MARIANA RANGEL PEREIRA

CARACTERIZAÇÃO FUNCIONAL E ESTRUTURAL DE ENZIMAS LIPOLÍTICAS DE UM CONSÓRCIO MICROBIANO DEGRADADOR DE ÓLEO DIESEL

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Orientadora:
Dra. Eliana Gertrudes de Macedo Lemos

Co-orientadora:
Dra. Andrea Balan Fernandes

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RESUMO

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O comércio mundial de enzimas industriais estava estimado em 2.3 bilhões de dólares entre detergentes (U$ 789 milhões), aplicações alimentícias (U$ 634 milhões), agricultura (U$ 237 milhões), entre outros. Neste contexto, as enzimas lipolíticas estão atraindo enorme atenção devido ao seu potencial biotecnológico, visto que estas podem catalisar múltiplas reações (hidrólise, acidólise, interesterificação e glicerólise). Enzimas lipolíticas de origem microbiana são economicamente atrativas por serem biodegradáveis, atuarem normalmente em condições brandas, e serem quimio-seletivas propiciando à indústria farmacêutica a obtenção de drogas com efeito colateral reduzido. Em trabalho anterior (dissertação de mestrado, número de processo 2009/06991-0), cinco genes potenciais codificadores de esterases/lipases foram identificados através de uma biblioteca metagenômica obtida de um consórcio microbiano degradador de óleo diesel. Em continuação, neste projeto, quatro destes genes foram clonados em vetores de expressão e expressos em *Escherichia coli* BL21 (DE3) a partir dos vetores pET28a ou pHAT2, e as proteínas correspondentes, denominadas como Est8, Est16, *ORF*17 e Est30, foram submetidas a ensaios funcionais e estruturais. Nossos resultados mostram que todas as proteínas puderam ser expressas e purificadas na forma solúvel e estável, sendo que a caracterização funcional da Est8, Est16 e Est30 mostrou que estas proteínas são esterases que possuem alta eficiência catalítica para *p*-nitrofenil acetato, *p*-nitrofenil butirato e *p*-nitrofenil valerato, respectivamente. Interessantemente, estas proteínas apresentaram elevada atividade em pH 9.0 e temperatura ótima entre 50-60 °C, em concordância com os ensaios de desnaturação térmica. Ressalta-se que a atividade da proteína Est16 aumentou na presença de dimetil sulfóxido (DMSO) e a da proteína Est8 não foi afetada por concentrações de até 10% deste solvente, tornando-as atrativas para serem usadas com solventes orgânicos e em elevadas temperaturas. Ainda, três estruturas cristalográficas foram resolvidas (Est8 a 1.8 Å, *ORF*17 a 1.4 Å; e Est30 a 1.7 Å e em complexo com butirato a 1.65 Å), o que permitiu a caracterização da cavidade interna destas proteínas, dos resíduos envolvidos na ligação com o substrato e o posicionamento das tríades catalíticas. Estes dados possibilitaram a construção de um conjunto de mutantes: o mutante Est8MF, onde se visou aumentar o canal que dá acesso à tríade catalítica da proteína Est8; e os mutantes Est30asp19 e Est30ala34, que foram construídos a fim de expor a tríade catalítica da proteína Est30. Os ensaios funcionais do mutante Est8MF mostraram que a sua velocidade de reação foi quatro vezes maior quando comparada com a proteína selvagem (Est8) usando como substrato o *p*-nitrofenil butirato, entretanto, para *p*-nitrofenil acetato a velocidade foi reduzida. Adicionalmente, as estruturas cristalográficas resolvidas neste trabalho, quando comparadas com as estruturas de esterase/lipase depositadas no Protein Data Bank (PDB), revelaram interessantes características para futuras aplicações biotecnológicas.

Palavras-chave: Metagenoma. Consórcio microbiano. Esterase. Lipase.

ABSTRACT

PEREIRA, M. R. **Functional and structural characterization of lipolytic enzymes from a microbe consortium specialized for diesel oil degradation**. 2015. 115 f. Thesis (Ph.D. in Biotechnology) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2015.

The global trade of industrial enzymes is estimated at 2.3 billion U.S. dollars, divided mainly between detergents (US$ 789 million), food applications (US$ 634 million), and agriculture (US$ 237 million). Within this trade, lipolytic enzymes have attracted enormous attention because of their biotechnological potential as catalysts of multiple reaction types (including hydrolysis, acidolysis, interesterification and glycerolysis). Lipolytic enzymes of microbial origin are economically attractive because they are easily biodegradable, usually act in mild conditions, and are chemo-selective, providing the pharmaceutical industry a method for obtaining drugs with reduced side effects. In a previous work (master's student project, process number 2009/06991-0), five genes encoding putative esterases/lipases were identified in a metagenomic library obtained from a microbe consortium isolated from diesel oil-contaminated soil. Here, four individual genes were cloned into the pET28a or pHAT2 vector and expressed in *Escherichia coli* BL21 (DE3), and their corresponding recombinant proteins, designated Est8, Est16, *ORF*17 and Est30, were used for functional and structural studies. Our results showed that all proteins could be expressed and purified as stable and soluble enzymes. Through functional characterization, it was possible to verify that Est8, Est16 and Est30 are esterases with the highest catalytic efficiency against *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate and *p*-nitrophenyl valerate, respectively. Interestingly, they displayed the highest activity at pH 9.0 and their optimal temperatures were approximately 50-60 °C, which are in agreement with the results of the thermal denaturation assay. In addition, Est16 had enhanced activity in the presence of dimethyl sulfoxide (DMSO), while Est8 showed no difference in activity in the presence of up to 10% DMSO. Thus, these thermostable enzymes are very attractive for use with organic solvents and at elevated temperatures. Crystal structures of the three proteins were also obtained (Est8 at 1.8 Å; *ORF*17 at 1.4 Å; and Est30 in a free form at 1.7 Å and in a complex with butyrate at 1.65 Å). The structures allowed for the characterization of the pocket of each esterase/lipase, the residues involved in the substrate binding, and the positioning of the catalytic triad. With this information, a set of mutants of these proteins were designed for the first time: in the Est8 protein, the channel for the passage of the substrate was increased in generating the Es8MF mutant, while in the Est30 protein, the catalytic triad was exposed in creating the Est30asp19 and Est30ala34 mutants. The reaction velocity of the Est8MF mutant was shown to be four times faster than that of the wild type (Est8) against *p*-nitrophenyl butyrate. However, it was noticed that Est8MF showed reduced activity toward *p*-nitrophenyl acetate compared to Est8. Interestingly, the comparison between the newly solved structures and the deposited esterase/lipase structures from the Protein Data Bank (PDB) revealed interesting features of the proteins for future biotechnological studies.

Keywords: Metagenome. Microbe consortium. Esterase. Lipase.

1 INTRODUCTION

The biosphere is dominated by microorganisms that are still mostly unknown. It is estimated that 99% of prokaryotic organisms have not been studied, which is probably due to the traditional methods of cultivation, which limit the development of these organisms in laboratory conditions (HARDEMAN; SJÖLING, 2007; HENNE et al., 2000; ROH; VILLATTE, 2008). The recent advances in molecular microbial ecology research have provided strong evidence of the existence of new microorganism species in the environment in large numbers and with high diversity (STEELE; STREIT, 2005), especially when compared to those few species that are cultivable in the laboratory. Confirmation of this evidence comes from the estimation of the DNA complexity and the discovery of several unique sequences of *16S rRNA* and *18S rRNA* from different environmental sources (RONDON et al., 2000; SILVEIRA et al., 2006).

To access the genetic resources of the vast numbers of microbial species that have thus far escaped detailed scientific testing, researchers have started using a new approach termed metagenomics (HANDELSMAN et al., 1998). This strategy includes the genomic DNA extraction from environmental samples, known as the collective genome, followed by the cloning of the obtained material (RONDON et al., 2000). The results are libraries composed of small or large DNA fragments which can be cloned into appropriate vectors to produce clones that can be used to examine metabolic routes, to analyze microbial diversity, to identify genes encoding proteins of biotechnological interest, and other purposes.

Previous studies have shown that these metagenomic analyses offer unlimited combinations for identifying new genes encoding potential enzymes such as esterases and lipases (COUTO et al, 2010; HU et al., 2010; WU; SUN, 2009). For example, in the last few years, several genes encoding lipolytic enzymes were identified from metagenomic libraries that were taken from various environmental samples, including soil (CHOW et al., 2012; LEE et al., 2004), soil collected from a wastewater treatment plant (GLOGAUER et al., 2011), mangrove sediment (COUTO et al., 2010), marine sediment (HU et al., 2010; JEON et al., 2012; PENG et al., 2014), swamp sediment (SEO et al., 2014), pond and lake water (RANJAN et al., 2005), sea water (CHU et al., 2008; FANG et al., 2014), and river water (WU; SUN, 2009).

Among these discoveries, Fang and collaborators (2014) identified the Est9x esterase from a marine microbial metagenome. This enzyme is a member of a new family of microbial lipolytic enzymes proposed by these authors that presents a high activity against *p*-nitrophenyl

acetate, an optimal temperature at 65 °C, and high salt tolerance, all of which make it an enzyme with potential for industrial applications. Another esterase termed Est23 was identified in a soil metagenomic library and demonstrated stability in the presence of up to 50% benzene or alkanes (JIN et al., 2012). In this context, the Est2K esterase showed activity in the presence of 30% methanol and was mostly active at pH 10.0, suggesting its potential as an alkaline enzyme (KIM et al., 2010).

In terms of the enzyme market, it is known that the global enzyme trade was estimated at 2.3 billion dollars, whose the main profit was divided into detergents (US$ 789 million), food applications (US$ 634 million), agriculture (US$ 237 million), among others (Global Industry Analysts, 2004). The current enzyme market is estimated at 3.4 billion euros, with an annual growth of 6.5 to 10% (CBDM.T, 2008). Within this market, lipolytic enzymes have attracted significant attention due to their biotechnological potential (VAKHLU; KOUR, 2006).

Most of the lipolytic enzymes used in industry are of microbial origins (JAEGER; DIJKSTRA; REETZ, 1999). The microorganism-derived enzymes are available for industrial application and the synthesis of new products, enabling high gains in different biotechnology processes. These enzymes have greater efficiency reducing expenses, and use fewer caustic chemicals and thus are beneficial to the environment (LORENZ; ECK, 2005). Nevertheless, the largest problem facing the use of lipases in industrial processes is the costly and time-consuming purification process, which generally result in a loss of enzymatic activity. However, this situation has the potential to change due to recent advances in technology, such as genetic engineering as well as protein modification and immobilization (KOBLITZ; PASTORE, 2004; SAXENA et al. 2003).

Based on their substrate preferences, these lipolytic enzymes are categorized as esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3), which catalyze the hydrolysis and synthesis of short (carbon chain length ≤10) and long-chain acylglycerols (carbon chain length >10), respectively. In addition, the bacterial lipolytic enzymes can be classified based on their amino acid sequences and some fundamental biological properties. According to Arpigny and Jaeger (1999), these enzymes are classified into eight major families: family I (true lipases); family II, which has members with the conserved GDSL motif; family III, which has representatives showing a 20% identity with the human PAF-AH protein; family IV, known as the hormone-sensitive lipases (HSL); family V, which has mesophilic, cold or heat-adapted representatives; family VI, consisting of enzymes that have a 40% sequence similarity with the eukaryotic lysophospholipases and presents the smallest esterases known (23-26 kDa);

family VII, which has enzymes with sequence similarity to eukaryotic acetylcholine esterases and carboxylesterases of approximately 30-40%; and family VIII, which has enzymes that present the conserved motif Ser-X-X-Gly (where X can be any amino acid) and enzymes displaying similarities to several class C β-lactamases. Despite this proposed classification, new lipases and esterases have been identified from different environmental samples using the metagenomic approach. According to Ranjan and collaborators (2005), the incessant search for esterases and lipases will increase the diversity of known lipolytic enzymes and consequently the number of families.

Esterases and lipases are members of the family of enzymes containing an alpha-beta hydrolase fold. This family includes proteases, dehalogenases, epoxide hydrolases and peroxidases, making it the most versatile enzyme family which contains the most common protein fold. Enzymes in this family do not share sequence similarity and substrate preferences, however, their structural arrangements are relatively homologous and they share a preserved catalytic site, suggesting a possible common ancestor (OLLIS et al., 1992). The folding of an alpha-beta hydrolase protein is characterized by a core of eight β-sheets connected by α-helices, which gives the arrangement α/β/α. In most members of this family, the β-sheets are parallel, but some proteins show reverse orientations of the β-sheets that result in an antiparallel orientation. This folding can provide a stable framework.

The catalytic residues are always composed of a highly conserved Ser-Asp-His triad. The nucleophilic residue is positioned after the β5-strand, the acid residue (Glu instead of Asp in some enzymes) is often positioned after the β7-strand, and the absolutely conserved histidine residue is located after the last β-strand (NARDINI; DIJSTRA, 1999), however, the location of the histidine residue (sequence length) may differ considerably between the different members in this family.

The nucleophilic residue is always located on a sharp bend called a "nucleophilic elbow", which facilitates the interaction with the substrate. The geometry of the "nucleophilic elbow" contributes to the formation of the oxy-anion binding site, which is necessary for the stabilization of the transition state during hydrolysis

Esterases can be differentiated from lipases mainly due to their substrate specificity and the lack of an interfacial activation (LONG, 1971), which is observed only in lipases. Lipases present a hydrophobic domain (lid) that covers the catalytic triad and moves to expose the active site in a minimal concentration of substrate (BORNSCHEUER, 2002). In contrast, esterases follow Michaelis-Menten kinetics. However, esterases and lipases do not differ in the mechanisms of ester hydrolysis and synthesis, which occur in a few steps. When

the substrate is bound to the serine residue, a tetrahedral intermediate is produced that is stabilized by the His and Asp residues. An acyl enzyme complex is formed after the release of alcohol, and then the nucleophile residue is attacked by water, alcohol, or an ester depending on the reaction (hydrolysis, transesterification or esterification, respectively). Again, the result is a tetrahedral intermediate that gives rise to the final product, which is either an acid or an ester, and the free enzyme (BORNSCHEUER, 2002).

Esterases and lipases present a broad applicability in different sectors, which justifies the incessant search for and research on these enzymes. In last few years, several esterases have become available from different companies, such as Amano, Diversa and Roche Diagnostics. Esterases can be used in the production of pure compounds, in the processing of food and beverages, as a flavor enhancer, in agricultural areas to detoxify insecticide compounds, in paper manufacturing, in cosmetics and perfumes, and in other areas. One such application is the use of a carboxyl esterase (32 kDa) from *Bacillus subtilis* in the development of Naproxen, an anti-inflammatory drug, which was carried out due to the high stereospecificity of S-naproxen [2-(6-methoxy-2-naphthyl)-propionic acid] esters and esters of related drugs (QUAX; BROEKHUIZEN, 1994).

In the food industry, a feruloyl esterase has been used to produce the pentylferulate ester, which is a precursor in food processing as well as in cosmetics (GIULIANI et al., 2001). In the production of sake, an esterase from *Saccharomyces cerevisiae* has been used for the production of isoamyl acetate, which determines the drink flavor (FUKUDA et al. 1998). Moreover, esterases and lipases from *Lactobacillus* sp. have been used to enhance the flavor of cheeses through the hydrolysis of milk fat (CHOI; LEE, 2001). Another important application is the use of phosphotriesterases from *Brevundimonas diminuta* and *Alteromonas* sp. to degrade organophosphorous compounds used in insecticides and nematicides, which have negative environmental impacts (HORNE et al. 2002). Finally, one of the most important applications for lipases is the production of detergents. Companies such as DuPont Industrial Biosciences have successfully developed detergents using bacterial lipases, including Lumafast™ from *Pseudomonas mendocina* and Lipomax™ from *Pseudomonas alcaligenes* (JAEGER; REETZ, 1998). These detergents have shown increased stability against detergent composition in the presence of boronic acid derivatives due to a reversible complex formation in the active site (patent number EP0478050A1).

To explore genes that encode proteins with biotechnological potential, a metagenomic library from a microbe consortia specializing in diesel oil degradation was used. This library is part of a library collection of different environmental sources and has been stored at the

Biochemistry Laboratory of Microorganisms and Plants (LBMP), Department of Technology, at the State University of São Paulo (UNESP), Jaboticabal Campus.

The metagenomic library from the microbial consortia contains approximately 4224 clones and has been used to prospect several genes, including genes that encode lipolytic enzymes. The soil that was used to develop the microbial consortia was obtained from an old lubricant factory in Ribeirão Preto town, São Paulo State, Brazil, which had been disposing of waste into the soil for a period of 15 years.

In the master's project entitled "Screening for lipolytic enzyme codification genes in a metagenomic library of consortia specializing in diesel oil degradation" (process number 2009/06991-0), five genes encoding lipolytic enzymes were identified. In the current Ph.D. project, four of these genes were cloned individually into the expression vector and the recombinant proteins were expressed and purified in order to characterize their functions and structures. One aim of this project was to evaluate the following for each enzyme: the substrate preference; the optimal pH and temperature; the enzyme performance in the presence of additives; the tolerance to organic solvents and detergents; the thermal stability; and the biotechnological potential. The second aim of this project was to design mutants in order to improve the enzymatic activity whenever necessary. The overall goal of this project was to obtain an enzyme collection that could be used in different industrial sectors, including bioremediation.

5 CONCLUSIONS

- In this project, it was possible to develop the functional characterization of three new esterases from a microbe consortia specializing in diesel oil degradation;
- Est16 proved to be an esterase capable of hydrolyzing acyl esters with chain lengths of up to 12 carbons. Moreover, this protein is a highly soluble enzyme that is more active in basic pH, thermally stable and DMSO tolerant;
- The structural model of Est16 revealed a highly conserved alpha-beta domain but different substrate pockets than those of patented enzymes, making Est16 a potential candidate for biotechnological applications;
- Est8 is a new alkaline esterase from family IV that displayed a substrate preference for acyl esters with short chain lengths. Interestingly, Est8 showed an optimal temperature at 60 °C and thermal denaturation above 55 °C. In addition, the activity of this protein did not change in the presence of up to 10% DMSO, suggesting its compatibility with organic solvents;
- Through the structure of Est8, it was possible to successfully design the Est8MF mutant. This mutant displayed activity against *p*-nitrophenyl butyrate that was four times higher compared to the wild type;
- Like Est16 and Est8, the Est30 protein is an alkaline esterase. However, Est30 had activity of greater than 60% over a broad temperature range (from 25 °C to 65 °C) and enhanced activity in the presence of DMF;
- In agreement with the functional characterization, the structures of Est30 both in the free form and in a complex with butyrate displayed a tunnel that crossed the protein from one side to another, indicating that long acyl esters could fit in this cavity. The interesting features of Est30 points to its potential for future biotechnological research;
- Although the functional characterization of the *ORF*17 protein was not performed, the high-resolution structure of this protein showed a tunnel that crosses the protein. Thus, it is expected that the *ORF*17 protein can hydrolyze different acyl esters, making it an attractive candidate for industrial applications.
- Altogether, the data presented in this thesis compile a set of results obtained from the metagenome and structural characterization of new enzymes. These results include the rational evolution of proteins, which reveal the enormous potential of this approach for discovering new potential enzymes with different applications.

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