Biochimica et Biophysica Acta 1851 (2015) 129–140

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Biochemical characterization of *Yarrowia lipolytica* LIP8, a secreted lipase with a cleavable C-terminal region

Jannet Kamoun [a], Mathieu Schué [b], Wala Messaoud [a], Justine Baignol [b], Vanessa Point [c], Eduardo Mateos-Diaz [c], Pascal Mansuelle [d], Youssef Gargouri [a], Goetz Parsiegla [c], Jean-François Cavalier [c], Frédéric Carrière [c,*], Ahmed Aloulou [a,c,**]

[a] University of Sfax, ENIS — Laboratory of Biochemistry and Enzymatic Engineering of Lipases, 3038 Sfax, Tunisia
[b] Proteabio Europe S.A.S., 290 Chemin de Saint-Dionysy, Bât A, 30980 Langlade, France
[c] CNRS, Aix-Marseille Université, UMR 7282 Enzymologie Interfaciale et Physiologie de la Lipolyse, 31 Chemin Joseph Aiguier, 13009 Marseille, France
[d] CNRS — Aix-Marseille Université, Plate-forme Protéomique FR3479, IBiSA Marseille-Protéomique, 31 Chemin Joseph Aiguier, 13009 Marseille, France

ARTICLE INFO

Article history:
Received 31 May 2014
Received in revised form 23 October 2014
Accepted 27 October 2014
Available online 1 November 2014

Keywords:
Enzyme
Lipolysis
Pichia pastoris
Regioselectivity
Signal peptide
Yeast

ABSTRACT

Yarrowia lipolytica is a lipolytic yeast possessing 16 paralog genes coding for lipases. Little information on these lipases has been obtained and only the major secreted lipase, namely YLLIP2, had been biochemically and structurally characterized. Another secreted lipase, YLLIP8, was isolated from *Y. lipolytica* culture medium and compared with the recombinant enzyme produced in *Pichia pastoris*. N-terminal sequencing showed that YLLIP8 is produced in its active form after the cleavage of a signal peptide. Mass spectrometry analysis revealed that YLLIP8 recovered from culture medium lacks a C-terminal part of 33 amino acids which are present in the coding sequence. A 3D model of YLLIP8 built from the X-ray structure of the homologous YLLIP2 lipase shows that these truncated amino acids in YLLIP8 belong to an additional C-terminal region predicted to be mainly helical. Western blot analysis shows that YLLIP8 C-tail is rapidly cleaved upon enzyme secretion since both cell-bound and culture supernatant lipases lack this extension. Mature recombinant YLLIP8 displays a true lipase activity on short-, medium- and long-chain triacylglycerols (TAG), with an optimum activity at alkaline pH on medium chain TAG. It has no apparent regioselectivity in TAG hydrolysis, thus generating glycerol and FFAs as final lipolysis products. YLLIP8 properties are distinct from those of the 1,3-regioselective YLLIP2, acting optimally at acidic pH. These lipases are tailored for complementary roles in fatty acid uptake by *Y. lipolytica*.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Lipases or triacylglycerol hydrolases (E.C. 3.1.1.3) are widespread in nature and are particularly common in the microbial world [1]. The production of lipases enables many microorganisms to assimilate lipids with ester bonds, *e.g.*, triacylglycerols (TAG), as carbon sources. TAG cannot passively cross cell membrane and have first to be broken down into partial glycerides and free fatty acids (FFAs) by lipases prior to the absorption of the free fatty acids (FFAs) by the cell [2].

The lipolytic yeast *Yarrowia lipolytica* is a "generally recognized as safe" (GRAS) microorganism and therefore used in various biotechnological applications [3]. *Y. lipolytica* also provides an interesting model organism to study extracellular lipolysis and fat uptake [2,4,5]. Since *Y. lipolytica* can use TAGs as carbon sources, increasing attention has been paid to the lipases produced by this yeast [6]. Its main secreted lipase is LIP2 (YLLIP2) which is produced in large amounts [7,8]. YLLIP2 shows one of the highest lipase activities on long-chain TAG ever characterized, and it maintains a high activity at low pH levels [9]. Among all lipases, YLLIP2 is also one of the few enzymes that hydrolyze long-chain TAG faster than tributyrin [9,10]. Moreover, at the physiological pH values found in the gastrointestinal tract (GI), YLLIP2 adsorption at the oil–water interface is not inhibited by bile salts, which is in contrary to observations reported from other microbial lipases [11]. YLLIP2 might thus be active in the conditions of the human GI tract like gastric and pancreatic lipases [12], two enzymes well characterized biochemically and structurally [13–16]. The crystal structures of the glycosylated wild-type YLLIP2 [17] and the non-glycosylated recombinant YLLIP2 produced in *Pichia pastoris* [18] have been reported and in both structures the active site is covered by a closed lid. The glycosylation of

Abbreviations: DAG, diacylglycerol; DLPC, 1,2-dilauryl-*sn*-glycero-3-phosphocholine; FFA, free fatty acid; GA, gum arabic; MAG, monoacylglycerol; NaTDC, sodium taurodeoxycholate; TAG, triacylglycerol; TMD, transmembrane domain; YLLIP8, *Yarrowia lipolytica* LIP8 lipase; YLLIP2, *Yarrowia lipolytica* LIP2 lipase; YPD, yeast-peptone-dextrose culture medium

* Corresponding author. Tel.: +33 491 164 134; fax: +33 491 715 857.
** Correspondence to: A. Aloulou, CNRS, Aix-Marseille Université, UMR 7282 Enzymologie Interfaciale et Physiologie de la Lipolyse, 31 Chemin Joseph Aiguier, 13009 Marseille, France. Tel./fax: +33 216 74 67 50 55.
E-mail addresses: carriere@imm.cnrs.fr (F. Carrière), ahmed.aloulou@enis.rnu.tn (A. Aloulou).

http://dx.doi.org/10.1016/j.bbalip.2014.10.012
1388-1981/© 2014 Elsevier B.V. All rights reserved.

YLLIP2 has been shown to impact enzyme activity and binding to lipids, and was found to be essential for preserving the lipase activity in the presence of proteases, particularly pepsin [18]. From this biochemical characterization, it is assumed that YLLIP2 is a good candidate for pancreatic enzyme replacement therapy in the treatment of pancreatic exocrine insufficiency [9].

Another extracellular lipase produced by *Y. lipolytica* is YLLIP8, which presents low sequence similarity with YLLIP2 [19] but 89.2% sequence identity with the uncharacterized CdLIP3 lipase from the closely related yeast *Candida deformans* [20]. From studies performed with *Y. lipolytica LIP2* and *LIP8* knock-out strains, it was estimated that the lipolytic activity of YLLIP8 might contribute to only 3% of the total extracellular lipase activity of *Y. lipolytica* wild-type strain [8,19]. It was also proposed that YLLIP8 would be mainly associated with the cell wall and could correspond to a cell-bound lipase [19], whereas YLLIP2 would be the main lipase released in the culture medium at the end of the growth phase [7].

Unlike the extensively characterized YLLIP2 lipase, the substrate specificity and kinetic properties of YLLIP8 were not studied in detail so far. A purified recombinant YLLIP8 produced in the yeast *P. pastoris* was characterized using *p*-nitrophenol esters as substrates and was found to display its maximum activity with C8 and C10 esters [21]. This is in agreement with similar results obtained with a crude preparation of YLLIP8 overproduced in *Y. lipolytica* [19] or displayed on *Saccharomyces cerevisiae* cell surface [22]. Only one article reported the activity of a recombinant IgG-tagged YLLIP8 produced in *Escherichia coli* on several TAG and vegetable oils, including olive oil [23]. This study revealed higher activity of IgG-YLLIP8 fusion protein on vegetable oils followed by pure TAGs and p-nitrophenyl esters, suggesting that YLLIP8 was a true lipase. Interestingly, this enzyme was found to be thermostable and showed a half inactivation time of 52 min at 80 °C, but this study was performed with the fusion protein and not with YLLIP8 itself.

The present study was undertaken to get more insight into the lipase activity of YLLIP8 and its substrate specificity towards short, medium and long chain TAGs. The combined effects of pH variation and surfactant (bile salts) concentration on the lipase activity were investigated, because these parameters are essential for understanding the mode of action of lipases and further link it with biological function. For this purpose, recombinant YLLIP8 (rYLLIP8) was produced in *P. pastoris*, purified and compared with the wild-type YLLIP8 (YLLIP8-wt) purified from crude *Y. lipolytica* culture medium. N-terminal sequencing, mass spectrometry analysis and 3D structural modeling suggest various post-translational modifications leading to the mature enzyme that was characterized here.

2. Materials and methods

2.1. Chemicals

1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) and 1,2-didecanoyl-*sn*-glycerol (1,2-dicaprin) from Avanti Polar Lipids, Inc. were purchased from COGER (Paris, France) and were >99% purity. Tributyrin, trioctanoin, triolein, diolein, monoolein, oleic acid, sodium taurodeoxycholate (NaTDC), NaCl, $CaCl_2$, Tris–HCl, anhydrous $MgSO_4$, cupric acetate monohydrate, orthophosphoric acid, iodine and HCl were purchased from Sigma-Fluka-Aldrich (St-Quentin-Fallavier, France) and were >99% purity. Endoglycosidase Endo H_f and amylose resin were from New England Biolabs (Ipswich, MA, USA). Thin layer silica gel 60 plates (10 × 20 cm) were from Merck KGaA (Darmstadt, Germany). All solvents were purchased from Carlo Erba Reactifs-SDS (Val de Reuil, France) and were of HPLC grade.

2.2. YLLIP8 DNA source and cloning

The cDNA encoding YLLIP8 was obtained from *Y. lipolytica* as previously reported [19]. A 1,116 bp XhoI/XbaI DNA fragment containing the entire YLLIP8 coding region was subcloned into the pGAPZαA *P. pastoris* transfer vector (Invitrogen, Saint Aubin, France), downstream of the GAP constitutive promoter and the sequence of the yeast secretion peptide α. Plasmids were produced in *E. coli* and DNA sequencing was performed by GATC Biotech (Mulhouse, France).

2.3. Production and purification of recombinant YLLIP8 (rYLLIP8)

Transformation of *P. pastoris* X33 strain and selection of recombinant yeast clones were performed as described previously [18,24]. Lipase activity measurement made it possible to easily identify the clones that efficiently secreted the heterologous protein into the culture medium. The time-course secretion of rYLLIP8 by the most efficient *P. pastoris* clone was monitored by measuring lipase activity and by performing western blot analysis with anti-YLLIP2 polyclonal antibodies cross-reacting with rYLLIP8. The best lipase-secreting clone was selected for the production of the recombinant enzyme in buffered YPD medium [18].

After yeast cell pellets had been discarded by centrifugation, all subsequent purification steps were conducted at 4 °C. Briefly, dithiothreitol was added to the culture supernatant at a final concentration of 1 mM to keep a reducing environment that prevents oxidation of the recombinant protein. The culture supernatant was concentrated using a 10 kDa Ultrasette™ tangential flow filtration device (Pall Corporation, St-Germain-en-Laye, France) to about 100 mL, and was then freeze dried. One gram of the remaining solid was dissolved in 2 mL of 10 mM MES-NaOH buffer, pH 7.0, containing 1 mM benzamidine (buffer A). After centrifugation at 15,000 *g* for 10 min, an insoluble pellet was discarded and the clear supernatant was filtrated using a 0.45 μm Acrodisc® syringe filter with Supor® membrane (Pall Corporation). The filtrate was loaded onto a Superdex 200 HR 26/60 gel filtration column (GE Healthcare, Velizy-Villacoublay, France) equilibrated in buffer A. The flow rate was adjusted to 1 mL min^{-1} and the pressure kept below 0.5 MPa using an ÄKTA explorer chromatographic device (GE Healthcare). Fractions eluted from the column showing lipase activity were pooled and applied to a Mono Q 5/50 GL anion exchanger column (GE Healthcare) equilibrated in buffer A. The flow rate was adjusted to 1 mL min^{-1} and the pressure was kept between 3.0 and 3.5 MP using the ÄKTA explorer device. A linear NaCl concentration gradient ranging from 0 to 500 mM NaCl was applied for 20 min. Again all fractions with lipase activity eluted from the column were pooled and concentrated using a 10,000 MWCO PES Sartorius™ Vivaspin™ centrifugal concentrator (Fisher Scientific, Strasbourg, France).

2.4. Production and purification of wild-type YLLIP8 (YLLIP8-wt)

Wild-type YLLIP8 was obtained as a side product of YLLIP2 production by *Y. lipolytica* using a genetically modified *Y. lipolytica* strain overproducing YLLIP2 were used in this study [15]. This strain had a mean number of 6 integrated copies of the lip2 gene under the control of the strong inducible POX2 promotor. The media and techniques used here to grow and handle *Y. lipolytica* have been described elsewhere [25]. The production 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. Briefly, 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 (at 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 activity level in the culture broth reached a maximum value. After discarding cell pellets, the culture supernatant was immediately

Download English Version:

https://daneshyari.com/en/article/8302125

Download Persian Version:

https://daneshyari.com/article/8302125

Daneshyari.com