STUDIES ON *PARA*-NITROBENZYL (*p*NB) ESTERASE FROM *BACILLUS SUBTILIS*: PURIFICATION, PROPERTIES, GENETIC CLONING, AND CHARACTERIZATION OF ACTIVE SITES

By

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STUDIES ON *PARA*-NITROBENZYL (*p*NB) ESTERASE FROM *BACILLUS SUBTILIS*: PURIFICATION, PROPERTIES, GENETIC CLONING, AND CHARACTERIZATION OF ACTIVE SITES

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INTRODUCTION

Penicillins and cephalosporins, β-lactam antibiotics, are naturally produced in the fungus *Cephalosporium acremonium.* The biosynthetic pathways of these two antibiotics have been established and shown in scheme I. Isopenicillin N epimerase catalyzes conversion of isopenicillin N (IPN) to penicillin N. IPN is produced from δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) in a reaction catalyzed by IPN synthetase. The penicillin N is ring expanded to deacetoxylcephalosporin C (DAOC), and DAOC is hydroxylated to deacetylcephalosporin C (DAC). In *C. acremonium*, these oxygen-dependent reactions are catalyzed by a bifunctional enzyme, penicillin N expandase/DAOC hydroxylase. DAC is converted to the end product, cephalosporin C, via acetylation catalyzed by acetyl CoA:DAC transferase.

To increase the pharmaceutical values of the naturally produced antibiotic, cephalosporin C is often converted to other β-lactam antibiotics, such as cephalexin, loracarbef and cephaclor nucleus, by a chemical method (see SCHEME I). The chemical structures of these β-lactam antibiotics are shown in Fig. 1. Protection of C_4-carboxyl group on the cephalosporin C molecule is absolutely necessary before the chemical conversion step can be carried out (1). This was usually achieved by esterification with alcohols, such as *p*-nitrobenzyl alcohol (*p*NB) (5), to produce cephalosporin C-*p*NB ester. The C_4- *p*NB ester group is stable throughout the chemical conversion step. The resulting *p*NB esters of cephalexin, loracarbef and cephaclor nucleus (For chemical structures see Fig. 1) are pharmaceutically inactive, and restoration of activity required regeneration of the carboxyl group through deesterification. Although numerous chemical methods (2-4) have

been successfully used for hydrolysis of β-lactam antibiotic-*p*NB esters to reform the free acid, all of them required the use of large volumes of environmentally toxic solvents and catalysts, such as zinc. The development of a low cost biocatalytic process, that is, hydrolysis of *p*NB esters of β-lactam antibiotics by an esterase, may provide a way to solve this environmental problem.

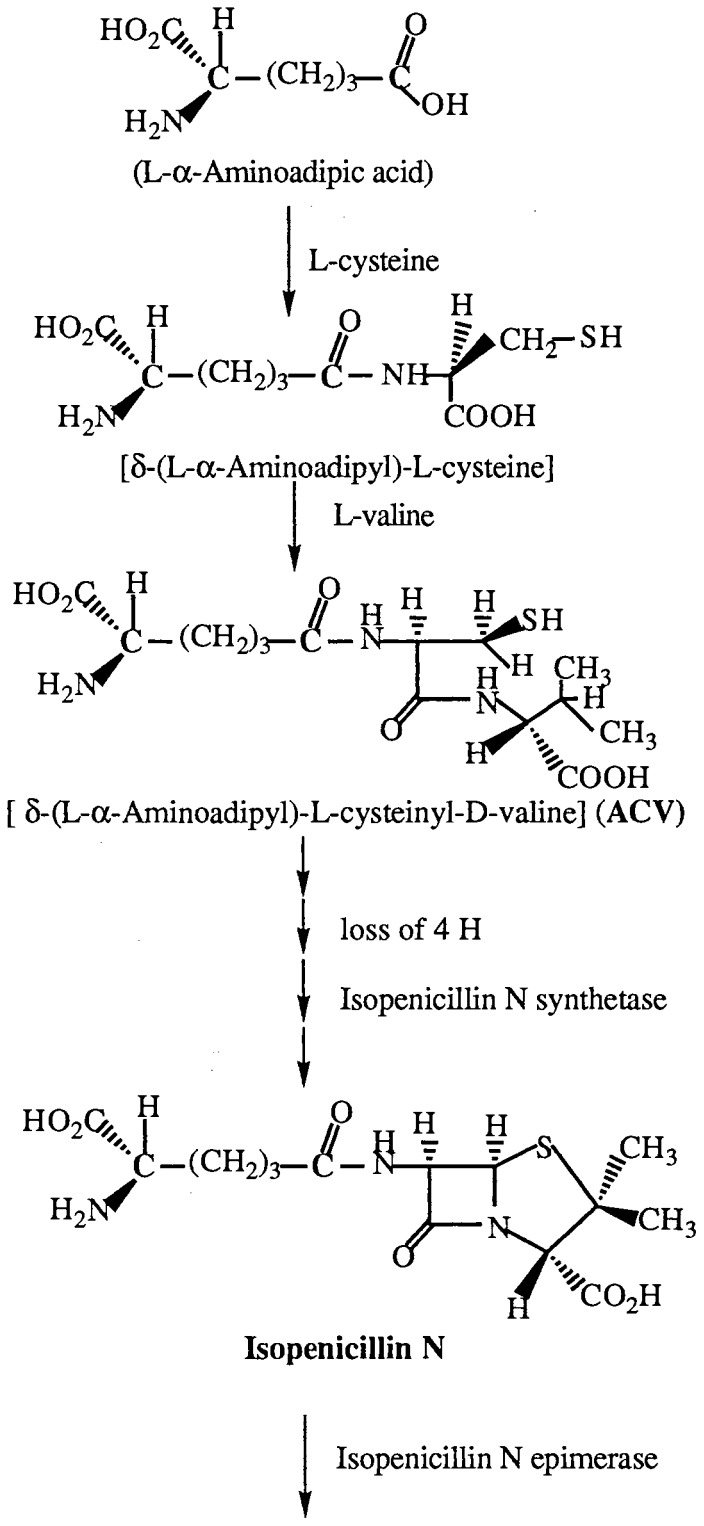
Esterases catalyze the hydrolysis of large number of uncharged carboxylic esters. Based on their behavior toward organophosphorus compounds, such as diethyl *p*-nitrophenyl phosphate, esterases are classified into three groups (6-7): A-esterases (EC 3.1.1.2) catalyze hydrolysis of organophosphorus compounds and are not inhibited by these substrates; B-esterases (EC 3.1. 1.1) do not hydrolyze organophosphorous compounds; the hydrolyses they catalyze are stoichiometrically inhibited by organophosphorus compounds; C-esterases do not catalyze hydrolysis of organophosphorus compounds nor are they inhibited by these compounds.

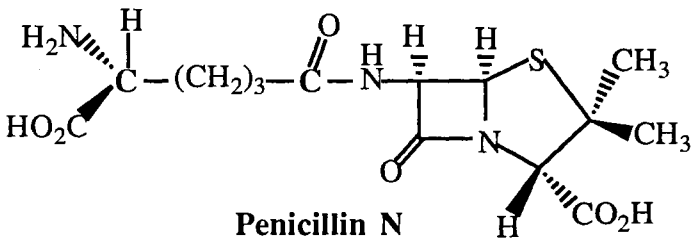
B-esterases have been formerly known as "ali-esterases" because of their broad substrate specificity(8). It is this group of esterases which were considered as logical candidates for developing an aqueous deesterification process for β-lactam-*p*NB ester intermediates. B−esterases are widely distributed in vertebrate tissues, blood serum, insects, plants, citrus fruits, and microorganisms. Several B-esterases have been purified and characterized from various sources (3, 5, 9-19). However, few of them can hydrolyze *p*NB esters of β-lactam antibiotics, such as cephalexin, cephaclor, or loracarbef. This type of esterase we have termed a *p*NB esterase. Among bacteria examined, *Bacillus subtilis* has the highest *p*NB-esterase activity (15). However, a *p*NB esterase has not been isolated and purified.

In this dissertation I report my work on *p*NB esterase in three sections. Purification and characterization of a *p*NB esterase from *B. subtilis* are detailed in Chapter I. The cloning and nucleotide sequencing of the DNA encoding this *p*NB esterase, and the over-

expression of the *p*NB esterase in *E. coli* are described in Chapter II. Purification of the *p*NB esterase from over-expressing *E. coli* cells, active site characterizations were reported in Chapter III.

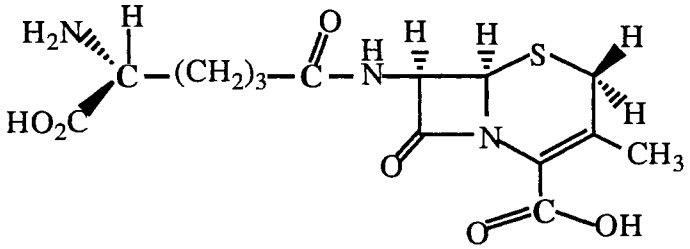
Scheme I: Biosynthetic Pathway of Penicillin N and Cephalosporin C in *Cephalosporium acremonium*





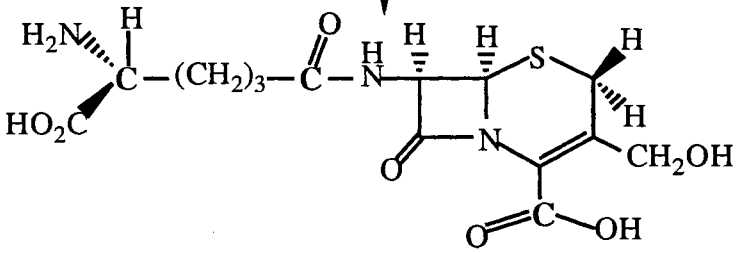
Penicillin N

Penicillin N expandase



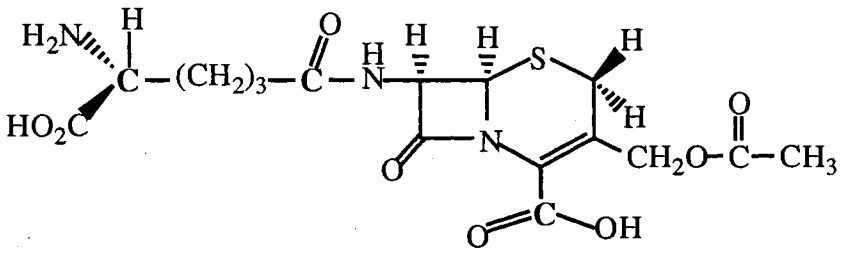
Deacetoxylcephalosporin C (**DAOC**)

O2
DAOC hydroxylase



Deacetylcephalosporin C (**DAC**)

Acetyl CoA:DAC transferase



Cephalosporin C

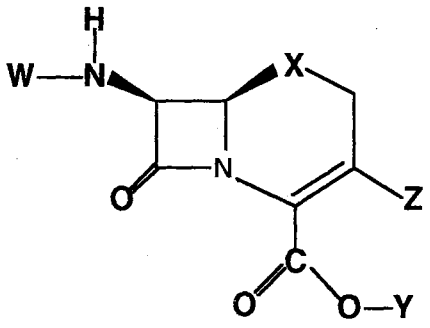
Protection of C_4-carboxylic group by *p*-nitrobenzyl alcohol (*p*NB)

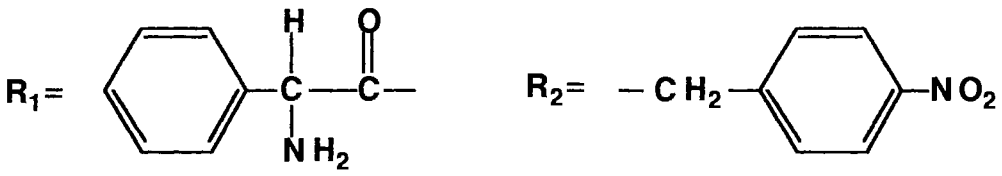
Chemical Synthesis

Cephalexin-*p*NB Loracarbef-*p*NB Cephaclor nucleus-*p*NB

Deprotection of *p*NB by either chemical method or *p*NB esterase

Cephalexin Loracarbef Cephaclor nucleus





Compound	W	X	Y	Z
Loracarbef	R_1	CH_2	H	Cl
Loracarbef-*p*NB	R_1	CH_2	R_2	Cl
Loracarbef nucleus-*p*NB	H	CH_2	R_2	Cl
Cephaclor	R_1	S	H	Cl
Cephaclor nucleus-*p*NB	H	S	R_2	Cl
Cephalexin	R_1	S	H	CH_3
Cephalexin-*p*NB	R_1	S	R_2	CH_3

Fig. 1. Structure of β-lactam antibiotics and *p*NB ester

CHAPTER I

PURIFICATION AND PROPERTIES OF *p*-NITROBENZYL ESTERASE FROM *BACILLUS SUBTILIS*

The cell-free extract from *Bacillus subtilis* NRRL B8079 was used as starting material for isolation and purification of the *p*NB esterase because it contains higher activity than other known sources. The *p*NB esterase activity was originally measured with a microbial plate assay method (15). This assay method is very time consuming; it requires 17 hrs to complete an assay and thus is not practical for use in enzyme purification process. We have developed two easy and accurate assay methods for *p*NB-esterase: one uses HPLC with β-lactam antibiotic-*p*NB esters as substrate, and another uses a spectrophotometer with *p*-nitrophenylacetate as a substrate.

MATERIALS AND EXPERIMENTAL PROCEDURES

Materials-- Loracarbef-*p*NB, loracarbef nucleus-*p*NB, cephalexin-*p*NB and cephalor nucleus-*p*NB were gifts for Dr. Bill Jackson of Eli Lilly and Co., Indianapolis, IN. *p*-nitrophenyl acetate was obtained from Aldrich. *p*-Aminobenzamidine agarose, β-mercaptoethanol and fast blue RR salt were obtained from Sigma. DEAE-cellulose (DE-52) was from Whatman. The molecular weight standard for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Bio-Lyte3/5, and isoelectric point standard for isoelectrofocusing, were obtained from Bio-Rad. Sephacryl S-200, Q-sepharose and the molecular weight standard proteins for gel filtration were from Pharmacia. Calcium phosphate was prepared by the method of Jenner (20) and mixed with cellulose powder in

the ratio of 3:1(w/w) before use. HPLC-grade methanol and acetonitrile were from J.T. Baker Chemical Company. Other chemicals were purchased commercially at the highest purity available.

Bacterial Growth-- *Bacillus subtilis* NRRL B8079 was grown in 500 ml Erlenmeyer flasks under the conditions described by Brannon (15). After cultivation for 48 hours, cells were harvested by centrifugation, washed with 15 mM Tris-HCl, pH 7.5. Washed cells were stored at -80 ^{o}C until use.

Protein was determined by the method of Lowry *et al.* (21) with bovine serum albumin as the standard.

Molecular Weight Determination-- The molecular weight of *p*NB-esterase was estimated by two methods: (1) gel filtration using a FPLC superose 12 column (1 x 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 (66,200), ovalbumin (45,000), chymotrypsinogen (25,000) and ribonuclease A (13,700) were used as molecular weight standards; (2) SDS-PAGE according to the method of Weber and Osborn (22). The protein standards used were phosphorylase B (97,400), bovine serum albumin, ovalbumin, carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme(14,400). The sample and standards were treated with 2% SDS and 5% β-mercaptoethanol at either 95 ^{o}C for 5 min or 55 ^{o}C for 1 hour before being subjected to electrophoresis.

Detection of Esterase by Activity Staining in Gel-- The detection of esterase-active bands on a non-denaturing polyacrylamide gel was carried out by a method described by Sobek & Gorisch (23) with modifications. The gel was soaked in 100 ml of 100 mM Tris-HCl buffer, pH 8.0, for 5 min at room temperature. Sixteen mg of α-naphthyl acetate in 0.5 ml acetonitrile was added to the soaking solution. The esterase activity band was

developed upon addition of 50 mg of Fast Blue RR salt (4-benzoylamino-2,5-dimethoxylbenzene diazonium chloride hemi[zinc chloride] salt). The background staining was removed by washing the gel with methanol-acetic acid-water (40:10:50).

Isoelectric Point (pI) Determination-- Analytical isoelectric-focusing was carried out in disk polyacrylamide gel electrophoresis with Bio-Lyte 3/5 (pH range 3.8-5.1) according to the protocol developed by O'Farrell (24) with modifications. A Bio-Rad isoelectric-focusing calibration kit was used as standard. Isoelectrofocusing gels were prepared with glass tubes (85 x 3.8 mm). The bottom of the tube was sealed with parafilm paper. To make 24 ml of 5% gel mixture (about 0.5 ml for each gel tube), 1.2 ml of 40% Bio-Lyte was added to 4 ml of 30% acrylamide and 18.6 ml of water. Two hundred µl of 10% ammonium persulfate was added, and the solution was degassed under vacuum for about 5 min. Immediately after addition of 15 µl of TEMED, the solution was loaded into the gel tube. The gel was overlaid with gel overlay solution containing 15% glycerol, 5% Bio-Lyte and 0.8%(w/v) glutamic acid. After the gel was solidified, the parafilm paper at the end of tube was removed and replaced with a dialysis membrane. The gel tube was placed in a standard tube gel electrophoresis chamber which was filled to the top with 1 M NaOH. The low reservoir was filled with 0.02 M H_3PO_4 and the upper reservoir was filled with 1 M NaOH. The gel was prerun at 4° C in the following sequence: (a) 200 volts for 0.25 hour, (b) 300 volts for 0.5 hour, and (c) 400 volt for 0.5 hour. The power was turned off and the upper reservoir was emptied. Fifty µl of *p*NB esterase in 30% glycerol and 2% Bio-Lyte was loaded onto the gel, the sample was overlaid with 10 µl of overlay solution, then the tube was filled with 1 M NaOH. After the upper chamber was refilled with 1 N NaOH, the electrophoresis was run at 400 volts for 12 hours and then at 800 volts for 1 hour at 4° C. When the electrophoresis was done, the gel was removed and stained for esterase activity..

Amino Acid Composition and Sequence Analysis-- Amino acids were analyzed by the methods of Heinrikson and Meredith (25) and Bidlingmeyer *et al.* (26) with a HPLC reversed-phase column after derivatization with phenylisothiocyanate (PITC) to phenylthiocarbamoyl amino acids (PTC-amino acids). The purified esterase was extensively dialyzed 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 quantitated by HPLC procedure with external amino acid standards, using an Ultrasphere-ODS column (0.46 x 25 cm) (Beckman, San Ramon, CA). The PTC-amino acids were eluted with 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).

For amino acid sequence analysis, the purified *p*NB esterase 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) (27,30-31). The analyses were done at the Molecular Biology Resource Facility, Saint Francis Hospital of Tulsa Medical Research Institute, University of Oklahoma Health Science Center, under the supervision of Dr. Ken Jackson.

Assay Procedures for *p*NB Esterase Activity-- Two assay methods were developed and used throughout the entire investigation.

(1) HPLC Assay with β-Lactam Antibiotic-*p*NB Esters as Substrates: The reaction mixture, 1 ml, containing 5 µmol Bis tris propane-HCl, pH 6.5, 0.5 µmol substrate (25 µl of 20 mM stock solution in DMSO for *p*NB esters of cephalexin, loracarbef and cephaclor nucleus) and appropriate amount of enzyme solution, was incubated at 37° C in a constant

temperature shaker for 30 minutes. The reaction was stopped by the addition of an equal volume of acetonitrile. The mixture was centrifuged and filtered through a 0.45 μm filter to remove the protein, and the supernatant solution was analyzed by HPLC for product formation and substrate disappearance. The HPLC was performed in a C-18 reversed-phase column (Nava-Pak C18 Radical Pak Cartridge, 0.8 x 10 cm, Millipore Corporation, Milford, MA) with a linear gradient formed from solvent A and solvent B at a flow rate of 1 ml/min. Solvent A is a mixture of 80% 1 mM Triethylamine-HCl, pH 2.5 and 20% of methanol; solvent B is 100% methanol. The product and substrate were detected at 254 nm and quantitated by integration of peak areas in the chromatogram. Figure 2 shows a typical HPLC chromatogram of this assay method.

(2) Spectrophotometric Assay with *p*-Nitrophenylacetate as Substrate-- By taking advantage of the absorption change resulting from the hydrolysis of *p*-nitrophenylacetate to *p*-nitrophenol and acetate, we have developed a spectrophotometric assay for esterase. Assays were carried out at room temperature in a 1 ml assay mixture containing 100 μmol Tris-HCl pH 7.0, 1.6 μmol *p*-nitrophenylacetate. One to 20 μl enzyme solution was added and the activity was measured by the absorption increase at 405 nm in a Cary spectrophotometer, model 219, or a Shimadzu UVPC 2010 spectrophotometer.

Purification of *p*NB Esterase-- All purification steps were carried out at 0-4 ^{o}C unless otherwise specified. Frozen *Bacillus subtilis* cells, 760 g, were thawed, homogenized in 2 liters of buffer A (10 mM potassium phosphate, pH 7.0 containing 1 mM β-mercaptoethanol and 0.5 mM EDTA) and disrupted by two passages through the French press at 1000 psi. The cell free extract was obtained by centrifugation of the homogenate at 24,000 x g for 30 min. Protamine sulfate was added to a final concentration of 2.0 mg/ml, the mixture was stirred for one hr, and centrifuged at 24,000xg for 30 min to remove the nucleic acid. The supernatant solution thus obtained was subjected to ammonium sulfate fractionation. The precipitate that formed between 45% and 80 %

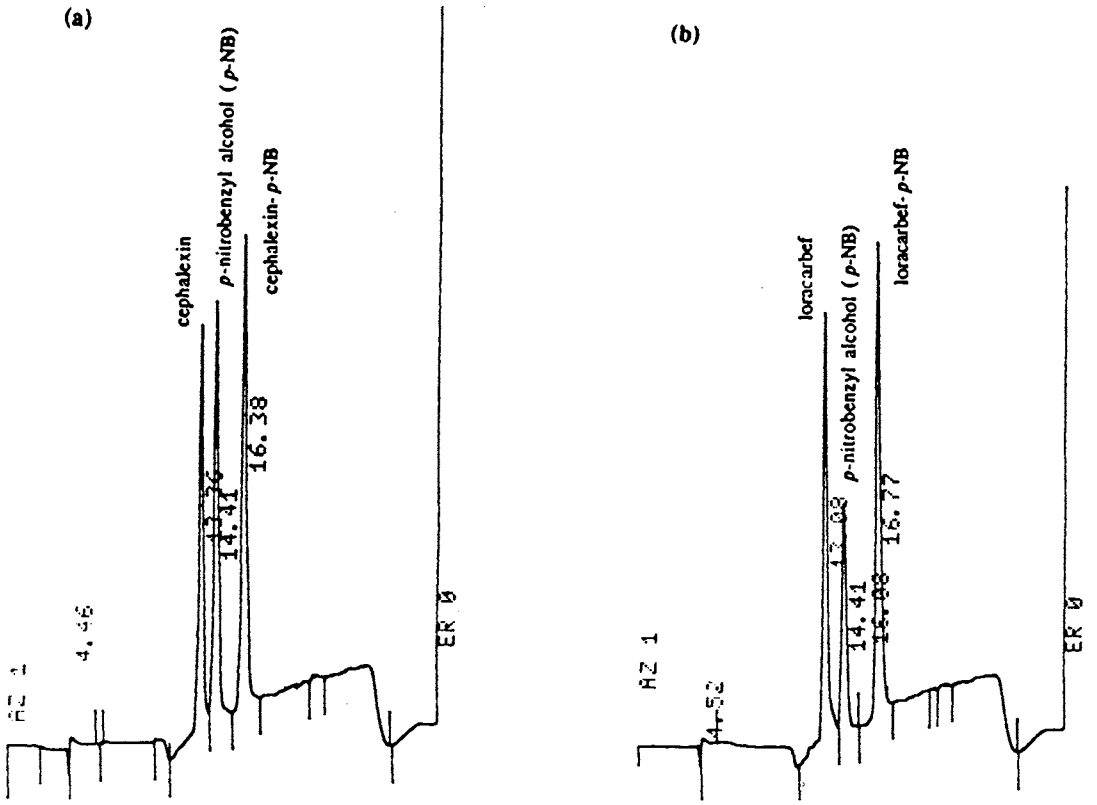


Fig. 2. HPLC chromatogram of mixtures of cephalexin-*p*NB, cephalexin and *p*-nitrobenzyl alcohol(a), and of loracarbef-*p*NB, loracarbef and *p*-nitrobenzyl alcohol (b). One ml assay mixture containing 5 µmoles Bis-Tris-propane-HCl, pH 6.5, 0.5 µmole substrate and an appropriate amount of enzyme solution was incubated at 37° C for 30 minutes. The reaction was stopped by addition of an equal volume of acetonitrile. The mixture was centrifuged to remove the protein and the supernant was analyzed by HPLC. The HPLC was performed in a C-18 reverse phase column with a linear gradient formed by solvent A [either a mixture of 80% 1 mM triethylamine-HCl, pH 2.5 and 20% methanol (a) or a mixture of 1 mM triethylamine-HCl, pH 2.5 and 5% methanol (b)] and solvent B [100% methanol]. The Arabic numerals indicate the retention times of peaks

ammonium sulfate saturation was collected by centrifugation at 45,000xg for 30 min, dissolved 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. After incubation for 10 min, the precipitate formed was removed by centrifugation. The supernatant was adjusted to pH 8.5 with 2 N NH_4OH and applied onto a DE-52 column (3.7 x 35 cm) equilibrated with buffer B (10 mM Tris-HCl, pH 8.5, containing 50 mM NaCl, 1 mM β-mercaptoethanol, and 0.5 mM EDTA). The column was washed with 350 ml of buffer B, followed with 1,750 ml of buffer C (10 mM Tris-HCl, pH 7.0, containing 50 mM NaCl, 1 mM β-mercaptoethanol, and 0.5 mM EDTA), and finally with a 3,500 ml of a linear gradient formed from 50 to 300 mM NaCl in buffer C. Fractions with *p*NB esterase activity, which eluted between 160 mM and 180 mM NaCl, were pooled and concentrated to about 30 ml with an Amicon PM-10 membrane.

The concentrated solution was loaded onto a Sephacryl S-200 HR column (5.0 x 95 cm) equilibrated with buffer D (10 mM Tris-HCl, pH 8.0, containing 0.1M NaCl, 1 mM β-mercaptoethanol and 0.5 mM EDTA). Fractions containing enzyme activity were combined, concentrated by ultrafiltration, and dialyzed against buffer B overnight . The dialyzed solution was applied onto a Q-Sepharose column (2.6 x 20 cm) equilibrated with buffer B. The column was washed, in sequence, with 100 ml of buffer B, 500 ml of buffer E (10 mM MES-NaOH, pH 6.0, containing 100 mM NaCl, 1 mM β-mercaptoethanol and 0.5 mM EDTA), and 1,500 ml of a linear gradient formed from 100 and 300 mM NaCl in buffer E. Fractions containing esterase activity, which eluted between 200 mM and 230 mM NaCl, were combined, concentrated by ultrafiltration, and dialyzed against 10 mM sodium acetate, pH 5.0, containing 1 mM β-mercaptoethanol and 0.5 mM EDTA overnight. After removing the precipitate by centrifugation, the supernatant solution was loaded onto a calcium phosphate-cellulose column (1.6 x 20 cm) equilibrated with 10 mM sodium acetate, pH 5.0, containing 1 mM β-mercaptoethanol and 0.5 mM

EDTA. The column was washed with 250 ml of the same buffer, then followed by 250 ml of a linear gradient formed from 10 to 50 mM potassium phosphate, pH 7.0, containing 1 mM β-mercaptoethanol and 0.5 mM EDTA. Fractions containing the esterase activity were pooled, concentrated by ultrafiltration, and dialyzed overnight against buffer D. The dialyzed enzyme solution was applied onto a *p*-aminobenzamidine-agarose column (1.0 x 20 cm) which has been activated with 70 mM sodium succinate buffer pH 5.6 containing 0.5 M KCl (28) and equilibrated with buffer D. The column was washed with 50 ml of buffer D, then with 100 ml of a linear gradient formed from 0 to 300 mM NaCl in buffer D. Fractions containing *p*NB esterase activity were pooled, concentrated by ultrafiltration, and stored at -80 ^{o}C until use. The purification procedure is outlined in SCHEME II.

RESULTS AND DISCUSSION

Purification Procedure, Specific Activity, Purity, Molecular Weight, and Stability of *p*NB Esterase- To facilitate the purification of *p*NB esterase, two assay methods were developed: one used HPLC with β-lactam antibiotic-*p*NB ester as substrate; another used spectrophotometer with *p*-nitrophenyl acetate as substrate. The HPLC method is specific for *p*NB esterase; the spectrophotometric method detects all esterases. The HPLC method is more time consuming than the spectrophotometric method. Therefore, during the purification of *p*NB esterase, especially when column chromatography was used, we used the spectrophotometric method to locate fractions containing esterase activity first, then used the HPLC method to identify the *p*NB esterase-containing fractions among the esterases containing fractions. For the purified esterase, the results of the spectrophotometric assay correlated well with those of the HPLC assay. Thus, this assay method is very valuable in stages of the purification endeavor.

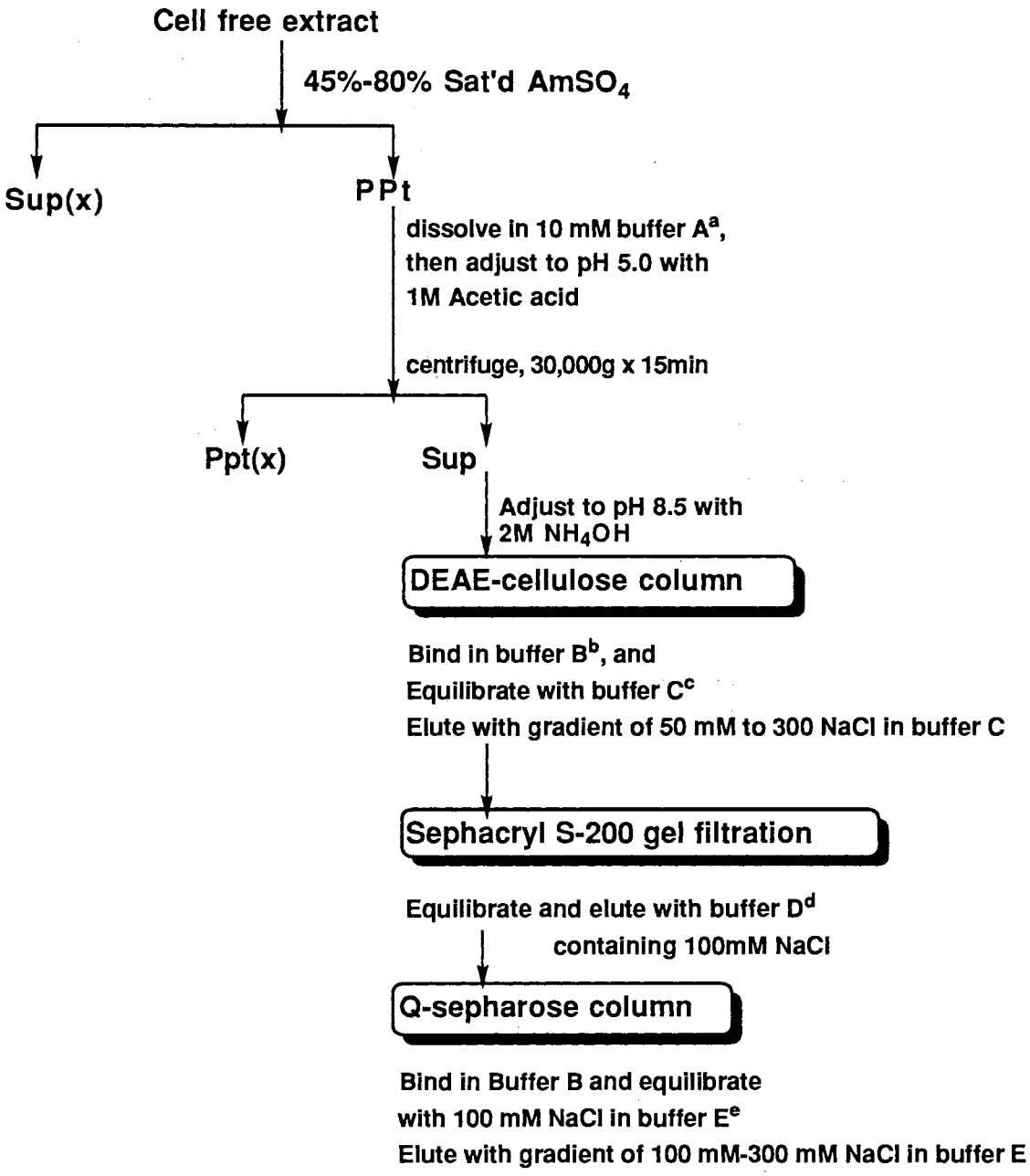
The *p*NB esterase was purified from a cell free extract of *B. subtilis* to homogeneity by a procedure involving ammonium sulfate precipitation; pH 5.0 treatment; DE-52 ion exchange, gel filtration, calcium phosphate and affinity column chromatography (see Scheme II). About 3 mg of the purified esterase was obtained from 760 g wet cells of *Bacillus subtilis* ; and about 9% of the *p*NB esterase activity present in the cell free extract was recovered in the final step (see Table I). One mg of purified enzyme catalyzed the hydrolysis of 2.13 μmoles of loracarbef-*p*NB to loracarbef and *p*NB in one minute and hydrolysis of 189 μmoles of *p*-nitrophenyl acetate to *p*-nitrophenol and acetic acid in one minute at room temperature. A 1180-fold purification was achieved with this purification procedure.

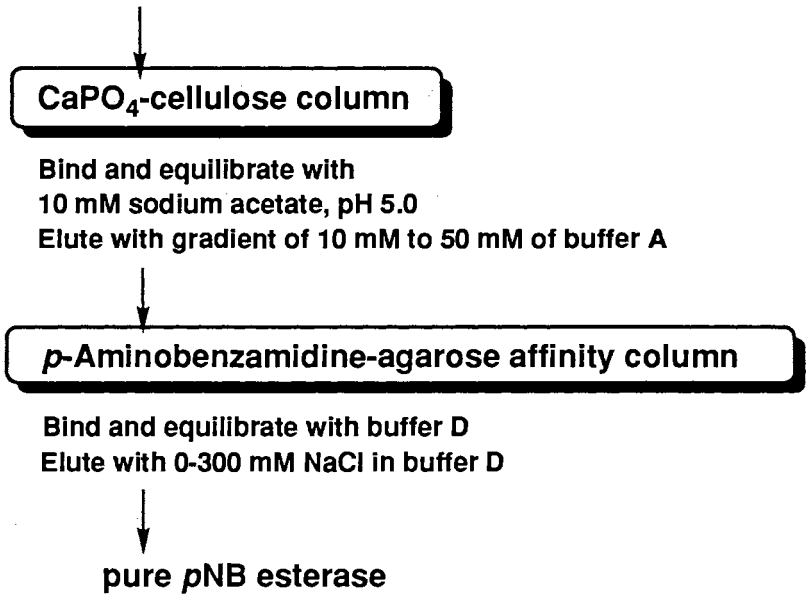
The ratio of activities observed with *p*-nitrophenyl acetate and loracarbef-*p*NB as substrates was about 88 for the pure enzyme and 68 for the crude extract. The different ratios suggest that there is more than one kind of esterase in *Bacillus subtilis*. DE-52 and Sephacryl S-200 column chromatography removed most esterases lacking β-lactam antibiotic-*p*NB ester hydrolyzing activity. In the DE-52 column elution, four esterase containing peaks (labeled as A, B,C, D in Fig. 3) were detected when assaying with the spectrophotometric method. However, only peak D contained *p*NB esterase.

Gel filtration with a Sephacryl S-200(HR) column removed the remaining trace amount of other esterases from *p*NB esterase, and the fractions were dominated by *p*NB esterase (peak B in figure 4). The absence of other esterases in the *p*NB esterase preparation after Sephacryl S-200 was confirmed by the single peak found in the activity profiles in the chromatograms (Figures 5, 6 and 7) of Q-sepharose, calcium phosphate-cellulose and *p*-aminobenzamidine-agarose columns using *p*-nitrophenyl acetate and loracarbef-*p*NB as substrates .

When isolated *p*NB esterase was subjected to SDS-PAGE (Fig. 8), a single protein band with a molecular weight of 54,000 was observed, indicating that the isolated protein

Scheme II: Purification of *p*NB esterase from *B. subtilis*





[a]Buffer A: (K/K)-PO4, pH7.0

[b]Buffer B: 10 mM Tris-HCl, pH8.5

[c]Buffer C: 10 mM Tris-HCl, pH7.0

[d]Buffer D: 10 mM Tris-HCl, pH8.0

[e]Buffer E: 10 mM MES-NaOH, pH6.0

TABLE I

PURIFICATION OF *p*NB ESTERASE FROM *BACILLUS SUBTILIS*

Step	Protein (mg)	Total activity (Unit[a])		Specific activity (mUnit/mg)		Purification (Folds)	
		Lora[b]	*p*-NPA[c]	Lora	*p*-NPA	Lora	*p*-NPA
Cell free extract	41900	74.0	5120	1.8	122	1.0	1.0
45-80% $AmSO_4$	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	448.0	34100	249.0	279.0
Calcium phosphate-cellulose	14	16.7	1930	1190.0	138000	661.0	1130.0
p-Aminobenzamidine agarose	3	6.4	567	2130.0	189000	1180.0	1550.0

[a] One Unit of enzyme activity is the amount of enzyme catalyzing the hydrolysis of 1 µmole loracarbef-*p*NB (oxalate salt) or *p*-nitrophenyl acetate per minute

[b] Lora, loracarbef-*p*NB oxalate salt

[c] *p*-NPA, *p*-nitrophenyl acetate

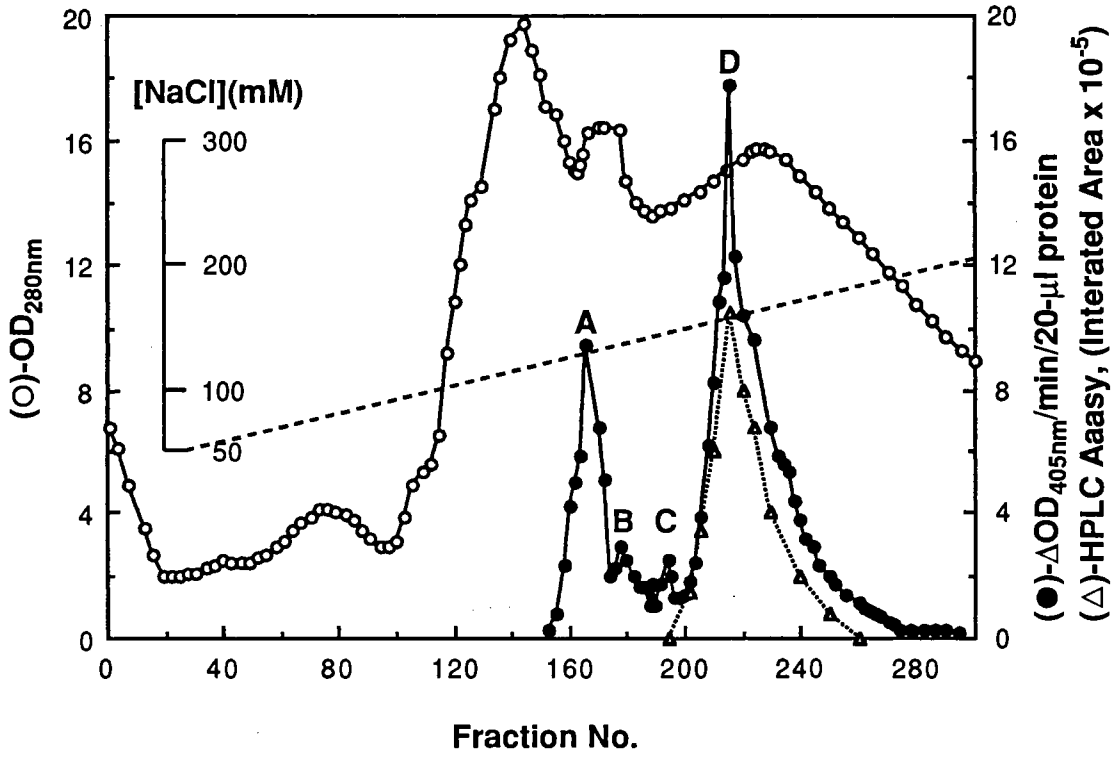


Fig. 3. DE-52 column chromatogram. The column eluting conditions were as described in "Materials and Experimental Procedures". The flow Rate was 35 ml/min, and 8 ml was collected for each fraction. Fractions were analyzed for esterase activity, using *p*-nitrophenyl acetate(●), and loracarbef-*p*NB as substrate(△), and for protein concentration, measured at OD_{280nm}, (O).

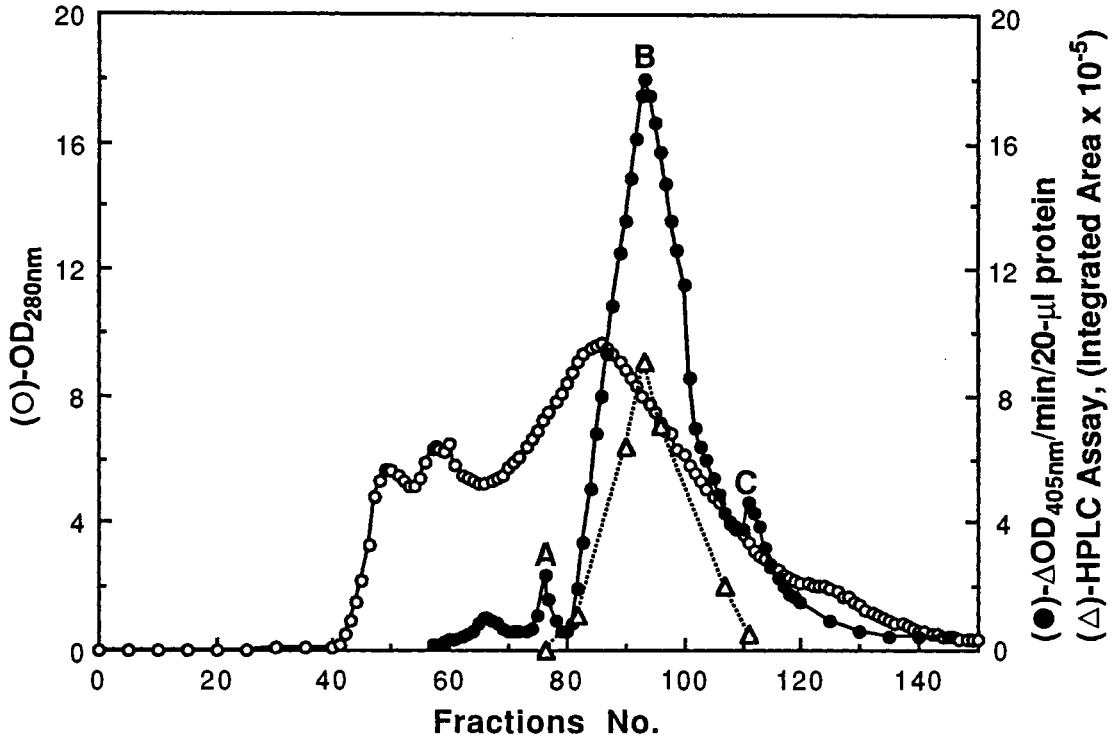


Fig. 4. Sephacryl S-200(HR) column chromatogram. The column eluting conditions were as described in "Materials and Experimental Procedures". Fractions were analyzed for esterase activity, using *p*-nitrophenyl acetate (●) and loracarbef-*p*NB (△) as substrates, respectively, and for protein concentration determined at $OD_{280\ nm}$ (○). The column flow rate was adjusted to 60 ml/min and 15 ml was collected for each fraction.

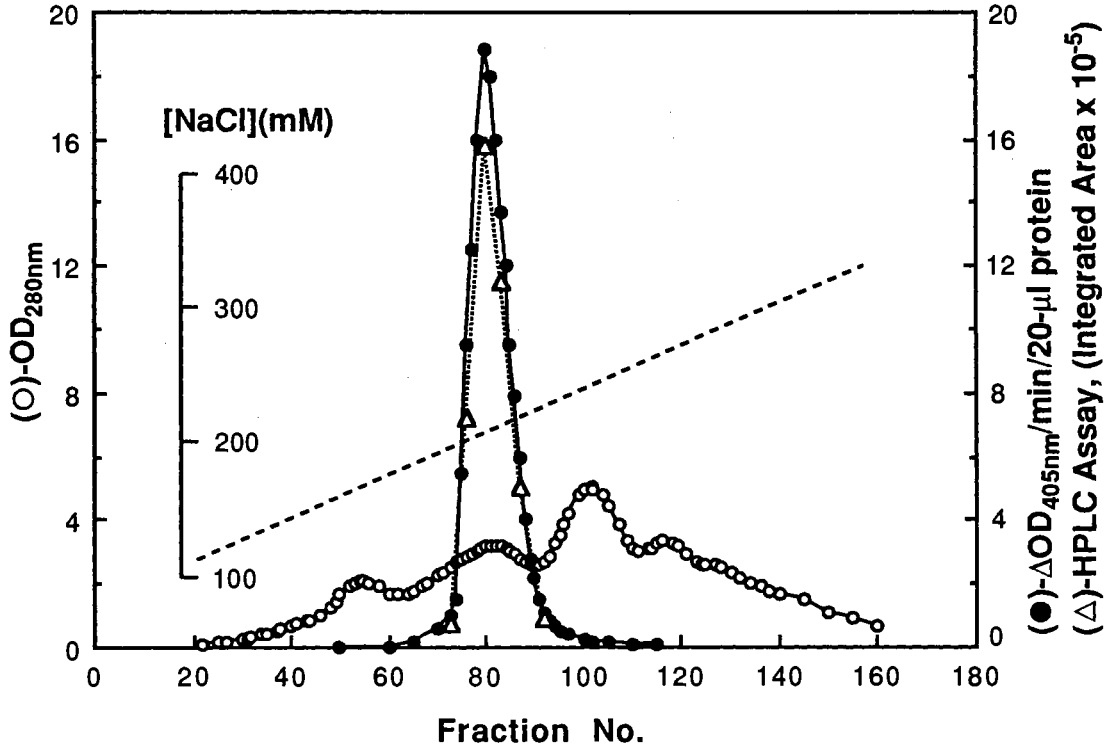


Fig. 5. Elution profile of Q-sepharose column. The column eluting conditions were as described in "Materials and Experimental Procedures". Fractions were analyzed for esterase activity with *p*-nitrophenyl acetate (●) and loracarbef-*p*NB(△) as a substrates, respectively, and for protein concentration determined at $OD_{280\ nm}$ (○). The flow rate of the column was controlled at 24 ml/hr, and 4 ml was collected for each fraction.

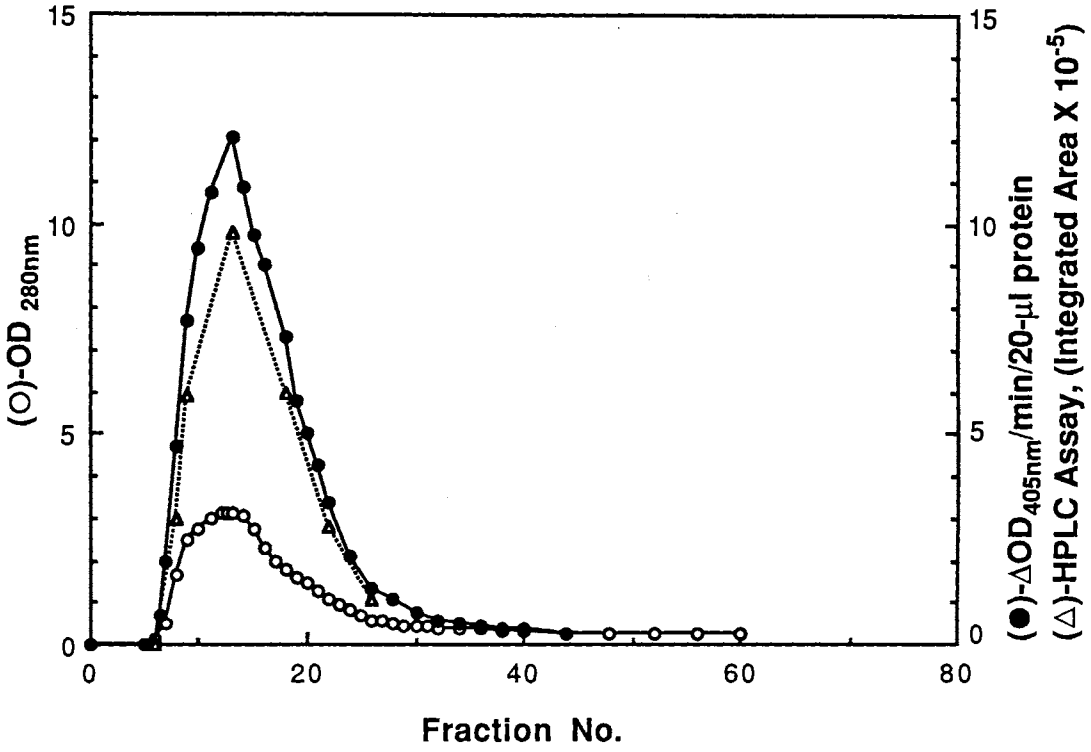


Fig. 6. Elution profile of calcium phosphate-cellulose column. The enzyme was eluted with a linear gradient in potassium phosphate buffer, at pH 7.0, from 10 mM to 50 mM, and the column flow rate was controlled at 24 ml/hr. 2.5 ml was collected for each fraction. Fractions were analyzed for esterase activity, with *p*-phenylacetate (●) and loracarbef-*p*NB (△) as substrates, respectively, and for protein concentration, OD_{280nm} (○).

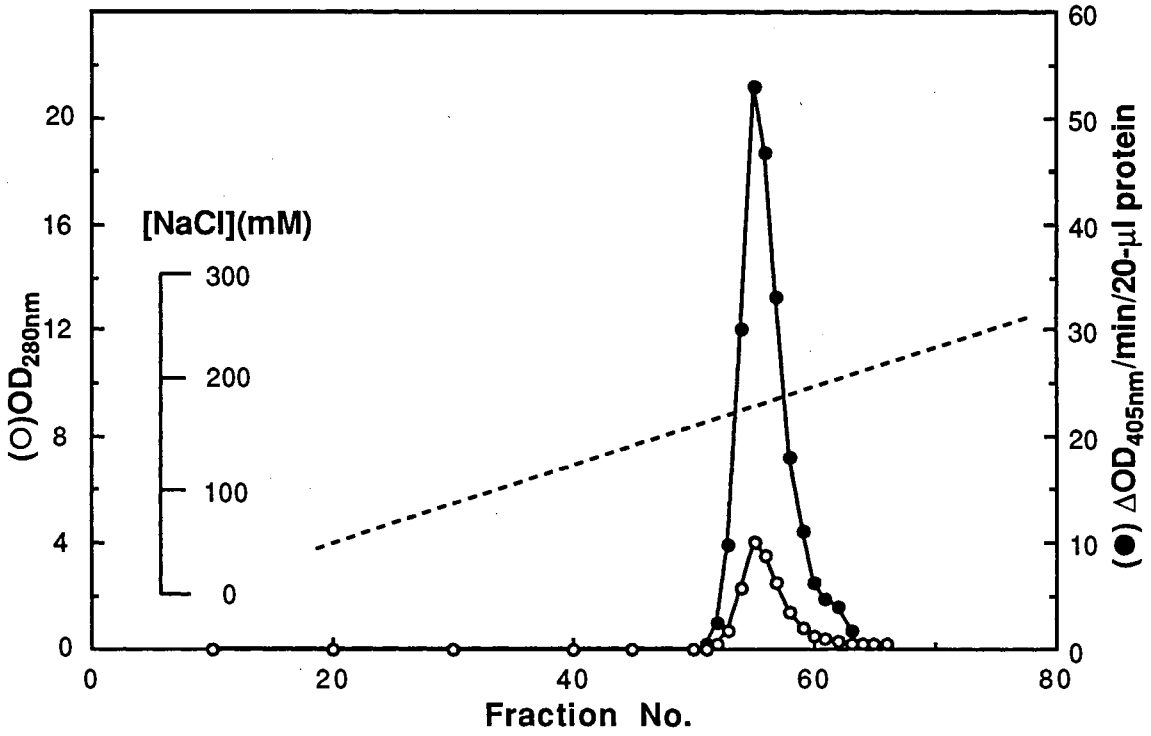


Fig. 7. Chromatogram of *p*-aminobenzamidine-agarose. Each fraction was measured for protein (O) and *p*NB esterase activity using *p*-nitrophenyl acetate as substrate (●). The flow rate of the column was controlled at 24 ml/hr, and 1.2 ml was collected for each fraction.

is electrophoretically pure. The purity of the isolated enzyme is also evident from the chromatogram of the *p*-aminobenzamidine-agarose column. Since the ratios between esterase activity (ΔOD_{405nm}/min/20-µl protein) and protein ($OD_{280\ nm}$) are equal across the fractions encompassed by the single peak in Figure 7, the preparation from affinity chromatography exhibits a constant specific activity. This criterium indicates the enzyme is homogeneous. The purified enzyme appears to be monomeric because the molecular weight determined from SDS-PAGE coincides with that determined from gel filtration (Fig. 9). There is no detectable prosthetic group in the purified enzyme.

Amino Acid Composition and N-terminal Amino Acid Sequence of *p*NB Esterase-- Table II shows the amino acid composition of purified *p*NB esterase. The predominant amino acid residues were leucine, alanine, glutamic acid and proline. The purified enzyme has an acidic pI of 4.1 (Fig.10). This is in line with the amino acid composition, which contains 61 acidic residues and 37 basic residues. The N-terminal amino acid sequence of the esterase was determined to be: NH_2- Met-Thr-His-Gln-Ile-Val-Thr-Thr-Gln-Tyr-Gly-Lys-Val-Lys-Gly-Thr-Thr-Glu-Asn-Gly-Val-His-.

Substrate Specificity of *p*NB Esterase-- Since most esterases isolated from various sources exhibit broad substrate specificity, it is of interest to see whether or not *p*NB esterase has the same property. The purified enzyme catalyzes hydrolysis of β-lactam antibiotic-*p*NB esters and also hydrolyzes simple organic esters (Table III). The esterase hydrolyzes simple organic esters about 100 times faster than the antibiotic-*p*NB esters that were tested, based on the estimated V_{max} for antibiotic esters. The higher K_m value for β-lactam antibiotic-*p*NB esters than for *p*-nitrophenylacetate indicates the *p*NB esterase has higher affinity for *p*-nitrophenylacetate than β-lactam antibiotics-*p*NB esters. It should be noted that the limited solubility of these antibiotic esters in aqueous solution made the estimation of the K_m and maximal enzyme activity for the antibiotic esterase difficult.

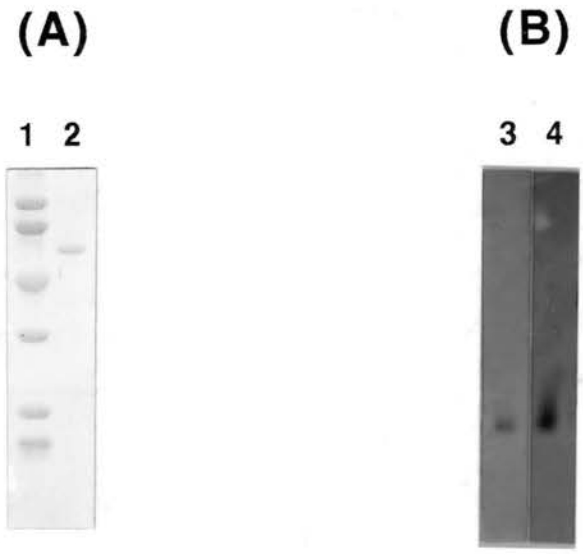


Fig. 8. SDS polyacrylamide gel- and non-denaturing gel-electrophoresis. (A), SDS-PAGE of protein molecular weight standard (lane 1) and purified esterase (lane 2). Proteins were stained with coomassie blue. A 12.5% polyacrylamide gel was used. (B) non-denaturing polyacrylamide gel electrophoresis of purified esterase stained for protein (lane 3, 2 μg) and stained for activity (lane 4, 0.1 μg). A 7% polyacrylamide gel was used. Electrophoresis was carried out at 4° C with a constant current, 20 mA, for 3 hours.

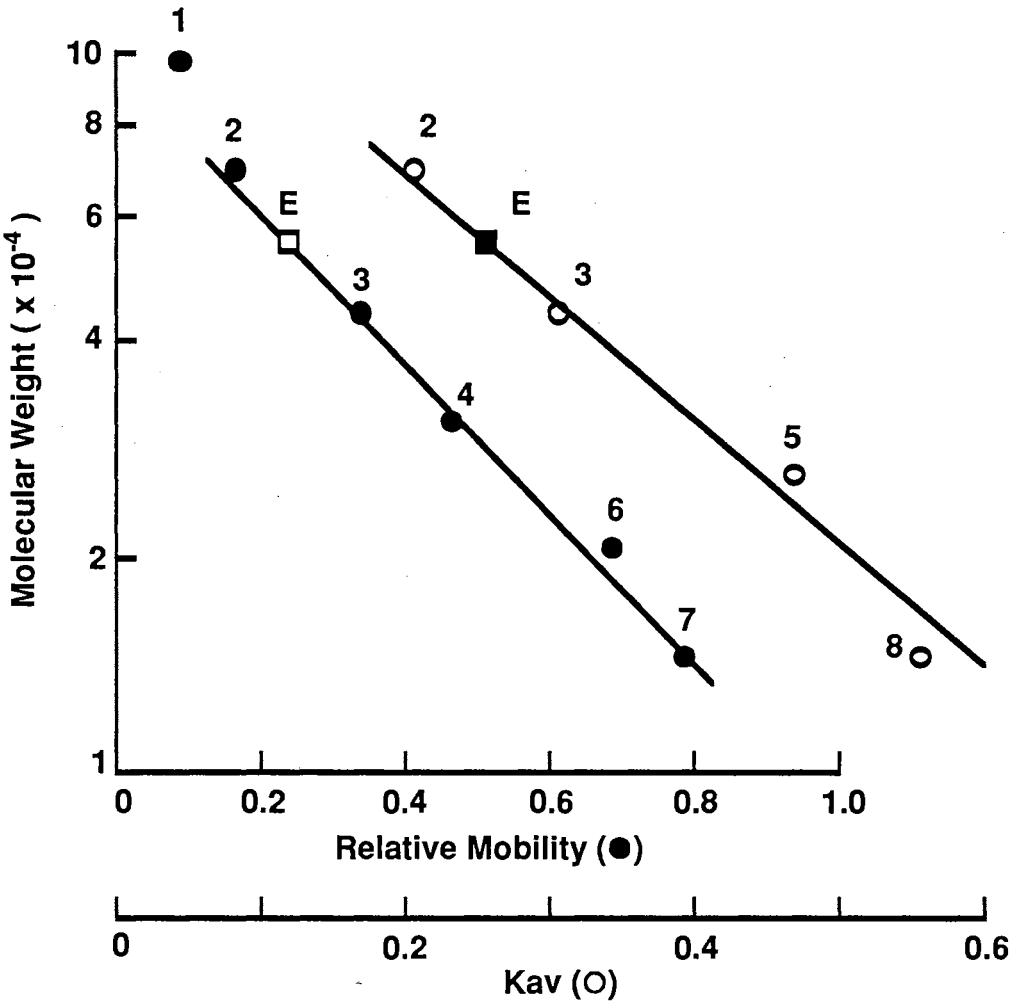


Fig. 9. Determination of the molecular weight of *p*NB esterase. A. By SDS-polyacrylamide gel electrophoresis. The purified *p*NB esterase (4 μg) and protein standards were treated with 2% SDS and 5% β-mercaptoethanol at 100 °C for 5 min and applied to a 12.5% gel according to the method of Weber and Osborn. The protein standards used were: (1) phosphorylase b (97,400); (2) bovine serum albumin (66,200); (3) ovalbumin (45,000); (4) carbonic anhydrase (31,000); (6) Trypsin inhibitor (21,500); (7) lysozyme (14,400). B. By FPLC. The purified esterase (0.1 mg in 0.2 ml) was applied to a FPLC superose 12 column (1 x 30 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.5, containing 0.15 M NaCl. Blue dextran 2000 was used for the estimation of void volume. The protein standards used were: (2) bovine serum albumin; (3) ovalbumin; (5) chymotrypsinogen; (8) ribonuclease A (13,700). *p*NB esterase is shown as E. The molecular weight was estimated from a semilogarithmic plot of molecular weight against mobility and K_{av} respectively.

TABLE II

AMINO ACID COMPOSITION OF *p*NB ESTERASE

The purified esterase was hydrolyzed in 6 N HCl at 110° C for 24 h as described under Experimental Procedures. Amino acids were derivatized with PITC and PTC-amino acids were analyzed by HPLC. The numbers of moles of amino acids per mole protein were calculated based on a protein molecular weight of 54,000.

Amino Acid	Moles residues /mole protein
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	N.D[a].
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	N.D[a].

[a] N.D., not determined

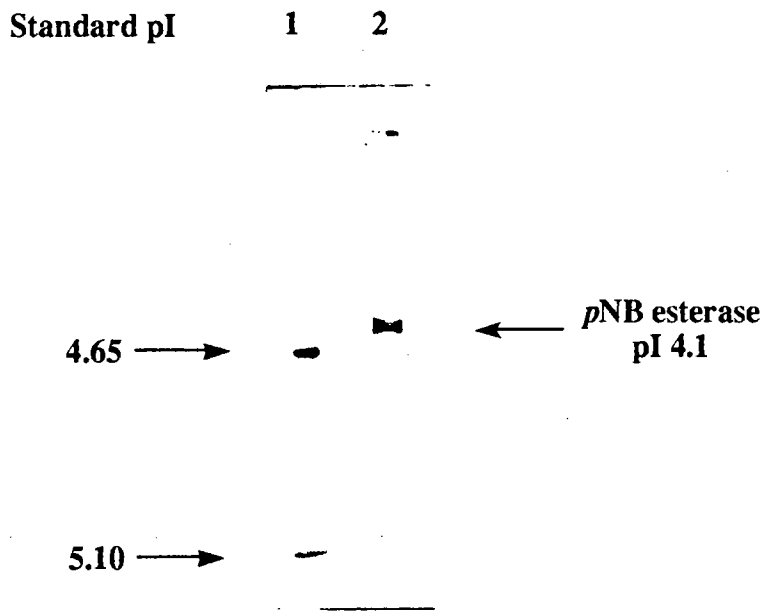


Fig. 10. Isoelectrofocusing of *p*NB esterase. The conditions of isoelectric focusing (IEF) are as described in "Materials and Experimental Procedures". Lane1, IEF standards: Phycocyanin with pI of 4.65 and β-lactoglobulin with pI of 5.10; lane 2, *p*NB esterase stained for activity.

TABLE III

SUBSTRATE SPECIFICITY OF *p*NB ESTERASE

100 % activity refers to the esterase specific activity of 2.1 μmol/min per mg, using 0.5 mM loracarbef-*p*NB (oxalate salt) as substrate. 0.5 mM of substrate [loracarbef-*p*NB(oxalate or tosylate salt), loracarbef nucleus-*p*NB, cephaclor nucleus-*p*NB, cephalexin-*pNB, or* N-benzoyl-DL-phenylalanine-β-naphthylester] in 5 mM Bis Tris propane-HCl buffer, pH 6.5. was incubated with esterase at 37° C for 30 min. The product formation and substrate disappearance were measured by HPLC. Loracarbef nucleus-*p*NB, *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.

Substrate	Concentration (mM)	Relative activity (%)	Km (mM)	Vmax (μmol/min/mg)
Loracarbef-*p*NB (oxalate)	0.5	100	1.1	12.8
Loracarbef-*p*NB (tosylate)	0.5	41	1.0	4.4
Loracarbef nucleus-*p*NB	1.0	59	1.1	6.8
	0.5	35		
Cephaclor nucleus-*p*NB	0.5	16	1.1	2.0
Cephalexin-*p*NB (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	N.D.	N.D.
	2.0	112		
α-Naphthyl acetate	5.0	318	N.D.	N.D.
	2.0	57		
N-benzoyl-DL-phenylalanine-β-naphthylester	0.5	43	N.D.	N.D.

Optimal pH and Stability of *p*NB Esterase-- The optimal pH for esterase activity was found to be around pH 8.3-8.5 when *p*-nitrophenyl acetate (Fig. 11A) or loracarbef-*p*NB (Fig.11B) was used as substrate. Purified *p*NB esterase was stable for several days at 4° C at neutral pH. When *p*NB esterase in 50 mM potassium phosphate buffer, pH 7.0, was incubated at the various temperatures for 10 min, the enzyme suffered only minor loss of activity up to 50° C. At temperatures higher than 50° C the enzyme became denatured rapidly (Fig.12). A 50% inactivation was observed when esterase was incubated at 55° C for 10 min. More than 80% of the esterase activity was lost after a 10 min incubation at 60° C. The thermodenaturation of esterase was not reversible even with prolonged incubation at low temperature.

Inhibitors For *p*NB Esterase -- Table IV lists various compounds (1 mM) which affected the esterase activity. The enzymatic activity of *p*NB esterase was completely inhibited by phenylmethylsulfonylfluoride (PMSF), diethyl *p*-nitrophenylphosphate (DNP), diisopropyl fluorophosphate(DFP), diethylpyrocarbonate(DEPC), mercuric chloride and ferrous sulfate. The inhibition of *p*NB esterase by organophosphorous compounds such as DNP and DFP is characteristic of B-type esterases . However, the complete inhibition of *p*NB esterase by ferrous sulfate is rather difficult to explain at present. The activity was strongly inhibited by phenylglyoxal because phenylglyoxal may act as suicide substrate of *p*NB esterase. The activity was partially inhibited by L-1-*p*-tosylamino-2-phenylethylchloromethyl ketone, dicyclohexyl carbodiimide, 5,5'-dithio-bis(2-nitrobenzoic acid), N-bromosuccimide, *p*-chloromercuribenzene sulfonic acid, methylglyoxal, chloramine T, N-chlorosuccimide, N-benzoyl phenylalanine naphthylamide and ferric chloride.

N-*p*-tosyl-L-lysine chloromethyl ketone, 1-ethyl-(3-dimethyl aminopropyl) carbodiimide, N-ethylmaleimide, iodoacetamide, 2,3-butanedione, cyclohexanedione and N-acetylimidazole do not affect the enzyme activity. Sulfhydryl reagents, such as β-

mercaptoethanol, dithiothreitol and N-acetyl-L-cysteine, also showed no effect on the activity. Since chelating reagents, such as EDTA or EGTA, do not affect the enzyme activity, apparently no metal ion is involved in catalysis by the esterase.

It is worth noting that when *p*NB esterase was treated with the amino group modifying reagent, succinic anhydride, no change in enzymatic activity was observed. This suggests that lysine is not essential for the activity. However, the pH profile of succinylated esterase differed from that of untreated enzyme (Fig. 13). The enzymatic activity of succinylated esterase was 3 times greater at pH 6.0 compared with that of native enzyme at the same pH. The optimal pHs for native esterase and succinylated esterase were about the same. This result suggests that some of the amino groups located on the enzyme surface may limit *p*NB esterase activity at low pH; that is, the protonation of some amino groups on the enzyme surface may affect esterase activity. The succinylation of esterase may prevent protonation of amino groups of the esterase at low pH, thus increasing the activity.

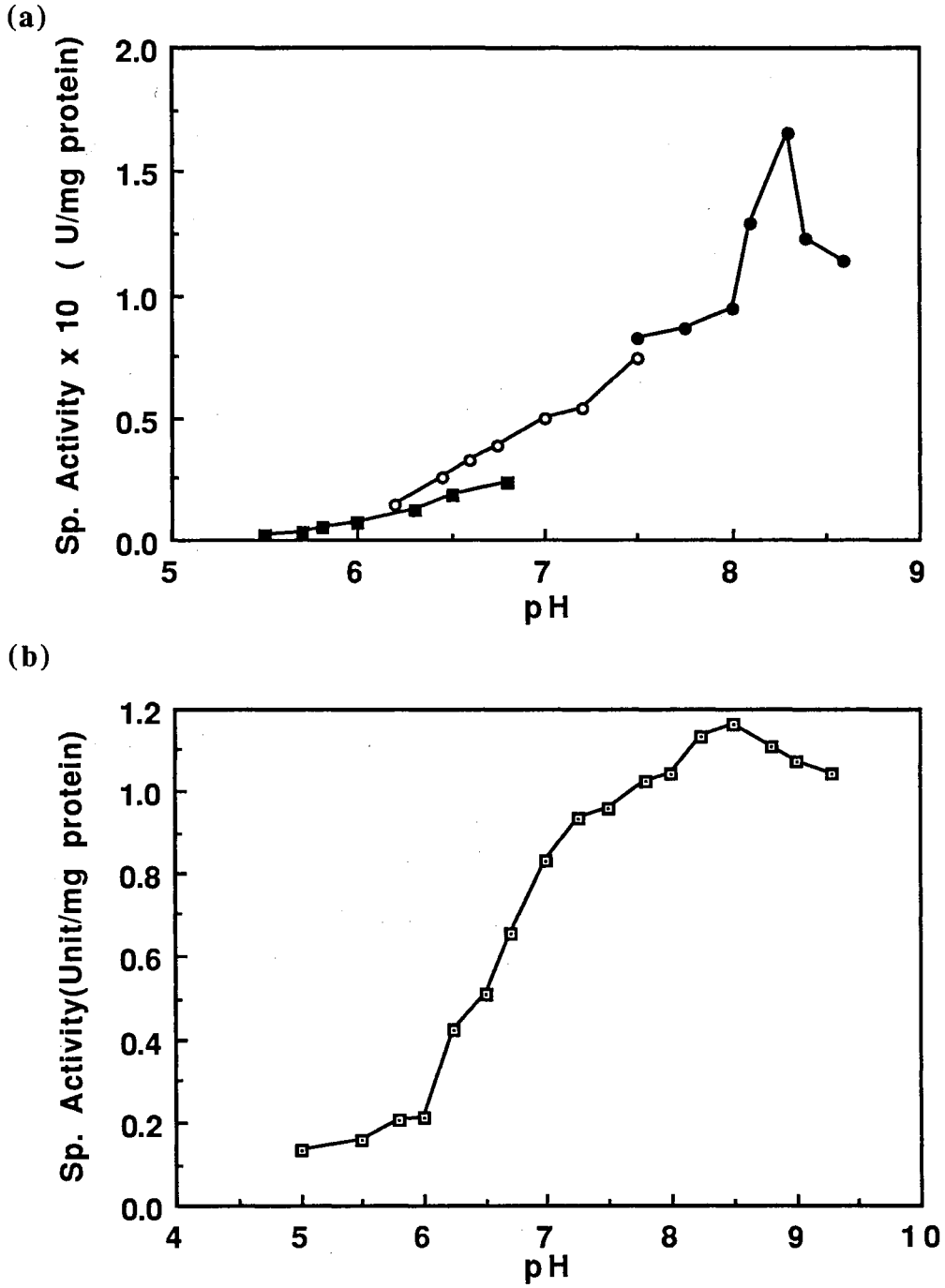


Fig. 11. pH dependent *p*NB esterase activity. (a) The esterase activity was assayed spectrophotometrically, using *p*-nitrophenyl acetate as substrate (25° C). The buffer systems used were as follows: 100 mM sodium acetate, pH 5.7-6.5; 100 mM MES-NaOH, pH 6.2-7.8; 100 mM Tris-HCl, pH 7.5-9.0. (b) The esterase activity was assayed by HPLC, using loracarbef nucleus-*p*NB ester as substrate(37° C). The buffer systems were: 50 mM sodium acetate, pH 5.0 -6.0 and 50 mM Bis-Tris-propane-HCl, pH 6.3-9.2.

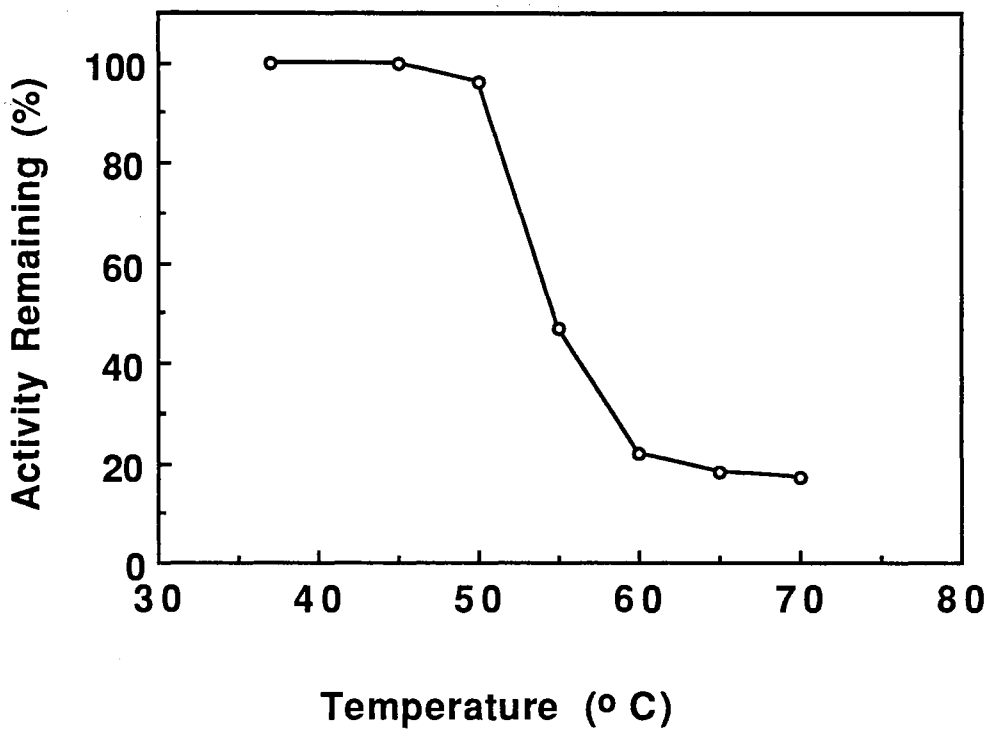


Fig. 12. Heat stability of *p*NB esterase activity. The purified esterase (1 mg/ml) was incubated in 50 mM potassium phosphate, pH 7.0, at the various temperatures indicated for 10 minutes. At the end of incubation, the enzyme solution was immediately chilled in ice water and 10 µl of enzyme solution was used for activity assay using loracarbef nucleus-*p*NB as substrate.

TABLE IV

EFFECTS OF VARIOUS COMPOUNDS ON *p*NB ESTERASE ACTIVITY

The purified esterase, 3.6 mg in 0.975 ml of 5 mM Bis Tris propane-HCl buffer, pH 6.5, was incubated with 1.0 mM of various compounds at 37° C for 5 min. The enzyme activity was subsequently measured by the addition of 25 ml of 20 mM loracarbef-*p*NB oxalate salt in DMSO. The 100 % activity represents the activity in the absence added compound, which was 2.1 µmol/min /mg.

Compound (Conc. 1 mM)	Inhibition(%)
Phenylmethylsulfonylfluoride	100
Diethyl *p*-nitrophenylphosphate	100
Diisopropyl fluorophosphate	100
Diethylpyrocarbonate	100
L-1-*p*-tosylamino-2-phenylethylchloromethyl ketone	29
N-*p*-tosyl-L-lysine chloromethyl ketone	0
Dicyclohexyl carbodiimide	78
1-Ethyl-(3-dimethyl aminopropyl) carbodiimide	0
5,5'-Dithio-bis(2-nitrobenzoic acid)	69
N-bromosuccimide	67
p-Chloromercuribenzene sulfonate	36
N-ethylmaleimide	0
Iodoacetamide	0
Phenylglyoxal	91
Methylglyoxal	13
2,3-butanedione	3
Cyclohexanedione	0
Chloramine T	44
N-chlorosuccimide	16
Maleic anhydride	26
N-acetylimidazole	3
Succinic anhydride	0
N-benzoyl phenylalanine naphtylamide	66
β-Mercaptoethanol	0
EDTA	0
$HgCl_2$	100
$FeSO_4$	100
$Zn(OCOCH_3)_2$	97
$FeCl_3$	87
$MgCl_2$	0
$MnCl_2$	0
$CuSO_4$	0

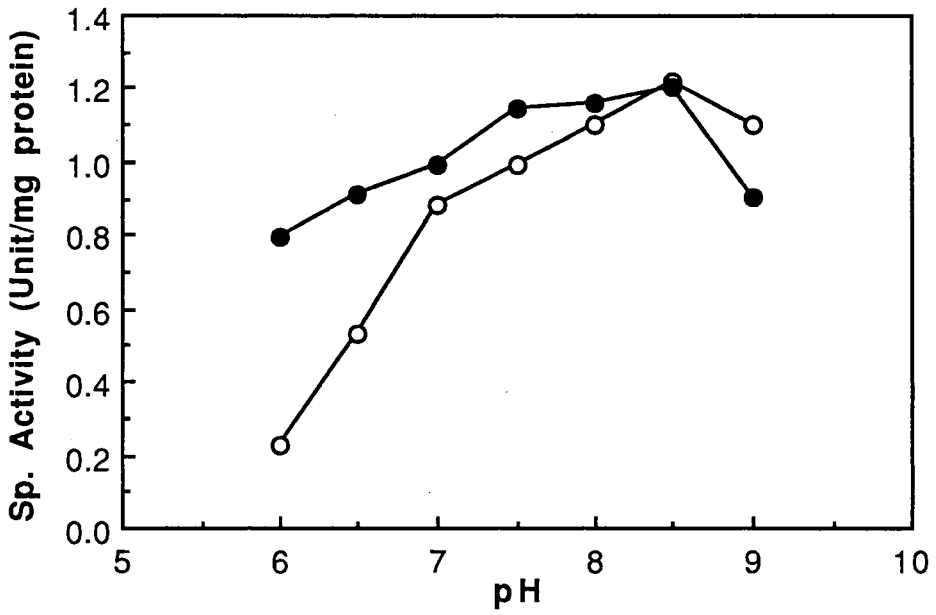


Fig. 13. Comparison of pH profiles between native *p*NB esterase(O) and succinylated *p*NB esterase(●). The esterase (5 mg/ml) in 50 mM potassium phosphate, pH 7.0 was used. Solid succinic anhydride was gradually added to enzyme with stirring, and the pH of succinylated enzyme was neutralized by 0.1 N NaOH. The esterase activity was assayed by HPLC, using loracarbef-*p*NB as substrate (37° C). The buffer system was 50 mM Bis-tris-propane-HCl.

CHAPTER II

CLONING, SEQUENCING, AND EXPRESSION OF *p*-NITROBENZYL ESTERASE

The *p*NB esterase from *Bacillis subtilis* strain NRRL B8079 was purified to homogeneity and characterized (Chapter I). However, the amount of *p*NB esterase present in wild type *B. subtilis* was insufficient for practical use in the manufacture of antibiotics. The cloning of the gene encoding *p*NB esterase and its overexpression in *E. coli* could remove this obstacle. Based on the previously determined partial N-terminal amino acid sequence of *p*NB esterase and codon usage for *B. substilis*, oligonucleotide probes can be designed and synthesized, and used for isolating the *B. substilis* DNA fragment encoding *p*NB esterase by southern hybridization. *E. coli* K12 DH5α, which is a recA$^-$ strain that has been developed to be highly transformable and provide a stable environment for maintenance of *E. coli.* plasmids, was used as host strain for the *B. subtilis* genomic library. The recA$^+$ *E. coli* K12 RV 308, was used for high level expression of the cloned *p*NB esterase gene and is a preferred host for expression of heterologous proteins in *E. coli* on an industrial scale.

MATERIALS AND EXPERIMENTAL PROCEDURES

Culture Media:

Trypticase-soy broth (TSB; Becton Dickinson microbiology systems) and Luria broth (L-broth) were used as growth medium for liquid cultures. LB agar, L-broth

supplemented with 2% w/v agar, was used as solid medium. Antibiotics were added to the medium where necessary, at the following concentrations: ampicillin (100 µg/ml), tetracycline (5 µg/ml). 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; from Sigma Co.) was added to medium at 50 µg/ml to detect β-galactosidase activity.

Transformation of Plasmids into *E. coli* K12 DH5α or *E coli* K12 Rv 308:

E. coli K12 DH5α and Rv308 competent cells were respectively obtained from BRL and Northern Region Research Laboratory of the USDA Service, Peoria, IL 61064. Two hundred µl aliquot of frozen competent *E. coli* cells were thawed on ice and immediately mixed with 20 µl (10-20 ng) of plasmid DNA in a polypropylene tube. The tube was placed on ice for 30 minutes, and transferred to a 42° C water bath for 90 seconds. After this heat-shock treatment, the tube was immediately placed on ice for 3 minutes, 2 ml of L-broth medium (room temperature) were added, and the tube was shaken for 1-1.5 hours at 37° C to allow the cells to recover. The cells were plated directly or concentrated before plating. To concentrate the cells, the culture was centrifuged for 1 min in a microcentrifuge. The supernatant was removed and the cells were suspended in 100 µl of LB-medium. Cells were plated on an LB-agar containing ampicillin and incubated at 37° C for 12-14 hrs. Transformants were confirmed by the size of the insert observed with horizontal agarose gel electrophoresis (29).

Isolation of Genomic DNA from *Bacillus subtilis*

Total DNA was isolated from a two liter culture of *Bacillus subtilis* strain NRRL B8079 grown to logarithmic phase in TSB at 25° C for 16 hrs. Cells were harvested by centrifugation and resuspended in 20 ml of buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA). Lysis of cells was accomplished in a two-step procedure: step 1, addition of lysozyme (1 mg/ml, from egg white, Calbiochem.) and incubation of the suspension at 37°

C for 20 minutes; step 2, addition of 4 ml of lysis buffer (0.5% SDS, 50 mM Tris-HCl pH 7.5, 0.4 M EDTA, 1 mg/ml proteinase K) and incubation at 50° C for 30 minutes. The DNA was purified by two repetitions of phenol extraction followed by one chloroform extraction, and then recovered by ethanol precipitation and resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA). DNase free RNase (bovine pancreatic ribonuclease from Sigma Chemical Co.) was added to a final concentration of 0.2 mg/ml, and the DNA sample was incubated at 37° C for 30 minutes. Further purification was accomplished by reextraction with phenol and chloroform, precipitation with ethanol, and resuspension in TE buffer.

Synthesis and End Labeling of Oligonucleotide Probes:

The partial amino terminal sequence of *p*NB esterase was determined to be : M-T-H-E-I-V-T-T-Y-G-K-K-V-K-G-T-Q-E-N-G-V-H- (chapter I). Based on this sequence and the known codon usage for ***B. subtilis*** (32), two oligonucleotide probes, 41 base "guessmers", called PNB1 and PNB2 were synthesized:

PNB1: 5'-ATGACACATCAAATTGTCACAACATATGGCAAAAAAGTCAA-3'

PNB2: 5'-TATGGCAAAAAAGTCAAAGGCACACAAGAAAATGGCGTCCA-3'

The PNB1 is the probe encoding M-T-H-E-I-V-T-T-Y-G-K-K-V-K, and the PNB2 is the probe encoding Y-G-K-K-V-K-G-T-Q-E-N-G-V-H.

The single stranded oligonucleotides were first column-purified (Oligonucleotide Purification Cartridge, Applied Biosystems. Inc.) and then end-labeled as follows: Ten picomoles of each probe were added to a 20 µl reaction mixture containing 12 µl [γ-^{32}P]-ATP (5000 Ci/ml) and 8 units of T4 polynucleotide kinase. The mixture was incubated at 37° C for 35 minutes, and then incubated at 70° C for 5 min to inactivate the kinase. The unincorporated [γ-^{32}P]-ATP was removed from the reaction mixture using a Sephadex G-

50 NICK Column® (Pharmacia Inc.). The NICK Columns were originally designed for purification of nick-translated DNA fragments but are suitable for separation of any labeled probe from unincorporated labeled nucleotides.

Southern Hybridization of *EcoR* I Restriction Fragments with Oligonucleotide Probes

Bacillus subtilis NRRL B 8079 genomic DNA was digested with *EcoR* I and subjected to a 1 % agarose gel electrophoresis. The size-fractionated DNA was then transferred to a Hybond-N^+ nylon membrane (from Amersham, Arlington, IL) by southern blotting(33), and cross-linked to the matrix by treatment with ultraviolet light for 5 minutes. After prehybridization of DNA at 70° C for 1-2 hours in hybridization buffer containing 6× SSC (1× SSC, pH 7.0, contains 0.15 M sodium chloride, 0.015 M sodium citrate), 5× Dendardt's solution (1x Denhardt's solution contains 0.2 g/l Ficoll 400, 0.2 g/l polyvinylpyrollidone and 0.2 g/l bovine serum albumin), 0.25% SDS, and 20 µg/ml calf thymus DNA, fragmented by treatment with ultrasound. Freshly prepared hybridization buffer containing (^{32}P)-labeled oligonucleotide was then added to produce a final probe concentration of 0.5 pmol/ml. The temperature of the incubation mixture was allowed to cool to 45° C during overnight incubation. The membranes were then washed for 25 minutes, 3 times in succession, at 45 °C in 4× SSC, 0.25% SDS. After washing, the membrane were dried at room temperature and exposed to film.

Polymerase Chain Reaction (PCR) Amplifications:

Polymerase chain reactions were performed using a DNA Thermal Cycler™ and the Gene-Amp™ reagent kit, according to the manufacturer's instructions. Thirty cycles of amplification were performed, with each cycle consisting of the following incubations: 1 minute at 93° C, followed by 2 minutes at 4° C, followed by 4 minutes at 72° C. Syntheses were completed by incubation at 72° C for 10 minutes.

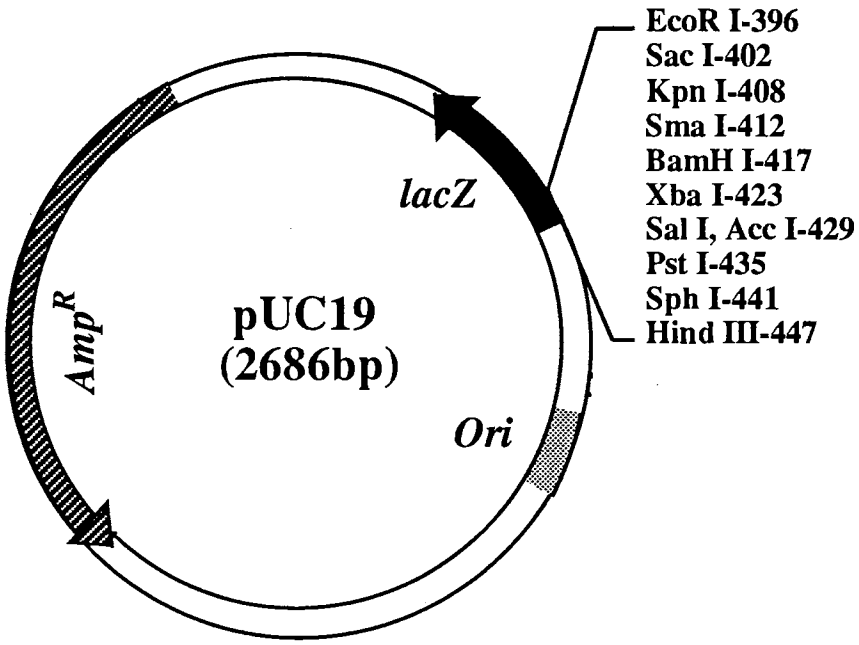


Fig. 14. Plasmid pUC19 circle map. The pUC plasmid contain the pBR322-derived ampicillin resistance gene and origin of replication, together with a portion of the *E. coli lac*Z gene encoding the α-complementation peptide. This *lac* Z fragment has been engineered to contain an array of unique restriction sites, or "multiple cloning site" (MCS). DNA inserted into the MCS inactivates the *lac*Z gene, providing blue/white selection for recombinants when appropriate hosts and indicator plates are used (36).

Construction of pNBE I plasmid:

Bacillus subtilis NRRL B8079 genomic DNA was completely digested with *EcoR* I and subjected to electrophoresis in a horizontal 1.2 % agarose gel (in 1x TAE buffer containing 40 mM Tris-acetate, 1 mM EDTA) at 2V/cm for 16 hours. The gel was stained in a dilute (1 mg/ml) ethidium bromide solution and the DNA bands was visualized under long-wavelength ultraviolet light. A slice was removed from the region of the gel that corresponded to DNA of approximately 6 kb in size, and that had previously been shown to hybridize to PNB 1 and PNB 2. Elution of the DNA fragment from the gel slice was performed according to the protocol of Sambrook *et al.*(34), with minor modification. Briefly, the gel slice was put into a dialysis bag with 200 µl 0.2× TAE buffer, sealed with clips, and electrophoresed in 0.2× TAE buffer for about 3 hours. The contents of the dialysis bag were diluted with 400 µl 0.2× TAE buffer and mixed with 2.4 ml of low salt buffer (0.2 M NaCl; 20 mM Tris-HCl, pH 7.5; 1.0 mM EDTA) and loaded onto the ELUTIP-d column (Schleicher & Schuell , Keene, NH). After washing with low salt buffer, the DNA was eluted from the column with two aliquots 400 µl of high salt buffer (1.0 M NaCl, 200 mM Tris-HCl, pH 7.5; 1 mM EDTA) and precipitated by addition of 800 µl of ice chilled ethanol followed by centrifugation at 14,000 rpm for 40 minutes. The air-dried pellet was dissolved in 20 µl TE buffer and the solution was stored at -20° C until ligation. The fragments were ligated into the vector pUC19 (from Pharmacia Co., see Figure 14) which was pre-treated by digestion with *EcoR* I followed by removal of the 5' phosphates using calf intestinal alkaline phosphatase (from Promega, Madison, WI). The resulting plasmid designated pNBE1 (Fig. 15) was used to transform competent *E. coli* K12 DH5α cells, which were then plated onto LB-agar plates containing ampicillin and X-Gal. The use of plasmid pUC19 as a vector allowed the specific selection of clones containing insert DNA using blue/white selection. Isolates containing an insert at the *EcoR* I site of pUC19 vector disrupt the reading frame of α-peptide of β-galactosidase encoded by *lacZ* gene, and are unable to convert the X-Gal, and thus remain white on media

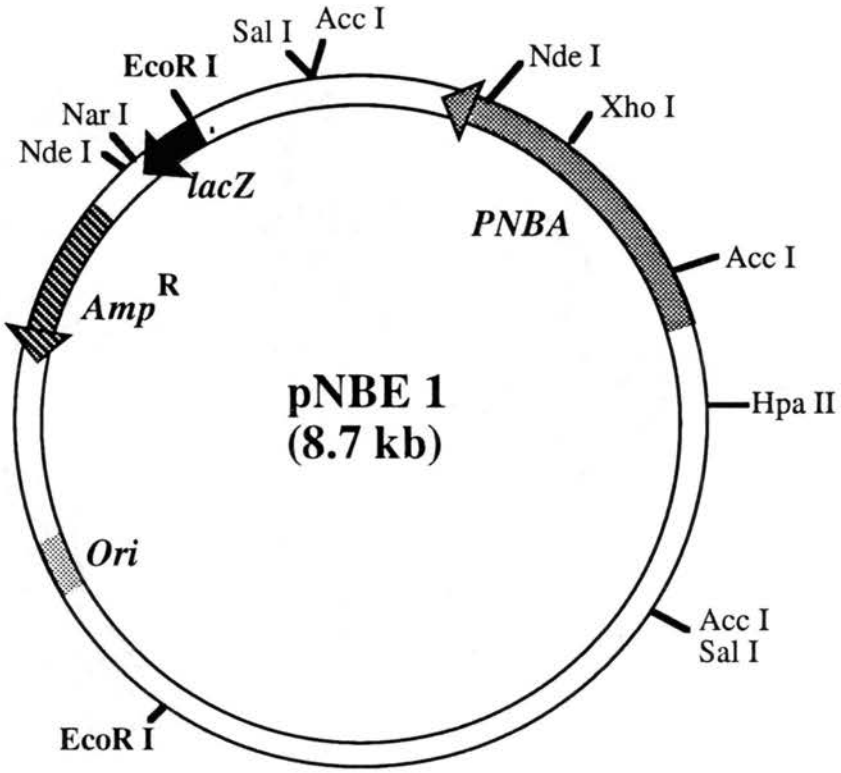


Fig. 15. Plasmid pNBE1 circle map. pNBE1 was constructed by cloning the 6 kb-*EcoRI B. substilis* DNA fragment containing the *pnb*A gene in the *Eco*RI site of pUC19 plasmid.

containing X-Gal. Plasmid DNA was isolated from white colonies using a standard alkaline-SDS procedure(35) and analyzed by gel electrophoresis.

Nucleotide Sequence Analysis

Supercoiled plasmid DNA templates for sequencing were purified from alkaline lysates of *E. coli* cultures. Dideoxynucleotide chain-termination sequencing reactions were performed according to the method of Sanger *et al.*(38) with fluorescent dye-labeled dideoxynucleotides, supercoiled plasmid templates, and sequence specific oligonucleotide primers. Sequence reactions were analyzed on an ABI model 373A automated DNA sequencer. Nucleotide sequences were compiled, edited, and analyzed with the GCG computer programs of Devereux *et al.*(39).

RESULTS AND DISCUSSION

Isolating a *B. subtilis* DNA Fragment Containing *p*NB Esterase Gene (*pnb*A)-- When *B. subtilis* NRRL B8079 DNA was digested with *EcoR* I and analyzed by southern blots, both probe PNB1 and probe PNB2 hybridized to a DNA band 6 kb in size (Figure 17). The 6-kb *EcoR* I fragment was ligated to pUC19 at its unique *EcoR* I site to generate pNBE1 plasmid (Figure 15). Digestion of pNBE1 with *EcoR* I yielded two fragments : one corresponds to linearized pUC19 (2.686 kb), and the other is about 6 kb in size, corresponding to the insert fragment. The 6-kb fragment from pNBE1 hybridized to PNB2 oligo probe. The restriction map of the 6-kb *B. subtilis* DNA fragment is shown in Figure 16.

E coli DH5α cells transformed with plasmid pNBE1 were assayed for esterase activity to determine if pNBE1 carried a complete *p*NB esterase gene. Transformed cells, pNBE1/DH5α, were transferred to a assay mixture containing *p*-nitrophenyl acetate. A

yellow color (indication of *p*-nitrophenol) formed within 5 minutes, whereas the same amount of DH5α or pUC19/DH5α cells produced no visually detectable change. The above observation confirmed that the 6 kb *B. subtilis* DNA fragment contains a functional *p*NB esterase gene.

Two PCR amplifications with pNBE1 as template were performed to determine the direction of *pnbA* transcription, relative to the *lacZ* gene in pNBE1, and to establish the approximate position of the ATG transcription start site of *pnb*A gene in insert DNA fragment. Primers used in the first amplification were PNB2 and pUC/M13 forward sequence primer (from New England Biolab). Primers used in the second amplification were PNB2 and the pUC/M13 reverse sequence primer. Only the reaction with the forward sequencing primer generated an amplified fragment. The size of the amplified fragment was 2.0 kb. Therefore, the ATG start site of *pnbA* is located approximately 2 kb downstream from the pUC19 multiple-cloning site, and *pnbA* is transcribed in the same direction as the *lacZ* gene.

To locate the *pnb*A gene in the 6-kb *Eco*RI fragment, a series of subclonings of pNBE1 were made (see SCHEME III):

(1). Construction of pNBHpE2.3 plasmid-- The 2.3 kb *Eco*RI-*Hpa*II fragment from pNBE plasmid was ligated into the *Eco*RI and *Acc*I sites of pUC19 plasmid to generate pNBHpE2.3 plasmid. In order for the 2.3 kb *Eco*RI-*Hpa*II fragment to be able to ligate into the *Eco*RI and *Acc*I sites of pUC19 plasmid, the *Hpa*II site of the *Hpa*II-*Eco*RI fragment and the *Acc*I site of pUC19 had to be converted to blunt-ends by treatment with Klenow fragment of DNA polymerase I. Therefore, the *Hpa*II site of the insert and the *Acc*I site of pUC19 were not regenerated after ligation. The pNBHpE2.3 plasmid was transformed into *E. coli* K12 DH5α cells. The resulting transformants showed *p*NB esterase activity, indicating that the *pnb*A gene is located in this DNA fragment.

(2). Construction of pNBH3S1.9 plasmid-- An additional subcloning experiment was performed in which the 1.9 kb *Sal* I-*Hin*d III fragment from pNBHpE2.3 was ligated into the *Hind* III and *Sal* I sites of pUC19 to generate pNBH3S1.9 plasmid. Activity assay indicated that this DNA fragment carries the complete *pnb*A gene. This construction established that *Sal* I-*Eco*RI fragment at the distal end of the 2.3 kb insert was not required for a functional *pnbA* gene.

Based on these subcloning and activity assay results, the relative location of *pnb*A gene in the 6-kb *B. subtilis* DNA fragment was established and indicated in Figure 16.

The Nucleotide sequence and Deduced Amino Acid Sequence of *p*NB Esterase-- The 1.9 kb insert from plasmid pNBH3S1.9 was subcloned into pBluescript SK(-) and used to determine the nucleotide sequence of *p*NB esterase(37). Figure 18 shows the nucleotide and deduced amino acid sequences of *p*NB esterase. The structural gene for *p*NB esterase (*pnb*A) has an open reading frame of 1470 nucleotides which encodes 489 amino acid residues. The molecular weight calculated from deduced amino acid sequence of *p*NB esterase is 53,947 Da which is very close to that obtained by physical determinations (54,000 Da estimated by SDS-PAGE and gel filtration).

The first 22 residue of the N-terminal amino acid sequence of *p*NB esterase deduced from the nucleotide sequence (A) was similar to that of the sequence obtained from peptide sequencing (B) except Q9 was Y; Y10 was G, G11 was K and T17 was Q.

(A) M-T-H-Q-I-V-T-T-Q-Y-G-K-V-K-G-T-T-E-N-G-V-H

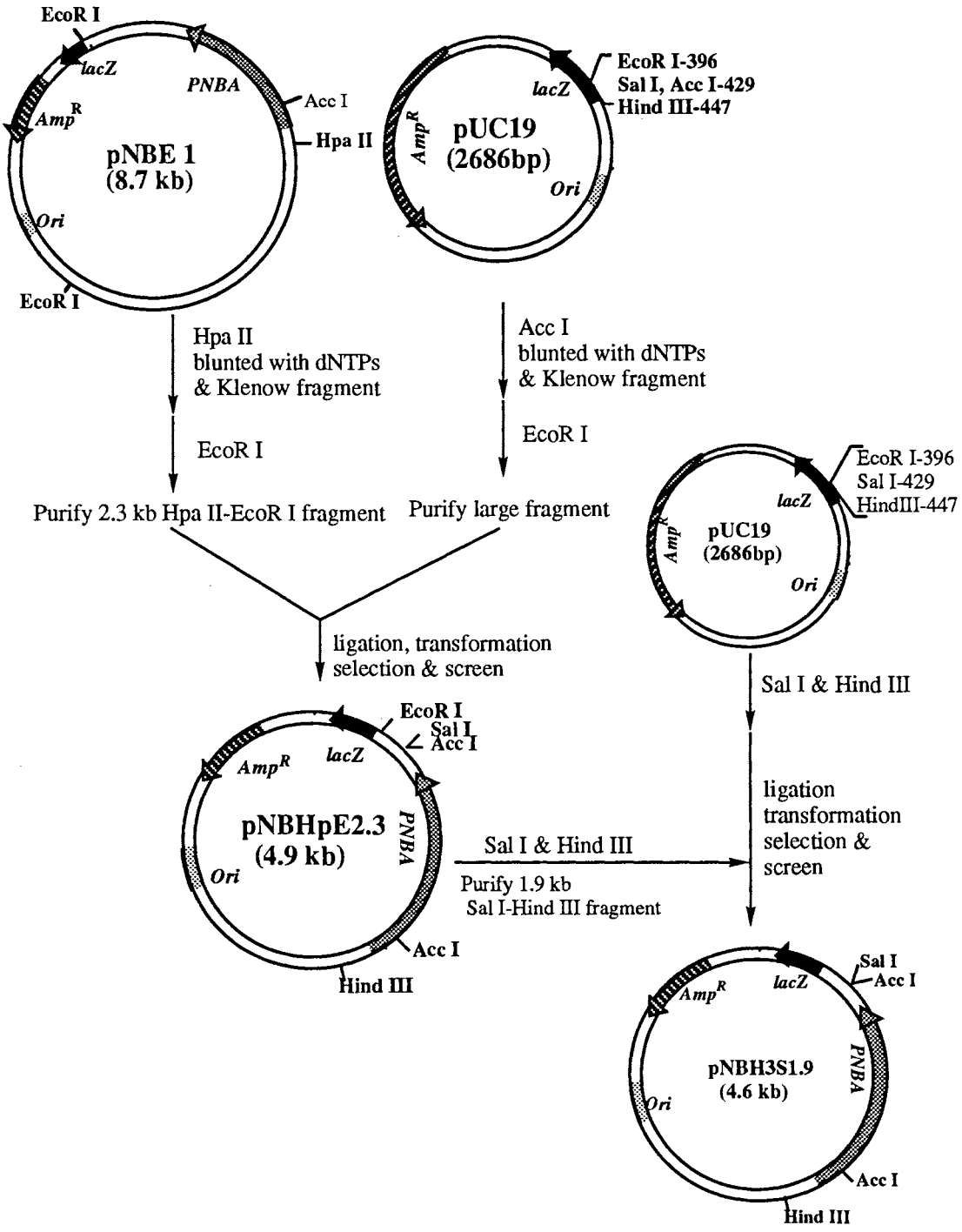
(B) M-T-H-Q-I-V-T-T-Y-G-K-K-V-K-G-T-Q-E-N-G-V-H

The several errors shown in sequence (B) resulted from the incorrect interpretation of the sequencing data. None of the interpretative errors corresponded to the ends of the oligonucleotide probes. Thus 18% error occurred in the chemically determined amino acid

sequence, but this error would not affect our use of this information to design the nucleotide probes.

The amino acid composition of *p*NB esterase based on the deduced amino acid sequence is also very similar to that obtained from chemical analysis except that there were 35 prolines in the deduced sequence versus 47 in the chemical analysis, and 54 alanines versus 35.

Scheme III: Subcloning of *pnbA* Gene



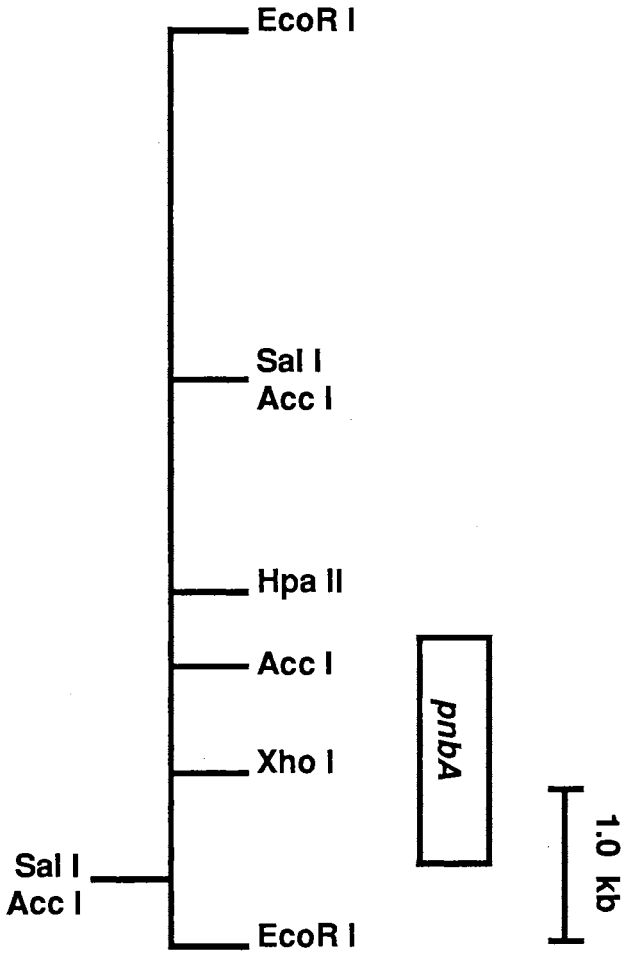


Fig. 16. Partial restriction map of the 6 kb *B. subtilis Eco*R I DNA fragment carrying the *p*NB esterase (*pnbA*) gene. The *open box* indicates the location of the *pnbA* gene.

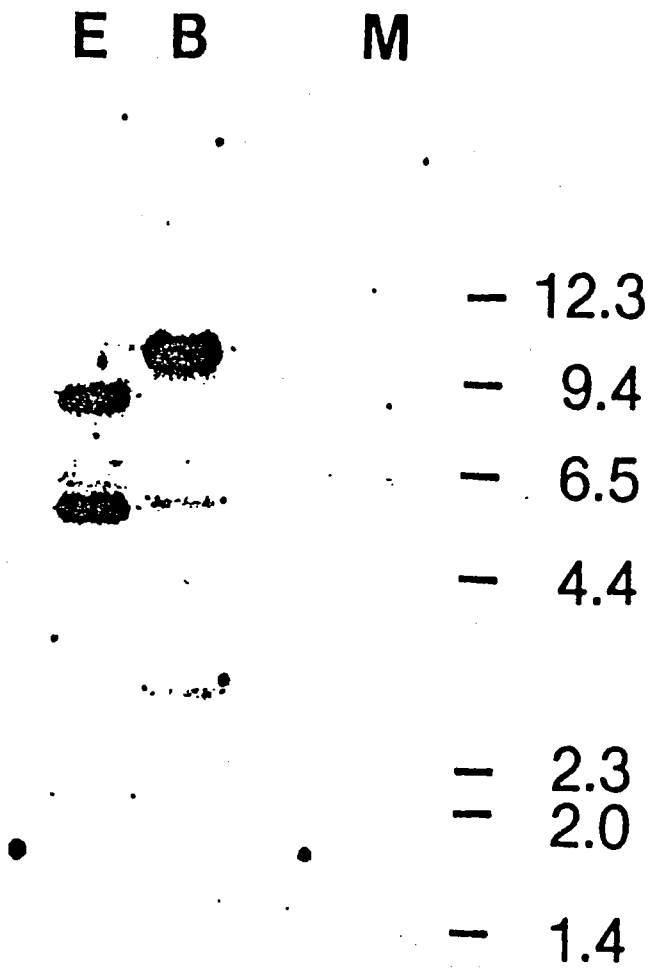


Fig. 17. Autoradiogram from southern blot of *B. subtilis* DNA digested with *EcoR* I(E) or *Bgl* II(B). The probe used for hybridization is PNB2.

```
ATG ACT CAT CAA ATA GTA ACG ACT CAA TAC GGC AAA GTA AAA GGC ACA ACG GAA
Met Thr His Gln Ile Val Thr Thr Gln Tyr Gly Lys Val Lys Gly Thr Thr Glu
1                                                                    18
AAC GGC GTA CAT AAG TGG AAA GGC ATC CCC TAT GCC AAG CCG CCT GTC GGA CAA
Asn Gly Val His Lys Trp Lys Gly Ile Pro Tyr Ala Lys Pro Pro Val Gly Gln
                                                                     36
TGG CGT TTT AAA GCA CCT GAG CCG CCT GAA GTG TGG GAA GAT GTC CTT GAT GCC
Trp Arg Phe Lys Ala Pro Glu Pro Pro Glu Val Trp Glu Asp Val Leu Asp Ala
                                                                     54
ACA GCG TAC GGT CCT ATT TGC CCG CAG CCG TCT GAT TTG CTC TCA CTG TCG TAT
Thr Ala Tyr Gly Pro Ile Cys Pro Gln Pro Ser Asp Leu Leu Ser Leu Ser Tyr
                                                                     72
ACA GAG CTG CCC CGC CAG TCC GAG GAT TGC TTG TAT GTC AAT GTA TTT GCG CCT
Thr Glu Leu Pro Arg Gln Ser Glu Asp Cys Leu Tyr Val Asn Val Phe Ala Pro
                                                                     90
GAC ACT CCA AGT CAA AAT CTT CCT GTC ATG GTG TGG ATT CAC GGA GGC GCT TTT
Asp Thr Pro Ser Gln Asn Leu Pro Val Met Val Trp Ile His Gly Gly Ala Phe
                                                                    108
TAT CTT GGA GCG GGC AGT GAG CCA TTG TAT GAC GGA TCA AAA CTT GCG GCA CAG
Ser Lys Leu Ala Ala Gln Gly Glu Val Ile Val Val Thr Leu Asn Tyr Arg Leu
                                                                    126
GGA GAA GTC ATT GTC GTT ACA TTG AAC TAT CGG CTG GGG CCG TTT GGC TTT TTG
Phe Gly Phe Leu His Leu Ser Ser Phe Asp Glu Ala Tyr Ser Asp Asn Leu Gly
                                                                    144
CAC TTG TCT TCG TTT GAT GAG GCG TAT TCC GAT AAC CTT GGG CTT TTA GAC CAA
Asp Gln Ala Ala Ala Leu Lys Trp Val Arg Glu Asn Ile Ser Ala Phe Gly Gly
                                                                    162
GCC GCC GCG CTG AAA TGG GTG CGG GAG AAT ATC TCA GCG TTT GGC GGT GAT CCC
Tyr Leu Gly Ala Gly Ser Glu Pro Leu Tyr Asp Gly Gly Pro Leu Leu Asp Pro
                                                                    180
GAT AAC GTA ACA GTA TTT GGA GAA TCC GCC GGC GGC ATG AGC ATT GCC GCG CTG
Asp Asn Val Thr Val Phe Gly Glu Ser Ala Gly Gly Met Ser Ile Ala Ala Leu
                                                                    198
CTC GCT ATG CCT GCG GCA AAA GGC CTG TTC CAG AAA GCG ATC ATG GAA AGC GGC
Leu Ala Met Pro Ala Ala Lys Gly Leu Phe Gln Lys Ala Ile Met Glu Ser Gly
                                                                    216
GCT TCC CGA ACA ATG ACA AAA GAA CAA GCG GCA AGC ACT GCG GCT GCC TTT TTA
Ala Ser Arg Thr Met Thr Lys Glu Gln Ala Ala Ser Thr Ala Ala Ala Phe Leu
                                                                    234
CAG GTC CTT GGG ATT AAT GAG AGC CAG CTG GAC AGA TTG CAT ACT GTA GCA GCG
Gln Val Leu Gly Ile Asn Glu Ser Gln Leu Asp Arg Leu His Thr Val Ala Ala
                                                                    252
GAA GAT TTG CTT AAA GCG GCC GAT CAG CTT CGG ATT GCA GAA AAA GAA AAT ATC
Glu Asp Leu Leu Lys Ala Ala Asp Gln Leu Arg Ile Ala Glu Lys Glu Asn Ile
                                                                    270
TTT CAG CTG TTC TTC CAG CCC GCC CTT GAT CCG AAA ACG CTG CCT GAA GAA CCA
Phe Gln Leu Phe Phe Gln Pro Ala Leu Asp Pro Lys Thr Leu Pro Glu Glu Pro
                                                                    288
GAA AAA TCG ATC GCA GAA GGG GCT GCT TCC GGC ATT CCG CTA TTG ATT GGA ACA
Glu Lys Ser Ile Ala Glu Gly Ala Ala Ser Gly Ile Pro Leu Leu Ile Gly Thr
                                                                    306
ACC CGT GAT GAA GGA TAT TTA TTT TTC ACC CCG GAT TCA GAC GTT CAT TCT CAG
Thr Arg Asp Glu Gly Tyr Leu Phe Phe Thr Pro Asp Ser Asp Val His Ser Gln
                                                                    324
GAA ACG CTT GAT GCA GCA CTC GAG TAT TTA CTA GGG AAG CCG CTG GCA GAG AAA
Glu Thr Leu Asp Ala Ala Leu Glu Tyr Leu Leu Gly Lys Pro Leu Ala Glu Lys
                                                                    342
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GCT GCC GAT TTG TAT CCG CGT TCT CTG GAA AGC CAA ATT CAT ATG ATG ACT GAT
Ala Ala Asp Leu Tyr Pro Arg Ser Leu Glu Ser Gln Ile His Met Met Thr Asp
                                                                    360
TTA TTA TTT TGG CGC CCT GCC GTC GCC TAT GCA TCC GCA CAG TCT CAT TAC GCC
Leu Leu Phe Trp Arg Pro Ala Val Ala Tyr Ala Ser Ala Gln Ser His Tyr Ala
                                                                    378
CCT GTC TGG ATG TAC CGG TTC GAT TGG CAC CCG GAG AAG CCG CCG TAC AAT AAA
Pro Val Trp Met Tyr Arg Phe Asp Trp His Pro Glu Lys Pro Pro Tyr Asn Lys
                                                                    396
GCG TTT CAC GCA TTA GAG CTT CCT TTT GTC TTT GGA AAT CTG GAC GGA TTG GAA
Ala Phe His Ala Leu Glu Leu Pro Phe Val Phe Gly Asn Leu Asp Gly Leu Glu
                                                                    414
CGA ATG GCA AAA GCG GAG ATT ACG GAT GAG GTG AAA CAG CTT TCT CAC ACG ATA
Arg Met Ala Lys Ala Glu Ile Thr Asp Glu Val Lys Gln Leu Ser His Thr Ile
                                                                    432
CAA TCC GCG TGG ATC ACG TTC GCT AAA ACA GGA AAC CCA AGC ACC GAA GCT GTG
Gln Ser Ala Trp Ile Thr Phe Ala Lys Thr Gly Asn Pro Ser Thr Glu Ala Val
                                                                    450
AAT TGG CCG GCG TAT CAT GAA GAA ACG AGA GAG ACG GTG ATT TTA GAC TCA GAG
Asn Trp Pro Ala Tyr His Glu Glu Thr Arg Glu Thr Val Ile Leu Asp Ser Glu
                                                                    468
ATT ACG ATC GAA AAC GAT CCC GAA TCT GAA AAA AGG CAG AAG CTA TTC CCT TCA
Ile Thr Ile Glu Asn Asp Pro Glu Ser Glu Lys Arg Gln Lys Leu Phe Pro Ser
                                                                    486

AAA GGA GAA TAA
Lys Gly Glu
        489
```

Fig.18. The nucleotide and deduced amino acid sequences of *p*NB esterase.

TABLE V

COMPARISON OF THE AMINO ACID COMPOSITION OF *p*NB ESTERASE OBTAINED FROM CHEMICAL ANALYSIS AND DEDUCED DNA SEQUENCE.

The purified esterase was hydrolyzed in 6N HCl at 110° C for 24 h as described under "Experimental Procedures". Amino acids were derivatized with PITC and PTC-amino acids were analyzed by HPLC. The numbers of moles of amino acids per mole protein were calculated based on a protein molecular weight of 54,000.

Amino Acid	Moles residues /mole protein	
	Chemical analysis	Deduced DNA sequence
Asx	39.6	40
Thr	31.2	28
Ser	28.9	31
Glx	61.3	61
Pro	46.6	35
Gly	31.6	32
Ala	46.9	54
Val	24.3	25
Cys	N.D[a].	2
Met	8.9	10
Ile	19.4	21
Leu	48.6	50
Tyr	15.7	17
Phe	22.1	22
Lys	23.3	25
His	12.3	12
Arg	15.3	14
Trp	N.D[a].	10

[a] N.D., not determined

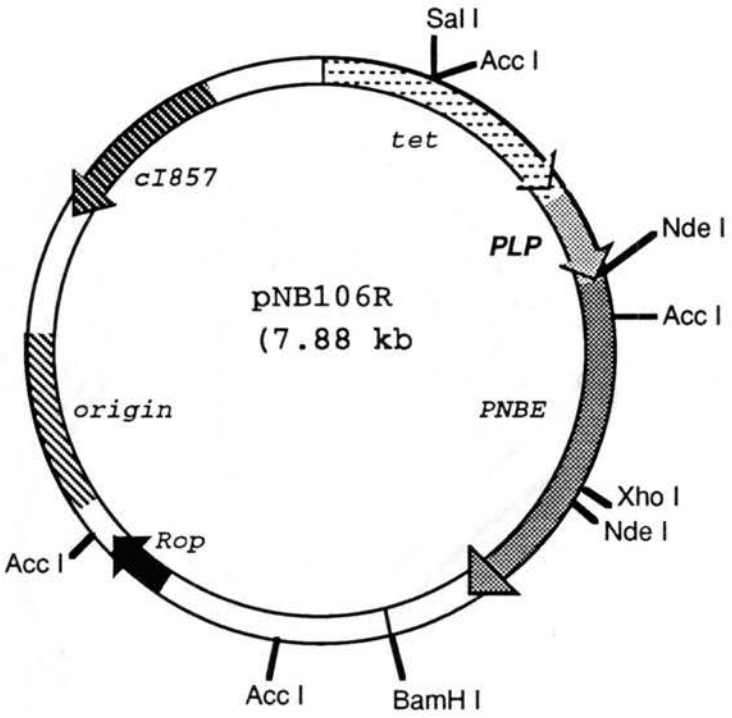


Fig. 19. Functional and restriction map of expression vector pNB106R. Construction of pNB106R is as described in the experimental procedures and scheme IV.

Expression of *p*NB Esterase in *E. Coli*:

(1). Construction of *p*NB esterase Expression Vector, pNB106R-- Expression of the cloned *p*NB esterase gene in *E. coli.* was achieved by construction of the expression vector pNB106R (Figure 19). The pNB106R plasmid contains a functional *rop* gene, responsible for control of plasmid copy number (40). The transcription of the *pnbA* gene is under control of the temperature inducible lambda pL106 promoter which is controlled by the cI857 repressor of λ. At low temperature (29-31° C) the cI857 repressor maintains the promoter in the repressed state. Raising the temperature destroys the repressor activity and allows extensive transcription from the pL106 promoter.

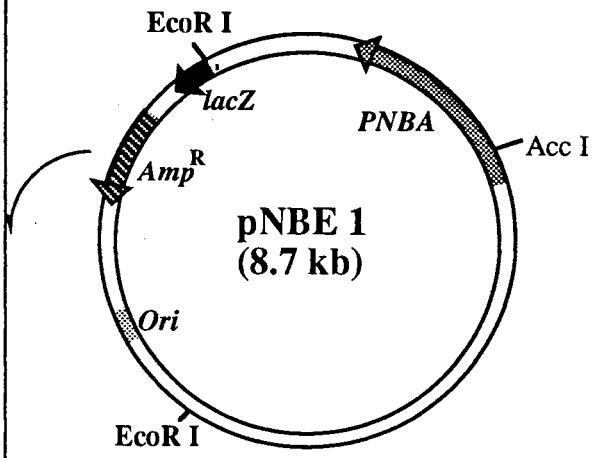
Scheme IV shows the strategy for construction of pNB106R plasmid. The pHKY338 plasmid, which is a temperature inducible high level expression vector (41-48), was used for the construction of the *p*NB esterase expression vector, pNB106R. Genetic elements of pHKY338 include: (i) a sequence derived from pBR322 which allows replication and maintenance of the plasmid in *E. coli,* and (ii) a lambda cI875 repressor gene that replaces the ampicillin resistance gene of pBR322; and (iii) a sequence in which a modified lambda promoter, pL106 (49), is situated downstream of the pBR322 tetracycline resistance gene and immediately adjacent to the ORF of a kanamycin resistance gene [see scheme IV-(c)]. The promoter controls transcription of this ORF via an "out-of-frame" two cistrom assembly (50). Plasmid pHKY338 was constructed so that the ORF of the kanamycin resistance gene can easily be removed by digestion with *Nde* I and *BamH* I. Any ORF with an *Nde*I restriction site at its ATG transcriptional start codon and a *BamH*I compatible restriction site downstream from its transcriptional termination sequences, can be inserted into pHKY338. The resulting inserted ORF will be aligned correctly for expression from the bacteriophage lambda pL promoter.

Since the cloned 1.9 kb *Sal* I- *Hind* III DNA fragment containing the *pnb*A gene in pBNH3S1.9 did not contain an *Nde*I site and *Bam*HI site at the desired locations, these

Scheme IV-(a): Construction of a 210 bp fragment coding for the amino-terminus of *p*NB esterase

AAAAAGGGAGAGAACCATATGACTCACCTAG

TTGACATACAAGCAATCCTC



Annealing
PCR Amplification
Purify 261 bp fragment

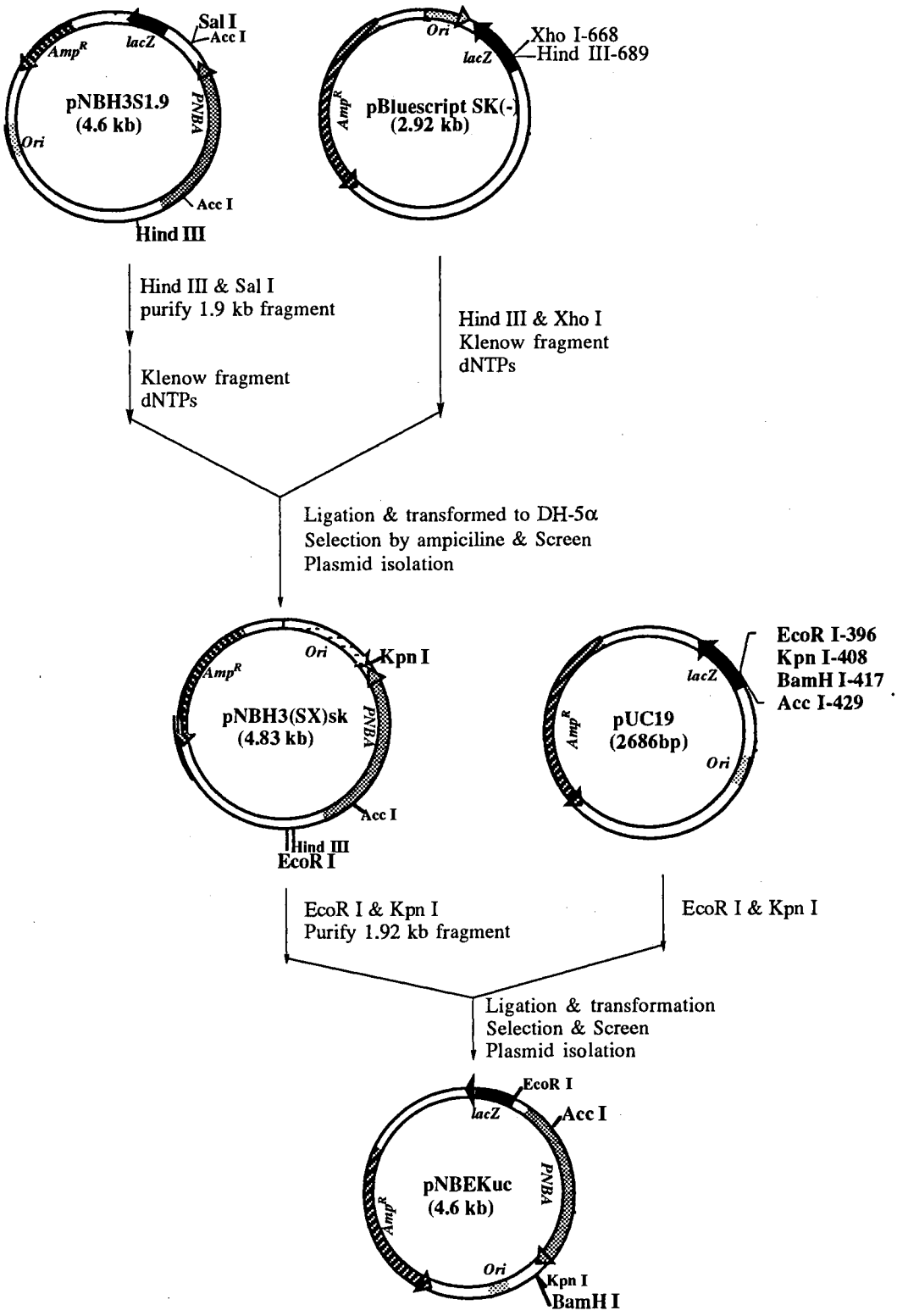
261 bp fragment

Nde I & Acc I

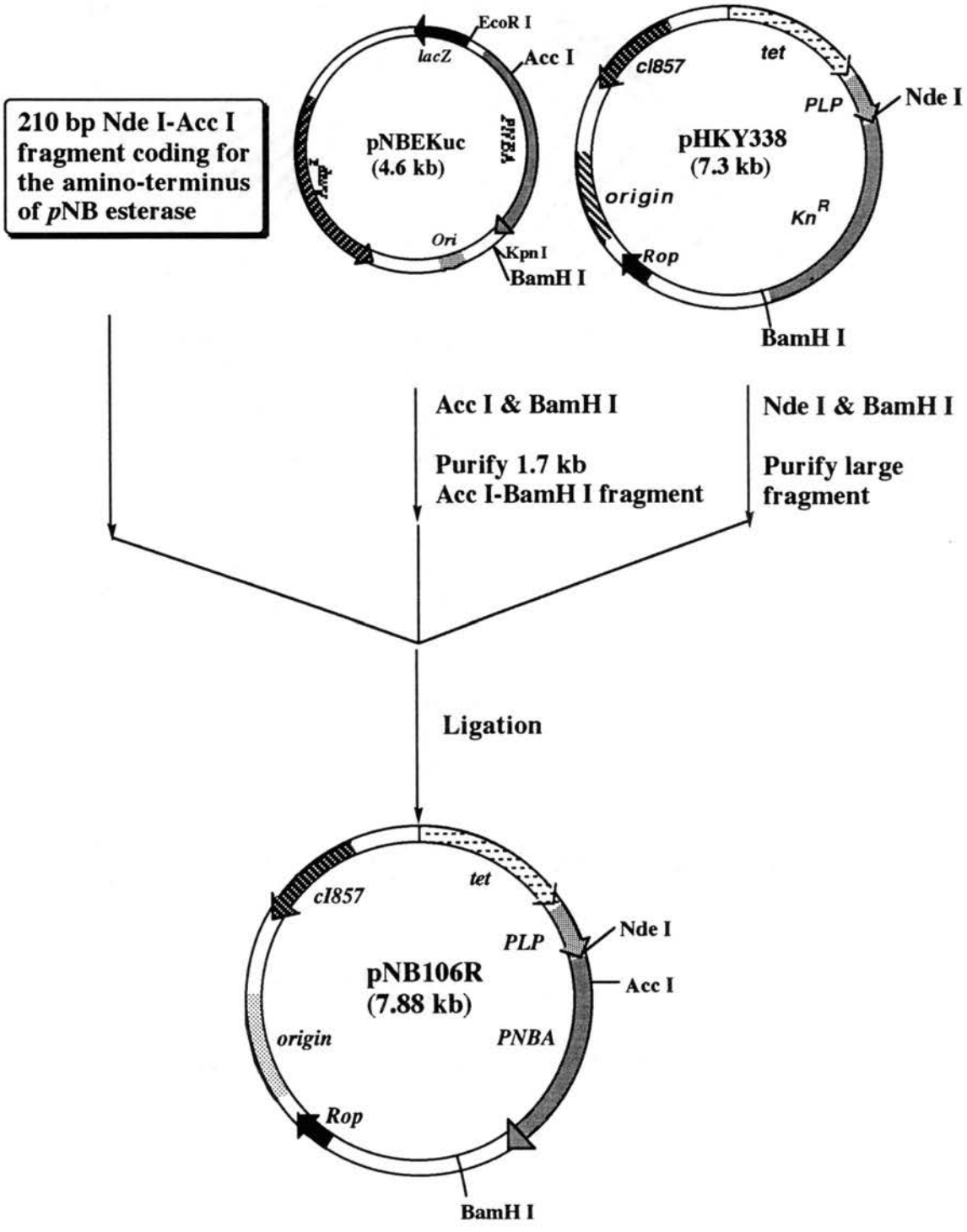
Purify 210 bp fragment

210 bp Nde I-Acc I fragment

Scheme IV-(b): Construction of pNBEKuc & 1.7 kb *Acc* I-*BamH* I fragment encoding the carboxyl terminal region of *p*NB esterase



Scheme IV-(c): Construction of pNB106R



two sites needed to be created in order to ligate into pHKY338. We used the *Acc*I site present in the *pnb*A gene to splice the *pnb*A gene into amino-terminal and carboxyl-terminal fragments. The *Nde*I recognition sequence was introduce on the first Met residue of the N-terminal fragment and the *Bam*HI site was introduced to the C-terminal fragment. These two fragments were rejoined through the *Acc*I site to generate the *Nde*I-*Bam*HI fragment containing the *pnbA* gene and ligated into the *Nde*I and *Bam*HI sites of pHKY338.

(a). Amino-terminal fragment : A 261 bp fragment encoding the amino-terminus of *p*NB esterase, containing an *Nde*I recognition sequence on the ATG transcription start site, was obtained by PCR amplification using two primers with the following sequences : PNBNDE1, 5'AAAAAGGGAGAGAACCATATGACTCATCAAATAG3'; and PNB5, 5'TTGACATACAAGCAATCCTC3'. PNB5 primer anneals approximately 25 base pairs downstream from the *Acc*I site located 215 bp downstream from the ATG start codon. The 261 bp fragment obtained from PCR amplification was treated with proteinase K (51) and subsequently digested with *Nde* I and *Acc*I, to generate 210 bp fragment with an *Nde*I site in the ATG transcription start site[see scheme IV-(a)].

(b). Carboxy-terminal fragment: Since the construction strategy required the use of the *Acc*I site internal to the ORF to splice the amino-terminal and carboxyl-terminal fragments, it was necessary to eliminate the other *Acc*I site (*Sal*I/*Acc*I) on the 1.9 kb fragment. This was accomplished as follows [Scheme IV(b)]: The pNBH3S1.9 plasmid was digested with *Hind* III and *Sal*I to release the *pnb*A-containing fragment, the protruding 5' end resulting from *Sal*I digestion was converted to a blunt end with Klenow fragment. This fragment was ligated into the *Hind* III and *Xho*I (converted to blunt end) sites of pBluescritp SK(-) plasmid. The resulting plasmid, pNBH3(SX)sk, eliminated all three sites, *Acc*I/*Sal*I, and *Xho*I.

The pNBH3(SX)sk plasmid was digested with *EcoR*I and *Kpn*I, to release the *pnb*A gene-containing fragment. The *EcoR*I-*Kpn*I fragment was ligated into the *EcoR*I and

*Kpn*I sites of pUC19 to generate pNBEKuc plasmid in which the *Bam*HI site is located just downstream from the *Kpn*I site (C-terminal end).

Plasmid pHKY338 was digested with *Nde*I and *BamH*I to remove the kanamycin resistance gene, and the large fragment was gel isolated. The 210-bp *Nde*I-*Acc*I fragment and 1.7-kb *Acc*I-*Bam*HI fragment from pNBEKuc were ligated together and inserted into the*Nde*I and *Bam*HI sites of pHKY338 to generate pNB106R as shown in scheme IV-(c).

(2). Production of *p*NB Esterase in *E. coli*-- The pNB106R plasmid was transformed into *E. coli* DH5α and *E. coli* RV308. *E. coli* RV308/pNB106R, or *E.coli* DH5α/pNB106R cells were grown in liquid medium at 30° C with vigorous shaking. The temperature of the cell culture was shifted from 30° C to 40° C to induce the production of *p*NB esterase. The production of esterase increased as the incubation time of cell culture at 40° C increased (Figure 20). Maximum production of *p*NB esterase was reached after 8 hours of temperature shift; after that, the production decreased. No *p*NB esterase activity was found in *E. coli* DH5α/pNB106R cells without temperature induction. *p*NB esterase activity was also not observed in *E. coli* DH5α/pHKY338 cells with or without temperature shift.

*p*NB esterase was highly expressed in *E. coli* cells. The *p*NB esterase activity, measured with loracarbef nucleus-*p*NB ester as substrate, in cell free extracts obtained from induced *E. coli* DH5α/pNB106R cells was 191 mU/mg protein and that from induced *E. coli.* RV308/pNB106R was 340 mU/mg protein. It should be noted that the specific activity of *p*NB esterase in the cell extract obtained from an optimized *B. subtilis* fermentation was about 1.1 mU/mg protein. Based on the specific activity of 1300 mU/mg for *p*NB esterase purified from *B. subtilis* using loracarbef nucleus-*p*NB ester as substrate, *p*NB esterase accounts for 15% and 26% of the protein in cell extracts of induced *E. coli* DH5α/pNB106R and RV308/pNB106R, respectively.

The enzymatic activity of expressed *p*NB esterase in cell-free extract toward various substrates was similar to those observed for the ***B. subtilis*** enzyme. The ratio of specific activities was: for cephalexin-*p*NB, 1; cephaclor nucleus-*p*NB, 1.4 ; loracarbef nucleus-*p*NB, 2.9 ; loracarbef-*p*NB, 8.3 ; *p*-nitrophenyl acetate, 739.

When the cell free extracts of ***E. coli*** DH5α/pNB106R or RV308/pNB106R were subjected to non-denaturing gel electrophoresis followed by activity staining, a band with molecular weight of 54,000 (confirmed by SDS-PAGE) was observed (see Figure 21), indicating that expressed *p*NB esterase has the same molecular mass as that of native protein from ***B. subtilis***.

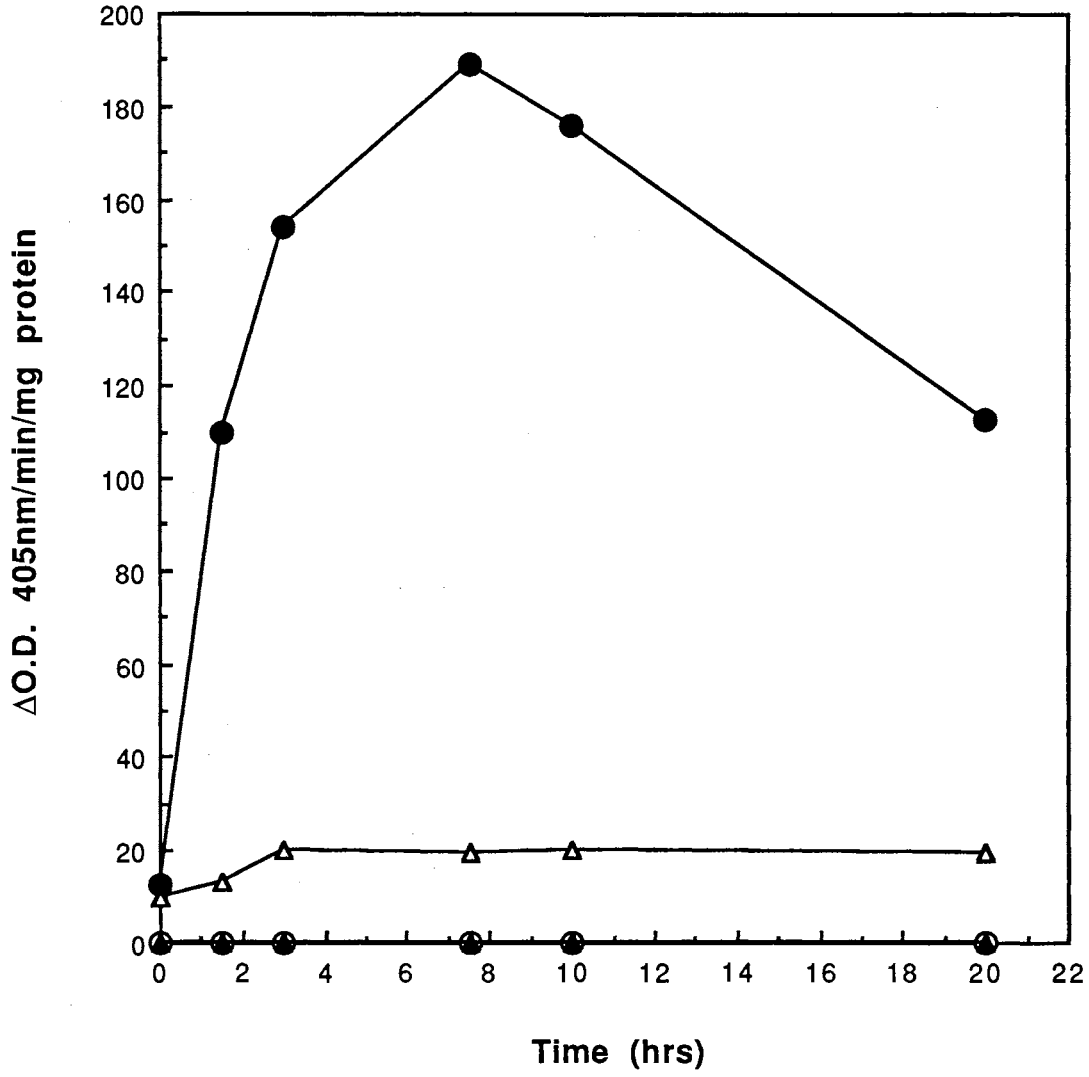


Fig. 20. Temperature induced expression of *p*NB esterase by *E. coli* transformed with pNB106R as measured by esterase activity. Expression was induced as follows: A frozen stock culture was inoculated into L-broth + 5μg/ml tetracycline. After 16 hr growth at 30° C, cell were subcultured (4% v/v) into fresh media and grown to mid-log phase. Induction was accomplished by raising the temperature of the culture to 40° C. The kinetics of synthesis was measured by sampling the culture periodically and assaying cell free extracts for *p*-nitrophenyl acetate esterase activity. The abscissa represents hours after temperature shift from 30° C to 40° C. Symbols are Rv308/pNB106R, induced (-●-), not induced (-Δ-); RV308/pHKY338 induced (-O-) and not induced (-▲-).

Fig. 21. Comparison of activity staining among *p*NBesterase from *B. subtilis*, high level expressed *E. coli* DH-5α and Rv 308. 7% polyacrylamide gel was used and *p*NB esterase activity was stained under non-denaturing conditions. (lane 1) 0.5 μg of purified *p*NB esterase from *B. subtilis.*; (lane 2) 5 μg of cell free extract of *E. coli* /DH 5α cells, and (lane 3) 5 μg of cell free extract of *E. coli* /Rv 308 cells.

CHAPTER III

PURIFICATION OF OVEREXPRESSED *p*NB ESTERASE FROM *E. COLI* AND CHARACTERIZATION OF ACTIVE SITE OF *p*NB ESTERASE

The functionally active *p*NB esterase has been sucessfully over-expressed in *E. coli* cells. This greatly eases our undertaking of sample-demanding experiments required for structure-function study of the *p*NB esterase, such as active site peptide isolation and sequencing, crystallization, and enzyme immobilization.

The complete inhibition of *p*NB esterase by organophosphates, such as diethyl *p*-nitrophenyl phosphate (DNP) and diisopropyl fluorophosphate (DFP), suggests the involvement of a hydroxyl group in its active site (Table IV). These organophosphate compounds have been used extensively for active site studies of B-esterases (52-60). The availability of these compounds in radioactive form, together with the fact that a stable covalent bond is formed between these organophosphate compounds and the hydroxyl group of amino acid residues of *p*NB esterase, enabled us to isolate an active site peptide through conventional peptide isolation and squencing techniques. The availability of the active site peptide can provide us a guideline for designing site-specific mutations to locate the amino acid residues involved in the active site of *p*NB esterase.

In this charpter, the detailed purification procedure for *p*NB esterase from overexpressing *E. coli*/pNB106R, isolation and sequencing of the active site peptide are described. The initial attempts for crystallization of *p*NB esterase using over-expressed protein are also discussed.

MATERIALS AND EXPERIMENTAL PROCEDURES

Materials-- The chromogenic cephalosporin substrate, nitrocefin, was a gift from Bill Jackson of Eli Lilly and Co. Diethyl *p*-nitrophenyl phosphate (DNP) was purchased from Aldrich Chemical Co. Diethyl pyrocarbonate (DEPC) and [^{14}C]-DEPC were obtained from Sigma. [^{3}H]-diisopropyl fluorophosphate (DFP) was purchased from New England Nuclear. Polyethylene glycol-8000 was obtained from Sigma, and the *CrystalPlate* ®, a vapor diffusion plate for protein crystallization, was purchased from Flow Laboratories, Inc.(McLean, VA 22102).

Method for detection of β-lactamase in *E. coli* cells using a chromogenic cephalosporin, nitrocefin, as substrate-- The chromogenic compound, nitrocefin, is a cephalosporin which allows rapid detection of β-lactamase activity (61). β-Lactamase hydrolyzes the amide bond in the ring of nitrocefin (see Fig. 22), resulting in a distinctive color change from yellow to red. In aqueous solution at pH 7.0, nitrocefin has a main absorption peak at about 390 nm when the β-lactam ring is intact. Ring opening results in the disappearance of this peak with the formation of a new peak at about 486 nm (see Fig. 23). Thus one may determine the β-lactamase activity in a cell free extract by spectrophotometric measurement of the absorption decrease at 390 nm or increase at 485 nm. Briefly, to a 1 ml of the assay mixture containing 50 µmol sodium phosphate, pH7.0, and 100 µmol of nitrocefin (10 mM stock solution in DMSO), an appropriate amount of enzyme solution was added to the assay mixture, and the mixture monitored for the abosrbance increase at 485 nm at room termperature with a Shimadzu UVPC 2010 or a Cary 219 spectrophotometer. The specific activity of β-lactamase was calculated by using a molar extinction coefficient of 15.9 $mM^{-1}cm^{-1}$ for hydrolyzed nitrocefin.

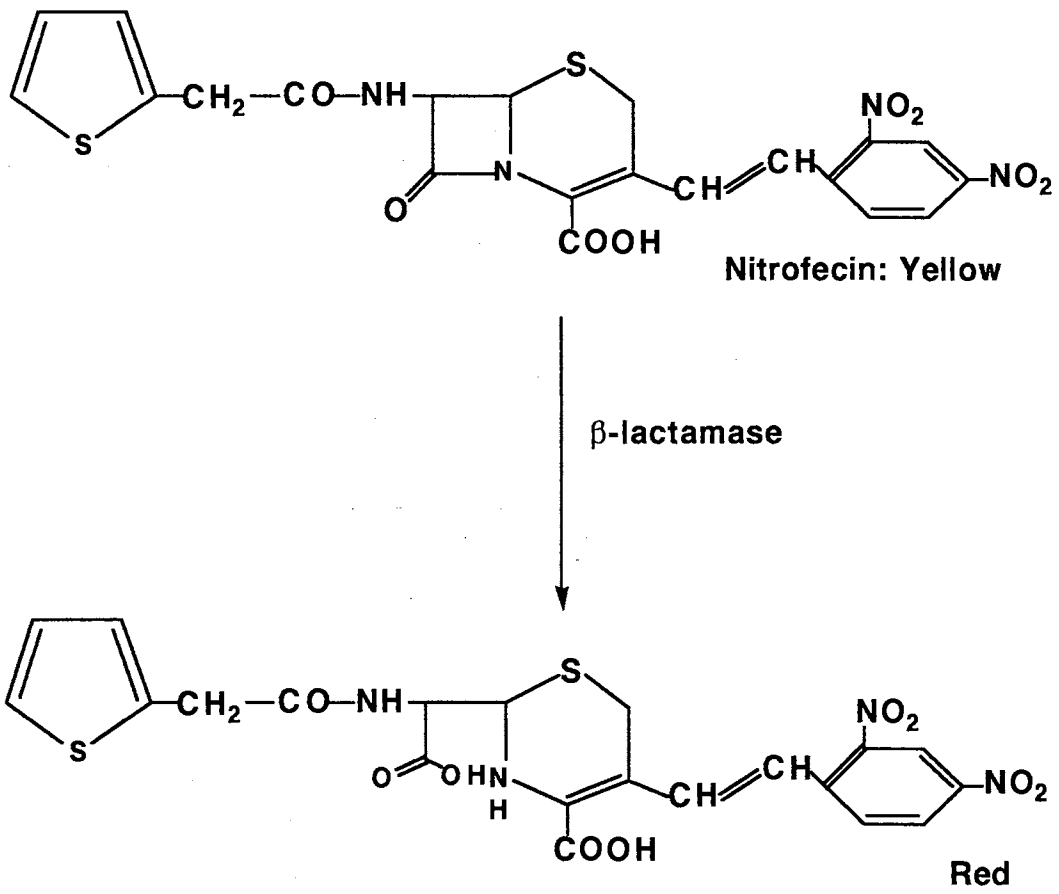


Fig. 22. Structure of chromogenic cephalosporin substrate: nitrocefin, and the hydrolysis reaction catalyzed by β-lactamase.

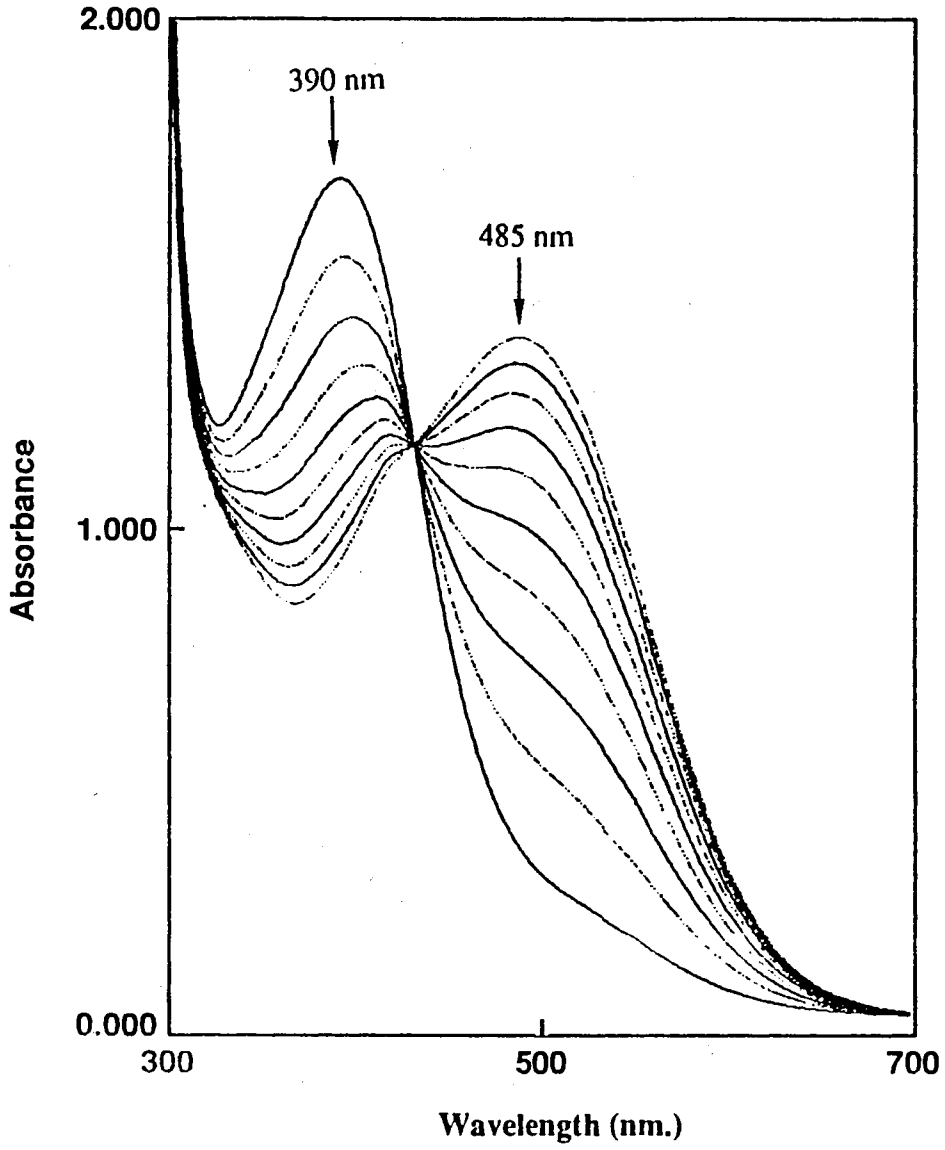
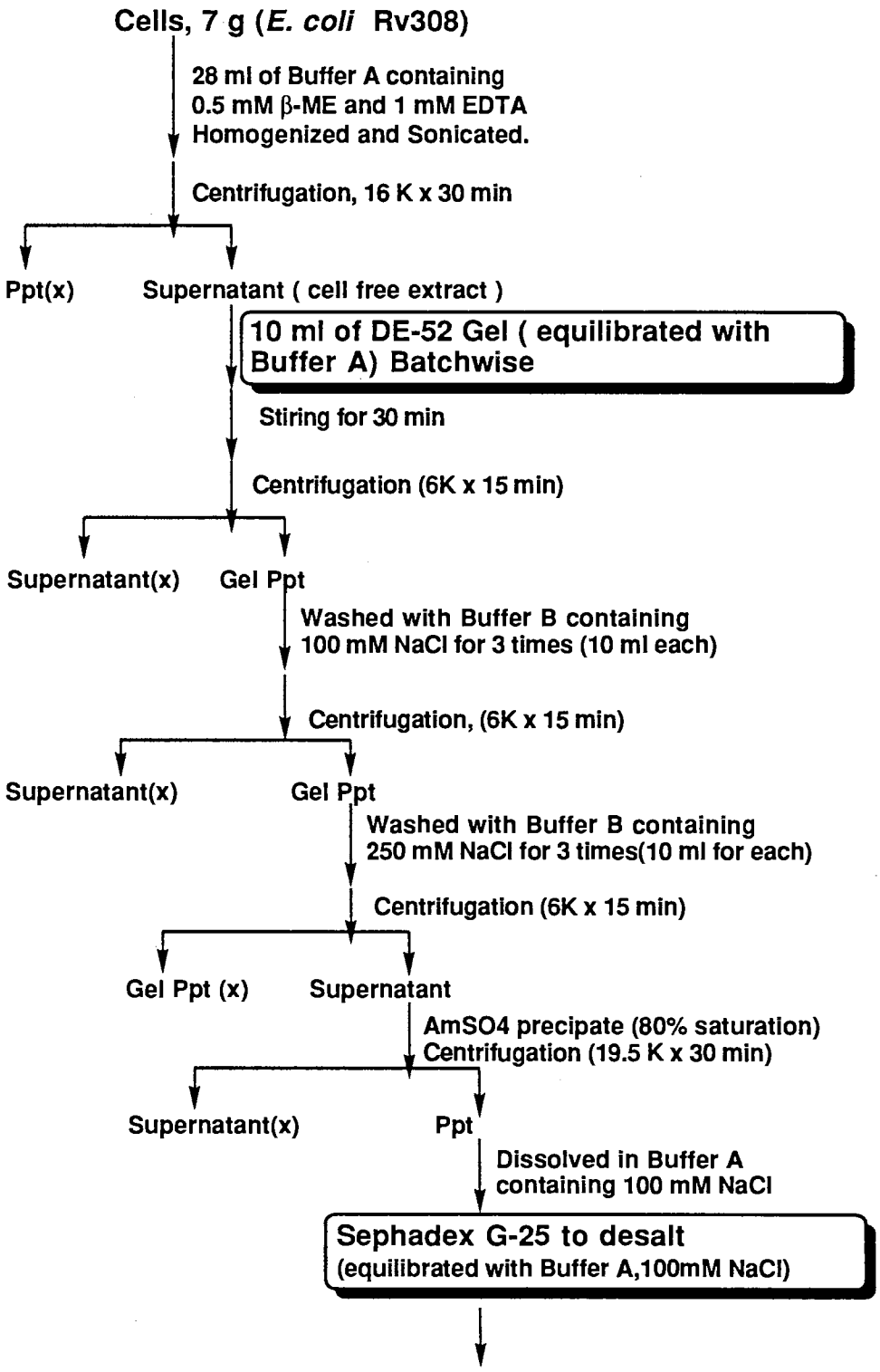
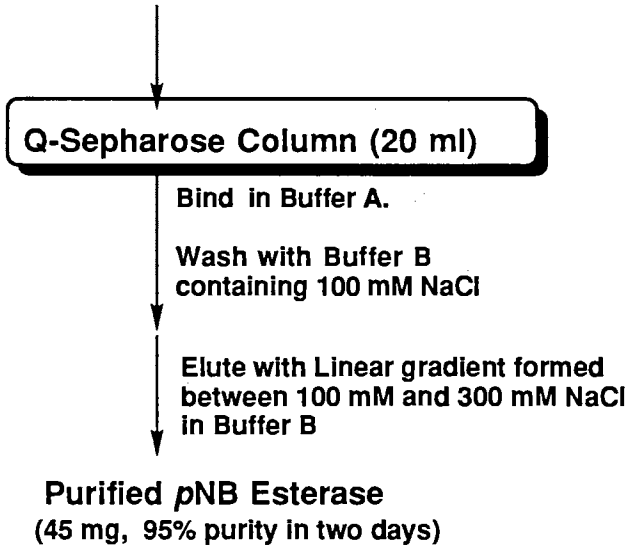


Fig. 23. Hydrolysis of nitrocefin by β-lactamase. 1.85 mg of *E. coli* DH5α/pNB106R cell free extract was added to 1 ml of reaction mixture containing 50 mM of sodium phosphate, pH 7.0 and 100 μmol of nitrocefin. The absolute spectra were recorded from 300nm to 700nm by repeat scanning at the interval of 2 min using a Shimadzu UVPC 2010 spectrophotometer. Nitrocefin has absorption peak at 390 nm, and its hydrolyzed product has absorbance at 485 nm.

Purification of *p*NB esterase from *E. coli.* Rv308/*p*NB106R-- Frozen cells (7 g) were thawed and homogenized in 28 ml of 10 mM Tris-HCl, pH 8.5, containing 0.5 mM β-mercaptoethanol and 1 mM EDTA (Buffer A). The cells were broken with sonication and subjected to centrifugation (28,000 x g, 15 min) to remove debris. The cell free extract obtained was mixed with 10 ml of DE-52 gel which was equilibrated with 10 mM Tris-HCl, pH 8.5 containing 0.5 mM β-mercaptoethanol and 1 mM EDTA (Buffer A), and stirred at 4° C for 30 min. The gel suspension was centrifuged (4300g x 15 min) to remove the supernatant. The resulting gel pellet was washed with 10 ml of 10 mM MES-NaOH, pH 6.5, containing 100 mM NaCl, 0.5 mM β-mercaptoethanol, and 1 mM EDTA (Buffer B) three times. Esterase was eluted from the gel with 10 ml of buffer B containing 250 mM NaCl; the elution wash was repeated three times. The supernatant obtained was concentrated by 80% ammonium sulfate precipitation. The precipitate formed was dissolved in buffer A and fast desalted using a Sephadex G-25 column (1.6 x 25 cm) equilibrated with buffer A containing 100 mM NaCl. Fractions containing esterase activity were combined and applied onto a Q-sepharose column (1.6 x 10 cm) equilibrated with buffer A . The column was washed with 100 ml of buffer B containing 100 mM NaCl, which was then followed with 300 ml of a linear gradient formed from 100 mM and 300 mM NaCl in buffer B. Fractions containing esterase activity were collected and concentrated by ultrafiltration. The procedure is summarized in Scheme V.

Scheme V: Purification of *pNB* Esterase from Over-expressed pNB106R/*E. coli* Rv308 Cells





[a]Buffer A: 10 mM Tris-HCl, pH 8.5 containing 0.5 mM β-ME and 1mM EDTA

[b]Buffer B: 10 mM MES-NaOH, pH 6.5 containing 0.5 mM β-ME and 1mM EDTA

Titration of *p*NB Esterase by Diethyl *p*-nitrophenyl phosphate(DNP) -- Titration of *p*NB esterase with diethyl *p*-nitrophenyl phosphate was performed as described by Krisch (54). One mg of purified *p*NB esterase (18.5 nmol) in 1 ml of 0.1 M of Tris-HCl buffer, pH 8.0, was mixed with 10 µl of acetonitrile containing various concentrations of diethyl *p*-nitrophenyl phosphate. The concentration of *p*-nitrophenol was determined spectrophotometrically using a molar extinction coefficient of $\varepsilon_{M,405nm}$=18,440 $mol^{-1}l$ cm^{-1}. The absorbance increase at 405 nm due to the release of *p*-nitrophenol was taken at 30 sec intervals for 3 min at room temperature. Immediately after the absorbance was measured, a 10 µl aliquot was withdrawn from the reaction mixture and used for an activity determination with *p*-nitrophenyl acetate as substrate.

Isolation of the [^{3}H]-Diisopropyl fluorophosphate (DFP) Labeled Peptide--100 µl of [^{3}H]-DFP (specific activity, 1 mCi/ml) was mixed with 1.2 mg of *p*NB esterase (1.5 mg/ml) and incubated at room temperature for 24 hours. The [^{3}H]-DFP treated-esterase was dialyzed against 0.1M Tris-HCl, pH 8.0, overnight, to remove the non-protein bound [^{3}H]-DFP. A one-µl aliquot was removed from the dialyzed protein solution and used for radioactivity measurement. About 10% of [^{3}H]-DFP was incorporated into the enzyme based on radioactivity recovery. 0.5 µl of β-mercaptoethanol was added to [^{3}H]-DFP labeled esterase to reduce the disulfide bonds in the protein. After overnight incubation at room temperature, the thiol groups of the reduced enzyme were protected by reacting with a 200-fold excess of iodoacetamide. Excess amounts of iodoacetamide were subsequently removed by reacting it with β-mercaptoethanol. The iodoacetamide modified [^{3}H]-DFP labeled esterase was dialyzed against 50 mM NH_4HCO_3, pH 8.0, overnight. The dialyzed protein solution was concentrated by ultrafiltration.

The reduced [^{3}H]-DFP-labeled *p*NB esterase (5mg/ml, 1.5 x 10^7 cpm/mg) in 50 mM ammonium bicarbonate, pH 8.0, containing 0.5 M urea, was digested with 24 µg of Lys C (endoproteinase, Boehringer Mannheim) at 37° C for 24 hours. 50 µl aliquots of

Lys C digested [^{3}H]-DFP-labeled esterase were separated by HPLC on a Synchropak RP-8 column (0.46 x 25 cm) using a gradient formed from 0.1% trifluoroacetic acid in 100% acetonitrile and 0.1% trifluoroacetic acid. The flow rate was adjusted to 1 ml/min. One-ml fractions were collected and the absorbance at 214 nm was measured. 50 µl aliquots were withdrawn for radioactivity determination in a Packard Tri-Carb 1900CA liquid scintillation analyzer.

Titration of *p*NB Esterase with Diethyl Pyrocarbonate (DEPC)-- Titration of *p*NB esterase with DEPC was carried out according to the method of Miles and Leskovac (62-63) with modifications. Two pairs of 0.2 cm light path cuvettes containing 0.4 ml of *p*NB esterase (11.6 mg/ml in 0.1 M potassium phosphate buffer, pH 6.0) and 0.4 ml of the same buffer, were respectively placed in the sample and reference compartments of a Shimadzu UVPC 2010 spectrophotometer. Difference spectra were recorded between 320 and 230 nm before and after each addition of 4 µl of ethanol containing a given concentration of DEPC to the esterase in the sample compartment and the buffer in the reference compartment. The number of histidines modified by DEPC was calculated from the absorbance at 240 nm using a molar extinction coefficient of 4327 $mol^{-1}l\ cm^{-1}$ for N-ethoxylcarbonylated histidine. One µl aliquots of DEPC treated esterase were withdrawn immediately after each difference spectrum was recorded and assayed for activity, using *p*-nitrophenyl acetate as substrate.

Correlation between DEPC incorporation and inactivation of *p*NB esterase-- 4 µl of [^{14}-C]-DEPC (1M in absolute alcohol with specific radioactivity 3.4 Ci/mol) were added to 0.6 ml of *p*NB esterase (0.55 mg/ml in 50 mM Tris-HCl buffer, pH 7.0), and the mixture was incubated at 0° C. At different time intervals, 15 µl aliquots were withdrawn and added to 15 µl of 20 mM L-histidine to stop further [^{14}C]-DEPC incorporation. The *p*NB esterase activity of each fraction was assayed with *p*-nitrophenyl acetate as substrate. For measuring [^{14}C]-DEPC incorporation, 2 µl aliquots were withdrawn from the sample at

different time intervals and spotted on Whatman No. 3 paper. When sampling was completed, the paper was developed with chloroform/methanol (2:1). The original spots in the paper were cut out, put into scintillation vials and 5 ml Insta-Gel scintillation counting solution was added. The radioactivity was determined in a Packard Tri-Card scintillation analyzer, model 1900A.

Crystallization of Over-expressed *p*NB esterase-- 10 µl of purified *p*NB esterase (23 mg/ml) in 10 mM MES-NaOH, pH 6.0, containing 20% glycerol was thawed and mixed with 11 µl of precipitating solution, containing 27% polyethylene glycol 8000 (PEG) and 90 mM MES-NaOH, pH 6.0. The mixture was incubated at 0° C for 15 min before centrifugation to remove the precipitate that formed. 15 µl of clear solution was placed in the lower presilylated glass slip (14 x 14 x 0.2 mm) of *Crystalplate*®. The equilibrating solution, which was composed of 90mM of MES-NaOH, pH 6.0, and 27% PEG, was placed in the buffer tank of *Crystalplate*®. The Upper glass slip (24 x 30 x 1.0 mm) covered the protein solution and equilibrating solution (see Figure 24). This vapor diffusion system between protein solution and equilibrating solution was kept in the cold room. Crystals were formed within 4 weeks and checked under a microscope equipped with polarized light.

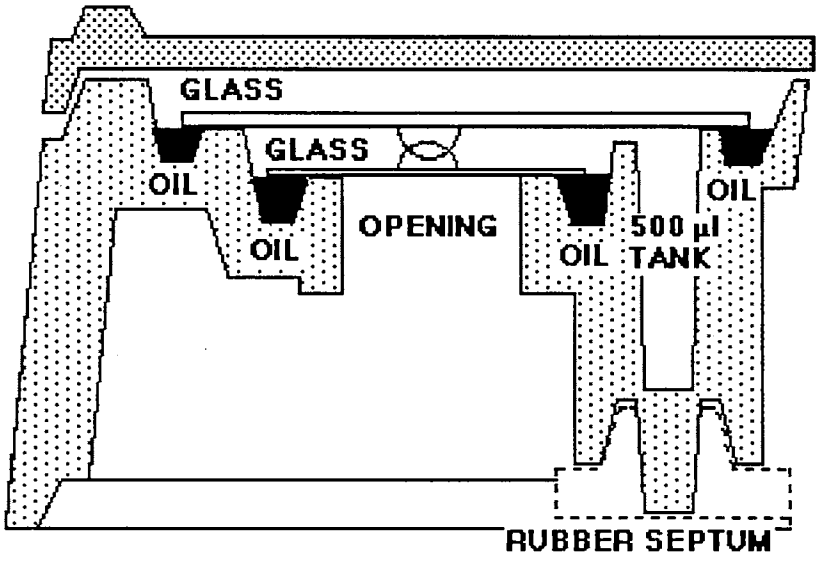


Fig. 24. Crystallization of *p*NB esterase by vapor diffusion on *CrystalPlate*® Each well on the plate contains one 24 x 30 x 1.0 mm upper and 14 x 14 x 0.2 mm lower glass slips. The slips were cleaned and silylated before use so that the protein solution would not wet the glass. A rubber septum was inserted to reduce vibration. Ordinary hydrocabon mineral oil filled the upper and lower troughs of each well. Protein solution was put either on the middle of the upper glass slip (for hanging drop) or the lower glass slip (for standing drop), and the desired equilibrating solution was put into the 500 µl tank.

RESULTS AND DISCUSSION

Purification and Properties of Over-Expressed *p*NB esterase from *E. coli* Cells -- In contrast to *B. subtilis,* the cell-free extract of *E. coli* /pNB106R contains a detectable amounts of β-lactamase. This enzyme hydrolyzes β-lactam antibiotic esters and their free acids and has to be removed from the *p*NB esterase preparation. The availability of a chromogenic substrate, nitrocefin, greatly eases the detection of β-lactamase during the process of purification of *p*NB esterase. The specific activities of β-lactamase in the cell-free extracts of *E. coli* DH5α/pNB106R and Rv308/pNB106R were 3.32 mUnits/min/mg and 3.23 mUnits/min/mg, respectively. The ratios between *p*NB esterase and β-lactamase in the cell-free extracts were 57.5 for DH5α and 105 for Rv308. The β-lactamase in the cell free extract was gradually removed during the process of enzyme purification. No β-lactamse activity was detected after the final purificaiton step (see Table VII).

Although the method developed for purification of *p*NB esterase from *B. subtilis* can be used to obtain pure *p*NB esterase from *E. coli* /pNB106R cells, the procedure is rather lengthy for over-expressed protein. Since *p*NB esterase is highly over-expressed in *E. coli* /pNB106R cells, about 26% of the total protein in cell-free extracts of *E. coli* Rv308 was *p*NB esterase as estimated by activity determination, the enzyme could be made homogenous by a 4-fold purification. A simplified procedure was developed for purification of *p*NB esterase from *E. coli* /pNB106R cells (Scheme V). While the original procedure involved seven purification steps: ammonium sulfate fractionation, pH 5.0 treatment, DE-52, Sephacryl S-200, Q-sepharose, calcium phosphate-cellulose, and *p*-aminobenzamidine agarose column chromatographies, the new method only needed three steps: DE-52, G-25, and Q-sepharose column chromatographies. The G-25 column chromatography was used to desalt the enzyme and required less than 30 min to complete with FPLC. Eluting *p*NB esterase from DE-52 gel by a batchwise elution method proved to be the most time saving modification. About 45 mg of purified *p*NB esterase was

obtained from 440 mg of crude extract, which was derived from 7 g of wet cells, in two days (Table VII). The enzyme obtained is electrophoretically pure and has a specific activity of 1250 mUnits/mg protein, using loracarbef nucleus-*p*NB as substrate. This is comparable to the activity obtained with the originally developed method. Since only 3 mg of pure *p*NB esterase was obtained from 41,400 mg of crude protein extract, obtained from 760 gm (wet weight) of *B. subtilis* cells, the process for obtaining pure *p*NB esterase using the recombinant *E. coli* source and simpler purification procedure was about 1000 times more efficient than the process using the natural source and the necessarily more expensive purification procedure.

Titration of *p*NB Estearse with Diethyl *p*-Nitrophenyl Phosphate (DNP)-- Since *p*NB esterase was completely inhibited by organophosphates such as DNP and DFP and PMSF, the involvement of a hydroxyl group in the active site is expected. When *p*NB esterase was titrated with varying amounts of DNP, the enzymatic activity decreased as the amount of DNP consumption increased (Figure 25). A complete inhibition was observed when one mole of DNP was consumed by one mole of the enzyme, suggesting that only one hydroxyl group is involved in the active site. Since DNP is readily reacted with *p*NB esterase, this hydroxyl group is probably located on the surface of the enzyme. Since radioisotope labeled DNP is not commerically available, study of active site domain with this compound is not practical. Although radioisotope labeled DFP and PMSF are both commerically available, DFP is preferred over PMSF for use in active site peptide isolation and sequencing for the economic reasons.

Identification of active site peptide by diisopropyl fluorophosphate (DFP) affinity labeling-- When purified esterase was treated with varying amounts of DFP, the enzymatic activity decreased as the amount of DFP increased. The titration pattern was similar to that observed for DNP. When [^{3}H]-DFP treated esterase was subjected to LysC digestion followed by HPLC separation, a single radioactive peptide was obtained (see Fig. 26).

The amino acid sequence of this labeled peptide was determined to be A-I-M-E-S-G-A-S-R-T-M-T-K-. This peptide corresponds to amino acid residues # 211 to 233 of the *p*NB esterase amino acid sequence. This DFP labeled peptide has similar features to those of active site peptides obtained from mammalian esterases (Table VIII). The general motif of the active site peptide of B-esterases is Glu-Ser-x-x-x-x-Ser. The isolated active site peptide of *p*NB esterase has only two amino acid residues instead of four between the two Ser residues. However, the fifth amino acid residue from the first Ser is a Thr, which can provide a hydroxyl group. The Thr may play the role of the second Ser.

Establishment of the Involvement of a Histidine Residue in the Catalytic Function of *p*NB Esterase by Diethyl Pyrocarbonate (DEPC) -- DEPC, a histidine modifying reagent, was found to be a inhibitor of *p*NB esterase of *B. subtilis* (chapter I). It is absolutely necesssary to establish a correlation between inactivation of enzymatic activity and modification of a histidine residue in order to show that a histidyl group is involved in the catalytic function of *p*NB esterase. We have established such a correlation by incubation of *p*NB esterase in the presence of various concentrations of DEPC (Figure 27) and for different lengths of time (Figure 28 with [^{14}C]-DEPC). When *p*NB esterase was titrated with varying concentrations of DEPC at 0° C for 10 min, the amount of histidine being modified correlated with activity loss until maximum inactivation was reached (1 mole of histidine/mole of protein) (Figure 27), suggesting that a histidine residue is involved in catalytic function of *p*NB esterase. When the concentrations of DEPC used were higher than those needed for maximum inactivation, histidine modification continued at a much slower rate without further loss of activity. This suggests nonspecific reacting of histidine residues with DEPC.

Figure 28 shows the effect of incubation time on the inactivation of and DEPC uptake by *p*NB esterase. When *p*NB esterase (10 μM) was incubated with 6.7 mM [^{14}C]-DEPC at 0° C for various lengths of time, the activity decreased as the incubation time

TABLE VI

PURIFICATION OF *p*NB ESTERASE FROM *E. COLI* RV308/pNB106R

Step	Protein (mg)	Activity[a] (Units)	Sp. Activity (Units/mg)	Fold	Yield (%)	β-lactamase (mU)
Cell free extract	440	150	0.34	1	100	1421
DE-52, batchwise	134	103	0.76	2.23	68	17
Q-sepharose column	45	56	1.25	3.67	37	0

[a] Avtivity was determined by using loracarbef nucleus-*p*NB as substrate. and one Unit of enzyme activity is the amount of enzyme catalyzing the hydrolysis of 1 μmol loracarbef nucleus-*p*NB per min.

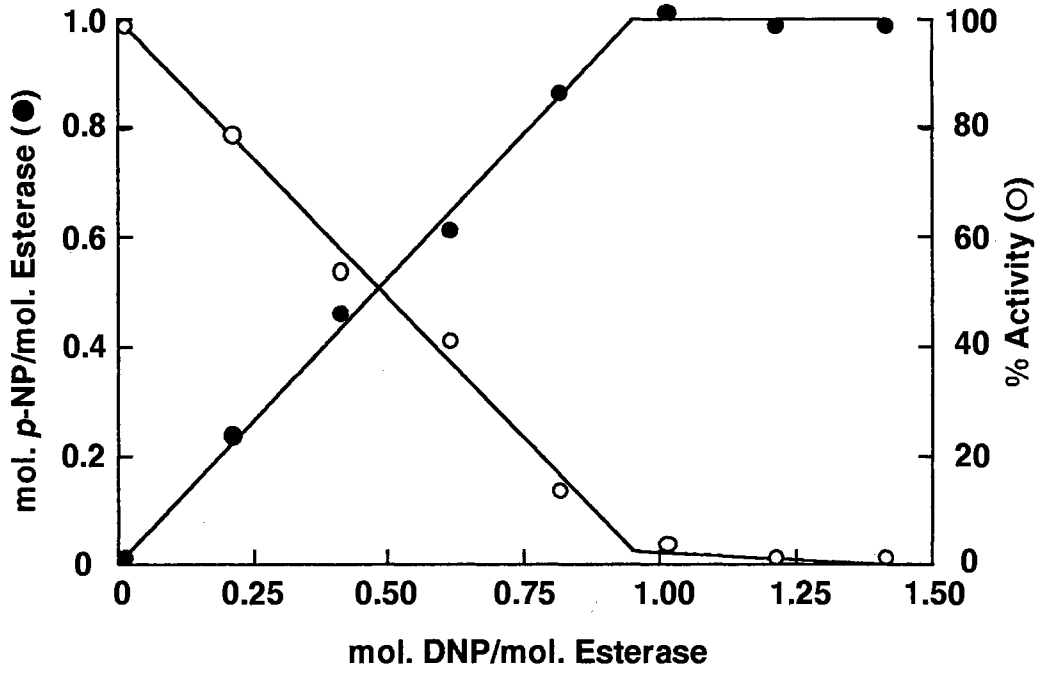


Fig. 25. Titration of *p*NB esterase by diethyl *p*-nitrophenyl phosphate (DNP). The consumption of DNP by pNB esterase (-●-) was measured by the absorbance increase at 405 nm due to the release of *p*-nitrophenol (*p*-NP). The % activity remaining (-○-) was determined with *p*-nitrophenyl acetate as a substrate. 100% activity refers to the untreated sample.

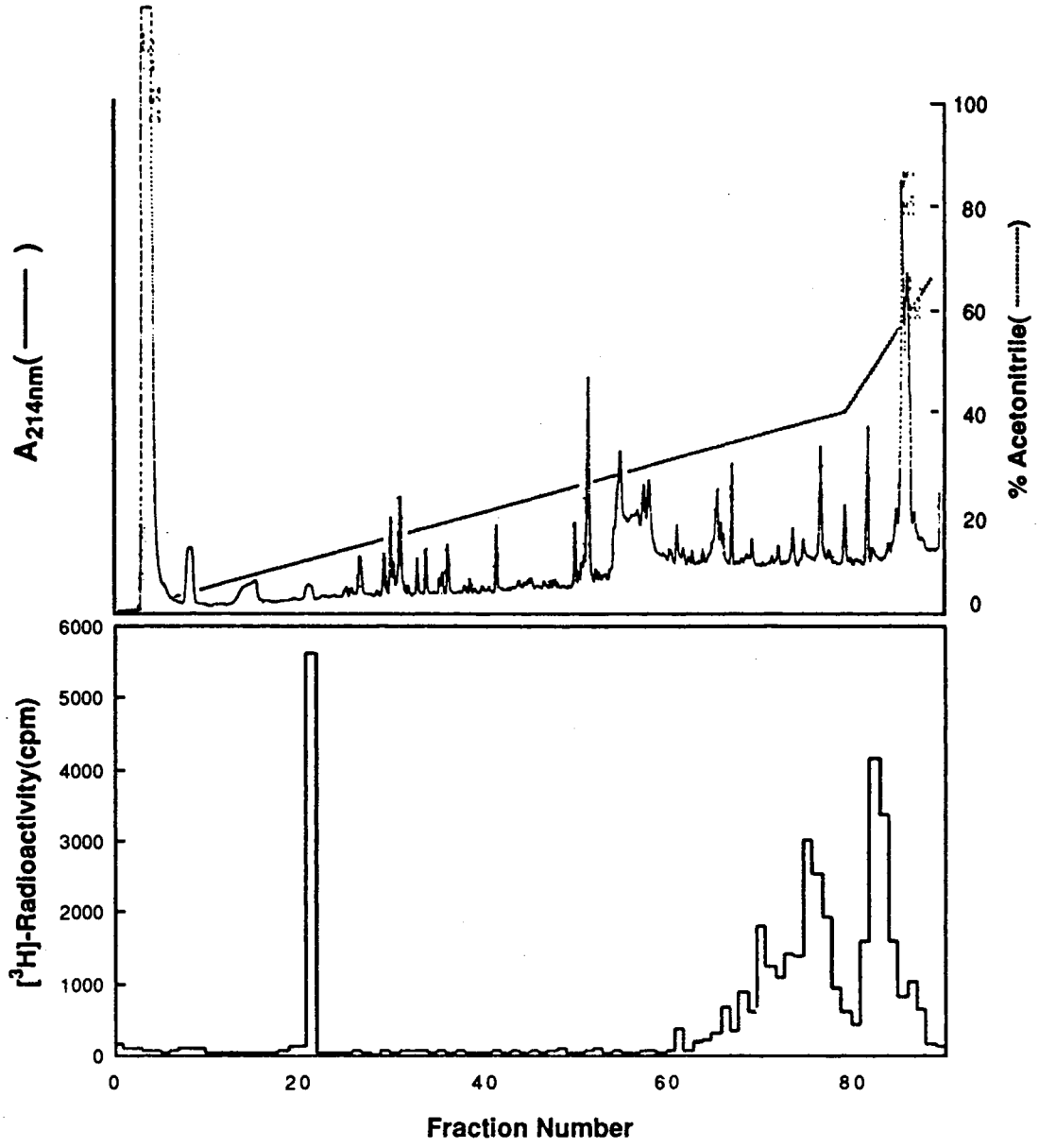


Fig. 26. [^{3}H] radioactivity distribution on an HPLC chromatogram of Lys C-digested [^{3}H]-diisopropyl fluorophosphate([^{3}H]-DFP) treated *p*NB esterase.

TABLE VII

AMINO ACID SEQUENCE AROUND THE ACTIVE SITE OF ESTERASES (58)

Source of Esterase	Sequence of active site peptide	Reference
Horse liver	-Gly-Glu-**Ser**-Ala-Gly-Gly-Glu-Ser-	58
Ox liver	-Gly-Glu-**Ser**-Ala-Gly-Ala-Glu-Ser-	56
Chicken liver	-Gly-Glu-**Ser**-Ala-Gly-Gly-Ile-Ser	56
Pig liver	-Gly-Glu-**Ser**-Ala-Gly-Asp-Gly-Ser	56
Rabbit	-Gly-Glu-**Ser**-Ala-Gly-Gly-Gln-Ser	64
Torpedo AChE[a]	-Gly-Glu-**Ser**-Ala-Gly-Gly-Ala-Ser	65
B. subtilis	-Ala-Ile-Met-Glu-**Ser**-Gly-Ala-Ser-Arg-Thr	This study

[a]. Acetylcholine esterase.

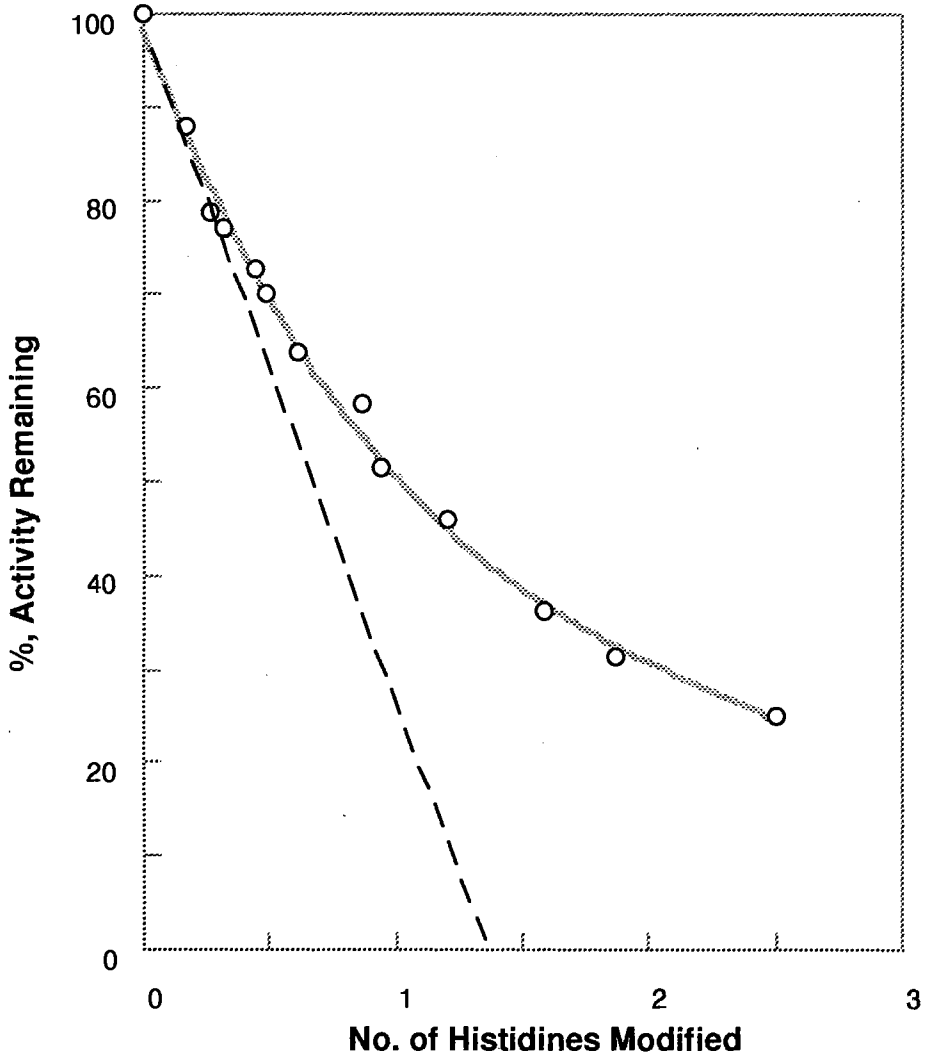


Fig. 27. Titration of *p*NB esterase with diethyl pyrocarbonate(DEPC). The conditions were detailed in "Materials and Experimental Procedures". The number of histidines modified by DEPC is calculated from the absorbance at 240 nm between DEPC-treated and untreated enzyme using a molar coefficient of 4327 $cm^{-1}l\ mol^{-1}$ for N-ethoxylcarbonylated histidine.

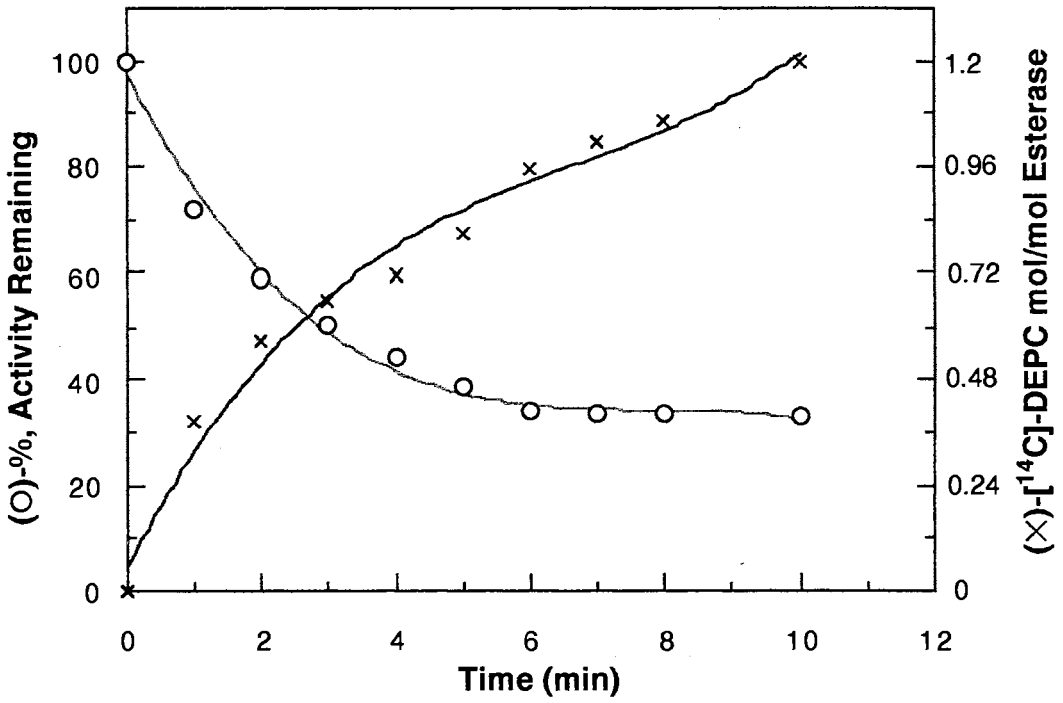


Fig. 28. Correlation between DEPC incorporation and inactivation of *p*NB esterase. 4 µl of [^{14}C]-DEPC (1M in acetonitrile with specific radioactivity of 3.4 Ci/mol) were added to 0.6 ml of *p*NB esterase (0.55 mg/ml in 50 mM Tris-HCl buffer, pH 7.0), and incubated at 0 °C. At the indicated time intervals, 15 µl aliquots were withdrawn, and 15 µl of 20 mM L-histidine was added to each fraction to stop further DEPC incorporation. *p*NB esterase activity was assayed with *p*-nitrophenyl acetate as substrate, following the absorbance change at 405 nm(O). For measurement of DEPC incorporation (×), 2 µl aliquots were withdrawn from the sample at the indicated time intervals and spotted to paper. When sampling was complete, the paper was developed with chloroform/methanol(2:1). The original spots in the paper were cut out, 5 ml of Insta gel scintillation counting gel was added , and the radioactivity was determined.

increased; maximum inactivation (66%) was reached at 6 min. The amount of DEPC uptake by the protein paralleled the extent of inactivation until the maximum is reached (1 mole of DEPC uptake per mole of protein), confirming that one histidyl residue is involved in the catalytic function of *p*NB esterase. Although incubation for longer than 6 min caused no further decrease in activity, DEPC uptake continued, but at a slower rate. This indicates that this uptake is due to nonspecific modification of histidine residiues by DEPC. It should be mentioned that a control sample containing the same amount of ethanol incubated under identical conditions, showed no activity loss over the time periods studied.

Putative catalytic triad function of esterase-- Since the inhibition behavior of DEPC on *p*NB esterase indicates non-competitive inhibition (Figure 29), it is expected that the substrate does not directly interact with the specific histidine of esterase in the deesterification reaction. Thus this specific histidine is distal from the active site serine^{215}. A hydrogen bond can be formed between the imidazole group of histidine and the β-hydroxyl group of serine^{215}. This hydrogen bond may contribute to stabilization of the enzyme-substrate complex and help the formation of a tetrahedral transition state during the process of substrate hydrolysis.

In addition to ser and his, an asp or a glu may also be involved in the catalysis of esterase because *p*NB esterase activity was inhibited by dicyclohexyl carbodiimide (DCCD). This asp or glu must be surrounded by an hydrophobic environment because the activity was only inhibited by DCCD but not by 1-ethyl-(3-dimethyl aminopropyl) carbodiimide. The carboxylate group of asp or glu could be in apposition with the imidazole nitrogen of the histidine to form a second hydrogen bridge. The hydrogen bridge formed between his and asp or glu may facilitate the formation of a tetrahedral transition state. Overall, the active site serine^{215}, histidine and an asparate or a glutamate of *p*NB esterase constitute the functional sites, which are involved in a putative catalytic triad function in a fashion similar to most serine hydrolases and serine proteases (66-69).

Figure 30 shows the proposed reaction model for hydrolysis of β-lactam antibiotic-*p*NB esters catalyzed by *p*NB esterase. A putative catalytic triad of ser^{215}, an unknown his, and an unknown asp or glu are shown in the model. First , the substrate β-lactam antibiotic-*p*NB ester associates with the enzyme, leading to formation of a tetrahedral transition state. Next the transition state collapses to form the trigonal acyl enzyme by linkage with the serine and release of *p*-nitrobenzyl alcohol. Finally, the acyl enzyme rapidly deacylates and yields active enzyme.

Crystallization of *p*NB esterase-- Figure 31 shows crystals of *p*NB esterase. The crystals are hexagonal in shape, and stable for several months at 4° C. When the crystals were re-dissolved in aqueous solution, full enzymatic activity was observed. Although the crystals exhibited a high degree of birefringence under polarized light, they were not suitable for X-ray crystallography work because the size of the crystals was too small (about 10 μm wide and 25 μm long).

Application of immobilized *p*NB esterase as biocatalyst-- The absence of amino group involvement in the enzyme activity, together with the property of having a low pI value, make *p*NB esterase suitable for immobilization on solid supports such as Affi-Gel 15 and DE-52. The purified *p*NB esterase was immobilized on Affi-Gel 15 (Figure 32), and the immobilized enzyme preparation was added to a suspension of loracarbef nucleus-*p*NB ester in 50 mM Tris bis propane-HCl buffer, pH 8.0. The reaction was maintained at 37° C for 3 hours. Sodium hydroxide solution was added periodically to maintain the pH at 8.0. Our preliminary results showed a 94% yield of loracarbef nucleus free acid for a 1% (w/v) substrate suspension and an 81% yield for a 2% (w/v) substrate suspension, based on HPLC analysis for that compound. The stability of the enzyme was apparently increased when compared to that of the free soluble enzyme.

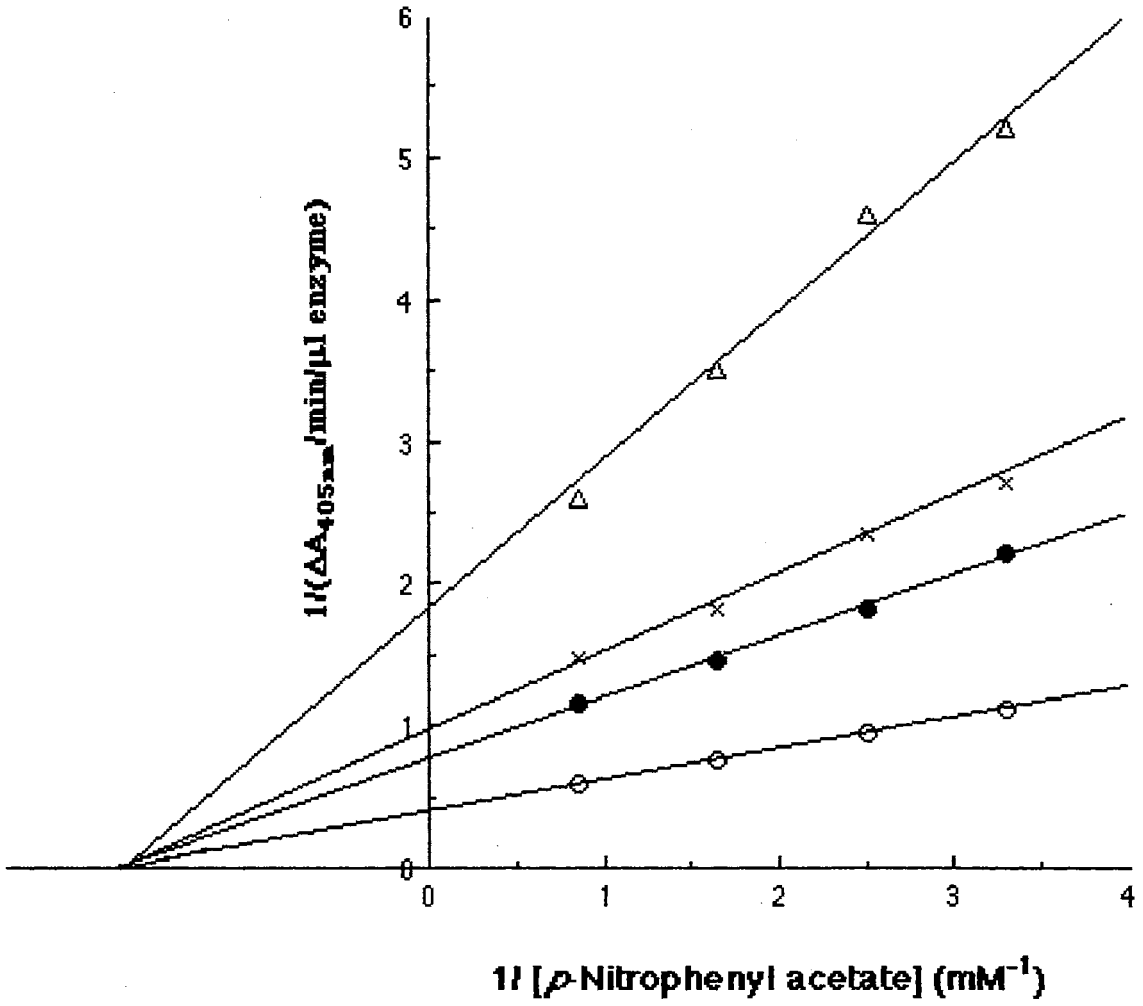


Fig. 29. Double reciprocal plots of substrate concentration,[*p*-nitrophenyl acetate], and activity of esterase, ΔA_{405nm}/min/μl enzyme, upon treatment with different concentrations of DEPC. The esterase (0.55 mg/ml, in 100 mM potassium phosphate buffer pH6.0) was incubated with 0 mM(O), 5 mM(●), 6.6 mM(×), and 10 mM(△) DEPC at 0 °C for 10 min. 5 μl of enzyme solution was withdrawn, and the activity determined using *p*-nitrophenylacetate as substrate.

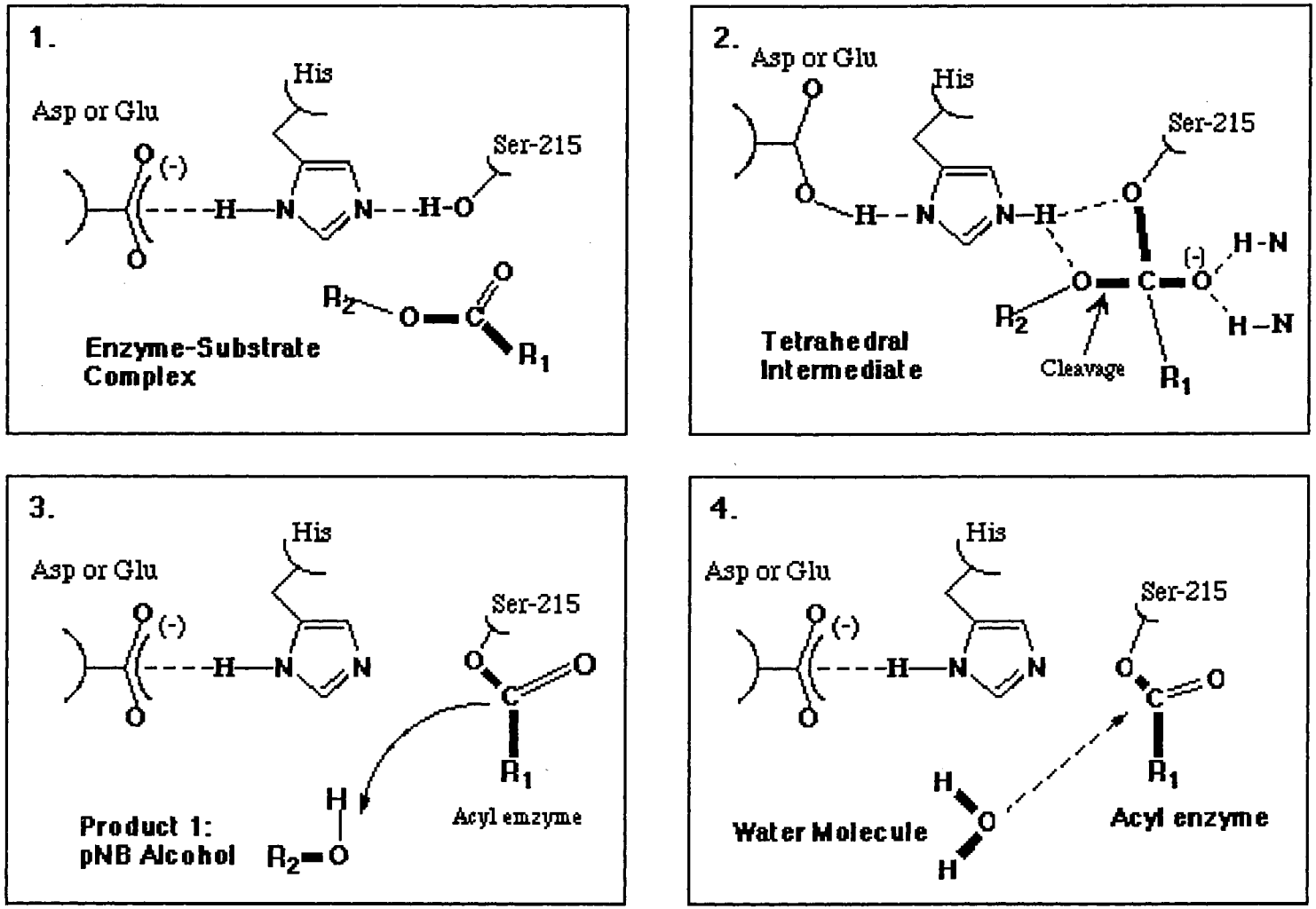
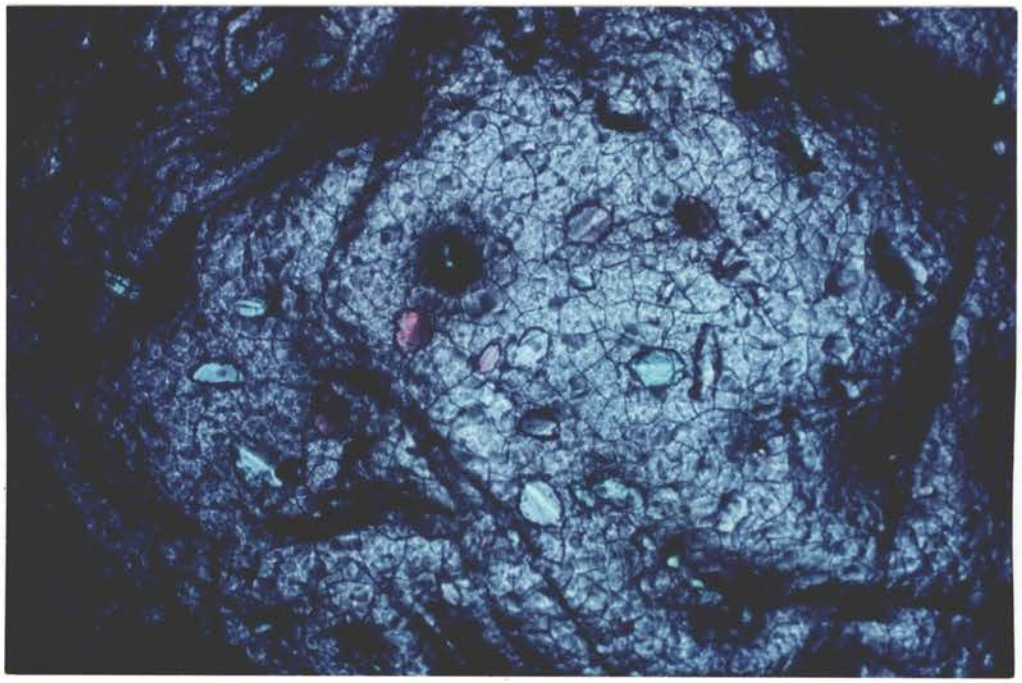


Fig. 30. Steps in substrate hydrolysis catalyzed by *p*NB esterase. **1.** The substrate is associated with enzyme **2.** leading to formation of tetrahedral transition state. **3.** The transition state collapses to form the trigonal acyl enzyme by linkage with the serine and release of *p*-nitrobenzyl alcohol. **4.** The acyl enzyme rapidly deacylates and yields active enzyme. R_1: β-lactam antibiotics as indicated in Figure 1, R_2: *p*-NB(*p*-nitrobenzyl group).

a.



b.

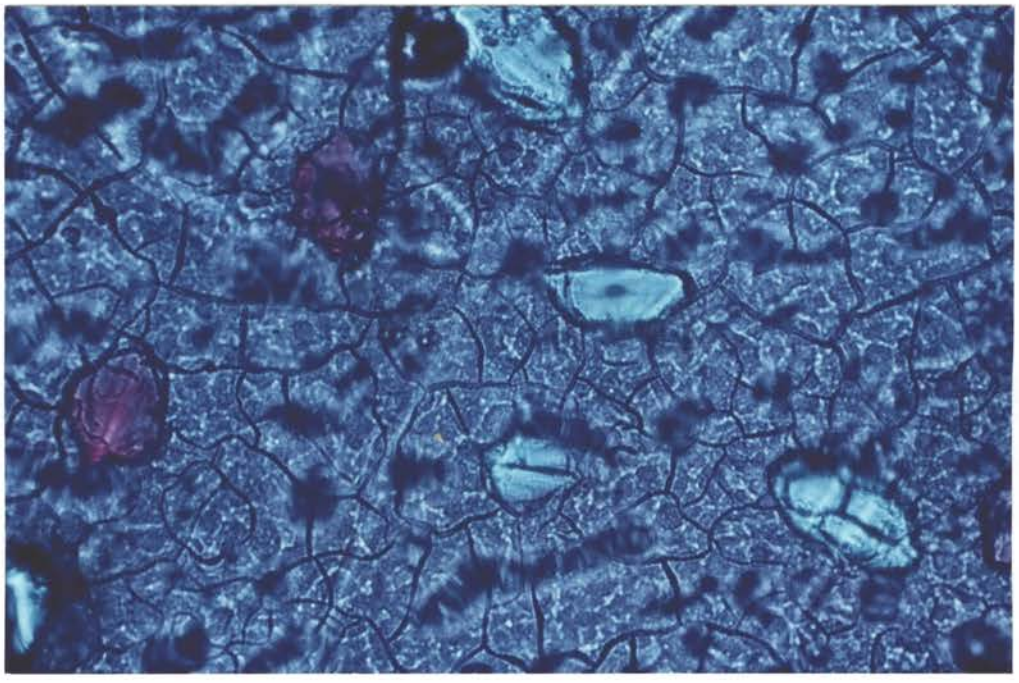


Fig. 31. Protein crystals of *p*NB esterase. Photomicrographs of crystals of *p*NB esterase grown from 27% polyethylene glycol 8000 at pH 6.0. The larger crystal measures 10 × 10 × 25 µm. (100× for **a**, and 400× for **b**)

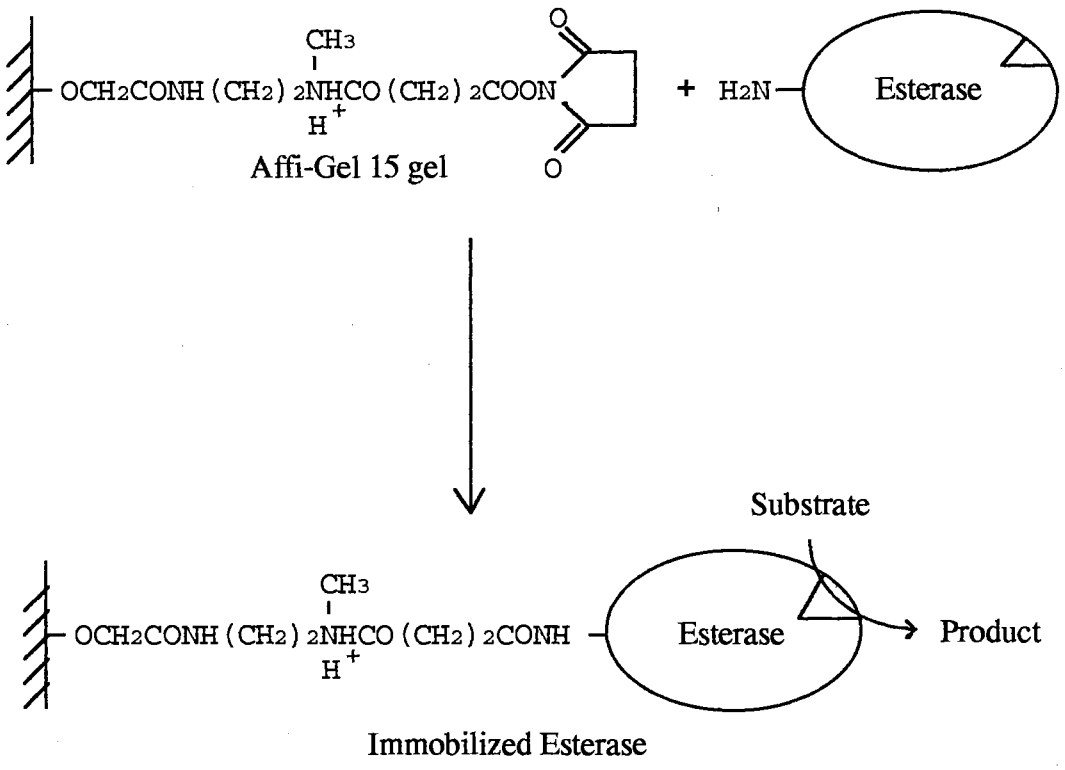


Fig. 32. Immobolization of *p*NB esterase on the Affi-Gel 15. The coupling reaction was carried out in 0.1 M MOPS-NaOH, pH7.5 buffer.

CONCLUSIONS

p-nitrobenzyl (*p*NB) esterase from *B. subtilis*, which catalyzes the hydrolysis of the *p*-nitrobenzyl carboxyl ester of several cephalosporin antibiotics, has been purified to homogeneity by a procedure involving ammonium sulfate fractionation; acidic treatment at pH 5; and DE-52-, Sephacryl S-200-, Q-Sepharose-, calcium phosphate-, and *p*-aminobenzamidine-agarose- column chromatographies. The purified esterase is soluble and monomeric in aqueous solution with a molecular weight of 54,000 estimated either by SDS-polyacrylamide electrophoresis or gel filtration. The enzyme has an acidic pI, 4.1, and shows a broad substrate specificity against various esters, such as cephalexin-*p*NB, loracarbef-*p*NB, *p*-nitrophenyl acetate, benzyl acetate, and α-naphthyl acetate. The turnover number of *p*NB estearse is 6.6 mole/mole/sec using loracarbef-*p*NB ester as substrate. The amino acid composition of the purified esterase has been determined; and the partial N-terminal amino acid sequence of *p*NB esterase was determined chemically as follows: NH_2-Met-Thr-His-Gln-Ile-Val-Thr-Thr-Gln-Trp-Gly-Lys-Val-Lys-Gly-Thr-Thr-Glu-Asn-Gly-Val-His-.

The *B. subtilis* gene encoding *p*-NB estearse (*pnbA*) has been cloned and sequenced. Two synthetic oligonucleotide probes were generated based on the N-terminal amino acid sequence and used to screen for *pnbA* by southern hybridization. The *pnbA* gene is 1470 base pairs long and encodes 489 amino acids. The molecular weight of *p*NB esterase, calculated from the amino acid sequence deduced from the DNA sequence, is 53,947, which is very close to that obtained from physical determination with the purified protein.

Functionally active *B. subtilis* *p*NB esterase has been successfully over-expressed in *E. coli* cells using a thermoinducible expression vector, pNB106R. The expression vector for *B. subtilis* *p*NB esterase (pNB106R) was constructed by ligating the *pnb*A gene into pHKY338 plasmid which contains the promoter of bacteriophage λ (pL106) and a temperature sensitive cI857 repressor. The expression of *p*NB esterase in *E. coli* was induced by raising the temperature from 30° C to 40° C. The amount of *p*NB esterase in recombinant *E. coli* cells is 300 times higher than that present in *B. subtilis.* *p*NB esterase was purified from recombinant *E. coli* cells to homogeneity by a simplified procedure involving batchwise DE-52 elution and Q-sepharose column chromatography. *p*NB esterase obtained from the recombinant source has enzymatic activity, substrate specificity, and a molecular size identical to those obtained with the enzyme from the natural source. The high amount of the *p*NB esterase obtained from the *E. coli* recombinant source makes it possible to develope a biocatalysis method to hydrolyze β-lactam antibiotic esters to the free acid form.

Inhibitor studies indicate that serine, histidine, and aspartic acid or glutamic acid are involved in the catalytic function of *p*NB esterase. Titration of purified *p*NB esterase by diethyl *p*-nitrophenyl phosphate indicates that one specific serine is involved in enzyme activity. When the [^{3}H]-diispropyl fluorophosphate treated *p*NB esterase is digested with LysC, followed by HPLC separation, a single [^{3}H]-labeled peptide is obtained. The amino acid sequence of the active site peptide is as follows: Ala-Ile-Met-Glu-Ser-Gly-Ala-Ser-Arg-Thr-Met-Thr-Lys- corresponding to the amino acid residues number 211 to 223 of the deduced sequence of the esterase. The results obtained from the nature of the inhibition by DEPC, titration with DEPC, and the correlation between [^{14}C]-DEPC incorporation and esterase inactivation suggest that one specific histidine distal from serine^{215} may also be involved in the catalysis. The involvement of serine, histidine and aspartic acid or glutamic acid in the catalysis of *p*NB esterase allows us to propose a putative catalytic triad function for the mechanism of enzyme catalysis.

Crystallization of the *p*NB estearse from overexpressed protein has been obtained using poly(ethylene glycol) 8000 as the precipitating reagent. Although the crystals show well-defined shape and a high degree of birefringence under polarized light, the size of the crystal appears to be too small to be suitable for X-ray crystallographic study. Thus improvement of crystallization conditions is needed in order to obtain a suitable crystal.

As listed in Table VIII, the *p*NB esterase shows a low turnover rate for *p*NB esters of β-lactam antibiotics. The effectiveness of catalysis is not sufficient for industrial manufacture when compared to traditional chemical methods. Since the stability and catalytic turnover number of *p*NB esterase are concerned, it would be profitable to explore genetic modification to improve the properties of the enzyme for industrial use.

TABLE VIII

MAXIMUM TURNOVER NUMBERS OF SOME ENZYMES

Enzyme		Turnover Number (mole/mole/sec.)
Carbonic anhydrase		600,000
3-Ketosteroid isomerase		280,000
Acetylcholinesterase		25,000
Penicillinase		2,000
Lactate dehydrogenase		1,000
Chymotrypsin		100
DNA polymerase I		15
Tryptophan synthetase		2
Lysozyme		0.5
*p*NB Esterase	(Loracarbef-*p*NB)	6.6
	(Loracarbef-nucleus-*p*NB)	3.9
	(Cephalexin-*p*NB)	0.8

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