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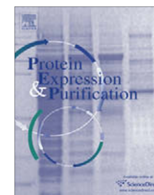

Extracellular secretion of *Pseudoalteromonas* sp. cold-adapted esterase in *Escherichia coli* in the presence of *Pseudoalteromonas* sp. components of ABC transport system

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ABSTRACT

Recently we described identification and characterization of GDSL esterase EstA from psychrotrophic bacterium *Pseudoalteromonas* sp. 643A. Attempts to obtain heterologous overexpression of this enzyme in *Escherichia coli* system were not satisfactory. The EstA protein was expressed as inclusion bodies, most of that were inactive after purification step, and the recovery of esterolytic activity was very low after refolding. Based on the sequence analysis we found that the esterase EstA gene is clustered with three genes encoding components of ABC transport system. These genes, designated *abc1*, *abc2*, and *abc3* encode an ATP-binding protein (ABC1) and two permease proteins (ABC2 and ABC3). In present study, to obtain larger amounts of the active cold-adapted EstA esterase from *Pseudoalteromonas* sp. 643A, we designed a two-plasmid *E. coli* expression system where the gene encoding EstA enzyme was cloned into pET30b(+) expression vector and three genes encoding components of ABC transport system were cloned into pACYC-pBAD vector. It was shown that the created expression system was useful for extracellular production of active EstA enzyme which was purified from the culture medium. In the presence of all the three transporter proteins the secretion of EstA was at the highest level. When one or two of these components were missing, EstA secretion was also possible, but not so effective. It indicates that ABC2 and ABC3 proteins of *Pseudoalteromonas* sp. 643A could be replaced with their homologous proteins of *E. coli*.

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Introduction

Lipolytic enzymes are important biocatalysts for various industrial applications due to some important features like no requirements for cofactors, remarkable stability in organic solvents, broad substrate specificity, regio- and stereo-selectivity. In the past few years, an increasing attention has been drawn to potential applications of cold-adapted lipolytic enzymes from microorganisms populating permanently cold environments [1–3]. These enzymes display attractive properties for some industrial purposes like the high catalytic activity at low temperature and low thermostability. Recently, we described a novel cold-adapted esterase (EstA) from a psychrotrophic bacterium *Pseudoalteromonas* sp. strain 643A [4] belonging to GDSL family of serine esterases/lipases, which can be further classified to a subgroup of this family, i.e. SGNH-hydrolases due to the presence of four strictly conserved residues Ser, Gly, Asn and His in four blocks (I, II, III, and V, respectively) of conserved sequences [5,6]. The subclass of GDSL esterases and lipases is characterized by a broad substrate specificity and regio-specificity, so these enzymes can be used in synthesis and hydrolysis reactions in many branches of industry. We have isolated and sequenced the gene of *Pseudoaltermonas* sp. 643A esterase and characterized the purified enzyme from the native source. The yield of this esterase synthesis by *Pseudoaltermonas* sp. 643A was very low and its heterologous overexpression in *Escherichia coli* cells resulted in formation of inclusion bodies deprived of the catalytic activity. To obtain larger amounts of active esterase EstA we decided to construct a new *E. coli* expression system. Upstream the *Pseudoaltermonas* sp. 643A *estA* gene we identified ORFs which probably encoded components of ABC transport system [4]. Some ABC exporters are known to take part in secretion of lipolytic enzymes such as lipases of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Serratia marcescens* [7–10]. Protein secretion using ABC exporters is one of five types of protein transport across two membranes of Gram-negative bacteria [11]. It is one of the largest paralogous protein families about 5% of the *E. coli* genome encodes components of ABC transporters [12]. The typical ABC transporter (ATP-binding

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Table 1
DNA primers used in this study

Primer name	Sequence	Restriction site
Abc1F	GGCTCCATGGGC**ATGAGTCATATGTCAGCGCTTTCTC**	NcoI
Abc1R	TATGAGCTC**TTAGCCCACATCAGCGGTGTCC**	SacI
Abc1RHis	TATGAGCTCTTAATGATGATGATGATGGTG**GCCCACATCAGCGGTGTCC**	SacI
Abc2F	ATAGAGCTCAACTTTAAG*AAGGAG*ATATACAT**ATGTGGGCTAAGTTAGCGCTAAAATTATTTGCG**	SacI
Abc2R	TATAGATCT**TCATAAAAGCGAGCGTATGTCTGCTTC**	BglII
Abc2RHis	TATAGATCTTCAATGATGATGATGATGGTG**TAAAAGCGAGCGTATGTCT GCTTC**	BglII
Abc3F	ATAAGATCTAACTTTAAG*AAGGAG*ATATACATA**TGATGAAAACGCTGGGCGGCA**	BglII
Abc3R	TATGAATTCT**TATGCTAAAGCACGTACCAAACC**	EcoRI
Abc3RHis	TATGAATTCTTAATGATGATGATGATGGAG**TGCTAAAGCACGTACCAAACC**	EcoRI

The bolded part of primer sequences are complementary to the nucleotide sequences of the amplified genes, the underlined sequences contain recognition sites for restriction endonucleases, and the italicized parts of the primer sequences are the added RBS sequences.

cassette, ABC-ATPase) consists of two transmembrane domains (TMDs)[1], and two nucleotide-binding domains (NBDs), encoded as separate polypeptides or fused into multidomain polypeptides [13,14]. ABC transporter TMDs are hydrophobic and span the membrane several times via predicted α-helical motifs. Hydrophilic ATP-binding domains (NBDs) contain Walker A and B motifs, linker peptide LSGGQQ, helical domain, and switch region [15,16]. The functional unit of an ABC transporter is a monomer of four core domains, but some additional proteins or domains might be required [17]. The transport cycle is initiated by the interaction of substrate with a specific binding site(s) on the TMDs. It induces conformational changes in the TMDs, which is transmitted to the NBDs to initiate ATP binding. After ATP hydrolysis and ADP/Pi releasing the transporter can start the next cycle [18–20]. ABC transporter is generally specific for its own particular substrate(s) or a group of chemically related substrates. However, some of them are multi-specific e.g. might have a broad specificity for hydrophobic compounds [20]. The ABC-ATPase is associated with two auxiliary proteins: a membrane fusion protein (MFP) and an outer membrane protein (OMP) [21,22]. ABC exporters of gram-negative bacteria are usually encoded by genes linked to the structural gene for the extracellular secretory protein [23,24].

In this study, we endeavored to produce the active EstA enzyme to the culture medium via the type I secretion pathway. The enzyme was then purified by ammonium sulfate precipitation followed by dialysis step. The effect of *Pseudoaltermonas* sp. 643A ABC transport system components on *Pseudoalteromonas* sp. 643A esterase EstA expression and secretion are shown.

Materials and methods

Bacterial strains, plasmids, growth conditions

Escherichia coli TOP10F' (Invitrogen) and BL21(DE3) (Novagen) were used as host strains for DNA manipulation and gene expression, respectively. Plasmid pUC19 (Invitrogen) was used for subcloning, and pET30b(+) (Novagen) and pACYC-pBAD [25] were used as expression vectors. Plasmid pLipo1 [4] was used for PCR amplifications of *abc1*, *abc2*, and *abc3* genes. pET22b-GDSL-643A [4] was used for *estA* gene cloning.

The *E. coli* strain was grown on LB medium (Sambrook and Russel 2001), supplemented with ampicillin ($100\,\mu g\,ml^{-1}$) and/or kanamycin ($50\,\mu g\,ml^{-1}$). Arabinose (Sigma) and IPTG (Sigma) were used as expression inducers.

[1] *Abbreviations used:* ABC, ATP-binding cassette; EstA, cold-adapted esterase of *Pseudoalteromonas* sp.643A; TMDs, transmembrane domains; NBDs, nucleotide-binding domains; NBDs, hydrophilic ATP-binding domains; MFP, membrane fusion protein; OMP, outer membrane protein; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; BLAST, basic local alignment search tool; IPTG, isopropyl-β-D-thiogalactopyranoside; ORF, open readings frames; Ap^R, ampicillin resistance gene; Kn^R, kanamycin resistance gene; IM, inner membrane, OM, outer membrane.

General DNA manipulations

DNA manipulations were carried out according to standard procedures [26] or manufacturer's recommendations. Restriction enzymes were purchased from Fermentas and DNA ligase was purchased from Epicentre. DNA polymerase *Pwo* and other PCR reagents were from DNA Gdańsk II s.c. Kits for plasmid isolation and DNA purification were purchased from A&A Biotechnology.

Electrophoresis and Western blot analysis

Protein fractions were analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on 15% gel slabs and stained with Commassie blue [27]. The amount of recombinant protein was evaluated by the optical densitometry of SDS–PAGE gels with Quantity One program (VersaDoc, Biorad) with the bovine serum albumin as a standard. Western blot analysis was performed as described previously [28].

Enzyme assay

Esterase activity was determined spectrophotometrically by measurements of concentration of *p*-nitrophenol (at 405 nm) released from *p*-nitrophenyl butyrate as described previously [4]. One unit of esterase activity is equivalent to 1 μmol of *p*-nitrophenol released from the *p*-nitrophenyl butyrate in 1 min at 35 °C.

ORFs analysis

Nucleotide and deduced amino acids sequence analysis, and ORFs search were performed with the ORF Finder program (NCBI). Sequence similarity analyzes were carried out using Basic Local Alignment Search Tool (BLAST) program from National Centre of Biotechnology server.

Construction of expression plasmids

Primers used for amplification of the *abc1*, *abc2*, and *abc3* *Pseudoalteromonas* sp. 643A genes were: abc1F and abc1R, abc2F and abc2R, abc3F and abc3R, respectively (Table 1). To amplify genes encoding ABC proteins with His-tag domains at their C-termini, abc1RHis, abc2His, and abc3His were used instead of abc1R, abc2R, and abc3R DNA primers (Table 1). The obtained PCR products were cloned into SmaI site of pUC19 vector, resulting in recombinant plasmids of pUC19-A, pUC19-B, pUC19-C, pUC19-AHis, pUC19-BHis, and pUC19-CHis, respectively. The correctness of the constructed plasmids was confirmed by DNA sequencing using ABI 3730 xl/ABI 3700 sequencing technology (AGOWA). Expression plasmids for production of native and His-tagged components of the ABC transport system were constructed in a three-step procedure. First, the *abc1* gene was excised from pUC19-A plasmid by NcoI and SacI and ligated into

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