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Production of stable isotope labelled lipase Lip2 from *Yarrowia lipolytica* for NMR: Investigation of several expression systems

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ABSTRACT

Extracellular lipase Lip2 from *Yarrowia lipolytica* is a promising biocatalyst with unusual structural features, as indicated by X-ray crystallography. These features comprise a mobile domain called the lid that controls access to the catalytic site. Conformational rearrangements of the lid have been suggested to regulate lipase enzymatic activities. We used nuclear magnetic resonance to investigate the dynamics of Lip2 by exploring four expression systems, *Escherichia coli*, cell-free, *Pichia pastoris* and *Y. lipolytica* to produce uniformly labelled enzyme. The expression of Lip2 was assessed by determining its specific activity and measuring ^{15}N–^{1}H HSQC spectra. *Y. lipolytica* turned out to be the most efficient expression system. Here, we report the first use of *Y. lipolytica* as an expression host for the production of uniform stable isotopic labelled protein for further structural and dynamics studies using NMR.

Introduction

Lipases (triacylglycerol acyl hydrolase EC 3.1.1.3) are enzymes that naturally hydrolyze the ester bonds of triglycerides. In favorable thermodynamic conditions (i.e. low water activity), they are also able to catalyze synthesis reactions on various substrates with high selectivity. The versatility of these enzymes makes them very attractive for many biotechnological applications. Amongst lipases, *Yarrowia lipolytica* lipase Lip2, a protein of 37 kDa, exhibits the highest activity on long-chain triglycerides reported to date [1]. It is also a good candidate for treatment of pancreatic exocrine insufficiency, as Lip2 remains highly active at low pH and it is not inhibited by bile salts. Its selectivity towards many different substrates and in particular, its ability to resolve racemic mixtures of 2-bromo-arylacetic acid esters [2], an important class of pharmaceutical intermediates, encourage its use in chemistry. Investigation of the structural determinants involved in its selectivity has already enabled the development of highly selective catalysts [3,4]. Recently, the three-dimensional structure of Lip2 was identified by X-ray crystallography [5,6] (PDB: 3O0D). Lip2 adopts the typical α/β hydrolase fold observed in lipases belonging to the fungal lipase family. An important structural feature of many lipases is the mobile α-helix, called lid. Depending on its conformation, the lid controls access to the catalytic site, which can be either closed or open. Unfortunately, all X-ray structures of Lip2 obtained to date showed the lid in the catalytically inactive closed conformation [5,6], consequently limiting insight into the catalytic mechanism. Indeed, the activation mechanism by which the lid switches from a closed to an open conformation is still not fully understood. Molecular dynamics studies suggested a complex lid opening motion involving both interactions with the substrate and with the lipid/water interface [5]. A better understanding of this process is crucial for the design of optimized lipase catalysts.

Further biophysical studies are required to be able to characterize the lid molecular motion of Lip2 lipase in more detail. To gain insight into protein dynamics, NMR spectroscopy provides a broad range of approaches that make it possible to sample protein dynamics at ps to ms time scales [7,8]. To do so, one relies heavily on 3D NMR and stable isotope labeling schemes, using ^{2}H, ^{13}C and ^{15}N, for determination of the structure and chemical shift assignment procedures, and for relaxation studies that enable access to spatially defined motion time scales and order parameters [9].

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For this purpose, well-defined minimal media are crucial for the production of recombinant proteins. Among the expression systems available for the production of labeled samples, recombinant *Escherichia coli* is the most widely used thanks to the high-level of protein expression and moderate cost of the growth media. However, as a prokaryotic expression system, it suffers from some drawbacks. The overproduction of eukaryotic recombinant protein often leads to misfolded protein aggregates in insoluble inclusion bodies. One possible way to overcome this limitation is the co-expression of molecular chaperones to favor correct folding and hence the solubility of the recombinant protein [10]. Another problem is that the formation of the disulfide bond, which requires an oxidative environment, is not compatible with the bacterial low intracellular redox state. Even if some strains have been engineered by deleting the glutathione reductase (*gor*)[1] and the thioredoxine reductase (*trxB*) leading to an altered cytoplasmic reducing potential [11], it remains challenging and often leads to the formation of insoluble inclusion bodies. An alternative strategy for the production of proteins containing disulfide bonds is the use of an acellular transcription/translation system. The cell-free reaction allows the direct supplementation of the reaction mixture with additives like metal ions, detergents, cofactors and/or binding partners. The redox potential can be fine tuned during the reaction to favor formation of the disulfide bond [12].

An alternative option is the use of recombinant eukaryotic hosts, including mammalian cells [13] or baculovirus-infected insect cells [14] with the necessary post-translational mechanisms. However, this generally leads to a low protein yield and the production of isotopic labeled samples for NMR studies would be expensive, because of the high cost of a rich culture medium. Yeasts thus appear to be the most attractive host as they are able to grow in low-cost minimal medium using ammonium chloride as nitrogen source, and glucose, glycerol, or methanol as carbon source. Yeasts combine the ease of use of *E. coli* with higher protein expression yields. Moreover, protein secretion and crucial post-translational modifications such as the formation of disulfide bonds and glycosylation are effective. The non-conventional methylotrophic yeast *Pichia pastoris* has already been used to produce large quantities of uniform $^{15}N^{13}C$ double labeled proteins using ^{15}N ammonium chloride and ^{13}C methanol [15]. Kluyveromyces lactis is another yeast that was recently used to produce uniform $^{15}N^{13}C$ double labeled proteins like in *P. pastoris* but by replacing the ^{13}C methanol carbon source by ^{13}C glucose [16].

Lip2 lipase is a glycosylated protein containing four disulfide bonds. Lip2 lipase is secreted by the non-conventional yeast *Y. lipolytica*, which has not been used for the production of isotopically labeled proteins to date. In the present study, we compared the expression of Lip2 in four different expression systems and checked correct folding and stability using $^{1}H–^{15}N$ HSQC, a prerequisite step for NMR structural studies and for the characterization of the lid motions upon activation of Lip2.

We tested and compared the four most promising strategies for protein expression and labelling: (i) homologous expression in *Y. lipolytica* [17]; (ii) heterologous expression in the yeast *P. pastoris* [1] and (iii) in *E. coli,* the most widely used expression system for labeled proteins [18]; and (iv) cell free expression, which has proven to be very efficient while allowing amino-acid specific labeling [19]. For reasons we discuss below, homologous expression turned out to be the most efficient strategy for Lip2.

[1] *Abbreviation used: gor*, glutathione reductase; *trxB*, thioredoxine reductase; YNB, yeast nitrogen base; PCR, polymerase chain reaction; CECF, continuous exchange cell-free expression; RM, reaction mixture; FM, feeding mixture; BSM, basal salt medium; *p*NPB, para-nitrophenolbutyrate.

Materials and methods

Chemicals

Restriction enzymes, phusion-HF polymerase, and T4-DNA-ligase were purchased from New England Biolabs (NEB) (Ipswich, MA, US); the primers came from Eurogentec (Liège, Belgium); tryptone, peptone, yeast extract, yeast nitrogen base (YNB) with or without ammonium sulfate and without amino acids were purchased from BD Difco (Franklin Lakes, NJ, US). Unless stated otherwise, other chemicals, were purchased from Sigma–Aldrich (St. Louis, MO, US). ^{15}N labeled ammonium sulfate was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, US).

Strains and culture conditions

E. coli strain DH5α (Life Technologies, Carlsbad, CA, US) was used for vector construction and amplification. Strains BL21(DE3) (Clontech, Mountain View, CA, US) and Shuffle T7 (NEB, Ipswich, MA, US) were used for expression. The BL21(Star) strain and Rosetta2 strains used for the preparation of cell-free extract were purchased respectively from Life Technology (Carlsbad, CA, US) and from Merck Millipore (Molsheim, France). *E. coli* strains were grown at 37 °C or 30 °C in LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl with 1.5% (w/v) solid agar) supplemented with the corresponding antibiotic, 40 mg/L of kanamycin, 25 mg/L of Zeocin, or 100 mg/L of Ampicillin.

P. pastoris strains X-33 and SMD1168H (Life Technologies, Carlsbad, CA, US) positive transformants were selected on YPDS solid medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose and 1 M sorbitol containing 1.5% (w/v) agar) supplemented with 100 mg/L of Zeocin. The transformants were screened to identify those producing the highest lipase activity using the following procedure: colonies were picked and cultured in 25 mL of BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM K_2HPO_4/KH_2PO_4 pH 6.0, 1.34% (w/v) YNB with ammonium sulfate and without amino acids, 4×10^{-5}% (w/v) biotin and 1% (v/v) glycerol) in a 250 mL baffled Erlenmeyer flask at 28 °C under stirring at 120 rpm. After the culture reached an $OD_{600} = 6$, cells were harvested by centrifugation and resuspended in 25 mL of BMMY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM K_2HPO_4/KH_2PO_4 pH 6.0, 1.34% (w/v) YNB with ammonium sulfate and without amino acids, 4×10^{-5}% (w/v) biotin and 1% (v/v) methanol) to trigger expression. One percent methanol (v/v) was added every day and OD_{600}, lipase activity and secretion were measured throughout the culture period.

The construction of the *Y. lipolytica* strain JMY1212 – JMP61-pTEF-LIP2 used in this work is described in [5].

Vector construction

Standard molecular genetic techniques were used [20]. The DNA sequence encoding the *Y. lipolytica* lipase Lip2 sequence (Uniprot: Q9P8F7) was amplified by polymerase chain reaction (PCR) using JMP61-LIP2 vector from [5] as template. Primers were designed to amplify the 906 bp sequence encoding the mature extracellular lipase of the LIP2 gene without the 99 bp sequence encoding the signal peptide from *Y. lipolytica*. All primer sequences are listed in Supplementary Table 1 and maps of the different vectors constructed are given in Supplementary Fig. S1. For all constructions, sequences were checked by DNA sequencing (GATC-Biotech, Konstanz, Germany) to ensure that no mutation was introduced during the PCR.

E. coli vector

For expression in *E. coli*, the forward primer aLip2_ECf and the reverse primer aLip2_ECr were used to introduce *Nde*I and *Not*I