material is frequently soluble in water (Freyvogel and Staubli, 1965; Richards and Richards, 1977) it may be easily overlooked. Perhaps this explains the assertions that a peritrophic membrane is lacking in the anterior midgut of several lower and higher Coleoptera such as Carabidae (Cheeseman and Pritchard, 1984b) and Curculionidae (Tristam, 1978; Baker *et al.*, 1984). Another explanation, at least for Curculionidae, is the use of animals not well fed, which may lack the anterior peritrophic membrane, as observed in *P. aequinoctialis*. It is not clear which molecular phenomena underlie condensation of the peritrophic membrane. Nevertheless, it is likely that the main event is the assemblage of microfibers from a finely granular secretory product, followed by aggregation of the microfibers into a protein–carbohydrate matrix (Richards and Richards, 1977). Since microfibers are chitin–protein structures (Richards and Richards, 1977; Peters and Latka, 1986), it is probable that the less chitin a peritrophic membrane has, the more fluid it becomes due to the presence of fewer microfibers. Such fluid peritrophic membranes are presumably more resistant to chitinase digestion than a condensed peritrophic membrane, which may not be formed in the presence of high chitinolytic activity. Thus, it is possible that fluid peritrophic membranes are usual among insects displaying a high luminal chitinase activity.

The majority of *P. aequinoctialis* digestive enzymes are found in the crop and luminal contents (Table 2). Taking into account the pH prevailing in gut contents and the pH–activity profiles of the soluble enzymes (Fig. 1, Table 3), there is no clear regional differentiation in digestion of protein and carbohydrates, as described for other beetles (see below). It is believed that most of the protein and carbohydrate digestion takes place in the crop under the action of digestive enzymes passed forward from the midgut. This hypothesis is supported by the lack of digestive enzymes in head homogenates, which indicates the absence of salivary enzymes, and in the identity of enzymes isolated from the crop contents and midgut tissue. Nevertheless, in view of the finding of a significant amount of maltase activity trapped into the midgut glycocalyx, and of the existence of an aminopeptidase, carboxypeptidase A and a dipeptidase bound to the membranes of the midgut cell microvilli (Figs 2 and 3), it is likely that at least part of the intermediate and final digestion occurs at the surface of the midgut cells.

Digestive enzymes are found in *P. aequinoctialis* hindgut in minor amounts (Table 2), suggesting the existence of a mechanism by which digestive enzymes are either recovered from the undigested food before it is passed into the hindgut or their loss is minimized by having the crop as the main site of digestion. In several insects, this mechanism is provided by an endo–ectoperitrophic circulation of digestive enzymes (Terra and Ferreira, 1981; Santos *et al.*, 1983; Terra *et al.*, 1985; Espinoza-Fuentes and Terra, 1987). According to this model, digestive enzymes diffuse out of the endoperitrophic space (inside the peritrophic membrane) into the ectoperitrophic space (outside the peritrophic membrane) as soon as the polymeric molecules they hydrolyze become sufficiently small to accompany them across the peritrophic membrane. A counter-current flux of fluid, resulting from the secretion of fluid in the posterior midgut and its absorption in the anterior midgut, displaces enzymes and products of the digestion towards the anterior midgut. This mechanism was questioned by Cheeseman and Gillott (1987) for Carabidae on the grounds that these beetles do not have a peritrophic membrane in the anterior midgut. According to them, a pyloric valve may restrict movement of midgut contents into the hindgut when the beetle is not undergoing diuresis. This may be an efficient mechanism mainly in beetles which ingest only fluids. Nevertheless, the finding of a fluid peritrophic membrane in *P. aequinoctialis* anterior midgut makes possible the existence of an endo–ectoperitrophic circulation of digestive enzymes in Carabidae. Even if the circulation system is not operative in Carabidae, their peritrophic membrane (both fluid and condensed region) is presumed to prevent unspecific binding of undigested material onto midgut membrane hydrolases and/or transporting proteins and, in those Carabidae beetles which ingest solid material, to safeguard the midgut epithelium as well.

There are three main secretory mechanisms known for cells. In holocrine and apocrine secretion, secretory products are stored in the cell cytoplasm until they are released at which time the whole (holocrine) or part (apocrine) of the secretory cell is lost to the extracellular space. In merocrine secretion (exocytosis), secretory products are contained in vesicles which eventually fuse with the plasma membrane releasing their contents. Coleoptera midguts display a large number of regenerative cells and mature cells which are being discharged into the lumen. Although this suggests a continuous cell renewal, many authors (e.g. Wigglesworth, 1984) interpret the cells being replaced as holocrine secretion. Nevertheless, Fig. 2 shows that soluble aminopeptidase, amylase, cellobiase and dipeptidase are found mainly bound to membranes in *P. aequinoctialis* midguts, which suggests they are secreted by exocytosis. Trypsin and maltase, which are found in major amounts in the soluble fraction of the midgut cells (Fig. 2), are presumably not present in the cytoplasm, but trapped in the cell glycocalyx, from which they are set free upon homogenizing.

Digestion by Carabidae: evolutionary aspects

The midgut of Coleoptera is frequently differentiated into a slightly acidic anterior midgut and a slightly alkaline posterior midgut (Crowson, 1981). Taking into account the amounts of hydrolases found in the two midgut regions, as well as their activity–pH relationships, one calculates that the carbohydrases are more active in anterior midgut, whereas the proteinases are more active in posterior midgut (e.g. Cerambycidae larvae: Martin, 1987; Curculionidae larvae: Baker *et al.*, 1984; Tenebrionidae larvae: Terra *et al.*, 1985). This differentiation may be regarded as a device to decrease the proteolytic inactivation of the animal's own digestive hydrolases (mainly carbohydrases), in spite of the fact that several digestive enzymes are stable in the presence of the animal's own proteinases (e.g. Diptera larvae:

Terra *et al.*, 1979; Lepidoptera larvae: Santos *et al.*, 1983; Coleoptera larvae: Terra *et al.*, 1985). Another possibility is that the anterior–posterior midgut differentiation is related to the acquisition of digestive enzymes from exogenous sources. Cerambycidae larvae, for example, ingest fungi cellulases along with cellulosic materials. The cellulases (acidic pH optimum) are stable and active in the anterior midgut of the beetle, and are inactivated only in the posterior midgut, where the beetle proteinase activity is higher (Martin, 1987).

According to cladism (see review in Cracraft, 1974), an ancestral character state is the evolutionary earlier state of a character, relative to its derived state. Species are grouped based on their shared derived characters, which are recognized by outgroup comparison. This consists in considering as ancestral the state of a character found in the studied taxon and in a related taxon (the outgroup) and as the derived character the state of the character which is not found in the outgroup.

The data discussed above and those presented in previous papers (Terra *et al.*, 1985; Terra, 1988) may be used in the proposal of an ancestral beetle digestive system, following cladistic-like techniques, whose characteristics are as follows: (a) digestive enzymes may pass forward from the anterior midgut to the crop, which is the main site of digestion; (b) polymer, oligomer and dimer hydrolases are free and small (<7.5 nm dia), and thus able to pass through the peritrophic membrane; (c) endo–ectoperitrophic circulation of digestive enzymes is caused by the secretion of fluid in the posterior midgut and its absorption by the anterior midgut; (d) differentiation of an acidic anterior midgut (with a fluid peritrophic membrane and high carbohydrase activity) and an alkaline posterior midgut (with a condensed peritrophic membrane and a high protease activity). This characteristic is thought to be an adaptation to a diet consisting mainly of cellulosic materials with associated fungi and bacteria, in which part of the beetle's enzymes are unstable in the presence of its own proteinases and/or in which ingested enzymes (including chitinase) play an important role in digestion. This proposal agrees with a hypothesis, based on a comparison of Permian fossils with modern forms of Coleoptera, according to which Coleoptera ancestors lived under the loose bark of dead trees, feeding mainly on fungi (Crowson, 1981). Carabidae ancestors appear to have lost the midgut differentiation in luminal pH and enzyme distribution, resulting in a uniform distribution of secreted enzymes in the midgut and crop. This may be an adaptation to a high-protein diet. The anterior fluid peritrophic membrane is probably an ancestral condition retained due to a high luminal chitinase activity. The occurrence in the crop of enzymes with high M_r values is probably related to an enhanced permeability of the fluid peritrophic membranes, which presumably display less chitin. Peritrophic membranes formed in the presence of inhibitors of chitin synthesis are more permeable than normal ones (Zimmermann and Peters, 1987). The highly permeable, non-chitinous anterior-midgut peritrophic membrane may be related to forward movement of enzymes, instead of being an ancestral beetle trait. Nevertheless, this is unlikely, since grasshoppers display a forward movement of enzymes and have condensed peritrophic membranes (Baines, 1978; Anstee and Charnely, 1977). Another derived character displayed by Carabidae is the occurrence of small amounts of membrane-bound enzymes involved in final digestion. Since the necessary midgut fluid fluxes have not been established, it is not possible to know if carabid beetles retained or not an endo–ectoperitrophic circulation of digestive enzymes.

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DIGESTIVE ENZYMES ASSOCIATED WITH THE GLYCOCALYX, MICROVILLAR MEMBRANES AND SECRETORY VESICLES FROM MIDGUT CELLS OF *TENEBRIO MOLITOR* LARVAE

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Abstract—Acetylglucosaminidase, amylase, cellobiase and maltase are more active in anterior midgut cells, whereas aminopeptidase, carboxypeptidase and trypsin are more active in posterior midgut cells of *Tenebrio molitor* larvae. Differential centrifugation of midgut homogenates prepared in saline (or mannitol) isotonic buffered solutions revealed that aminopeptidase is associated with membranes, which occur in subcellular fractions displaying many microvilli. Carboxypeptidase, trypsin and the carbohydrases are mostly found in the soluble fraction, although significant amounts sediment together with cell vesicles. Data on differential calcium precipitation of midgut homogenates and on partial ultrasound disruption of midgut tissue suggest that aminopeptidase is a microvillar enzyme and that the digestive enzymes recovered in the soluble fraction of cells are loosely bound to the cell glycocalyx. About 5% of the non-absorbable dye amaranth fed to *T. molitor* larvae remains in the midgut tissue after rinsing. Most dye was recovered in the soluble fraction of midgut cells. This provided further support for the hypothesis that the digestive enzymes found in the soluble fraction are actually extracellular and that the true intracellular enzymes are those associated with cell vesicles. The results suggest that the carbohydrases are secreted by exocytosis from the anterior midgut and carboxypeptidase and trypsin from the posterior midgut.

Key Word Index: microvillar enzymes, exocytosis, carbohydrase secretion, protease secretion, ultrasound disruption, differential centrifugation, differential precipitation, mealworm .

INTRODUCTION

Studies of the compartmentalization of aminopeptidase, amylase, cellobiase, trehalase and trypsin along the midgut of *Tenebrio molitor* larvae led Terra *et al.* (1985) to propose that most carbohydrate digestion occurs in the lumen of the anterior midgut of this insect, whereas protein digestion takes place partly in the lumen and partly at the cell surface of the posterior midgut. This proposal assumes that the aminopeptidase found associated with membranes is a microvillar enzyme occurring mainly in the posterior midgut cells and does not advance any hypothesis on the site and mechanism of secretion of the soluble digestive enzymes.

T. molitor larval midgut displays many mature cells rich in morphological signs of exocytosis (rough endoplasmic reticulum, Golgi complex and secretory vesicles) and some cells which are entirely or partly discharging into the lumen (Koefoed and Zerahn, 1972; Berdan *et al.*, 1985; Tano *et al.*, 1987; Ferreira, Ribeiro and Terra, unpublished observations). The first cells suggest that the main route of secretion is exocytosis, whereas the latter cells support a holocrine (or apocrine) route, in spite of many authors interpreting them as an artifact or as part of the cell renewal process (for review see Chapman, 1985).

Subcellular fractionation of midgut cells, followed by enzyme assays, may be used to distinguish enzymes present in the cytosol from those occurring in secretory vesicles. Thus, this technique provides useful information to support proposals on the mechanisms by which midgut cells secrete digestive enzymes. Using these techniques, it was possible to support a model for the secretion of digestive enzymes by *Erinnyis ello* (Lepidoptera) (Santos *et al.*, 1986) and *Musca domestica* (Diptera) (Espinoza-Fuentes *et al.*, 1987) larval midgut cells, which included exocytosis and a microapocrine mechanism of secretion by extrusion of vesicles from the sides and tips of the cell microvilli. Based on the same techniques, digestive enzymes are only secreted in *Pheropsophus aequinoctialis* (Coleoptera) by exocytosis (Ferreira and Terra, 1989).

In this paper we showed, by several criteria, that aminopeptidase is a microvillar enzyme of posterior midgut cells and that carbohydrases are secreted by exocytosis from the anterior midgut cells, whereas carboxypeptidase and trypsin are secreted by exocytosis from the posterior midgut cells of *T. molitor* larvae. Furthermore, we propose from our data that the dye amaranth may be used as a marker for molecules associated with the cell glycocalyx in subcellular fractionation experiments.

MATERIALS AND METHODS

Animals

Stock cultures of the yellow mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae), were cultured under natural photoregime conditions on wheat bran at 24–26°C and a relative humidity of 70–75%. Fully grown larvae (each weighing about 0.12 g), having midguts full of food, of both sexes were used.

Preparation of samples

Larvae were immobilized on ice, after which they were dissected in cold 342 mM NaCl. After removal, the midgut was divided into three sections (anterior, middle and posterior) of identical length. The peritrophic membrane, with contents, was removed from each section and the remaining tissue was thoroughly rinsed with saline. In most experiments, only the anterior and posterior midguts were used, middle midguts being discarded.

Differential centrifugation of midgut tissue homogenates was accomplished as follows. Anterior or posterior midgut tissue was homogenized with the aid of a Potter–Elvehjem homogenizer (15 strokes) in either 328 mM KCl, 5 mM EDTA in 2 mM Tris–hydroxymethyl aminomethane (Tris)–HCl buffer, pH 7.0 or 656 mM mannitol, 5 mM EDTA in 2 mM Tris–HCl buffer, pH 7.0. The pH of the buffers was adjusted at room temperature. The homogenates displaying about 3 mg of protein/ml, after being filtered through a piece of nylon mesh of 45 μm pore size, were adjusted to contain about 2 mg of protein/ml and centrifuged at 4°C. The following fractions were collected: P_1, pellet resulting from centrifugation at 600 *g* for 10 min; P_2, pellet from 3300 *g*, 10 min; P_3, pellet from 25,000 *g*, 10 min; P_4, pellet from 100,000 *g*, 60 min (in media with mannitol the centrifugation lasted for 180 min); S, supernatant from 100,000 *g*, 60 min (or 180 min). Electron microscopy was performed in fractions obtained from homogenates prepared in buffered saline, as described in Espinoza-Fuentes *et al.* (1987). Whichever midgut section was used, electron microscopy revealed that fraction P_1 is characterized mainly by large vesicles displaying microvilli and plentiful mitochondria. Fraction P_2 displays very large amounts of mitochondria and unidentified vesicles. Fraction P_3 shows many recognizable cell microvilli, and other structures, whereas in fraction P_4 microsomes are visible. After differential centrifugation the pellets were collected and homogenized in the previously used buffer, and, following 3 freezing and thawing cycles, they were centrifuged at 100,000 *g* for 60 min (in media with mannitol the centrifugation lasted for 180 min). The supernatants and pellets corresponding to each fraction were assayed for several enzymes. The following enzyme markers for subcellular fractions were assayed: succinate dehydrogenase (mitochondria), lactate dehydrogenase (cytosol) and alkaline phosphatase and γ-glutamyl transferase, which are usually associated with the microvillar membranes.

Differential precipitation for the purification of microvilli was performed according to procedures based on Schmitz *et al.* (1973) as modified by Ferreira and Terra (1980). The procedure is based on the fact that calcium ions cause the aggregation of mitochondria, modified microvilli (such as those from lepidopteran goblet cells), baso-lateral membranes and part of the endoplasmic reticulum which then sediment at a low gravity value. The supernatant free from these structures is centrifuged at a higher speed to pellet the microvillar membranes, leaving lysosomes, the majority of endoplasmic reticulum and soluble proteins in the supernatant (see Terra, 1988, for a review dealing with insect material). Midgut tissue was homogenized with either an Omni-mixer (Sorvall) at 15,000 rpm for 20 s or with a Potter–Elvehjem homogenizer (15 strokes) in a hypotonic medium containing 50 mM mannitol in 2 mM Tris–HCl buffer, pH 7.1. The homogenates were passed through a 45 μm pore nylon mesh. Solid $CaCl_2$ was added to the filtrates, diluted to a concentration of 15 gut portions/ml, to give a final concentration of 10 mM and samples were left for 10 min at 4°C prior to centrifugation. The following fractions were collected: C_1, pellet resulting from centrifugation at 3300 *g* for 10 min; C_2, pellet from 20,000 *g*, 15 min; C_3, supernatant 20,000 *g*, 15 min. In some experiments, the homogenizations were accomplished with an Omni-mixer (Sorvall) at 15,000 rpm for 20 s in an isotonic medium containing 675 mM mannitol in 2 mM Tris–HCl buffer, pH 7.1. The homogenates were filtered after which identical volumes of 20 mM $CaCl_2$ in water were added to them. After standing 10 min, the homogenates were centrifuged. The following fractions were collected: M_1, pellet from 3300 *g*, 10 min; M_2, pellet from 20,000 *g*, 15 min; M_3, pellet from 25,000 *g*, 45 min; SM, supernatant from 25,000 *g*, 45 min.

Partial ultrasonic disruption of midgut tissues was performed according to the procedure of Cioffi and Wolfersberger (1983). Midgut tissue pieces from 50 animals were put in 1.5 ml plastic tubes with 1 ml of 656 mannitol, 5 mM EDTA in 2 mM Tris–HCl buffer, pH 7.0. Sonication was carried out for 5 s using a Branson B-2 sonicator at its lowest setting, with a semimicroprobe. Following sonication, the material was centrifuged for 15 min at 5000 *g* and 4°C. The pellet was labelled US_1 and the supernatant was centrifuged again for 15 min at 5000 *g* and 4°C. The resulting pellet was labelled US_2 and the supernatant was centrifuged again for 30 min at 12,000 *g* and 4°C to pellet the cell microvilli (US_3). All the other material that went into solution during sonication remained in the final supernatant (US_4). In some experiments, the whole procedure was identical except that sonication was carried out for 1 min in the water tank of Branson SX-10 sonicator.

Subcellular fractions could be stored for at least one month at −20°C without noticeable change in the activity of the enzymes assayed.

Adsorption of dyes to subcellular fractions

One hundred parts of wheat bran were mixed with one part of amaranth and the mixture was fed to *T. molitor* larvae for 4 h. After this period, the larvae were dissected and the anterior and posterior midgut tissue and peritrophic membrane contents were isolated as described above. Midgut tissue was homogenized with a Potter–Elvehjem homogenizer in buffered saline and submitted to differential centrifugation as previously described. Absorbance readings of the final supernatant and of the pellets resuspended in the homogenization buffer were at 510 and 650 nm. Amaranth does not absorb at 650 nm, whereas 510 nm is its absorption maximum. Thus, absorbance readings at 650 nm are proportional to the background absorbance of the samples and may be used to correct the absorption maximum taking into account the absorbance ratio A510/A650. The absorbance ratios were calculated from control experiments in which no dye is present in the wheat bran. Protein was also determined in the subcellular fractions obtained in the control experiments. The data were displayed as histogram bars corresponding to each subcellular fraction. The length of the horizontal bars is proportional to the percentage of total protein. The height of the vertical bars is proportional to the ratio of the percentage of total absorbance and the percentage of total protein. Thus, the area of the histogram is proportional to the amount of dye in that fraction and its height is proportional to the concentration of the dye in the fraction in relation to the concentration of the dye in the initial homogenate.

The anterior and the posterior peritrophic membrane with contents were homogenized as the midgut tissue and centrifuged at 10,000 *g*, 10 min at 4°C, to clear the preparations. The pellets were rehomogenized twice in 1% Triton X-100 and after centrifugation all supernatants were combined. Triton X-100 is necessary to remove all the dye adsorbed

Table 1. Assay conditions and methods used in the determination of enzymes in *T. molitor* larvae

Enzyme	Substrate	Concentration	pH	Substance or group determined	Reference
β-*N*-Acetylglucosaminidase	NPNAG	25 mM	4.5	Nitrophenolate	See text
Acid phosphatase	NPP	4 mM	5.0	Nitrophenolate	See text
Alkaline phosphatase	NPP	4 mM	10.4	Nitrophenolate	See text
Aminopeptidase	LpNA	1 mM	7.8	Nitroaniline	Erlanger *et al.* (1961)
Amylase	Starch	0.5%	5.8	Reducing groups	Noelting and Bernfeld (1948)
Carboxypeptidase A	ZGlyPhe	15 mM	8.0	Phenylalanine	Nicholson and Kim (1975)
Cellobiase	Cellobiase	7 mM	5.3	Glucose	Dahlqvist (1968)
G6P DH	G6P	1.5 mM	7.4	Dye reduction	Santos and Terra (1984)
γ-Glutamyl transferase	GpNA,GlyGly	1 and 100 mM	5–9	Nitroaniline	Erlanger *et al.* (1961)
Lactate DH	Lactate	50 mM	7.4	Dye reduction	Bergmeyer and Bernt (1974)
Maltase	Maltose	7 mM	5.3	Glucose	Dalhqvist (1968)
Succinate DH	Succinate	20 mM	7.5	Dye reduction	Santos and Terra (1984)
Trypsin	BAPA	0.8 mM	8.0	Nitroaniline	Erlanger *et al.* (1961)

All assays were performed at 30°C at the indicated pH values. The buffers (50 mM) used were: citrate–sodium phosphate (pH 4.5–5.8), with NPP acid sodium acetate, Tris–HCl (7.0–8.0) and glycine–NaOH (10.4). The reaction medium with starch contained 10 mM NaCl besides buffer; with ZGlyPhe, 50 mM NaCl; with NPP (alkaline), 1 mM $MgSO_4$. Incubations have been carried out for at least four different periods of time and the initial rates of hydrolysis calculated. All assays were performed under conditions such that activity was proportional to protein concentration and to time. A unit (U) of enzyme is defined as the amount that catalyses the cleavage of 1 μmol of substrate (or bond)/min. Abbreviations: BAPA, α-*N*-benzoyl-DL-arginine *p*-nitroanilide; DH, dehydrogenase; G6P, glucose-6-phosphate; GpNA, L-γ-glutamyl-*p*-nitroanilide; LpNA, L-leucine *p*-nitroanilide; NPNAG, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide; NPP, *p*-nitrophenyl phosphate; ZGlyPhe, *N*-carbobenzoxy-glycyl-L-phenylalanine.

onto the contents of the peritrophic membrane. Absorbance readings and corrections were performed as described above for subcellular fractions.

Hydrolase assays and protein determination

Protein was determined according to Bradford (1976) using ovalbumin as a standard. Serum bovine albumin binds more dye on a mass basis than most other proteins causing underestimation of protein content if used as a standard. Enzymatic assays were accomplished as described in Table 1. Hydrolytic activities on nitrophenol derivatives were determined using an incubation volume of 0.2 ml; the reaction was stopped by the addition of 1 ml 0.25 M bicarbonate–0.25 M carbonate 1% SDS (with NPNAG as substrate) or 1 ml of 40 mM NaOH (with NPP as substrate). The molar extinction coefficient at 420 nm of *p*-nitrophenolate under these conditions is 18,000.

RESULTS

Enzyme activities in cells of different regions of the T. molitor larval midgut

Acid phosphatase and carbohydrase are found mainly in anterior midgut cells, whereas the proteases occur mostly in posterior midgut cells (Table 2). Glucose 6-phosphate dehydrogenase, lactate dehydrogenase and succinate dehydrogenase occur in similar amounts along the whole midgut, although their specific activities are higher in posterior midgut (Table 2). This suggests that posterior midgut is metabolically more active than other midgut regions. Middle midgut seems to be a transition region between the anterior and posterior midgut (Table 2). For this reason, in most experiments only the anterior and posterior midgut were studied.

Differential centrifugation of midgut homogenates

The intracellular distribution of enzymes in anterior midgut cells (homogenized in isotonic saline buffer) is displayed in Fig. 1.

Aminopeptidase activity is membrane bound and is recovered mainly in P_3 and P_4, suggesting that it is bound to the microvillar membranes which occur in these fractions. Attempts to confirm this by using other insect microvillar enzyme markers such as γ-glutamyl transferase (Espinoza-Fuentes *et al.*, 1987) and alkaline phosphatase (Ferreira and Terra, 1980; Santos and Terra, 1984; Ferreira *et al.*, 1988) were unsuccessful. γ-Glutamyl transferase displayed a very low activity in pH values from 5 to 9 and alkaline phosphatase was found to be a soluble enzyme, since 94.0 ± 0.3% ($n = 3$) of this enzyme is recovered in the supernatant of midgut homogenates centrifuged at 100,000 *g* for 60 min. Nevertheless,

Table 2. Enzyme activities in cells of different midgut regions of *T. molitor* larvae

Enzyme	Anterior midgut	Middle midgut	Posterior midgut
Acetylglucosaminidase	48 (130)	32 (149)	20 (128)
Acid phosphatase	49 (47)	30 (50)	21 (48)
Aminopeptidase	1 (0.2)	2 (2.0)	97 (122)
Amylase	51 (11,800)	36 (14,100)	13 (6900)
Carboxypeptidase A	5 (0.7)	18 (3.7)	77 (23)
Cellobiase	45 (35)	46 (63)	9 (17)
G6P DH	27 (0.4)	36 (1.0)	37 (1.4)
Lactate DH	32 (0.3)	31 (0.6)	37 (0.9)
Maltase	50 (67)	31 (72)	19 (60)
Succinate DH	28 (0.4)	33 (0.8)	39 (1.3)
Trypsin	7 (0.5)	28 (4.0)	65 (13)

Results are relative activities displayed as percentage of the sum of activities found in midgut and specific activities (in parentheses) displayed as mUnits/mg protein. Figures are means based on determinations carried out in 3 different preparations from 50 larvae each. SEM were found to be 5–30% of the means. The amount of protein found in each region (μg/animal, mean and SEM, $n = 3$) was: anterior midgut, 187 ± 8; middle midgut, 110 ± 10; posterior midgut, 88 ± 8.

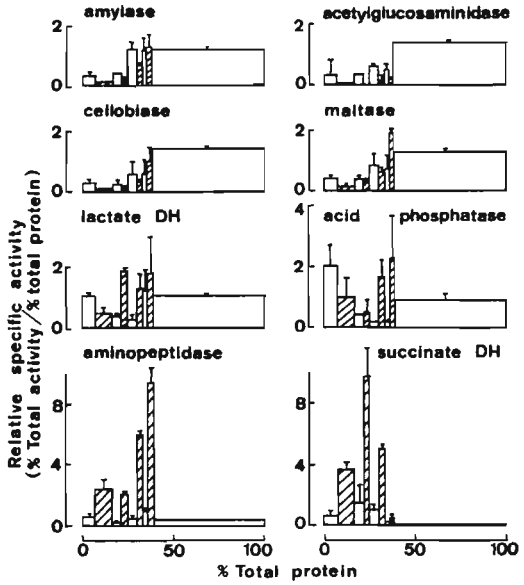

Fig. 1. Differential centrifugation of homogenates from *T. molitor* anterior midgut. Homogenizing medium: 328 mM KCl, 5 mM EDTA, 2 mM Tris–HCl buffer, pH 7.0. The enzyme markers of subcellular fractions were lactate dehydrogenase, cytosol; succinate dehydrogenase, mitochondria. The following fractions were collected: P_1, pellet resulting from centrifuging at 600 *g* for 10 min; P_2, pellet from 3300 *g* for 10 min; P_3, pellet from 25,000 *g* for 10 min; P_4, pellet from 100,000 *g* for 60 min; S, final supernatant. After differential centrifugation, all fractions were homogenized, and after three freezing–thawing cycles they were centrifuged at 100,000 *g* for 1 h. The supernatants and pellets corresponding to each fraction were assayed. Clear areas correspond to supernatants and shaded ones to pellets. The data are means and ranges based on determinations carried out in two independent preparations obtained from 300 larvae each. Fractions (supernatants and pellets) are displayed from left to right in the order they were obtained (P_1, P_2, P_3, P_4 and S, respectively). The recovery of each hydrolase activity in subcellular fractions was between 80 and 105% of the homogenate activity. The length of horizontal bars (with or without histogram bars) is proportional to the percentage of total protein. The absence of a histogram means that no activity was found in the corresponding sample.

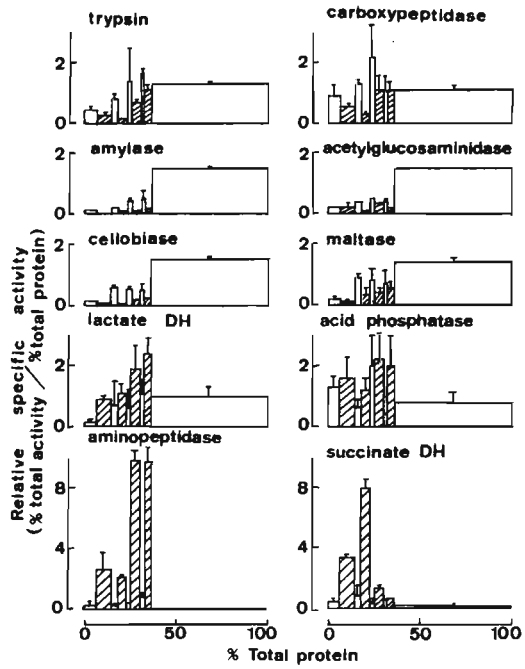

Fig. 2. Differential centrifugation of homogenates from *T. molitor* posterior midgut. The recovery of each hydrolase activity in subcellular fractions was between 80 and 105% of the homogenate activity. Other details are as in the legend to Fig. 1.

acid phosphatase is mostly soluble (clear areas in Figs 1 and 2) and much more active than alkaline phosphatase. The specific activities of these enzymes in midgut cells were (mU/mg protein, $n = 3$): alkaline phosphatase, 0.70 ± 0.03; acid phosphatase, 49 ± 2. Thus, the possibility remained that the observed alkaline phosphatase is actually the residual activity of the acid phosphatase. For this reason, alkaline phosphatase assays were performed in the absence and presence of 10 mM NaF, which completely inhibits the *T. molitor* acid phosphatase (Table 3), as previously shown for insects (Santos and Terra, 1984; Terra *et al.*, 1988) and mammals (Araujo *et al.*, 1976). The subcellular distribution of alkaline phosphatase is identical in the absence and presence of fluoride (Figs 4 and 5), suggesting that the enzyme assayed is actually alkaline phosphatase. Further support for this conclusion is that the enzyme depends on magnesium ions (the activity in non-dialysed homogenates increases 2-fold in the presence of 1 mM $MgSO_4$) and is inhibited by Cys (Table 3). These properties are usual among known alkaline phosphatases (Fernley, 1971). Thus, the data demonstrate that our assay procedures are able to determine both acid phosphatase and alkaline phosphatase.

Succinate dehydrogenase is mostly recovered in the membranes (shaded areas in Fig. 1) of P_1 and P_3. Lactate dehydrogenase occurs in several fractions membrane bound and in the final supernatant (Fig. 1). The subcellular distribution of succinate dehydrogenase agrees with the observed distribution of mitochondria, whereas membrane bound lactate dehydrogenase suggests the occurrence of soluble proteins sedimenting with membranes. Acetylglucosaminidase, amylase, cellobiase and maltase are found mainly in the final supernatant and are also found in significant amounts in fractions P_3 and P_4 (Fig. 1). Soluble activities (clear areas) recovered in P_3 and P_4 may correspond to the contents of secretory vesicles, whereas those in the final supernatant may be derived from cytosol or cell glycocalyx (see below for details).

Table 3. Effect of fluoride and cysteine on *T. molitor* midgut phosphatases

	% of Control	
Enzyme	Fluoride (10 mM)	Cysteine (10 mM)
Acid phosphatase	3 ± 2	107 ± 10
Alkaline phosphatase	100 ± 5	6 ± 2

The chemicals were added to the reaction media without a pre-incubation step. Figures are means and ranges based on determinations carried out in two different midgut homogenates obtained from 50 animals each.

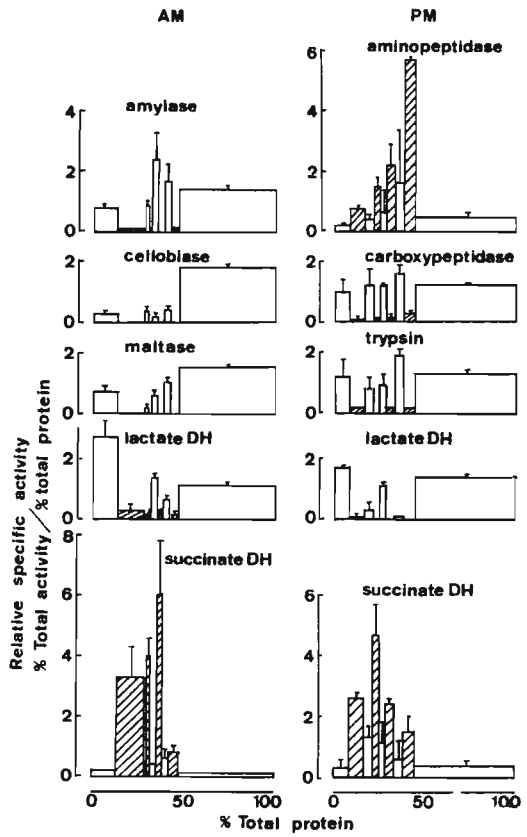

Fig. 3. Differential centrifugation of homogenates from *T. molitor* anterior midgut (AM) and posterior midgut (PM). Homogenizing medium: 656 mM mannitol, 5 mM EDTA, 2 mM Tris–HCl buffer, pH 7.0. The other details were identical to those described in the legend to Fig. 1, except for the centrifugations at 100,000 *g*, which lasted 180 min because of the higher density and viscosity of mannitol solutions in comparison to KCl solutions. The recovery of each hydrolase activity in subcellular fractions was between 70 and 95% of the homogenate activity.

The intracellular distribution of enzymes in the cells of the posterior midgut is similar to that in the anterior midgut, except for amylase, which is almost entirely recovered in the final supernatant (Fig. 2). Since the activities of carboxypeptidase and trypsin are high in posterior midgut, their subcellular distributions were studied in this tissue. The results showed that carboxypeptidase and trypsin display a high specific activity in the soluble portion of the fraction P_3 (Fig. 2). This suggests that these enzymes occur at least in part inside secretory vesicles.

Secretory vesicles may lyse in the presence of KCl (Arvan *et al.*, 1983), thus decreasing the recovery of digestive enzymes in fractions P_2, P_3 and P_4 and increasing their recovery in the final supernatant. To test this possibility, *T. molitor* midguts were homogenized and centrifuged in media displaying 656 mM mannitol instead of KCl. The results (Fig. 3) were similar to those in Figs 1 and 2 with amylase, carboxypeptidase, cellobiase, lactate dehydrogenase, maltase and trypsin displaying much less activity in the membrane portion of the fractions and with amylase, carboxypeptidase and trypsin being recovered in higher amounts in the soluble portion of the fractions P_2, P_3 and P_4. Except for lactate dehydrogenase, the increase in soluble activities in the referred fractions may be accounted for by the decrease in the membrane-bound activities. Thus, the data suggest that the association of enzymes with membranes, observed in Figs 1 and 2, was due to unspecific adsorption and that the lysis of secretory vesicles by KCl did not occur at a significant extent. Nevertheless, subcellular fractionation in mannitol, in contrast to other procedures, suggests that a significant part of aminopeptidase and succinate dehydrogenase is soluble (clear areas in Fig. 3). This is probably a consequence of the high density and viscosity of mannitol solutions which prevent complete membrane pelleting even after 180 min at 100,000 *g*. Thus, taking into account these results and the fact that subcellular fractionation in KCl is performed in a time shorter than in mannitol, the KCl technique should be preferred for most cases.

Differential precipitation of midgut homogenates

Only aminopeptidase is enriched in fraction C_2, the microvillar fraction, in both anterior and posterior midgut (Figs 4 and 5). This confirms that only this enzyme is present in the microvillar membranes. Succinate dehydrogenase is recovered mainly in fraction C_3, probably due to the vigorous homogenizing technique (Omni-mixer) employed. If tissue is

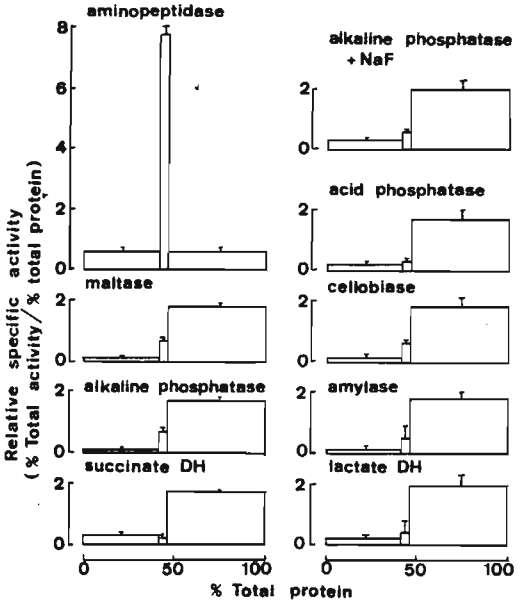

Fig. 4. Differential calcium precipitation of homogenates from *T. molitor* anterior midgut. Midgut cells were homogenized with an Omni-mixer (Sorvall) at 5000 rpm for 2 × 15 s in 50 mM mannitol, 2 mM Tris, pH 7.1 and passed through a 45 μm pore nylon net. The filtrate was made up to 10 mM in $CaCl_2$ and was centrifuged after 10 min. The following fractions were collected: C_1, pellet 3300 *g* for 10 min; C_2, pellet 20,000 *g* for 15 min; C_3, final supernatant. The data are means and range based on determinations carried out in 2 independent preparations obtained from 50 animals each. Fractions are displayed from left to right in the order they were obtained (C_1, C_2 and C_3, respectively). The recovery of each hydrolase activity in subcellular fractions was between 75 and 110% of the homogenate activity.

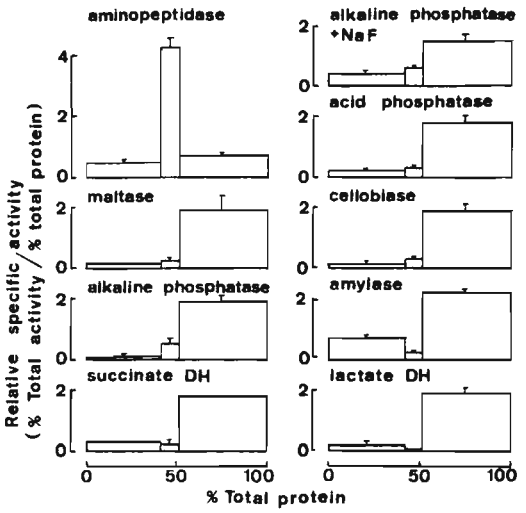

Fig. 5. Differential calcium precipitation of homogenates from *T. molitor* posterior midgut. The recovery of each hydrolase activity in subcellular fractions was between 70 and 110% of the homogenate activity. Other details are as in the legend to Fig. 4.

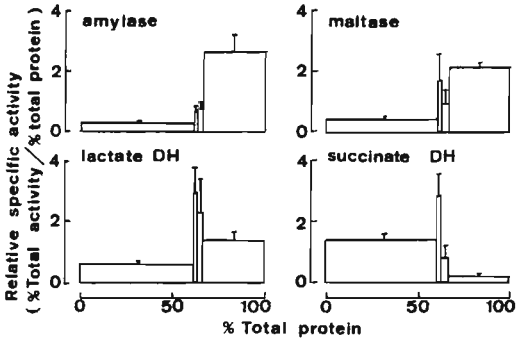

Fig. 7. Partial ultrasonic disruption of *T. molitor* anterior midgut. Sonication of midgut tissue was carried out for 5 s using a Branson B-2 sonicator at its lowest setting, with a semimicroprobe. Following sonication, the material was centrifuged. The following fractions were collected: US_1 pellet from 5000 *g* for 15 min; US_2, other pellet from 5000 *g* 15 min; US_3, pellet from 12,000 *g* for 30 min; US_4, final supernatant. The data are means and SEM based on determinations carried out in 3 independent preparations obtained from 50 animals each. Fractions are displayed from left to right in the order they were obtained (US_1, US_2, US_3 and US_4, respectively).

homogenized with a Potter–Elvehjem homogenizer, instead of an Omni-mixer, succinate dehydrogenase is recovered mainly in C_1 and aminopeptidase is enriched about 15-fold in C_2 (Fig. 6), whereas the results for the other enzymes are identical to those shown in Figs 4 and 5 (data not shown). Homogenizing tissue with an Omni-mixer in isotonic media (see Materials and Methods for details) results in profiles similar to those displayed in Figs 4 and 5 (data not shown). This suggests that *T. molitor* midgut mitochondria are more sensitive to shearing forces than to osmotic shock.

Ultrasonic disruption of midgut homogenates

Figure 8 shows the results obtained after ultrasonic disruption of *T. molitor* posterior midgut with a semimicroprobe. Most succinate dehydrogenase is found in US_1, indicating that few mitochondria leave the tissue on ultrasonication. Only aminopeptidase is enriched in US_3, which indicates that ultrasonic disruption applies satisfactorily well to coleopteran tissue, as well supports the assumption that this enzyme is bound to microvillar membranes. Lactate dehydrogenase is found in major amounts both in the tissue remaining after ultrasonic disruption (US_1) and in the material leaked from the cells (US_4). Amylase, carboxypeptidase and maltase are found in much larger amounts in US_4 than in US_1. This supports the assertion that these enzymes are associated in major

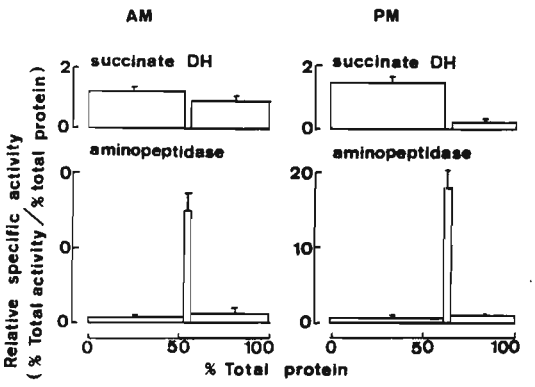

Fig. 6. Differential calcium precipitation of aminopeptidase and succinate dehydrogenase from *T. molitor* midgut homogenates. Midgut cells were homogenized with the aid of a Potter–Elvehjem homogenizer in 50 mM mannitol, 2 mM Tris, pH 7.1. Other details as in the legend to Fig. 4. The recovery of aminopeptidase and succinate dehydrogenase activity in subcellular fractions was between 70 and 90% of the homogenate activity. (AM) Anterior midgut; (PM) posterior midgut.

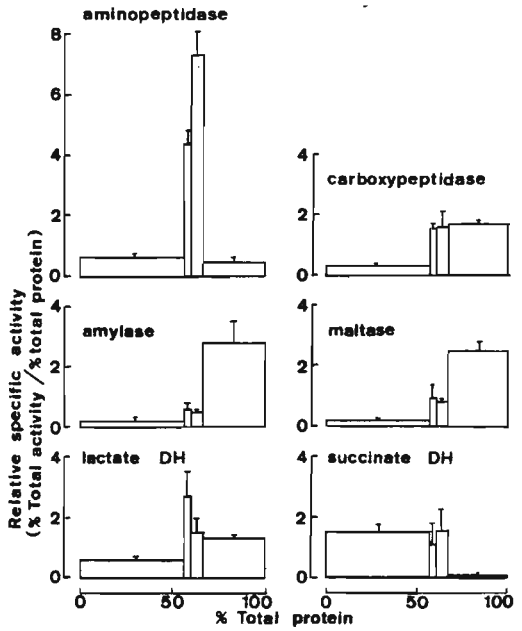

Fig. 8. Partial ultrasonic disruption of *T. molitor* posterior midgut. Other details are as in the legend to Fig. 7.

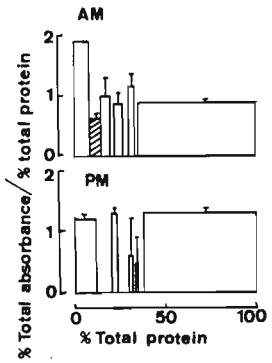

Fig. 9. Distribution of the dye amaranth among subcellular fractions from the anterior midgut (AM) and posterior midgut (PM) of *T. molitor* larvae. Tissue homogenization and centrifugation were performed as described in the legend to Fig. 1. Data are means and SEM based on determinations carried out in 3 independent preparations obtained from 30 animals each.

amounts to the cell glycocalyx (see below for details). Results for anterior midgut cells (Fig. 7) are similar to those for posterior midgut cells (Fig. 8).

Data obtained after ultrasonic disruption of tissue in a water tank (see Materials and Methods for details) were similar to those obtained with a semi-microprobe (Figs 7 and 8), except that the results were somewhat more variable, the enzyme activity recoveries in US_1 were higher, and those in US_4 were lower (results not shown).

Dye adsorption to midgut cells

A non-absorbable dye fed to larvae is expected to be, in part, associated to the outside surface of midgut cells. Thus, the dye distribution among subcellular fractions obtained from rinsed midgut tissue should parallel the subcellular distribution of digestive enzymes associated to the outside surface of midgut cells.

The *T. molitor* larval midgut tissue retains about 9% of the ingested amaranth dye. The subcellular distribution of the dye in the anterior and posterior midgut is shown in Fig. 9.

DISCUSSION

Microvillar enzymes from T. molitor midgut cells

Aminopeptidase was enriched, whereas succinate and lactate dehydrogenase were not, in fractions P_3 and P_4 (differential centrifugation), C_2 (calcium differential precipitation) and US_3 (ultrasonic disruption) (Figs 1, 2, 3, 4, 5, 6, 7 and 8). Furthermore, in the electron microscope, P_3 displays mainly microvilli from midgut cells. These results, together with the finding that aminopeptidase is membrane bound, led to the conclusion that aminopeptidase is bound only to the plasma membranes covering the microvilli of *T. molitor* midgut cells. Although aminopeptidase occurs in the cell microvilli along the whole *T. molitor* midgut, its amount is only significant in the last third of the midgut.

Aminopeptidase has been found in the microvillar membranes of the midguts of larvae and adults of Diptera and Lepidoptera (reviews: Terra, 1988, 1990). The same seems true for the coleopteran larvae *Cylas formicarius* (Curculionidade) (Baker *et al.*, 1984). In other coleopteran larvae, aminopeptidase occurs in soluble form in addition to the microvillar form, as in *Pheropsophus aequinoctialis* (Carabidae) (Ferreira and Terra, 1989) and possibly also in *Pyrearinus termitilluminans* (Elateridae) (Colepicolo-Neto *et al.*, 1986).

Alkaline phosphatase, which is a microvillar enzyme in several insects (Ferreira and Terra, 1980, 1988; Santos and Terra, 1984), is a soluble enzyme in *T. molitor* midgut cells. In spite of being soluble, it is activated by magnesium ions and is inhibited by cysteine, as is the other insect alkaline phosphatases.

Surface bound and vesicular enzymes from T. molitor midgut cells

Santos and Terra (1984) and Santos *et al.* (1986) were the first to propose that large amounts of digestive enzymes may be trapped into the glycocalyx of insect midgut cells, thus becoming bound to the tissue surface. They based their assertion on the finding that some soluble digestive enzymes from *Erinnyis ello* larvae (Lepidoptera) occur in major amounts both in P_1 and in the final supernatant resulting from the differential centrifugation of midgut homogenates. This contrasted with lactate dehydrogenase (a cytosolic marker) which abounds only in the final supernatant. Furthermore, they found that the majority of the cytosol of the midgut tissue leaks from the cells during sonication (judged by lactate dehydrogenase data), whereas the soluble digestive enzymes mostly remained in the tissue. Based on this, they interpreted their results as follows. Soluble activities in P_1 resulted from enzymes trapped in the intermicrovillar glycocalyx space of midgut cells (electron microscope examination of this fraction showed large vesicles displaying brush borders). After freezing and thawing, these vesicles probably change in structure, the enzymes formerly present in the intermicrovillar glycocalyx space being released. Soluble activities in the final supernatant may result from the release during homogenization of enzymes loosely bound to the cell glycocalyx. Thus, depending on how much an enzyme is bound to the glycocalyx it is supposed to abound in P_1, final supernatant or at both fractions.

Despite the rationale presented, a direct demonstration of the distribution of a glycocalyx-associated molecule among fractions obtained by differential centrifugation is lacking. A non-absorbable dye fed to larvae is expected to be in part associated to the surface of midgut cells, since these cells are not covered with a mucus. This association may be due to a macroscopical trapping of dye molecules in tissue foldings, or in a microscopical scale, due to trapping in the cell glycocalyx, electrostatical association with the plasma cell lipid bilayer and/or with the integral proteins. Since the free surface area of integral proteins is small, interactions of dye molecules with them should be quantitatively not important. Cell glycocalyx trapping of dye molecules is expected to occur in preference to interaction with the cell lipid bilayer if the dye molecules are negatively charged. In these conditions, dye molecules will not electrostatically interact with the polar heads of the membrane

phospholipids, but may diffuse through the carbohydrate chains (the cell glycocalyx) of the plasma membrane integral proteins and become mechanically trapped in them. Another possibility is that, due to the varied chemical groups in the carbohydrate chains of the cell glycocalyx, part of the dye molecules become somehow associated with the glycocalyx. On homogenizing, dye molecules, in tissue foldings and those loosely bound to the glycocalyx will be set free, whereas those tight-bound to the glycocalyx will tend to follow cell membrane fragments on differential centrifugation. Thus, the distribution among cell fractions of dye molecules should parallel that of loosely-bound glycocalyx-associated digestive enzymes (and enzymes trapped in tissue foldings) or tight-bound glycocalyx-associated digestive enzymes.

With this aim, we fed *T. molitor* larvae with amaranth, which is not absorbed by midgut cells (Nijhout, 1975), and which displays a negative charge. Amaranth seems to be loosely bound to the glycocalyx, since it is recovered mostly in soluble fraction (Fig. 9.).

Only soluble acetylglucosaminidase, amylase, carboxypeptidase, cellobiase, maltase and trypsin occur in midgut cells of *T. molitor* larvae. The membrane-associated forms of these enzymes (shaded areas in Figs 1, 2 and 3) are actually soluble enzymes unspecifically adsorbed onto the membranes (see Results). The highest specific activities of soluble (clear areas in Figs 1, 2 and 3) amylase (from anterior midgut cells), carboxypeptidase and trypsin (from posterior midgut cells) are found in fractions P_3, P_4 and final supernatant, although the last mentioned fraction contain most of the activities. Since those soluble enzymes are more enriched in fractions P_3 and P_4 than amaranth, which estimates molecules associated with the glycocalyx, they are at least in part present inside vesicles sedimenting in fractions P_3 and P_4. These enzymes are set free from the vesicles on freezing and thawing. The major amounts of amylase, carboxypeptidase and trypsin found in the final supernatant (Figs 1 and 2) may be cytosolic, judging by the subcellular distribution of lactate dehydrogenase (Figs 1 and 2), or may be enzymes trapped in the cell surface from which they are set free upon homogenizing. Since ultrasonic disruption releases more amylase, carboxypeptidase and trypsin than lactate dehydrogenase (Figs 7 and 8), it is more likely that most of those enzymes are not cytosolic but glycocalyx-associated. This hypothesis is supported by the finding that most amaranth is recovered in the final supernatant after differential centrifugation.

There are no ultrasonic disruption data for acetylglucosaminidase and cellobiase. Nevertheless, it is probable that these enzymes are found mainly trapped in the cell glycocalyx.

Secretory mechanisms in T. molitor larval midgut

There are three main secretory mechanisms known for cells. In holocrine and apocrine secretion, secretory products are stored in the cell cytoplasm until they are released at which time the whole (holocrine) or part (apocrine) of the secretory cell is lost to the extracellular space. In merocrine secretion (exocytosis), secretory products are contained in vesicles which eventually fuse with the plasma membrane releasing their contents.

T. molitor larval midgut displays a large number of regenerative cells and mature cells which are entirely or partly being discharged into the lumen (Koefoed and Zerahn, 1982; Berdan *et al.*, 1985; Tano *et al.*, 1987; Ferreira, Ribeiro and Terra, unpublished results). Although this suggests a continuous cell renewal, many authors (e.g. Wigglesworth, 1984) interpret the cells being replaced as holocrine secretion. Nevertheless, we presented the above evidence supporting the proposal that most of the tissue soluble amylase, carboxypeptidase and trypsin is actually glycocalyx-associated, that is, extracellular. The activities of amylase found bound to membranes in *T. molitor* anterior midgut cells, and the activities of carboxypeptidase and trypsin which occur bound to membranes in *T. molitor* posterior midgut cells, seem to be truly intracellular. The results suggest that these intracellular membrane-bound enzymes are secreted by exocytosis: amylase from anterior midgut, whereas carboxypeptidase and trypsin from posterior midgut. Despite the lack of sufficient data, the previous discussion and the finding that acetylglucosaminidase, cellobiase and maltase abound in anterior midgut cells (Table 2) suggest that these enzymes are secreted by exocytosis from the anterior midgut.

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Midgut dipeptidases from Rhynchosciara americana (Diptera) larvae. Properties of soluble and membrane-bound forms.

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Running title: Midgut dipeptidases

Abstract

Dipeptidase activity in Rhynchosciara americana (Diptera: Sciaridae) is found mainly in midgut caeca cells. The caecal dipeptidase activity is partly soluble and partly membrane bound. Differential centrifugation of midgut caeca homogenates, followed by assays of enzyme markers and dipeptidase, suggest that soluble dipeptidase is cytosolic or weakly associated with the cell glycocalyx. Membrane-bound dipeptidase is likely a microvillar enzyme. Soluble dipeptidase activity is resolved by gel filtration and ion exchange chromatography into two enzymes (Mr 63,000 and 73,000), which hydrolyze both Gly-Leu and Pro-Gly, although with different efficiency. The two enzymes also differ in their stability in the presence of EDTA and degree of inhibition by phenanthroline and aminoacyl hydroxamates. Dipeptidase inhibition by phenanthroline is reversed by dialysis. Membrane-bound dipeptidase activity was solubilized by Triton X-100 and papain. Density-gradient ultracentrifugation, gel filtration, and ion-exchange chromatography suggest that there is only one detergent (Mr 86,000) form of this enzyme, which is active on Gly-Leu and Pro-Gly. No activity upon Gly-Pro was found in R. americana midguts, whereas the weak activity observed upon carnosine is independent of the enzymes hydrolyzing Gly-Leu and Pro-Gly. Thus, R. americana midguts seem to have major soluble and membrane-bound dipeptide hydrolases (EC 3.4.13.11), which in contrast to the mammalian enzyme, are very active

upon Pro-Gly. R. americana also has a minor carnosinase (EC 3.4.13.3).

Key words: Soluble dipeptidases, microvillar dipeptidase, dipeptidase purification, carnosinase, dipeptide hydrolase.

Introduction

Dipeptidases (EC 3.4.13.) hydrolyze dipeptides and are classified according to their substrate specificities. Digestive dipeptidases have been studied extensively in vertebrates. In these animals, the following dipeptidases are usually described: carnosinase (EC 3.4.13.3), which acts on certain aminoacyl-L-histidine dipeptides; prolyl dipeptidase (EC 3.4.13.8), which hydrolyzes Pro-X dipeptides; proline dipeptidase (EC 3.4.13.9), active on X-Pro dipeptides and dipeptide hydrolase (EC 3.4.13.11), which displays a broad specificity, although cleaving preferentially hydrophobic dipeptides (Nomenclature Committee, 1992).

Dipeptidases comprise the poorest known of the insect peptide hydrolases. There are few works with insects in which dipeptidase assays were performed and even fewer attempts to characterize the enzymes. Hydrolase activity upon Ala-Gly was detected in the luminal contents and midgut cells of Locusta migratoria and Dysdercus fasciatus (Khan, 1962). In Pheropsophus aequinoctialis, a midgut activity hydrolyzing Gly-Leu was found as a soluble and as a membrane-bound enzyme, the latter apparently associated with cell microvilli (Ferreira and Terra, 1989). Drosophila melanogaster midgut homogenates hydrolyze Leu-Pro and Phe-Leu (Hall, 1988). Although the data suggest the occurrence of dipeptidases in these insects, part of the observed activity could have resulted from residual aminopeptidase

activity.

Chromatographic data showed the occurrence of a true dipeptidase in Rhodnius prolixus midguts, although its substrate specificity was not studied (Garcia and Guimarães, 1979). This unambiguous demonstration of the existence of a true insect midgut dipeptidase, as an activity distinct from aminopeptidase, was followed by studies in other insects. Musca domestica larval midgut dipeptidase (substrate: Gly-Leu) is found only in cell microvillar membranes and has a pH optimum of 8.0, Mr 111,000 and Km of 3.7 mM for Gly-Leu (Jordão and Terra, 1989). In Spodoptera frugiperda larvae there are two dipeptidases (substrate: Gly-Leu) restricted to midgut cells: one (pH optimum 8.0; Mr 95,000) is soluble and glycocalyx-associated and the other (pH optimum 8.2; Mr 130,000) is membrane bound to cell microvilli (Ferreira et al., 1994).

This paper describes properties of soluble and membrane-bound dipeptidases found in Rhynchosciara americana (Diptera: Sciaridae) larval midguts, and also reports on their midgut and intracellular distribution. Furthermore, specificities of the dipeptidases are reported. The results suggest that specificities of insect dipeptidases differ from those of the vertebrate enzymes.

Materials and Methods

Animals

R. americana was reared as described by Lara et al. (1965). Mature feeding female larvae at the end of the second period of the fourth instar were used (Terra et al., 1973). Second period starts when the larvae become reddish-brown and ends when the larvae stop feeding.

Preparation of samples

Larvae were dissected in ice cold 0.1 M NaCl. After removal of the gut, luminal fluid was collected from the caeca with the aid of a capillary and the gut was cut at the proventriculus and at the opening of the Malpighian tubules. The peritrophic membrane with its paste-like contents and the midgut caeca were then removed and the remaining ventriculus divided into an anterior and a posterior section. The entire midgut, as well as the midgut caeca, anterior and posterior ventriculus was rinsed thoroughly with saline and homogenized in cold double-distilled water using a Potter-Elvehjem homogenizer. The luminal contents of the caeca were solubilized in a known volume of double distilled water. All the above preparations were passed through a piece of nylon mesh of 100 μm pore size. The peritrophic membrane with its contents was homogenized like the other preparations, except there was no saline rinse, and centrifuged at 10,000 g for 10 min at 4^{o}C. All

homogenates could be stored for at least five years at -20°C without a loss of activity.

Differential centrifugation of homogenates

Midgut caeca tissue corresponding to 20 larvae were homogenized in a tight-fitting Dounce homogenizer (5-10 strokes) in 1.5 ml of isotonic 0.11 M KCl pH 7.0 containing 5 mM EDTA. The homogenates, after being filtered through a piece of nylon mesh of 45 µm pore size, were adjusted to contain material corresponding to 10 animals per ml and centrifuged at 4°C. The following fractions were collected: P_1, pellet 600 g, 10 min; P_2, pellet 3300 g, 10 min; P_3, pellet 25,000 g, 10 min; P_4, pellet 100,000g, 60 min; FS, final supernatant. P_1 was suspended in the homogenization medium, recentrifuged (600g, 10 min) and the supernatants combined before fraction P_2 was collected. After differential centrifugation the pellets were collected and homogenized with the aid of a Potter-Elvehjem homogenizer in the previously used medium and, following three freezing and thawing cycles, they were centrifuged at 100,000g for 60 min. Thus, fraction P_1 resulted in a supernatant SP_1 and a pellet PP_1; fraction P_2 in SP_2 and PP_2; fraction P_3 in SP_3, and PP_3 and fraction P_4 in SP_4 and PP_4. The supernatants and pellets corresponding to each fraction were assayed for several enzymes. The following enzyme markers for subcellular fractions were assayed: succinate dehydrogenase (mitochondria), lactate dehydrogenase (cytosol) and alkaline

phosphatase (plasma membrane) (Ferreira and Terra, 1980). Results are presented according to De Duve et al. (1955). Thus, the height of the histogram is proportional to the specific activity and the area of the histogram is proportional to the total activity of the enzyme.

Solubilization of midgut caeca plasma membranes in Triton X-100

R.americana midgut caeca were homogenized in iso-osmotic saline (0.11 M KCl, pH 7.0) in an Omni-Mixer (Sorvall) at 15,000 rev./min for 20s. The homogenates, after being filtered through a piece of nylon mesh of 45 μm of pore size, were centrifuged at 25,000g for 30 min at 4°C. The sediment, which contains the major part of the plasma membranes (Ferreira and Terra, 1980), was washed by centrifugation and then exposed to Triton X-100 for 20h at 4°C. (10 mg of Triton X-100/mg of protein) before being centrifuged at 25,000g for 30 min. No sediment was visible after the centrifugation of this supernatant at 100,000g for 60 min. The activity of the enzymes remains unchanged, at -20°C, for periods of at least a month.

Solubilization of membrane hydrolases in papain

To midgut caecal membranes (see previous item) suspended in 0.1 M potassium phosphate buffer, pH 7.0, activated (previously incubated with 60 mM cysteine) papain

was added in a ratio of 1 mg of papain to 10 mg of membrane protein. After 30 min at 30°C, samples were diluted twice with cold double-distilled water, centrifuged at 100,000g for 60 min and the resulting supernatants used as enzyme sources. Activity of the enzymes remains constant for at least one year at -20°C.

Protein determination and hydrolase assays

Protein was determined according to Bradford (1976) using ovalbumin as a standard. Alkaline phosphatase was assayed in 50 mM glycine-NaOH buffer pH 10.4 containing 1 mM $MgSO_4$ and 2.5mM $ZnCl_2$, using 4mM p-nitrophenyl phosphate as substrate (Terra et al. 1979). Aminopeptidase was assayed in 50 mM Tris-HCl buffer pH 7.5, by using 1 mM leucine p-nitroanilide (LpNA) as substrate (Erlanger et al. 1961). Dipeptidase activity was assayed using 5 mM Gly-Leu, 2.5 mM Pro-Gly, 1 mM carnosine (β-alanyl-histidine) or 1 mM Gly-Pro as substrates. When Gly-Leu was used, assays were conducted in 50 mM Tris-HCl pH 7.5 and the release of leucine was followed according to Nicholson and Kim (1975). When other substrates were used, dipeptidase assays were performed in 60 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 7.0 and the release of amino groups was followed according to Rosen (1957). Lactate dehydrogenase and succinate dehydrogenase were assayed according to Bergmeyer and Bernt (1974) and Ackrell et al. (1978), respectivelly, as detailed elsewhere (Santos and Terra, 1984).

Incubations were carried out for at least four different time periods, and initial rates of hydrolysis were calculated. All assays were performed under conditions such that activity was proportional to protein concentration and to time. Controls without enzyme or without substrate were included. One unit of enzyme is defined as the amount that hydrolyzes 1 µmol of substrate (or bond) per min.

Density-gradient ultracentrifugation

Samples (0.2 ml) of midgut preparations were added to 1.5 mg of bovine hemoglobin and 50 µg of bovine liver catalase and were then layered onto 4.6 ml glycerol gradients (10-30%, w/v) made up in 50 mM sodium phosphate buffer pH 6.2. When samples solubilized in detergent were used, the buffer contained 0.1% Triton X-100. Centrifugation and collection of fractions were performed as described previously (Terra and Ferreira, 1983). Mr values (relative molecular weights) of enzymes assayed in the fractions were calculated by the method of Martin and Ames (1961), by using sedimentation rates of bovine hemoglobin (Mr 64,500) and bovine liver catalase (Mr 232,000) as reference standards. Recoveries of enzyme activities applied to the gradients were about 85%.

Gel filtration

Gel filtration was performed in a FPLC system (Pharmacia-LKB Biotechnology, Sweden) using a Superose HR

10/30 column equilibrated and eluted with 20 mM MES pH 7.0. When samples solubilized in detergent were used, the buffer contained 0.1% Triton X-100. The flow rate used was 0.4 ml/min. Mr values were calculated using the following proteins as standards: cytochrome c (Mr 12,400), ovalbumin (Mr 45,000), aldolase (Mr 65,000) and L-amino acid oxidase (Mr 89,000). Recoveries of enzyme activities applied to the column were about 95%.

Ion-exchange chromatography

Ion-exchange chromatography was performed in a FPLC system using a Mono-Q HR 5/5 column equilibrated with 20 mM MES buffer pH 7.0. When samples solubilized in detergent were used, the buffer contained 0.1% Triton X-100. A dialysate containing the proteins to be separated was applied to the column, which was washed with 5 ml of the same buffer, and then eluted with 0.1-0.5M NaCl in the same buffer. The flow rate used was 1 ml/min. Recoveries of enzyme activities applied to the column were about 50%.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out in a 6% polyacrylamide gel prepared as described by Hedrick and Smith (1968), using the system of Davis (1964), in glass tubes of 5 mm id and 100 mm length. Other details have been described elsewhere (Terra and Ferreira, 1983). The recoveries of the enzyme

activities applied to the gels were about 65%.

Results

Distribution of dipeptidase activities in midgut regions

Dipeptidase activities were assayed in several midgut compartments of R.americana larvae and the results found are displayed in Table 1. Activities determined in peritrophic membrane contents and in anterior midgut cells amounted to less than 5% of the sum of activities in different sections of the midgut. Thus, most dipeptidase activity occurs in soluble or membrane bound form in midgut caeca cells, with minor amounts being recovered from posterior ventricular cells. The small dipeptidase activity recovered in midgut caecal contents may result from cell desquamation and/or cell disruption during dissection.

In addition to Gly-Leu and Pro-Gly, other dipeptidase substrates were used in assaying dialysed midgut caeca cell homogenates. Whereas no activity was found upon Gly-Pro (detection limit: 1 mU/mg protein), the following activities were determined with carnosine (β-alanyl-histidine): soluble fraction, 0.55±0.09 mU/mg protein; membrane fraction, 4.0±0.7 mU/mg protein. Assays with carnosine as substrate were also accomplished with dialysed soluble and membrane fractions of caeca, in the absence and presence of 2 mM

dithiothreitol and 50 μM Mn^{++}, which is known to activate vertebrate carnosinase (Lenney 1990a, b). The results showed that R.americana carnosinase activity is inhibited by 25% in these conditions. Attempts to assay carnosinase after any separation method (chromatography, ultracentrifugation or electrophoresis) were unsuccessful. Because of these results, all subsequent work on dipeptidases was done only with midgut caeca cell homogenates as the enzyme source and with Gly-Leu and Pro-Gly as substrates.

Differential centrifugation of midgut caeca homogenates

The subcellular distribution of enzymes in midgut caeca cells is displayed in Fig. 1. Most lactate dehydrogenase is recovered in the final supernatant (Fig. 1), although some activity is found associated with membranes. Similar results were found in subcellular fractionation of Tenebrio molitor midgut cells and were shown to result from unspecific adsorption caused by high-salt homogenizing medium (Ferreira et al., 1990). Soluble dipeptidases predominate in the final supernatant, whereas membrane-bound dipeptidases are recovered mainly in PP_2, PP_3 and PP_4 (Fig. 1).

Soluble dipeptidases from midgut caeca

Only one activity against Gly-Leu is resolved when the soluble fraction of midgut caeca cells is submitted to density-gradient ultracentrifugation (Fig. 2a) or to gel electrophoresis (Fig. 2c). This activity seems to be

distinct from that of aminopeptidase (Fig. 2b, d) and corresponds to a protein with a Mr value of 100,000 ± 10,000, as calculated from data in Fig. 2a.

Gel filtration of the soluble fraction of midgut caeca cells results in resolution of the activity upon Gly-Leu into two peaks and a shoulder (Fig. 3a), with Mr values of 117,00, 73,000 and 63,000. Except for the enzyme with Mr 117,000, the other enzymes are also active upon Pro-Gly (Fig. 3b). The enzyme which hydrolyzes Gly-Leu, but not Pro-Gly, is supposed to be an aminopeptidase, as it is eluted with this enzyme (Fig. 3). It should be recalled that R.americana midgut soluble aminopeptidase are able to hydrolyze some dipeptides (Ferreira and Terra, 1984). The observed minor peak of activity on LpNA is due to an aminopeptidase A, which is enriched in these preparations (Fig. 3c). Data on this aminopeptidase will appear elsewhere (Klinkowstrom et al., 1994). The Mr 63,000 dipeptidase is more active on Pro-Gly than on Gly-Leu, whereas the contrary is true for the Mr 73,000 dipeptidase. The soluble dipeptidases are well resolved by ion-exchange chromatography (Fig. 4). Nevertheless, dipeptidase recoveries are lower with this method than with the others. The dipeptidase which recovery is worse seems to be that which is more active on Gly-Leu. This explains the fact that after ion exchange chromatography the activities of the two dipeptidases on Gly-Leu become similar, and one of the activities on Pro-Gly almost disappears. The dipeptidases

partially purified by ion-exchange chromatography are differentially inhibited by aminoacyl hydroxamates (Table 2). Both dipeptidases are more inhibited by 1,10-phenanthroline than by EDTA (Table 2). Dipeptidase inhibition by phenanthroline is completely reversed by dialysis (data not shown).

Membrane-bound dipeptidases from midgut caeca

About 90% of the dipeptidase activity originally present in the midgut caecal membranes of R. americana is solubilized in Triton X-100, and about 40% of the original activity is released into solution by treatment with papain. In each case, the solubilized and the non-solubilized activity add up to the activity found originally in the membrane preparation.

Density-gradient centrifugation suggests that there is one detergent (Mr 130,000 ± 14,000) form and one papain (Mr 89,000 ± 8000) form of the membrane-bound dipeptidase. These enzymes are distinct from the aminopeptidase (Fig. 5). The activities on Gly-Leu other than that of dipeptidase are apparently due to aminopeptidase. Gel filtration of detergent forms confirms that there is only one molecular form of dipeptidase (Mr 86,000), which is active on Gly-Leu and Pro-Gly and is distinct from the aminopeptidase, which has some activity on Gly-Leu but not on Pro-Gly (Fig. 6). Identical conclusions are obtained from ion-exchange chromatography data with detergent forms (Fig. 7).

Discussion

Midgut distribution of dipeptidase activity in R. americana larvae

Dipeptidase activity in R. americana is found mainly in midgut caeca cells (Table 1), the major site of terminal digestion in this insect (Terra et al., 1979). The caecal dipeptidase activity is partly soluble and partly membrane bound. Differential centrifugation of midgut caeca homogenates, followed by assays of enzyme markers and dipeptidase, showed that soluble dipeptidase occurs primarily in the final supernatant, as observed for lactate dehydrogenase (Fig. 1). This suggests that soluble dipeptidase is a cytosolic enzyme. Another possibility is that the enzyme is extracellular, but weakly associated with the cell glycocalyx. When the tissue was homogenized, the enzyme may have been released in a manner similar to that previously shown to occur with dyes fed to Tenebrio molitor larvae and which subsequently became associated with the midgut cell surface (Ferreira et al., 1990). Present data are not sufficient to decide between the two possibilities, as intracellular dipeptide hydrolysis is known to occur in some animals, as mammals (Ugolev et al., 1990).

Membrane-bound dipeptidases are recovered mainly in PP_2, PP_3 and PP_4 (Fig. 1). This subcellular distribution resembles that of alkaline phosphatase, although dipeptidase activity in PP_1 is significantly different from that of

alkaline phosphatase. This suggests that dipeptidases and alkaline phosphatase occur in somewhat different membranes. It is probable that dipeptidases are associated with microvillar membranes, whereas alkaline phosphatase occurs both in microvillar and in baso-lateral membranes. Alkaline phosphatase occurring in microvillar and baso-lateral membranes of vertebrate enterocytes is known (Schmitz et al., 1973).

Properties of soluble and membrane-bound R. americana midgut caeca dipeptidases

The soluble caecal dipeptidase activity is resolved by gel filtration into two enzymes hydrolyzing both Gly-Leu and Pro-Gly, although with different efficiency. The two enzymes, which may also be resolved by ion-exchange chromatography, also differ in their stability in the presence of EDTA and degree of inhibition by phenanthroline and aminoacyl hydroxamates.

R. americana soluble dipeptidases are more affected by phenanthroline than by EDTA and the inhibition caused by phenanthroline is completely reversed by dialysis. Chelating agents are usually considered to inhibit metalloenzymes by removing metal ions from the active site or by forming an inactive ternary complex with the metal and the enzyme. In the first type, enzyme activity cannot be restored on dialysis, but in the second type restoration of activity is possible. The action of EDTA on insect midgut

aminopeptidases seems to be of the first type (Ferreira and Terra, 1984, 1986), whereas the action of phenanthroline may be of the second type (Ferreira and Terra, 1984) or of a third type (Ferreira and Terra, 1986). In the latter, phenanthroline binds in the neighborhood of the hydrophobic substrate decreasing its binding. The action of phenanthroline on soluble caecal dipeptidases may be of the third type described above, since these dipeptidases are able to bind hydrophobic leucine residues in their active sites.

The caecal membrane-bound dipeptidase was solubilized by Triton X-100 and papain. Density-gradient centrifugation, gel filtration and ion-exchange chromatography reveal that there is only one molecular species of dipeptidase which is active on Gly-Leu and Pro-Gly. Papain treatment decreases the Mr value (measured by ultracentrifugation) of membrane-bound dipeptidase in the presence of detergent from 130,000 to 89,000. This suggests that papain (in accordance with the findings obtained by other authors with other systems; see review by Kenny and Maroux, 1982) separates a catalytic and hydrophilic domain of the dipeptidase from a non-catalytic and presumably hydrophobic domain, which might be responsible for insertion of the enzyme into the microvillus plasma membrane.

The soluble and membrane-bound caecal dipeptidases described above are able to hydrolyze Gly-Leu and resemble dipeptide hydrolase (dipeptidase, EC 3.4.13.11) although, in

contrast to the mammalian enzyme (Norén et al., 1973) it is very active upon Pro-Gly. R. americana caecal cells seem to have in addition to dipeptide hydrolase a soluble and a membrane-bound aminoacyl-histidine dipeptidase (carnosinase, EC 3.4.13.3) which, in contrast to the mammalian enzymes are inhibited (instead of activated, see Lenney 1990 a,b) by dithiothreitol and Mn^{++}.

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Legends of figures

Fig. 1. Distribution of hydrolases among the subcellular fractions of Rhynchosciara americana midgut caeca. The enzyme markers of subcellular fractions were alkaline phosphatase (AP), plasma membrane; lactate dehydrogenase (LDH), cytosol; and succinate dehydrogenase (SDH), mitochondria. After differential centrifugation, the fractions P_1, P_2, P_3, and P_4 were collected and homogenized, and after 3 freezing and thawing cycles they were centrifuged at 100,000 g for 60 min. The supernatants (SP) and pellets (PP) corresponding to each fraction were assayed. Clear areas correspond to supernatants and shaded ones to pellets. The data are means and range based on four assays performed in each of two different preparations obtained from 100 larvae. Fractions (supernatants and pellets) are displayed from left to right in the order they were obtained (SP_1, PP_1, SP_2, PP_2, SP_3, PP_3, SP_4, PP_4 and final supernatant, respectively). The recovery of each hydrolase activity in subcellular fractions was between 80 and 100% of the homogenate activity. The length of horizontal bars (with or without histogram bars) is proportional to the percentage of total protein. The height of the histogram is proportional to the relative specific activity (ratio of the specific

activity of the sample to that of the homogenate, which is established as one), whereas the area of the histogram is proportional to the percentage amount (% total activity) of the enzyme. The absence of a histogram means that no activity was found in the corresponding sample. Soluble (S) and membrane (M) bound dipeptidase was assayed with Pro-Gly or Gly-Leu as substrate.

Fig. 2. Resolution of soluble aminopeptidases and dipeptidases from R.americana midgut caeca cells. Sedimentation profiles in a linear glycerol gradient: (a) assay with Gly-Leu and (b) assay with LpNA. Electrophoretic separation in 6% polyacrylamide gel: (c) assay with Gly-Leu and (d) assay with LpNA. Mr markers: C, catalase (Mr 232,000); H, bovine hemoglobin (Mr 64,500).

Fig. 3. Gel filtration of soluble aminopeptidases and dipeptidases from R.americana midgut caeca cells in a Superose HR 10/30 column. The column was equilibrated and eluted with 20 mM MES pH 7.0. Activity on (a) Gly-Leu; (b) Pro-Gly and (c) LpNA.

Fig. 4. Ion exchange chromatography of soluble aminopeptidase and dipeptidases from R.americana midgut caeca cells in a Mono Q HR 5/5 column equilibrated with 20 mM MES buffer pH 7.0. After applying the sample, the column was washed with 5 ml of 20 mM MES buffer pH 7.0 and then eluted with

a 0.1-0.5 M NaCl gradient. Fractions were collected at a flow rate of 1 ml/min. Activity on (a) Gly-Leu, (b) Pro-Gly and (c) LpNA.

Fig. 5. Sedimentation profiles of membrane-bound aminopeptidases and dipeptidases from R.americana midgut caeca cells. The enzymes were solubilized in Triton X-100 (a and b) or released into solution by papain (c and d). Activity on (a) Gly-Leu; (b) LpNa; (c) Gly-Leu and (d) LpNa.

Fig. 6. Gel filtration of detergent-solubilized membrane-bound aminopeptidases and dipeptidases from R.americana midgut caeca cells in a Superose HR 10/30 column. The column was equilibrated and eluted with 20 mM MES buffer pH 7.0 containing 0.1% Triton X-100. Activity on (a) Gly-Leu, (b) Pro-Gly and (c) LpNA.

Fig. 7. Ion exchange chromatography of detergent-solubilized membrane-bound aminopeptidases and dipeptidases from R.americana midgut cells in a Mono Q HR 5/5 column equilibrated with 20 mM MES buffer pH 7.0, containing 0.1% Triton x-100. Other details as in the legend of Fig. 4. Activity on (a) Gly-Leu; (b) Pro-Gly and (c) LpNa.

TABLE 1. Dipeptidases and protein present in different midgut sites of R.americana larvae*

Substrate	Caecal Contents	Caecal cells		Anterior Ventricular cells	Posterior ventricular cells		PM Contents
		Soluble	Membrane		Soluble	Membrane	
Gly-Leu	8.1(68)	39(107)	38(169)	1.6(13)	8.9(38)	2.5(27)	1.8(6.0)
Pro-Gly	4.0(21)	51(113)	21(69)	3.2(19)	15(52)	4.2(37)	1.8(4.3)
Protein (µg/animal)	80	110	70	46	70	25	63

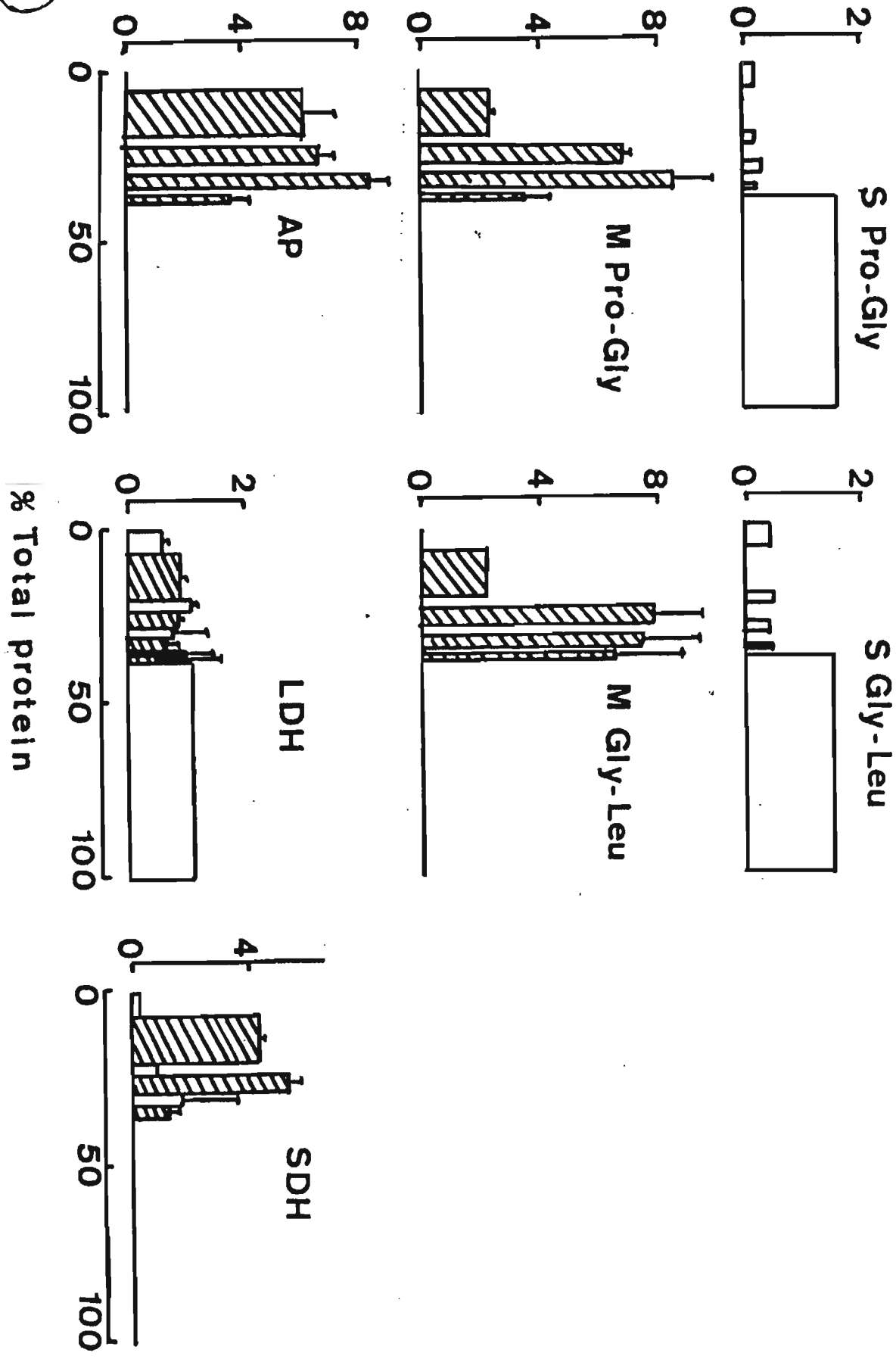
* Enzyme results are relative activities displayed as percentage of the activities found in the different sections of the midgut and specific activities (in parentheses) displayed as mUnits/mg. Soluble and membrane enzymes are those remaining in supernatant and pellet, respectivelly, of homogenates centrifuged at 100,000 g for 60 min at 4°C. Figures are means based on 4 determinations carried out in each of 3 different preparations of 100 animals. SEM were found to be about 25% of the means. The activities and protein found in the different midgut sections sum 50-70% of the activities and protein determined in whole midguts. PM, peritrophic membrane.

TABLE 2. Inhibition (%) of R.americana midgut caeca soluble dipeptidases by different compounds*.

Compound(mM)	D_1	D_2
EDTA (30)	16±4	40±10
1,10-Phenanthroline (2.7)	65±4	98±1
Arginine hydroxamate (1.25)	59±6	53±4
Aspartate hydroxamate (1.25)	50±10	93±1
Glycine hydroxamate (1.25)	90±4	82±7
Leucine hydroxamate (1.25)	66±8	79±5

*D_1 and D_2 correspond to the dipeptidases eluting from the ion exchange column (see Fig.4) with lower and higher NaCl concentration, respectivelly. Figures are means and SEM (n=3).

Fig. 1


Relative specific activity
(% Total activity / % total protein)
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M Pro-Gly
S Pro-Gly
LDH
M Gly-Leu
S Gly-Leu
SDH
% Total protein
0
50
100

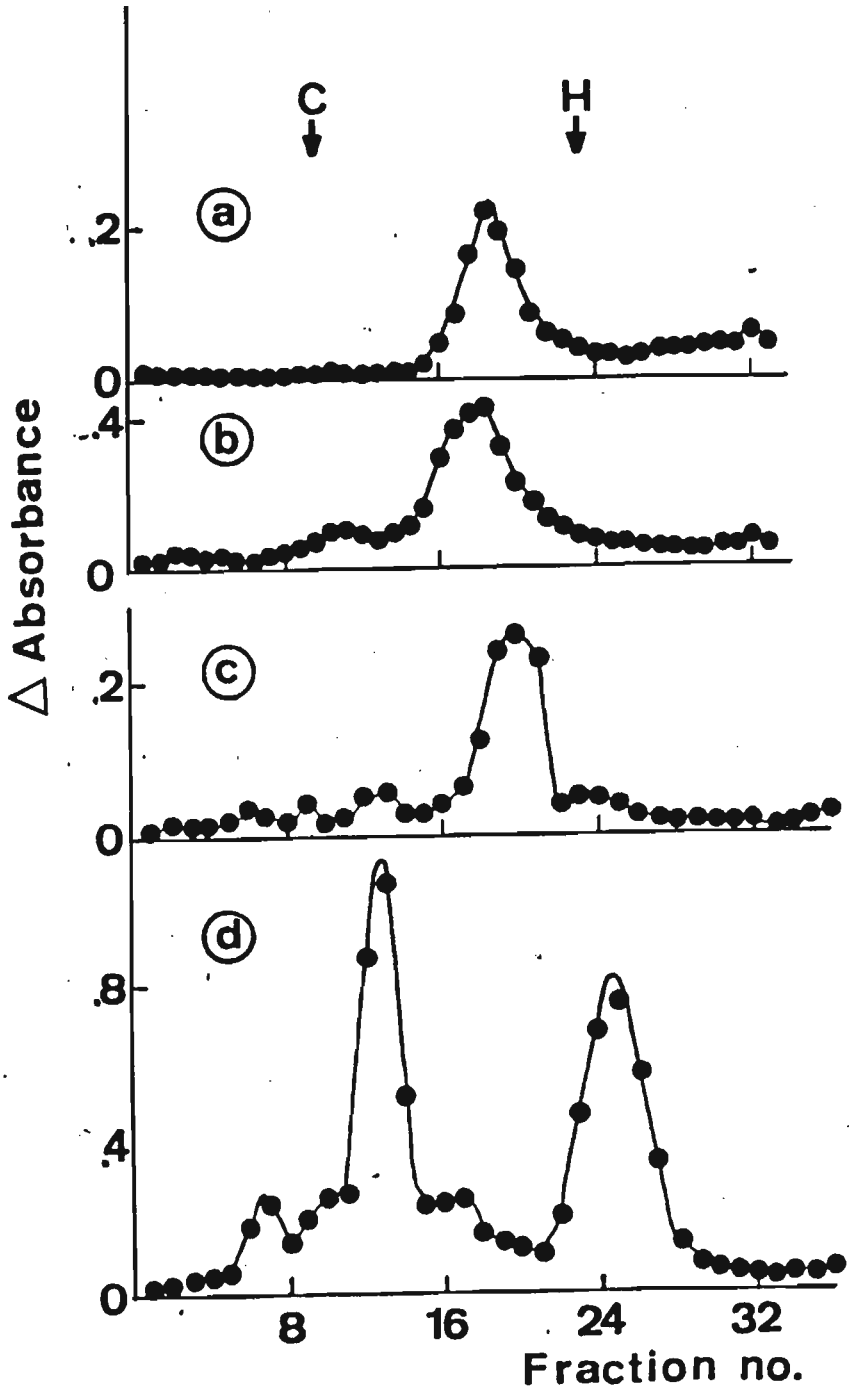


Fig. 2

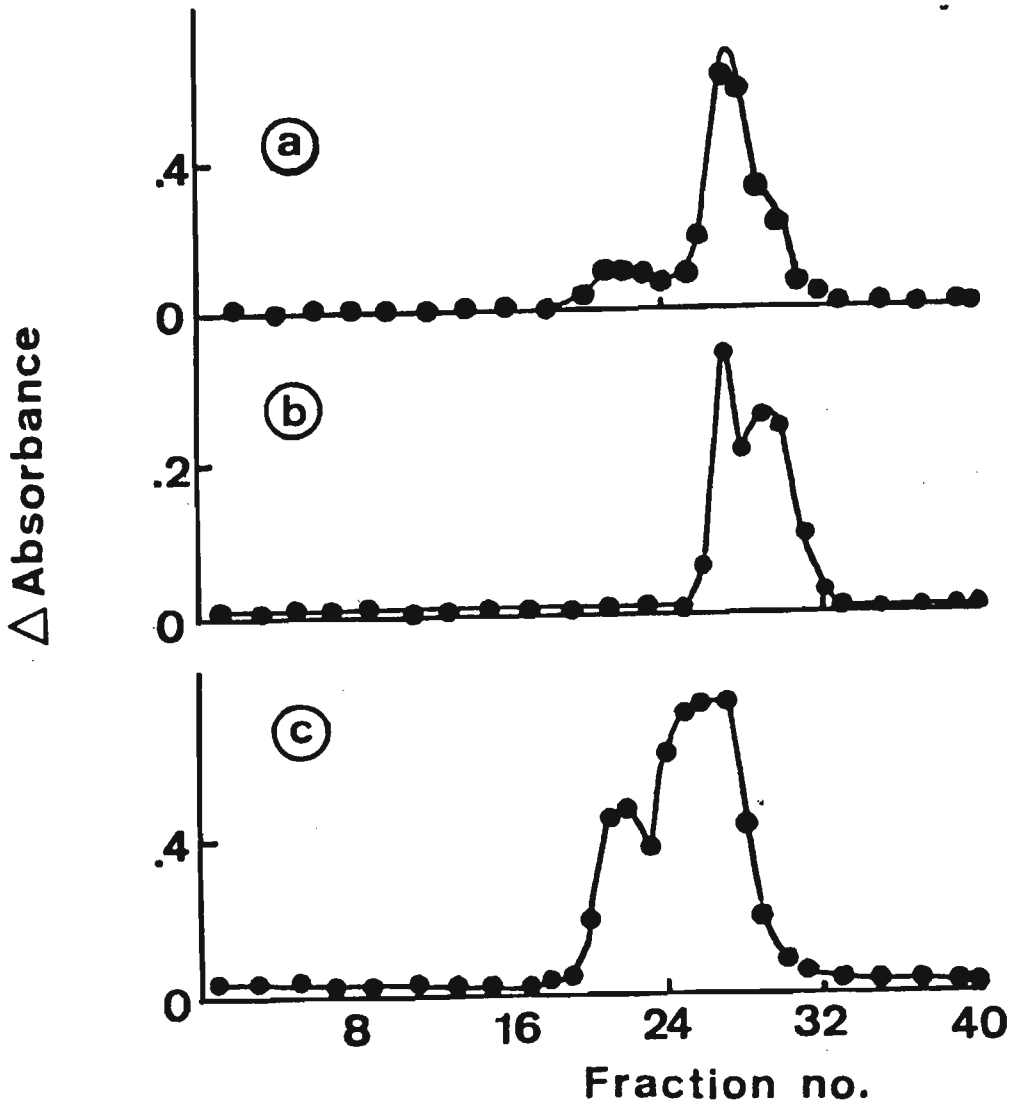
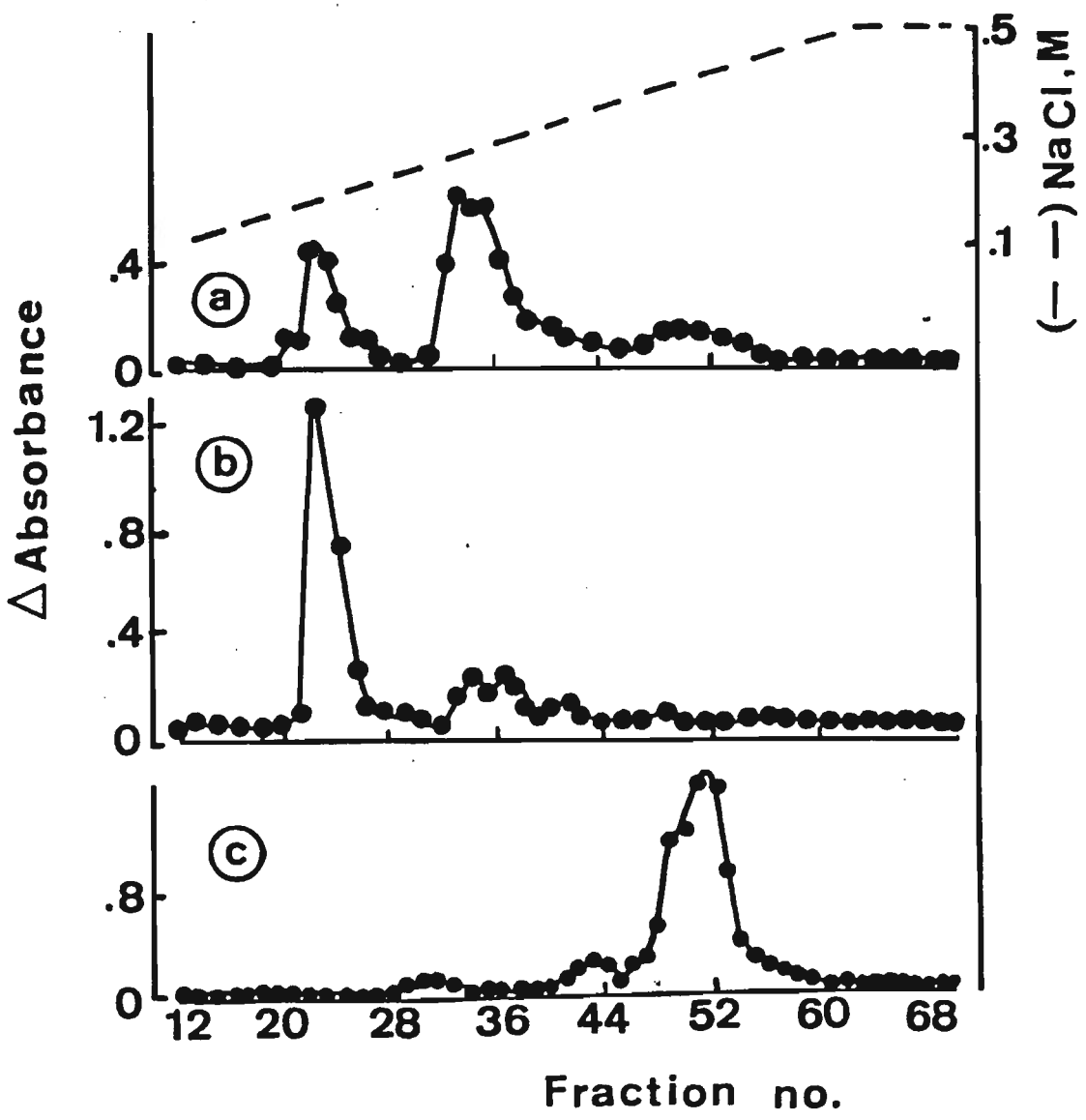


Fig. 3


Δ Absorbance
(— —) NaCl, M
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.3
.1
.4
0
a
1.2
.8
.4
0
b
.8
0
c
12
20
28
36
44
52
60
68
Fraction no.

Fig. 4

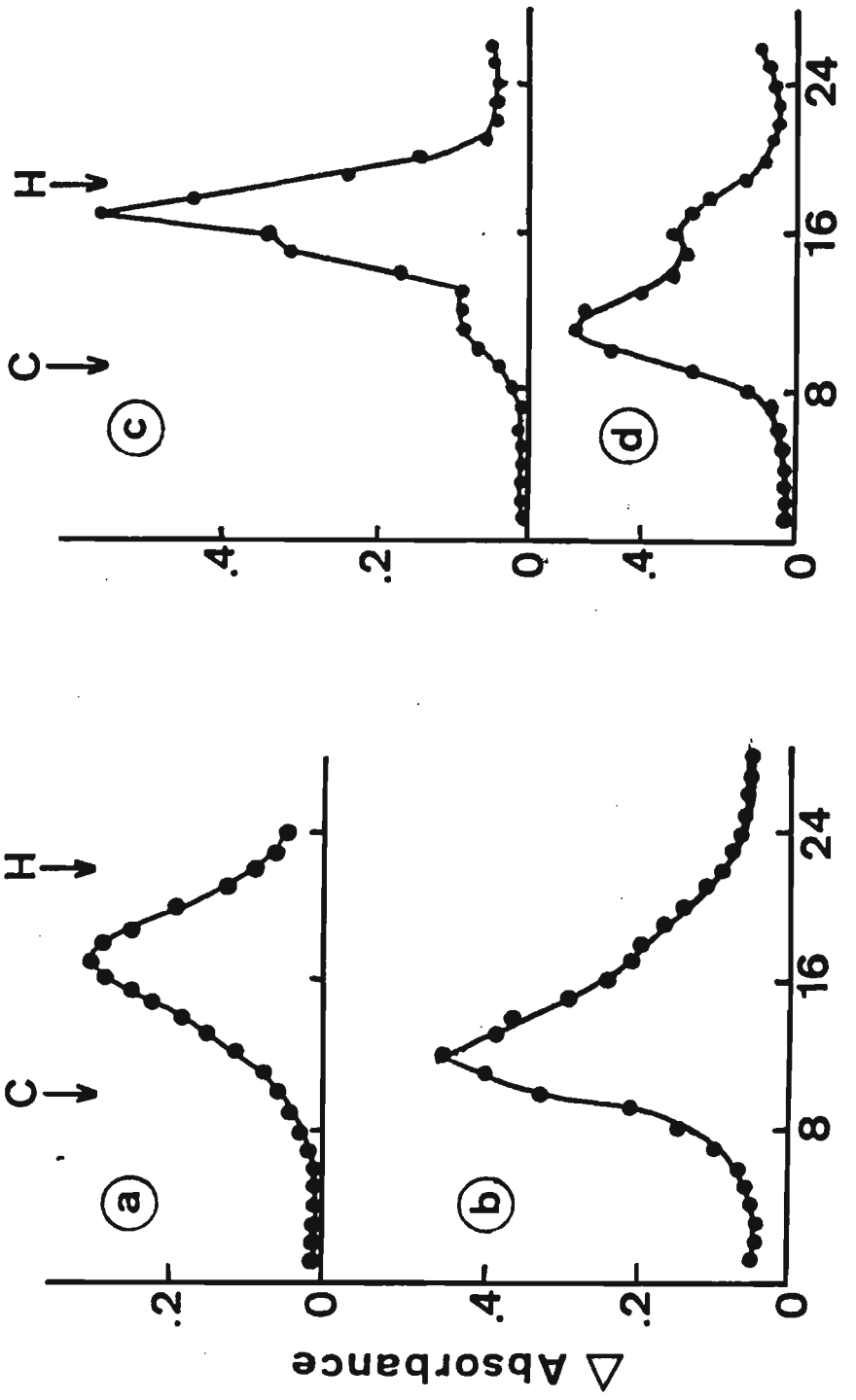

Fig. 5

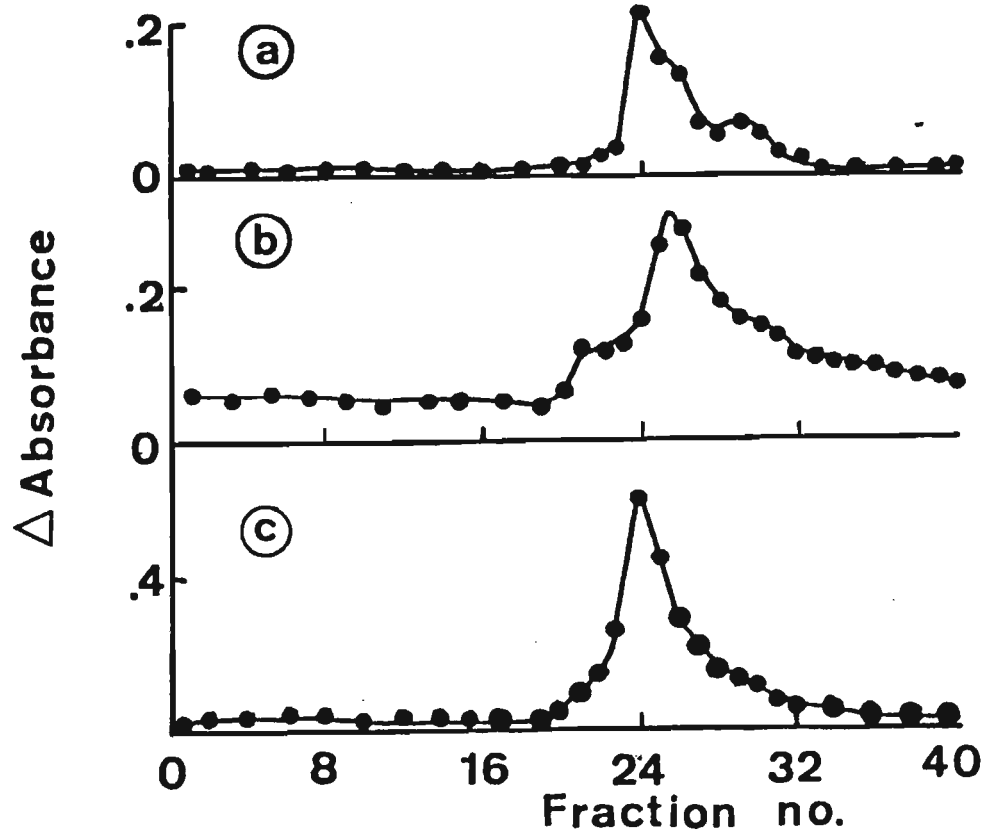

Fig. 6

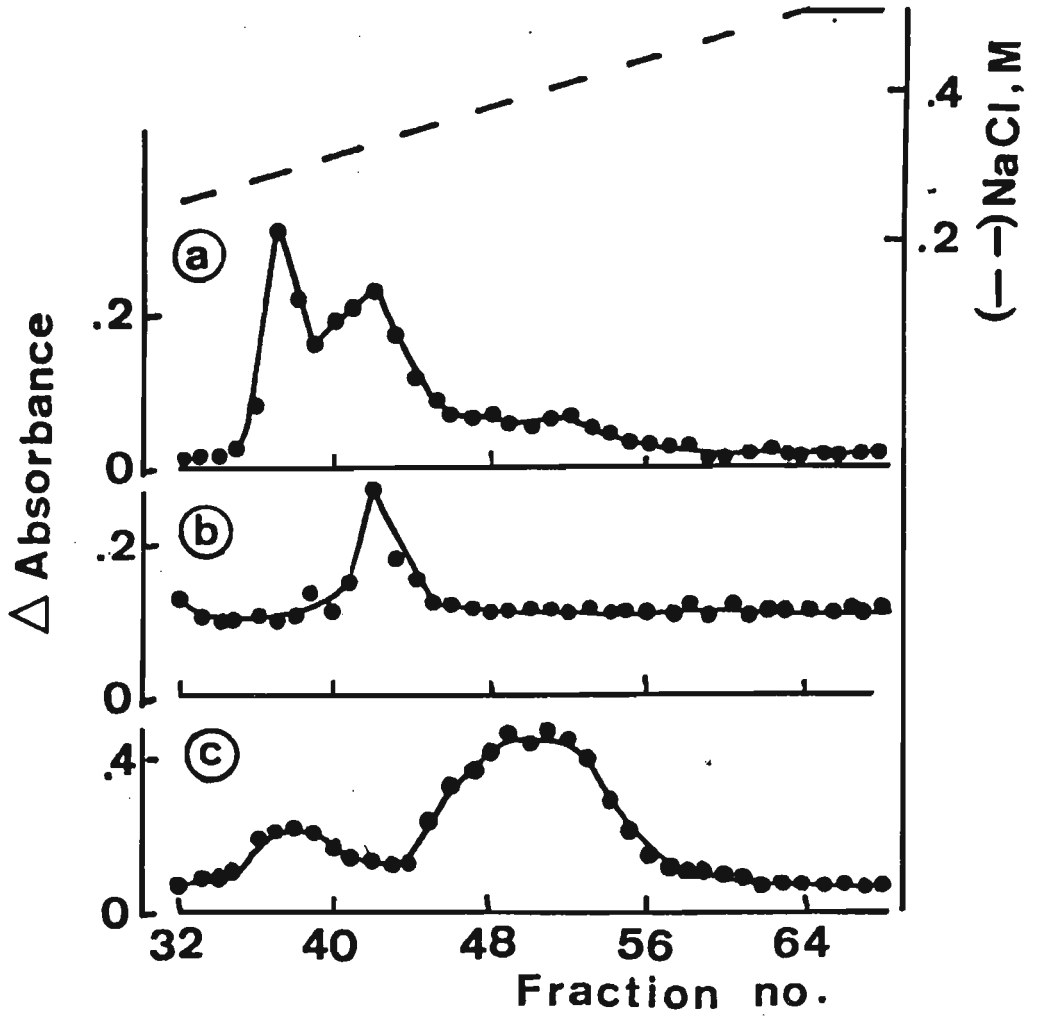

Fig. 7

Aminopeptidase A from Rhynchosciara americana (Diptera) larval midguts. Properties and midgut distribution.

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Abstract

L-Aspartic acid α-(β-naphthylamide) (AspβNA) hydrolase activity is almost restricted to the midgut caeca of R. americana larvae. The membrane-bound activity is solubilized in detergent and, after electrophoretic separation, proved to be identical to leucine p-nitroanilide (LpNA) hydrolases previously described. Differential centrifugation of midgut caeca homogenates, followed by assays of enzyme markers and aminopeptidase, suggest that the soluble AspβNA hydrolase is associated to the cell glycocalyx. R. americana midgut caeca soluble aminopeptidades are resolved into three activities by gel electrophoresis. The slow migrating activity hydrolyzes AspβNA well and displays a low activity on LpNA and Proline β-naphthylamide (ProβNA). Thus, this enzyme is an aminopeptidase A (EC 3.4.11.7). It has a pH optimum of 7.5, Mr 117,000 (gel filtration) and is competitively inhibited by aspartate hydroxamate (K_i 0.1 mM). Nevertheless, this enzyme, in constrast to the vertebrate enzyme, is not activated by calcium ions. The aminopeptidase A seems to have a charge variant that displays an intermediate migration and is not resolved from an aminopeptidase N (enzyme very active on LpNA). These two activities are not resolved also by either gel filtration or ion-exchange chromatography. The aminopeptidades N with intermediate and high migration, previously reported to be charge variants,

were shown in this paper to differ in substrate specificities and in the strength they associate to the cell glycocalyx.

key words: aminopeptidase A, aminopeptidase N, terminal digestion, aminopeptidase properties, soluble aminopeptidases.

Footnote to Introduction

Abbreviations: AlaβNA, L-alanine-β-naphthylamide; ArgβNA, L-arginine-β-naphthylamide; ArgpNA, L-arginine-p-nitroanilide; AspβNa, L-aspartic acid α-(β-naphthylamide); LeuβNA, L-leucine-β-naphthylamide; LpNA, L-leucine-p-nitroanilide; MES, 2-(N-morpholino) ethanesulfonic acid; MetβNA, DL-methionine-β-naphthylamide; ProβNA, L-proline-β-naphthylamide.

Introduction

Aminopeptidases (EC 3.4.11.) hydrolyze single amino acids from the N-terminus of the peptide chain and are classified on the basis of their dependence on metal ions and substrate specificity. The best known digestive aminopeptidases are those found in mammalian guts associated to the microvillar membranes (aminopeptidase N and A) or in the cytosol (leucine aminopeptidase, aminopeptidase B, and tripeptide aminopeptidase) of enterocytes. Aminopetpidase N (EC 3.4.11.2) has a broad specificity, although it removes preferentially alanine and leucine from peptides, whereas aminopeptidase A (EC 3.4.11.7) prefers aspartyl (or glutamyl)-peptides as substrates (Norén et al., 1986). Leucine aminopeptidase (EC 3.4.11.1) has a broad specificity towards peptides and displays a low activity upon LpNA; aminopeptidase B (EC 3.4.11.6) removes only N-terminal arginine or lysine from peptides, and tripeptide aminopeptidase (EC 3.4.11.4) cleaves exclusively unsubstituted tripeptides, except those displaying a charged N-terminal amino acid (Sjostrom and Norén, 1986).

There are few studies with insect midgut aminopeptidases that include substrate specificity data. Thus, detailed substrate specificity studies are known only for the Mr 115,700 soluble (Ferreira and Terra, 1984) and the two membrane-bound (Mr 107,000 and 169,000) aminopeptidases of R. americana (Ferreira and Terra, 1985), and for the midgut aminopeptidases of A. megatoma (Baker and

Woo, 1981) and T. bisselliella (Ward 1975 a, b). These enzymes have a wide specificity, although they differ in relation to their action on di-, tri- and tetrapeptides. In spite of the differences found, all those enzymes resemble vertebrate digestive aminopeptidase N. R.. americana midgut aminopeptidases were initially detected using LpNA as substrate. Thus, in spite of a detailed study of these aminopeptidases (Ferreira and Terra 1984, 1985, 1986a,b), the possibility remains that this insect displays aminopeptidases that hydrolyze substrates other than LpNA.

This paper describes the properties of a soluble aminopeptidase A found in R. americana larval midguts, and also reports on their midgut and intracellular distribution. This is the first report of an aminopeptidase A in insect midguts, and the data reveal that it differs from the mammalian enzyme in not being activated by calcium ions.

Materials and Methods

Animals

Rhynchosciara americana (Diptera: Sciaridae) were reared as described by Lara et al. (1965). We used only mature feeding female larvae at the end of the second period of the fourth instar (Terra et al., 1973).

Preparation of samples

Larvae were dissected in ice cold 0.1 M NaCl. After the removal of the gut, the luminal fluid was collected from the caeca with the aid of a capillary and the gut was cut at the

proventriculus and at the opening of the Malpighian tubules. The peritrophic membrane with its paste-like contents and the midgut caeca were then removed and the remaining ventriculus divided into an anterior and a posterior section. The entire midgut, as well as the midgut caeca, anterior and posterior ventriculus were rinsed thoroughly with saline, and then homogenized in cold double-distilled water using a Potter-Elvehjem homogenizer. The luminal contents of the caeca were solubilized in a known volume of double distilled water. All the above preparations were then passed through a piece of nylon mesh of 100 µm pore size. The peritrophic membrane with its contents was homogenized like the other preparations, though without rinsing with saline, and centrifuged at 10,000 g for 10 min at 4°C. All the homogenates could be stored for at least five years at -20°C without noticeable change in the activity of the enzymes.

Differential centrifugation of homogenates

Midgut caeca tissue corresponding to 20 larvae were homogenized in a tight-fitting Dounce homogenizer (5-10 strokes) in 1.5 ml of isotonic 0.11 M KCl pH 7.0 containing 5 mM EDTA. The homogenates, after being filtered through a piece of nylon mesh of 45 µm pore size, were adjusted to contain material corresponding to 10 animals per ml and centrifuged at 4°C. The following fractions were collected: P_1, pellet 600 g, 10 min; P_2, pellet 3300 g, 10 min; P_3,

pellet 25,000 g, 10 min; P_4, pellet 100,000g, 60 min; FS, final supernatant. P_1 was suspended in the homogenization medium, recentrifuged (600g, 10 min) and the supernatants combined before fraction P_2 was collected. After differential centrifugation the pellets were collected and homogenized with the aid of a Potter-Elvehjem homogenizer in the previously used medium and, following three freezing and thawing cycles, they were centrifuged at 100,000g for 60 min. Thus, fraction P_1 resulted in a supernatant SP_1 and a pellet PP_1; fraction P_2 in SP_2 and PP_2; fraction P_3 in SP_3, and PP_3 and fraction P_4 in SP_4 and PP_4. The supernatants and pellets corresponding to each fraction were assayed for several enzymes. The following enzyme markers for subcellular fractions were assayed: succinate dehydrogenase (mitochondria), lactate dehydrogenase (cytosol) and alkaline phosphatase (plasma membrane) (Ferreira and Terra, 1980). Results are displayed according to De Duve et al. (1955). Thus, the height of the histogram is proportional to the specific ativity and the area of the histogram is proportional to the total activity of the enzyme.

Solubilization of midgut caeca plasma membranes in Triton X-100

R.americana midgut caeca were homogenized in iso-osmotic saline (0.11 M KCl, pH 7.0) in an Omni-Mixer (Sorvall) at 15,000 rev./min for 20s. The homogenates after being filtered through a piece of nylon mesh of 45 μm of

pore size, were centrifuged at 25,000g for 30 min at 4°C. The sediment, which contains the major part of the plasma membranes (Ferreira and Terra, 1980), was washed by centrifugation and then exposed to Triton X-100 for 20h at 4°C, in a ratio of 10 mg of Triton X-100/mg of protein, before being centrifuged at 25,000g for 30 min. No sediment was visible after the centrifugation of this supernatant at 100,000g for 60 min. The activity of the enzymes remains unchanged, at -20°C, for periods of at least a month.

Solubilization of membrane hydrolases in papain

To midgut caecal membranes (see previous item) suspended in 0.1 M potassium phosphate buffer, pH 7.0, activated (previously incubated with 60 mM-cysteine) papain was added in a ratio of 1 mg of papain to 10 mg of membrane protein. After 30 min at 30°C, samples were diluted twice with cold double-distiled water, centrifuged at 100,000g for 60 min and the resulting supernatants used as enzyme sources. The activity of the enzymes remains constant for at least a year at -20°C.

Protein determination and hydrolase assays

Protein was determined according to Bradford (1976) using ovalbumin as a standard. Alkaline phosphatase was assayed in 50 mM glycine-NaOH buffer pH 10.4 containing 1 mM $MgSO_4$ and 2.5 mM $ZnCl_2$, using 4mM p-nitrophenyl phosphate as

substrate, and following the release of nitrophenol according to Terra et al. (1979). Aminopeptidase was assayed in 50 mM Tris-HCl buffer pH 7.5 (its pH optimum) using one of several different substrates: AlaβNA, ArgβNA, AspβNA, LeuβNA, LpNA, MetβNA, and ProβNA. In aminopeptidase assays with LpNA as substrate (1 mM, final concentration), the release of p-nitroaniline was followed according to Erlanger et al. (1961). When any of the other substrates were used (0.5 mM, final cooncentration), the release of β-naphthylamine was determined according to Hopsu et al. (1966). Lactate dehydrogenase and succinate dehydrogenase were assayed according to Bergmeyer and Bernt (1974) and Ackrell et al. (1978), respectivelly, as detailed elsewhere (Santos and Terra, 1984).

Incubations were carried out for at least four different time periods, and initial rates of hydrolysis were calculated. All assays were performed under conditions such that activity was proportional to protein concentration and to time. Controls without enzyme and others without substrate were included. One unit of enzyme is defined as the amount that hydrolyzes 1 μmol of substrate per min.

Inhibition studies were accomplished by incubating different concentrations of inhibitor in each of several different concentrations of the substrate. K_i values were determined from replots of slopes and intercepts of Lineweaver-Burk plots against inhibition concentration (Segel, 1975).

Aminopeptidase heat inactivation was studied by incubating samples at 50°C, followed by the determination of the activity remaining at different times.

Density-gradient ultracentrifugation

Samples (0.2 ml) of midgut preparations were added to 1.5 mg of bovine hemoglobin and 50 µg of bovine liver catalase and were then layered onto 10 ml glycerol gradients (10-30%, w/v) made up in 50 mM sodium phosphate buffer pH 6.2. When samples solubilized in detergent were used, the buffer contained 0.1% Triton X-100. Centrifugation and collection of fractions were performed as described previously (Terra and Ferreira, 1983). Mr values (relative molecular weights) of enzymes assayed in the fractions were calculated by the method of Martin and Ames (1961), by using the sedimentation rates of bovine hemoglobin (Mr 64,500) and bovine liver catalase (Mr 232,000) as reference standards. Revoveries of the enzyme activities applied to the gradients were about 85%.

Gel filtration

Gel filtration was performed in a FPLC system (Pharmacia-LKB Biotechnology, Sweden) using a Superose HR 10/30 column equilibrated and eluted with 20 mM MES pH 7.0. When samples solubilized in detergent were used, the buffer contained 0.1% Triton X-100. The flow rate used was 0.4 ml/min. Mr values were calculated using the following

proteins as standards: cytochrome c (Mr 12,400), ovalbumin (Mr 45,000), aldolase (Mr 65,000) and L-amino acid oxidase (Mr 89,000). Recoveries of the enzyme activities applied to the column were about 95%.

Ion-exchange chromatography

Ion-exchange chromatography was performed in a FPLC system using a Mono-Q HR 5/5 column equilibrated with 20 mM MES buffer pH 7.0. When samples solubilized in detergent were used, the buffer contained 0.1% Triton X-100. A dialysate containing the proteins to be separated was applied to the column, which was washed with 5 ml of the same buffer, and then eluted with 0.1-0.5M NaCl in the same buffer. The flow rate used was 1 ml/min. The recoveries of the enzyme activities applied to the column were about 80%.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out in polyacrylamide gels prepared as described by Hedrick and Smith (1968), using the system of Davis (1964), in glass tubes of 5 mm id and 100 mm length. Other details have been described elsewhere (Terra and Ferreira, 1983). The recoveries of the enzyme activities applied to the gels were about 65%.

Results

Distribution of aminopeptidase activities in midgut regions

Aminopeptidase activity assayed with LpNA is found mainly in midgut caeca contents and in midgut caeca cells, where they occur both soluble and membrane bound (Table 1). In contrast, most activity assayed with AspβNA is found in midgut caeca cells, mainly membrane bound (Table 1).

The subcellular distribution of enzymes in midgut caeca cells is displayed in Fig. 1. Most lactate dehydrogenase is recovered in the final supernatant (Fig. 1), although some activity is found associated with membranes. Similar results were found in subcellular fractionation of Tenebrio molitor midgut cells and shown to result from unspecific adsorption caused by high-salt homogenizing medium (Ferreira et al. 1990). Membrane-bound aminopeptidase activities with both substrates are recovered mainly in PP_3 and PP_4 (Fig. 1). Soluble aminopeptidases display higher specific activities in SP_1, mainly the enzyme active on AspβNA, which is also very active on SP_2, SP_3 and SP_4.

Membrane-bound aminopeptidases from midgut caeca

About 90% of the aminopeptidase activity originally present in the midgut caecal membranes of R. americana is solubilized in Triton X-100, and about 40% of the original activity is released into solution by treatment with papain. In each case, the solubilized activity add up to the activity found originally in the preparation of membranes.

Detergent-solubilized membrane-bound aminopeptidases are resolved by gel electrophoresis into 5 peaks active on

LpNA (Fig. 2). Peaks T_1, T_2, T_3, and T_4 have been described before (Ferreira and Terra, 1985). Peak T_5 was probably resolved due to a larger number of gel fractions used in this work in comparison to that used previously. The substrate specificity of T_5 is similar to that of T_1, except that it does not hydrolyze AspβNA with efficiency.

Electrophoresis of papain-released membrane-bound aminopeptidases, followed by assays with the same substrates described in Fig. 2, results in all cases in three peaks of activity (results not shown). These peaks are identical to those reported previously as acting on LpNA (Ferreira and Terra, 1986a, b).

Soluble aminopeptidases from midgut caeca

Three aminopeptidases activities (AP_1, AP_2 and AP_3) are resolved when the soluble fraction of midgut caeca cells is submitted to gel electrophoresis (Fig. 3). AP_1 accounts for most activity on AspβNA and do not hydrolyze well LpNA (Fig. 3a, d, Table 2). The amount of AP_1 changes among different midgut caeca samples. This and the fact AP_1 is poorly active on LpNA has led to it beeing missed previously (Ferreira and Terra, 1984). AP_2 and AP_3 correspond to the aminopeptidases previously studied (Ferreira and Terra, 1984) and are able to hydrolyze, in addition to LpNA, AlaβNA, ArgβNA, MetβNA and LeuβNA (Fig. 3, Table 2). AP_2, but not AP_3, is also active on AspβNA and ProβNA (Fig. 3, Table 2). The subcellular distribution of AP_2 and AP_3 differs. AP_2 is

found mainly in SP_1, whereas AP_3 predominates in SF (Fig.4).

Attempts to resolve the activity on LpNA from the activity on AspβNA were carried out using electrophoresis in gels prepared with different acrylamide concentrations. The results were always similar to those shown in Fig. 5a, b. The activity profiles associated with A_1 were identical for LpNA and AspβNA, whereas for A_2 the profiles were slightly different (Fig. 5 a, b). Electrophoretic behavior (parallel lines in Fig. 5 c) suggest that AP_1 and AP_2 activity on AspβNA correspond to molecules with similar Mr values (187,000 and 180,000, respectively, calculated from Fig. 5c and 5e), but with different pI values (see theoretical aspects on Hedrick and Smith, 1968). Mr values calculated for AP_1 and AP_2 activities on LpNA (using data in Fig. 5d and 5e) were found to be 187,000 and 157,000, respectively.

The activities on LpNA and AspβNA were not resolved by gel filtration (Fig. 6) nor by ion-exchange chromatography (Fig. 7). Using both procedures, the major activity on AspβNA is eluted with the minor activity on LpNA, whereas the activities on ProβNA follows approximately those on LpNA (Figs. 6 and 7). Mr values determined for the two activities on LpNA were as follows: major activity: 82,000; minor activity: 117,000.

The results suggest that associated to A_1 there is only one aminopeptidase that hydrolyzes both LpNA and AspβNA, whereas A_2 seems to include at least two different aminopeptidases. To test this hypothesis, fractions

corresponding to A_1 and A_2 obtained from electrophoretical separations similar to that described in Fig. 5 were pooled. When pooled fractions corresponding to A_1 is submitted to ultracentrifugation in glycerol gradients, only one coincident activity is observed (Mr 200,000 ± 13,000) with LpNA, AspβNA, and ProβNA (Fig. 8a, b, c). In contrast, with material derived from A_2, there is a major activity on LpNA (Mr 109,000 ± 7,000) coincident with the major activity on ProβNA, which is resolved from two activities on AspβNA (Fig. 8 d, e, f). The activities on AspβNA, correspond to proteins with Mr 155,000 ± 10,000 (major activity) and Mr 220,000 ± 15,000 (minor activity). It seems that the aminopeptidases active on AspβNA display also a minor activity on LpNA and ProβNA (Fig. 8d, e, f).

Fractions containing A_1 and A_2 obtained by electrophoresis were subjected to inhibition by hydroxamates. Aspartate hydroxamate is a simple intersecting linear competitive inhibitor of the activity on AspβNA present both in A_1 and A_2, with similar K_i values of 0.10 mM and 0.09 mM, respectively (Fig. 9). When leucine hydroxamate is used as a inhibitor, and LpNA is the substrate, the activities present in A_1 and A_2 are inhibited (simple intersecting linear competitive inhibition) with distinct K_i values of 0.072 mM and 0.017 mM, respectively (Fig. 10).

Samples of A_1 and A_2 obtained as before were heated at 50°C, for different periods of time, and the remaining activity on AspβNA, LpNA and ProβNA determined (Fig. 11).

The heat inactivation kinetics of the activity on AspβNA is the same whichever sample (A_1 or A_2) was used (Fig. 11). Using A_1, the inactivation of the activity on LpNA follows that on AspβNA, whereas with A_2 the kinetics is different (Fig. 11). The activity on ProβNA inactivates as those on AspβNA and LpNA, if A_1 is used, or as that on LpNA, if A_2 is employed (Fig. 11). The inactivation kinetics of the activity on AspβNA supports the view that there is only one enzyme active on this substrate in A_1 and A_2, and that the enzymes in A_1 and A_2 seem to differ only in net charge. Thus, the activities on AspβNA found in A_2, and which are resolved by ultracentrifugation (Mr 220,000 and 155,000) (Fig. 8e), must be different aggregation states of the same enzyme. The two activities should not correspond to different enzymes. The minor activity has a Mr value identical, whereas the major activity necessarily have properties similar to that of the enzyme of A_1, as all the kinectical properties determined using AspβNA as substrates are the same with samples of A_1 and A_2.

The effect of calcium ions on the soluble activity that hydrolyzes AspβNA was studied. For this, samples dialysed and non-dialysed of the soluble fraction of midgut caeca homogenates were incubated with AspβNA in the presence and absence of 0.1 mM or 1.0 mM $CaCl_2$. No difference was found among assays.

Discussion

Midgut distribution of aminopeptidase activity in R. americana larvae

The major aminopeptidases that hydrolyze LpNA are secreted into the caecal contents and are also found in significant amounts both as soluble and membrane-bound forms in caecal cells (Terra et al., 1979; Ferreira and Terra, 1982, 1983, Table 1). The midgut caeca are the major site of terminal digestion in R. americana larvae (Terra et al., 1979). The soluble activity on AspβNA is almost restricted to caeca cells and seems not to be secreted (Table 1). The small activity observed in the caecal contents may result from cell desquamation and/or cell disruption during dissection, whereas the high activity recovered associated to caecal membranes are supposed to result from the same enzyme that hydrolyzes LpNA (Table 1 and text).

Differential centrifugation of midgut caeca homogenates, followed by assays of enzyme markers and aminopeptidase, showed that soluble aminopeptidase are more enriched in SP_1 (mainly the enzyme more active on AspβNA), although a large amount of the enzymes are recovered in SF, which corresponds to the cell cytosol (Fig. 1). Nevertheless, the presence of a high aminopeptidase activity in the cell cytosol has no biological significance, as only amino acids and dipeptides are supposed to be absorbed by midgut cells (Ugolev et al., 1990). The major amount of aminopeptidase activity recovered in fraction SP_1 (Fig. 1) must also be explained. One possibility is that the

aminopeptidase in this fraction is a cytosolic enzyme bound to cell fragments, but this has no support from intracellular distribution data on lactate dehydrogenase, a cytosolic marker, which relative activity in SP_1 is less than one (Fig. 1). It seems more likely that soluble activities in SP_1 resulted from enzymes trapped in the intermicrovillar glycocalyx space of midgut caeca cells. After freezing and thawing, these vesicles probably change in structure, the enzymes formerly present in the intermicrovillar glycocalyx space being released. Soluble activities in the final supernatant may result from the release during homogenization of enzymes loosely bound to the cell glycocalyx. These hypothesis are supported by the finding that non-absorbable dyes fed to Tenebrio molitor larvae become partly associated to the midgut cell surface and, on tissue homogenization and differential centrifugation, are recovered mostly in fraction SF and SP_i (Ferreira et al., 1990). Thus, depending on how much an enzyme is bound to the glycocalyx it is supposed to abound in P_1, final supernatant or at both fractions.

Properties of soluble and membrane-bound R. americana midgut caeca aminopeptidases.

Detergent-solubilized and papain-released membrane bound aminopeptidases from R. americana midgut caeca were resolved by gel electrophoresis and assayed with several different substrates. None aminopeptidase different from

those previously described (Ferreira and Terra 1986a, b) was found. Therefore, they will not be referred to anymore.

R. americana midgut caeca soluble aminopeptidases are resolved into three activities by gel electrophoresis. Associated with the activity displaying low electrophoretic migration (A_1), there is only one molecular species which hydrolyzes AspβNA well, and displays a low activity on LpNA and ProβNA. This enzyme presents different Mr values depending on the technique employed in the determination (gel filtration, 117,000; electrophoresis in native conditions, 187,000; gradient ultracentrifugation, 200,000). It is likely that the differences found result from different aggregation states of the enzyme. A charge variant of the enzyme that hydrolyzes AspβNA has an intermediate electrophoretic migration and is responsible for all activity on AspβNA (and part of the activity on LpNA and ProβNA) observed associated with A_2. This enzyme is not resolved from the major activity on LpNA and ProβNA. Another aminopeptidase has high electrophoretic migration (A_3) and is similar to the major LpNA hydrolase found in A_2, although differing somewhat in substrate specificity. The fact that rectilinear curves are observed when inhibition (by hydroxamates) and heat inactivation data are ploted using A_2 as enzyme source and LpNA as substrate is a consequence of only one enzyme in A_2 has high activity on LpNA. The same is true for heat inactivation data obtained using A_2 with ProβNA as substrate.

Ferreira and Terra (1984) using semi-preparative gel electrophoresis isolated from R. americana midgut caeca the major two soluble aminopeptidases active on LpNA. Employing LpNA and ArgpNA as substrates, they found that all physical and kinetic properties of the two enzymes, except for pI values, were identical. They concluded that the enzymes were charge isomers and the differences observed were thought to result from differential glycosilation. Nevertheless, in this work we have shown that the two enzymes have different substrate specificities (only one of them is able to hydrolyze AspβNA and ProβNA), suggesting they are different enzymes. Furthermore, evidence is presented in this paper that the two LpNA hydrolases have different subcellular distributions. The enzyme which is also active on ProβNA is found mainly in SP_1, whereas the other predominates in SF. Thus, based on subcellular distribution data previously discussed, the enzyme hydrolyzing ProβNA (p^I, 8.7; Ferreira and Terra, 1984) is more firmly bound to the glycocalyx than the enzyme inactive on ProβNA (p^I, 7.8; Ferreira and Terra, 1984). The differential binding of the LpNA hydrolases seems to depend on their net charges. The higher the p^I value is, the more positive the enzyme is supposed to be in physiological media, and hence more bound to the negatively charged glycocalyx.

The aminopeptidases present in the microvillar membranes of R. americana midgut caeca (Ferreira and Terra, 1985, 1986 a,b and this paper), as well as those soluble and

highly active on LpNA (Ferreira and Terra, 1984, this paper) resemble aminopeptidase N (EC 3.4.11.2), although displaying a somewhat broader specificity. The R. americana midgut soluble aminopeptidase highly active on AspβNA resembles the vertebrate aminopeptidase A (EC 3.4.11.7), in spite of not being activated by calcium ions. As much as we know this is the first report on the existence of an aminopeptidase A in insects.

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Legends of figures

Fig. 1. Distribution of hydrolases among the subcellular fractions of Rhynchosciara americana midgut caeca. The enzyme markers of subcellular fractions were alkaline phosphatase (AP), plasma membrane; lactate dehydrogenase (LDH), cytosol; succinate dehydrogenase (SDH), mitochondria. After differential centrifugation, the fractions (P_1, P_2, P_3, P_4) were collected and homogenized, and after 3 freezing and thawing cycles they were centrifuged at 100,000 g for 60 min. The supernatants (SP) and pellets (PP) corresponding to each fraction were assayed. Clear areas correspond to supernatants and shaded ones to pellets. The data are means and range based on four assays performed in each of two different preparations obtained from 100 larvae. Fractions (supernatants and pellets) are displayed from left to right in the order they were obtained (SP_1, PP_1, SP_2, PP_2, SP_3, PP_3, SP_4, PP_4 and final supernatant, respectively). The recovery of each hydrolase activity in subcellular fractions was between 80 and 100% of the homogenate activity. The length of horizontal bars (with or without histogram bars) is proportional to the percentage of total protein. The height of the histogram is

proportional to the relative specific activity (ratio of the specific activity of the sample to that of the homogenate, which is established as one), whereas the area of the histogram is proportional to the percentual amount (% total activity) of the enzyme. The absence of a histogram means that no activity was found in the corresponding sample. Soluble (S) and membrane (M) bound aminopeptidase was assayed with LpNA (L) or AspβNA (A) as substrate.

Fig. 2. Electrophoretic separation in 5.5% polyacrylamide gel of Triton X-100-solubilized membrane-bound aminopeptidases of R. americana midgut caecal cells. Assays with: (a) LpNA; (b) LeuβNA; (c) AlaβNA; (d) ArgβNA; (e) MetβNA; (f) AspβNA; (g) ProβNA.

Fig. 3. Electrophoretic separation in 6% polyacrylamide gel of soluble aminopeptidases from R. americana midgut caecal cells. Assays with: (a) LpNA; (b) MetβNA; (c) AlaβNA; (d) AspβNA; (e) ArgβNA; (f) LeuβNA; (g) ProβNA.

Fig. 4. Electrophoretic separation in 5% polycrylamide gel of soluble aminopeptidases from two subcellular fractions (SP_1 and SF) of R. americana midgut

caecal cells. (a) Sample, SP_1; substrate, LpNA; (b) sample, SP_1; substrate, ProβNA; (c) sample, SF; substrate, LpNA; (d) sample, SF; substrate, ProβNA.

Fig. 5. Electrophoretic properties of soluble aminopeptidases from R. americana midgut caeca cells. (a) and (b) electrophoretic separation of aminopeptidases in 6% polyacrylamide gels assayed with LpNA (a) and AspβNA (b). The most active fractions (✕) of A_1 and A_2 were pooled for later use. (c) Effect of different polyacrylamide gel concentrations on the electrophoretical migration of soluble AspβNA hydrolase present in A_1 (○) or A_2 (●). Rm, electrophoretic migration of the enzyme in relation to the tracking dye. (d) Same as (c) for the LpNA hydrolases present in A_1 (■) and A_2 (△). (e) Standard curve for the determination of the Mr values by electrophoresis in different concentrations of polyacrylamide gel using the method of Hedrick and Smith (1968). The slopes of the plots displayed in Figs. 5c and 5d are M values. Mr markers: M, myoglobin (Mr 17,800); O, ovalbumin (Mr 45,000); H, bovine hemoglobin (Mr 64,500); C, catalase (Mr 232,000); and F, ferritin (Mr 450,000).

Fig. 6. Gel filtration of soluble aminopeptidases from R. americana midgut caeca cells in a Superose HR 10/30 column. The column was equilibrated and eluted with 20 mM MES buffer pH 7.0. Assays on: (a) LpNA; (b) AspβNA; and (c) ProβNA.

Fig. 7. Ion exchange chromatography of soluble aminopeptidases from R. americana midgut caeca cells in a Mono Q HR 5/5 column equilibrated with 20 mM MES buffer pH 7.0. After applying the sample, the column was washed with 5 ml of 20 mM MES buffer pH 7.0 and then eluted with 0.1-0.5 M NaCl. Fractions were collected at a flow rate of 1 ml/min. Assays on: (a) LpNA; (b) AspβNA; and (c) ProβNA.

Fig. 8. Sedimentation profiles of purified soluble aminopeptidases from R. americana midgut caeca cells in a linear glycerol gradient. Samples were pooled fractions corresponding to either A_1 (○) or A_2 (●) from the experiment described in Fig. 5a. Assays on: (a) LpNA; (b) AspβNA; (c) ProβNA; (d) LpNA; (e) AspβNA; and (f) ProβNA. Mr markers: C, catalase (Mr 232,000); H, hemoglobin (Mr 64,500).

Fig. 9. Inhibition of purified soluble aminopeptidases from R. americana midgut caeca cells by aspartate hydroxamate. Lineweaver-Burk plots for different

concentrations of hydroxamate (a and c) and replots (b and d) of slopes calculated from Lineweaver-Burk plots against the concentration of hydroxamate. The enzyme sources were pooled fractions corresponding to either A_1 (a and b) or A_2 (c and d) from the experiment described in Fig. 5a. Assays were performed with AspβNA as substrate.

Fig.10. Inhibition of purified soluble aminopeptidases from R. americana midgut caeca cells by leucine hydroxamate. Lineweaver-Burk plots for different concentrations of hydroxamate; insets, replots of slopes calculated from Lineweaver-Burk plots against the concentration of hydroxamate. The enzyme sources were pooled fractions corresponding to either A_1 (a) or A_2 (b) from the experiment described in Fig. 5a. Assays were performed with LpNA as substrate.

Fig. 11. Thermal inactivation, at 50°C, of purified soluble aminopeptidases from R. americana midgut caeca cells. The enzyme sources were pooled fractions corresponding to either A_1 (○, □, △) or A_2 (●, ■, ▲) from the experiment described in Fig. 5a. Assays on: (△, ▲) LpNA; (○, ●) AspβNA; and (□, ■) ProβNA.

TABLE 1. Aminopeptidases and protein present in different gut sites of R.americana larvae*

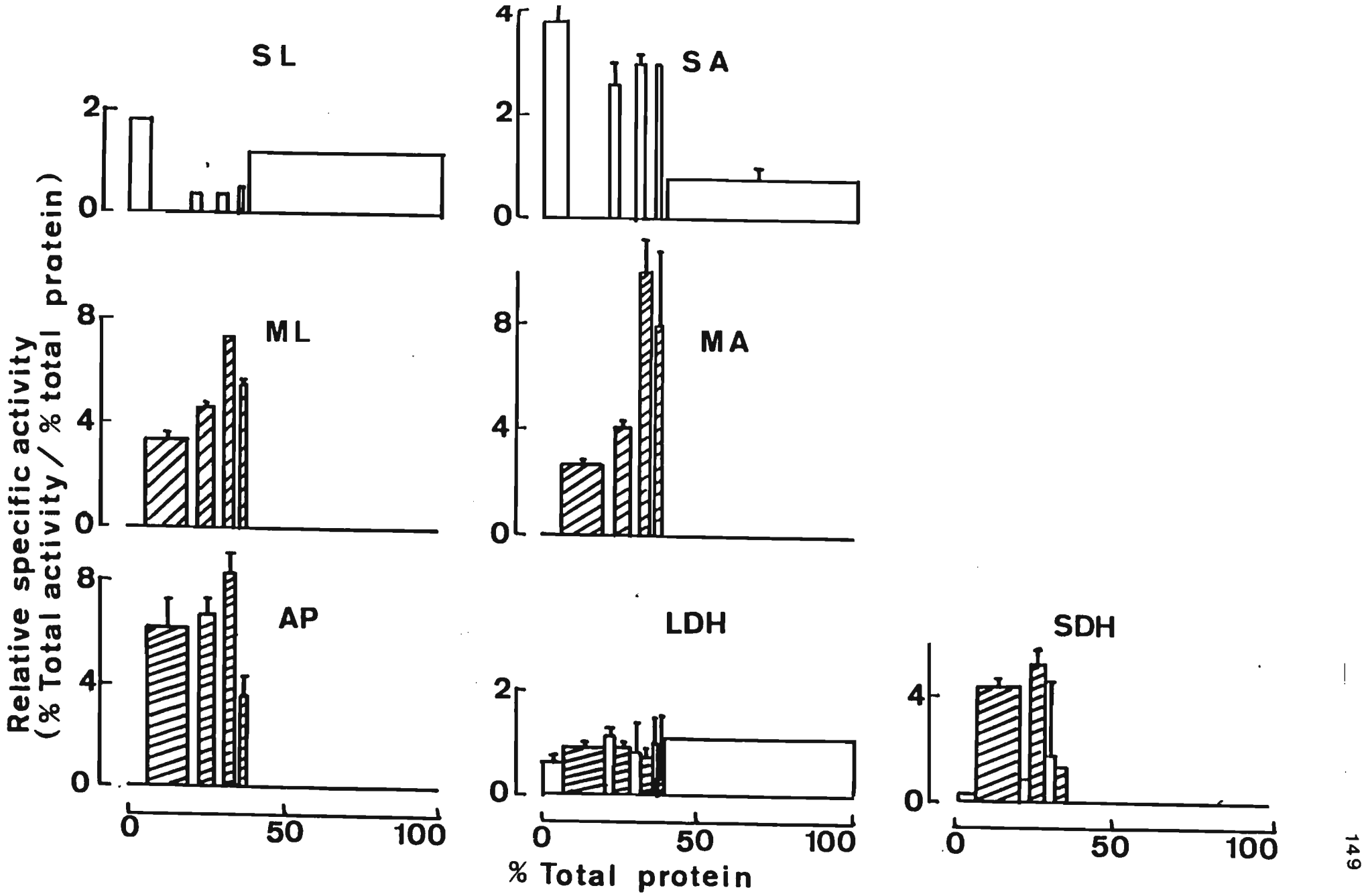
Substrate	Caecal Contents	Caecal cells Soluble	Caecal cells Membrane	Anterior Ventricular cells	Posterior ventricular cells Soluble	Posterior ventricular cells Membrane	PM Contents
AspβNA	1.2 (10)	13 (60)	80 (430)	1.0 (10)	1.2(14)	3.5 (57)	0.6 (0.6)
LpNA	33 (3500)	16 (960)	23 (2200)	4.1 (680)	12 (1200)	4.5 (1100)	7.0 (820)
Protein (μg/animal)	80	110	70	46	70	25	63

* Enzyme results are relative activities displayed as percentage of the activities found in the different sections of the midgut and specific activities (in parentheses) displayed as mUnits/mg. Soluble and membrane enzymes are those remaining in supernatant and pellet, respectivelly, of homogenates centrifuged at 100,000 g for 60 min at 4°C. Figures are means based on 4 determinations carried out in 3 different preparations of 100 animals each. SEM were found to be about 25% of the means. The activities and protein found in the different midgut sections sum 70-90% of the activities and protein determined in whole midguts. PM, peritrophic membrane.

TABLE 2. Substrate specificity of soluble aminopeptidases from R.americana midgut caeca.

Substrate	AP_1	AP_2	AP_3
LpNA	3±1	50±1	47±1
AlaβNA	3±2	78±2	19±4
AspβNA	53±4	47±4	0
ArgβNA	3.0±0.1	66±2	31±1
LeuβNA	4±1	61±3	35±3
MetβNA	3±3	66±8	31±4
ProβNA	1±1	99±1	0

Figures are percentage of the total aminopeptidase activity on each substrate, followed by range (n=2). Calculations were performed from two sets of profiles similar to those presented in Fig.3.


SL
SA
ML
MA
AP
LDH
SDH
Relative specific activity
(% Total activity / % total protein)
% Total protein
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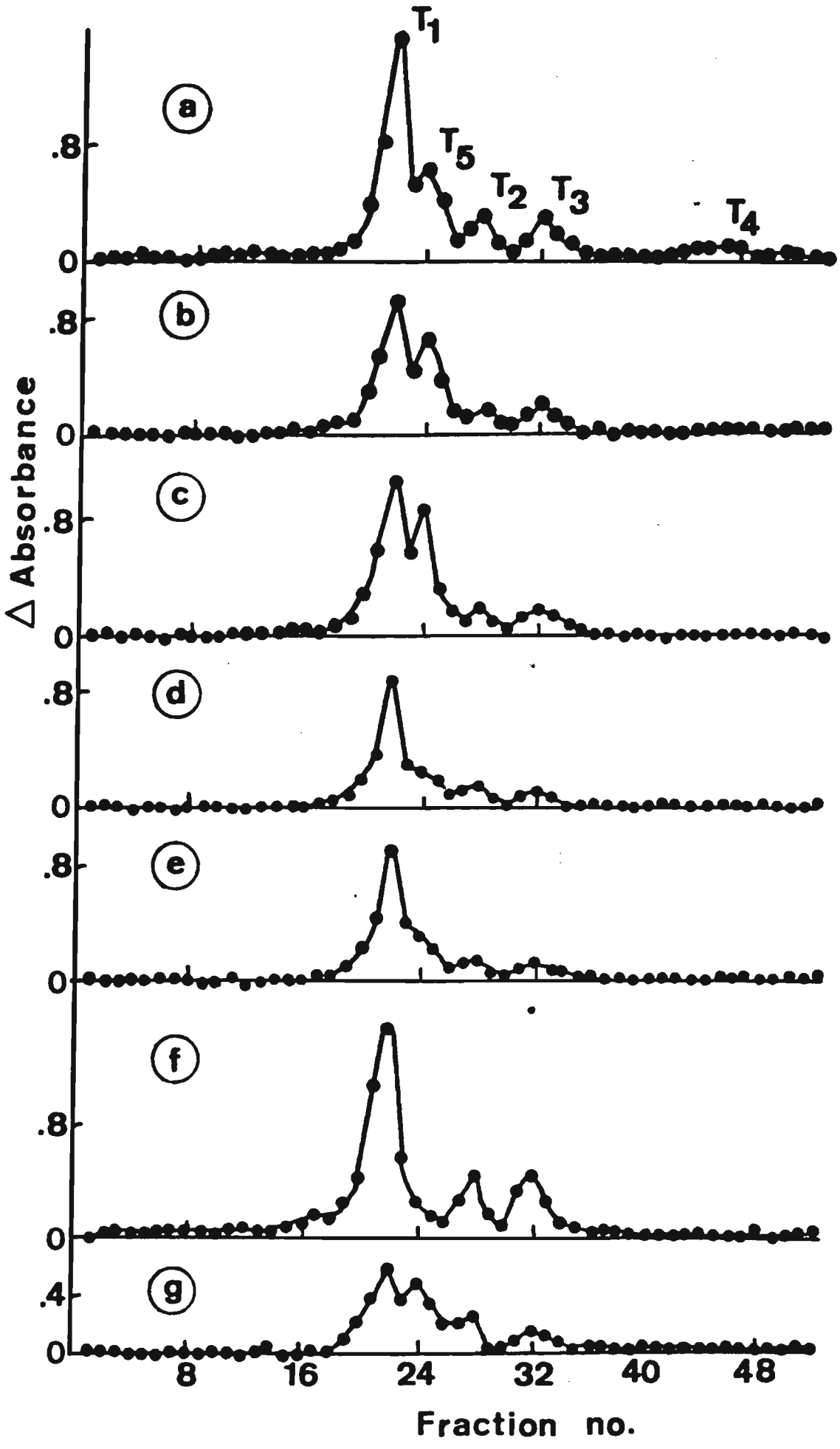
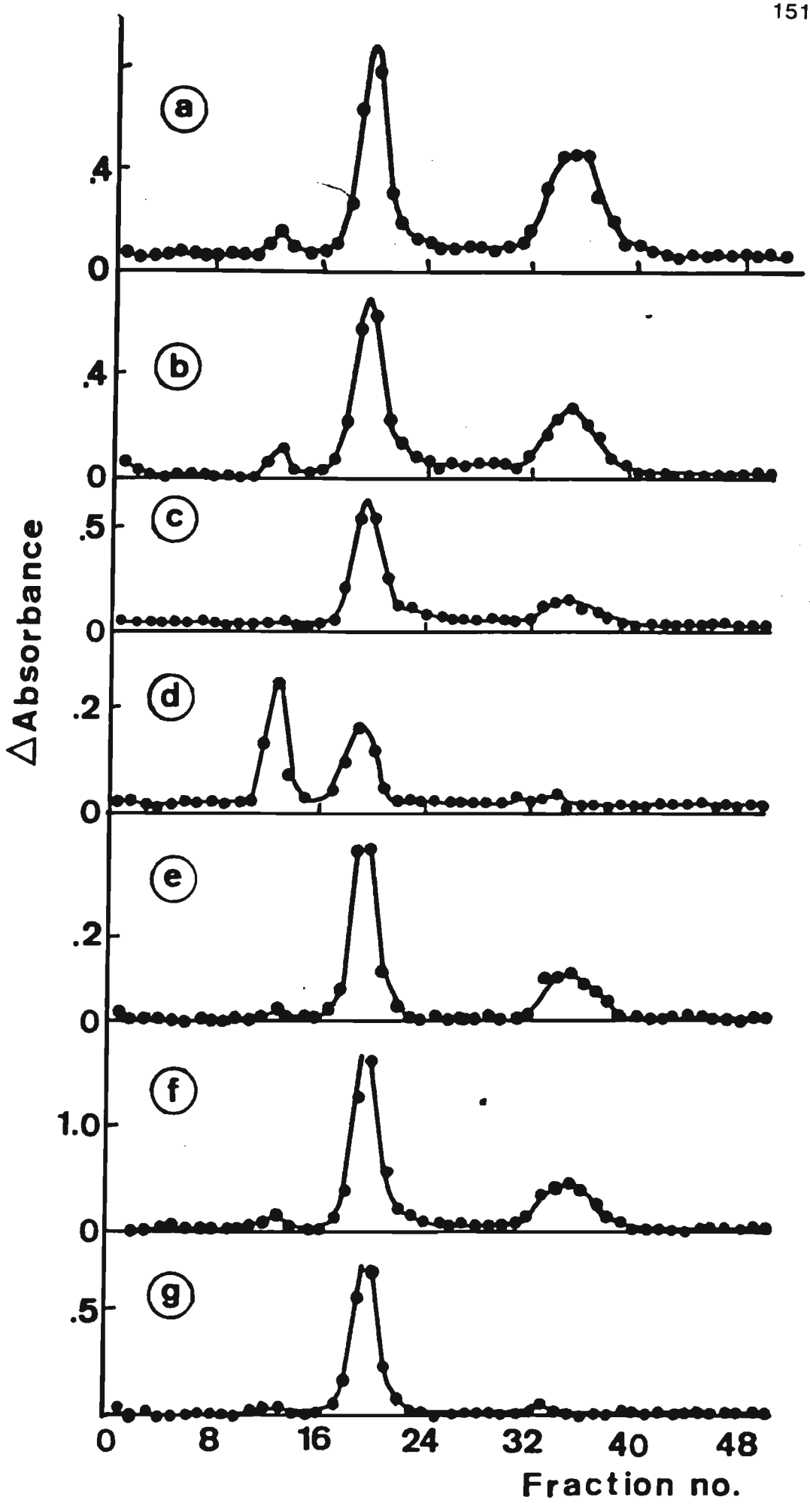
50
100
2
4
8


T1
T5
T2
T3
T4
a
b
c
d
e
f
g
.8
.4
0
Δ Absorbance
8
16
24
32
40
48
Fraction no.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c
d
e
f
g
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.4
0
.5
0
.2
0
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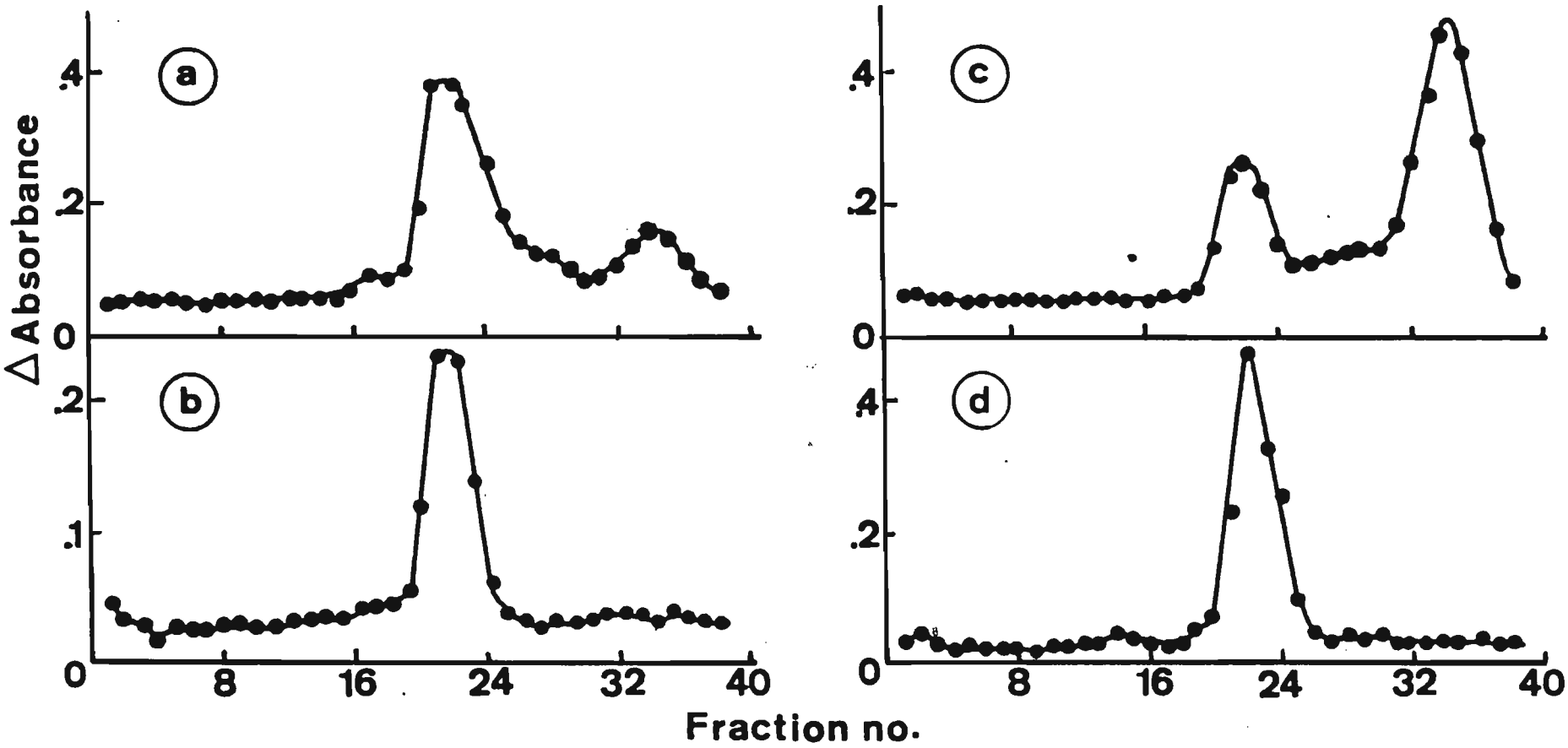
0
.5
ΔAbsorbance
0
8
16
24
32
40
48
Fraction no.

Fig 3


a
b
c
d
Δ Absorbance
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0
8
16
24
32
40
8
16
24
32
40
Fraction no.

Fig 4

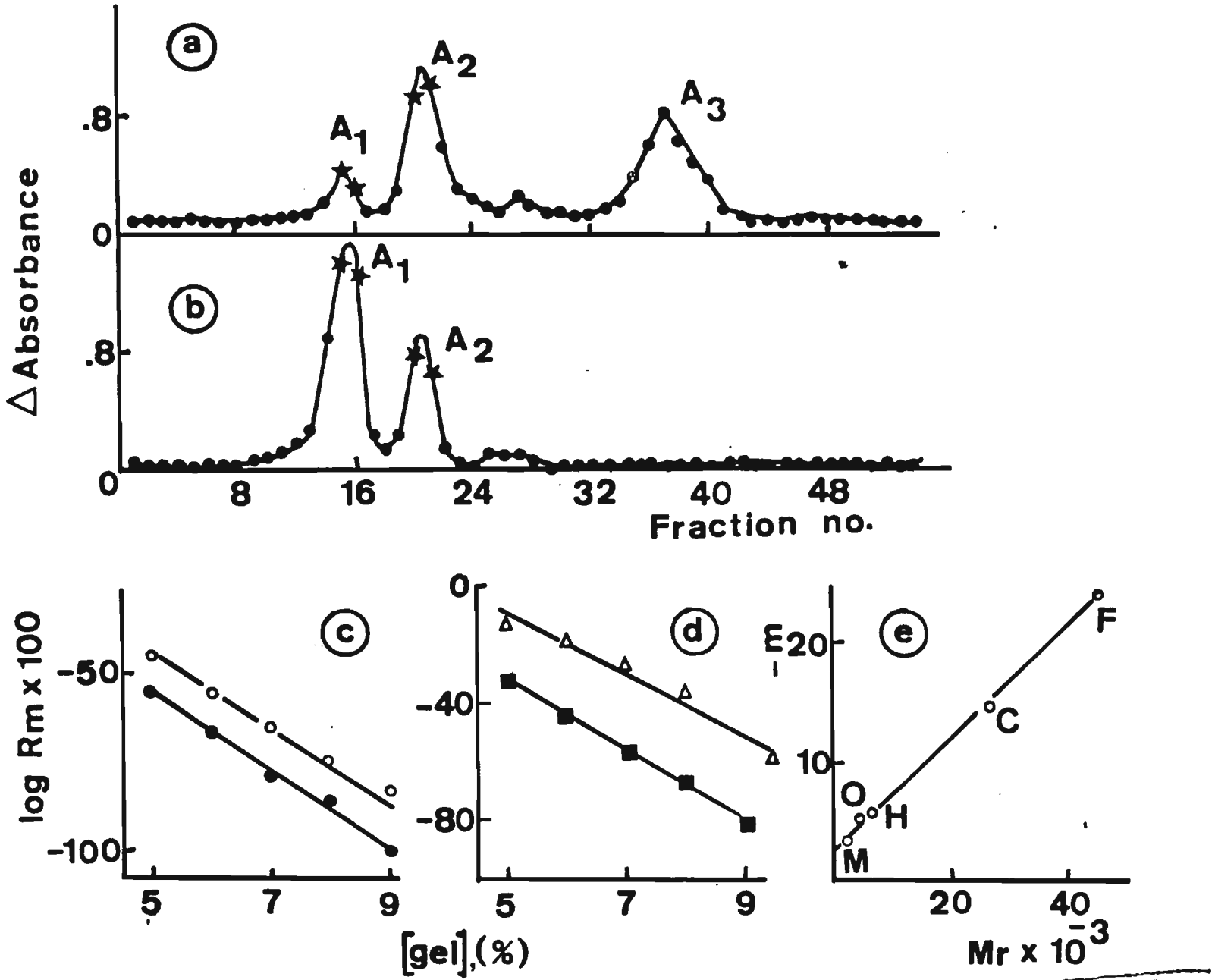

a
b
c
d
e
A1
A2
A3
.8
0
ΔAbsorbance
8
16
24
32
40
48
Fraction no.
log Rm x 100
-50
-100
5
7
9
[gel],(%)
-40
-80
E20
10
O
H
M
C
F
20
40
Mr x 10-3

Fig 5

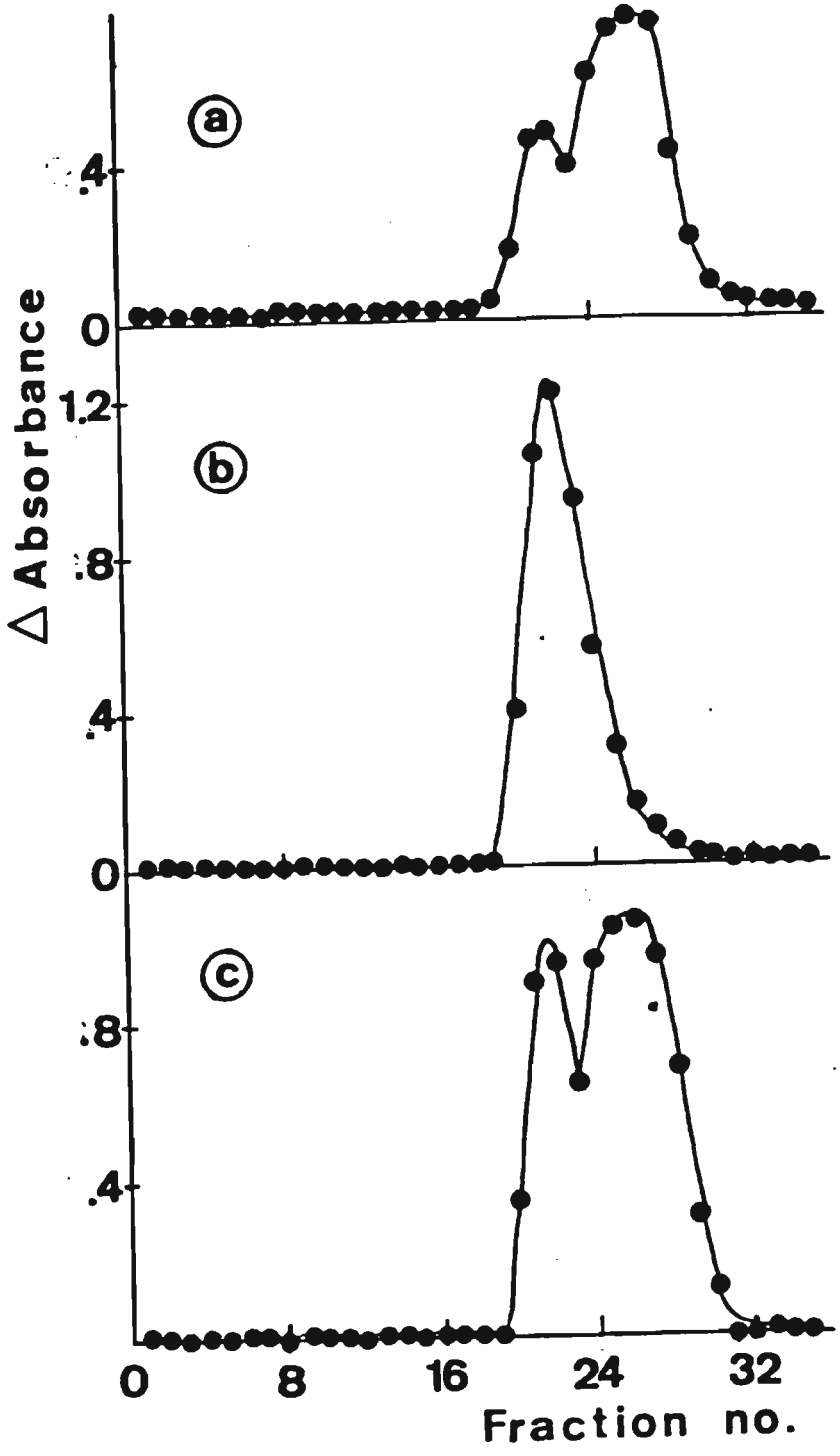
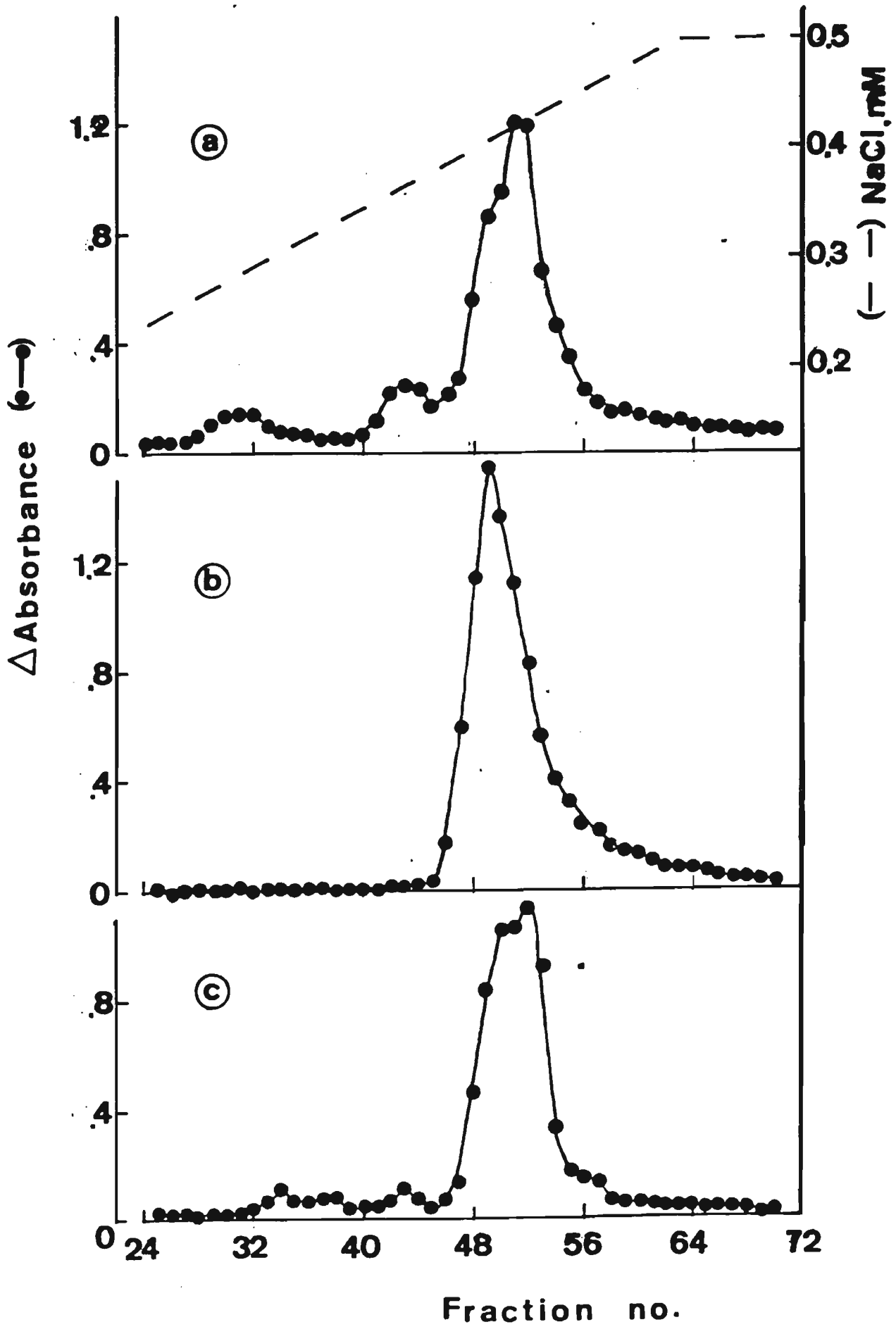


Fig 6


ΔAbsorbance (●—●)
(—) NaCl, mM
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0.4
0.3
0.2
1.2
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.4
0
a
b
c
24
32
40
48
56
64
72
Fraction no.

Fig 7

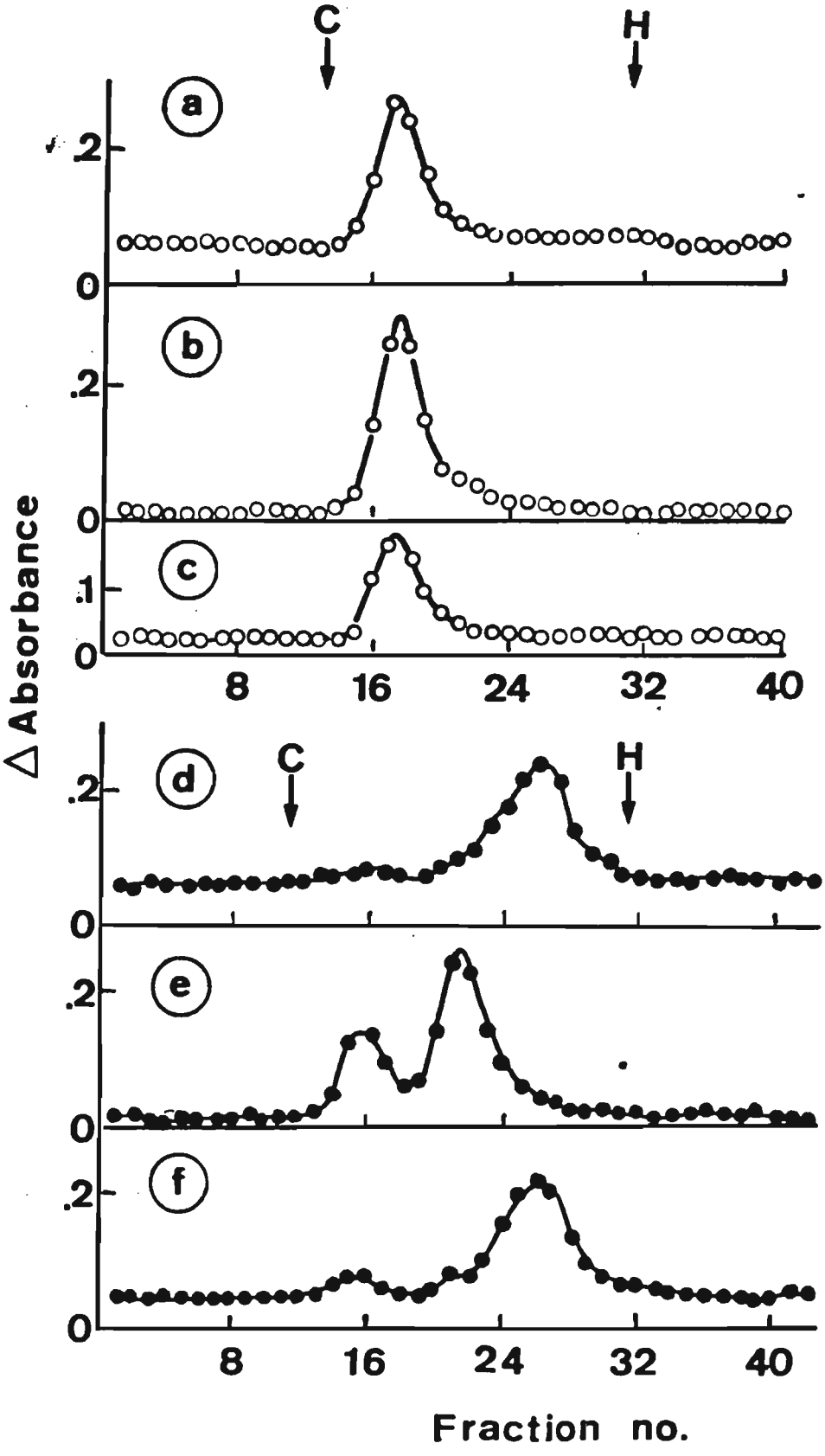
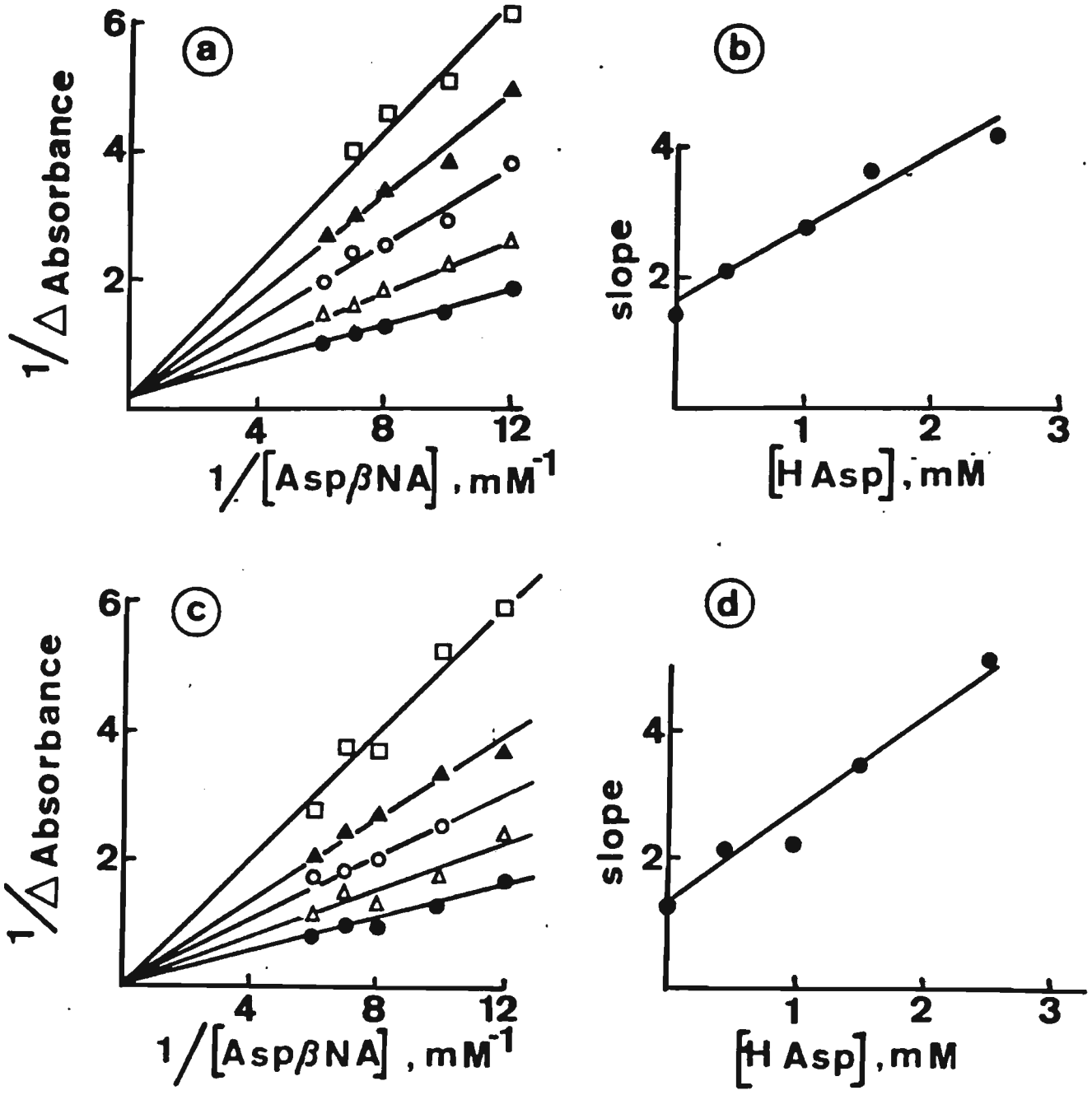
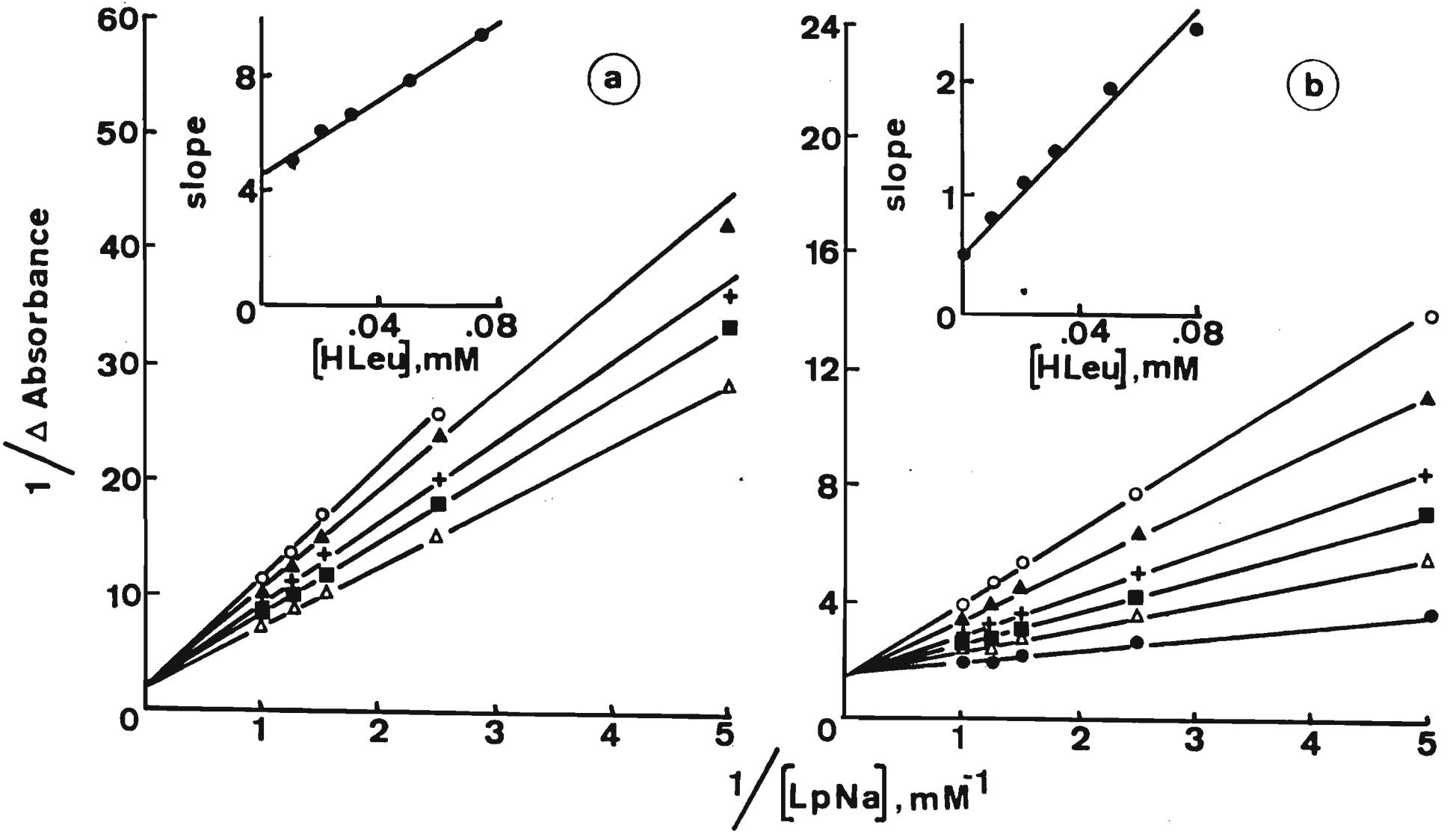


Fig 8


a
1/Δ Absorbance
6
4
2
4
8
12
1/[AspβNA], mM⁻¹
b
slope
4
2
1
2
3
[HAsp], mM
c
1/Δ Absorbance
6
4
2
4
8
12
1/[AspβNA], mM⁻¹
d
slope
4
2
1
2
3
[HAsp], mM


a
b
slope
[HLeu],mM
1/Δ Absorbance
1/[LpNa],mM⁻¹

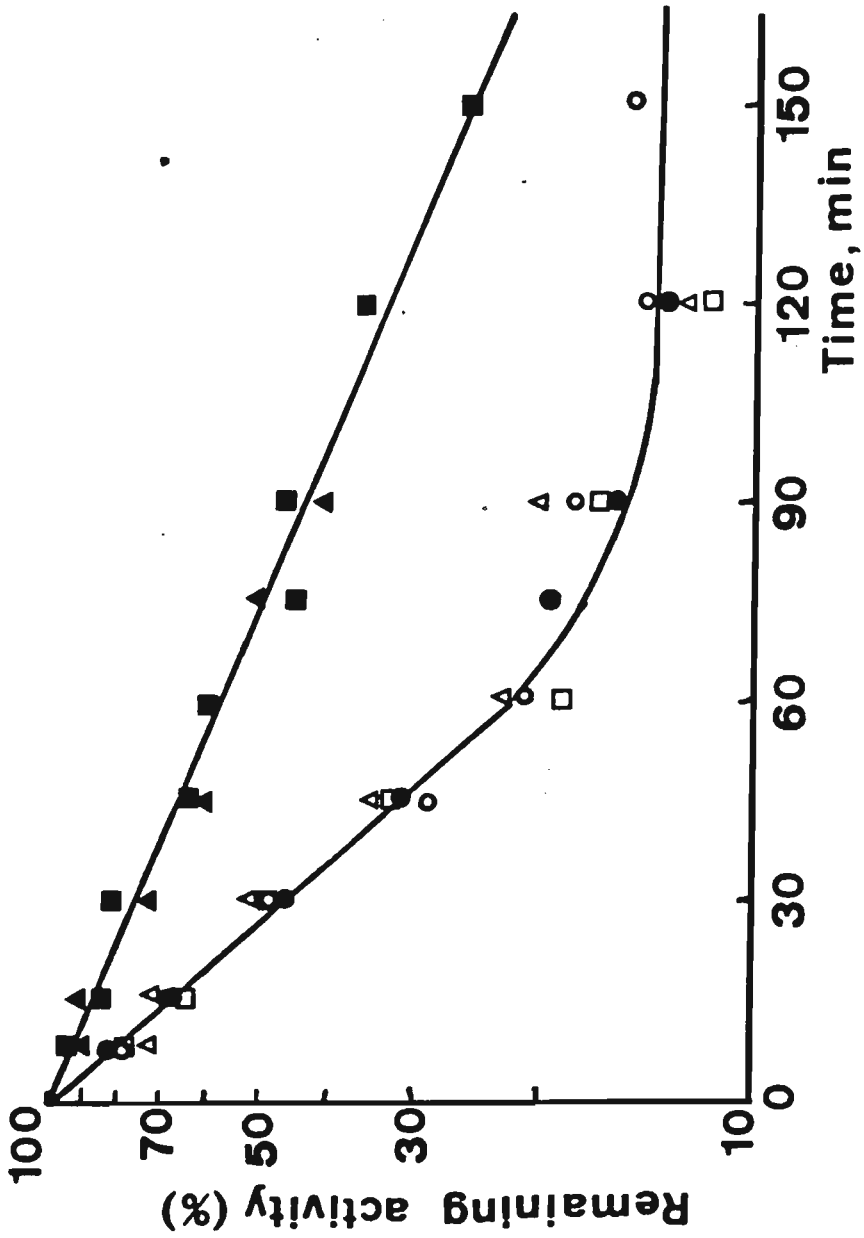


Fig 11

Chemistry of microvillar membranes purified from brush-borders isolated from the larval midgut of Coleoptera and Diptera.

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Running title: chemistry of microvillar membranes.

Abstract

Brush-borders (microvilli) were isolated from different midgut regions of larvae of Tenebrio molitor (Coleoptera), Rhynchosciara americana (lower Diptera), and Musca domestica (higher Diptera) by differential precipitation from homogenates prepared as previously described. The microvilli preparations were treated with the chaotropic salts lithium diiodosalicylate and sodium thiocyanate in order to disrupt microvilli into microvillar membranes and core (cytoskeleton) material. Marker enzymes were extensively inactivated and there was not a selective release of cytoskeleton elements from the microvillar membranes. Insect microvilli preparations were also treated with hyperosmotic Tris buffer,diluted.centrifuged,and purified microvillar membranes were recovered from the resulting pellet. Specific activities of marker enzymes in purified membranes were 1.5 to 2.5-fold higher than in the original microvilli preparations with a final yield of about 20%. Contamination by soluble proteins is under 0.3% and by other membranes never exceed 5%,as judged by chromatography in Sepharose 4B and sucrose gradient ultracentrifugation. In comparison with mammals, insect membranes are rich in carbohydrates (all insects studied), cholesterol (T.molitor), lipids (T.molitor), and protein (M.domestica). The densities, and the ratio of lipid to protein in the microvillar membranes of Coleoptera is lower than that in the two species of Diptera. This agrees with the fact that

microvillar hydrolases are more important in Diptera than in Coleoptera digestion.

Key words - Microvillar carbohydrates, microvillar cholesterol, microvillar lipids, microvillar proteins.

Introduction

The first preparation of insect microvilli was performed by Ferreira and Terra (1980), using larval midgut caeca tissue of Rhynchosciara americana. The method employed was the differential calcium (or magnesium) precipitation technique developed for vertebrate enterocytes by Schmitz et al. (1973). This technique was also used by Hanozet et al. (1980) to purify microvilli from columnar cells of midgut tissue (composed of columnar and goblet cells) of a lepidopteran larvae (Philosamia cynthia). Nevertheless, only in 1986 was it shown that this procedure results in preparations free from the modified microvilli of goblet cells (Santos et al., 1986). After the pioneering work with R. americana and P. cynthia, several other preparations were obtained by using procedures based on Schmitz et al. (1973), but that differed in the homogenization method (rotating blades, Potter-Elvejhem homogenizer, ultrasound), cation used (Ca^{++} or Mg^{++}) and centrifugation speeds (Espinoza-Fuentes et al., 1984; Santos et al., 1986; Parenti et al., 1986; Houk et al., 1986; Wolfersberger et al., 1987; Eisen et al., 1989; Terra et al., 1990; Ferreira et al., 1990; Minami et al., 1991; Lemos and Terra, 1992; Reuveni et al., 1993). Most preparations used frozen tissue, which gave identical results as fresh tissue, except for a 3-fold increase in contaminating mitochondria (Eisen et al., 1989).

A different approach to prepare lepidopterous columnar cell microvilli was introduced by Cioffi and Wolfersberger (1983). The method is based on the stepwise disruption of tissue by ultrasound. Preparations are almost free from contaminants, however the yield of microvillar membranes is very low when compared with the divalent cation differential precipitation methods (Santos et al., 1986; Eisen et al, 1989).

Early attempts to study the biochemistry of microvillar membranes consisted of the determination of the ratio of phosphorus to protein content of microvilli preparations from Lepidoptera larvae (Wolfersberger et al., 1987) and SDS-PAGE of these proteins (Wolfersberger et al., 1987) and in similar preparations from Diptera adults (Houk et al., 1986). Nevertheless, electron microscopy of insect midgut microvilli preparations (Houk et al., 1986; Santos et al., 1986) demonstrates, as previously observed for vertebrates enterocytes (Schmitz et al., 1973), that they are substantially free from other cell structures, although the microvilli still contain some cytoskeleton elements.

In this paper, the preparation and chemical characterization of purified microvillar membranes from larval midguts of Musca domestica (Diptera: Cyclorrhapha), R. americana (Diptera: Nematocera), and Tenebrio molitor (Coleoptera: Polyphaga) are described. The results showed that microvillar membrane composition varies along the midgut in one insect and among different insects.

Materials and Methods

Animals

Larvae of M. domestica were reared in a mixture of fermented pig food and rice hull (1:2, v/v). The larvae used in this study were actively feeding individuals at the third larval instar. R. americana were reared as described by Lara et al. (1965). We only used mature feeding female larvae. Stock culture of the yellow mealworm, T. molitor, were cultured under natural photoregime condition on wheat bran at 24-26°C and a relative humidity of 70-75%. Fully grown larvae (each weighing about 0.12g) with midguts full of food, of both sexes, were used.

Isolation of microvilli (brush border) from M. domestica midgut sections

Larvae fed on 10% starch for 100 min were dissected in cold 150 mM NaCl. The midguts were divided in the following sections: midgut caeca (discarded), anterior midgut (also called fore-midgut), middle midgut (discarded) and posterior midgut (also called hind-midgut). Sometimes the posterior midgut was divided into three sections of equal length: proximal posterior midgut, middle posterior midgut and distal posterior midgut. Microvilli were prepared by sonication of midgut sections (tissue plus contents) in isotonic buffered medium, followed by differential precipitation in the presence of 12 mM $MgCl_2$ as described by Lemos and Terra (1992).

Isolation of microvilli (brush border) from R. americana midgut caeca

Larvae were dissected in ice cold 0.1 M NaCl. After the removal of the gut, midgut caeca were cut out and thoroughly rinsed with saline. Microvilli were prepared from midgut caeca by homogenization with Omni-mixer (Omni, USA) in hypotonic buffered medium, followed by differential precipitation in the presence of 10 mM $CaCl_2$ according to Ferreira and Terra (1980).

Isolation of microvilli (brush border) from T. molitor midgut sections

Larvae were immobilized on ice, after which they were dissected in cold 342 mM NaCl. After removal, the midgut was divided into three sections (anterior, middle and posterior) of identical length. Middle midguts were discarded, whereas anterior and posterior midguts were thoroughly rinsed with saline. Microvilli were prepared from anterior and posterior midguts as described above for R. americana midgut caeca,except that tissues were homogenized in a Potter-Elvehjem homogenizer (Ferreira et al.,1990).

Treatment of microvilli (brush border) of M. domestica and T. molitor with sodium thiocyanate

M. domestica and T. molitor microvilli prepared as described above were treated with sodium thiocyanate essentially according to Hopfer et al. (1983). The microvilli preparation was

centrifuged at 20,600 g for 30 min at 4°C and the pellet was suspended in 0.1 M mannitol in 1 mM tris-(hydroxymethyl) aminomethane (Tris) and 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer pH 7.4. To the suspension, sufficient sodium thiocyanate in the same buffer was added to become 0.41 M. The sample was homogenized at 2000 rpm for 12 strokes in a Potter-Elvehjem homogenizer. The homogenate was diluted twice with the mannitol buffer and centrifuged at 4°C. The following fractions were collected: P_1, pellet resulting from centrifugation at 6,000 g for 10 min; P_2, pellet resulting from the centrifugation at 34,000 g for 25 min (microvillar membranes) and final supernatant. The preparations were stored at -20°C until used.

Treatment of microvilli (brush border) of M. domestica and T. molitor with lithium 3,5-diiodosalicylate

M. domestica and T. molitor microvilli prepared as described above were treated with lithium 3,5-diiodosalicylate following Riendeau et al. (1986). The microvilli preparation was centrifuged (4°C) at 25,000 g for 30 min, and the pellet was suspended in 10 mM Tris-HCl buffer pH 7.5. To the suspension, sufficient lithium diiodosalicylate in the same buffer was added to become 20 mM. After standing on ice for 1 h, with periodical stirring, the sample was centrifuged at 25,000 g for 30 min. The resulting pellet (microvillar membranes) and supernatant were dialysed for 20 h at 4°C against 1,000 vol of 10 mM Tris-HCl

buffer pH 7.5, with one change of buffer. The preparations were stored at -20°C until used.

Purification of midgut microvillar membranes of M. domestica, T. molitor and R. americana with hyperosmotic Tris

Microvilli prepared as described above were suspended in 1 M Tris-HCl buffer pH 7.0. After standing 1 h on ice, with periodical stirring, tha sample was diluted to 50 mM Tris-HCl with cold distilled water and centrifuged (4°C) at 25,000 g for 30 min. The pellet (microvillar membranes) was suspended in 2 mM Tris-HCl buffer pH 7.4, containing 10 mM NaCl. The preparations were maintained at -20°C until used.

Chromatography of purified microvillar membranes in Sepharose 4B

M. domestica and T. molitor Tris-purified (see above) microvillar membranes were chromatographied at 8°C according to Carlsen et al. (1983). For this, samples were applied to a column (15 cm x 1 cm i.d.) of Sepharose 4B (Pharmacia, Sweden) equilibrated with 50 mM Tris-HCl buffer pH 7.4, containing 150 mM NaCl. The non-retained membranes were eluted with 3 column volumes of the same buffer. The flux was 5 ml/ h and the fractions of 0.5 ml were collected. The retained membranes were eluted with 3 column volumes of 2 mM Tris-HCl buffer pH 7.4.

Gradient ultracentrifugation of purified microvillar membranes

Tris-purified (see above) microvillar membranes from midgut sections of M. domestica, R. americana and T. molitor were

layered on the top of sucrose gradients (20-60% for M. domestica; 4.5-45% for R. americana; and 10-50% for T. molitor) prepared in 50 mM citrate-sodium phosphate buffer pH 6.5. The gradients were centrifuged (4°C) at 100,000 g for 25 h and fractions (0.2 ml) were collected from the bottom of each gradient with the aid of a peristaltic pump. Marker enzyme activity and the refractive index were determined in each fraction. The density of each fraction was interpolated in a plot of refractive index and sucrose densities.

Chemical determinations in purified microvillar membranes

Tris-purified microvillar membranes (see above) were used in all chemical determinations.

Carbohydrates were determined using the phenol-H_2SO_4 reagent according to Dubois et al (1965), using reduced volumes of samples and reagent. Each determination was performed in the presence and absence of phenol. The accepted value was the difference between the two determinations. This procedure permits to discount the unspecific colour which may result from heating samples with H_2SO_4.

Cholesterol was enzymatically determined using a kit (Sigma Chemical Co., kit no. 352-20), following the supplier's instructions.

Lipids were determined by a modification of the procedure of Frings and Dunn (1970), using as standard triolein solubilized in water and serially diluted in ethanol or water (similar results were obtained). To 50 µl of sample, 0.2 ml of concentrated H_2SO_4

was added and the mixture heated to 95°C for 10 min in a water bath. After cooling, 1 ml of the chromogenic reagent [0.12% (w/v) vanillin in 80% (v/v) concentrated phosphoric acid] was added to the mixture which was left 5 min at 37°C. Absorbance readings (540 nm) were made within 10 min from the end of the incubation period.

Total phosphorus was determined according to Baginski et al. (1967) with reduced volumes.

SDS-Polyacrylamide gel electrophoresis

Samples were combined with sample buffer containing 60 mM Tris-HCl buffer pH 6.8, 2.5% (w/v) SDS, 0.36 mM β-mercaptoethanol, 0.5 mM EDTA, 10% (v/v) glycerol and 0.005% (w/v) bromophenol blue. The samples were heated for 2 min at 95°C in a water bath before being loaded onto a 7% or 7.5% (w/v) polyacrylamide gel slab containing 0.1% (w/v) SDS (Laemmli, 1970). The gels were run at 0.2 mA/ cm^2 of gel and stained for protein using a silver stain procedure (Blum et al., 1987). Polypeptide Mr values were calculated from a plot of log Mr against Rm values (Shapiro et al., 1967) determined for known proteins with Mr values 14,000-116,000 purchased from Sigma (USA) and Bio-Rad (USA).

If necessary, protein samples were concentrated by trichloroacetic acid (TCA) precipitation before further processing. For this, a TCA solution was added to the protein sample to become 10%. After standing 1 h on ice, the suspension was centrifuged at 10,000 g for 10 min. The pellet was washed by

centrifugation with 95% ethanol and acetone to remove TCA. After drying at 55°C in a water bath, the pellet was suspended in a two-fold diluted sample buffer and then processed as usual samples.

Protein determination and hydrolase assays

Protein was determined according to Smith et al. (1985), as modified by Morton and Evans (1992), using bovine serum albumin as standard.

Aminopeptidase was assayed in 50 mM glycine-NaOH buffer pH 8.5 using 1 mM L-leucine p-nitroanilide, and following the release of p-nitroaniline according to Erlanger et al. (1961).

β-Glucosidase activity was measured determining the appearence of p-nitrophenol (Terra et al., 1979) from 10 mM p-nitrophenil β-D-glucoside in 50 mM citrate-sodium phosphate buffer pH 6.0.

Dipeptidase activity was determined measuring the release of leucine (Nicholson and Kim, 1975) from 5 mM glycyl-leucine in 50 mM Tris-HCl buffer pH 8.0.

γ-Glutamyl transferase was assayed in 50 mM glycine-NaOH buffer pH 8.8 using 1 mM L-γ-glutamyl-p-nitroanilide plus 100mM glycyl-glycine, and following the release of p-nitroaniline according to Erlanger et al., 1961.

Maltase was assayed according to Dahlqvist (1968) using 7 mM maltose in 50 mM citrate-sodium phosphate buffer pH 6.5.

Incubations were carried out for at least four different time periods, and initial rates of hydrolysis were calculated.

All assays were performed under conditions such that activity was proportional to protein concentration and to time. Controls without enzyme and others without substrate were included. One unit of enzyme is defined as the amount that hydrolyzes 1 µmol of substrate per min.

Results

Treatment of midgut microvillar preparations with lithium 3,5-diiodosalicylate and sodium thiocyanate

The yield and enrichment of microvilli from M. domestica midgut cells were similar to those previously described (Lemos and Terra, 1992). Attempts to isolate cytoskeleton-free microvillar membranes from these preparations by treatment with lithium 3,5-diiodosalicylate or sodium thiocyanate as described for mammals were unsuccessful. The membranes isolated after treatment have a lower specific activity and recovery of the marker enzyme aminopeptidase (Espinoza-Fuentes et al., 1987) than the original microvilli preparation (Table 1). Although this may result from aminopeptidase inactivation, other data suggest that behavior of insect microvilli in relation to those salts differs from mammalian microvilli. Thus, only 20 ± 2% (mean and SEM, n=3) of the M. domestica microvilli protein was solubilized in the presence of diiodosalicylate in contrast to 40% from mammalian microvilli (Riendeau et al., 1986), and only 35 ± 9% (mean and SEM, n=3, calculated from the total activity recovered) of the marker enzyme remains in the M. domestica microvillar membranes

after sodium thiocyanate treatment, whereas in mammals the recovery is 65% (Hopfer et al., 1983).

The results obtained with microvilli prepared from T. molitor midgut sections are not detailed here because they were as unsatisfactory as those found for M. domestica.

Purification of microvillar membranes with hyperosmotic Tris

Microvilli were prepared from midgut sections of M. domestica, R. americana and T. molitor (Tables 2, 3 and 4). The yield and enrichment of marker enzymes in these preparations were similar to those previously described (Ferreira and Terra, 1980; Ferreira et al., 1990; Lemos and Terra, 1992). Treatment of microvilli preparations with hyperosmotic Tris, followed by dilution and centrifugation resulted in membranes which will be called microvillar membranes. These membranes have marker enzyme specific activities 1.5-2.5 fold higher than the original microvillar preparation isolated from each of the midgut regions tested (Tables 2,3, and 4).

The enrichment and yield of microvillar membranes differ significantly between T.molitor anterior and posterior midgut (Table 4). Alhough this may result from the cells differing significantly between the two regions, the fact that aminopeptidase activity is very low in anterior midgut in comparison to posterior midgut may result in artifacts. Thus, the determinations were repeated assaying dipeptidase. This enzyme is evenly distributed along the T.molitor midgut and is bound to the

microvillar membranes (Jordão, Terra and Ferreira, unpublished results). The results were identical to those shown in Table 4.

SDS-PAGE of proteins solubilized by Tris and from microvillar membranes showed that Tris seems to preferentially remove low-Mr proteins (Fig. 1). This suggests that cytoskeletal elements are being released by this treatment.

Most soluble midgut enzymes in the insects studied have a too low activity to be useful in the evaluation of microvillar membrane contamination by soluble proteins. An exception is found for maltase in T. molitor midguts. The following recoveries were found for maltase in microvilli preparations in relation to the original midgut homogenate (mean and SEM, 4 preparations of 400 animals each): anterior midgut, 0.7±0.2%; posterior midgut, 1.2±0.4%. After washing the microvilli in 10mM Tris - HCl buffer pH 7.5 (hyperosmotic Tris was not used because it inhibits maltase),the maltase recoveries were (4 assays in a single preparation obtained from 400 animals): anterior midgut, 0.23%; posterior midgut, 0.30%.

Microvillar membranes may be resolved from their contaminating membranes by chromatography in Sepharose 4B (Carlsen et al.,1983). Microvillar membranes from anterior midgut and distal posterior midgut of M.domestica, and from T.molitor posterior midguts were chromatographied in Sepharose 4B.. In both samples,the marker enzyme (aminopeptidase) was eluted at high ionic strengh without increase in specific activity (results not shown). This suggests that membrane contaminants in our preparations are very low. Nevertheless, this method is not

sensitive enough to be practical for minute insect samples. Protein peaks are barely visible even loading the column with material from as much as 300 insects.

Sucrose density gradient ultracentrifugation proved to be more useful than chromatography on Sepharose 4B for our samples. Microvillar membranes from midgut sections of R. americana (Fig. 2a), T. molitor (Fig. 2b,c) and from M. domestica distal posterior midgut (Fig. 2g) sediment in sucrose gradients as a single peak. Microvillar membranes from M. domestica anteior midgut (Fig. 2d), proximal posterior midgut (Fig. 2e), and middle posterior midgut (Fig. 2f) are resolved in a major and a minor peak. The minor peak varies from one sample to another (data not shown), but never exceeds that shown on Fig. 2e. Membranes sedimenting in the major peak have a higher density and should represent the microvillar membranes, . whereas membranes corresponding to the minor peak have a lower density and are supposed to represent basolateral membranes. The fact that only the major peak possess γ-glutamyl transferase activity (another marker enzyme, see Espinoza-Fuentes et al., 1987) (Fig. 2e) supports this hypothesis. In agreement with these conclusions, work done in mammals showed that aminopeptidase is found both in microvillar and basolateral membranes of enterocytes (Maroux et al., 1988). Anyhow, the contamination of the microvillar membranes by basolateral membranes exceptionally attains 5% in some M. domestica samples, whereas in R. americana and T. molitor this contamination is not detected.

Density and chemical composition of purified microvillar membranes

Densities of purified microvillar membranes were calculated from the major peaks observed in Fig 2 and are shown in Table 5. Microvillar membrane densities vary depending on insect (T. molitor < R. americana < M. domestica) and on midgut section (anterior section < posterior section) (Table 5). Densities vary inversely to lipid content (Table 5), which means that the major determinant of membrane density is the lipid-protein ratio.

Cholesterol content seems to depend more on the insect (M. domestica < R. americana < T. molitor) than on midgut section (Table 5). T. molitor microvillar membranes are richer in cholesterol even if cholesterol content is calculated in relation to total lipid.

The amount of phospholipids calculated from the phosphorus content approximatelly agrees with or exceeds the lipid content minus cholesterol content. Hence phosphorus should be associated with proteins in addition to lipids.

The results suggest that R. americana midgut caeca and the more anterior sections of M. domestica midgut have microvillar membranes poorer in carbohydrate than the other membranes (Table 5). The carbohydrate richer membranes are those from T. molitor posterior midgut (Table 5).

Fig.3 shows that the polypeptide pattern of microvillar membranes change along the midgut of M. domestica and T. molitor. In T. molitor membranes, few polypeptide chains are resolved by

SDS-PAGE (Fig. 3), in agreement with the low protein content found in them.

Discussion

Purification of midgut microvillar membranes

Midgut microvillar membranes are prepared in two steps. The first consists in the isolation of microvilli (brush borders) from midgut cells by one of the methods reviewed in the Introduction section. Although enrichments and recoveries obtained in a microvilli preparation depend on the procedures used, the highest enrichment obtainable depends on the tissue used. The enrichment of a microvilli in a preparation depends on the ratio of total cellular protein to microvillar protein. The lower the microvillar protein concentration relative to total protein, the more enrichment of microvilli can occur. Thus, the smaller enrichments observed in Diptera preparations (3 to 7.5) (this paper, Houk et al., 1986), compared with Lepidoptera preparations (4.5 to 10) (Hanozet et al., 1980; Santos et al., 1986; Wolfersberger et al., 1987; Eisen et al., 1989; Minami et al., 1991) may result from the fact that all Diptera midgut cells have microvilli (Ferreira et al., 1981; Terra et al., 1988), whereas only columnar cells display true microvilli in Lepidoptera larval midgut tissue (Cioffi, 1979; Santos et al, 1984). High enrichments observed in Coleoptera preparations (10 to 13) (this paper, Reuveni et al., 1993) may be because the protein content of Coleoptera midgut microvilli is lower than

those of Diptera (see below) and probably also of Lepidoptera. A similar explanation may also hold for the Dictyioptera preparation (enrichment 40, Parenti et al., 1986).

The second step in the purification of microvillar membranes include the treatment of microvilli in such a way that microvilli are disrupted into microvillar membrane and core material. Several compounds have been used with this purpose.

Lithium diiodosalicylate and sodium thiocyanate are chaotropic salts which are thought to affect the structure of proteins by weakening hydrophobic interactions through their influence on water structure. These salts have been used successfuly to produce a selective release of cytoskeletal proteins from the membranes of mammalian intestinal brush borders (Hopfer et al., 1983; Riendeau et al., 1986). Treatment of M. domestica and T. molitor preparations with those chaotropic salts, in the conditions used for mammalian brush borders, resulted in poor or excess protein solubilization, in addition to an extensive inactivation of marker enzymes. This suggests that the attachment pattern of cytoskeleton elements in midgut cells differ betwen mammals and insects.

Tris at high concentration disrupts brush border fragments into microvillar membrane and core material which may be resolved by discontinuous gradient ultracentrifugation (Schmitz et al., 1973). Preliminary work with R. americana brush borders showed that this procedure has a too low yield to be useful in insect work (Ferreira and Terra, unpublished results). Critchley et al. (1975) treated mammalian brush borders with Tris and centrifuged.

The particulate material was then washed with EDTA buffered solution to dissociate cytoskeletal elements. Enrichments of marker enzymes in this procedure were, however, smaller than those based on chaotropic salts. This led us to consider the possibility that a dilution step introduced after Tris disruption would permit to pellet the microvillar membranes, while while leaving cytoskeleton elements in the supernatant. The results (Tables 2, 3, 4 and Fig. 1) showed that this procedure is equivalent to the best methods developed for mammalian cells (see for example Hopfer et al., 1983; Riendeau et al., 1986), as the marker enzymes were enriched 1.5-2.5 fold in the final membranes over the midgut brush border preparation, with a yield of about 20%. Our procedure gave better results than the Tris procedure of Critchley et al. (1975) suggesting that the dilution step after Tris treatment is more effective in releasing cytoskeleton elements than EDTA washing of the particulate material after Tris treatment. Contamination of our microvillar membranes are under 0.3% for soluble proteins and, except for a rare 5% contamination of basolateral membranes in some M. domestica samples, membranes seem not to contaminate our preparations. If necessary, the low basolateral membrane contamination may be eliminated by sucrose gradient ultracentrifugation.

The enrichment of marker enzymes in microvillar membranes over the initial microvilli, as noted above, are similar among mammals (Hopfer et al., 1983; Riendeau et al., 1986) and insects (Tables 2, 3 and 4). This suggests that our microvillar membranes are as pure as the best ones from mammals and that the ratio of

microvillar protein to core protein are similar among mammals and different insects.

Chemistry of microvillar membranes and evolutionary implications

This is the first paper to describe chemical determinations performed in insect midgut microvillar membranes. Thus, our results will be discussed only in the light of data on mammals.

Microvillar membranes from mammalian enterocytes have 410-650 µg lipid/ mg of protein (Proux,1991). In comparison with those values, microvillar membranes from M.domestica posterior midgut have low ratios, whereas those from T.molitor (mainly from anterior midgut) have high ratios. The high ratios found for T.molitor membranes are similar to basolateral membranes of mammalian enterocytes (Proulx,1991) and their densities are as low as those found for the myelin ' sheat (Evans,1978). Nevertheless, in contrast to basolateral membranes, T.molitor microvillar membranes have high cholesterol content,whereas the membranes of the other insects are in the range 50-121 µg cholesterol/mg protein found in mammalian microvillar membranes (Proulx,1991).

The cell glycocalyx corresponds to the carbohydrate moieties of the integral proteins and glycolipids. The width of the cell glycocalyx visible on electron micrographs is reduced during the purification of microvillar membranes (Critchley et al.,1975). Thus,carbohydrate determinations in purified microvillar membranes are expected to be underestimated and somewhat variable, depending on more or less gentle methods were used

during membrane isolation. In spite of this, the results obtained in this paper for insect microvillar membranes (190-700 µg carbohydrate/.mg of protein, Table 5) contrast to that obtained for rabbit small intestinal microvillar membranes (112-116 µg carbohydrate/ mg protein, Cooper and Kent,1978). Perhaps this means that the cell glycocalyx in insect midgut cells are larger than on mammalian enterocytes, or that glycolipids is a more comon component of insect microvillar membranes than of mammalian ones.

In mammals, glycolipids (mainly sphingolipids) account for 30-60% of the microvillar membrane lipids (Proulx,1991). As commented above, glycolipids may be even more important in insect microvillar membranes. Thus, the finding in insect microvillar membranes that phospholipids (calculated from total phosphorus) together with cholesterol account for all membrane lipids (Table 5) suggest that phosphorus must occur in proteins and carbohydrates, in addition to lipids. Pieris brassicae (Lepidoptera) midgut microvilli has 19.8µg of phosphorus/mg of protein (Wolfersberger et al.,1987) which taking into account core protein, probably corresponds to 30-40 µg phosphorus/mg of protein in purified microvillar membranes. If confirmed, this suggest that the phosphorus content of Lepidoptera midgut microvillar membranes are closer to those of Coleoptera than of Diptera.

The chemical determinations of insect midgut microvillar membranes may be summarized as follows. Coleoptera (T.molitor) membranes have low densities,are poor in protein and rich in

cholesterol, phosphorus and carbohydrate. Lower Diptera (R.americana) membranes show intermediate densities, protein and cholesterol contents and are poor in carbohydrate. Higher Diptera (M.domestica) membranes have high densities,are rich in proteins and poor in cholesterol and phosphorus, whereas carbohydrates vary (low content in anterior midgut, high content in posterior midgut). The chemical characteristics of the insect midgut microvillar membranes, mainly the protein content, are in accordance with the presumed role of these membranes in digestion. Thus, in Coleoptera most digestion occurs inside the peritrophic membrane with few or none digestion being carried out by enzymes associated with the microvillar membranes. in contrast, in Diptera the initial and intermediate stages of digestion occur in the midgut lumen, whereas most terminal digestion is carried out by microvillar enzymes. Furthermore, there is a differentiation along the Coleoptera and higher Diptera midguts so that most terminal digestion takes place at the posterior midgut, which in higher Diptera functionally corresponds to the whole midgut of other insect species (reviews: Terra,1988;1990).

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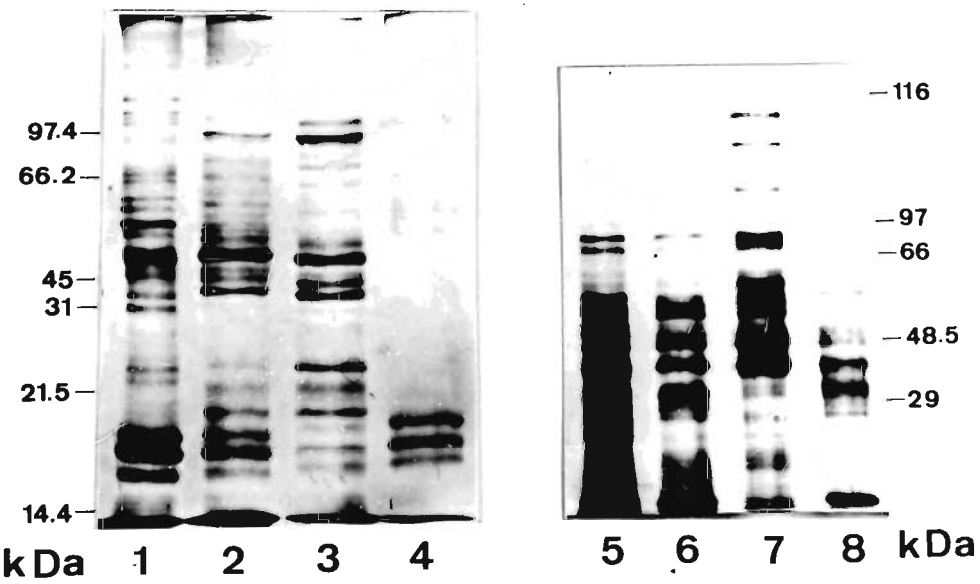
Legends of Figures

Figure 1. SDS-PAGE of polypeptides from fractions obtained during the purification of insect midgut microvillar membranes. M.domestica anterior midgut (lanes 1,2,3, and 4): 1,homogenate; 2, microvilli preparation; 3, purified microvillar membrane; 4, Tris-solubilized material. R.americana midgut caeca (lanes 5,6,7, and 8): 5, homogenate; 6, microvilli preparation; 7, purified microvillar membrane; 8, Tris-solubilized material. Two μg of protein were loaded at each lane. Migration of molecular weight markers is indicated at left and right of the figure.

Figure 2. SDS-PAGE of polypeptides from purified insect midgut microvillar membranes. M.domestica (lanes 1,2,3, and 4): 1, anterior midgut; 2, proximal posterior midgut; 3, middle posterior midgut; 4, distal posterior midgut. T.molitor (lanes 5 and 6): 5, anterior midgut; 6, posterior midgut. Two μg of protein were loaded at each lane. Major characteristic polypeptide chains in each midgut region are marked by points at the left of the lanes. Migration of molecular weight markers is indicated at left and right of the figure.

Figure 3. Typical sedimentation profiles of purified insect midgut microvillar membranes in sucrose gradients. Gradient fractions were assayed for enzyme markers and their densities were determined with the aid of refractometer. R.americana (marker: β-glucosidase): a, midgut caeca. T.molitor (marker;

dipeptidase): b, anterior midgut; c, posterior midgut. M.domestica (markers: aminopeptidase,• • ;γ- glutamyl transferase, o o): d,anterior midgut; e,proximal posterior midgut; f, middle posteior midgut; g, distal posterior midgut.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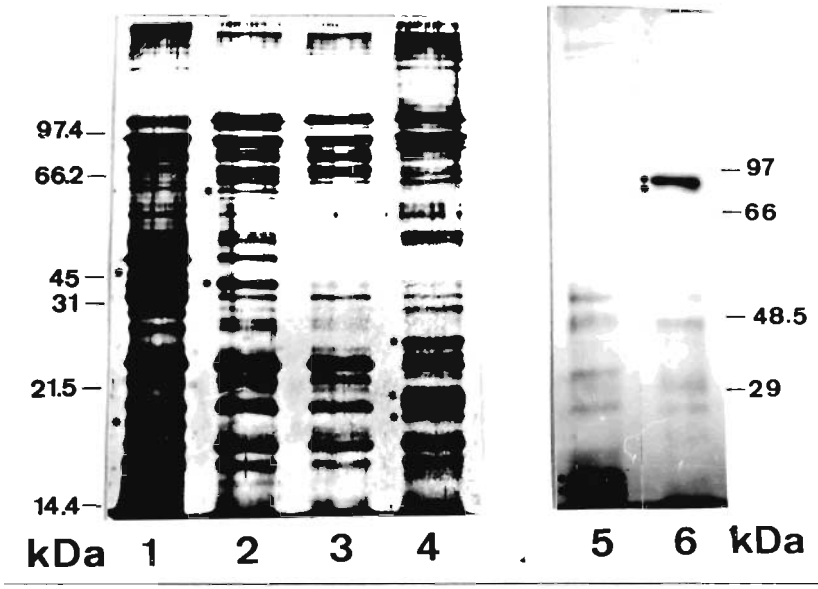
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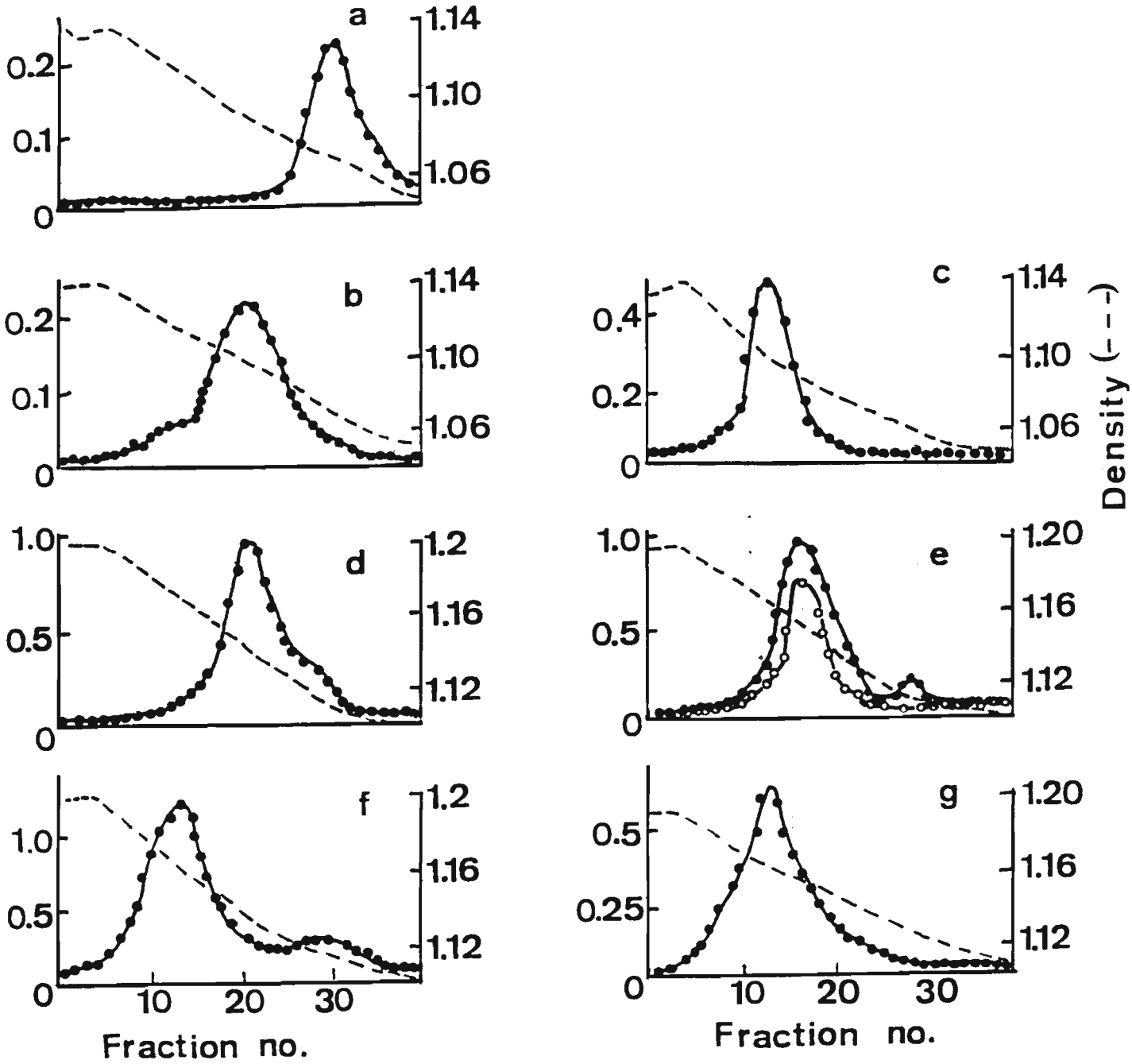
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Table 1. Enrichment and recovery of aminopeptidase during the purification of M. domestica midgut microvillar membranes with lithium diiodosalicylate and sodium thiocyanate.

Fraction	Lithium diiodosalicylate			Sodium thiocyanate		
	mU/mg protein	Enrichment	Recovery	mU/mg protein	Enrichment	Recovery
AM Homogenate	950 ± 60	1.0	100	1400 ± 300	1.0	100
AM Microvilli	6000 ± 2000	6.0 ± 2.0	23 ± 1	8000 ± 900	6 ± 1	20 ± 1
AM Microvillar membranes	4000 ± 3000	4 ± 2	13 ± 3	2000 ± 1000	1.4 ± 0.9	2 ± 1
PM Homogenate	1900 ± 100	1.0	100	1600 ± 300	1.0	100
PM Microvilli	7000 ± 1000	4 ± 1	40 ± 4	7000 ± 1000	4 ± 1	39 ± 2
PM Microvillar membranes	5000 ± 1000	2.6 ± 0.1	21 ± 2	5000 ± 1000	3.1 ± 0.4	9 ± 4

Microvillar membranes are the membranes obtained from the microvilli (brush border) preparations after treatment with lithium diiosalicylate or sodium thiocyanate.
The figures are means and SEM of determinations performed on three different preparations obtained from 240 animals each.
AM, anterior midgut; PM, posterior midgut.

Table 2. Enrichment and recovery of aminopeptidase during the purification of *M. domestica* midgut microvillar membranes with hyperosmotic Tris.

Midgut region Fraction	mU/mg protein	Enrichment	Recovery
Anterior midgut			
Homogenate	700 ± 100	1.0	100
Microvilli	4000 ± 1000	6 ± 1	26 ± 7
Microvillar membranes	8000 ± 1000	11 ± 4	20 ± 5
Proximal posterior midgut			
Homogenate	1200 ± 100	1.0	100
Microvilli	6000 ± 1000	5 ± 1	30 ± 10
Microvillar membranes	8400 ± 600	7.0 ± 0.3	24 ± 1
Middle posterior midgut			
Homogenate	900 ± 200	1.0	100
Microvilli	3500 ± 400	3.9 ± 0.5	22 ± 3
Microvillar membranes	7000 ± 900	8 ± 2	15 ± 2
Distal posterior midgut			
Homogenate	900 ± 100	1.0	100
Microvilli	3100 ± 500	3.4 ± 0.6	27 ± 3
Microvillar membranes	5300 ± 200	5.8 ± 0.3	20 ± 2

Microvillar membranes are the membranes obtained from the microvilli (brush border) preparations after treatment with hyperosmotic Tris.
The figures are means and SEM of determinations performed on three different preparations abtained from 240 animals each.

Table 3. Enrichment and recovery of β-glucosidase during the purification of R. americana midgut caeca microvillar membranes with hyperosmotic Tris.

Fraction	mU/mg protein	Enrichment	Recovery
Homogenate	50 ± 10	1.0	100
Microvilli	219 ± 2	4 ± 1	13 ± 3
Microvillar membranes	300 ± 30	6 ± 1	8 ± 1

The figures are means and SEM of determinations performed on three different preparations obtained from 300 animals each.

Table 4. Enrichment and recovery of aminopeptidase during the purification of T. molitor midgut microvillar membranes with hyperosmotic Tris.

Midgut region	mU/mg protein	Enrichment	Recovery
AM Homogenate	0.28 ± 0.03	1.0	100
AM Microvilli	2.7 ± 0.5	10 ± 2	20 ± 4
AM Microvillar membranes	4.3 ± 0.8	15 ± 6	18 ± 7
PM Homogenate	150 ± 50	1.0	100
PM Microvilli	2000 ± 200	13 ± 6	50 ± 20
PM Microvillar membranes	6000 ± 1000	40 ± 10	33 ± 8

The figures are means and SEM of determinations performed on three different preparations obtained from 300 animals each.
AM, anterior midgut; PM, posterior midgut.

Table 5. Density and chemical composition of microvillar membranes purified from brush borders from different insect species.

Species Midgut region	µg/mg protein Total Lipid	Phosphorus	Phospholipid	Cholesterol	Carbohydrate	Density (g/cm^3)
T. molitor						
AM	710 ± 80	31 ± 3	740 ± 70	140 ± 10	400 ± 100	1.072 ± 0.002
PM	600 ± 100	30 ± 2	720 ± 50	110 ± 20	700 ± 200	1.098 ± 0.002
R. americana						
Caeca	580 ± 60	n.d.	n.d.	70 ± 40	190 ± 80	1.105 ± 0.002
M. domestica						
AM	500 ± 100	13.9 ± 0.6	340 ± 40	59 ± 1	240 ± 60	1.136 ± 0.005
Proximal PM	380 ± 70	12.9 ± 0.9	310 ± 50	50 ± 4	300 ± 100	1.152 ± 0.004
Middle PM	380 ± 60	13 ± 3	310 ± 80	40 ± 10	400 ± 200	1.150 ± 0.010
Distal PM	380 ± 100	17.1 ± 0.7	410 ± 50	52 ± 8	410 ± 200	1.163 ± 0.004

Phospholipid was calculated assuming that all phosphorus are present in phosphatidyl ethanolamine molecules (Mr 747). AM, anterior midgut; PM, posterior midgut. n.d., not determined.
The figures are means and SEM of determinations performed on four different preparations obtained from 240 (*M. domestica*), 300 (*T. molitor*) or 400 (*R. americana*) animals each.