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METABOLISM OF FUCOSE, ALPHA-L-FUCOSIDASES AND FUCOSYLTRANSFERASES: ENZYMATIC CHARACTERISATION, MECHANISM OF CATALYSIS AND PHYSIOLOGICAL ROLE

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ABSTRACT

Perrella NN. **Metabolism of fucose, alpha-L-fucosidases, and fucosyltransferases: Enzymatic characterisation, mechanism of catalysis and physiological role**. [Ph. D. thesis (Biotechnology)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo, 2018.

Carbohydrates are diverse and complex molecules being employed by living organisms in biological functions such as energetic, structural and signalling events. L-Fucose is a monosaccharide component of many glycans present in a variety of biological groups, such as mammals, insects, and plants. Some of the best-known examples of post-translational modified molecules containing L-Fucose are the ABO blood antigen system and human milk. Changes in the fucosylation pattern are related to pathologies like cancer and fucosidosis. These changes are related to the balance between the activities of α-L-fucosidases and fucosyltransferases. α-L-fucosidases are glycoside hydrolases that catalyse the hydrolysis of glycosidic bonds between residues of L-Fucose to other molecules. Fucosyltransferases are glycosyltransferases which transfer L-Fucose from a GDP-fucose to a specific acceptor. Although the importance of fucose metabolism, there is few information about this subject in Arthropoda. Literature indicates that fucose, α-L-fucosidases and fucosyltransferases are involved in host-pathogen interaction in ticks, suggesting that fucose metabolism is essential to Arachnida. Our aim was to study the metabolism of fucose in two Arachnida species: the spider *Nephilingis cruentata* and the tick *Amblyomma sculptum*. This was accomplished through the characterisation of native and recombinant fucosidases, structure and *in silico* analyses, site-directed mutagenesis, expression pattern, specificity and, effect on tumour cells. Besides that, we analysed fucosyltransferases sequences and expression by qPCR. The enzymes involved in fucose metabolic pathways were also investigated. NcFuc and AsFuc have a pH optimum of 5.0, are inhibited by fucose and fuconojirimycin and present an oligomerisation process pH dependent. We successfully produced the recombinant form of these enzymes, and they have the same kinetic properties of native forms. Natural substrate hydrolysis by NcFucr and AsFucr suggest different specificities and they were able to remove fucose residues from tumour cell lines, reducing cell invasion. Moreover, they catalysed transfucosylation reactions. The recombinant production allowed the identification of the residues D214 and E59 as the catalytic dyad in NcFuc. Phylogenetic analysis, kinetic data, molecular modelling, and specificity assays suggest that α-L-fucosidase active sites are different to each Arachnida species and indicated that the physiological significance of fucose removal is different among organisms. qPCR assays evidenced that although fucosidases might be lysosomal enzymes, they are mainly expressed at the digestive system in Arachnida and are involved in digestion. Representatives of all known families of fucosyltransferases were identified in the spider MG transcriptome data. However, POFUT1 was also identified at the proteomic level and its expression analysis indicated a higher expression at MG. This might be related to the regeneration of cells after secretion of digestive enzymes. Transcriptomic and proteomic analysis indicate that Arachnida uses both salvage and *de novo* pathways to fucose synthesis. Considering all the obtained data we concluded that fucose metabolism is related to digestion in Arachnida since they are able to salvage fucose from diet due to the presence of very active α-L-fucosidases.

Keywords: Fucosidase. Fucose. Glycosidases. Spider. Catalysis.

RESUMO

Perrella NN. **Metabolismo de fucose, alfa-L-fucosidases e fucosiltransferases: Caracterização enzimática, mecanismo de catálise e papel fisiológico**. [Tese de doutorado (Biotecnologia)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo, 2018.

Os carboidratos são moléculas diversas e complexas que são empregadas por organismos vivos em funções biológicas, como eventos energéticos, estruturais e de sinalização. L-Fucose é um monossacarídeo presente em diversos grupos biológicos, como mamíferos, insetos e plantas. Umas das modificações pós-traducionais mais conhecidas contendo L-Fucose são os antígenos do sistema sanguíneo ABO e o leite humano. Alterações no padrão de fucosilação estão relacionadas a patologias como câncer e fucosidose. Essas alterações estão relacionadas ao balanço entre as atividades de α-L-fucosidases e fucosiltransferases. As α-L-fucosidases são glicosídeo hidrolases que catalisam a hidrólise de ligações entre resíduos de L-Fucose ligados a outras moléculas. Fucosiltransferases são glicosiltransferases que transferem L-Fucose de GDP-fucose para um substrato receptor específico. Embora a importância do metabolismo da fucose, existem poucas informações sobre este assunto em Arthropoda. A literatura indica que fucose, α-L-fucosidases e fucosiltransferases estão envolvidas na interação parasita-hospedeiro em carrapatos, sugerindo que o metabolismo da fucose é essencial para Arachnida. Nosso objetivo foi estudar o metabolismo de fucose em duas espécies de Arachnida: a aranha *Nephilingis cruentata* e o carrapato *Amblyomma sculptum*. Ele foi realizado através da caracterização das fucosidases nativas e recombinantes, estrutura e análise *in silico*, mutagênese sitio-dirigida, padrão de expressão, especificidade e efeito em células tumorais. Além disso, analisamos as sequências de fucosiltransferases e a expressão por qPCR. As enzimas envolvidas em caminhos metabólicos de fucose também foram investigadas. NcFuc e AsFuc têm um pH ótimo de 5, são inibidas por fucose e fuconojirimicina e apresentam processo de oligomerização dependente de pH. Nós produzimos com sucesso as formas recombinantes dessas enzimas, e elas apresentam as mesmas propriedades cinéticas das formas nativas. A hidrólise de substratos naturais por NcFucr e AsFucr sugerem especificidades diferentes, e ambas conseguiram remover resíduos de fucose de celulares tumorais, reduzindo a invasão celular. Além disso, elas catalisaram reações de transfucosilação. A produção recombinante permitiu a identificação dos resíduos D214 e E59 como díade catalítica em NcFuc. As análises filogenéticas, os dados cinéticos, a modelagem molecular e a especificidade sugerem que os sítios ativos das α-L-fucosidases são diferentes em cada espécie de Arachnida e indicaram que o significado fisiológico da remoção de fucose é diferente entre os organismos. Os ensaios de qPCR evidenciaram que, embora as α-L-fucosidases possam ser enzimas lisossômicas, elas são principalmente expressas no sistema digestório em Arachnida e estão envolvidas na digestão. Representantes de todas as famílias conhecidas de fucosiltransferases foram identificados nos dados do transcriptoma de aranha. No entanto, POFUT1 também foi identificado no nível proteômico e sua análise de expressão indicou uma maior expressão no MG. Isso pode estar relacionado à regeneração de células após a secreção de enzimas digestivas. A análise transcriptômica e proteômica indica que o Arachnida usa vias salvage e *de novo* para a síntese de fucose. Considerando todos os dados obtidos, concluímos

que o metabolismo da fucose está relacionado à digestão em Arachnida, uma vez que eles podem obter a fucose da dieta devido à presença de α-L-fucosidases muito ativas.

Palavras-chave: Fucosidase. Fucose. Glicosidases. Aranha. Catálise.

PREFACE

Carbohydrates (monosaccharides, oligosaccharides and polysaccharides) are extremely diverse and complex molecules being employed by living organisms in many biological functions such as energetic, structural and signalling events (1).

Monosaccharides differ from one another by subtle stereochemical variations. Due to that, enzymes that process carbohydrates have acquired their specificity through structural differences and evolved from a limited number of ancestors (2). Glycoside hydrolases (GH), glycosyltransferases (GT), polysaccharide lyases (PL) and carbohydrate esterases (CE) are classified into families based on sequence homology (Carbohydrate-Active enzymes database - CAZy - www.cazy.org) (3). The enzymes involved in the digestion of carbohydrates obtained from diet are the glycoside hydrolases. GH or glycosidases constitute a large superfamily of enzymes that catalyses the hydrolysis of glycosidic bonds. Genome analyses have revealed that 1 to 3% of an organism's genes are normally dedicated to carbohydrate degradation, a proof of the importance of carbohydrate metabolism to living organisms (2). Glycoconjugates present on the cell surface play a key role in many physiological and pathological processes including cell-to-cell adhesion, cell differentiation, immune response, fertilization, viral and bacterial infection, and tumor progression (4, 5). The absence of the enzymes that process these glycoconjugates, mainly in the lysosome, is associated with a series of deficiencies such as mucolipidosis, fucosidosis and, Fabry disease, among others (6).

L-Fucose (6-deoxy-L-galactose) is a monosaccharide component of many glycans and glycolipids present in a variety of biological groups, such as mammals, insects, and plants. Two structural features distinguish L-Fucose from other six-carbon sugars: the absence of a hydroxyl group on carbon six and the L configuration (7).

Fucosylation is often one of the final steps in the biosynthesis of glycoconjugates. One of the best-known examples of post-translational modified molecules containing L-Fucose is the ABO blood antigen system (8). Usually, in N-glycans, L-Fucose residues are linked to galactose residues at α-1,2 glycosidic bonds or bound to N-acetylglucosamine residues at α-1,3 links; 1,4 or 1,6 (9).

L-fucosylation is also observed in the oligosaccharides present in human milk, which represent a natural protection due to prebiotic properties to the infant

microbiota (10-12), modulating the growth and pathogenicity of enterohaemorrhagic *Escherichia coli* in the gastrointestinal tract. Changes in the fucosylation of glycoproteins and glycolipids are observed in some tumours such as lung, colon, liver, breast and ovarian carcinomas and metastatic processes, and these alterations are often promoted by alterations in the activity of fucosyltransferases and α-L-fucosidase (4, 7, 9, 13-17). In addition, L-fucose containing glycans can serve as ligands for some pathogens such as *Campylobacter jejuni, Helicobacter pylori, Pseudomonas aeruginosa, Anaplasma marginali* and *Schistosoma mansoni* (10, 18-20). It is also possible to directly or indirectly attribute the involvement of L-fucose residues to deficiency of type II leukocytes adhesion, cystic fibrosis, and rheumatoid arthritis (9, 13, 16).

Beyond the biological importance, L-fucose has the potential to biotechnological application. Rare sugars, such as L-fucose, have many interesting properties, making them attractive to various applications, such as anti-inflammatory substances, antioxidants, and antiviral agents, justifying the effort to produce them synthetically. The advantages of the enzymatic synthesis are the simplicity of the process, stereospecificity and the absence of the secondary synthesis of toxic compounds, which often occur in the chemical synthesis. For example, Fucogel (Solabia - France), is a linear polymer produced by the bacteria *Klebsiella pneumoniae*, consisting of units of galacturonic acid, L-Fucose, and D-Galactose used as a dermocosmetic agent (21). Owing to that, the study of α-L-fucosidases and fucosyltransferases is valuable to glycoconjugate synthesis.

In general, glycosidases are capable of catalysing transglycosylation reactions. Then, both fucosidases and fucosyltransferases can be used for synthesis purposes (10).

Several studies in literature demonstrate the wide distribution of α-L-fucosidases in diverse organisms and the importance of this enzyme in animal physiology.

At the present thesis we will show the study of α-L-fucosidases and fucosyltransferases present in the digestive system of arachnids and the importance of fucose in the metabolism of these animals.

This thesis is presented in three different chapters: Chapter 1: Fucosidases, Chapter 2: Fucosyltransferases and Chapter 3: Fucose metabolism in Arachnida, for a better approach of each subject.

CHAPTER 1 - FUCOSIDASES

1.1 Introduction

α-L-fucosidases (EC 3.2.1.51) are glycoside hydrolases (GH 29 and GH 95 - CAZY- Carbohydrate-active enzymes) that catalyse the hydrolysis of glycosidic bonds between residues of L-Fucose linked α-1,2, α -1,3, α-1,4 or α-1,6, to other molecules, such as carbohydrates, lipids or proteins (3).

The α-L-fucosidases are divided into two subgroups: α-L-fucosidases that present a retaining catalytic mechanism, which belongs to the family GH29 (22) and the inverting type, GH95. Among the GH29 are mammalian α-L-fucosidases and some bacterial α-L-fucosidases, and among the GH95 are α-L-fucosidases from plants, fungi, and bacteria (23).

The hydrolysis by the retaining glycosidases is conducted in two steps. The first one, glycosylation, involves the formation of a covalent glycosyl-enzyme intermediate. The covalent intermediate is subsequently hydrolysed in the deglycosylation step, realising the free sugar with retained stereochemistry and the enzyme. Retaining glycosidases present two amino acid residues involved in catalysis. One acts as a nucleophile by attacking the anomeric centre of the covalent glycosyl-enzyme intermediate and the other residue functions as an acid catalyst (proton donor), which in the glycosylation step protonates the glycosidic oxygen to activate the aglycone as leaving group. The same residue then acts as a basic catalyst in the deglycosylation step, deprotonating the water molecule and then hydrolysing the intermediate glycosyl-enzyme (Figure 1.1) (2).

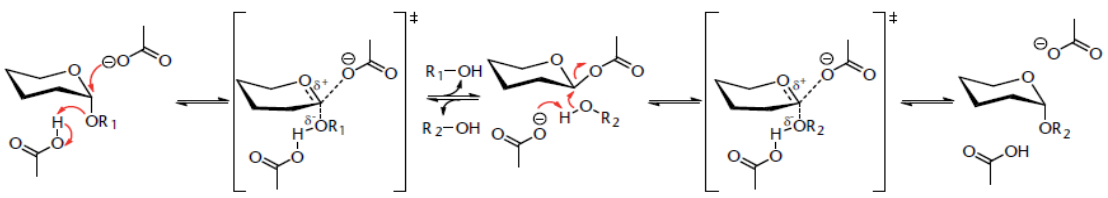

Figure 1.1 – **Mechanism of hydrolysis by the retaining α-glycosidases**. Retrieved from Kotzler et al. (2014) (2).

The first studies with α-L-fucosidases were performed with mammalian enzymes, particularly studies of the human lysosomal α-L-fucosidase. The absence of this enzyme is associated with fucosidosis, an autosomal recessive disease that causes the accumulation of fucoglycoconjugates in the lysosomes of the cells in different tissues, causing various severe phenotypes like neurological deterioration, growth retardation, and even being fatal (24). The lysosomal α-L-fucosidase activity was associated with a number of pathological conditions, such as inflammation (25), cancer (26) and cystic fibrosis (27).

Two genes encoding α-L-fucosidases have been described in humans. One encoding a lysosomal α-L-fucosidase, FUCA1, and one encoding a secreted α-L-fucosidase, FUCA2. For FUCA1, several pathological conditions have already been described due to its altered activity. So far, only a few functions have been described for FUCA2. Liu et al. (2009) have described that FUCA2 is essential for *H. pylori* adhesion in gastric and duodenal cancer patients (18).

Alhadeff et al. (1975) characterised the properties of human hepatic α-L-fucosidase, which has a molecular mass of 50 kDa determined by SDS-PAGE and 175 kDa determined by gel filtration, indicating oligomerization under physiological conditions (28).

Among the bacterial α-L-fucosidases, the main species studied is the α-L-fucosidase from the thermophilic bacteria *Thermotoga maritima* (TmFuc), which was the first α-L-fucosidase to be crystallized (24). In its monomeric form, TmFuc has 52 kDa and it aggregates in a hexamer at the crystalline structure.

The crystallization of TmFuc with L-Fucose allowed the identification of the enzyme active site as well as the substrate binding site, which fits only one fucose residue, being compatible with the exoglycosidic activity performed by TmFuc (24). Through kinetic characterisation and site-directed mutagenesis assays, it was possible to identify the residues involved in catalysis (29). The nucleophile is an aspartic acid at position 244 and the proton donor, residue involved in acid/base catalysis, a glutamic acid at position 266. Until now only bacterial and fungi α-L-fucosidases have been crystallized (Table 1.1).

Table 1.1 – Crystal structures of α-L-fucosidases

Family	Specie	Kingdom	Reference*
GH29	*Thermotoga maritima*	Bacteria	(24)
	Fusarium graminearum	Fungi	(30)
	Bacteroides thetaiotaomicron	Bacteria	(31)
	Bifidobacterium longum Subsp. infantis	Bacteria	(32)
GH95	*Bifidobacterium bifidum*	Bacteria	(33)
	Bacteroides ovatus	Bacteria	No reference available

*Source: NCBI (34)

Liu et al. (2009) used alignments of *Homo sapiens* and TmFuc α-L-fucosidases, which show 38% identity, and molecular homology modelling studies to identify the catalytic residues of human α-L-fucosidase (35). Nine residues were chosen and replaced by site-directed mutagenesis. The authors verified that the Asp225 residue (corresponding to residue 224 of TmFuc) is actually the residue involved in the nucleophilic attack. However, to acid/base catalysis Glu289 was the only residue that resulted in a decrease of catalytic efficiency. The authors demonstrated that the residue responsible for acid/base catalysis in human α-L-fucosidase does not retain the same position as the residue indicated as acid/base in TmFuc (Glu266). Shaikh et al. (2013) have done a similar study, combining alignment and site direct mutagenesis, to compare bacterial and human α-L-fucosidases from the GH29 family (36). This study confirmed the non-conservative position of the acid/base catalytic residue to the α-L-fucosidases from *Thermotoga maritima*, *Homo sapiens, Bifidobacterium infantalis* and *Bacteroides thetaiotaomicron*. Based on these results the authors suggested the subdivision of the family GH29 in A and B. In subfamily A are the less specific α-L-fucosidases as *Thermotoga maritima* and *Homo sapiens* and at subfamily B are the more specific enzymes.

Fucoidan is a sulphated polymer of L-Fucose, present in the cell wall of brown algae and it is a natural substrate for fucosidases. Fucoidan and derivatives of its hydrolysis have a number of pharmacological uses including antibacterial,

anticoagulant, antiviral and antitumoral (37-39). Thus, the enzymes involved in their processing or synthesis are quite interesting (40). Several species of mollusks feed with brown algae and therefore have become important species of study of α-L-fucosidases. It was verified that the digestive enzymes of these molluscs have high efficiency in the processing of the sulphated fucans (41, 42). Besides fucoidan, 2-fucosyllactose, a glycan present in human milk and lacto-N-fucopentaose I, II and III which are antigens of the ABO blood system, respectively known as H antigen, Lewis A, and Lewis X are natural substrates for α-L-fucosidases. These substrates have L-Fucose bound via α1,2; α1,3; α1,4 and α1,6; being of great importance for the study of α-L-fucosidases specificity. Inhibitors of α-L-fucosidase have also been applied to characterise the specificity and the active site of α-L-fucosidases. Additionally, these inhibitors are of great interest to the development of potential therapeutic agents (43).

L-Fucose and fuconojirimycin are inhibitors of fucosidase commonly used for specificity determination. L-Fucose is a competitive inhibitor of α-L-fucosidase and fuconojirimycin derivatives are strong competitive inhibitors of several α-L-fucosidase such as the *Homo sapiens* (44) and *Pecten maximus* (42) α-L-fucosidases.

Synthetic substrates and inhibitors, as well as natural substrates such as fucoidan and fucosylated oligosaccharides, are used for α-L-fucosidases specificity characterisation. α-L-fucosidases from different organisms have different substrate specificities and this characteristic is variable according to the biological species to which they belong, making the study of new models fundamental.

In addition to the hydrolytic role of α-L-fucosidases, the ability of these enzymes to synthesize glycosidic linkages via transfucosylation using different types of acceptor molecules has been described in several articles (45, 46). Thus, the importance of the study of α-L-fucosidases in new biological groups is very clear. One of these new groups is the Arthropoda. Arthropods contain about 80% of the living species described and occupy all ecological niches (47). Such diversity reflects a very efficient adaptive process. One of the factors of this success is associated with the digestion capacity of different sources of food. Such diversity makes this group of animals quite suitable for the study of different α-L-fucosidases and fucosyltransferases.

Our group identified biochemically the presence of α-L-fucosidase activity in the digestive system of the tick *Amblyomma (cajennense) sculptum* (48), the harvestman *Neosadocus sp*., the spider *Nephilingis cruentata* and the scorpion

Tityus serrulatus. For molluscs, the role of α-L-fucosidases as digestive enzymes is very clear; and the specificity of these enzymes is already well described (41, 42) . However, for ticks we proposed that the digestive α-L-fucosidases have, in addition to the role of removal of L-Fucose residues from ingested food, that these enzymes play a defensive role, possibly being involved in the removal of residues of fucose involved in the interaction of pathogenic microorganisms such as *Anaplasma marginali* with the midgut epithelium (48). Pedra et al. (2010) (49) suggested the hypothesis that the pathogen *Anaplasma phagocytophilum* modulates the expression of α-1,3-fucosyltransferases to utilize α-1,3 fucosylation to colonize ticks. They found that, during the infection, there is a super-expression of α-1,3-fucosyltransferases and the silencing of genes encoding these enzymes caused a reduction in infection. They concluded that α-1,3-Fucose is essential for colonization despite not knowing the mechanism. The possible defensive role of α-L-fucosidases may be related to the removal of L-Fucose residues that are essential for the colonization of pathogenic microorganisms. These studies demonstrate the wide distribution of α-L-fucosidases in the most diverse organisms; however, for arthropods, the physiological role of fucosidase present in the digestive system and its specificity is still unknown.

1.5 Conclusions

The combination of biochemical characterisation and protein isolation, proteomic, *in silico* and transcriptomic analysis allowed the identification of digestive fucosidases in the spider *Nephilingis cruentata* and in the tick *Amblyomma sculptum*. Spiders apparently have only one α-L-fucosidase gene while tick's genome evidence more than one α-L-fucosidase. The characterisation of these enzymes indicated an oligomerisation process pH dependent and suggests different specificities in comparison to other fucosidases previously characterised.

We successfully produced the recombinant form of NcFucr and AsFucr. Both were characterised kinetically and exhibited same properties of native forms, besides that they showed potential to catalyse transfucosylation reactions.

The recombinant production allowed us to characterise the first Arthropoda α-L-fucosidase catalytic mutants. The mutation of the glutamic acid at position 59 resulted in a significant change of k_{cat} value and the chemical rescue provided by azide further supported this, suggesting that D214N and E59A function as catalytic

dyad. Kinetic data, molecular modelling, and specificity assays suggest that α-L-fucosidase active sites are different to each Arachnida species, indicating that more studies are necessary to completely comprehend catalysis in different α-L-fucosidases.

Crystallographic structure of Arachnida fucosidases has not been successful so far.

All these data combined evidence the importance of crystallographic studies of other α-L-fucosidases to enhance the comprehension of these anomalous enzymes.

CHAPTER 2 - FUCOSYLTRANSFERASES

2.1 Introduction

Glycosyltransferases (GT) catalyse the formation of glycosidic linkage using as substrate a sugar donor containing a phosphate group as leaving group (73).

Fucosyltransferases are GT which transfer L-Fucose from a GDP-fucose to a specific acceptor substrate such as galactose (Gal), N-acetyl-glucosamine (GlcNAc) residues or to serine and threonine in proteins. Since all fucosyltransferases utilize the same nucleotide sugar, their specificity will probably reside in the recognition of the acceptor molecule and in the type of linkage formed (74). Fucosyltransferases are divided into five families GT10, GT11, GT23, GT65 and GT68 according to their sequence and specificity. All the fucosyltransferases present inverting mechanism; they use GDP-β-fucose as substrate donor (Carbohydrate-Active enzymes database - CAZy - www.cazy.org) (3).

Inverting GTs have a carboxylic residue that acts as a basic catalytic that deprotonates the receptor nucleophile, releasing the phosphate group and then forming the glycosidic linkage (Figure 2.1) (73).

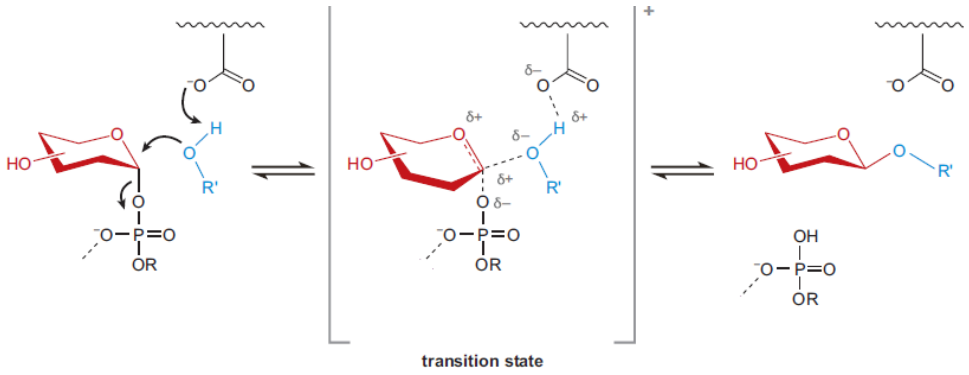

Figure 2.1 - **Mechanism of catalysis proposed for inverting glycosyltransferases**. Adapted from Lairson et al. (73)

Fucosyltransferases have been described in humans, bacteria, insects, and plants. Thirteen genes encoding fucosyltransferases have been identified in humans. Among them are the fucosyltransferases responsible for the synthesis of the antigens A, B, and H present in the blood groups of the ABO system, the synthesis of Lewis antigens and that add L-Fucose directly to polypeptide chains (7) for example, O-

fucosylation of the Notch receptor. Fucosylation occurs from the transfer of L-Fucose by GDP-L-fucose, which is synthesized *in vivo* via a *de novo* pathway from GDP-mannose or via salvage pathway from L-Fucose. The α-1,2; α-1,3/4 and α-1,6-fucosyltransferases are enzymes present in the Golgi whereas O-fucosyltransferases are enzymes present in the endoplasmic reticulum (4).

The presence of the Notch receptor, as well as its role, has also been proposed for Arachnida. Stollewerk (75) and Stollewerk et al. (76) described the role of Notch during the neurogenesis and cellular differentiation of the spider *Cupiennius salei*.

Lei et al. (77) characterise the N-glycan structure of dengue virus envelope glycoprotein E. The E protein is involved in the entry of the virus into the host cell when it binds to a particular receptor. N-glycans of protein E influence folding, cell localization, and receptor interactions. Among the monosaccharides identified in these glycans is L-Fucose, added by a fucosyltransferase.

Moreover, aberrant protein glycosylation is known to be associated with the development of cancers. The aberrant glycans are produced by the combined actions of changed glycosylation enzymes, substrates and transporters in glycosylation synthesis pathways in cancer cells (68, 78, 79). It has been reported that α-1,6-fucosyltransferase (FUT8) activity and expression is increased in several human cancers, suggesting a role for this enzyme in tumour development and progression, such as hepatocellular carcinoma, colorectal, lung, ovarian serous adenocarcinoma, and prostate cancer. On the other hand, decreased core-fucosylation contributes to malignancy in gastric cancer (71, 79). Due to its great importance in different types of cancer fucosylation is a promising target for cancer diagnosis and therapy.

2.1.1 α-1,2-Fucosyltransferase

α-1,2-fucosyltransferases belong to the GT11 family. These enzymes catalyse the transfer of L-Fucose to residues of β-D-galactose presents at the end of glycoconjugates, through a α-1,2 linked. In humans, there are two genes encoding α-1,2-fucosyltransferases, FUT1 and FUT2. FUT1 is responsible for the production of H antigen in red blood cells, since FUT2 is expressed in the epithelium of secretory tissues individuals called "secretors" have at least one functional copy of this gene;

they secrete the H antigen, which is subsequently transformed into A and/or B antigen depending on the ABO genotype (NCBI BioSystems database - NCBI - http://www.ncbi.nlm.nih.gov/biosystems) (34).

2.1.2 α-1,3/4-Fucosyltransferase

α-1,3/4-fucosyltransferases are GT belonging to family 10. They transfer L-Fucose to GlcNAc residues. In humans, there are eight genes encoding α-1,3/ 4-fucosyltransferases: FUT3, FUT4, FUT5, FUT6, FUT7, FUT9, FUT10, and FUT11.

The Lewis blood system comprises a set of fucosylated glycosphingolipids which are synthesized by exocrine epithelial cells and circulate in body fluids. These molecules have important roles in embryogenesis, tissue differentiation, tumour metastasis, inflammation, and bacterial adhesion. α-1,3/4-fucosyltransferases catalyse the addition of L-Fucose to precursor polysaccharides in the last step of biosynthesis of Lewis antigens, α-1,3/4-fucosylation is essential for the correct functioning of these molecules. Mutations in these genes are responsible for most of the Lewis negative phenotypes (The NCBI BioSystems database - NCBI - http://www.ncbi.nlm.nih.gov/biosystems/) (34).

2.1.3 α-1,6-Fucosyltransferase

α-1,6-fucosyltransferases (FUT8) belong to the GT23 family. These enzymes transfer L-Fucose from GDP-fucose to N-acetylglucosamine presents at the reducing end of N-glycans. α -1,6 fucosylation is essential for the function of growth factor receptors.

The expression of this gene may contribute to the malignancy of cancer cells and to their invasive and metastatic capacities (The NCBI BioSystems database - NCBI - http://www.ncbi.nlm.nih.gov/biosystems) (34).

2.1.4 O-Fucosyltransferase 1

O-fucosyltransferase 1 (POFUT1), encoded by the fut12 gene in *Homo sapiens*, belongs to the family GT65 and is responsible for O-fucosylation of proteins, transferring L-Fucose directly to the hydroxyl group of serine and threonine residues. It is responsible for fucosylation of the EGF (epidermal growth factor) domain. The

EGF repeat sequence was found at the Notch receptor, in Notch receptor ligands, and in coagulation factors VII, IX, and VII (5). Notch is a transmembrane receptor that mediates cell-cell signalling that plays crucial roles in several developmental processes in multicellular organisms, such as cell differentiation and neurogenesis. In humans, aberrant signaling by Notch involves numerous diseases, including multiple sclerosis and various types of cancer. This receptor has repeated extracellular growth factor epidermal (EGF) motifs, with fucosylation being essential for signalling (80).

2.1.5 O-Fucosyltransferase 2

O-fucosyltransferase 2 (POFUT2), encoded by the fut13 gene in *Homo sapiens*, belongs to the 68 family of GTs. POFUT2 also transfers L-Fucose directly to proteins. It is responsible for O-fucosylation of TRS (thrombospondin repeat domain). The TSR motif is present in extracellular matrix proteins involved in the cell-cell interaction (5).

Fucosyltransferases from all families have been crystallized (Table 2.1) and will be used as models in sequences analyses.

Table 2.1 – Fucosyltransferases that have obtained their structure solved.

Enzyme	Family	Specie	Kingdon	Reference
α-1,3/1,4-fucosyltransferase	GT10	*Arabidopsis thaliana*	Plantae	(81)
		Helicobacter pylori	Bacteria	(82)
α-1,6-fucosyltransferase	GT23	*Bradyrhizobium sp. WM9*	Bacteria	(83)
		Homo sapiens	Animalia	(84)
O-fucosyltransferase 1	GT65	*Mus musculus*	Animalia	(85)
		Caenorhabditis elegans	Animalia	(86)
		Homo sapiens	Animalia	(87)
O-fucosyltransferase 2	GT68	*Homo sapiens*	Animalia	(88)

Source: NCBI (34)

2.5 Conclusions

Transcriptomic analyses allowed us to identify the sequences of fucosyltransferases from all families in the digestive system of the scorpion *Tityus serrulatus* and the spider *Nephilingis cruentata*. However, just POFUT1 from *Nephiligis cruentata* was identified by proteomic analysis.

Alignment analysis of Arachnids and crystallized FucTs indicated conserved catalytic and interaction residues.

Quantitative PCR assays indicated NcFuc POFUT1 is most expressed in the midgut, however, is not expressed exclusively in the digestive system of the spider. The higher expression of POFUT1 at midgut may be related to the regeneration of the intestine cells after secretion of digestive enzymes.

CHAPTER 3 - FUCOSE METABOLISM AND FUCOSIDASE PHYSIOLOGICAL ROLE IN ARACHNIDA

3.1 Introduction

Fucose is one of the sugars added to proteins during the posttranslational modification process. Fucose-containing glycans have important roles in humans like blood transfusion reactions, host/microbe interactions, and numerous other events, including Notch pathway signalling events (4, 7, 18, 26, 55). Alterations in the synthesis of fucosylated oligosaccharides have also been observed in several pathological processes, including cancer and atherosclerosis (17, 72, 91). Although the fucose addiction is a ubiquitous modification, the meaning of protein-fucose ornamentation is not completely understood due to the diversity of fucose-containing glycoconjugates and the difficulties inherent in studying the biology of carbohydrates (92). Due to that, it is likely that many additional functions for fucosylated glycans remain to be uncovered (4, 9, 93). The glycosylation metabolic pathways have provided valuable information on the role of N-/O-glycoproteins in the control of growth, morphogenesis and another physiological process in diverse organisms and cells (94). The pathways to glycosylation and even fucosylation are widespread among eukaryotes. However, some groups of organisms have particular repertoires of glycosylation and particularly fucosylation. Insects, for example, have the ability to add core 1,3-fucose even to an already previously 1,6-fucosylated substrate. Although this kind of fucosylation is shared with plants and nematodes, it is distinct from mammalian fucosylation. This modification is crucial in insects. However, there is no study of this ability throughout Arthropoda. Arachnida is the second most specious and diverse group in Arthropoda.

Our interest in the metabolism of fucose in Arachnida came from the advent of the biochemical identification of unusual fucosidase activity in the digestive process of ticks (*Amblyomma sculptum*), spiders (*Nephilingis cruentata*) and scorpions (*Tityus serrulatus*) (48) and (58) (in press). Alpha-L-fucosidases are, in general, lysosomal enzymes responsible for the removal of fucose residues from glycoconjugates.

Some questions popped into our minds: Why are fucosidases present in the digestive system of Arachnida species? Which would be the natural substrates to Arachnida digestive fucosidases? Are Arachnida, distinctly from insects, able to

salvaged fucose obtained from the diet? Which are the fucose metabolic pathways present in Arachnida? Which are the fucose metabolic pathways present in Arthropoda? Are the fucosyltransferases present in Arachnida similar to other Arthropoda enzymes?

Recently, Arachnida DNA and protein high throughput analysis have allowed the comprehension of different aspects of Arachnida physiology (95, 96). One of these is the fucose metabolism. In addition, Pedra et al. (49) showed that *Anaplasma phagocytophilum* induces the expression of three distinct fucosyltransferases and that fucose is essential to host/parasite interaction, suggesting that fucosidase might be a digestive/defensive enzyme.

Fucose is one of the eight monosaccharides present in insects (97). α1,6-linked fucose is among the major N-linked glycan species in insects (93, 97). Similar to humans, insects present a diverse set of fucosyltransferases since the distinct enzymes will interact with different GDP-fucose acceptors (74). All of them use GDP-fucose as substrate. In insects, the synthesis of GDP-fucose uses the *de novo* pathway in which the synthesis of fucose starts from mannose residues. However, mammalian uses the two known different pathways to GDP-fucose synthesis, the *de novo* and the salvage pathways. Until now, there was no report of enzymes involved in any of the fucose pathways in Arachnida and this will be addressed.

3.5 Conclusions

The presented data indicates that Arachnida uses both salvage and *de novo* fucose synthesis.

Phylogenetic analysis evidences that the spider fucosidases are closer to mollusk digestive fucosidases and are separated from tick fucosidases. Although all analysed sequences are orthologs, it seems that the physiological significance of fucose removal may change among different organisms.

Fucosidases are not exclusively expressed in the digestive system of the arachnids in study, suggesting that, fucosidases are lysosomal enzymes that could be required in extracellular digestion.

The recombinant expression allowed us the obtainment of an antibody which will be essential to intracellular location of fucosidases. The experiments are under way with the collaboration of Dr. Marta Antoniazzi.

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