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Purification and Biochemical Characteristic of a Cold-Active Recombinant Esterase from *Pseudoalteromonas* sp. 643A under Denaturing Conditions

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Abstract

In this paper production of a cold-active esterase EstA from the Antarctic bacterium *Pseudoalteromonas* sp. 643A in *E. coli* expression system was described. The purification and biochemical characteristic of EstA were performed in the presence of urea and then compared with results obtained for the esterase with no addition of urea and isolated from the native source. In both cases the cold-active enzyme displayed similar properties. However, the differences concerning thermal activity were observed. The optimal temperature for recombinant esterase in the presence of urea (1 M) was about 15°C lower in comparison with enzyme isolated from the native source. Furthermore, the EstA was found to be more thermolabile in denaturant conditions. The differences were presumably caused by slightly changed protein structure in the presence of urea. The preservation of activity of EstA dissolved in buffer containing 8M urea suggests that the protein structure is retained and it does not undergo dramatic changes due to high urea concentration. This thesis was confirmed with FT-IR data.

Key words: *Pseudoalteromonas* sp., cold-active enzyme; esterase, denaturing conditions, urea

Introduction

Lipases and esterases, collectively known as lipolytic enzymes, hydrolyze hydrophobic long- and short-chain carboxylic acid esters, respectively (Arpigny and Jaeger, 1999; Fojan *et al*., 2000; Singh *et al*., 2006). They are also able to catalyze esterification, transesterification and enantioselective hydrolysis reactions (Pandey *et al.*, 1999). Due to the lack requirement for cofactors, stability in organic solvents, chemo-, regio- and stereoselectivity lipolytic enzymes are important biocatalysts in many branches of industry. They are widely used in food industry, manufacturing of cosmetics and detergents, fats and oils processing, syntheses of fine chemicals and pharmaceuticals, in paper making, sewage treatment plants, and in polymer decomposition (Jaeger and Reetz, 1998; Jaeger *et al*., 1999; Pandey *et al*., 1999; Sharma *et al*., 2001; Jaeger and Eggert, 2002). Nowadays an increasing attention has been drawn to potential applications of cold-active lipolytic enzymes isolated from psychrophilic and psychrotrophic microorganisms (Rashid *et al.*, 2001; Kulakova *et al.*, 2004; Ryu *et al*., 2006) due to the high catalytic activity at low temperature and low thermostability.

Our group has isolated a psychrotrophic bacterium *Pseudoalteromonas* sp. strain 643A, producing a novel cold-active esterase EstA belonging to the GDSL family of bacterial lipolytic enzymes (Cieśliński *et al.*, 2007). The subclass of GDSL esterases and lipases is characterized by a broad substrate specificity and regiospecificity, so these enzymes could be used in synthesis and hydrolysis reactions in many branches of industry (Lo *et al*., 2003; Akoh *et al*., 2004). We have isolated and sequenced the gene encoding esterase EstA of *Pseudoalteromonas* sp. 643A and characterized the purified enzyme from the native source. However, its heterologous overexpression in *E. coli* was not satisfying either due to formation of inclusion bodies and its probable integration in *E. coli* cell membrane (Cieśliński *et al*., 2007), or ineffective extracellular secretion (Długołęcka *et al*., 2008).

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In this study we describe a new *E. coli* expression system in which the cold-active esterase was produced without its signal sequence to be accumulated in *E. coli* cells and to avoid its integration into the cell membrane. The recombinant esterase was purified under denaturing conditions (8 M urea) and its biochemical characteristics were assayed without removing the urea from the reaction mixture. Moreover, we estimated the optimal concentration of urea with respect to purified enzyme activity and the changes in the enzyme structure. To this end, the EstA protein structure was determined using the FT-IR technique in the presence and absence of urea.

Experimental

Material and Methods

Bacterial strains, plasmids, growth conditions. *E. coli* TOP10F' (Invitrogen) and BL21(DE3) (Novagen) were used as the host strains for DNA manipulation and gene expression, respectively. Plasmid pUC19 (Invitrogen) was used for subcloning, and pET22b(+) (Novagen) was used as expression vector. Plasmid pLipo1 (Cieśliński *et al*., 2007) was used for PCR amplifications of *estA* gene. The *E. coli* strain was grown on LB medium (Sambrook and Russel, 2001), supplemented with ampicillin (100 μg/ml), and IPTG (0,5 mM) (Sigma). IPTG (isopropyl-β-D-thiogalactopyranoside) was used as expression inducer.

General DNA manipulations. DNA manipulations were carried out according to the standard procedures (Sambrook and Russel, 2001) 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 purchased from DNA Gdańsk II s.c. Kits for plasmid isolation and DNA purification were purchased from A&A Biotechnology.

Electrophoresis. Protein fractions were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gel slabs and stained with Commassie blue (Walker, 1996). The amount of recombinant protein was evaluated by the optical densitometry of SDS-PAGE gels using the Quantity One program (Bio-Rad) with the bovine serum albumin (Sigma) as a standard.

Expression of the recombinant esterase gene in *E. coli* and protein purification. Primers used for amplification of the *estA* gene of *Pseudoalteromonas* sp. 643A were: EstHis-F 5'-ATACATATGCACCATCAT CATCATCAT**GACAACACGATTTTAATAC ACGGAG**-3' (containing NdeI recognition site and sequence encoding poliHis domain) and Est-R 5'-CCCAAG**CTT**TTAGACGTTATTTAACCAC-3' (containing HindIII recognition site) were used. The boldface parts of primer sequences are complementary to the nucleotide sequences of the *Pseudoalteromonas* sp. 643A EstA esterase gene, whereas added recognition sites for restriction endonucleases are underlined. The obtained PCR product, encoded EstA esterase devoid of its signal sequence and with His-tag domain at the N-termini, was cloned into SmaI site of pUC19 vector, resulting in recombinant plasmids of pUC19-EstAHis. The correctness of the constructed plasmid was confirmed by DNA sequencing using ABI 3730 xl/ABI 3700 sequencing technology (AGOWA GmbH). Expression plasmid for production of His-tagged esterase EstA was constructed by excising the *estA* gene from pUC19-EstAHis by NdeI and HindIII and ligated into the pET22b(+) plasmid digested with the same endonucleases to give the expression plasmid pET22b(+)-EstAHis. The *E. coli* strain Bl21(DE3) cells transformed with pET22b(+)-EstAHis were grown for 16 h at 37°C in LB medium containing ampicillin (0.1 mg/ml). The preculture was inoculated (1%) into fresh LB medium containing ampicillin and cultivation was continued at 37°C to OD_{600} of 0.5. The culture was then supplemented with 0.5 mM IPTG and grown for 3 h at 30°C to achieve the overexpression of GDSL-esterase gene. Cells were harvested by centrifugation at 16 000 × g for 10 min, resuspended 50 mM Tris-HCl buffer pH 8.0 containing 300 mM NaCl, 5 mM imidazole, and 8 M urea, disrupted by sonification, and cell debris was removed by centrifugation at 16 000 × g for 10 min. The enzyme was purified on His-Bind Resin (Novagen) using denaturing conditions, with a gravity flow of 2 ml min^{-1}. For washing 50 mM Tris-HCl buffer pH 8.0 containing 300 mM NaCl, 5 mM imidazole, and 8 M urea was used and esterase EstA-His was eluted with a 50 mM Tris-HCl buffer pH 8.0 containing 300 mM NaCl, 250 mM imidazole, and 8 M urea. Protein was then dialyzed against 50 mM Tris-HCl buffer pH 8.0 containing 8 M urea (for activity assays) or against 20 mM phosphate buffer pH 8.0 (for FT-IR).

Enzyme assays. Esterase activity was determined spectrophotometrically by measurements of concentration of *p*-nitrophenol (at 405 nm) released from *p*-nitrophenyl butyrate. Reaction mixtures contained 5 μl of 50 mM substrate solution and 5 μl of enzyme solution buffered in 100 μl of 50 mM Tris-HCl pH 7.5 were incubated at 20°C for 5 min. To determine urea influence on EstA activity substrate solution and enzyme solution were buffered in 50 mM Tris-HCl pH 7.5 containing urea at different concentrations. However the characterization of EstA esterase under denaturing conditions was performed for the final concentration 0.4 M of urea in the reaction mixture.

One unit of esterase activity is equivalent to 1 mmol of *p*-nitrophenol released from the *p*-nitrophenyl bu-

tyrate in 1 min at 20°C. For routine enzyme assay, *p*-nitrophenyl butyrate (pNPB) (Sigma) was used as esterase substrate. To determine enzyme specificity various *p*-nirophenyl derivatives were used. Short-chain fatty acid esters of *p*-nitrophenol (*p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl caprylate, and *p*-nitrophenyl decanoate) were dissolved in acetonitrile whereas long-chain fatty acid esters (*p*-nitrophenyl palmitate and *p*-nitrophenyl stearate) were dissolved in *n*-hexane.

FT-IR measurements and analysis. Protein pellet suspension (1ml) dialyzed against 20 mM phosphate buffer pH 8.0 was resuspended (not solubilized) in 100 µl of 20 mM phosphate buffer pH 8.0 or completely solubilized in 20 mM phosphate buffer pH 8.0 containing 8 M urea. To prepare the calibration curve ten solutions of urea were prepared in the range 0 to 9 M urea (pH 8.0). The refractive index of each sample was measured with thermostated refractometer (PZO). High urea concentration excluded the usage of classical transmission cell. Instead, attenuated total reflection accessory (ATR) had been used, which allowed to obtain satisfactory spectra of protein and protein-urea solutions. The spectra were recorded on Nicolet 8700 spectrometer (Thermo Electron Co.), using the Golden Gate single-reflection ATR accessory (Specac). The temperature during measurements was kept at 25 ± 0.1°C using electronic temperature controller (Specac). For each spectrum 128 scans were collected with a resolution of 4 cm^{-1}. The spectrometer source was on Turbo mode during measurement. The spectrometer and the ATR accessory were purged with dry nitrogen to diminish water vapour contamination of spectra. All ATR spectra were water vapour subtracted and corrected using advanced ATR correction algorithm (part of the OMNIC software). The absorbance value at 1160 cm^{-1} was used to determine the urea concentration in protein sample. The corresponding urea spectrum was interpolated using Yanusz.ab program run under Grams/32 (Galactic Industry Corporation) and subtracted from protein spectrum. The reference water spectrum was subtracted from the protein spectra to obtain straight horizontal line in the range of 1900–1720 cm^{-1}. The spectra were smoothed with a 13-point Savitzky-Golay algorithm. The difference spectra were analysed using the OMNIC (Thermo Electron Corporation) and Grams/32 programs (Galactic Industry Corporation).

Results and Discussion

Gene cloning, expression, and purification of EstA. EstA esterase was produced as a His-tagged protein at its N-terminus. Additionally, to avoid accumulating in cell membrane or in periplasm of *E. coli*, the enzyme was devoid of its signal sequence. However, the enzyme accumulated in the cytoplasm as inclusion bodies, which were easily solubilized in buffers containing 8 M urea. Surprisingly, esterase activity was at high level and was found to precipitate during dialysis against buffers without urea. The EstA esterase shown the highest activity in the presence of 1 M urea whereas it partially lost the activity at lower or higher denaturant concentrations (Table I). The applied expression system was quite efficient, giving about 140 mg of esterase from 1 L induced culture. As a result of purification in 'denaturant' conditions about 80 mg of electrophoretically homogenous protein was obtained, with a specific activity of 420 U mg_{EstA}^{-1} (Fig. 1),which was the highest activity in comparison with all previous attempts (Table II).

Table I
EstA activity at different urea concentrations.

Urea concentation [M]	Specific activity [U × mg_{EstA}^{-1}]	Relative activity [%]
0.08	387.0 ± 2.34	58
0.1	431.0 ± 2.53	65
0.2	464.0 ± 2.19	70
0.3	464.0 ± 2.50	70
0.4	420.0 ± 2.78	63
0.5	420.0 ± 2.11	63
0.6	409.0 ± 4.19	62
0.7	464.0 ± 2.43	70
0.8	442.0 ± 2.32	67
0.9	464.0 ± 2.17	70
1	663.0 ± 2.45	100
2	420.0 ± 2.54	63
4	132.0 ± 2.03	20
6	126.0 ± 2.80	19
8	99.0 ± 3.23	15

Average ± SD, (n = 3).

The enhancement of activity in the presence of urea at low concentration was also observed for some other enzymes (Kaufman, 1968; Narayanasami *et al.*, 1997; Zhang *et al.*, 1997; Kumar *et al.*, 2003; Shahnawaz *et al.*, 2007). The presence of urea (at low concentration) in the environment of the protein causes some slight conformal changes in the neighborhood of the active site. These changes in the secondary and tertiary protein structure might promote the more open enzyme conformation (Deshpande *et al.*, 2001; Kumar *et al.*, 2003) or make the active site more flexible (Zhang *et al.*, 1997).

The esterase EstA *Pseudoalteromonas* sp. 643A which possesses its esterolytic activity in the presence of urea might be useful due to its simple purification procedure for some biotechnological applications: there is no need to remove the denaturant after solubilisation of inclusion bodies. However, it is important

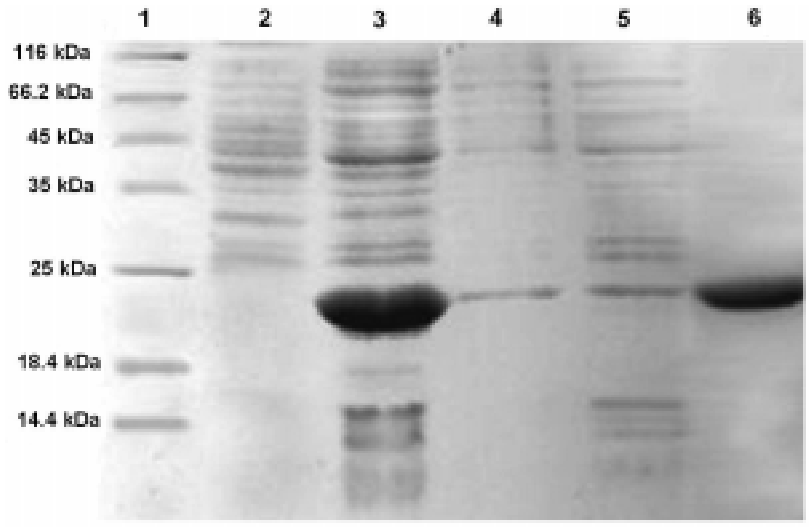

Fig. 1. Purification of EstA esterase.

Lane 1 – Unstained Protein MW Marker (Fermentas); lane 2 – *E. coli* BL(DE3), cell fraction; lane 3 – *E. coli* BL(DE3) + pET22b(+)-EstAHis, soluble fraction of total lysate; lane 4 – fraction of unbounded proteins; lane 5 – fraction of protein collected during washing; lane 6 – protein fraction after purification and dialysis.

to evaluate the optimal concentration of urea with respect to enzyme concentration in the reaction condition *e.g.* 1 M urea for recombinant EstA enzyme. On the other hand, for some applications the purification of EstA might be unnecessary – the *E. coli* proteins coproduced with EstA should be inactive in the presence of denaturing agent.

Characterization of EstA esterase at denaturing conditions. The optimum temperature for activity of recombinant enzyme was 20°C. Esterase retained above 40% of maximum activity at 0°C and lost it rapidly above 20°C (Fig. 2a). It was found to be stable at the temperature near to 0°C and it was completely unstable after 1 h incubation at 30°C (Fig. 2b). The optimum pH range for the enzyme activity and stability is from 7.0 to 8.5 (Fig. 2c, d). Studies of the substrate specificity of purified recombinant EstA esterase performed by comparing enzymatic activity towards various chromogens (*p*-nitrophenyl derivatives) revealed that the preferred substrate for EstA esterase is *p*-nitrophenyl butyrate, but it hydrolyses also esters of shorter- (*p*-nitrophenyl acetate) and longer-chain (*p*-nitrophenyl caprylate) fatty acids (Table III). Enzyme was strongly or completely inhibited by 5 mM Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} ions, whereas Ca^{2+} and Mg^{2+} ions were found to activate it (Table IV). Furthermore, 5 mM thiol compounds strongly decreased esterase activity (Table V). Recombinant esterase was also activated by EDTA,

Table II
Comparison of various methods for EstA isolation and purification. Data relate to protein purified from 1 L culture.

Isolation and purification	Total protein [mg]	Total activity [U]	Specific activity [U/mg_{EstA}]	Reference
EstA from a native source – *Pseudoalteromonas* sp. 643A	3.78	10.8	51	Cieśliński *et al*., 2007
Recombinant EstA produced in *E. coli* cells, purified from inclusion bodies	23	0.21	0.01	Cieśliński *et al*., 2007; unpublished data
Recombinant EstA co-produced in *E. coli* with ABC transporters of *Pseudoalteromonas* sp., purified from culture medium	28	362	10.3	Długołęcka *et al*., 2008; unpublished data
Recombinant EstA produced in *E. coli* cells, purified in urea-containing buffer	80	$3.3\ 10^4$	420	This study

Table III
EstA activity for *p*-nitrophenyl esters.

Substrate	No. of C atoms in alkyl chain	Relative activity [%]
p-nitrophenyl acetate	2	62
p-nitrophenyl butyrate	4	100
p-nitrophenyl caprylate	8	21
p-nitrophenyl decanoate	10	3
p-nitrophenyl palmitate	16	1
p-nitrophenyl stearate	18	0

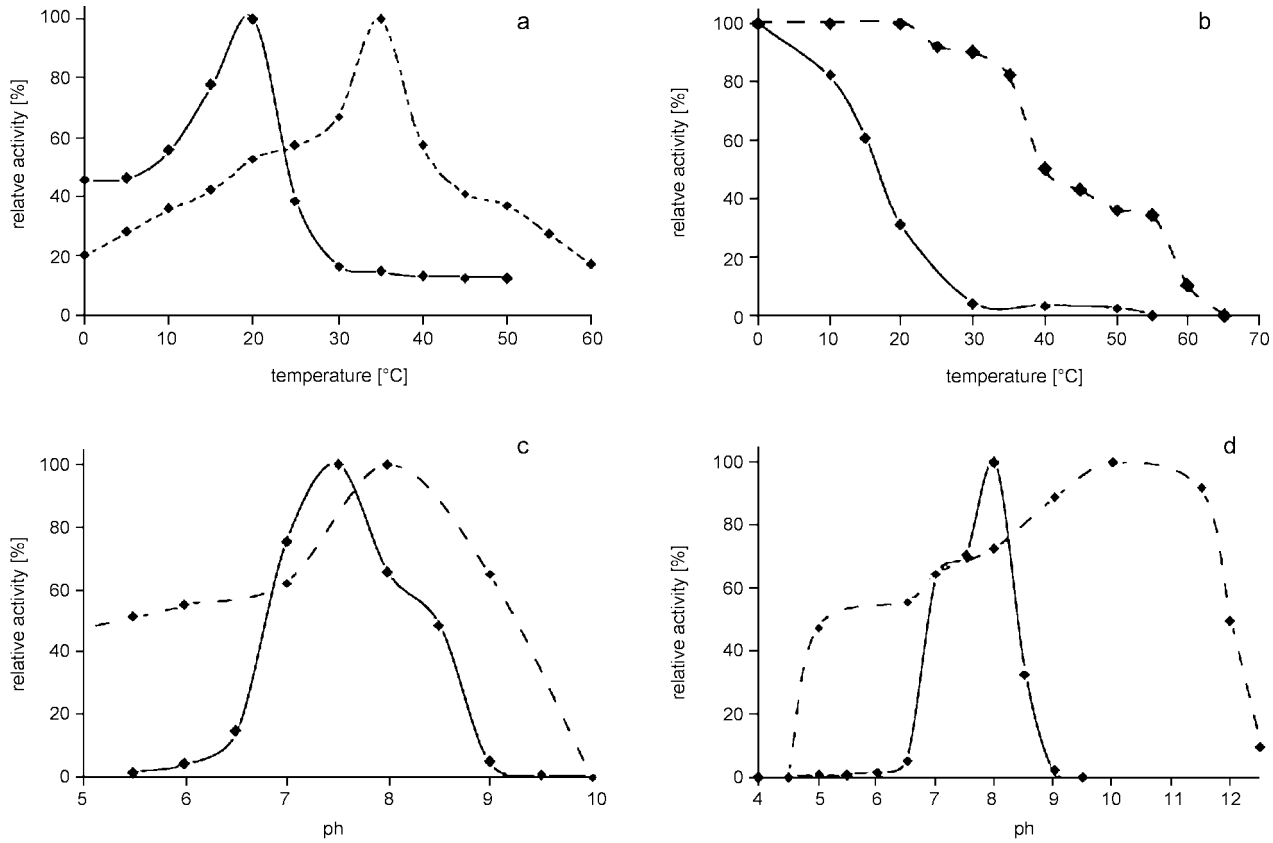

Fig. 2. Effects of temperature and pH on EstA activity and stability.

(a) – temperature dependence of EstA activity. (b) – thermal stability after 1 h incubation of EstA at various temperatures; (c) – pH dependence of EstA activity; (d) – pH-stability after 1 h incubation of EstA at various pH; comparatively, results for native EstA esterase (Cieśliński *et al*., 2007) were added and indicated with dashed lines, results for recombinant EstA esterase with continuous line.

but it can be easily explained by chelating Ni^{2+} residues used in purification step.

The performed biochemical characteristic of recombinant EstA revealed that enzyme produced in *E. coli* cells and dissolved in buffer with urea possesses similar properties (Tables III–V) to esterase isolated from the native source without addition of urea (Cieśliński *et al.*, 2007). The main difference is the temperature dependence activity (Fig. 2a) and thermal stability (Fig. 2b), respectively. Recombinant EstA in the presence of urea was found to show the highest activity at the temperature of 20°C and it completely lost its stability after 1 h incubation at 30°C. In contrast, enzyme isolated from a native source without addition of urea displayed its maximal activity at the temperature of 35°C and was much more stable at higher temperatures- the complete inactivation occurred after 1 h incubation at 65°C. Another difference is that the recombinant enzyme in the presence of urea is active and stable in more narrow range (Fig. 2c, 2d). These differences were probably connected with the esterase EstA structure, which was slightly changed in the

Table IV

Effects of selected chemicals on EstA activity. The enzyme was incubated for 1 h at 20°C with the reagent (5 mM) and the residual activity was assayed under standard conditions.

Reagent	Ca^{2+}	Cd^{2+}	Co^{2+}	Cu^{2+}	Mg^{2+}	Mn^{2+}	Ni^{2+}	Zn^{2+}	None
Residual activity [%]	103	18	30	12	189	69	4	8	100

Table V

Effects of selected chemicals on EstA activity. The enzyme was incubated for 1 h at 20°C with the reagent (5 mM) and the residual activity was assayed under standard conditions.

Reagent	Residual activity [%]
2-mercaptoethanol	13
DTT	10
EDTA	281
Glutathione-SH	20
PMSF	23
None	100

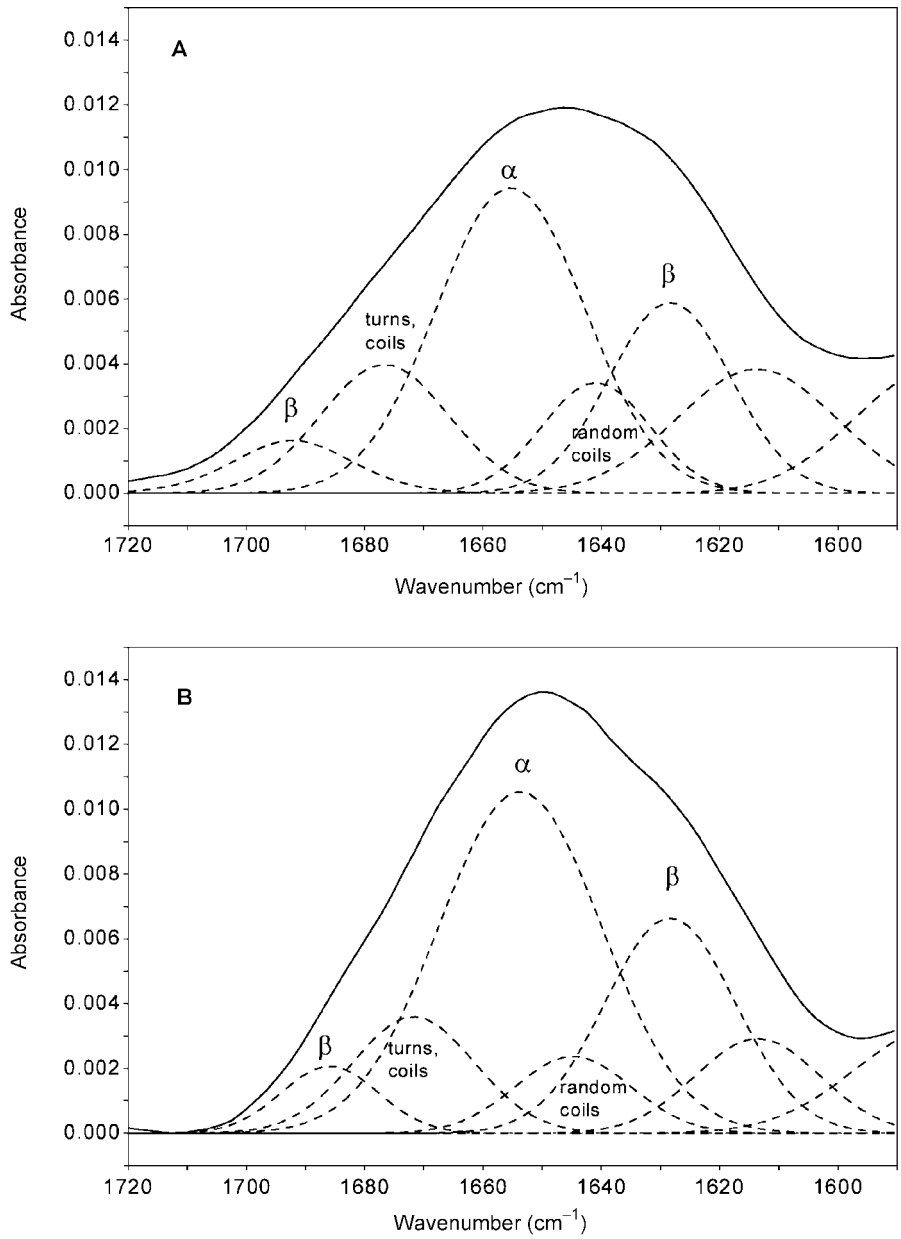

Fig. 3. Decomposition of amide I band into Gaussian-Lorentzian product components.
(a) the amid I band (solid line) of EstA in 20 mM phosphate buffer pH 8.0; (b) EstA dissolved in 8M urea in 20 mM phosphate buffer, pH 8.0; the components (dashed line) are described with corresponding secondary structures (Arrondo and Goni, 1999; Barth and Zscherp, 2002; Natalello *et al*., 2005).

presence of urea (Desphande *et al*., 2001; Grinberg *et al*., 2008). The decreasing of the optimum temperature for the EstA activity in the presence of urea might be another advantage of using the enzyme for some specific applications. However, to explain the role of this denaturant in decreasing of the optimal temperature for enzyme activity, the esterase EstA structure without addition of urea has to be solved.

Analysis of the EstA structure in the presence of urea. In the amide I region of protein spectra (1600–1700 cm^{-1}) it is possible to distinguish several overlapped bands characteristic to various structures (*e.g.* β-sheets, α-helix) (Arrondo and Goni, 1999; Barth and Zscherp, 2002). By means of different mathematical methods (second derivative, Fourier self-deconvolution) the amide I band can be resolved into individual components, allowing describing the secondary structure of a given protein. The peak-fitting technique allows describing the amide I band shape with a few synthetic bands of specified shape. Such resolved amide I band and its components allow to ascribe the percentage participation of particular secondary structure in the overall structure of a given protein (Barth and Zscherp, 2002). Figure 3a reveals that the main structural components of the EstA protein in 20 mM phosphate buffer are both α-helices (band near 1655 cm^{-1})

and β-sheets (band near 1630 cm^{-1}). The data would seem to suggest that the protein consists of twice as many α-helices as β-sheets (34% and 17%, respectively). This is not quite consistent with available structure data of analogous *E. coli* thioesterase I/protease I/lysophospholipase L1 (PDB no.: 1IVN_A) which consists of 47–56% α-helices and 11–14% β-sheets, depending on secondary structure assignment algorithm. The arising differences probably result from the form of measured sample which was a precipitated protein suspended in phosphate buffer, not dissolved. It is possible that intermolecular interactions contribute in the β-sheet characteristic bandwidth (van de Weert *et al.*, 2001). However, the spectrum of this protein still exhibits a shape characteristic for α/β proteins. The preservation EstA activity dissolved in phosphate buffer containing 8 M urea suggests that the structure of this protein is retained and it does not undergo dramatic changes due to high urea concentration. This thesis can be confirmed with the FT-IR data. No band characteristic for protein denaturation can be seen in the spectra (Haris and Severcan, 1999; van de Weert *et al.*, 2001; Natalello *et al.*, 2005). Figure 3b suggests that secondary structure of the protein still can be described as consisting of α and β structures. The proportions of both are similar to the protein suspended only in buffer, though exact analysis of bands area indicates, that participation of α-helix and β-sheet bands in the whole amide I band area is higher (42% and 20%, respectively). These are closer to the available structure of *E. coli* thioesterase I, particularly the α-helix content. Although the products of amide I band decomposition must be taken with great care, the results can suggest that structure of EstA protein might be slightly different than the *E. coli* thioesterase, especially the amount of β-sheet structure seems to be a little higher. Though, these results must be confirmed with other techniques.

Conclusions. Research of recombinant lipolytic enzymes is significantly limited by their tendency to accumulate in the cytoplasm as inactive inclusion bodies (Chung *et al.*, 1991; Zhuo *et al.*, 2005). An active lipase or esterase can be then obtained by dissolving the inclusion bodies and then refolding the enzyme in buffer with denaturing agent, *e.g.* urea. In standard enzyme purification procedures the denaturing agent is removed at the next purification step to promote protein renaturation. However, in many cases the efficiency of the refolding procedure is low. Therefore, in our study we decided to modify the standard procedure of refolding the EstA esterase. In this aim we analyzed the impact of urea concentration on the structure (FT-IR measurements and analysis) and activity of the analyzed esterase. We found that the active EstA esterase could be effectively retrieved from inclusion bodies and purified by immobilized metal affinity chromatography under denaturing conditions without removing the denaturing agent completely, which might enable the use of the EstA enzyme for some biotechnological applications.

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