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A Novel Alkaline Esterase from *Sporosarcina* sp. nov. Strain eSP04 Catalyzing the Hydrolysis of a Wide Variety of Aryl-carboxylic Acid Esters

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A novel esterase showing activity specific for esters of aryl-carboxylic acids was discovered in *Sporosarcina* sp. nov., which was identified by the 16S rDNA sequencing method in addition to morphological and physiological analyses. The aryl-carboxylesterase (named EstAC) was purified 780-fold from crude cell extracts by a 5-step procedure. EstAC was characterized as a monomeric protein with a molecular weight of 43,000, an optimum pH of around 9.0, and an optimum temperature of 40 °C. The pH optimum and the effects of inhibitors together with an internal amino acid sequence suggested that EstAC is a member of family VIII esterases. EstAC was found to be highly active on a wide variety of substrates such as alkyl benzoates, alkyl phenylacetates, ethyl α- or β-substituted phenylpropionates, dialkyl terephthalates, dimethyl isophthalate, and ethylene glycol dibenzoate. However, monomethyl terephthalate was not hydrolyzed. It was suggested that EstAC had 4-hydroxybenzoyl and cinnamoyl esterase activities as well.

Key words: aryl-carboxylesterase; benzoyl esterase activity; diethyl terephthalate; substrate specificity; *Sporosarcina* sp.

Esterases (EC 3.1.1.x) are ubiquitous hydrolases in all of the kingdoms of life. They catalyze the hydrolysis and formation of ester bonds and act on a wide variety of natural and xenobiotic substrates.[1–3] Carboxylesterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are two major classes of esterases. Both enzymes are relatively stable in organic solvents[4] and have numerous applications in organic synthesis. They are useful catalysts for regio- and stereoselective reactions in fine-chemical synthesis,[3,5] and also used as bulk enzymes in a variety of chemical and other industries.[2] In general, the esterases are specific for either the alcohol or the acid moiety on the substrate, but not for both.[6] Many carboxylesterases have specificity for the relatively short-chain acid moiety of the carboxylic ester, different from almost all the lipases that are specific for the long-chain acid moiety or the alcohol one. The substrate specificities of a large number of carboxylesterases have been documented.[3,5]

Reports on aryl-carboxylesterases showing activities for the esters of aryl-carboxylic acids containing an aromatic ring next to the ester carboxyl group, such as ethyl benzoate (EBz), are very limited[7–11] compared with those on ordinary aliphatic carboxylesterases. The enzymes hydrolyzing phthalic acid esters, which are potential environmental pollutants, may be one exceptional aryl-carboxylesterase studied in many research groups.[12–16] In addition, the aryl-carboxylesterases thus far reported appeared to have rather narrow substrate specificity, or the substrates studied with the enzymes are limited in structure,[7–11] when one considers the occurrence of a large number of structurally analogous aryl-carboxylic acid esters. Enzymes hydrolyzing 4-hydroxybenzoic acid esters are considered to belong to a different esterase family from benzoyl esterases.[17,18] Aryl-carboxylesterases, however, have many potential applications in various fields. They are attractive biocatalysts for the production of various aromas, fragrances,[19,20] and preservatives,[17,18] for the selective removal of protecting groups,[21,22] and in the field of health[23–26] as well as being potentially beneficial for the environment. It is thus of great interest to obtain a new aryl-carboxylesterase with broad or unusual substrate specificities. In addition, if such an esterase has remarkable activities for a large variety of substrates, it might become a useful biocatalyst in various applications.

Here we report a characterization of strain eSP04, a soil bacterial strain that produces an esterase capable of hydrolyzing a wide variety of aryl-carboxylic acid esters with substantial activity. We also characterize this aryl-carboxylesterase, focusing on its substrate specificity.

Materials and Methods

Chemicals. Three substrates, ethyl 2-(4-isobutylphenyl)propionate, ethyl 2-methyl-3-phenylpropionate, and ethyl 3-phenylbutanoate, were prepared from the corresponding carboxylic acids and ethanol using H_2SO_4 as catalyst. The products were washed, evaporated, and purified by silica gel column chromatography (hexane:ethyl acetate = 20:1). 1-Phenoxy-2-propyl acetate was synthesized by esterification of 1-phenoxy-2-propanol with acetic anhydride and was distilled under reduced pressure (92 °C/1.0 mmHg). The structures of the four substrates were ascertained using proton nuclear magnetic resonance (^{1}H-NMR) spectra in $CDCl_3$ with tetramethylsilane recorded on a FT NMR spectrometer (JNM-LA400, JEOL, Tokyo) at 400 MHz. All other chemicals used, including the other substrates examined, were from commercial sources.

Media and culture conditions. A dozen bacterial strains, maintained in our laboratory's collection for general screening to find organisms

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Abbreviations: BSA, bovine serum albumin; DET, diethyl terephthalate; DMSO, dimethyl sulfoxide; EBz, ethyl benzoate; EGDB, ethylene glycol dibenzoate; EstAC, aryl-carboxylesterase from *Sporosarcina* sp. nov. strain eSP04; M (in nucleic acid), A or C; Y (in nucleic acid), C or T; PAGE, polyacrylamide gel electrophoresis

having esterase activity, were examined for the hydrolysis of diethyl terephthalate (DET). They were cultured on DET minimal medium modified from Kurane *et al.*,[27] as follows: 2 g of DET, 1 g of $(NH_4)_2SO_4$, 1.6 g of K_2HPO_4, 0.2 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 20 mg of $CaCl_2 \cdot 2H_2O$, 0.1 g of Plysurf A210G (a detergent, Dai-ichi Kogyo Seiyaku, Kyoto, Japan), and 15 g of agar per liter (pH 7.5), supplemented with 20 mL of metal I and 10 mL of vitamin solutions. The metal I solution contained 0.5 g of $FeSO_4 \cdot 7H_2O$, 25 mg of $CuSO_4 \cdot 5H_2O$, 25 mg of $MnSO_4 \cdot 5H_2O$, 25 mg of $ZnSO_4 \cdot 7H_2O$, 25 mg of $Na_2MoO_4 \cdot 2H_2O$, and 25 mg of $Na_2WO_4 \cdot 2H_2O$ per liter; and the vitamin solution consisted of 0.2 g of *myo*-inositol, 0.2 g of biotin, 50 mg of vitamin B_{12}, 40 mg of calcium D-pantothenate, 40 mg of nicotinic acid, 40 mg of pyridoxine hydrochloride, 40 mg of thiamine hydrochloride, 40 mg of riboflavin, and 20 mg of *p*-aminobenzoic acid per liter (vitamins from Nacalai Tesque, Kyoto, Japan). One strain was found to form a large translucent halo around its colony through incubation for 2 d at 37 °C. This strain, eSP04, was selected as source of aryl-carboxylesterase after confirmation of its hydrolyzing ability toward EBz as well. Subculture of the strain was conducted for 5 d at 35 °C with 100 mL of fresh DET minimal medium, replacing the vitamin solution and the detergent with 0.3 g L^{-1} yeast extract (Nacalai Tesque) in a 500-mL Sakaguchi flask containing several stainless steel coils, as described previously,[28] and this was repeated sequentially for 5 months.

For enzyme preparation, cells of the strain inoculated on Luria-Bertani (LB) agar plates (0.5% NaCl instead of 1%: 10 g of peptone from Mikuni Chemical, Tokyo, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar per liter) were grown for 16 h at 37 °C with 30 mL of soluble starch media in 300-mL Erlenmeyer flasks with rotational shaking (150 rpm, 32 mm in diameter). Then 90 mL of pre-cultivated broth was inoculated into 2 L of the same fresh medium in a 5-L jar fermentor for 6 h at 37 °C, 300 rpm, and 2 L min^{-1} aeration. After the addition of 5 g of DET to the culture, incubation was continued for a further 13 h. The soluble starch media was composed of 10 g of soluble starch, 5 g of peptone, 5 g of yeast extract, 1 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 2 mL of metal II solution per liter (pH 8.2). The metal II solution was composed of 2.5 g of $CuSO_4 \cdot 5H_2O$, 2.5 g of $MnCl_2 \cdot 4H_2O$, 1.0 g of $FeSO_4 \cdot 7H_2O$, and 0.5 g of $ZnSO_4 \cdot 7H_2O$ per liter.

Characterization of the bacterium. The strain was examined physiologically, analyzed morphologically with a scanning electron microscope (XL20, Philips Electron Optics, Eindhoven, Netherlands), and 16S rRNA gene analysis was performed for its taxonomical identification. The culture was heated for 10 min at 95 °C and total genomic DNA was extracted. The 16S rDNA sequence of the bacterium was amplified by polymerase chain reaction (PCR) using universal bacterial primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′).[29] The 5′ end from position 75 to 682 (*E. coli* numbers) of the 16S rRNA gene was sequenced on both strands and compared with the bacterial gene sequences at DDBJ/GenBank. Sequence homology searches were performed with the BLAST program.[30] A phylogenetic tree was constructed by the neighbor-joining method[31] using MEGA (version 5) software.[32]

Purification of the enzyme. Purification of the enzyme from strain eSP04 cells was conducted by ammonium sulfate precipitation and then a four-step column chromatography procedure. All procedures were carried out at 4 °C throughout purification, and esterase activity was monitored with EBz as substrate. The washed cells were suspended in 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM EDTA, disrupted using a sonic oscillator (Model 201M, Kubota, Tokyo) at 9 kHz and 150 W for 10 min, and centrifuged at 12,000 × *g* for 30 min. The precipitate obtained through fractionation with $(NH_4)_2SO_4$ (33–60% saturation) was dissolved in 25 mM of the above Tris–HCl buffer, dialyzed against the same buffer, and applied to ion-exchange chromatography (BioLogic HR System, BioRad Laboratories, Munich, Germany) on a DEAE Sepharose Fast Flow column (2.6 × 20 cm, GE Healthcare, Uppsala, Sweden) equilibrated with 20 mM Tris–HCl buffer (pH 7.5). The active fractions eluted with a linear gradient of NaCl (150–250 mM) were collected, brought to 15% $(NH_4)_2SO_4$ saturation, and applied to a TSKgel Phenyl 650-S column (2.6 × 4.6 cm, Tosoh, Tokyo) that had been equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 20% saturated $(NH_4)_2SO_4$. The column was washed with the same buffer containing 10% saturated $(NH_4)_2SO_4$, and then the active fractions were eluted with a linear gradient of $(NH_4)_2SO_4$ (10–2% saturation), dialyzed against 25 mM Tris–HCl buffer (pH 8.0), and concentrated by ultrafiltration (Centricon Plus-20, Millipore, Bedford, MA). The concentrate was put on a DEAE-5PW column (7.5 × 75 mm) equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 120 mM NaCl, and then eluted with a linear gradient of NaCl (120–200 mM). The active fractions were concentrated by ultrafiltration, and were designated partially purified enzyme. Then the concentrated active fractions were subjected to a Superdex 75 pg column (2.6 × 60 cm, GE Healthcare) equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl, desalted, concentrated by ultrafiltration, and pooled as purified enzyme.

The purity of the enzyme was analyzed by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli[33] and stained with Coomassie Brilliant Blue R-250. Gel filtration for molecular weight determination was performed on the BioLogic HR System using a Superdex 75 pg column in 20 mM Tris–HCl buffer containing 100 mM NaCl.

Enzyme and protein assays. Esterase activities were determined by measuring the initial rates of hydrolysis of the substrates. Initial rate measurements were carried out using a pH-stat autotitration system (718 STAT Titrino Autotitrator, Metrohm, Herisau, Switzerland; the capillary nozzle replaced with that of a Radiometer Analytical System, Radiometer, Lyon, France), as described previously,[34] with some modifications. The substrate solution consisted of a suitable amount of each substrate, dimethyl sulfoxide (DMSO) in which the substrate was first dissolved at 30 °C or a higher temperature, and then mixed into 25 mM Tris–HCl buffer (pH 8.0). After 10 min of incubation of the solution at 35 °C, the reaction was started by the addition of an enzyme solution (0.5 mL) containing 0.1% bovine serum albumin (BSA) and 10 mM EDTA at a stirring rate of about 400 rpm. In the case of insoluble substrates, agitation was conducted at more than 700 rpm to achieve a sufficiently emulsified reaction mixture.[35] The total volume of the reaction solution, and the concentrations of the substrate and DMSO at the starting point of the reaction, were adjusted to 15.0 mL, 2 or 4 mM, and 5% respectively, as per the solubility of the substrate, irrespective of the presence or absence of inhibitor or detergent. The carboxylic acid liberated from the substrate was titrated with 20–50 mM NaOH back to the initial pH of 8.0 unless otherwise stated. Spontaneous hydrolysis was subtracted at every run, although it was not substantial.

One unit (U) of esterase activity was defined as the amount of enzyme that liberated 1 μmol equivalent of carboxylic acid in 1 min at pH 8.0 and 35 °C. Specific activity was defined as units per mg of protein. The protein concentrations were determined by the method of Lowry, with BSA as the standard. pH and temperature dependences were measured in ranges of pH 4.0–10.0 at 35 °C and 25–55 °C at pH 8.0 respectively, with EBz as substrate. All the experiments were performed at least twice, and most were replicated 3 times or more. All the data are represented as means ± standard deviation.

N-Terminal and internal amino acid sequencing. The purified enzyme was blotted onto a polyvinylidene difluoride membrane (BioRad Laboratories), and its N-terminal amino acid sequence was determined by the Edman degradation method using a protein sequencer (Model 492 Precise Sequencing System, Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The purified enzyme was digested with *Staphylococcus aureus* V8 protease (Roche, Mannheim, Germany), and the resulting peptides were separated by SDS–PAGE. The resulting major peptide, molecular weight 33,000 on SDS–PAGE, was subjected to amino acid sequencing as described above. The sequences determined were compared with protein sequence databases using the BLAST program.[30]

Accession numbers. Strain eSP04 has been deposited in the collection of the NITE Biological Resource Center (Chiba, Japan) as strain NBRC 108908. The 16S rDNA sequence of the isolate eSP04 has been deposited in DDBJ/GenBank under accession no. AB700599.

Table 1. Taxonomical Properties of Carboxylesterase-Producing Bacterium Strain eSP04

Morphological characteristics:		Cultural characteristics:	
Gram staining	+	Growth in NaCl	0–7%
Cell morphology		Growth temperature	5–45 °C
Rod-shaped, *ca.* 0.6 × 1.9 μm			
Motility	+	16S rRNA gene sequence similarity	(%)
Spore		*Bacillales* bacterium PDD-12b-7 (DQ512782)	97.6
Round, terminal, sporangium swollen		*Sporosarcina luteola*^{T} (AB473560)	97.6
		Sporosarcina sp. IDA0953 (AJ544773)	97.4
Biochemical characteristics:		*Sporosarcina* sp. B3 (GU397443)	97.4
Catalase	+	*Sporosarcina saromensis*^{T} (AB243859)	97.4
Anaerobic growth	−	*Bacillus* sp. CPB 5 (AF548878)	97.4
Voges-Proskauer test	−	*Sporosarcina* sp. 5-4 (FJ795663)	97.3
NO_3^- to NO_2^-	+	*Sporosarcina* sp. MHS026 (DQ993301)	97.2
Tyrosine degradation	+	*Sporosarcina* sp. IDA3546 (AJ544776)	97.2
Egg yolk agar opacity	−	*Sporosarcina thermotolerans*^{T} (FN298445)	97.2
Acid from D-glucose	−		
Hydrolysis of casein	−		
gelatin	+		
starch	−		

+, positive; −, negative; *T*, type strain

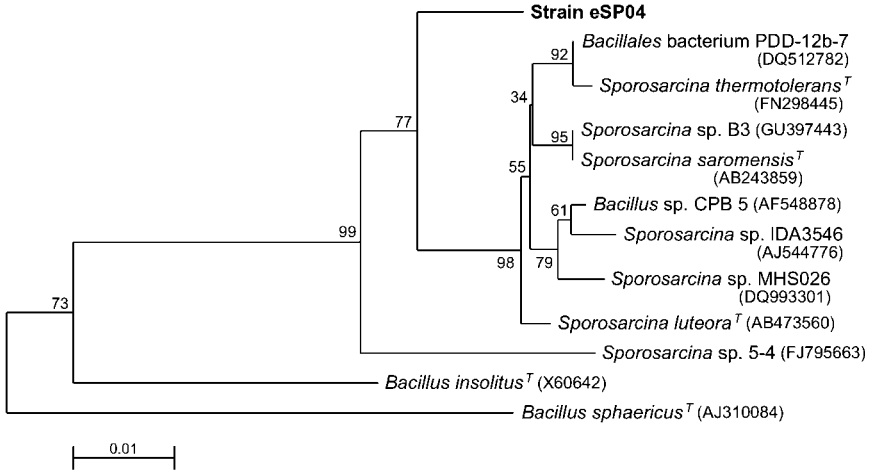

Fig. 1. Phylogenetic Analysis of Bacterial Strain eSP04 Based on 16S rRNA Gene Sequences.
This phylogenetic tree was generated by the neighbor-joining method (MEGA ver. 5). Numbers at the nodes indicate bootstrap percentages for 1,000 replicates. DDBJ accession numbers are given in parentheses. Bar, one substitution per 100 nucleotide positions. *T*, type strain.

Results and Discussion

Identification of the bacterium

Bacterial strain eSP04 that produced a DET- and EBz-hydrolyzing enzyme (an aryl-carboxylesterase, EstAC) was isolated from an enrichment culture with DET. The culture supernatant of the strain did not hydrolyze either DET or EBz, indicating that EstAC is an intracellular enzyme. The sequential subculture with DET minimal medium appeared to contribute to stabilizing the enzyme production. DET was consumed during the 5-d culture.

Morphological and physiological analyses of strain eSP04 are summarized in the left column of Table 1, which shows atypical results for several likely candidate species. The bacterium was further characterized taxonomically and phylogenetically by the amplified 16S rDNA sequencing method.[36] The top 10 organisms with high percentage sequence similarity are shown in the right-hand column of the table. The neighbor-joining tree is shown in Fig. 1. The results indicate that the best overall match was with the genus *Sporosarcina*. This genus consists of a recently emended large cluster[37] including former *Bacillus* species that belong to rRNA group 2 and some non-*Bacillus*-type organisms.[38] However, identification to the species level was not possible owing to certain features in the phylogenetic tree, as well as insufficiently high percentage similarity of less than 98%. The *Sporosarcina* strains might be classified into three or four phylogenetic groups, and strain eSP04 appeared to belong to a different group than most of the displayed species or *Sporosarcina* sp. 5-4. It is concluded that strain eSP04 producing EstAC is a strain of *Sporosarcina* species. We designated it *Sporosarcina* sp. nov. strain eSP04.

Purification and molecular weight of the enzyme

EstAC was purified 780-fold from crude cell extracts of strain eSP04 to a specific activity of 150 U/mg of protein at a yield of 7.1% by a 5-step procedure of salting-out and column chromatography (Table 2). The homogeneity of the purified enzyme was examined by SDS–PAGE under reducing conditions (Fig. 2A). The purified enzyme gave a single protein band correspond-

Table 2. Purification of an Aryl-carboxylesterase (EstAC) from *Sporosarcina* sp. nov. Strain eSP04

Purification step[a]	Total protein (mg)	Activity (U)[b]	Yield (%)	Specific activity (U/mg)	Purification (fold)
Crude extract	1,310	260	100	0.20	1
Ammonium sulfate precipitation	1,070	240	92	0.22	1.1
DEAE Sepharose FF	139	196	75	1.4	7.1
Phenyl-650S	7.30	75.0	29	10	52
DEAE-5PW	0.46	34.0	13	74	370
Superdex 75 pg	0.12	18.5	7.1	150	780

[a]Steps carried out with 2,000 mL of culture broth.
[b]Enzyme activity was measured for ethyl benzoate (EBz) at pH 8.0 and 35 °C, as described in "Materials and Methods."

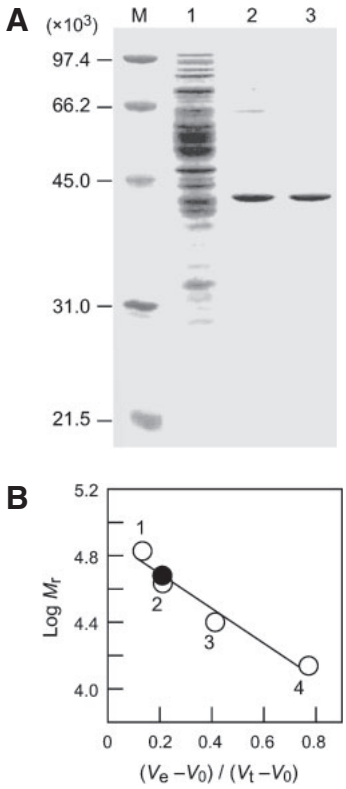

Fig. 2. Gel Electrophoresis Analysis of the Purified Fraction of EstAC from *Sporosarcina* sp. nov. eSP04 and Estimation of the Molecular Weight of the Esterase.

(A) SDS–PAGE analysis of EstAC. Lane 1, crude cell extract; lane 2, DEAE-5PW fraction; lane 3, Superdex 75 pg fraction; M, molecular weight markers (BioRad Laboratories). (B) Gel filtration (Superdex 75 pg) chromatogram the purified EstAC (solid circle) under non-denaturing conditions. Hollow circles, labeled 1 to 4, represent the standard proteins (bovine serum albumin, 67,000; ovalbumin, 43,000; chymotrypsinogen A, 25,000; ribonuclease, 13,700). V_e, elution volume for the protein; V_0, column void volume; V_t, column total bed volume.

ing to a molecular weight of approximately 43,000. Gel filtration indicated a molecular weight of 48,000 ± 6,000 under non-denaturing conditions (Fig. 2B), suggesting EstAC should be present as a monomer.

Since the amount of purified enzyme obtained was 0.12 mg with 7.1% recovery, the content of the enzyme in the cells of strain eSP04 was assessed to be at most 0.13% of total soluble proteins. This low level of the enzyme in the cells would make it difficult to study the essential enzymatic properties of EstAC. The cloning of the esterase gene from *Sporosarcina* sp. nov. and its overexpression is one attractive way to overcome this problem. As the first step of such an approach, we determined an internal sequence as well as the N-terminal amino acid sequence from the purified enzyme by Edman degradation, as described below.

Characterization of the enzyme

Partially purified enzyme instead of purified enzyme was used for activity measurements for the most part (see "Materials and Methods"). Partially purified EstAC from the DEAE-5PW column always showed just half the activity of the purified enzyme. For instance, the former enzyme preparation gave a specific activity of 73 ± 5 U/mg for EBz at pH 8.0 and 35 °C, whereas the latter gave 150 ± 8 U/mg under the same experimental conditions. The use of the partially purified enzyme was not thought to have any adverse effects in this study, although a faint band at around 60,000 was associated with this enzyme preparation (Fig. 2A), the origin of which was not clear.

The benzoyl esterase activity of EstAC displayed an optimum pH at around 9 (Fig. 3A), an unexpectedly alkaline pH for carboxylesterase. Protein electrostatics investigation suggested that the active site of esterases and lipases displayed a negative potential in the pH range associated with their maximum activity,[39] and that the esterases showed their optimum charge at pH 6 to 7, which correlated with their usually lower pH-activity optimum than that of the lipases around pH 8.[1,6] These investigations were carried out using esterases and lipases of which the 3D-structures and amino acid sequences had been experimentally determined. All the enzymes used in these studies had the catalytic triad of S/H/D (or E) and a conserved sequence motif of GXSXG around the active site serine residue. Hence, the observed maximum activity at around pH 9 suggests that EstAC had a catalytic site different from S/H/D. After the enzyme was maintained without substrate at pH 4.0–10.0 and 4 °C for 50 h, more than 65% activity was retained, and more than 90% was retained between pH 8.0 and 10.0 (Fig. 3C). Thus EstAC can be said to have been fairly stable over a wide range of pH. The temperature optimum was between 40 °C and 45 °C, with a rapid decrease in activity at higher temperatures (Fig. 3B). EstAC appeared to be somewhat heat-labile: it lost 5% and 45% of its activity at pH 8.0 after 60 min incubation without substrate at 40 °C and 45 °C respectively (Fig. 3D).

The effects of inhibitors on activity are summarized in Table 3. The activity of EstAC was completely inhibited

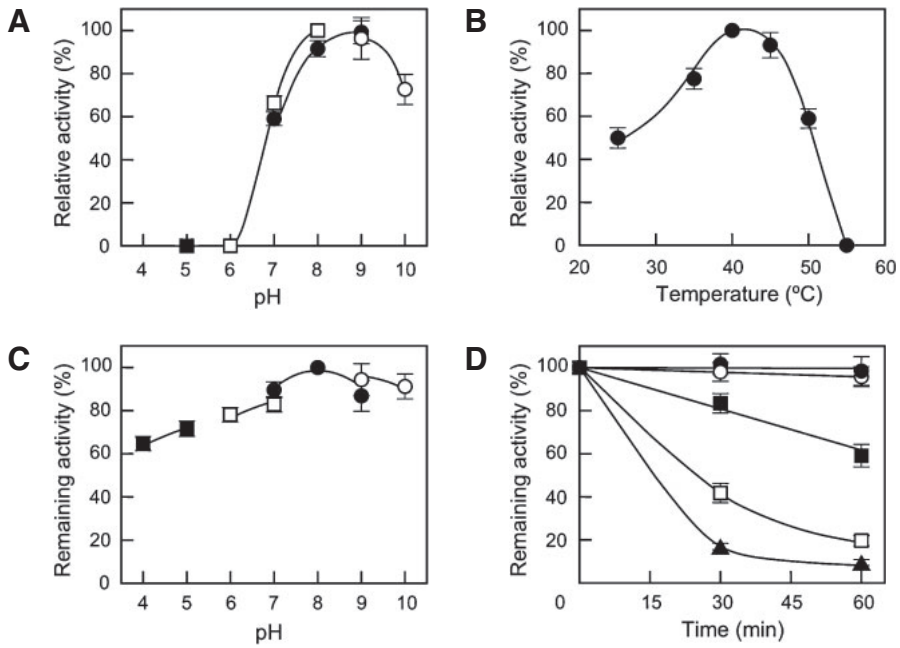

Fig. 3. Optimum pH (A) and Temperature (B) for EstAC, and Effects of pH (C) and Temperature (D) on the Enzyme Stability.

Enzyme activity was measured for EBz at pH 8.0 and 35 °C as described in "Materials and Methods," except for the following: the buffers used in (A) and (C) were 25 mM citric acid-sodium citrate buffer (■, pH 4.0–5.0), sodium phosphate buffer (□, pH 6.0–8.0), Tris–HCl buffer (●, pH 7.0–9.0), and glycine-NaOH buffer (○, pH 9.0–10.0). (B) pH stability was determined after maintaining the enzyme solution for 50 h at 4 °C at a given pH. (D) To measure thermal stability, the enzyme solution was incubated at 35 °C (●), 40 °C (○), 45 °C (■), 50 °C (□), and 55 °C (▲) at pH 8.0, and then the remaining activity was assayed.

Table 3. Effects of Enzyme Inhibitor on the Activity of EstAC

Inhibitor	Concentration (mM)	Relative activity (%)
None		100
Phenylmethanesulfonyl fluoride	0.1	0
Acetic anhydride	1.0	85 ± 5
p-Chloromercuribenzoate	1.0	84 ± 7
2-Mercaptoethanol	1.0	82 ± 5
EDTA	10	101 ± 6
o-Phenanthroline	1.0	81 ± 9
N-Bromosuccinimide	1.0	60 ± 7

Enzyme activity was measured for EBz using a Metrohm pH-stat at pH 8.0 and 35 °C (see "Materials and Methods").
The total volume, 15 mL, of the reaction mixture initially consisted of 4 mM EBz, 5.5 μg of enzyme, 5% DMSO, and 25 mM Tris–HCl buffer solution besides the inhibitor.

in the presence of 0.1 mM phenylmethylsulfonyl fluoride, suggesting that serine residues were involved in the enzyme activity. Compared with the enzyme concentration, the presence of extremely high concentrations of acetic anhydride, *p*-chloromercuribenzoate, 2-mercaptoethanol, EDTA, and *o*-phenanthroline did not significantly inhibit the activity, suggesting that amino groups, sulfhydryl groups, disulfide bonds, and metal ions were not significantly involved in it. *N*-Bromosuccinimide inhibited the activity more significantly than these five chemicals. Since this particular compound cleaves tryptophanyl and tyrosyl peptide bonds and oxidizes sulfhydryl groups,[40,41] this result suggests the involvement of tryptophan or tyrosine residues in the enzyme activity. The presence of detergents, which are denaturing agents, also inhibited the activity to a considerable extent: for example, 0.1% Triton X-100 reduced the activity to 49 ± 5%.

The determined N-terminal and internal amino acid sequences of EstAC were (M?)GGNVEMKTKLQETLDQLAN (or R) and IADIMVLDGFDENGNDKLRK respectively. The first methionine of the N-terminal might have been digested by a methionine aminopeptidase of *Sporosarcina* sp. nov. The comparison of these sequences with those of proteins in the databases revealed that the internal sequence had 58% (11/19) and 53% (10/19) homology with two bacterial esterases, and 58% (11/19) with one putative esterase. The former two esterases are 4-chloro-3-hydroxybutyrate hydrolase from *Rhizobium* DS-S-51, showing hydrolytic activity toward (*R*)-4-chloro-3-hydroxybutyrate,[42] and 1,4-butanediol diacrylate esterase from *Brevibacterium linens*, giving 4-hydroxybutyl acrylate.[43] The latter was deduced from the genome of a bacterium, *Rhodopseudomonas palustris*.[44] Aryl-carboxylesterase activity remains undetermined for all these enzymes. The N-terminal sequence did not show significant similarity to any known hydrolases.

Bacterial esterases are classified into eight families according to conserved sequence motifs and biological properties.[45] The two esterases described above are classified in family VIII. Esterases in family VIII have the catalytic serine residue in the conserved sequence motif SXXK, which is different from the case of GXSXG around the nucleophilic serine, and usually show an alkaline pH-activity optimum. In addition, they have also been reported to have a tyrosine residue that can be regarded as acting as the general base[46] in a consensus motif of YXXN. The putative esterase also had the same motifs, SXXK and YXXN. Taking this homology in internal amino acid sequence, the alkaline pH-activity optimum, and the effects of the inhibitors into consideration, EstAC might be a member of family VIII esterases.

Table 4. Substrate Specificity of an Aryl-carboxylesterase EstAC

Substrate	Concentration (mM)	Specific activity		Relative activity[a] (%)
		purified enzyme (U/mg)	partially purified enzyme (U/mg)	
n-Butyl benzoate	4		63 ± 7	86 ± 10
Ethyl benzoate	4	150 ± 8	73 ± 5	100
Methyl benzoate	4		83 ± 16	113 ± 20
Ethyl phenylacetate	4	43 ± 10	18 ± 5	25 ± 6
Methyl phenylacetate	4		24 ± 3	33 ± 4
(±)-Ethyl mandelate	4	45 ± 9	16 ± 4	22 ± 5
(±)-Ethyl 2-(4-isobutylphenyl)propionate	4*		1.0 ± 0.4	1.4 ± 0.6
(±)-Ethyl 2-methyl-3-phenylpropionate	4		22 ± 5	30 ± 7
(±)-Ethyl 3-phenylbutanoate	4		4.0 ± 1.6	5.4 ± 2
Diethyl terephthalate	2	160 ± 13	74 ± 3	101 ± 4
Dimethyl terephthalate	2		51 ± 2	69 ± 3
Monomethyl terephthalate	2		~0	0
Dimethyl isophthalate	2	35 ± 7	17 ± 2	24 ± 2
Diethyl phthalate	2	~0	~0	0
Dimethyl phthalate	2	~0	~0	0
Ethylene glycol dibenzoate	2*		22 ± 4	30 ± 5
Tributyrin	4*	13 ± 5	6.0 ± 3	8.1 ± 4
1-Phenoxy-2-propyl acetate	4		1.8 ± 0.7	2.5 ± 0.9
p-Nitrophenyl acetate	4		45 ± 13	62 ± 18

Specific activity was measured at pH 8.0 and 35 °C using a Metrohm pH-stat system. The total volume, 15 mL, of the reaction mixture initially consisted of 6.7–22.0 μg of partially purified enzyme or 2.7–8.0 μg of purified enzyme, 5% DMSO, and 25 mM Tris–HCl buffer solution besides the substrate.

[a]Relative activity is expressed as a percentage of the specific activity obtained with ethyl benzoate.

*The substrate was insoluble under the experimental conditions. The reaction mixture was emulsified by vigorous stirring (see "Materials and Methods").

Substrate specificity

The specific activities for a wide variety of aryl-carboxylic acid esters are summarized in Table 4. Several of the substrates were also examined with the purified enzyme to compare results. This confirmed the appropriateness of using the partially purified EstAC. The pH and temperature for the specific activity measurements were determined by considering spontaneous hydrolysis, the solubility of the substrates, and the stability of the enzyme. EstAC displayed the highest activity not only for EBz and methyl benzoate, but also for DET, of all the substrates examined. Our examination revealed that among previously reported aryl-carboxylesterases, an esterase from *Aspergillus nomius* was the most highly active, especially for EBz, *n*-butyl benzoate, and ethylene glycol dibenzoate (EGDB). The purified EstAC activity for EBz (Table 4) was almost the same as the *A. nomius* esterase activity for EBz and for *n*-butyl benzoate under similar experimental conditions. EstAC was also active on not only ethyl and methyl phenylacetates but also on the ethyl ester of α-substituted phenylacetic acid. Furthermore, the enzyme had some activities for ethyl esters of α- or β-substituted phenylpropionic acids as well, whereas the *A. nomius* esterase showed no activity for either ethyl phenylacetate or DET.[9)]

EstAC also showed high activities for dialkyl esters of dibasic aryl-dicarboxylic acids and dihydric alcohol esters of aryl-carboxylic acids in addition to ordinary esters of aryl-carboxylic acid, as described above. It catalyzed the hydrolysis of DET, dimethyl terephthalate, dimethyl isophthalate, and EGDB, although it did not hydrolyze phthalates. DET and EGDB appear to have chemical structures that are the inverse of each other. The specific activity for EGDB was 30% of DET and of EBz. Nevertheless, the activity value for EGDB (Table 4) was substantially high and even greater than that of a remarkable benzoyl esterase from *Rhodotorula mucilaginosa*, previously reported.[10)] EstAC showed no activity on monomethyl terephthalate. This suggests that *Sporosarcina* sp. nov. strain eSP04 has another enzyme to hydrolyze monoalkyl terephthalate, since it grew on DET as sole carbon source and consumed it during the production of the enzyme.

EstAC was highly active on *p*-nitrophenyl acetate, as previously reported for various esterases, including aryl-carboxylesterases. However, it showed a relatively low activity for an ester having a phenoxy group in an alcohol moiety, such as 1-phenoxy-2-propyl acetate, which was easily hydrolyzed with various lipases.[47,48)] This again confirmed that EstAC is specific for the aromatic acid moiety. A preliminary experiment on the enantioselectivity of the enzyme indicated that EstAC preferentially hydrolyzed the (*R*)- and (*S*)-enantiomers of ethyl 2-(4-isobutylphenyl)propionate and ethyl 3-phenylbutanoate respectively. The enantiomer excesses of the two products were 40% and 30% at 20% yield, respectively. This indicates that the enzyme prefers the same structure with regard to the bulkiness of substituents.

Aryl-carboxylesterase EstAC from *Sporosarcina* sp. nov. was found to be an attractive biocatalyst having substantial activities for a broad variety of aryl-carboxylic acid ester substrates. If it proves possible to obtain a sufficient amount of purified EstAC without great difficulty, the present study should lead to further investigation of the enzyme, which has scientifically interesting features and various potential applications, as briefly reviewed towards the start of this report. Studies of the molecular structure, reaction mechanism, and stereoselectivity of the enzyme are in progress using recombinant EstAC overexpressed in *E. coli* cells, and will be reported soon. Here, however, we mention an observation concerning the substrate specificity of the

enzyme. Purified recombinant EstAC hydrolyzed ethyl 4-hydroxybenzoate and ethyl cinnamate with 1.2% and 65% activities of EBz respectively (unpublished results, our laboratory). This indicates that the enzyme has 4-hydroxybenzoyl and cinnamoyl esterase activities as well. The former activity might be regarded as being too low for practical use, but it is much higher than that of a recently reported new 4-hydroxybenzoyl esterase from *Aspergillus oryzae*, although the experimental conditions were slightly different.[18)]

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