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Purification and properties of a *p*-nitrobenzyl esterase from *Bacillus subtilis*

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A procedure for purifying to homogeneity a microbially produced biocatalyst useful for deblocking intermediates in the manufacture of beta-lactam antibiotics is reported. In aqueous solution the purified *p*-nitrobenzyl (PNB) carboxy-esterase was soluble, monomeric (molecular weight: 54000 by SDS-PAGE or by gel filtration) and exhibited an acidic pI, 4.1. The PNB carboxy-esterase catalyzed rapid ester hydrolysis for simple organic esters such as PNB-acetate, benzyl acetate and α-naphthyl acetate and catalyzed deblocking (ester hydrolysis) of beta-lactam antibiotic PNB esters such as cephalexin-PNB and loracarbef-PNB. The *N*-terminal amino acid sequence and the amino acid composition are reported. A serine residue is involved in ester hydrolysis: the PNB carboxy esterase was inhibited by phenylmethylsulfonyl fluoride and diethyl *p*-nitrophenyl phosphate; one mole of diisopropyl fluorophosphate titration was required per mole of PNB carboxy-esterase for complete inhibition. When the [^{3}H]-diisopropyl fluorophosphate-treated biocatalyst was digested with Lys C and the resulting peptides separated by HPLC, a single [^{3}H]-labeled peptide was obtained; its amino acid sequence is reported. Inhibition of the PNB carboxy esterase by diethyl pyrocarbonate suggests that a histidinyl residue (or residues) is (are) also involved in the catalytic site of the esterase.

Keywords: *p*-nitrobenzyl carboxy esterase; biocatalyst; beta-lactam antibiotic *p*-nitrobenzyl ester; *Bacillus subtilis*; deesterification; carboxyl protection

Introduction

Chemical conditions used in the preparation of the α-aminobenzyl β-lactam antibiotics cephalexin (Cp), cefaclor (Cf), and loracarbef (Lc) (Figure 1) require that carboxyl moieties of early intermediates be esterified to prevent their degradation. The *p*-nitrobenzyl (PNB) ester is more stable than trimethylsilyl esters in protecting these intermediates [5] and efficient methods have been developed for ring-expansion of the PNB carboxy esters of penicillin V and penicillin G into the corresponding 3-methyl-cephalosporins [10] and 3-methyl-carbacephalosporins. After formation of the bicyclic cephem or carbacephem ring system (bicyclic rings in Figure 1) the PNB group must be removed. Removal in organic solvent, [7, 8, 17] can involve the use of environmental toxicants such as zinc and acid that are expensive to dispose properly. Biocatalytic hydrolysis by a PNB carboxy-esterase (PNBCE) in aqueous buffer may improve yield, avoid metal catalysts and reduce use of solvents and acid. Previously microbial extracts and known esterases and lipases were screened for PNBCE activity with the PNB carboxy-ester of cephalexin (Cp-PNB) [4] and with the PNB carboxy-ester of loracarbef (Lc-PNB) (unpublished study, Eli Lilly and Company). For both substrates *Bacillus subtilis* NRRL B8079 was found to be a superior source of PNBCE to hydrolyze these carboxy-esters to their corresponding free acids and PNB alcohol. Here we report purification to homogeneity of the PNBCE from *B. subtilis* NRRL B8079 together with physical and kinetic characterization of this biocatalyst.

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Abbreviations used: β-ME, β-mercaptoethanol; Cf, cefaclor; Cf nucleus-PNB, (6*R*, 7*R*)-7-amino-3-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0]-oct-2-ene-2-carboxylic acid, (4-nitrophenyl)methyl ester; Cp, cephalexin; Cp-PNB, *p*-nitrobenzyl carboxy-ester of cephalexin; DEPC, diethyl pyrocarbonate; DFP, diisopropyl fluorophosphate; DMSO, dimethyl sulfoxide; DNP, diethyl *p*-nitrophenyl phosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(aminoethyl ether) *N,N,N'N'*-tetracetic acid; Lc, loracarbef; Lc-PNB, *p*-nitrobenzyl carboxy-ester of loracarbef; Lc nucleus-PNB, (6*R*, 7*S*)-7-amino-3-chloro-8-oxo-1-azabicyclo[4.2.0]-oct-2-ene-2-carboxylic acid, (4-nitrophenyl)methyl ester; Lys C, an endoproteinase specifically cleaving at C terminal lysine residues; MW_r, relative molecular weight; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonylfluoride; PNB, *p*-nitrobenzyl; PNBCE, *p*-nitrobenzyl carboxy-esterase; SDS, sodium dodecyl sulfate

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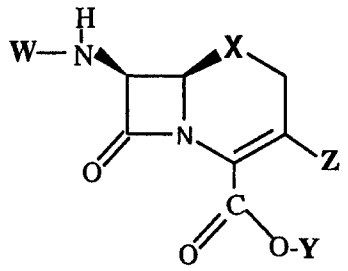
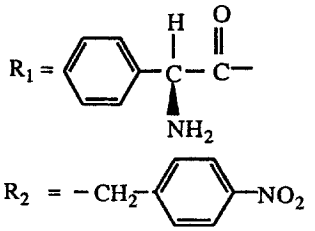
Materials and methods

Materials

Lc-PNB, Lc nucleus-PNB, Cp-PNB and Cf nucleus-PNB were gifts from Dr Bill Jackson of Eli Lilly and Co, Indianapolis, IN, USA. *p*-Aminobenzamidine agarose and *p*-nitrophenyl acetate were obtained from Sigma (St Louis, MO, USA). Dithiothreitol, the MW_r standard proteins for SDS-PAGE and DEAE Affi-Gel Blue were purchased from Bio-Rad Lab (Hercules, CA, USA). DEAE-cellulose (DE 52) was obtained from Whatman (Hillsboro, OR, USA). Sephacryl S-200, Q-Sepharose, and the MW_r standard proteins for gel filtration were purchased from Pharmacia (Uppsala, Sweden). Calcium phosphate was prepared by the method of Jenner [12]. HPLC-grade methanol and acetonitrile were obtained from J.T. Baker Chemical Co (Phillipsburg, NJ, USA). Other chemicals were obtained commercially at the highest available grade.



Compound	W	X	Y	Z
Lc*	R_1	CH_2	H	Cl
Lc-PNB	R_1	CH_2	R_2	Cl
Lc nucleus*	H	CH_2	H	Cl
Lc nucleus-PNB	H	CH_2	R_2	Cl
Cf*	R_1	S	H	Cl
Cp nucleus*	H	S	H	Cl
Cp nucleus-PNB	H	S	R_2	Cl
Cp*	R_1	S	H	CH_3
Cp-PNB	R_1	S	R_2	CH_3



*free acid

Figure 1 Structures of β-lactam antibiotics and PNB esters

Culture conditions

Bacillus subtilis NRRL B8079 was grown in 500-ml Erlenmeyer flasks under conditions described by Brannon *et al* [4]. After cultivation for 48 h, cells were harvested by centrifugation, washed with 15 mM Tris-HCl, pH 7.5, containing 1.0 M KCl and 20% (w/w) ethanol and then with 15 mM Tris-HCl buffer, pH 7.5. Washed cells were stored at −80° C until used. Protein was determined by the method of Lowry *et al* [18] with bovine serum albumin as a standard.

Molecular weight determination

The MW_r of the PNBCE was estimated by gel filtration using a FPLC Superose 12 column (1 × 30 cm) equilibrated with 10 mM Tris-HCl, pH 8.5, containing 0.15 M NaCl. Blue dextran 2000 was used for estimation of void volume. Bovine serum albumin (66200), ovalbumin (45000), chymotrypsinogen (25000) and ribonuclease A (13700) were used as MW_r standards. The MW_r of denatured PNBCE was determined by SDS (12.5%)-PAGE according to the method of Weber and Osborn [24]. Protein standards were phosphorylase B (97400), bovine serum albumin, ovalbumin, carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and lysozyme (14400). Sample and standards were treated with 2% SDS and 5% β-ME at 100° C for 5 min before they were subjected to electrophoresis.

For activity staining of purified PNBCE under non-denaturing conditions, 1 μg of the purified esterase was used; for protein staining, 0.1 μg of the purified esterase was used. Electrophoresis was carried out at 4° C with a constant current, 20 mA, for 3 h. After electrophoresis the gel was soaked in 100 ml of 100 mM Tris-HCl buffer, pH 8.0, at room temperature for 5 min and then 16 mg of α-naphthyl acetate dissolved in 0.5 ml acetonitrile was added. The esterase activity band was developed by addition of 50 mg of Fast Blue RR salt. Background staining was removed by washing the gel with methanol-acetic acid-water (40 : 10 : 50). Gel for protein staining was stained with Coomassie blue.

Amino acid composition and sequence analysis

Amino acids were analyzed by the methods of Heinrikson and Meredith [11] and Bidlingmeyer *et al* [3] with a HPLC reversed-phase column after derivatization with phenylisothiocyanate (PITC) to phenylthiocarbamoyl amino acids (PTC-amino acids). The purified esterase was dialyzed extensively against water, lyophilized, and hydrolyzed with 6 N HCl at 110° C for 24 h. The hydrolysate was reacted with PITC and PTC-amino acids were separated by HPLC using a gradient formed from solvent A and solvent B. Solvent A is 50 mM ammonium acetate, pH 6.0, and solvent B is a mixture of acetonitrile-methanol-0.22 M ammonium acetate, pH 6.0, (44 : 10 : 46). PTC-amino acids were quantified with external PTC-amino acid standards.

For amino acid sequence analysis, the purified PNBCE was extensively dialyzed against water, lyophilized, dissolved in 5% acetonitrile containing 0.1% TFA, and then absorbed into the polybrene-coated glass microfiber filter. The analysis was performed by automated Edman degradation using model 470 gas phase protein sequencer with released amino acid phenylthiohydantoin derivatives (PTH-amino acids) detected on-line by model 120A PTH-amino acid analyzer (Applied Biosystems, Foster City, CA, USA) [1]. The analyses were done at the Molecular Biology Resource Facility, St Francis Hospital of Tulsa Medical Research Institute, University of Oklahoma Health Science Center, under the supervision of Dr Ken Jackson.

Assay procedures

Two assay methods were used:

(1) HPLC assay with the antibiotics substrates Unless otherwise noted, 1 ml of a reaction mixture containing 5 μmol bis-tris-propane-HCl, pH 6.5, 0.5 μmol substrate (25 μl of 20 mM stock solution in DMSO) and appropriate amounts of enzyme solution, was incubated with shaking at 37° C for 30 min. The reaction was stopped by adding an equal volume of acetonitrile. The mixture was centrifuged and filtered through a 0.45-μm pore size filter to remove protein and the supernatant solution was analyzed

by HPLC for product formation and substrate disappearance. The HPLC was performed in a C-18 reverse-phase column (Nava-Pak C18 Radial-Pak Cartridge, 0.8 × 10 cm, Waters, Milford, MA, USA) with a linear gradient formed by solvent A and solvent B at a flow rate of 1 ml min^{-1}. Solvent A is a mixture of 80% of 1 mM triethylamine-HCl, pH 2.5 and 20% of methanol; solvent B is methanol. The products and substrate were detected at 254 nm. Figure 2 shows a typical HPLC chromatogram of substrate and products.

(2) Spectrophotometric assay with p*-nitrophenylacetate as substrate* PNBCE catalyzes hydrolysis of *p*-nitrophenylacetate to acetate and *p*-nitrophenol. For purified esterase, quantitation of this hydrolysis by the HPLC method and a spectrophotometric assay give equivalent results. The latter assay is useful in stages of the purification after other esterases have been removed and strict substrate specificity is not required to identify PNBCE. The assay is carried out at room temperature in a 1-ml mixture containing 100 μmol Tris-HCl, pH 7.0, 1.6 μmol *p*-nitrophenylacetate and 1–20 μl enzyme solution. Esterase activity is followed by the absorption change at 405 nm in either a Cary spectrophotometer, model 219 (Varian Optical Instruments, Mulgrave, Victoria, Australia), or a Beckman DU-50 (Fullerton, CA, USA) at room temperature.

Inhibition of PNBCE activity by selected compounds Purified PNBCE (3.6 μg in 0.975 ml of 5 mM bis-tris-propane-HCl buffer, pH 6.5) was incubated at 37° C for 5 min in the presence of 1.0 mM concentrations of compounds listed in Table 1; 25 μl of 20 mM Lc-PNB oxalate salt in DMSO was then added and PNBCE activity measured by HPLC.

Purification of PNBCE from Bacillus subtilis All purification steps were carried out at 0–4° C unless otherwise specified. Purification data are summarized in Table 2. Frozen *B. subtilis* cells (760 g) were thawed and homogenized in 2 L of buffer A (10 mM potassium phosphate, pH 7.0 containing 1 mM β-ME and 0.5 mM EDTA). The cell-free extract was obtained by centrifugation of the homogenate at 24000 × *g* for 30 min without further

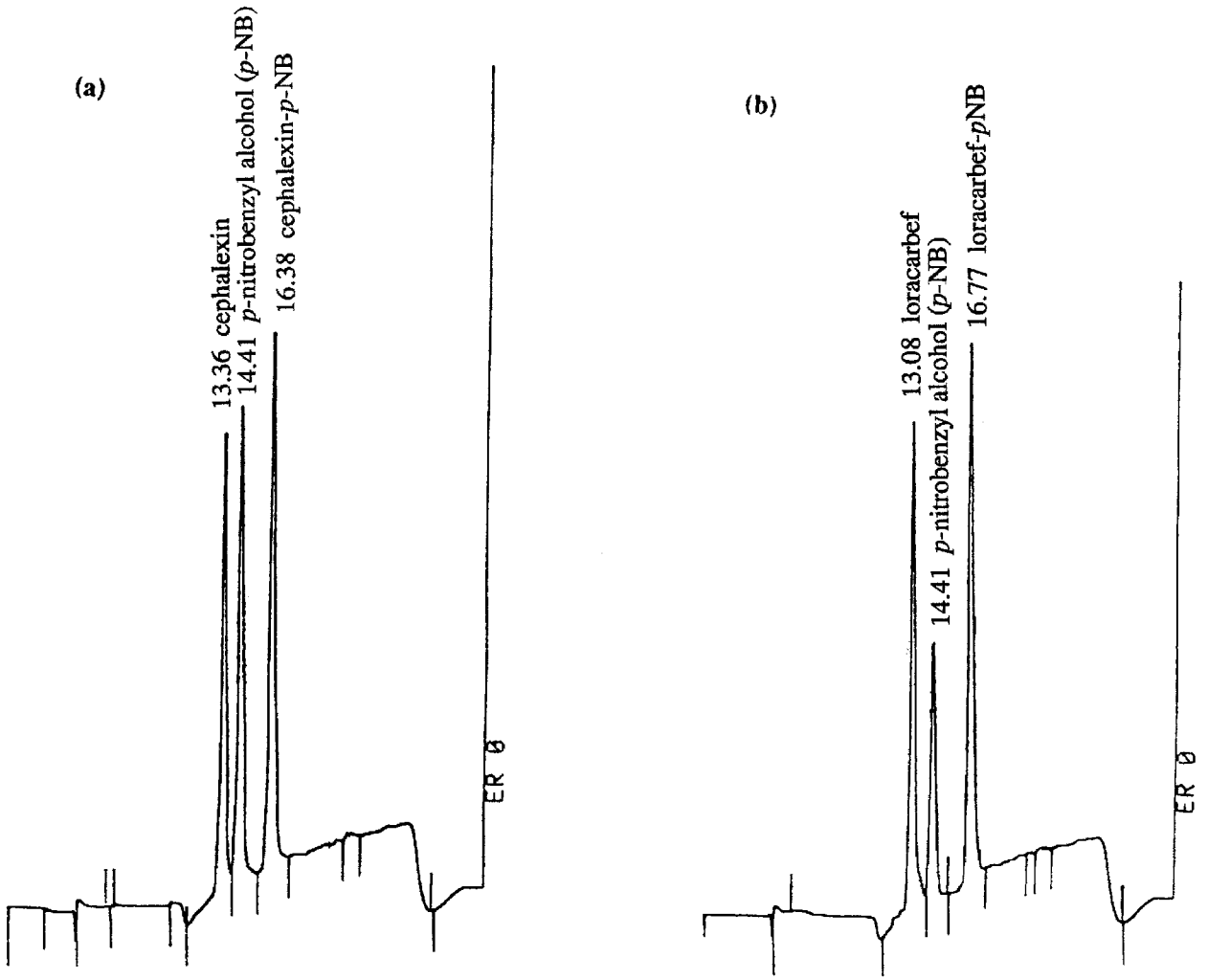


Figure 2 HPLC chromatogram of mixtures of Cp-PNB, Cp and *p*-nitrobenzyl alcohol (**a**), and of Lc-PNB, Lc and *p*-nitrobenzyl alcohol (**b**). The detailed assay conditions are described in the Methods section. The Arabic numerals indicate elution times of peaks

Table 1 Compounds that inhibit PNBCE activity when present at 1 mM concentration

Compound	Inhibition(%)
Phenylmethylsulfonylfluoride	100
Diethyl *p*-nitrophenylphosphate	100
Diisopropyl fluorophosphate	100
Diethylpyrocarbonate	100
$HgCl_2$	100
$FeSO_4$	100
$Zn(OCOCH_3)_2$	97
Phenylglyoxal	91
$FeCl_3$	87
Dicyclohexyl carbodiimide	78
5,5′-Dithio-bis(2-nitrobenzoic acid)	69
N-Bromosuccimide	67
N-Benzoyl phenylalanine naphtylamide	66
Chloramine T	44
p-Chloromercuribenzene sulfonate	36
L-1-*p*-Tosylamino-2-phenylethylchloromethyl ketone	29
Maleic anhydride	26
N-Chlorosuccimide	16
Methylglyoxal	13
2,3-butanedione	3
N-Acetylimidazole	3
Other[a]	0[b]

[a]At 1 mM *N*-*p*-Tosyk-L-lysine chloromethyl ketone, 1-ethyl-(-3-dimethyl aminopropyl) carbodiimide, *N*-ethylmaleimide, iodoacetamide, cyclohexanedione, succinic anhydride, β-mercaptoethanol, EDTA, EGTA, $MgCl_2$, $MnCl_2$, and $CuSO_4$ did not inhibit PNBCE activity. For conditions, see *Inhibition of PNBCE* in Methods
[b]0% inhibition: activity is 2.1 μmol min^{-1} mg^{-1} protein in the absence of any added compound

mechanical disruption. Protamine sulfate was added to the cell-free extract (final concentration 2.0 mg ml^{-1}) and the mixture was stirred for 1 h. Precipitate that formed was removed by centrifugation and the resulting supernatant phase was subjected to ammonium sulfate fractionation. Precipitate that formed between 45 and 80% ammonium sulfate saturation was collected by centrifugation, redissolved in buffer A, and then dialyzed overnight against the same buffer. The dialyzed sample was acidified to pH 5.0 with 1 N acetic acid and incubated for 10 min. The precipitate formed was removed by centrifugation. The supernatant phase was adjusted to pH 8.5 with 2 N NH_4OH and applied onto a DE 52 column (3.7 × 35 cm) equilibrated with buffer B (10 mM Tris-Cl, pH 8.5, containing 50 mM NaCl, 1 mM β-ME, and 0.5 mM EDTA). The column was washed with 350 ml buffer B and subsequently with 1750 ml buffer C (10 mM Tris-Cl, pH 7.0, containing 50 mM NaCl, 1 mM β-ME, and 0.5 mM EDTA). The esterase was eluted with a 3500-ml linear gradient formed from 50 and 300 mM NaCl in buffer C. Fractions with the esterase activity were pooled and concentrated by ultrafiltration with an Amicon PM-10 membrane (Beverly, MA, USA). The concentrated crude esterase solution was loaded onto a Sephacryl S-200 HR column (5.0 × 95 cm) (Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer D (10 mM Tris-Cl, pH 8.0, containing 0.1 M NaCl, 1 mM β-ME, and 0.5 mM EDTA). The fractions containing enzyme activity were combined, concentrated by ultrafiltration, and dialyzed overnight against buffer B. The dialyzed solution was applied onto a Q-Sepharose column (2.6 × 20 cm) (Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer B. The column was washed with 100 ml of buffer B and subsequently with 500 ml of buffer E (10 mM MES-NaOH, pH 6.0, containing 100 mM NaCl, 1 mM β-ME and 0.5 mM EDTA). The esterase was eluted with a 1500-ml linear gradient formed from 100 and 300 mM NaCl in buffer E. The fractions containing the esterase were combined, concentrated by ultrafiltration, and dialyzed overnight against 10 mM sodium acetate, pH 5.0, containing 1 mM β-ME and 0.5 mM EDTA. After removing the precipitate by centrifugation, the enzyme solution was loaded onto a calcium phosphate-cellulose column (1.6 × 20 cm) equilibrated with 10 mM sodium acetate, pH 5.0, containing 1 mM β-ME and 0.5 mM EDTA. After the column was washed with 250 ml of the same buffer, the esterase was eluted with a 250-ml linear gradient formed from 10 and 50 mM potassium phosphate, pH 7.0, containing 1 mM β-ME and 0.5 mM EDTA. The fractions containing the esterase activity were pooled, concentrated by ultrafiltration, and then dialyzed overnight against buffer D. The dialyzed enzyme solution was applied onto a *p*-aminobenzamidine-agarose column (1.0 × 20 cm) equilibrated with buffer D. After washing the column with 50 ml of buffer D, the ester-

Table 2 Purification of PNB carboxyl-esterase from *Bacillus subtilis*

Step	Protein (mg)	Total activity (Unit[a])		Specific activity (mUnit mg^{-1})		Purification (folds)	
		Lc-PNB[b]	*p*-NPA[c]	Lc-PNB	*p*-NPA	Lc-PNB	*p*-NPA
Cell-free extract	41900	74.0	5120	1.8	122	1.0	1.0
45–80% NH_4SO_4	24500	53.3	4510	2.2	184	1.2	1.5
pH treatment (pH 5)	17200	51.5	3720	3.0	216	1.7	1.8
DE-52	1780	45.5	3120	25.6	1750	14.2	14.3
Sephacryl S-200	1100	42.4	3010	38.5	2740	21.4	22.5
Q-Sepharose	67	30.1	2290	448	34100	249	279
Calcium phosphate-cellulose	14	16.7	1930	1190	138000	661	1130
p-Aminobenzamidine agarose	3	6.4	567	2130	189000	1180	1550

[a]One Unit of enzyme activity is the amount of enzyme catalyzing the hydrolysis of 1 μmol (HPLC assay) or *p*-nitrophenyl acetate (spectrophotometric assay) per min
[b]Oxalate salt of Lc-PNB
[c]*p*-NPA, *p*-nitrophenyl acetate

ase was eluted with a 100-ml linear gradient formed from 0 and 300 mM NaCl in buffer D. The fractions with esterase activity eluting between *ca* 160 and 220 mM NaCl in buffer D were pooled, concentrated by ultrafiltration, and stored in −80° C as the purified enzyme until used.

Results

Purification of PNBCE from B. subtilis *NRRL B8079*

The PNBCE is intracellular; cells were lysed by freezing and thawing. Purification from crude extract to electrophoretic homogeneity involved sequentially: ammonium sulfate precipitation, pH treatment, anionic ion exchange-, gel filtration-, calcium phosphate- and affinity-chromatographies. About 3 mg of the purified esterase was obtained from 760 g wet cells. The overall yield was *ca* 9%. Using Lc-PNB as a substrate, an 1180-fold purification was obtained (Table 2). The ratio of activities observed with *p*-nitrophenyl acetate and Lc-PNB as substrates was *ca* 88 for the pure enzyme and 68 for the crude extract. The different ratios indicate the existence of more than one esterase in this bacterium. The purified PNBCE is soluble in aqueous buffer and stable at neutral pH for several days. There is no detectable prosthetic group in the purified enzyme.

The MW_r of denatured and native PNBCE was estimated to be 54000 Da by SDS-PAGE (Figure 3) and Superose 12 gel filtration column chromatography (Figure 4). The esterase appears to be monomeric: the activity staining band coincides with the protein band in the native PAGE. A similar MW_r was observed for partially purified PNBCE during Sephacryl S-200 gel filtration (step 5, Table 2).

Amino acid composition and N-terminal amino acid sequence of PNBCE

A chemically determined amino acid composition for the PNBCE is presented in Table 3. Leucine, alanine, glutamic acid and proline residues were predominant; there were 61 acidic and 37 basic residues. Purified PNBCE exhibited an acidic pI, 4.1. This pI is consistent with the amino acid composition data. The N-terminal amino acid sequence of the esterase is: Met-Thr-His-Gln-Ile-Val-Thr-Gln-Try-Gly-Lys-Val-Lys-Gly-Thr-Thr-Glu-Asn-Gly-Val-His-.

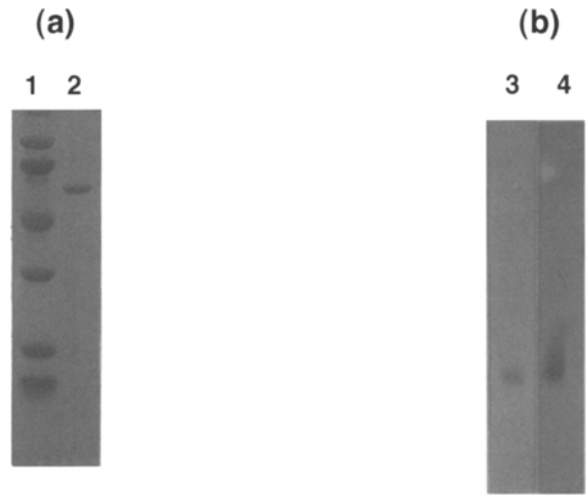


Figure 3 SDS polyacrylamide gel- and native gel-electrophoreses. Gels 1 and 2 in panel (**a**) are SDS-PAGE of protein MW_r standard and purified esterase stained for protein with Coomassie blue. 12.5% polyacrylamide gel was used. Gels 3 and 4 in panel (**b**) are non-denatured PAGE of purified esterase stained for protein and activity. 7% polyacrylamide gel was used

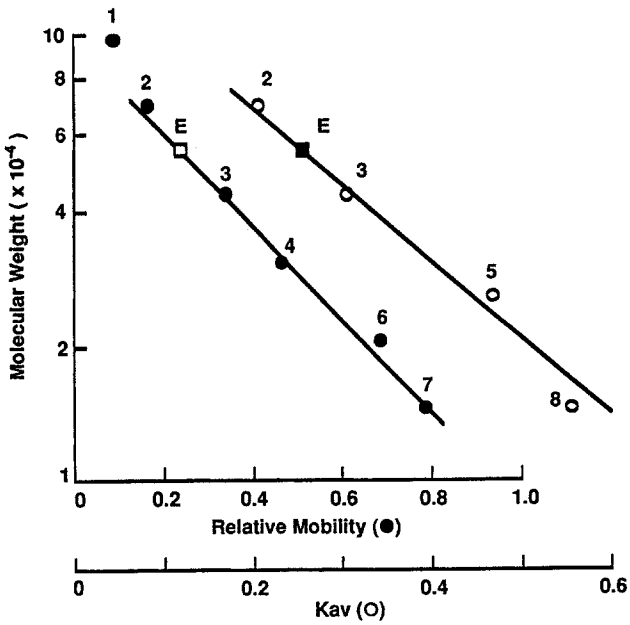


Figure 4 Determination of MW_r of PNBCE. Curve with solid circles (●) indicates MW_r determined by SDS-PAGE. The purified PNBCE (4 μg) and protein standards were used. Curve with open circles (○) indicates MW_r determined by FPLC. The purified esterase (0.1 mg in 0.2 ml) was applied to FPLC Superose 12 column (1 × 30 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.5, containing 0.15 M NaCl. The protein standards used were: (1) phosphorylase; (2) bovine serum albumin; (3) ovalbumin; (5) chymotrypsinogen; (8) ribonuclease A (13700). PNBCE is shown as E (■,□). The MW_r was estimated from a semilogarithmic plot of MW_r against mobility and K_{av} respectively

Table 3 Amino acid composition of PNBCE

Amino acid	Moles residues[a] per mole PNBCE
Asx	39.6
Thr	31.2
Ser	28.9
Glx	61.3
Pro	46.6
Gly	31.6
Ala	46.9
Val	24.3
Cys	ND[b]
Met	8.9
Ile	19.4
Leu	48.6
Tyr	15.7
Phe	22.1
Lys	23.3
His	12.3
Arg	15.3
Trp	ND[b]

Analysis described in Methods
[a]Based on MW_r of 54000
[b]ND, not determined

Catalytic properties of PNBCE

Purified PNBCE exhibited broad substrate specificity which included hydrolysis of simple organic esters and the PNB esters of several beta-lactam antibiotics (Table 4). As noted above, the ability to catalyze hydrolysis of PNB esters of these antibiotics at these rates is rare among esterases. Not surprisingly, the esterase hydrolyzed simple organic esters faster than the antibiotic PNB esters that were tested. The estimated V_{max} values for antibiotic esters were *ca* 100-fold less. It should be noted, however, that the low solubility of most of these antibiotic esters in aqueous solution limited the reaction rate and only *apparent* V_{max}s can be determined for these compounds. Comparisons of relative PNBCE activity for most antibiotic esters were carried out at pH 6.5. This maximized the reaction rate. The esters were more soluble at pH 6.5 than at pH 8.0.

Optimal pH and heat stability of the PNBCE activity

The optimal pH of the esterase activity was *ca* 8.3 in 100 mM Tris-HCl buffer (Figure 5). The esterase was stable for several days at 4° C at neutral pH. When the esterase was incubated at the various temperatures for 5 min in 50 mM of potassium phosphate buffer, pH 7.0, the enzyme suffered only minor loss of activity up to 50° C. Above 50° C the enzyme was denatured rapidly (Figure 6); 50% inactivation was observed upon 10 min incubation at 55° C and >80% inactivation occurred with 10 min at 60° C. The thermodenaturation was not reversible by prolonged incubation at low temperature.

Inhibitors of PNBCE activity

Table 1 lists compounds that, at 1 mM concentration, inhibited PNBCE activity. Activity was completely inhibited by organophosphate compounds DNP and DFP. Activity was completely inhibited by the serinyl-reactive reagent, PMSF, and the histidinyl-reactive reagent, DEPC. Mercuric chloride completely inhibited. Ferrous sulfate and ferric chloride inhibited 100% and 87%, respectively. Activity was partially inhibited by dicyclohexyl carbodiimide (reagents for Asp and Glu residues), 5,5-dithiobis (2-nitrobenzoic acid) and chloromercuribenzene sulfonic acid (reagents for Cys residues), *N*-bromosuccimide and *N*-chlorosuccimide (reagents primarily for Trp residues; secondarily for His, Arg, Lys and Tyr residues), phenylglyoxal (reagent for Arg residues). The indicated residues probably affect PNBCE catalysis indirectly. The 66% inhibition observed with *N*-benzoyl phenylalanine naphthylamide, a substrate for chymotrypsin, may reflect ability of the amide to compete at the ester substrate binding site. Sulfhydryl reagents such as β-ME, dithiothreitol had no effect on activity of esterase at the tested condition. Chelating reagents, such as EDTA or EGTA, did not affect enzyme activity: apparently no metal ion is involved in catalysis.

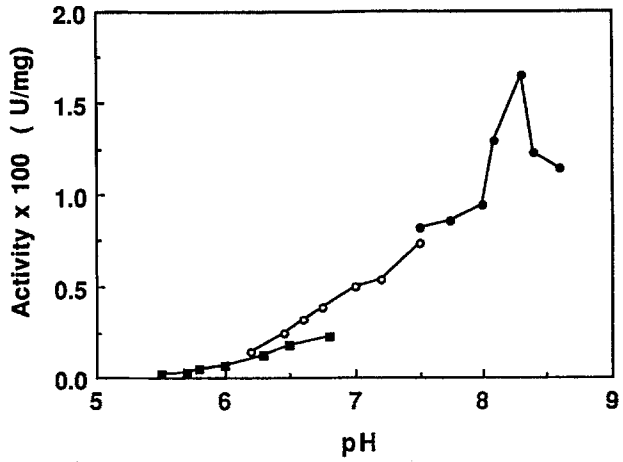


Figure 5 pH-dependent PNBCE activity. The esterase activity was assayed spectrophotometrically, using *p*-nitrophenyl acetate as substrate (25° C). The buffer systems used were: 100 mM sodium acetate, pH 5.7–6.5; 100 mM MES-NaOH, pH 6.2–7.8; 100 mM Tris-HCl, pH 7.5–9.0

Table 4 Substrate specificity of PNBCE[a]

Substrate	Concentration (mM)	Relative activity[b] (%)	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
Lc PNB (oxalate)	0.5	100	1.1	12.8
Lc PNB (tosylate)	0.5	41	1.0	4.4
Lc nucleus-PNB	1.0	52	1.1	6.8
	0.5	35		
Cf nucleus PNB	0.5	16	1.1	2.0
Cp PNB (tosylate)	0.5	12	1.3	1.7
p-Nitrophenyl acetate	1.6	8870	0.56	381.7
p-Nitrobenzyl acetate	5.0	268		
Benzyl acetate	10.0	521	ND	ND
	2.0	112		
α-Naphthyl acetate	5.0	318	ND	ND
	2.0	57		
N-Benzoyl-DL-phenylalanine-β-naphthylester	0.5	43	ND	ND

[a]Specific activity of purified PNBCE used in this experiment is 2.1 μmol min^{-1} per mg with 0.5 mM Lc-PNB (oxalate salt) as substrate
[b]Assay was by HPLC procedure; product formation and substrate disappearance were measured and were in agreement. Lc nucleus-PNB, *p*-nitrobenzyl acetate, benzyl acetate, α-naphthyl acetate were assayed in the same condition except 100 mM bis-tris-propane-HCl buffer, pH 8.0 and 1 mM substrate concentration were used

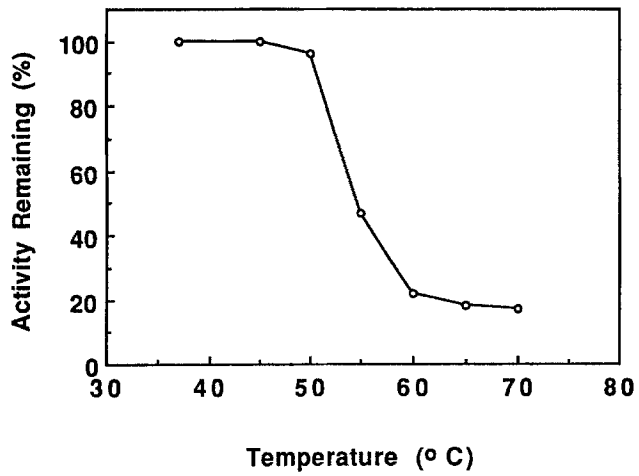


Figure 6 Heat stability of PNBCE activity. The purified esterase (1 mg ml^{-1}) was incubated in 50 mM potassium phosphate, pH 7.0, at various temperatures indicated for 10 min. At the end of incubation, the enzyme solution was immediately chilled in ice and 10 μl of enzyme solution was used for activity assay using Lc-PNB as substrate

Succinic anhydride, a water soluble amino group modifier, showed no inhibitory effect on PNBCE.

Titration with diethyl p-*nitrophenyl phosphate*

The sensitivity of the purified esterase to organophosphate indicates involvement of a hydroxyl group in its active site. Titration with DNP [15] caused purified PNBCE to be inactivated with the degree of inactivation of enzyme directly proportional to the consumption of the reagent (Figure 7). Complete inhibition was observed at one mole reagent per mole enzyme. The linear relationship between inactivation and consumption of DNP suggests that DNP is reacting with an active site hydroxyl. The rapid rate for the reaction of DNP with PNBCE suggests that the hydroxyl is near a surface of the enzyme and is readily accessible to the enzyme's surrounding aqueous environment.

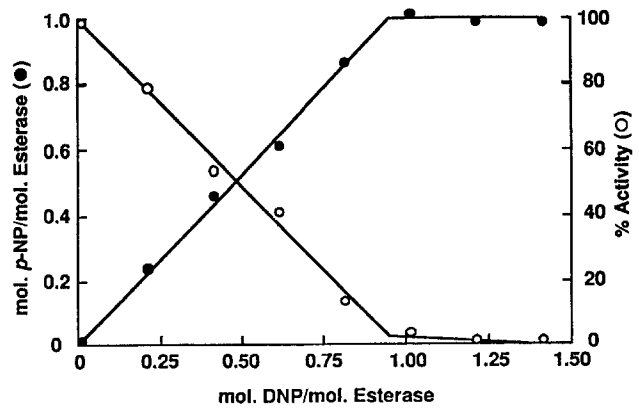


Figure 7 Titration of PNBCE by DNP. Titration of PNBCE with DNP was performed as described by Krisch [15]. One mg (18.5 nmol) purified PNBCE in 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, was mixed with 10 μl of DNP solution. The concentrations of DNP were determined spectrophotometrically using a molar extinction coefficient of $\epsilon_{M,\,405nm}$ = 18440 M^{-1} cm^{-1}. The absorbance increase at 405 nm due to release of *p*-nitrophenol (*p*-NP) was taken at 30-s intervals for 3 min, at 25° C. Immediately after the OD was taken, a 10-μl sample was withdrawn from the reaction mixture and used for the remaining activity determination by spectrophotometric assay

Identification of active site peptide

When purified PNBCE was treated with DFP, the enzyme activity decreased as the amount of reagent increased. The titration pattern was similar to that observed for DNP. When [^{3}H]-DFP was used, uptake of radioactivity by PNBCE was directly proportional to activity loss. When the [^{3}H]-DFP treated esterase was subjected to Lys C digestion followed by HPLC separation, a single radioactive peptide was obtained (Figure 8). The amino acid sequence of this labelled peptide was determined to be Ala-Ile-Met-Glu-Ser-Gly-Ala-Ser-Arg-Thr-Met-Thr-Lys-.

Titration with DEPC

Titration of the PNBCE with DEPC indicated that a histidine adjacent to the active site peptide may also be involved in the catalysis. The esterase was completely inhibited at one mole DEPC per mole enzyme (data not shown).

Immobilization of esterase

Since amino groups are not involved in the catalytic activity (succinic anhydride at 1 mM did not inhibit PNBCE), immobilization of PNBCE through the formation of covalent bonds between surface amino groups of PNBCE and an activated aldehyde group of a solid support such as Affi-Gel 15 is possible. The low pI value of the purified PNBCE facilitated its ionic binding to DE-52. PNBCE covalently immobilized on Affi-Gel 15 and PNBCE non-covalently immobilized on DE-52 had the same catalytic activity as that of the free enzyme. The immobilized enzymes were marginally more stable (less than 2-fold) than the free enzyme (data not shown).

Discussion

Esterases which catalyze hydrolysis of many different uncharged carboxylic esters have been classified into three groups [16]: A-carboxy esterases catalyze hydrolysis of organophosphorous compounds, such as DNP, and are not inhibited by these substrates; B-carboxy esterases do not hydrolyze organophosphate compounds; the hydrolyses that they catalyze are strongly inhibited by organophosphate compounds; C-carboxy esterases do not catalyze hydrolysis of organophosphate compounds nor are they inhibited by these compounds.

The PNBCE of *B. subtilis* NRRL B8079 is a B-type carboxy esterase: it did not hydrolyze DNP but ester hydrolyses catalyzed by the enzyme were strongly inhibited by this organophosphate compound. The very broad substrate specificity of PNBCE (Table 4) is consistent with the fact that, in general, B-carboxy esterases exhibit broad substrate specificity. Significantly, the specificity of this B-carboxy esterase extends to PNB ester intermediates of labile β-lactam antibiotics. Numerous B-esterases have been purified and characterized from various sources [2, 4, 6, 12, 14, 19–23]; however, very few hydrolyze PNB esters of β-lactam antibiotics, such as Cp, Cf, or Lc. We speculate that this rare ability may be related to an unusual

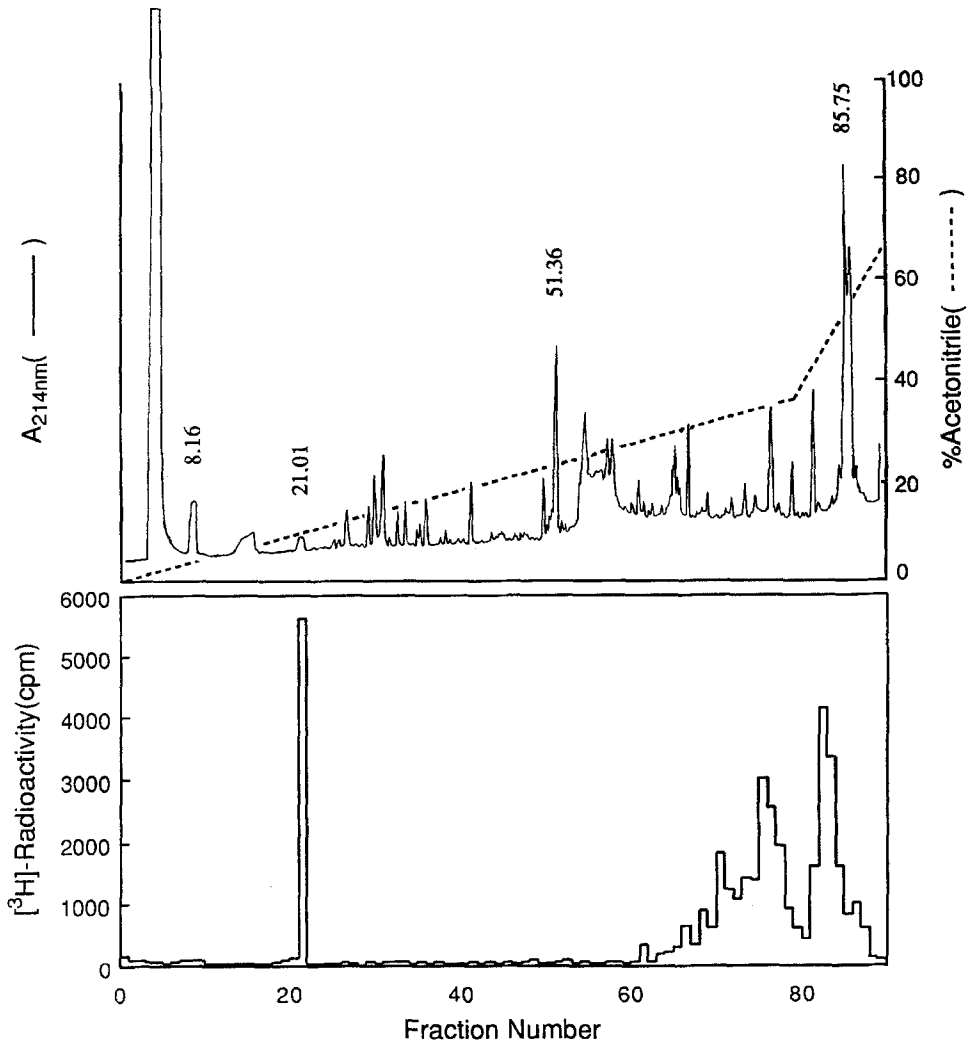


Figure 8 [^{3}H] radioactivity distribution on HPLC chromatogram of Lys C-digested [^{3}H]-DFP treated PNBCE. The purified [^{3}H]-DFP-labeled PNBCE (5 mg ml^{-1}, 1.5 × 10^7 cpm mg^{-1}) in 50 mM ammonium bicarbonate, pH 8.0, containing 0.5 M urea, was digested with 24 μg of Lys C (endoproteinase, Boehringer Mannheim Corporation, Indianapolis, IN, USA) at 37° C for 24 h; 50-μl aliquots of Lys C digested [^{3}H]-DFP-labeled esterase were separated by HPLC on a Synchropak RP-8 column (0.46 × 25 cm) (SynChrom Inc, Lafayette, IN, USA) using a gradient of acetonitrile in 0.1% trifluoroacetic acid with a flow rate of 1 ml min^{-1}. One-milliliter fractions were collected and the absorbance at 214 nm was measured. Fifty-milliliter aliquots of each fraction were withdrawn for radioactivity determination in a Packard Tri-Carb 1900CA liquid scintillation analyzer (Packard, Meriden, CT, USA)

active site amino acid sequence motif observed in PNBCE. Active site peptides have been sequenced from a number of mammalian esterases. The motif Glu-Ser x-x-x-x-Ser is observed. The active site peptide Ala-Ile-Met-Glu-Ser-Gly-Ala-Ser-Arg-Thr-Met-Thr-Lys- in PNBCE has only two amino acid residues instead of four between the two Ser residues. In the active site peptide of PNBCE, the fifth amino acid residue from the first Ser is a Thr; this Thr might provide a hydroxyl group involved in catalysis, ie this Thr may play the role of the second Ser in the Glu-Ser x-x-x-x-Ser of other B-esterases.

The 1550-fold purification necessary to achieve homogeneity for the PNBCE *B. subtilis* NRRL B8079 in this study indicates that PNBCE represents <0.1% of the soluble intracellular protein of the bacterium. This information makes clear the utility of cloning and overexpression of the gene for PNBCE in a suitable host. The amino acid sequences of two peptides from PNBCE serve as aids for cloning this gene. In fact, using this information industrial collaborators have recently cloned the gene and overexpressed its open reading frame in *Escherichia coli* [25].

The kinetic data of the current study demonstrates utility for increasing the tolerance of PNBCE to an organic solvent miscible with water and able to increase the solubility of PNB-antibiotic esters. For practical results a solvent-resistant form of PNBCE would have to be fully active in water containing a moderate amount of the appropriate solvent. The general feasibility of modifying enzymes in this manner has been established by Frances Arnold at the California Institute of Technology [9].

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