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Organic Solvent-Tolerant Esterase from *Sphingomonas glacialis* Based on Amino Acid Composition Analysis: Cloning and Characterization of EstSP2 S

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Organic solvent-tolerant (OST) enzymes are widely applied in various industries for their activity and stability in organic solvents, for their higher substrate solubility, and for their greater stero-selectivity. However, the criteria for identifying OST enzymes largely remain undefined. In this study, we compared the amino acid composition of 19 OST esterases with that of 19 non OST esterases. OST esterases have increased the ratio of Ala and Arg residues and decreased the ratio of Asn, Ile, Tyr, Lys, and Phe residues. Based on our amino acid composition analysis, we cloned a carboxylesterase (EstSP2) from a psychrophilic bacterium, *Sphingomonas glacialis* PAMC 26605, and characterized its recombinant protein. EstSP2 is a substrate specific to *p*-nitrophenyl acetate and hydrolyzed aspirin, with optimal activity at 40°C; at 4°C, the activity is approximately 50% of its maximum. As expected, EstSP2 showed tolerance in up to 40% concentration of polar organic solvents, including dimethyl sulfoxide, methanol, and ethanol. The results of this study suggest that selecting OST esterases based on their amino acid composition could be a novel approach to identifying OST esterases produced from bacterial genomes.

Keywords: Amino acid composition, cold-adapted enzymes, esterases, organic solvent-tolerant enzymes, *Sphingomonas glacialis*

Introduction

Enzymes are generally inactivated in organic solvents due to the changes in polarity that result in disruption of the hydrophobic core [1]. However, certain enzymes from organic solvent-tolerant (OST) bacteria, such as *Pseudomonas aeruginosa* LST-03 [2, 3] and *P. aeruginosa* PST-01 [4], as well as from non-OST bacteria [5] showed enzymatic activity in organic solvents. The OST enzymes have advantages when used in industrial applications, because of their greater solubility in non-polar substrates; their seteroselctivity; their tendency to shift thermodynamic equilibrium as a response to synthesis reaction; and their ability to eliminate microbial contaminants from reaction mixtures [6–8]. The OST enzymes also undergo conformational changes in organic solvents: they can bind themselves with organic solvent molecules at specific sites of the enzyme; they can strip water molecules from the protein surface [9, 10]; they can rearrange surface amino acids [11, 12]; they can weaken hydrophobic interactions [1, 13]; and they can enhance structural flexibility [12].

Many efforts have been made to increase the stability of non-OST enzymes in organic solvents via site-directed mutagenesis or directed evolution, which introduces salt-bridges, hydrogen bonds, and hydrophobic interactions in different parts of the native enzymes [14–17]. Although enzyme immobilization [18, 19], chemical modification [20], and physical modifications [21] also enhance the stability of enzymes in organic solvents, these methods may hinder the activity of the enzymes or change substrate specificities [19]. Hence, selecting an enzyme that possesses good organic solvent stability while avoiding the complications in substrate binding and enzyme kinetics remains a challenge. With increasing demands for industrially

important OST enzymes, it becomes essential to identify a method of quickly screening and validating organic solvent-tolerant, natural enzymes [22]. However, the criteria for identifying OST enzymes have largely remained unknown.

Esterases hydrolyze esters into an acid and an alcohol, and are capable of performing synthetic reactions in the presence of organic solvents by adjusting the thermodynamic equilibrium [22]. In this study, we identified the amino acid composition unique to OST esterases by comparing the amino acid composition of 19 OST esterases with that of 19 non-OST esterases. We further validated our approach by cloning a 948-bp carboxylesterase gene *estSP2* from a psychrophilic bacterium, *Sphingomonas glacialis* PAMC 26605, and characterizing the recombinant EstSP2 protein. As expected, we have found that EstSP2 was tolerant in polar organic solvents. We propose that our method of amino acid composition analysis be a novel approach to identifying OST esterases from bacterial genomes.

Materials and Methods

Materials

Sphingomonas glacialis PAMC 26605, isolated from the Arctic lichen *Ochrolechia* sp., was provided by the Polar and Alpine Microbial Collection (PAMC) of Korea Polar Research Institute (Korea) [23]. The strain, formerly known as *Sphingomonas* sp. PAMC 26605 [24], was renamed *S. glacialis* PAMC 26605 in the EzTaxon database [25], based on its 16S rRNA sequence homology to *S. glacialis* C16y^T (99.78%) [26]. *Taq* polymerase, TA vector, and restriction enzymes were purchased from Enzynomics (Korea). pET28a(+) expression vector was acquired from Novagen (USA). HisTrap, Q-Sepharose, and Superdex-200 HR 10/300 gel filtration columns were purchased from GE Healthcare (Piscataway, USA), and *p*-nitrophenyl (*p*NP) esters were purchased from Sigma (USA). Aspirin (acetylsalicylic acid), salicylic acid, acetaminophen, and 4-aminophenol were acquired from TCI (Japan). All other reagents were from Sigma unless noted otherwise.

Amino Acid Composition Analysis

Thirty-eight carboxylesterases (19 OST esterases and 19 non-OST esterases) were selected from the literatures published during the period 1960–2017 in PubMed. And their amino acid sequences were retrieved from UniProt and NCBI (Please see Table S1). The ProParam web server (https://web.expasy.org/protparam/) was used to analyze the amino acid composition of each enzyme. Statistical analysis by student's *t*-test was used to determine the significance of each amino acid in the OST and non-OST esterases.

Gene Cloning of *estSP2*

Based on the amino acid composition analysis, the 948-bp *estSP2* gene (GenBank ID: WP_010183419) was selected from the genome of *S. glacialis* PAMC 26605. The *estSP2* gene was amplified by polymerase chain reaction, cloned into a TA vector, and subsequently into a pET28a(+) vector. The primers used for polymerase chain reaction are listed in Table S3.

Protein Expression and Purification

Escherichia coli BL21 (DE3) transformed with the pET28a (+) vector containing the *estSP2* gene, was cultured in a shaking incubator at 37°C up to the mid-log phase (OD_{600} = 0.6–0.8). Cells were then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside and cultured for additional 16 h at 18°C, and harvested by centrifugating at 12,000 ×*g* for 10 min. Following cell lysis by sonication, the crude enzyme was purified to homogeneity by nickel-chelate affinity chromatography using a 1-ml HisTrap column and a step gradient of imidazole (20 mM and 500 mM) in buffer A (20 mM Tris·HCl, pH 7.5, 0.1 M NaCl, 5 mM imidazole, and 5% glycerol). This was followed by anion-exchange chromatography using a 1-ml Q-FF column and a linear gradient of KCl (50–1,000 mM) in buffer B (20 mM Tris·HCl, pH 8.0, 50 mM KCl, and 5% glycerol). Protein concentration was determined by the Bradford method. The purified enzyme was frozen in liquid nitrogen and stored at −80°C.

Macromolecular Complex Analysis

The macromolecular complexity of the protein was determined by polyacrylamide gel electrophoresis under non-denaturing conditions, as well as by Superdex-200 gel filtration chromatography on AKTA Explorer System with a flow rate of 0.1 ml per min in buffer B. Molecular weight of EstSP2 in macromolecular complex was determined using the standard curve plotted from the elution volume of proteins with known molecular weight using a Superdex-200 gel filtration column.

Enzymatic Assay

The enzyme activity of EstSP2 was determined using a reaction buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5% glycerol) with *p*NP esters by Shimadzu UV-1800 spectrophotometer at 405 nm. Background hydrolysis of *p*NP esters was subtracted from each reading. The optimal temperature for enzymes was determined to range from 4°C to 60°C. The optimal pH was determined at room temperature for sodium phosphate buffer (pH 6 to 7), Tris buffer (pH 7 to 9), and sodium carbonate buffer (pH 9 to 11). The thermal stability of EstSP2 was determined by the residual enzyme activity measured at the optimal temperature (40°C), after incubation period at varying temperatures (4°C to 45°C) at the indicated time point.

Aspirin Hydrolysis

Aspirin hydrolysis was determined by measuring the formation of salicylic acid in 1 ml of reaction buffer. The reaction was stopped when 0.25 ml of 3 M HCl was added. Pure salicylic acid was used as a quantitative standard. Salicylic acid was determined

Table 1. Amino acid composition of OST esterases, non-OST esterases, and EstSP2.

Amino acid	OST esterases (%)	non-OST esterases (%)	EstSP2 (%)
Ala	16.1 ± 3.1*	8.7 ± 1.8	15.6
Arg	6.1 ± 2.3*	4.5 ± 1.7	8.3
Asn	1.9 ± 1.5*	4.2 ± 1.8	1.6
Asp	5.9 ± 1.3	5.7 ± 1.1	8.6
Cys	0.6 ± 0.5	1.3 ± 0.9	1.6
Gln	4.6 ± 2.5	3.2 ± 1.0	1.6
Glu	4.3 ± 1.5	5.3 ± 1.8	3.2
Gly	9.3 ± 1.2	8.4 ± 1.8	8.9
His	2.0 ± 0.8	2.3 ± 1.1	2.2
Ile	3.4 ± 1.3*	5.7 ± 2.0	3.5
Leu	9.6 ± 1.9	9.7 ± 1.4	10.8
Lys	2.8 ± 2.0*	5.0 ± 2.4	0.3
Met	1.9 ± 0.7	1.9 ± 0.9	2.2
Phe	2.9 ± 1.0*	4.2 ± 1.3	3.5
Pro	6.0 ± 1.8	5.3 ± 1.5	8.9
Ser	5.6 ± 1.4	6.6 ± 1.7	2.5
Thr	5.1 ± 2.8	5.4 ± 1.6	6.0
Trp	1.4 ± 0.6	1.3 ± 0.6	1.3
Tyr	3.1 ± 0.8*	4.5 ± 1.1	3.2
Val	7.3 ± 1.7	6.8 ± 1.2	6.3

Data of OST and non-OST esterases correspond to the mean ± SD.
Significance of the amino acid residues was analyzed using student's *t*-test. *P* value lower than 0.05 was significant (denoted with asterisk).

for absorbance at a wavelength of 300 nm.

Fluorescence Quenching of Trp Fluorescence

A SCINCO FS-2 fluorescence spectrometer was used to measure the fluorescence emission spectra of EstSP2 at room temperature at an excitation of 295 nm. Fluorescence quenching of the protein by acrylamide was measured in the presence of increasing concentrations of acrylamide (0–0.5 M) mixed with 8 μmol of protein in reaction buffer with different concentrations of organic solvent. Quenching data are presented as the ratio of intrinsic fluorescence intensity (F_0) to the fluorescence intensity in the presence of 0–0.5 M acrylamide (F).

Results

Amino Acid Composition Analysis

The mean values of the amino acid composition for 19 OST esterases and 19 non-OST esterases are shown in Table 1. OST esterases showed an increased ratio of Ala and Arg residues by 1.8- and 1.4-fold, respectively, and a reduced ratio of Asn, Lys, Ile, Phe, and Tyr residues by 2.2-, 1.8-, 1.7-, 1.5-, and 1.5-fold, respectively, as compared with non-OST esterases. Ala and Arg had low hydropathy index values [27].

Selection of EstSP2 Gene from *S. glacialis* PAMC 26605 and Sequence Analysis

The integrated microbial genome server [28] predicted that *S. glacialis* PAMC 26605 would have 60 different esterase and lipase sequences. Considering our criteria of a medium size esterase that is approximately 30 to 35 kDa, we identified 3 putative OST esterases from the genome of *S. glacialis* PAMC 26605 (Table S2); from these, we selected EstSP2 (Table 1) to evaluate our hypothesis on OST esterases. EstSP2 consists of 315 amino acids with the molecular weight of 33.7 kDa. Based on the pentapeptide sequence of GESAG, EstSP2 belongs to the esterase family VII, which is known to have multimeric structures [29]. Ser158, Asp256, and His286 residues form the catalytic triad of EstSP2 (Fig. S1). Multiple sequence alignments revealed that EstSP2 exhibits 43% amino acid sequence identity with a heroin-degrading esterase HerE (GenBank ID: WP_003938520.1) [30], and 41% sequence identity with an esterase Est8 (GenBank ID: KP699699.1) [31] (Fig. S2). EstSP2 also exhibited 28% sequence identities with human carboxylesterase 1 (hCE1) and carboxylesterase 2 (hCE2) (data not shown), which hydrolyze therapeutically active compounds to their inactive metabolites in the liver and intestinal organs, respectively [32].

Expression and Purification

Expressed as a soluble protein in *E. coli* BL21 (DE3), the recombinant EstSP2 was purified to homogeneity by nickel-chelate affinity chromatography, followed by anion-exchange chromatography. EstSP2 appears as a single band

Table 2. Purification summary table of EstSP2.

	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purity (%)
Cell lysate	143	6258	63	100	31
HisTrap column	41	4856	192	78	93
Q-FF column	33	4452	206	71	100

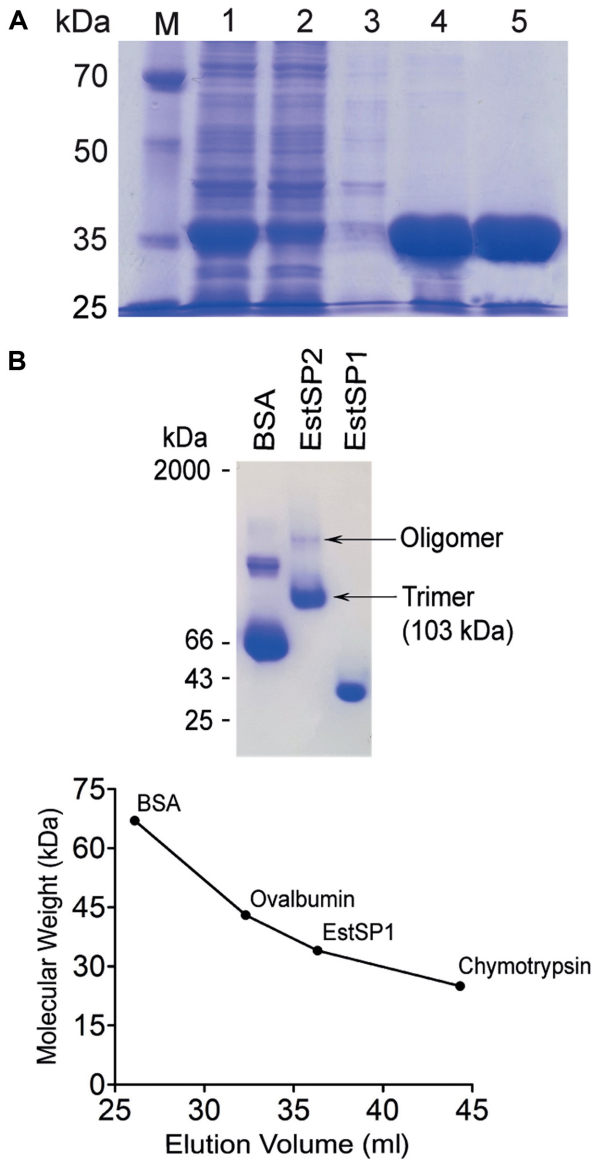

Fig. 1. Purification and molecular weight determination of EstSP2.

(**A**) EstSP2 was purified to homogeneity by nickel-chelate affinity chromatography with a 1-ml HisTrap column, using a step gradient of imidazole (20 and 500 mM) in buffer A, followed by anion-exchange chromatography with a 1-ml Q-FF column using a linear gradient of KCl (50–1,000 mM) in buffer B. M, Molecular weight marker; Lane 1, Cell lysate; Lane 2–4, Flow through, Wash, and Elution from HisTrap column, respectively; Lane 5, Elution from Q-FF column. (**B**) Native polyacrylamide gel electrophoresis for BSA, EstSP2, and EstSP1 (upper panel). BSA and EstSP1 were used as control proteins. Molecular weight markers on the native polyacrylamide gel were placed based on the elution profile of the proteins with known molecular weight using Superdex-200 gel filtration column (lower panel). Chymotrypsin (25 kDa), EstSP1 (34 kDa), Ovalbumin (43 kDa), and BSA (66 kDa)

of approximately 34 kDa on a Coomassie blue-stained SDS gel (Fig. 1). The purification summary of EstSP2 is shown in Table 2.

Macromolecular Complexity

To investigate the multimeric complex formation of EstSP2, we used gel filtration chromatography and native polyacrylamide gel electrophoresis. EstSP2 was eluted from Superdex-200 gel filtration column at an elution volume of 10.1 ml (Fig. S3). From the standard curve using the elution volume of proteins with known molecular weight (Fig. 1B lower panel), the molecular weight of EstSP2 was estimated to be approximately 103 kDa, suggesting that EstSP2 exists in a trimeric form. The native polyacrylamide gel data also show that EstSP2 exists as a trimer and a high molecular weight oligomeric form compared with the molecular weight of bovine serum albumin and a monomeric esterase EstSP1 isolated from this bacterium [33] (Fig. 1B upper panel). These results indicate that EstSP2 possesses the esterase family VII characteristics.

Substrate Specificity

Of the *p*NP esters (C2 to C10), EstSP2 showed substrate specificity to short-chain esters, especially *p*NP acetate (Fig. 2A). The hydrolysis of *p*NP butyrate is only 5% of that measured for *p*NP acetate (Fig. 2A), with no activity being measured for C6 to C10 (data not shown). Contrary to the long list of microbial lipases, only a few microbial esterases are known, with fewer reports of cold-adapted esterases [34]. Many esterases show substrate preference for *p*NP butyrate [35], with limited esterases having a substrate preference for two-carbon molecules [36, 37]. As EstSP2 belongs to the esterase family VII like hCE1 and hCE2, we measured the hydrolysis of aspirin by EstSP2. As shown in Fig. 2B, EstSP2 hydrolyzes aspirin to salicylic acid and acetate. However, acetaminophen, another non-steroidal, anti-inflammatory drug that contains an amide bond, is not hydrolyzed by EstSP2 (data not shown). The overall data indicates that EstSP2 is capable of hydrolyzing acetyl groups. To the best of our knowledge, EstSP2 is the first bacterial carboxylesterase that hydrolyzes aspirin.

Optimum pH and Temperature, and Thermal Stability

Many cold-adapted enzymes, including EstSP2, have the optimum temperature in the range of 15–45°C [38]. Using *p*NP acetate as a substrate, EstSP2 showed optimum activity at pH 8.5 (Fig. 3A) and 40°C (Fig. 3B), whereas activity of EstSP2 at 4°C was approximately 50% of that measured at

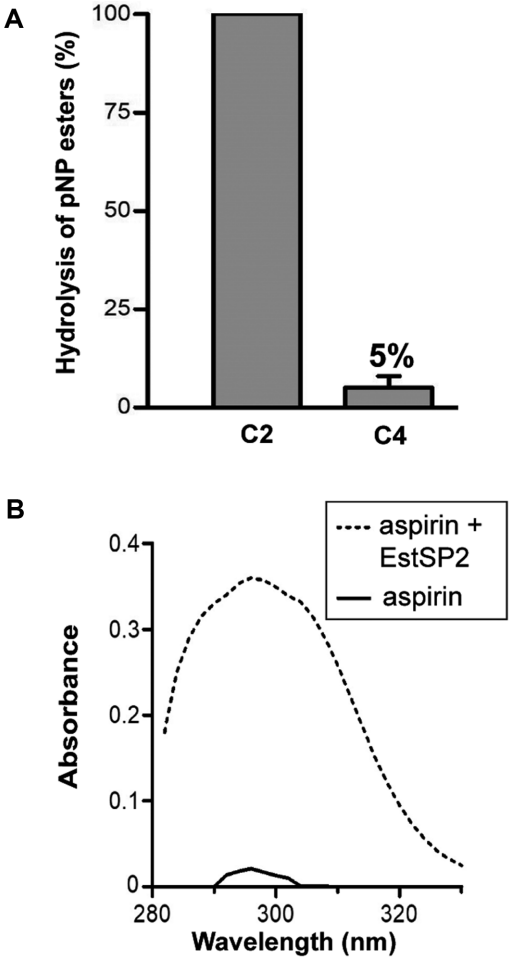

Fig. 2. Substrate specificity of EstSP2.
(**A**) The hydrolysis of *p*NP esters. (**B**) The hydrolysis of aspirin. Data corresponds to the mean ± SD of three experiments.

40°C. EstSP2 showed stability between 4–25°C for 2 h, but rapidly lost activity with increasing temperature (Fig. 3C), thus suggesting that EstSP2 is a psychrophilic enzyme.

Organic Solvent Stability

EstSP2 is exceptionally stable in the buffer containing DMSO, ethanol, and methanol (Fig. 4). Activity of EstSP2 is maintained in 20% DMSO, ethanol, and methanol, while 75% activity was retained at organic solvent concentrations of 40%. However, activity was lost with increasing isopropanol and acetone concentrations. Organic solvent-induced structural changes determined by acrylamide-induced fluorescence quenching revealed that EstSP2 attained flexible structure in organic solvents (Fig. 5). In the 40% organic solvents, EstSP2 showed enhanced flexibility, ranging from 1.2- to 1.6-fold. A higher degree of flexibility

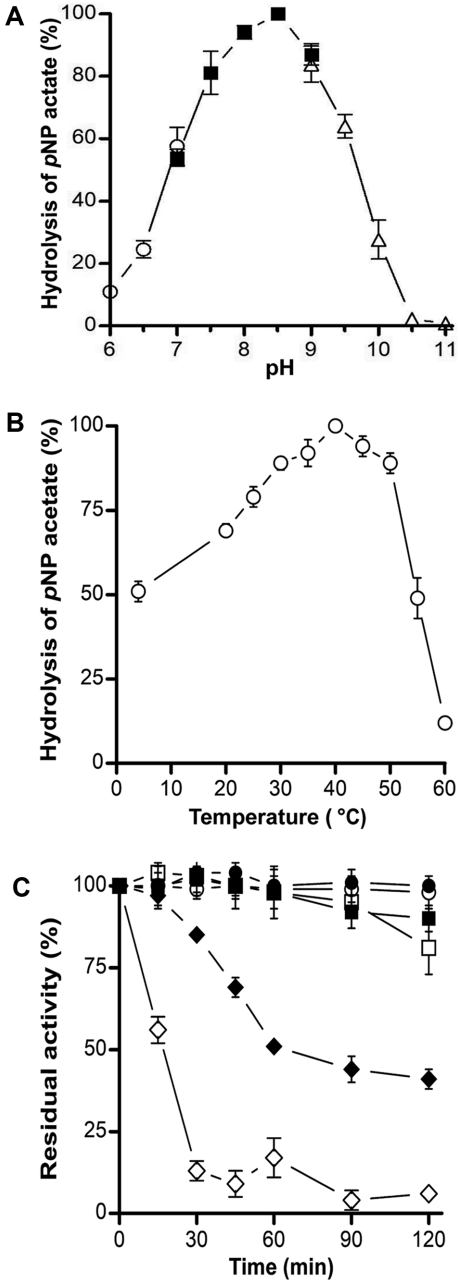

Fig. 3. Enzymatic properties of EstSP2.
(**A**) The optimum pH of EstSP2 was measured with sodium phosphate buffer (pH 6–7) (○), Tris buffer (pH 7–9) (■), and sodium carbonate buffer (pH 9–11) (△). 100% = 36 μM/min. (**B**) The apparent optimum temperature was determined by the activity of EstSP2 measured at the temperatures ranging from 4°C to 60°C with reaction buffer. 100% = 36 μM/min. (**C**) Thermal stability of EstSP2 was determined by the residual activity measured at 40°C, after enzyme incubation period at (●) 4°C, (○) 25°C, (■) 30°C, (□) 35°C, (◆) 40°C, or (◇) 45°C at the indicated time point. The enzyme activity before incubation was 100% (36 μM/min). Data corresponds to the mean ± SD of three experiments.

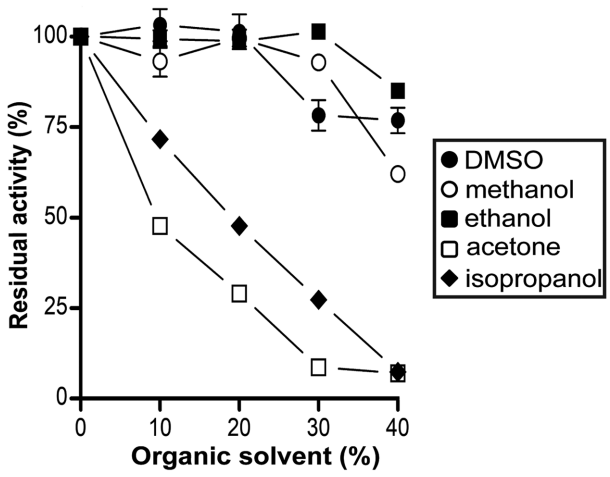

Fig. 4. Organic solvent tolerance.
Enzymatic activity in organic solvents was measured with *p*NP acetate. Prior to the measurement, EstSP2 was incubated for 1 h in different concentrations of organic solvents, and subsequently diluted with reaction buffer; the final concentration of the organic solvent was maintained as 5% while determining the activity. The activity of EstSP2 without organic solvent is 100% (22 μM/min). Data corresponds to the mean ± SD of three experiments.

was induced by DMSO, followed by ethanol, isopropanol, and methanol. However, the structural flexibility of EstSP2 could not be determined with 30–40% acetone as the enzyme was completely inactive and the intrinsic fluorescence was weakened.

Kinetic Analysis

The kinetic parameters of EstSP2 were measured using either *p*NP acetate or aspirin at 40°C. The K_m and k_{cat} values of EstSP2 for *p*NP acetate were 141 μM and 136 s^{-1}, respectively. However, the catalytic efficiency of EstSP2 for aspirin hydrolysis was approximately 1000-fold lower than that for *p*NP acetate hydrolysis, with a K_m of 6,700 μM and a k_{cat} of 6 s^{-1} for aspirin.

Discussion

Although OST enzymes exhibit good stability and activity in organic solvents, the mechanism of organic solvent tolerance is poorly understood. Conformational changes, such as stripping water molecules from the protein surface and binding organic solvent molecules to the enzymes, did not confer stability on the natural OST enzymes [22]. Although the stability of native lipases in organic solvents was improved with new intramolecular interactions in the protein structure, it also helped reduce the enzymatic activity [16]. Introduction of basic amino acid residues on the surface of LST-03 lipase improved the stability of the enzyme due to the formation of additional hydrogen bonds and ionic interactions [39, 40]. To maintain structural stability at high temperatures, thermophilic enzymes are naturally packed with a large number of ionic interactions and hydrogen bonds, but they are not necessarily OST enzymes. Therefore, identifying differences in amino acid sequences could be a novel way to distinguish OST esterases from non-OST esterases.

Our amino acid composition analysis clearly indicates that OST enzymes exhibit an increased ratio of Ala and Arg residues and a reduced ratio of Asn, Ile, Lys, Phe and Tyr residues. The changes are related to the decrease of hydrophobic interactions based on the hydropathy index values [27]. The increase in the Arg to Lys ratio helps the enzyme to form ionic interaction or hydrogen bonds by which the tertiary structure of the enzyme is maintained in the presence of organic solvents. However, the role of Asn and Tyr residues in OST enzymes needs further investigation. Although EstSP2 is an OST esterase, different levels of enzymatic activity were observed for different organic solvents. Depending on the dielectric constant of the solvent, there are variations in the structural dynamics of EstSP2 as previously reported for chymotrypsin [41]. We previously cloned and characterized an OST carboxylesterase, EstSP1, which belongs to the esterase family IV, from *S. glacialis* PAMC 26605 [33]. EstSP1 showed the ratio of Ile to be 2-fold higher compared to the OST esterase group, but the ratio of other amino acids were similar. The results of the current study support the hypothesis that enzymes undergo conformational changes with different organic solvents, which is consistent with our previous observations on the conformational flexibility of cold-adapted, OST enzymes [33, 42]. There are esterases that show reduced Ala and Arg ratio compared with non-OST esterases, including an acetylesterase LaAcE from *Lactobacillus acidophilus* [43], LipJ from *Bacillus* sp. JR3 [44], and 5 esterases found in the genome of *S. glacialis* PAMC 26605 (Table S4). Interestingly, the enzymatic activity of LaAcE was reduced in the presence of 10–30% ethanol [43]. However, the OST nature of these enzymes need further investigation.

In humans, aspirin is hydrolyzed into hCE1 and hCE2, both of which belong to the esterase family VII. EstSP2 hydrolyzes aspirin with a low catalytic efficiency as compared to *p*NP acetate (Fig. 2B). The structural model of EstSP2 suggests that a short-chain ester group is preferred

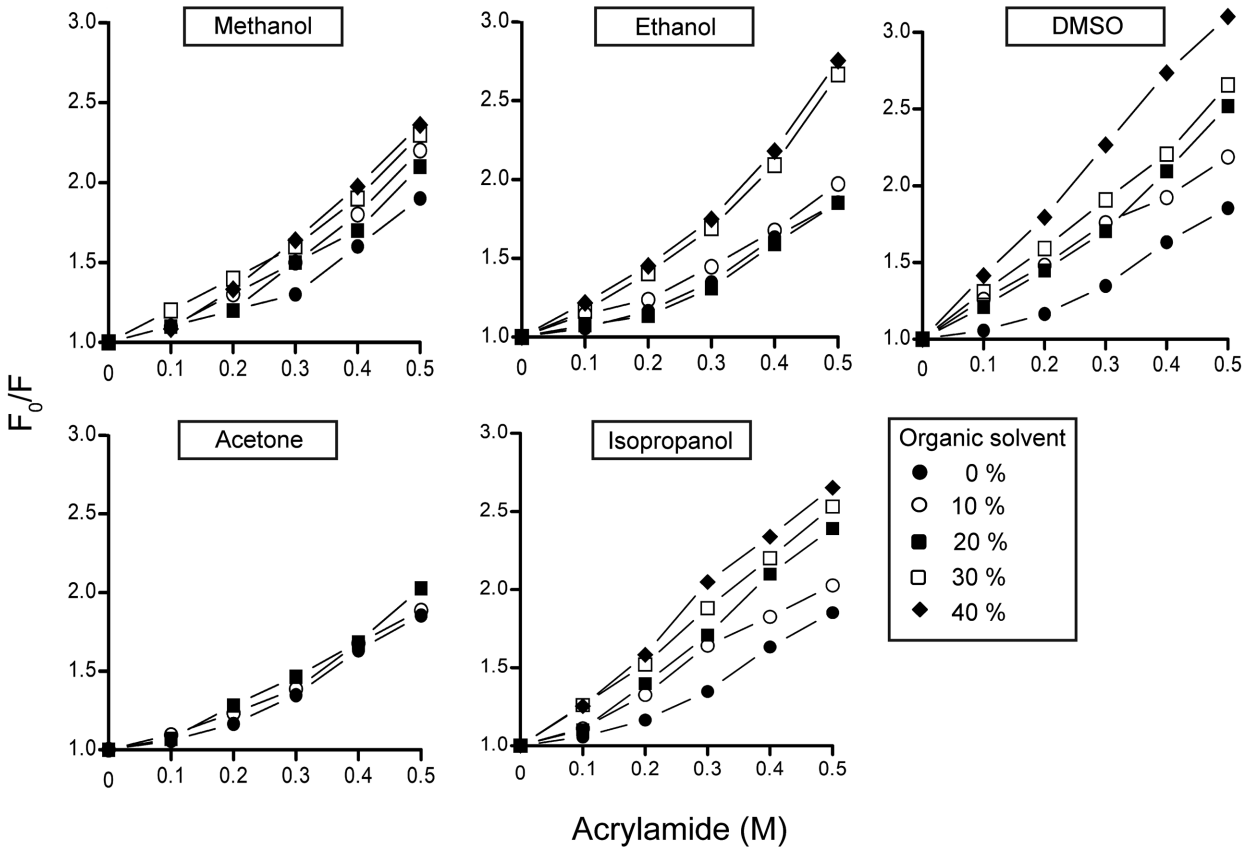

Fig. 5. Effects of organic solvents on conformational flexibility of EstSP2.
Changes in the conformational flexibility of EstSP2 were measured at 25°C by acrylamide-induced fluorescence quenching of Trp fluorescence in the presence of organic solvent upon excitation at 295 nm. F_0 is the fluorescence intensity in the absence of acrylamide, and F is the fluorescence intensity in the presence of 0–0.5 M acrylamide. Fluorescence quenching was not determined for acetone, since EstSP2 lost its fluorescence signal in 30%–40% acetone.

in the substrate binding pocket of EstSP2, but the carboxyl group hinders the binding of aspirin to the EstSP2 active site. The acetyl group in acetaminophen fits into the EstSP2 active site, but the amide bond is usually stronger than ester bond and requires an amidase for hydrolysis. Interestingly, EstSP2 showed a similar level of activity with *p*NP acetate and aspirin in varying concentrations of DMSO, methanol, and ethanol, indicating inherent OST nature of the enzyme (data not shown).

In conclusion, we cloned a psychrophilic carboxylesterase EstSP2 from *S. glacialis* PAMC 26605 based on the amino acid composition of OST and non-OST esterases. The results of this study indicate that the amino acid composition analysis could be used to identify uncharacterized OST esterases from many bacterial genomes and have a potential in industrial applications.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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