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N-terminal peptide of *Rhizopus oryzae* lipase is important for its catalytic properties

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Abstract In a culture medium, the *Rhizopus oryzae* strain produces only one form of lipase, ROL32. When the concentrated culture medium was stored at 0 °C during several months or kept at 6 °C during a few days, we noticed the appearance of a second shorter form of ROL32 lacking its N-terminal 28 amino acid (ROL29). ROL29 was purified to homogeneity and its 21 N-terminal amino acid residues were found to be identical to the 29–49 sequence of ROL32. The cleavage of the N-terminal peptide reduced the specific activity of ROL29 by 50% using either triolein or tributyrin as substrates. In order to explain this decrease of the specific activity of ROL29, we measured its critical surface pressure of penetration into phosphatidyl choline from egg yolk films which was found to be 10 mN/m, in contrast to a value of 23 mN/m found in ROL32. A kinetic study on the surface pressure dependency, stereoselectivity and regioselectivity of ROL29 was performed using the three dicaprin isomers spread as monomolecular films at the air–water interface. Our results showed that in contrast to ROL32, ROL29 presented a preference for the distal ester groups of one diglyceride isomer (1,3-*sn*-dicaprin). Furthermore, ROL32 was markedly more stereoselective than ROL29 for the *sn*-3 position of the 2,3-*sn*-enantiomer of dicaprin. A structural explanation of the enhanced penetration capacity as well as the catalytic activity of ROL32 was proposed by molecular modeling. We concluded that the N-terminal peptide of ROL32 can play an important role in the specific activity, the regioselectivity, the stereoselectivity and the binding of the enzyme to its substrate.

Keywords: *Rhizopus oryzae* lipase; Nucleotide sequencing; Monomolecular films; Critical pressure; 3D structure modeling; Binding

1. Introduction

The isolation of lipases (glycerol ester hydrolases EC 3.1.1.3) from various microorganisms revealed that all these enzymes degrade the ester bonds present in triacylglycerols, but their substrate selectivity is often relaxed. In addition to natural glycerides, some lipases can hydrolyse synthetic lipids and/or phospholipids [1]. The maximum lipase catalytic activity is usually expressed in the presence of a lipid–water interface and the physico-chemical properties of the interface play an important role in lipolysis [2].

Over the past few years, more than twenty lipase structures have been determined [3,4]. Although the overall homology between lipases is low and their molecular masses vary from 20 to 60 kDa, all known lipases share a comparable three-dimensional structure which is common to the α/β hydrolase family [5]. Apart from this highly conserved fold of the central core, diversity among lipases mainly results from extra-structural extensions displaying low homology. The region having the highest conservation is the active site, which contains a ''classical'' Ser-His-Asp catalytic triad and residues involved in the oxyanion hole. Interestingly, in most lipase structures, the active site is inaccessible due to coverage by one or more surface loops or helical structures. The structures of lipases bound to substrate [6] and of lipases inhibited by transition-state analogues [7,8] showed that the active site becomes exposed to substrate upon interaction with micelles or substrate molecules. These studies provided a structural basis for the well-known phenomenon of interfacial activation as it was discovered nearly forty years ago [9,10].

In previous works, the lipase of *Rhizopus oryzae* (ROL32) was produced, purified to homogeneity from the culture medium and some kinetic properties were determined using classical emulsified system [11]. The purified enzyme was shown to be a monomeric protein with a molecular mass of 32 kDa. Interestingly, specific activities of 10 000 and 2000 U/mg were obtained using triolein and tributyrin as substrates, respectively [11].

The ROL32 presents the interfacial activation phenomenon and its p*I* value is 6.85 [12]. Purified ROL32 possesses the same N-terminal sequence as the mature *Rhizopus niveus* lipase which is also found in the last 28 amino acids of the propeptide sequence derived from the cDNA of *R. oryzae* lipase [13] or *Rhizopus delemar* lipase [14]. According to Beer et al. [13] and Haas et al. [14], this propeptide should be removed in order to give rise to the mature enzymes.

As described in previous studies [11,12], *R. oryzae* strain produces only one form of lipase, ROL32. When the concentrated culture medium was stored at 0 °C during several months or

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Abbreviations: RDL, *Rhizopus delemar* lipase; RNL, *Rhizopus niveus* lipase; ROL, *Rhizopus oryzae* lipase; rROL, recombinant *Rhizopus oryzae* lipase; Egg-PC, phosphatidyl choline from egg yolk; PCR, polymerase chain reaction; S.I., Stereoselectivity index; TC_3, tripropionin; V.I., Vicinity index

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kept at 6 °C during a few days, we noticed the appearance of a second shorter form of ROL lacking its first 28 amino acids and named ROL29.

In this work we purified to homogeneity ROL29. The N-terminal sequence of ROL29 clearly indicated that this enzyme was generated by the cleavage of the first 28 amino acids of ROL32. Using the monomolecular film technique, we assessed the influence of the N-terminal sequence from ROL32 on its specific activity, regioselectivity, stereoselectivity and its binding to a lipidic interface. Molecular modelling of ROL32 and ROL29 was proposed to clarify the role played by this sequence.

2. Materials and methods

2.1. Chemicals

Benzamidine was from Fluka (Buchs, Switzerland); sodium deoxycholate (NaDC) and sodium taurodeoxycholate (NaTDC) were from Sigma Chemical (St. Louis, USA); gum arabic was from Mayaud Baker LTD (Dagenham, UK); acrylamide and electrophoresis grade, were from BDH (Poole, UK); marker proteins was from Pharmacia (Uppsala, Sweden); PVDF membrane was purchased from Applied Biosystems (Roissy, France); trans blot cell apparatus was from Bio-Rad (Paris, France); pH-stat was from Metrohm (Switzerland).

2.2. Bacterial strains, plasmids and media

Escherichia coli strain DH5α was used as cloning host for the part of the gene encoding the mature lipase. *E. coli* strain was grown in Luria–Bertani medium, supplemented with 100 μg/ml ampicillin when plasmid maintenance was required. The plasmid pMOS*Blue* (Amersham–Pharmacia Biotech) was used as cloning vector.

2.3. Lipids

1,2-*sn*- and 1,3-*sn*-Dicaprin were from Sigma; 2,3-*sn*-dicaprin was prepared from tricaprin (Sigma Chemical) by stereospecific enzymatic hydrolysis of the *sn*-1 ester bond as described previously [15]. As expected, the surface pressure–molecular area curves of 1,2-*sn*- and 2,3-*sn*-dicaprin are superimposable (data not shown). They are characteristic of the liquid expanded state and show no sign of discontinuity in the full range of surface pressure. The collapse pressure of 1,2-*sn*- and 2,3-*sn*-dicaprin is 40 mN/m and that of 1,3-*sn*-dicaprin is 32 mN/m [15]. Egg-phosphatidyl choline (egg-PC), *rac*-dicaprin, tributyrin were from Fluka.

2.4. Enzymes and proteins

Bovine serum albumin fraction IV (BSA) was from Sigma (St. Louis, USA). *R. oryzae* lipase (ROL32) was purified from growth medium according to the procedures described in previous work [11].

2.5. Determination of protein concentration

Protein concentration was determined using BSA as standard as described by Bradford [16].

2.6. Lipase activity determination

The lipase activity was measured titrimetrically at pH 8.2 and at 37 °C with pH-stat under the standard assay conditions as described previously [11]. When TC_3 was used as substrate, solutions were systematically prepared by mixing (3×30 s in a warring blender) a given amount of TC_3 in 30 ml of 0.33% GA and 0.15 M NaCl. The release of propionic acid was recorded continuously at pH 7 and at 37 °C [17].

2.7. Analytical methods

Analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (0.3 M) and β-mercaptoethanol (0.25 M) or DTT (0.5 M) (SDS/PAGE) was performed by the method of Laemmli [18]. Samples for sequencing were electroblotted according to Bergman and Jörnvall [19]. Protein transfer was performed during 1 h at 1 mA/cm^2 at room temperature.

2.8. Amino acid sequencing

The N-terminal sequences of purified ROL29 (present study) was determined by automated Edman's degradation, using an Applied Biosystems 470 A protein sequencer equipped with PTH 120A analyser [20]. The sequence was kindly determined by Dr. Reinbolt (IBMC, UPR 9002, CNRS-Strasbourg, France).

2.9. DNA preparation and transformation procedure

Rhizopus DNA was prepared as described previously [21]. Cells were lysed by the addition of lysostaphin (Sigma) at 4.5 U/ml. *E. coli* supercoiled plasmid DNA was prepared by the modified alkaline lysis method [22]. *E. coli* was transformed by the $CaCl_2$ method. Enzymes for molecular cloning were obtained from Boehringer (Mannheim), BRL and Pharmacia LKB. Assay conditions were as recommended by the suppliers. PCR products were purified using Wizard PCR Preps DNA Purification system (Promega).

2.10. Cloning of the mature lipase gene region

The part of the gene encoding the mature ROL32 was amplified by PCR from genomic DNA of *R. oryzae* with primers, oligo#24: 5′-GAT GATAACTTGGTTGGTGGC-3′, and oligo#25: 5′-TTACAAACAG CTTCCTTCGTTGAT-3′. The primers were predicted, respectively, from the N-terminal sequence of the purified ROL32 [12] and the conserved region of ROL32 with the gene encoding the RDL (nucleotides from 1201 to 1224) [14].

The PCR product (894 pb) was isolated and ligated into the *Eco*RV-linearised and dephosphorylated pMOS*Blue* vector, using the pMOS*Blue* blunt ended cloning kit RPN 5110, according to manufacturer's protocol (Amersham–Pharmacia Biotech). Protoplasts of *E. coli* DH5α were transformed with the ligation mixture. The resulting recombinant plasmid was named pROL32. The presence of the appropriate insert was determined by PCR and by restriction analysis. DNA products were analysed on a standard 1% agarose gel containing ethidium bromide. DNA sequences were elucidated by the dideoxynucleotide chain termination method according to a cycle sequencing protocol using thermosequenase (Amersham–Pharmacia Biotech). The sequencing reactions were analysed with the DNA sequencor (Genom express, Grenoble, France).

The sequencing was performed using the recombinant vector (pROL32) as template using the T7 promoter primers (Amersham–Pharmacia Biotech).

2.11. Nucleotide sequence access number

The nucleotide sequence of mature ROL32, determined in this study, was deposited in the GenBank database under Accession No. AY513724.

2.12. Measurement of the lipase penetration into egg-PC monolayer

The surface pressure increase due to the penetration of lipase into egg-PC/water interface was measured in a cylindrical trough drilled in a Teflon block (surface area 7 cm^2, the total volume was 5 ml of 10 mM Tris–HCl, pH 8, 150 mM NaCl, 21 mM $CaCl_2$ and 1 mM EDTA).

The aqueous subphase was continuously stirred at 250 rpm with a magnetic rod. Measurements of penetration were estimated as described previously [23].

2.13. Monomolecular film techniques

Measurements were performed with KSV-2000 Baro-stat equipment (KSV-Helsinki). The principle of the method was described previously by Verger and de Haas [24]. It involves the use of a "zero-order" trough with two compartments: a reaction compartment and a reservoir compartment, which were connected to each other by a small surface channel [12,24].

The kinetic data were analysed as described previously [24,25]. Activities are expressed as the number of moles of substrate hydrolyzed per unit time and unit surface of the reaction compartment of the "zero order" trough for an arbitrary lipase concentration of 1 M.

2.14. 3D structure prediction

The secondary structure of the 28 N-terminal amino acids of ROL32 was predicted using the Deep View/Swiss-Pdb Viewer v 3.7 (http://www.expasy.org/spdbv/). The rest of the molecule