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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.