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Heterologous expression of a fungal sterol esterase/lipase in different hosts: Effect on solubility, glycosylation and production

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Ophiostoma piceae **secretes a versatile sterol-esterase (OPE) that shows high efficiency in both hydrolysis and synthesis of triglycerides and sterol esters. This enzyme produces aggregates in aqueous solutions, but the recombinant protein, expressed in *Komagataella* (synonym *Pichia*) *pastoris*, showed higher catalytic efficiency because of its higher solubility. This fact owes to a modification in the N-terminal sequence of the protein expressed in *Pichia pastoris*, which incorporated 4–8 additional amino acids, affecting its aggregation behavior. In this study we present a newly engineered *P. pastoris* strain with improved protein production. We also produced the recombinant protein in the yeast *Saccharomyces cerevisiae* and in the prokaryotic host *Escherichia coli*, corroborating that the presence of these N-terminal extra amino acids affected the protein's solubility. The OPE produced in the new *P. pastoris* strain presented the same physicochemical properties than the old one. An inactive form of the enzyme was produced by the bacterium, but the recombinant esterase from both yeasts was active even after its enzymatic deglycosylation, suggesting that the presence of *N*-linked carbohydrates in the mature protein is not essential for enzyme activity. Although the yield in *S. cerevisiae* was lower than that obtained in *P. pastoris*, this work demonstrates the importance of the choice of the heterologous host for successful production of soluble and active recombinant protein. In addition, *S. cerevisiae* constitutes a good engineering platform for improving the properties of this biocatalyst.**

[**Key words:** *Escherichia coli*; *Saccharomyces cerevisiae*; *Pichia pastoris*; Lipase; Sterol esterase; Aggregation behavior; Glycosylation]

Carboxylic ester hydrolases (EC 3.1.1) are a heterogeneous group of enzymes catalyzing the cleavage of ester bonds, including carboxylesterases (EC 3.1.1.1), triacylglycerol lipases (EC 3.1.1.3) and sterol esterases (EC 3.1.1.13). Triacylglycerol lipases, also known as lipases, have acylglycerols as their natural substrates. In aqueous media, these enzymes catalyze the hydrolysis of triglycerides to free fatty acids, diglycerides and monoglycerides but they are also able to carry out synthesis reactions in the presence of organic solvents (1). Similarly, sterol esterases hydrolyze fatty acid esters of sterols (2) and carry out the opposite reaction in organic media (3). They are widespread in nature, being the human cholesterol esterase one of the best studied among this group of enzymes (4–6). However, those from microorganisms have gained special interest due to their broad substrate specificity and their possible use for biotechnological purposes since they can be produced in bulk at low cost.

Both kinds of enzymes, lipases and sterol esterases, belong to the α/β-hydrolase superfamily, where residues responsible for its catalytic activity are highly conserved and form the so-called catalytic triad Ser–Asp/Glu–His (7,8), with the serine as the nucleophile residue participating directly in catalysis. For this reason, they are also known as serine hydrolases. They display a wide range of molecular mass, usually from 20 to 80 kDa, although enzymes with lower masses have been reported (9) and, in general, their active site is characterized for having a hydrophobic cavity covered by an amphipathic loop named "flap". Being considerably hydrophobic, these proteins tend to aggregate in dimeric, tetrameric, and even hexameric or more aggregated forms, displaying pseudo-quaternary structures (10–12).

Some lipases show broad substrate specificity, including triglycerides and water insoluble sterol esters. This is the case of the *Candida rugosa* (synonym *Candida cylindracea*) lipase family (abH03.01) that comprises a variety of closely related enzymes from which at least three of them (Lip1, Lip2 and Lip3), display activity on both triglycerides and cholesterol esters, although differing in their substrate specificity (13). Among sterol esterases, this promiscuity has been reported for the one secreted by the ascomycete *Ophiostoma piceae* (OPE), which shows more than 40% sequence identity with *C. rugosa* lipases and similar substrate-binding sites, as suggested by its structural model (2). These properties have also been reported for the *Melanocarpus albomyces* sterol esterase (14).

The versatile use of these enzymes in hydrolysis or synthesis reactions made them interesting alternative biocatalysts in different industrial sectors. Concerning its hydrolytic ability, the use of OPE for pitch biocontrol during hardwood or softwood pulp production (15) or to decrease the problems caused by stickies during recycled paper manufacture (16) have been reported. But OPE can also catalyze the synthesis of phytosterol or phytostanol

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esters, nutraceuticals currently added to dairy products because of the hypocholesterolemic effect they exert (3). However, the use of any enzyme for biotechnological purposes requires its production in the adequate system for obtaining high amounts of the biocatalyst at low cost. In this sense, the use of the recombinant DNA technology for the improvement of enzyme production by using different heterologous expression hosts is mandatory. Prokaryotic hosts have proven useful for expression of eukaryotic proteins despite the absence of glycosylation routes (17), but the advantage of eukaryotic systems is their ability to carry out post-translational modifications (18), which can be essential to express functional recombinant proteins. In addition, for specific applications such as food industry, the production of recombinant proteins in prokaryotic or eukaryotic hosts Generally Recognized as Safe (GRAS) is compulsory.

We have previously reported that *Komagataella* (synonym *Pichia*) *pastoris* is the optimal biofactory for the heterologous production of OPE (19). The aim of this work was to test and compare the yields and activity of the OPE produced in several recombinant hosts. Here we present the results for OPE cloning and expression in the prokaryotic model organism *Escherichia coli*, that synthesizes non-glycosylated protein, as well as in two eukaryotic hosts: an overproducer strain of *Pichia pastoris* and the GRAS yeast *Saccharomyces cerevisiae*. The production and properties of the recombinant proteins are discussed, keeping in mind the initial goal of finding the best heterologous expression system for OPE, what would be the starting point for the exploitation of this enzyme in biocatalysis applications.

MATERIALS AND METHODS

Strains, plasmids, culture media and materials All heterologous host strains and plasmids used in the present study are summarized in Table 1. *E. coli* DH5α and the plasmid pGEM-T Easy (Promega, Madison, WI, USA) were used for the general cloning procedures. The plasmids pET29a(+), pET28a(+) (Novagen, Merck KGaA, Darmstadt, Germany), pTYB12, pTYB1 (New England Biolabs, Hertfordshire, UK), pGEX-6P-2 (GE Healthcare, Uppsala, Sweden) and pFj1, provided by Dr. F. J. Medrano (CIB–CSIC, Spain), were used for expression in *E. coli*. The *E. coli* strains used for OPE expression were obtained from different providers: C43(DE3) from Lucigen, Middleton, WI, USA; BL21(DE3), BL21(DE3) pLysS, Rosetta(DE3)pLysS and Tuner(DE3) from Novagen Merck; BL21(DE3)pT-GroE was kindly donated by Dr. F. J. Medrano; K12 (*dam*$^{-}$/*dcm*$^{-}$) and SHuffle T7 express were from New England Biolabs, Hertfordshire, UK.

Three replicative shuttle vectors were used for expression in *S. cerevisiae*: pJRoC30, p426ADH and p426GPD (20,21). The yeast strains are summarized in Table 1 and were obtained from different sources: BJ5465 and Lalvin T73-4a were kindly donated by Dr. S. Camarero (CIB–CSIC, Spain) and Prof. A. Querol (IATA-CSIC, Spain), respectively, and BY4741 was from GE Healthcare. Finally, the recombinant *P. pastoris* KM71 strain previously obtained in our laboratory (19), carrying the construct pPIC9OPE, was re-transformed with the integrative expression vector pPIC9OPE.

E. coli was grown in two different media: LB (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) and 2× Tryptone-Yeast Extract (2× TY) (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl).

Yeast Extract-Peptone-Dextrose (YPD) plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar were used for general growing of the yeast strains. Minimal selection medium for *S. cerevisiae* contained 6.7 g/L Yeast Nitrogen Base without amino acids (Becton, Dickinson and Company, Sparks, MD, USA), 1.92 g/L Yeast Synthetic Drop-out Medium Supplement without uracil (Sigma–Aldrich, Steinheim, Germany), 20 g/L raffinose, 25 mg/L chloramphenicol and 20 g/L agar in the case of minimal selection plates. Expression medium for *S. cerevisiae* contained Bacto Peptone 20 g/L, yeast extract 10 g/L, 100 mM KH_2PO_4 pH 6.0 buffer, 20 g/L galactose or 20 g/L glucose in the case of inducible or constitutive promoters, respectively, and 25 mg/L chloramphenicol. Minimal selection plates for *P. pastoris* contained 6.7 g/L Yeast Nitrogen Base without amino acids, 1.92 g/L Yeast Synthetic Drop-out Medium Supplement without histidine (Sigma–Aldrich), 20 g/L glucose and 20 g/L agar. Expression medium for *P. pastoris* contained 20 g/L Bacto Peptone, 10 g/L yeast extract, 10 g/L sorbitol, 100 mM KH_2PO_4 pH 6.0 buffer and 0.5 % (w/v) methanol.

Restriction enzymes were from New England Biolabs (Hertfordshire, UK) while the primers were obtained from Sigma–Aldrich. Taq DNA polymerase was purchased from Invitrogen (Carlsbad, CA, USA). T4 DNA ligase was provided by Promega and the purification kits from Qiagen (Valencia, CA, USA). CHAPS was purchased from Thermo Scientific (Rockford, IL, USA) and Triton X-100 and Sarkosyl (Sodium lauroyl sarcosinate) were obtained from Sigma–Aldrich.

Cloning procedures Cloning and transformation procedures were performed according to established techniques (22) and suppliers' manuals.

For cloning in *E. coli,* the gene of sterol esterase from *O. piceae* (*ope*) was amplified from the expression vector pPIC9OPE (19), using the reverse primer OPER and the forward primers OPEF1 or OPE4F1, to add 4 extra hydrophobic amino acids to the N-terminus (Table 2). The amplifications were carried out in a Mastercycler

TABLE 1. Constructs and strains used in this study.

Strain	Strain features	Vector	Vector features promoter/inducer	Extra N-t amino acids	Fusion tag and location	Expression
E. coli						
BL21(DE3)	Deficient in both *lon* and *ompT* proteases	pET29a (+)	T7*lac*/IPTG	–/4/6/8	–	+/+/+/+
		pET29a (+)	T7*lac*/IPTG	6	nHis C-t	+
		pET28a (+)	T7*lac*/IPTG	–/4	nHis N-t	+/+
		pTYB12	T7*lac*/IPTG	–/4	Intein N-t	+/+
		pFJ1	PhoA/Constitutive	–/4	–	–/–
BL21(DE3)pLysS	High-stringency, reduces basal expression level	pTYB1	T7*lac*/IPTG	–/4	Intein C-t	–/–
		pGEX-6P-2	Ptac/IPTG	–	GST N-t	+
		pET29a (+)	T7*lac*/IPTG	4	–	+
BL21(DE3)pT-GroE	Contains chaperone GroESL, enhances protein folding	pGEX-6P-2	Ptac/IPTG	–/4	GST N-t	+/+
Rosetta(DE3)pLysS	Provides rare codons tRNAs	pET29a (+)	T7*lac*/IPTG	–/4	–	–/+
Tuner(DE3)	Allows control of expression levels	pET29a (+)	T7*lac*/IPTG	–	–	+
C43(DE3)	Enhances expression of toxic proteins	pET29a (+)	T7*lac*/IPTG	–/4	–	–/–
K12	No DNA methylation	pGEX-6P-2	Ptac/IPTG	–	GST N-t	–
		pFJ1	PhoA/Constitutive	–/4	–	–/–
SHuffle T7 express	Allows disulfide bond formation in the cytoplasm	pET28a (+)	T7*lac*/IPTG	–	nHis N-t	+
		pET29a (+)	T7*lac*/IPTG	6	–	+
S. cerevisiae						
BJ5465	Protease-deficient strain	pJRoC30	*GAL1*/Galactose	4–8	–	+
		P426GPD	*GPD1*/Constitutive	4–8	–	+
		P426ADH	*ADH1*/Constitutive	4–8	–	+
BY4741	Presents four detectable marker genes	pJRoC30	*GAL1*/Galactose	4–8	–	+
		P426GPD	*GPD1*/Constitutive	4–8	–	+
Lalvin T73-4a	Wine-growing yeast	pJRoC30	*GAL1*/Galactose	4–8	–	+
		P426GPD	*GPD1*/Constitutive	4–8	–	+
P. pastoris						
KM71 (pPIC9OPE)	Strain previously transformed	pPIC9	*AOX1*/Methanol	4–8	–	+

n-His, Histidine tag; N-t, N-terminal; C-t, C-terminal; +, positive expression; –, no expression.

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TABLE 2. Primers used in this study.

Primers	Sequence (5′→3′)	Restriction site
E. coli		
OPEF1	CATATGACAACCGTGAATGTAAACTACC	*Nde*I
OPE4F1	CATATGGAGCTCTACGTAACAACCGTGAATGTAAACTACC	*Nde*I
OPEF2	GGATCCACAACCGTGAATGTAAACTACC	*Bam*HI
OPE4F2	GGATCCGAGCTCTACGTAACAACCGTGAATGTAAACTACC	*Bam*HI
OPE8F1	CATATGGGATCCGAAGCGGAAGCGTATGTGGAATTTACAACCGTGAATGTAAACTACC	*Nde*I
OPE6F2	CATATGGGATCCGAAGCGTATGTGGAATTTACAACCGTGAATGTAAACTACC	*Nde*I
OPER	CTCGAGGATGCGGAAGATGCCAATGTTG	*Xho*I
OPE2R	CTCGAGTTAGATGCGGAAGATGCCAATG	*Xho*I
S. cerevisiae		
SCF1	CGGGGATCCATGAGATTTCCTTCAATTTT	*Bam*HI
SCF2	CCCGGGTTAGATGCGGAAGATGCCAATGTTG	*Xma*I
SCF3	TAAGCGGCCGCTTAGATGCGGAAGATGCCAATG	*Not*I

Pro S (Eppendorf, Hamburg, Germany). The PCR products were purified and ligated into the pGEM T-Easy vector, digested with the restriction enzymes *Nde*I and *Xho*I and subcloned into the expression vectors, pET29a(+), pET28a(+), pTYB12 and pFj1, with the same restriction sites. To create a recombinant GST-OPE protein in the N-terminus we used OPEF2 or OPE4F2, to add the 4 extra hydrophobic amino acids, and OPER as reverse primer (Table 2). The PCR fragments were ligated into the pGEM T-Easy vector and subcloned in the expression vector pGEX-6P-2, using *Bam*HI and *Xho*I as restriction sites. To produce the OPE with the Intein protein in the C-terminus, *ope* was amplified with the forward primers OPEF1 or OPEF2 and the reverse primer without stop codon OPE2R. The PCR product was cloned in pGEM T-Easy and then subcloned into the expression vector pTYB1.

Previous reports described an improved solubility of the recombinant protein expressed in *P. pastoris* (19) and the reason for this behavior was attributed to the presence of 4, 6 or 8 extra amino acids in its N-terminus due to the cloning strategy. Then, these 4, 6 or 8 extra amino acids were added in this construct. For gene amplification, the forward primers OPE6F1 or OPE8F1 were used with the reverse primers OPER or OPE2R when a 6× histidine tag was added to the C-terminus of the recombinant protein (Table 2). The PCR product was purified and ligated into the pGEM T-easy and, after digestion with *Nde*I and *Xho*I, subcloned into the expression plasmid pET29a(+). All recombinant plasmids were verified by sequencing before use.

The cloning strategy to produce the recombinant OPE in *S. cerevisiae* consisted on amplifying the α-factor pre-pro-leader sequence and the OPE gene from pPIC9OPE, using the pair of primers SCF1 and SCR1 (Table 2). The purified PCR product was ligated into pGEM T-Easy plasmid, digested with *Bam*HI and *Xma*I and subcloned into the episomal vectors p426ADH and p426GPD. The construct for recombinant expression in the plasmid pJRoC30 was developed according to a similar strategy using the primers SCF1 and SCR2, and subcloning in pJRoC30 using *Bam*HI and *Not*I like restriction sites.

Protein expression and analytical procedures The constructs were introduced into their corresponding host. In the case of *E. coli* different electrocompetent strains (Table 1) were transformed using a MicroPulser electroporator (Bio-Rad, Hércules, CA, USA), following the manufacturer's protocol. For the expression screening, several clones were grown overnight in 4 mL of 2× TY medium with the corresponding antibiotic at 37°C and 250 rpm. To induce the expression, 0.5 mM of IPTG was added to the culture. Protein expression was checked by SDS-PAGE using 10% polyacrylamide gels, staining with Coomassie R-250. To evaluate the solubility of the protein, positive clones were grown at 37°C in 50 mL of 2× TY medium, with the corresponding amount of antibiotic, until optical density at 600 nm reached 0.6. Then, the culture was induced with 0.5 mM IPTG and kept overnight at different temperatures (16°C, 30°C and 37°C). The cells were harvested by centrifugation (6000 ×*g*, 20 min, 4°C) and resuspended in 5 mL lysis buffer (10 mM Tris–HCl, pH 8, with 150 mM NaCl). Cell pellets were sonicated (Misonix S4000; Qsonica, Newtown, CT, USA), separated from the supernatant fraction and checked for protein solubility using SDS-PAGE gels (as described above). To increase the solubility, three different detergents were assayed: the zwitterionic surfactant CHAPS (20–80 mM), the non-ionic detergent Triton X-100 (1%), and the alkyl anionic surfactant Sarkosyl (7.5–30 mM).

In the case of *S. cerevisiae*, three different episomal vectors (p426ADH, p426GPD and pJRoC30) were introduced into the strains BJ5465, BY4741 and Lalvin T73 following the *S. cerevisiae* transformation kit instructions (Sigma–Aldrich). Single colonies of the transformed *S. cerevisiae* clones were picked from minimal selection plates, used to inoculate 3 mL of minimal medium and incubated for 48 h at 30°C and 220 rpm. An aliquot of the cells was used to inoculate a final volume of 20 mL in 100 mL flasks, adjusting the OD_{600} to 0.25, and then incubating for two complete cell divisions (6–8 h). Thereafter, the cells were diluted to $OD_{600} = 0.1$ in a final volume of 20 mL of expression medium in 100 mL flasks (23). The strain of *P. pastoris* containing the construct pPIC9OPE (19) was retransformed with the construct PIC9OPE, following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), to obtain improved expression levels. Semi-quantitative PCR with four serial dilutions of genomic DNA was carried out to check the number of copies of the *ope* gene in the new strain. Single colonies of the new *P. pastoris* strain were picked from minimal selection plates, used to inoculate 5 mL of YEPS medium without 0.5 % (w/v) methanol and incubated overnight at 28°C and 250 rpm. An aliquot of the cells was used to inoculate a final volume of 20 mL of expression medium in 100 mL flasks. Methanol (0.5 %) was added daily for maintaining the induction and counteracting evaporation.

Enzyme activity was routinely measured by monitoring *p*-nitrophenol release from 1.5 mM *p*-nitrophenyl-butyrate (*p*NPB) (Sigma–Aldrich) (24) in 20 mM Tris–HCl pH 7 buffer at 25°C in a Shimadzu UV-1800. One unit of activity (1U) is defined as the amount of enzyme releasing 1 μmol of substrate per minute under the defined conditions.

Kinetic studies by using *p*NPB as substrate were assayed in the presence of 1% (v/v) Genapol X-100 (Sigma–Aldrich) (25). Experimental data were fitted to hyperbolic Michaelis–Menten curves and statistically analyzed with the Sigma Plot 11.0 software.

Protein concentration was determined by the BCA bioassay (Thermo Scientific, Rockford, IL, USA), using bovine serum albumin as standard. For *N*-deglycosylation, the purified OPE was dialyzed against sodium citrate buffer, pH 5.5, and then incubated with Endo H at 37°C for 24 h, following the manufacturer's instructions. Both the glycosylated and the deglycosylated proteins (around 5 μg each) were analyzed by SDS-PAGE and *N*-linked-carbohydrate content was estimated based on visual analysis of the stained gel, from the difference between the molecular mass of the sterol esterase before and after deglycosylation with Endoglycosidase H (2) (Endo H; Roche, Mannheim, Germany).

Protein production and purification A His-tagged version of OPE was expressed in strain *E. coli* BL21(DE3) in order to purify the protein via His-tag affinity purification. 500 mL of the 2× TY medium in 2 L flasks were inoculated and grown at 37°C, until optical density at 600 nm reached 0.6. Then, the culture was induced with 0.5 mM IPTG and kept at 16°C and 250 rpm overnight. The cell cultures were harvested by centrifugation (6000 ×*g*, 20 min, 4°C), resuspended in 10 mL of lysis buffer with 20 mM Sarkosyl and sonicated. Bacterial lysates were pelleted at 50,000 ×*g* for 1 h at 4°C in a 60Ti rotor. The supernatant was loaded onto a His-Trap HP 5 mL column (GE Healthcare) equilibrated with binding buffer (10 mM Tris–HCl, pH 8.0, with 5 mM imidazole, 500 mM NaCl and 18 mM CHAPS). The target proteins were eluted with 50 mL elution buffer (10 mM Tris–HCl, pH 8.0, with 150 mM imidazole, 500 mM NaCl and 18 mM CHAPS). The purified enzyme was dialyzed against 10 mM Tris–HCl, pH 8, with 18 mM CHAPS to reduce the salt and imidazole content.

The sterol esterase expressed in *S. cerevisiae* BJ5465 strain with pJRoC30OPE, was produced in 1 L flasks with 200 mL of expression medium at 22°C and 220 rpm, and inoculated as described above. The sterol esterase overexpressed in the new strain of *P. pastoris* was produced in 1 L flasks with 100 mL of expression medium at 28°C and 250 rpm. When maximum activity was reached (generally after 72–96 h in both cases), the cells were harvested by centrifugation (6000 ×*g*, 4°C) and the supernatants concentrated in 10,000 MWCO Amicon-Ultra Centrifugal filters (Merck Millipore, Darmstadt, Germany). The esterase purification was carried out in a single hydrophobic chromatography step (Octyl-Sepharose cartridge, GE Healthcare) as previously reported (25). The purified enzyme was dialyzed against 25 mM Tris–HCl, pH 7, to remove the detergent. The fractions containing the purified protein from the different hosts were further concentrated by ultrafiltration (30 kDa cut-off, Merck Millipore).

To evaluate the relevance of *N*-linked glycosylation in OPE, the doubly-transformed *P. pastoris* strain was grown for 6 days in 250 mL flasks with 25 mL expression medium at 28°C and 250 rpm in the presence of 20 μg/mL of tunicamycin (Sigma–Aldrich). Culture samples (1 mL) were withdrawn periodically and centrifuged (6000 ×*g*). Esterase activity was evaluated in the supernatant as described above. Yeast biomass was determined gravimetrically after drying yeast pellets until constant weight in an aeration oven at 65°C.

N-terminal sequencing and analytical ultracentrifugation The N-terminal sequence of the recombinant protein was obtained by automated Edman degradation of 10 μg of purified sample as previously described (19).

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A sedimentation velocity assay was used to check the aggregation behavior of recombinant proteins. Solutions of the purified protein (0.5 mg/mL) in 20 mM Tris–HCl, pH 7.0, were used for this experiment. Measurements were performed as previously reported (25). Differential sedimentation coefficient distributions c(s) were calculated by least-squares boundary modeling of sedimentation velocity data using the continuous distribution c(s) Lamm equation model as implemented by SEDFIT (version 14.1). Experimental *s* values were corrected to standard conditions using the program SEDNTERP.

Circular dichroism spectroscopy Measurements were carried out using a JASCO J-720 spectropolarimeter. Far-UV spectra were recorded in a 0.1 cm path length quartz cell at a protein concentration of 0.1 mg/mL in 20 mM sodium phosphate buffer, pH 7.0. The spectra from five scans were averaged and corrected for the baseline contribution of the buffer. The observed ellipticities were converted into mean residue ellipticities (θ) based on a mean molecular mass per residue of 110 Da.

RESULTS

Expression in *E. coli* The expression of OPE using *E. coli* as heterologous host (Table 1) was only achieved using vectors carrying inducible promoters such as pGEX-6P-2 or pET family vectors, although the protein produced was not soluble. Many strategies were attempted (Table 1) trying to improve protein solubility: (i) fusing the target protein to tags such as 6x-His, GST or Intein; (ii) using *E. coli* strains such as C43, designed for the expression of toxic proteins, Tuner, that allow modulating the induction level, or SHuffle T7, engineered to favor disulfide bond formation in the cytoplasm; (iii) improving host cell codon usage by expressing the protein in *E. coli* strain Rosetta; or (iv) enhance protein folding using as host the pT-GroE strain. Furthermore, different growth conditions were assayed, using several temperatures to optimize the expression and solubility of the recombinant proteins. However, in all cases, the protein expressed formed big aggregates commonly referred to as inclusion bodies.

With the aim of solubilizing the OPE produced in *E. coli*, three different surfactants were tested on the inclusion bodies produced from pET28a(+) 6x-His in the BL21(DE3) strain. Only Sarkosyl above the critical micelle concentration (CMC = 14.6 mM) was effective in solubilizing OPE, as observed by SDS-PAGE (Fig. 1). Protein aggregates obtained from overnight cultures was dissolved using Sarkosyl and purified in a single step in a prepacked cartridge for His-tagged recombinant proteins, after replacing Sarkosyl by CHAPS, a detergent easily removable by dialysis (26). Fifteen milligrams of pure enzyme, with a molecular mass around 60 kDa, corresponding to the mass determined using the amino acid composition, were obtained from 1 L of 2× TY medium (Fig. 2). The circular dichroism spectrum of the purified protein in the far-UV region differed significantly from those registered for the native OPE as well as for the recombinant OPE expressed in the eukaryotic systems (Fig. 3). The purified enzyme revealed to be inactive after checking its performance in *p*NPB hydrolysis.

A second approach to avoid the formation of inclusion bodies, producing soluble OPE from the prokaryotic host, consisted on adding to the sequence of the native protein 4, 6 or 8 extra amino acids in the N-terminus of the protein, emulating the N-terminal region of the very soluble OPE populations expressed in *P. pastoris* (19). A soluble form of the recombinant protein from *E. coli* was exclusively produced when 6 or 8 extra amino acids were added to

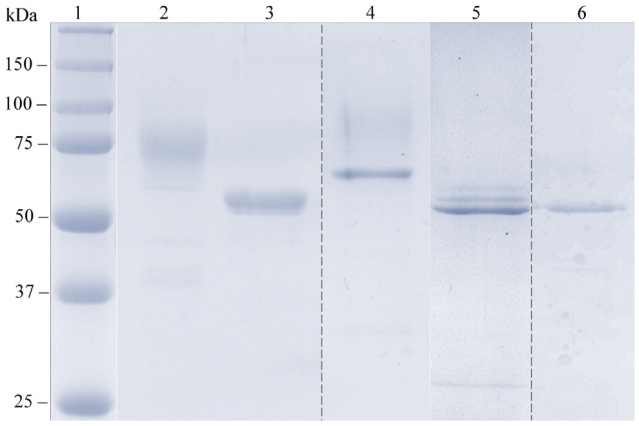

FIG. 2. SDS-PAGE of purified OPE expressed in different heterologous hosts. Lane 1, molecular mass markers. Lane 2, OPE expressed in *P. pastoris*. Lane 3, OPE expressed in *P. pastoris* and deglycosylated with Endo-H. Lane 4, OPE produced in *S. cerevisiae*. Lane 5, OPE produced in *S. cerevisiae* and deglycosylated with Endo-H. Lane 6, OPE produced in *E. coli*.

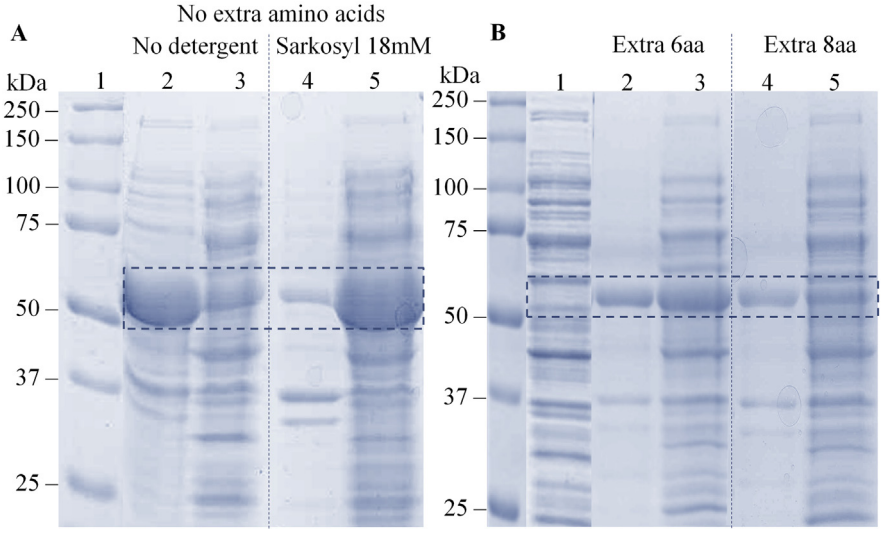

FIG. 1. SDS-PAGE of the crude extract of *E. coli* BL21(DE3) cultures containing the *ope* gene. The dashed boxes indicate OPE expression. (A) OPE overexpressed without N-terminal extra amino acids. Lane 1, molecular mass markers. Lanes 2 and 3, pellet and supernatant, respectively, of the culture induced with IPTG without addition of surfactant. Lanes 4 and 5, pellet and supernatant, respectively, of the culture induced with IPTG and solubilized with 18 mM of Sarkosyl. (B) OPE overexpressed with 6 or 8 extra N-terminal amino acids. Lane 1, molecular mass markers. Lane 2, supernatant from an uninduced culture. Lanes 3 and 4, pellet and supernatant, respectively, of the culture expressing OPE with 6 extra N-terminal amino acids. Lanes 5 and 6, pellet and supernatant, respectively, the culture expressing OPE with 8 extra N-terminal amino acids.

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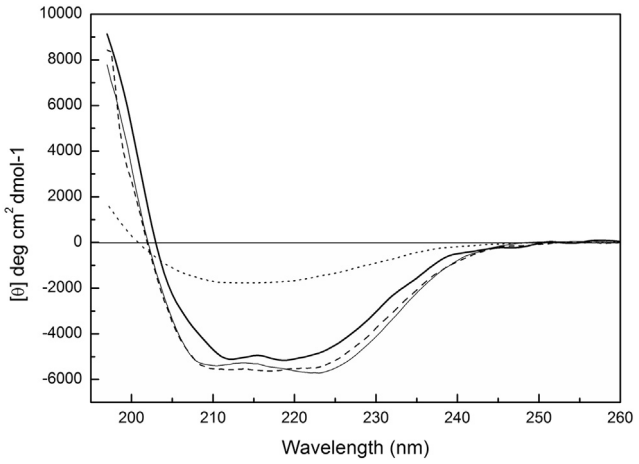

FIG. 3. Circular dichroism spectra (far UV spectrum) of purified OPE expressed in different heterologous hosts. Bold line, native protein secreted from the fungus *O. piceae*. Thin line, OPE expressed in *P. pastoris*. Dashed line, OPE expressed in *S. cerevisiae*. Dotted line, OPE expressed in *E. coli*.

the N-terminus (Fig. 1), but none of the proteins were active against the substrate assayed.

Expression in *S. cerevisiae* and *P. pastoris* Screening OPE production in the yeast *S. cerevisiae* (Fig. 4) demonstrated the expression of soluble and active protein in all constructs and strains assayed (Table 1). The greatest activity (0.9 U/mL) was achieved in the protease deficient strain BJ5465, with the galactose-inducible promoter *GAL1*, after 3 days of incubation at 22°C in 100 mL Erlenmeyer flasks. The clone of the retransformed *P. pastoris* strain giving maximal production yielded 42 U/mL after 4 days of incubation at 28°C, 2.4-fold higher than the previous clone (19). Semi-quantitative PCR suggested that the new clone carried two copies of the OPE construct (data not shown).

The recombinant proteins were purified to homogeneity. As expected, the OPE expressed in the new strain of *P. pastoris* was similar in molecular mass, *N*-carbohydrate content and comparable activity levels on *p*NPB to the protein previously produced in the same host (19). Nevertheless, the calculated specific activity

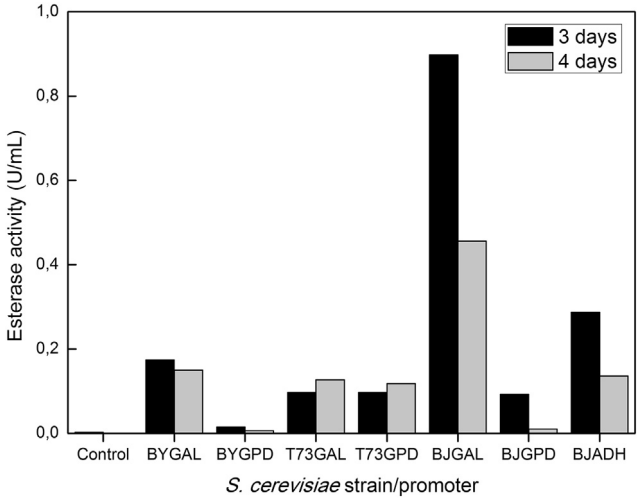

FIG. 4. Comparison of the esterase activity from different crude extracts from *S. cerevisiae*. The hydrolytic activity was checked against 1.5 mM of *p*NPB as the substrate. The OPE gene was expressed using the following constructs: the yeast strains Lalvin T73, BJ5465 and BY4741 carrying either the p426 plasmid with the constitutive promoters *GPD* or *ADH*, or the pJRoC30 vector with the inducible promoter *GAL1*.

differed since protein concentration was now determined by the BCA bioassay, as recommended for very hydrophobic proteins, due to the fact that these proteins are underestimated by the Bradford method (27).

Expression in *S. cerevisiae* yielded 4.1 mg/L of purified OPE, whereas in the new strain of *P. pastoris* 66 mg/L were obtained. Based on visual analysis of the stained gel, both proteins showed a similar molecular mass (around 75 kDa), with around 28% *N*-linked sugars (Fig. 2). In addition, both had a molecular mass of around 60 kDa after deglycosylation with EndoH, which coincides with the value expected from the amino acid sequence of OPE. The specific activities of the OPE synthesized by *S. cerevisiae*, before and after deglycosylation, were 34 U/mg and 25 U/mg, while for the OPE produced in *P. pastoris*, they were 120 U/mg and 98 U/mg respectively, indicating that most of the activity is retained in the secreted protein in spite of lacking the sugar moiety. The addition of tunicamycin, an inhibitor of *N*-linked glycosylation, to *P. pastoris* cultures, drastically reduced the OPE production, obtaining 0.4 U per mg of yeast biomass against the 3 U per mg of yeast biomass obtained in untreated cultures after 6 days of incubation.

The K_m value against *p*NPB was 0.28 mM for the OPE from *S. cerevisiae* and similar values were found for the protein produced in *P. pastoris* (Table 3). However, the turnover frequency and catalytic efficiency (k_{cat}/K_m) against this substrate were about 3-fold lower in the OPE expressed in *S. cerevisiae* (Table 3).

In this work, the N-terminal sequence of the recombinant OPE expressed in the two eukaryotic hosts contained the same 4–8 extra amino acids described in a previous paper for the protein produced in *P. pastoris* (19). These additional residues derive from a wrong processing of the EAEA repetition by signal peptidase STE13 and from the cloning procedure that leaves a residual sequence YVEF from the *SnaB*I and *Eco*RI restriction sites. Due to the presence of different protein populations in the sample, we are not able to discriminate if the main population contains 4, 6 or 8 extra amino acids, the ratio between the different populations, or the particular behavior of each one of them in terms of solubility.

The circular dichroism spectrum of the OPE expressed in *S. cerevisiae* revealed a secondary structure similar to those from the native protein and the recombinant form secreted by *P. pastoris* (Fig. 3). However, the measure of the sedimentation velocity of the former by analytical ultracentrifugation (Fig. 5) showed an intermediate aggregation state between those displayed for the native OPE and the recombinant protein expressed in *P. pastoris*. Whereas the native protein from *O. piceae* showed a tendency to form big aggregates in aqueous solution (19), the recombinant form expressed in *P. pastoris* was a combination of monomeric (66%) and dimeric (27%) forms with a small amount of big aggregates (7%), while OPE from *S. cerevisiae* appeared as a mixture of monomers (53 %), dimers (12%), and big aggregates (35%).

DISCUSSION

The *O. piceae* sterol esterase has been expressed in two new hosts, the bacterium *E. coli* and the GRAS yeast *S. cerevisiae*, and its production has been improved in the yeast *P. pastoris*. The characteristics of the recombinant proteins have been analyzed and compared with those from the native protein.

TABLE 3. Comparative heterologous expression levels and apparent kinetics parameters of recombinant protein against *p*NPB as substrate.

Host	Production (mg/L)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
S. cerevisiae	4.1	0.28 ± 0.05	50.0 ± 2.9	175.5 ± 27.6
P. pastoris	66.0	0.26 ± 0.08	146.9 ± 12.1	567.5 ± 142.6

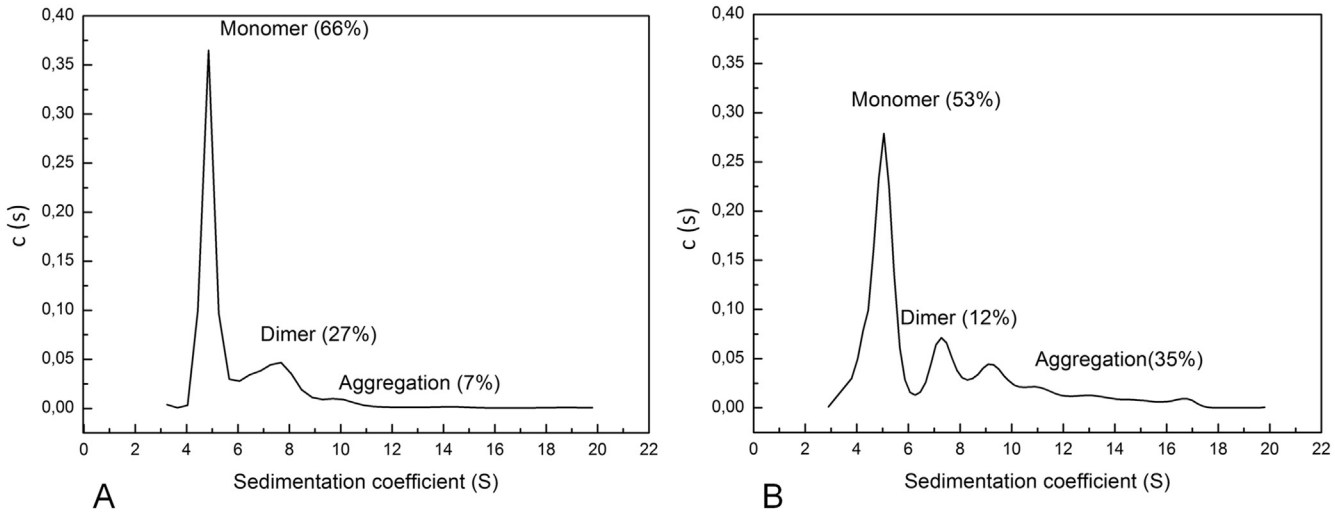

FIG. 5. Sedimentation velocities obtained by analytical centrifugation of the recombinant OPE expressed in (A) *P. pastoris* and (B) *S. cerevisiae*. The percentages of the different species are represented in brackets. Conditions are described in the experimental section.

Regardless of the *E. coli* strain assayed, the production of recombinant OPE in this bacterial host rendered an insoluble and inactive protein stored inside inclusion bodies. The insolubility of the foreign proteins expressed in this microorganism, is an ordinary problem found when a eukaryotic protein is expressed in a prokaryotic host. Inclusion bodies are formed by deposition of unfolded or partially misfolded protein species that interact through hydrophobic patches, unusually exposed to the solvent (28). Highly hydrophobic proteins, as OPE is, are more prone to accumulation in inclusion bodies (29). This phenomenon can be minimized through the control of parameters such as temperature, the use of fusion tags, the co-expression of plasmid-encoded chaperones (30) or by using surfactants to solubilize inclusion bodies (31). In this particular case, and after assaying several of the aforementioned strategies, recombinant soluble OPE was obtained by addition of Sarkosyl, an alkyl anionic detergent, to *E. coli* cultures. Nevertheless, the soluble protein showed no activity on the substrate assayed. *In vitro* folding is usually required to obtain active recombinant proteins from inclusion bodies, although it has been reported the expression in *E. coli* of Lip4 from *C. rugosa* fused with thioredoxin gene, yielded 10 % of the total recombinant protein in its soluble form (32).

On the other hand, previous results from OPE expression in *P. pastoris* (19), described the increased solubility of the recombinant protein. This interesting feature was related to the presence of 4–8 extra amino acids in its N-terminus, although the reasons underlying the observed effect of the extra sequence were not explained. The recently elucidated crystal structures of that protein, in its closed and open conformations, shed light on this point suggesting that the presence of the extra N-terminal residues disrupts a hydrophobic patch in the protein surface, preventing its aggregation (25). Then, in a last trial to obtain a soluble and active form of OPE in *E. coli*, we tested the effect of adding these extra amino acids at the N-terminal region of the protein. Only the incorporation of 6 or 8 extra amino acids rendered partially soluble protein in *E. coli*, corroborating that they avoid aggregation, albeit the protein was inactive.

N-linked glycosylation is one of the most common post-translational modifications of proteins. Since *E. coli* cannot perform this complex posttranslational modification, the synthesis of a non-glycosylated recombinant protein could cause the failure to produce an active form of OPE. However, it has been demonstrated that both the native and the recombinant OPE from yeasts maintain their activity levels after enzymatic deglycosylation (19) indicating that the presence of the glycan moiety is not necessary to preserve the enzymatic activity of the glycoprotein once folded and secreted. Then, the lack of activity of the recombinant protein synthesized by *E. coli* could be more related to its misfolding, taking into account the relevant role played by sugars in the first steps of protein folding inside the endoplasmic reticulum (33,34). The far-UV CD spectrum data of OPE from *E. coli* (Fig. 3) support this theory since they suggest an incorrect protein folding in this heterologous expression system. This hypothesis was tested by adding tunicamycin, a drug that prevents the first committed step of *N*-linked glycosylation of proteins in the endoplasmic reticulum, to *P. pastoris* cultures. The drastic reduction of the amount of OPE per biomass unit secreted in this yeast as a consequence of this treatment upholds the essential role of the initial glycosylation steps for the correct folding and secretion of the active protein (35,36).

The recombinant protein expressed in *S. cerevisiae* was soluble and active, showing similar molecular mass, glycosylation degree and circular dichroism spectrum than OPE from *P. pastoris*. Regarding the solubility of the protein expressed in *S. cerevisiae*, it was more soluble than the native one (19), but less than that produced in *P. pastoris*, as can be inferred from analytical ultracentrifugation experiments (Fig. 5). The expression levels achieved in *S. cerevisiae* were considerably lower than those obtained in *P. pastoris* in the conditions assayed. Among the strains tested, the best *S. cerevisiae* producer was the protease-deficient strain carrying the inducible promoter. Although being replicative, the plasmid used in this construct showed to have high stability after several evolution cycles in experiments of directed evolution of fungal laccases (21).

Despite the similarity of the OPE expressed in the two eukaryotic hosts, the efficiency of the enzyme produced in *S. cerevisiae* was three times lower than that of the protein expressed in the doubly-transformed *P. pastoris* strain (Table 3). Although both proteins contain the extra N-terminal amino acids, their differences in yield, aggregation behavior and kinetic constants may be explained from having a different ratio of species with 4, 6 or 8 N-terminal residues. In *S. cerevisiae*, the protein is produced at 22°C and there is a low protein secretion level. Under these conditions, the peptidase STE13 is likely to process the EAEA repetition more efficiently than it does in *P. pastoris* cultures, where the environment is less favorable in terms of temperature and protein amount. Then, the population of proteins with 4 extra amino acids would be higher in

the former situation, but this fact would impact negatively in OPE solubility, as can be deduced from the lack of solubility observed for the recombinant OPE form with only 4 extra amino acids in its N-terminus produced in *E. coli*.

Although we are aware of the low yields of the OPE produced in *S. cerevisiae*, it is interesting to remark that the success in achieving the functional expression of OPE in this yeast, used as platform of directed evolution (21), opens new strategies to produce more robust tailor-made enzymes. Further experiments would be required to optimize OPE expression in this host.

In summary, here we analyzed the expression, in prokaryotic and eukaryotic hosts, of the sterol esterase from *O. piceae*, an enzyme with potential biotechnological interest. The results suggest that the first steps of *N*-glycosylation during OPE synthesis may condition its final folding and are crucial to secrete an active protein. The choice of the heterologous host and growth conditions seem also to be very important to obtain soluble forms (monomeric or dimeric) of OPE, enhancing its catalytic efficiency and preventing its aggregation.

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