APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2005, p. 8974–8977 Vol. 71, No. 12
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.12.8974–8977.2005

Heterologous Production of Functional Forms of *Rhizopus oryzae* Lipase in *Escherichia coli*

Mirella Di Lorenzo,[1,2] Aurelio Hidalgo,[1] Michael Haas,[3] and Uwe T. Bornscheuer[1]*

Department of Technical Chemistry and Biotechnology, Institute of Chemistry and Biochemistry, Greifswald University, Greifswald, Germany[1]; Department of Chemical Engineering, Federico II University, 80125 Napoli, Italy[2]; and United States Department of Agriculture, ERRC, ARS, Wyndmoor, Pennsylvania[3]

Received 17 May 2005/Accepted 10 September 2005

To date, expression of the lipase from *Rhizopus oryzae* (ROL) in *Escherichia coli* always led to the formation of inclusion bodies and inactive protein. However, the production of active ROL and its precursor ProROL in soluble form was achieved when *E. coli* Origami(DE3) and pET-11d were used as expression systems.

Lipases (triacylglycerol ester hydrolases; E.C. 3.1.1.3) have multiple applications in a wide range of biotechnological processes (11, 13, 19, 20, 25). Lipases from the genus *Rhizopus* are attractive catalysts in lipid modification processes, since they are active only against esters of primary alcohols and positionally selective, acting only at the *sn1* and *sn3* locations (2). Both the structure (5, 22) and function of the lipase from the fungus *Rhizopus oryzae* ATCC 34612 (formerly *Rhizopus delemar*) (18) have been deeply investigated. The *Rhizopus delemar* lipase is initially synthesized as a preproenzyme, consisting of the 269 amino acids of the mature enzyme, a 97-amino-acid propeptide fused to its amino terminus, and a 26-amino-acid-long export signal peptide at the amino terminus of the propeptide (8). Additionally, it contains six cysteine residues, which form three disulfide bridges (6). Since *Escherichia coli* lacks the necessary proteases to process fungal maturation signals, the *Rhizopus delemar* lipase cDNAs were previously expressed in *E. coli* for both the unprocessed lipase precursor and the mature product in insoluble form (10). The production of an active mature *Rhizopus* lipase has been performed in *Pichia pastoris* (4, 14) and in *Saccharomyces cerevisiae* (23).

In this work we present, for the first time, the expression of the *Rhizopus oryzae* lipase gene in *E. coli* to yield a correctly folded product, present only in the cytoplasm fraction.

Methods, results, and discussion. Cloning of the cDNA coding for the prolipase and mature lipase from the fungus *Rhizopus delemar* (renamed as *Rhizopus oryzae* in accordance with the literature [18]) in pET11-d has been previously reported (15). *E. coli* strain DH5α [*supE44* d*lac*U169(ϕ80*lac*ZΔM15)*hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used as host for genetic manipulation of plasmids. *E. coli* BL21(DE3) [F^- *ompT hsd*S_B(r_B^- m_B) *gal dcm* (DE3)], Rosetta(DE3)[F^- *ompT hsd*S_B(r_B^- m_B) *gal dcm* (DE3) pRARE^2 (Cm^r)] and Origami(DE3) [Δ*ara-leu7697* d*lacX74* Δ*phoAPvuII phoR araD139 ahpC galE galK rpsL* (Sm^r)$^4F^1$ [lac^+(*lacI*^{q}) *pro*] *gor522*::Tn*10* (Tc^r) *trx*B::kan (DE3)] strains were used for the overexpression of proteins. The *E. coli* strains were grown in Luria-Bertani medium containing 100 mg/liter ampicillin, 30 mg/liter kanamycin, 10 mg/liter tetracycline, 34 mg/liter chloramphenicol, as required. Plasmids pET-11d, pET-15b, pET-28b(+), and pET-22b(+) (Novagen) were used for cloning and protein expression. Transformation of *E. coli* was performed as described previously (9). All molecular biology protocols were performed using standard methods (17).

For PCR amplification of the genes of interest, the following oligonucleotides were used: 1F (5′-AAGGAGATATCATATGGTTCCTGT-3′), 2F (5′-GAGATATCATATGGATGGTGGTA-3′), and 3R (5′-AACACGTCAAGAATTCTTCAAACA-3′) (underlined portions of sequences are NdeI restriction sites introduced for cloning purposes). To obtain an N-terminal His-tagged product, the prolipase and mature lipase genes were amplified by PCR by using, respectively, oligonucleotides 1F or 2F and the T7 terminator primer. To obtain a C-terminal His-tagged product, the amplification by PCR was also performed with primer 1F or 2F and primer 3R.

The PCR products were purified and digested with NdeI and EcoRI and ligated into pET-28b(+) and pET-22b(+) vectors. The pET-28b(+) constructs carrying the prolipase and mature lipase genes were digested with NdeI and XhoI, purified, and ligated into the empty pET-15b vector. To evaluate the influence of the His tag on expression, the prolipase gene preceded by a six-His tag sequence was cloned into pET-11d. For this purpose the pET-15b vector containing the prolipase gene was digested with NcoI and BamHI, and the fragment was purified and ligated into the pET-11d vector.

The *E. coli* strains harboring the pET recombinant plasmids were grown in 100 ml Luria-Bertani medium supplemented with the required antibiotics using isopropyl-β-D-thiogalactopyranoside as inducer to a final concentration of 0.1 mM. At different time intervals, aliquots (equivalent to 5 ml at an optical density at 600 nm [OD_{600}] of 1) were centrifuged for 10 min at 800 × *g* to harvest the cells. The cells were then resuspended in 300 μl 50 mM phosphate buffer, pH 7.5, and disrupted by sonication (20 s, 50% pulse). The soluble fraction and the particulate material were separated by centrifugation, and 10 μl from these preparations was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% polyacrylamide gels and stained with Coomassie blue, as described by Laemmli

* Corresponding author. Mailing address: Department of Technical Chemistry and Biotechnology, Institute of Chemistry and Biochemistry, Greifswald University, Soldmannstr. 16, D-17487 Greifswald, Germany. Phone: 49 3834 86-4367. Fax: 49 3834 86-80066. E-mail: uwe.bornscheuer@uni-greifswald.de.

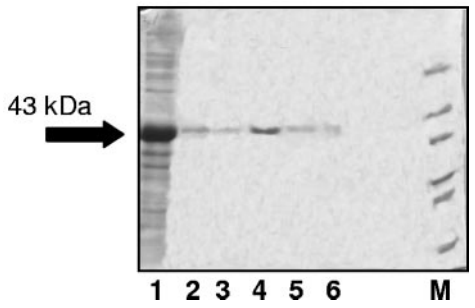

FIG. 1. Purification of the prolipase from inclusion bodies with a cobalt-based resin. Lane 1, pellet solution; lanes 2 and 3, washings; lanes 4, 5, and 6, elution; lane M, molecular mass marker (66, 45, 36, 29, 24, and 20.1 kDa). Ten-microliter aliquots of the soluble and insoluble fraction samples, prepared as described in the text, were loaded onto each lane. The arrow indicates the prolipase.

(12). The soluble fraction was also subjected to activity measurement by monitoring the amount of *p*-nitrophenol released upon hydrolysis of a 1 mM solution of *p*-nitrophenyl butyrate in 50 mM phosphate buffer, pH 7.5 at room temperature. Aliquots (100 μl) of the cell fraction assayed were added to 900 μl of the reaction mixture, and the increase in absorbance at 410 nm was measured for 1 min. One unit of hydrolase activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min at room temperature. The protein concentration of the samples was determined according to the method of Bradford (3).

The expression of the pET-11d prolipase and mature lipase constructs in *E. coli* BL21(DE3) or Rosetta led to an insoluble and inactive product (data not shown). The insoluble protein pellet obtained from a 100-ml culture was resuspended in 15 ml of 0.1% (vol/vol) Triton X-100, incubated at 37°C for 10 min, centrifuged at 16,000 × *g*, and washed with 20 ml of 50 mM phosphate buffer, pH 7.5. The inclusion bodies were resuspended in 2 ml of sodium phosphate buffer, pH 7, containing 8 M urea and incubated at 37°C for 20 min. The total sample volume was then purified with 2 ml of Talon cellThru IMAC resin (BD Biosciences, Palo Alto, Calif.) according to the manufacturer's instructions in the presence of 8 M urea (Fig. 1). The eluted fractions containing the target protein were pooled, concentrated, and refolded according to a previously described protocol (7). The purified prolipase was refolded in the presence of cysteine, leading to an active enzyme preparation (0.645 U/ml). Unfortunately, the enzyme was inactivated after storage of the refolded protein either at −20°C, at 4°C, or when lyophilized.

When the *E. coli* Origami(DE3) strain was used, the expression was successful and the target protein was expressed as soluble and active forms (Fig. 2A and B). Although their specific activities are comparable, the yield of prolipase was higher than for the mature lipase, probably due to the toxicity of the latter towards the host cells (8). The prosequence has been reported to modulate the enzyme activity of the mature lipase so that it can be synthesized without damaging the host, in this case as a result of a decreased affinity of the prolipase for phospholipids in comparison to the mature lipase (1). This modulation also causes the prolipase and the

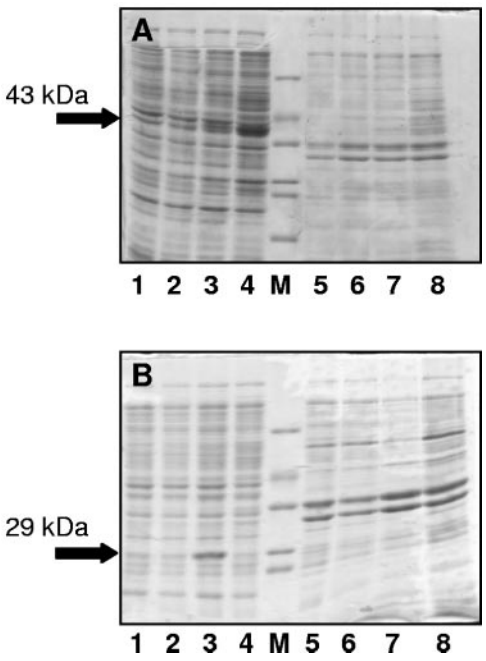

FIG. 2. A. Expression at 25°C of *E. coli* Origami harboring the pET11-d prolipase. Lane 1, prolipase before induction, supernatant; lane 2, prolipase after 1 h of induction, supernatant; lane 3, prolipase after 3 h of induction, supernatant; lane 4, prolipase after 24 h of induction, supernatant; lane M, molecular mass marker (66, 45, 36, 29, 24, and 20.1 kDa); lane 5, prolipase before induction, pellet; lane 6, prolipase after 1 h of induction, pellet; lane 7, prolipase after 3 h of induction, pellet; lane 8, prolipase after 24 h of induction, pellet. Ten microliters of the soluble and insoluble fraction samples, prepared as described in the text, was loaded onto each lane. The arrow indicates the prolipase. B. Expression at 25°C of *E. coli* Origami harboring the pET11-d mature lipase. Lane 1, lipase before induction, supernatant; lane 2, lipase after 1 h of induction, supernatant; lane 3, lipase after 3 h of induction, supernatant; lane 4, lipase after 24 h of induction, supernatant; lane M, molecular mass marker (66, 45, 36, 29, 24, and 20.1 kDa); lane 5, lipase before induction, pellet; lane 6, lipase after 1 h of induction, pellet; lane 7, lipase after 3 h of induction, pellet; lane 8, lipase after 24 h of induction, pellet. Ten microliters of the soluble and insoluble fraction samples, prepared as described in the text, was loaded onto each lane. The arrow indicates the mature lipase.

mature lipase to have different affinities for their substrates (24), although whether this is caused by an interaction between the prosequence and the peptide lid that sits atop the active site in an inactive form of the mature enzyme (5) has not been confirmed, since a resolved crystal structure of the prolipase is not available. However, the fact that the pro-

TABLE 1. Influence of growth temperature and OD_{600} of induction on yield of prolipase from *R. oryzae* produced in *E. coli* Origami (pET 11-d construct)

Growth temp (°C)	OD_{600} at induction	Activity[a] (U/ml)	Protein concn (mg/ml)	Sp act (U/mg)
20	0.5	40.7	0.7	60.4
20	1	166	1.5	110.7
25	0.5	45.1	0.95	47.3
25	1	28.4	0.7	42.4

[a] Determined using *p*-nitrophenyl butyrate as substrate.

TABLE 2. Influence of vector and OD_{600} at induction time on activity of prolipase from *R. oryzae* expressed in *E. coli* Origami at 20°C

Vector	OD_{600} at induction	Activity[a] (U/ml)	Protein concn (mg/ml)	Sp act (U/mg)
pET11	0.5	40.7	0.7	60.4
pET11	1	166	1.5	110.7
pET15	0.5	53.1	1.6	32.5
pET15	1	35.7	0.875	40.8
pET11 + His tag	0.5	34	1.64	20.7
pET11 + His tag	1	33	1.75	18.8
pET22	0.5	82	0.7	116
pET22	1	50	0.7	70.7

[a] Determined using *p*-nitrophenyl butyrate as substrate.

lipase is active implies that the part of the expression product corresponding to the mature peptide is already correctly folded.

In order to improve the amount of prolipase produced, several temperatures and cell densities at the time of induction were analyzed (Table 1). Only at 25°C and 20°C was an active product obtained, and under optimal conditions, the expression of *E. coli* Origami pET-11d prolipase gave 110.7 U/mg, at a growth temperature of 20°C with induction at an OD_{600} of 1. Although the functional expression of *Rhizopus* sp. lipase has been already performed in *Pichia pastoris* (14–16) and *Saccharomyces cerevisiae* (21, 23), we hesitate to compare the data, as different activity assays with different substrates and conditions have been used. In addition, the productivity is difficult to compare, as a system using *Pichia pastoris* has the advantage—in contrast to an *E. coli* expression system—that the lipase is in the supernatant and cell disruption is not necessary, but the enzyme is highly diluted. On the other hand, high-cell-density cultivation of *E. coli* can also yield large amounts of recombinant protein, and no background lipase (or esterase) activity is present in crude cell extracts. Thus, a purification of the lipase is not necessary.

The influences of several vectors in the prolipase production were studied too. pET15 and pET22 were considered for a simplified purification of the His-tagged, recombinant product. The expressions were performed at 20°C and at OD_{600} values of 1 and 0.5. A deeper evaluation of the influence of the His tag on the expression was carried out by cloning the prolipase gene preceded by a six-His tag sequence into vector pET-11d and comparing the expression results between this construct and that without an N-terminal His tag. Table 2 shows that the His tag in the N-terminal position negatively influenced the protein activity. On the other hand, the His tag in the C-terminal position did not influence the activity. The fact that the C-terminal position of the His tag does not have an effect on activity, while the N-terminal markedly does, may be related to the role of the N-terminal prosequence as an intramolecular chaperone assisting in the folding of the mature peptide. Although no structural data on the prolipase are available, it seems logical that the environment of the prosequence should be kept as unmodified as possible, since it has been previously reported to influence the formation of disulfide bonds (1).

In summary, we have demonstrated that the lipase from *Rhizopus oryzae* can now be functionally expressed in *E. coli* without the need for inclusion body purification and a tedious refolding process. The prolipase could be efficiently produced in high yield at high specific activity.

A. Hidalgo acknowledges the financial support provided through the European Community's Human Potential Programme under contract HPRN-CT2002-00239.

M. Di Lorenzo thanks D. Pirozzi and G. Greco for giving her the possibility to work in Germany.

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