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Purification and biochemical characterization of the LIP2 lipase from *Yarrowia lipolytica*

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Abstract

The LIP2 lipase from the yeast *Yarrowia lipolytica* (YLLIP2) was obtained from two genetically modified strains with multi-copies of the *lip2* gene and further purified using gel filtration and cation exchange chromatography. Four YLLIP2 isoforms were identified and subjected to N-terminal amino-acid sequencing and mass spectrometry analysis. These isoforms differed in their glycosylation patterns and their molecular masses ranged from 36,874 to 38,481 Da, whereas the polypeptide mass was 33,385 Da. YLLIP2 substrate specificity was investigated using short (tributyrin), medium (trioctanoin) and long (olive oil) chain triglyceride substrates at various pH and bile salt concentrations, and compared with those of human gastric and pancreatic lipases. YLLIP2 was not inhibited by bile salts at micellar concentrations with any of the substrates tested, and maximum specific activities were found to be 10,760±115 U/mg on tributyrin, 16,920±480 U/mg on trioctanoin and 12,260±700 U/mg on olive oil at pH 6.0. YLLIP2 was found to be fairly stable and still active on long chain triglycerides (1590±430 U/mg) at pH 4.0, in the presence of bile salts. It is therefore a good candidate for use in enzyme replacement therapy as a means of treating pancreatic exocrine insufficiency.

Keywords: Microbial lipase; Lipolysis; pH-dependency; Bile salts; Yeast; Triglycerides

1. Introduction

The yeast *Yarrowia lipolytica*, formerly known as *Candida*, *Endomycopsis* or *Saccharomycopsis lipolytica*, is one of the most extensively studied non-conventional yeasts. This yeast, the only species recognized so far in the *Yarrowia* genus, is quite different from the other frequently studied yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* in terms of its phylogenetic evolution, physiology, genetics, and molecular biology. *Y. lipolytica* is non-pathogenic to humans and has been approved for several Generally Recognized As Safe (GRAS) industrial processes. *Y. lipolytica is* an obligate aerobic dimorphic ascomycete that naturally secretes large amounts of various metabolites (such as organic acids and extracellular proteins) [1]. Since an increasing number of studies have clearly shown that *Y. lipolytica* occurs naturally in various kinds of food, information about this yeast is of great importance to the agro-food industry [2]. Strains of this species have mostly been isolated from lipid-rich food such as cheese and olive oil, as well as from sewage, and this yeast grows readily on hydrophobic substrates such as alkanes, fatty acids and oils [2–4]. Its substrate preferences and physiological properties have been attributed to the fact that this yeast efficiently produces and secretes proteolytic and lipolytic enzymes [1]. Several processes have been developed involving the use of *Y. lipolytica* in bioconversion reactions with hydrophobic substrates [2], some of which have been patented in the fields of bioremediation, fine chemistry and the food industry. For instance, *Y. lipolytica* can be used for alkane and fatty-acid bioconversion, the production of aromas, single-cell protein (SCP), single-cell oil (SCO) and citric acid, as well as for steroid biotransformation.

Since *Y. lipolytica* can use fat and oils as carbon sources, increasing attention has been paid to the lipases produced by this yeast. Lipases (EC 3.1.1.3) catalyze the hydrolysis of long-chain

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triacylglycerols and play an important physiological role in several metabolic processes (fat digestion in the gastro-intestinal tract, lipolysis in the adipose tissue, lipolysis of lipoproteins) in the conversion of oils and fats into free fatty acids and partial acylglycerols. Considerable efforts have been made to elucidate their role in dietary fat digestion [5–7] and to describe in detail the molecular and kinetic events occurring during the catalytic process at lipid/water interfaces [8]. Lipases with new properties (substrate specificity, stereoselectivity, thermostability) are of particular interest, and these can be obtained either by isolating them from various natural sources, by using protein engineering methods, or by performing directed evolution [9]. Increasing attention has been paid to lipases of microbial origin, due to their wide range of biochemical properties and because they are relatively easy to produce in bioreactors and to recover from the culture medium [10]. The catalytic properties of microbial lipases have made it possible to develop many biotechnological applications in the food, cosmetic, detergent, and pharmaceutical industries [11–14].

In *Y. lipolytica*, the *lip2* gene which codes for a 301-amino acid extracellular lipase YLLIP2 has been cloned and amplified and it was established that most of the extracellular lipase activity results from *lip2* expression [15,16]. The authors of recent studies reported that methyl oleate modulates YLLIP2 expression in *Y. lipolytica* [2]. Although YLLIP2 seems involved in the use of triglycerides as a carbon source for the yeast, the finding that a *lip2* knock-out strain is still able to use this source suggested the presence of other lipases in *Y. lipolytica*. The cloning and functional analysis of the *lip7* and *lip8* genes encoding two predicted lipases were recently performed [2]. Unlike YLLIP2, which is secreted into the culture medium, it was observed that YLLIP7 and YLLIP8 are mainly associated with the cell wall.

Fungal lipases mainly belong to two families: (i) lipases which are homologous to acetylcholine esterase (i.e. *Geotrichum candidum* and *Candida rugosa* lipases) and (ii) triacylglycerol lipases including *Rhizopus oryzae*, *Rhizomucor miehei*, *Candida antarctica* and *Thermomyces lanuginosus* [17]. Amino acid sequence comparisons have shown that YLLIP2 belongs to the second group (Fig. 1). The highest amino acid sequence similarity was 91.6% with *Candida deformans* lipase LIP1. Great similarities were also found to exist with the lipases from *Thermomyces lanuginosus* (30.3%), *Candida ernobii* (27.7%) and *Rhizomucor miehei* (25.1%).

Unlike these extensively characterized fungal lipases, no purified YLLIP2 has been biochemically characterized so far, to our knowledge. In the present study, we therefore purified this enzyme for the first time, and determined its lipase activity on triglycerides with various chain lengths. The effects of pH and bile salts were tested and compared with those observed with human pancreatic and gastric lipases.

2. Materials and methods

2.1. YLLIP2 production

Two genetically modified *Y. lipolytica* strains overproducing YLLIP2 were used in this study [15]. These strains had a mean number of 6 and 20 integrated copies of the *lip2* gene under the control of the strong inducible *POX2* promotor.

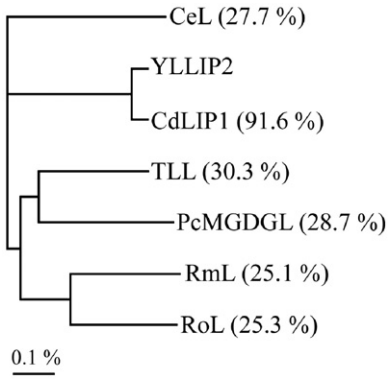

Fig. 1. Dendrogram of fungal lipase sequence alignment showing the homologies between YLLIP2 and other lipases. YLLIP2 (*Yarrowia lipolytica* lipase, access no CAB91111), TLL (*Thermomyces lanuginosus* lipase, AAC08588), RmL (*Rhizomucor miehei* lipase, CAA00250), CdLIP1 (*Candida deformans* lipase LIP1, CAD21428), PcMGDGL (*Penicillium camemberti* mono- and diacylglycerol lipase, BAA14345), CeL (*Candida ernobii* lipase, CAC15040) and RoL (*Rhizopus oryzae* lipase, AAF32408). Accession numbers are those of the NCBI protein data base. Sequence alignment was performed using the ClustalW program [42]. Identity percentages versus YLLIP2 are in brackets.

The 20-copy strain produces more active lipase than the 6-copy strain under lipase-inducing conditions (see below). The media and techniques used here to grow and handle *Y. lipolytica* have been described elsewhere [18]. The production of YLLIP2 was performed using fed-batch cultures under controlled conditions at 28 °C in a 6 L bioreactor (BIOSTAT®A-DCU, Sartorius BBI systems) with a working volume of 4.5 L. Dissolved oxygen was continuously monitored with an oxygen probe (Ingold, Urdorf, Switzerland). The stirrer was used at a speed of 1000 rpm to ensure that the dissolved oxygen reached a saturation level of at least 30%. Air flow was set at 2 L/min and the pH was automatically held at 5.6±0.1 by adding 28% NH_4OH in water. In order to prevent excess foam formation, a level sensor triggering the addition of an antifoam compound (J673, Struktol, Germany) was placed 10 cm from the top of the vessel. Initially, the bioreactor was filled with 3 L of YPD culture medium and then inoculated at 12.5% (v/v) with *Y. lipolytica* liquid pre-culture, having an optical density (600 nm) of 1, in order to start the cell growth directly in the exponential growth phase, as well as to establish reproducible cell culture conditions. During the first 25-h fermentation phase, the biomass level was increased using the glucose (10 g/L) present in the YPD culture medium. Then, the lipase expression was induced by feeding the bioreactor with culture medium containing 1% (v/v) of oleic acid as the only source of carbon during the following 35 h. Biomass and lipase activity were monitored as a function of time until the lipase production level in the culture broth reached a maximum value, which was generally around 3 g lipase/L of culture supernatant after 60 h of fermentation. The lipase mass concentration was estimated from the lipase activity measurement (32,280 U/mL using tributyrin as substrate) and the specific activity of the lipase (10,760±115 U/mg) determined in the present study. The culture (4.5 L) was then stopped to avoid any proteolysis and the cells were pelleted by centrifugation at 3000×*g* for 20 min at 4 °C. Benzamidin was then added to the supernatant at a final concentration of 2 mM in order to limit the proteolysis of YLLIP2. The culture supernatant (2.5 L) was immediately filtered by tangential flow ultrafiltration using hollow cartridges (Millipore) with a fiber diameter of 0.1 μm. The filtrate was then lyophilised and stored at −20 °C.

2.2. YLLIP2 purification

All subsequent purifications steps were performed at 4 °C. One hundred mg of the resulting powder containing 120,640 lipase U (tributyrin as substrate) were suspended in a minimum volume (~10 mL) of 10 mM Tris buffer, pH 7.5, containing 150 mM NaCl and 2% (v/v) protease inhibitors (Complete®, Roche), and filtered. Gel filtration chromatography was performed after loading the previous solutions on a Superdex 200 HR 26/60 column (Pharmacia), using