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Molecular rules for chemo- and regio-selectivity of *Candida antarctica* lipase B in peptide acylation reactions

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ABSTRACT

The chemo- and regio-selectivity of the lipase B of *Candida antarctica* (CALB) in peptide acylation by oleic acid was investigated combining experimental and theoretical methodologies. Molecular dynamics and docking simulations were performed to study the selectivity of CALB toward the dipeptide Lysine-Serine at the molecular level. To this end, a model that mimics the acyl-enzyme system was built from CALB crystallographic structure and optimized then to be used as docking target. One main orientation of the peptide within the catalytic cavity was obtained. The lysine side chain was observed to enter the cavity, placing the ε-amino group as to be acylated near the catalytic residues. This result was consistent with the N-acylation experimentally observed, showing the robustness of the model. Docking simulations were then applied to the peptides Lysine-Tyrosine-Serine, Serine-Tyrosine-Lysine and Leucine-Glutamine-Lysine-Tryptophan aiming to predict the selectivity of the reaction. Whatever the peptidic sequence and its constitutive amino acids, the models suggested the preferential N-acylation of the lysine side chain. These theoretical results were in perfect accordance with experimental data showing that Nε-oleoyl-Lys derivatives were the major products.

1. Introduction

Peptides are molecules presenting various beneficial effects such as antioxidant [1,2], anti-hypertensive [3,4], neuroprotective [5], antiviral [6] or antimicrobial activities [7]. However their use can be limited by their short half-life due to potential hydrolysis by endo-proteases and their polarity that can restrict their transfer through biological membranes [8]. One solution to overcome these drawbacks is the acylation of peptides with fatty acids [9–11]. The main challenge when acylating polyfunctional substrates like peptides is the selectivity of the reaction. Conversely to chemical pathways, enzymatic acylation allows grafting acyl chains on specific positions, leading to specific structures. Understanding the factors that regulate selectivity constitutes a scientific challenge that can be undertaken by both experimental and theoretical approaches such as molecular modeling simulations. Experiments provide valuable insights on the efficiency of the reaction and the structure of the products, allowing to draw conclusions about selectivity, but without any understanding of the mechanism. One major difficulty of this approach is the purification and the structural determination of the products that can be laborious. Molecular modeling simulations provide additional information about the possible binding modes of the substrates within the catalytic cavity. Accessibility of the substrate functional groups can be investigated, and assumptions about selectivity can be then formulated. Such an approach is expected to deepen understanding of enzyme selectivity and even to give elements to predict selectivity before experimental verification.

Several enzymes are able to catalyze acylation reaction. The most studied and industrially used are lipases (EC 3.1.1.3). These are able to catalyze the acylation of both hydroxyl (O-acylation) and amine groups (N-acylation). Few studies reported the selective acylation of polyfunctional molecules like amino-alcohols [12–15]. Other authors described the enzymatic acylation of peptides and amino acids catalyzed by CALB. The results demonstrated that the regio- and the chemo-selectivity of the reaction strongly depend on the structure of the acyl acceptor. When using the L-lysine as acyl acceptor, the N-acylation was shown to occur specifically on the ε-amino group, with oleic and palmitic acid as acyl donors [16]. The same tendency was observed for the acylation of the modified peptide L-phe-α-L-lys-O-t-Bu and L-ala-α-L-lys-O-t-Bu with

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trifluoroethyl acetate, catalyzed by lipases from *Pseudomonas* sp., *Aspergillus niger* and *Chromobacterium viscosum* [17]. Other studies reported the acylation of a serine that was modified on either its N- or its C-terminal extremity. Results showed the efficient O-acylation of the serine side chain when acylating the L-serine-amide with lauric acid [18]. Similar results were observed for the acylation of the N-carbobenzyloxy-L-serine with myristic acid [19]. Nevertheless when lysine and serine are linked together like in the peptide Lys-Ser (KS) the specific N-acylation of the lysine side chain was obtained [20]. The O-acylation of the serine side chain was never observed. A possible explanation was the weak nucleophilicity of the serine hydroxyl group due to the electrophilic effect of the C-terminal carboxylic group.

All these results were obtained from experimental approach. Until now, only assumptions were made to explain the selectivity of CALB toward amino-acids and peptides but no extensive explanation has been given so far.

For quite a number of years now, molecular modeling simulations have been recognized as powerful tools to explain enzymatic selectivity. Most of molecular simulations studies related to enantio-selectivity of enzymes. Only few works reported the use of molecular modeling methodologies to address the topic of regio- and chemo-selectivity toward polyfunctional substrates. Two recent studies combining molecular dynamics and docking simulations provided a possible explanation for the regioselective acetylation of flavonoids catalyzed by CALB and *Pseudomonas cepacia* lipase [21,22]. Interaction modes between the catalytic cavity of the enzyme and the substrates were shown to be directly responsible for functional group accessibility and consequently for selectivity. Few other studies concerned the chemo-selectivity of CALB toward various amino alcohols. The structure of both the acyl donor and the acyl acceptor was shown to be the main factor influencing selectivity, favoring either the O- or the N-acylation [23,24]. To our knowledge, no study has ever been conducted to explain the selectivity of lipases toward peptide acylation.

The present work aimed to gain deeper insight into the selectivity of CALB in acylation reactions involving short peptides and oleic acid as acyl donor. Molecular modeling simulations were carried out to explain the selectivity experimentally observed during the acylation of KS. To achieve this goal, a model of acyl-enzyme was developed and used as target to dock the peptide within the catalytic cavity. The binding modes of the peptide were studied to draw conclusions about the chemo- and the regio-selectivity of the reaction. Simulation results were compared to experimental data and then the model was applied to predict the selectivity of the reaction in the case of short peptides presenting a lysine residue but differing from each other by their sequence. Finally, acylation reactions were achieved in order to check the concordance between theoretical results and experimental evidence.

2. Materials and methods

2.1. Enzymatic reactions

2.1.1. Syntheses

Novozym 435® (lipase B from *Candida antarctica* immobilized on an acrylic resin) with propyl laurate synthesis activity of 7000 PLU g^{-1} and protein grade of 1–10% was from Novo Nordisk A/S (Bagsværd, Denmark). Peptides KS-HCl, Lys-Tyr-Ser (KYS), Ser-Tyr-Lys (SYK) with 99% of purity were acquired from Bachem (Switzerland). The peptide Leu-Gln-Lys-Trp (LQKW) (99% purity) was produced by Genosphere (France). Oleic acid used as acyl donor substrate for all syntheses (M = 282.49 g.mol^{-1}, purity: 99%) was from Sigma–Aldrich (France). 2-Methyl-2-butanol (M_2B_2) and triethylamine of HPLC quality were purchased from Carlo Erba (France).

The enzymatic acylation of peptides was carried out in test tubes. In a typical reaction, the peptide (0.12 M) and oleic acid (0.24 M) were added in 2 mL of M_2B_2 previously dehydrated on 4 Å molecular sieves. The initial water activity of the media was decreased below 0.1 to favor the synthetic activity of the lipase and prevent hydrolysis. Triethylamine was added to the reaction media in a large excess (2.4 mol L^{-1}) to favor the neutral form of amino groups [25,26]. Peptides exhibit low solubility in organic medium; consequently few hours are necessary to allow the formation of an ionic complex between the peptidic substrate and oleic acid. This complex favored the solubilization of the peptide. After solubilization of the substrates for 12 h at 55 °C, the acylation was started by the addition of 10 g L^{-1} of enzyme preparation. Reaction media were kept at 55 °C and stirred at 250 rpm. 50 μL samples were withdrawn for analyses. The enzyme was removed from the reaction medium by filtration. Then, samples were diluted with methanol/water (80/20, v/v) and stored at −18 °C before LC–MS analysis. Each reaction was repeated twice.

2.1.2. Analyses of reaction media

Qualitative and semi quantitative analysis of peptide derivatives was carried out on a HPLC–MS system (ThermoFisher Scientific, San Jose, CA, USA) consisting in a binary delivery pump connected to a photodiode array detector (PDA) and a LTQ ion trap as mass analyzer (Linear Trap Quadrupole) equipped with an atmospheric pressure ionization interface operating in positive electrospray mode (ESI^+). Chromatographic separation was performed on a C18 amide column (150 mm × 2.1 mm, 5 μm porosity – Grace/Alltech, Darmstadt, Germany) equipped with a C18 amide pre-column (7.5 mm × 2.1 mm, 5 μm porosity – Grace/Alltech Darmstadt, Germany) at 25 °C. Mobile phases consisted in methanol/water/TFA (80:20:0.1, v/v/v) for A and methanol/TFA (100:0.1, v/v) for B. Acylated peptides were eluted using a linear gradient from 0% to 100% of B for 5 min and then an isocratic step at 100% of B for 10 min, at a flow rate of 0.2 mL min^{-1}. Mass spectrometric conditions were as follows: spray voltage was set at +4.5 kV; source gases were set for sheath gas, auxiliary gas and sweep gas at 30, 10 and 10, respectively (in arbitrary units min^{-1}); capillary temperature was set at 250 °C; capillary voltage was set at 48 V; tube lens, split lens and front lens voltages were set at 120 V, −34 V and −4.25 V, respectively. Ion optic parameters were optimized by automatic tuning using a standard solution of oleoyl KS at 0.1 g L^{-1} infused in mobile phase (A/B, 50:50) at a flow rate of 5 μL min^{-1}. Full scan MS spectra were performed from 100 to 1000 m/z and additional MS^2 scans were realized in order to get structural information based on daughter ions elucidation. Raw data were processed using Xcalibur software (version 2.1 Thermo Scientific).

2.2. Molecular modeling simulations

2.2.1. Computational resources

Construction and relaxation of the systems as well as docking experiments were carried out on a bi-processor AMD Dual Core 280, 2.4 GHz. Docking and Scoring were performed using respectively the LigandFit [27] and the Consensus Score modules of the program-package Discovery Studio version 3.5 (Accelrys, Inc.). Molecular dynamics simulations were carried out on a computer cluster equipped with 2 Quad Core Intel Xeon processors L5420, 2.5 GHz and 16 GB of RAM on a Linux Platform, using NAMD (v.2.7) [28]. Visualization and analysis of trajectories were performed with VMD (v.1.8.7) [29]. All molecular mechanics and molecular dynamics calculations were performed with the CHARMm force field [30].