Characterization of the 6-aminohexanoate-dimer hydrolase from *Pseudomonas* sp. NK87

KAZUO KANAGAWA, MAYUMI OISHI, SEIJI NEGORO,* ITARU URABE and HIROSUKE OKADA†

Department of Biotechnology, Osaka University, 2-1 Yamada-oka, Suita-shi, Osaka 565, Japan

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The DNA base sequence of the *Pseudomonas* sp. NK87 gene (*P-nylB*) for 6-aminohexanoate-dimer hydrolase (P-EII), a xenobiotic-compound-degrading enzyme, was determined. It has an open reading frame of 1188 bp, initiated by ATG and terminated by TAG, and coding for 396 amino acids. The base sequence of the open reading frame has 53 % sequence similarity to that of the gene for the same enzyme of *Flavobacterium* sp. KI72 (*F-nylB*) and 35 % sequence similarity with respect to the deduced amino acid sequence. The P-EII enzyme was purified from an *Escherichia coli* clone in which the P-EII gene was highly expressed. The P-EII enzyme was inhibited by a serine protease inhibitor, diisopropyl fluorophosphate, as was the F-EII enzyme. Double reciprocal plots obtained from various concentrations of 6-aminohexanoate-dimer indicated that the k_{cat} value of the P-EII enzyme (9.2 s^{-1}) was approximately half that of the F-EII enzyme (19 s^{-1}), and the P-EII enzyme had higher affinity toward this substrate (K_m for P-EII, 0.6 mM; K_m for F-EII, 15 mM). The P-EII enzyme had a temperature optimum of 48 °C, and a pH optimum of 7.5. It is speculated that since the *P-nylB* and *F-nylB* genes are more diverged from each other than the corresponding *nylA* genes, the latter may have evolved more recently.

Introduction

Micro-organisms are believed to be highly adaptive toward environmental conditions. Such adaptability is implied from the observation that micro-organisms which metabolize various xenobiotic compounds can be easily isolated from nature (Hicks *et al.*, 1990; Cork & Krueger, 1991). We have isolated two bacterial strains, *Flavobacterium* sp. KI72 (Kinoshita *et al.*, 1977) and *Pseudomonas* sp. NK87 (Kanagawa *et al.*, 1989), which

can degrade 6-aminohexanoate-cyclic dimer (AC2), a by-product of the nylon-6 industry. In both strains, 6-aminohexanoate-cyclic-dimer hydrolase (EI, EC 3.5.2.12) (Kinoshita *et al.*, 1977) and, 6-aminohexanoate-dimer hydrolase (EII, EC 3.5.1.46) (Kinoshita *et al.*, 1981) are responsible for the degradation of the cyclic dimer. In *Flavobacterium* sp. KI72 the EI gene (*F-nylA*) and the EII gene (*F-nylB*) are encoded on the same plasmid, pOAD2 (Negoro *et al.*, 1980, 1983), while in *Pseudomonas* sp. NK87 the EI gene (*P-nylA*) and the EII gene (*P-nylB*) are encoded on different plasmids, pNAD2 and pNAD6, respectively (Kanagawa *et al.*, 1989). These results suggest that gene transfer mediated by plasmids plays an important role in the distribution of the ability to degrade this new xenobiotic compound.

We have compared the sequences of *F-nylA* and *P-nylA* genes and found that they show 99 % similarity with respect to the DNA base sequence and the deduced amino acid sequence (Tsuchiya *et al.*, 1989). This suggests that the *nylA* genes diverged only recently. Anti-F-EII serum does not cross-react with P-EII enzyme, whereas anti-F-EI serum reacts with P-EI. These results suggest that the *nylB* genes diverged earlier than the *nylA* genes. To obtain direct information about the evolutionary distance between the two EII enzymes, in this study we

* Author for correspondence. Tel. 06 877 5111; fax 06 876 2250.

† Present address: Department of Applied Microbial Technology, Kumamoto Institute of Technology, 4-22-1 Ikeda, Kumamoto 860, Japan.

Abbreviations: EI, 6-aminohexanoate-cyclic-dimer hydrolase; EII, 6-aminohexanoate-dimer hydrolase; *nylA*, EI gene; *nylB*, EII gene (the prefixes F- and P- denote the enzymes and genes from *Flavobacterium* and *Pseudomonas*, respectively); LB medium, Luria-Bertani medium; AL2, 3, 4 and 5, 6-aminohexanoate-dimer, -trimer, -tetramer and -pentamer; AC2, 6-aminohexanoate-cyclic dimer; Z-AL2 and 3, *N*-carbobenzoxymethyl-6-aminohexanoate-dimer and -trimer; DFP, diisopropyl fluorophosphate; TNBS, trinitrobenzene sulphonate.

The sequences reported in this paper have been submitted to GenBank and have been assigned the accession number D10678.

determined the DNA base sequence of the *P-nylB* gene and compared it with the sequence of the *F-nylB* gene reported previously (Okada *et al.*, 1983). We also purified the P-EII enzyme from an *Escherichia coli* clone in which the *P-nylB* gene was highly expressed, and examined the basic characteristics of the enzyme.

Methods

Enzymes and chemicals. Restriction endonucleases (*Bam*HI, *Eco*RI, *Eco*RV, *Hinc*II, *Hind*III, *Nru*I, *Pvu*II, *Pst*I, *Rsa*I, *Sal*I, *Sma*I and *Sph*I), T4 DNA ligase, DNA polymerase I (Klenow fragment), exonuclease III and mung bean nuclease were obtained from Toyobo Co. Other chemicals used were certified reagent grade and were purchased from Nacalai Tesque Co. (Kyoto). The 6-aminohexanoate-oligomer (AL2-AL5), and its *N*-carbobenzoxy derivatives (Z-AL2, Z-AL3) were synthesized in our laboratory by the mixed anhydride method (Kinoshita *et al.*, 1975). AC2 was purified by crystallization from hot water (Kinoshita *et al.*, 1975). The following commercial dipeptides were used for the enzyme assay: L-Ala-Gly, L-Ala-L-Phe, L-Ala-L-Asp, L-Ala-L-Tyr, L-Ala-L-Ile, L-Ala-L-Thr, DL-Ala-DL-Asn, L-Ala-L-Pro, L-Ala-L-Ala, L-Ala-L-Trp, L-Ala-L-Glu, L-Ala-L-Lys, L-Ala-L-Met, Gly-L-Ala, L-His-L-Ala, L-Val-L-Ala, L-Pro-L-Ala, L-Glu-L-Ala, L-Met-L-Ala, L-Phe-L-Ala, L-Trp-L-Ala, L-Tyr-L-Ala, L-Ser-L-Ala, L-His-Gly, L-Val-Gly, L-Val-L-Phe, L-Leu-Gly, L-Phe-Gly, L-Asp-Gly, L-Tyr-Gly, L-Met-Gly, L-Lys-Gly, L-Lys-L-Ala, L-Lys-L-Asp, L-Lys-L-Phe, L-Lys-L-Lys, L-Lys-L-Leu, L-Lys-L-Val, L-Trp-Gly, L-Pro-Gly, L-Ser-Gly, L-Leu-L-Leu, L-Glu-L-Val (Sigma); DL-Ala-DL-Leu, Gly-DL-Leu (Nutritional Biochemicals); DL-Ala-Gly, Gly-Gly, L-Leu-L-Ala (Nacalai Tesque); Gly-L-Val, Gly-L-Phe, Gly-L-Pro, L-His-L-Leu (Protein Research Foundation); Gly-L-Leu, DL-Ala-L-Ala (Tokyo Kasei-Kogyo Co.); L-Leu-L-Tyr (Calbiochem).

Plasmids, phages and bacterial strains. pNK2 (Kanagawa *et al.*, 1989), a hybrid plasmid consisting of a 2.5 kb *Pst*I fragment containing the *P-nylB* gene and 2.6 kb of pUC12 vector DNA, was used. M13mp18 and mp19 (Yanisch-Perron *et al.*, 1985) were used as vectors for DNA sequencing. Plasmids pKP1500 (Miki *et al.*, 1987) and pKK 223-3 (Amann *et al.*, 1983), which contain the *tac* promoter, were used as vectors. *E. coli* JM103 [$\Delta(lac-pro)$ *thi-1* *strA* *supE* *endA* *sbclB15* *hsdR4*, *F'**traD36* *proA*⁺ *lacI*^q *lacZ* Δ M15] (Messing, 1983) and KP3998 (*F*⁻, *hsdS20* (*r_B* *m_B*) *ara-14* *proA2* *lacI*^q *galK2* *rpsL2* *xyl-5* *mtl-1* *supE44*, λ ⁻) (Miki *et al.*, 1987) were used as the recipients for transformation by M13 phage DNAs.

DNA preparation and electrophoresis. Plasmid DNA was prepared by the alkaline extraction method (Birnboim & Doly, 1979). M13 single-stranded DNA and double-stranded DNA were prepared from the culture supernatant and pellets of the M13-infected cells as described by Sambrook *et al.* (1989). Agarose gel electrophoresis of DNA was done by conventional methods (Sambrook *et al.*, 1989). SDS-PAGE of proteins was done by the method of Laemmli (1970).

Nucleotide sequencing. This was done by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using 7-deaza-2'-deoxyguanosine triphosphate (Mizusawa *et al.*, 1986) and a sequencing kit (Toyobo Co.).

Enzyme assay. Enzyme activities were quantitatively measured by determining the increase of amino groups by the trinitrobenzene sulphate (TNBS) method (Kinoshita *et al.*, 1977). Unless otherwise specified, enzyme reactions were done at 30 °C, using 1.5 mM-AL2 as substrate (standard assay condition) in 20 mM-potassium phosphate buffer containing 10% (v/v) glycerol (pH 7.2) (buffer A) (Kinoshita *et*

al., 1977). For qualitative detection of the activity of purified enzyme toward various dipeptides, the enzyme (0.3 mg ml⁻¹) was incubated with dipeptides (5 mg ml⁻¹) in buffer A for 20 h, and reaction products were spotted on to thin-layer silica gel sheets (60F₂₅₄, Merck), developed with *n*-propanol/water/ethyl acetate/aqueous ammonia (24:12:4:1, by vol.) and detected with ninhydrin (0.2% in *n*-butanol) (Negoro *et al.*, 1983). Proteins were measured from the absorbance at 280 nm. For F-EII, A₂₈₀ 1% was taken as 15 (Negoro *et al.*, 1989), and this value was used for the estimation of the P-EII enzyme concentration.

The pH stability of P-EII was tested by incubating the enzyme (0.3 mg ml⁻¹) at 30 °C for 2 h in either 50 mM-phosphate buffer (pH 6.2–7.8) or 50 mM-Tris/HCl buffer (pH 7.8–8.6). Samples were diluted 100-fold in buffer A before being assayed.

For the substrate specificity tests (Table 2), to confirm that the enzyme had no detectable activity toward the 6-aminohexanoate-cyclic dimer and *N*-carbobenzoxy-6-aminohexanoate dimer, the purified enzyme (1.7 mg ml⁻¹) was incubated with 10 mM of these substrates at 30 °C in buffer A, and the reaction products (0.3 µl) were spotted on thin-layer silica gel sheets (60F₂₅₄, Merck) developed using *n*-butanol/acetic acid/water (4:1:2, by vol.). However, no products were detected even after 20 h of reaction with ninhydrin (0.2% in *n*-butanol). However, 6-aminohexanoate was detected after 1 h of reaction from a reaction mixture containing 10 mM-6-aminohexanoate-dimer and the purified enzyme (0.02 mg ml⁻¹).

Enzyme purification. Cell extracts were prepared from parental *Pseudomonas* sp. NK87 cells grown in 20 litres of CDY medium (Negoro *et al.*, 1980). The cells were disintegrated in a French press (400 kg cm⁻²), and the suspension was centrifuged (10000 g). The supernatant obtained (200 ml) was used as crude enzyme solution. From the extracts the P-EII enzyme was purified by two passages through a DEAE-Sephadex A50 column, gel filtration chromatography using Toyopearl HW55, DEAE-Toyopearl 650S chromatography, and preparative PAGE. (See Table 1*a*).

To purify the P-EII enzyme from *E. coli* KP3998 harbouring pNK3, the cells were cultivated at 37 °C in 2.4 litres of LB medium containing 50 µg ampicillin ml⁻¹, using a reciprocal shaker. When the culture density reached 3 × 10⁹ cells ml⁻¹, isopropyl β-D-thiogalactoside (final concentration, 0.1 mM) was added to the culture broth. After a further 2 h of cultivation, cells (wet weight, 36 g) were harvested, washed with 50 mM-potassium phosphate buffer (pH 7.2) (buffer B), and suspended in 30 ml buffer B. The cells were disintegrated in the French press (900 kg cm⁻²), and the suspension was centrifuged (10000 g). The supernatant obtained (49 ml) was used as crude enzyme solution. The enzyme precipitated with 30% saturation of ammonium sulphate was dissolved in 40 ml buffer A, and dialysed against buffer A for 16 h. The sample (41 ml) was applied to a DEAE-Sephacrose CL-6B column (4.2 × 34 cm) equilibrated with buffer A, and eluted with a linear gradient of NaCl (0.05–0.5 M) in a total volume of 1.8 litres. The active fractions were concentrated by ultrafiltration (UP-20, Advantech). The sample (49 ml) was applied to a Q-Sepharose column (2.8 × 18 cm) equilibrated with buffer A, and eluted with a linear gradient of NaCl (0.1–0.5 M) in a total volume of 1.2 litres. For amino acid analysis and sequencing of the N-terminal region of the enzyme, samples were desalted on a Sephadex G50 column equilibrated with distilled water, and lyophilized (see Table 1*b*).

F-EII enzyme which had been purified from *E. coli* 294(pNL4) previously and stored at –80 °C (Negoro *et al.*, 1989) was used for the inhibition experiment (see Fig. 6).

Amino acid composition and N-terminal amino acid sequence. The amino acids of the purified enzyme were sequenced by sequential Edman degradation using an automated protein sequencer (Applied Biosystems model 470A). The purified sample was hydrolysed at 110 °C in 6 M-HCl containing 0.2% phenol for 24 h. The amino acid

Fig. 1. Construction of plasmid pNK3, which strongly expresses the *P-nylB* gene (a), and sequencing strategy of the *P-nylB* gene (b). (a) To eliminate an upstream region of *P-nylB* included in pNK2, pNK2 was digested with *Sa*I, followed by exonuclease III. The 5'-protruding ends were removed by S1 nuclease treatment and repaired by DNA polymerase (Klenow fragment) treatment. This fragment was digested with *Hind*III and fractionated by agarose gel electrophoresis. The 2.0 kb fragment containing *P-nylB* was recovered and integrated between the *Hind*III and *Sma*I sites in a multilinker region of pKK223-3. The resulting plasmid was named pNO39-4. pNO39-4 was partially digested with *Eco*RI, and then completely digested with *Hind*III, and the 2.0 kb *Eco*RI-*Hind*III fragment was recovered from the agarose gel. This fragment was ligated to pKP1500 which had been digested

composition was measured by an amino acid analyser (Hitachi model L8500).

Results

Sequence of the *P-nylB* gene

The DNA base sequence of a 2049 bp region containing the *P-nylB* gene starting from the *Pst*I site in the multilinker region (see Fig. 1) was determined by the dideoxynucleotide chain-termination method. An open reading frame (ORF) of 1188-bp, beginning at ATG (611 bp) and terminating at TAG (1799 bp), and coding for 396 amino acids, was observed (Fig. 2). At 6 bp upstream of the translational initiation codon a consensus Shine-Delgarno sequence of GAGG was observed, and downstream of the termination codon there were two short (5 bp) inverted repeats and one long (13 bp) inverted repeat.

The P-EII enzymes purified from *Pseudomonas* sp. NK87 and from *E. coli* harbouring pNK3 containing the *P-nylB* gene in expression vector pKP1500 (described below) were subjected to Edman degradation in order to determine the amino acid sequence of the N-terminal region. The 10 amino acid sequence of the enzyme from *E. coli* KP3998(pNK3) was identical to the sequence which starts from the expected initiation Met codon. However, for the enzyme derived from *Pseudomonas* sp. NK87, in addition to the major sequence of Phe-Arg-Asp-Pro-(Thr)-Val-Pro-Gly-Asn-Ser-His-Ile-Pro-Arg-Gln-Asp-, minor sequences of (Pro, Val)-Pro-(Val, Gly)-(Pro, Phe, Asn)-(Gly, Arg)-Asp were observed. The major sequence coincides with the sequence from the seventh to twenty-second amino acids deduced from the base sequence. The minor sequences are considered to be a mixture of Val-Pro-X-Phe-Arg-Asp-, Pro-X-Val-Pro-Gly-Asn- and Val-Pro-Gly-Asn- which would have originated from the *P-nylB* gene product having 3, 9 and 11 amino acids respectively eliminated from its N-

with *Eco*RI and *Hind*III, and treated with bacterial alkaline phosphatase, and then used to transform *E. coli* KP3998. The transformants were selected and the structure of the plasmid pNK3 harboured in the transformant was confirmed from its restriction map. Inserted fragments are shown as boxes, in which the *P-nylB* regions are shaded. Vector regions are shown as thin lines, in which ampicillin resistance genes are marked by black boxes. The *tac* promoter regions are shown as arrowheads. (b) The top line shows the locations of restriction sites in the inserted DNA fragment of pNK2. E, *Eco*RI; H, *Hind*III; Hc, *Hinc*II; Nr, *Nru*I; P, *Pst*I; Pv, *Pvu*II; R, *Rsa*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; and V, *Eco*RV. The horizontal dashed and solid lines below the restriction map represent the DNA fragments subcloned in M13mp18 or mp19, in which the sequences represented by the solid lines were determined. The direction of sequencing is shown by the arrows.

10 20 30 40 50 60 70 80 90
 CTGCAGGAGACCTGCGGCTGGAGCTGGATGCCATCGTCAAGGCCTATGCCCTGACCACGTCCGAAGTGTGGGACGAGGCCGCGCGCTG
 L Q E S L R L E L D A I V K A Y A L T T S E V W D E A A G L
 A G E P A A G A G C H R Q G L C P D H V R S V G R G R R P A
 180
 CTGGCGACGACCCGACCATCTATGTCTCGGTTTCCAGGCCTCGAAGGGCTGGCGCTGGATTTCGCCAGCCGCTGCTCTGGGCGCGT
 L A T T R T I Y V V G F Q A S K G L A L D F A S R L L W A R
 G D D P H H L C R R F P G L E G P G A G F R Q P P A L G A F
 270
 TCCAACGTCAATTTTCATCGACAACGCCCTCGGGCACCTTCGGCGAGATCATCAATGCCGATCCCGGCAGAGCGTCTGCTCCTCGTCGAC
 S N V I F I D N A S G T F G E I I N A D P R Q S V V V L V D
 Q R H F H R Q R L G H L R R D H Q C G S P A E R R R P R R H
 360
 ACCGCTCCTATGCGACGCGCGCATCAAGCTGGTGGAGAAGCTGAAGTCGATGGACATGCCGCTGGTTCATCGTCACAGACAAGTTTCAGC
 T A S Y A T R G I K L V E K L K S M D M P L V I V T D K F S
 R L L C D A R H Q A G G E A E V D G H A A G H R H R Q V Q P
 450
 CACTGGCCCTTCGCTATACGCGCTTCGTATTGAGCCGATACGCTTGTAAAACTTCTGGGATTTCGACGGCGAGCCTCAGTGTCTG
 H W A F A Y T R F V F E P H T L V K T F W D S T A S L S V V
 L G L R L Y A L R I R A A Y A C K N L L G F D G E P Q C R A
 540
 CTCAACCTGTGATCGACGCCGTGCCACCAAGCTCGGGCGAAGGCCAAGCGCAACTTCGCCATGATGAGCGATATGGGCACCTCTTC
 L N L L I D A V A T K L G P K A K R N F A M M S D M G T L F
 Q P A D R R R R H Q A R A E G Q A Q L R H D E R Y G H P L R
 630
 GGAGAAATTCGTCGGCGGCACTTATCTTCGCCGTAACGATTCATTCGCTCCAACCGCAAAACGAGCCCAAGCATGACACCGTTCCGCCCTT
 G E F V G G S Y L R R N D * M N T V P P F
 R I R R R Q L S S P *
 720
 CCGGATCCGACCGTACCCGGAATTCCCATATTCCAAGGCAGGATTGGGACCGGGCGCCTGGAATCGCTGGACCTTCAGCATGTCCG
 R D P T V P G N S H I P R Q D W D R A P W N R W T F Q H V R
 810
 CGAGCTTCTGCCGACCACGAAGTCTGGCGCGGAACGGGGCGGCAAGCCCTCTGCCGCTGATCTGCCGGATATCGATGCCGTTTCTCT
 E L L P T T K V W R G T G P A S P L P V D L R D I D A V S F
 900
 CGCGCGGAGGGGAGAGCCATACCATCGCGGCTTCTCGAAACGAGCTATGCCGATGGCTTCCTGGTGTCCACGGCGGAAAGATCGT
 A A E G Q S H T I A G F L E T S Y A D G F L V L H G G K I V
 990
 GGCCGAGCGCTACCTCAACGGAATGGCGCGCACACCCAGCACCTGTGCAATCGGTGCCAAATCGGTCTGCGCACCGTGGCGGGCAT
 A E R Y L N G M A P H T Q H L S Q S V A K S V V G T V A G I
 1080
 CCTGATCGATCGCGGCGTGGTCAACCCCGCGCGCTCTGACGCACTACCTTCGGAACTGGAGCGACAGCCTATCGCGGCGGACCGT
 L I D R G V V N P A A L L T H Y L P E L E A T A Y R G A T V
 1170
 CCAGCAGTACTCGACATGACCAGCGCGTGGTCTTCGACGAGACCTATACCGCGCTCGATTCCACATGGCGCAGCTGGATGTGCCAG
 Q H V L D M T S G V V F D E T Y T A L D S H M A Q L D V A S
 1260
 CGGCTGGAAGGATAGCCCAACCCGACTGGCCACGATGTCTGGGATCTGATCCTGTCCCTGAAGGATCTGGAATGCCCGCACGGCGC
 G W K D S P N P D W P T H V W D L I L S L K D L E C P H G A
 1350
 CTCCTTCGCTATCGCTCGATCGAGACCGATGTCTGGCCTTCGTCTGCAACGCGCGCGCGCGCGCTTCGCGAACTCGTCAGCCG
 S F R Y R S I E T D V L A F V L Q R A A A A P L A E L V S R
 1440
 CGAATTGTGGGCGCGATGGGGCGGAGGAAGACGCTATTTACCGTGGATACCGCGGCTATCGCTGGGTGACGGCGGTTCAACGC
 E L W A P M G A E E D A Y F T V D T A G Y A L G D G G F N A
 1530
 GACCCTTCGCGACTATGCGGCTTTCGCTTGTGTCATCTTCGCGCGCGGAGATCGATGGCCGGCGTATCGTGTGCGCCGCTGGATTGC
 T L R D Y A R F A L L H L R G G E I D G R R I V S P G W I A
 1620
 CGCAACGCGCTTCGCTGCGATCCGGCGCTCTTCGGCGACATTTATCGAGAGGCCCTGCCCGCGCGCCTATCACAACGATTCTGGAT
 A T R F G A D P A L F G D I Y R E A L P A G A Y H N Q F W I
 1710
 CGAGGATACCGACGCGCGCCTATATGGCGCGCGGCTCTTCGGACAACGATCTACATCGACCCGGAAGCGGATTTTCGGCGCGGTCAT
 E D T A R G A Y M A R G V F G Q L I Y I D P E A D F A A V I
 1800
 CCTGTGAGCTGGCCGGAATTCGTGAGCAGCAGCGGCTGCGGACGGCGCTTGGCGCGGTACGGGCCGTTTCGCGAGGCGCTGTACGGCTA
 L S S W P E F V S T T R L R T A L A A V R A V R E A L S A *
 1890
 GCGAGCGGCGGATTACCGCTTTGTGCGGGATAGCCCCGGCGCAGGCCAAAGCGCAACACAAAAGAGAGCATTTCCGGCGAACCCGT
 1980
 GTTACCGGAAATGTTCAAGTCGGATCACACTACGCGACCTCCATCCAACCCGCACGAAAACGCAATACTCCGCACGTAGCGTCCCTCG
 2049
 GAAACCATGCGCGCGCAAGCCGGCTAAACAAGGATGACCGGTGACGGCAACATTTCCAGGACGCC

Fig. 2.

terminus. Although it is possible that this non-uniformity of the N-termini is due to artifacts occurring during the purification processes, it may also mean that the P-*nylB* gene product is processed in several different ways in *Pseudomonas* sp. NK87. Since the P-EII enzyme from *E. coli* KP3998(pNK3) has slightly higher specific activity toward 6-aminohexanoate-dimer than the enzyme from strain NK87 (described below), the processing in the *Pseudomonas* strain seems not to be essential for maturation of the protein but rather to be due to partial degradation by endogenous proteases.

The number of amino acid residues per subunit predicted from this ORF agreed well with that calculated from the results of amino acid analysis of the purified protein from *E. coli* KP3998(pNK3) (numbers in parentheses) except for unstable amino acids such as Trp: Lys, 5 (6.0); His, 13 (13.3); Trp, 11 (0); Arg, 27 (27.5); Asp+Asn, 35 (35.6); Thr, 26 (25.8); Ser, 21 (20.4); Glu+Gln, 29 (32.0); Pro, 24 (23.0); Gly, 31 (30.3); Ala, 53 (54.6); Cys, 1 (0); Val, 30 (30.9); Met, 6 (5.9); Ile, 16 (15.9); Leu 38 (39.6); Tyr, 13 (13.0); Phe, 17 (17.2). From these results, we concluded that this ORF encodes P-EII enzyme.

Alignment of the nucleotide sequences of the P-*nylB* and F-*nylB* genes by the method of Wirbur & Lipman (1983) showed that these genes have 53% sequence similarity in the coding region. This similarity is less than that observed for the P-*nylA* and F-*nylA* (99%) (Tsuchiya *et al.*, 1989). Alignment of the amino acid sequences by the method of Dayhoff *et al.* (1978) revealed that the sequence similarity between the P-EII and F-EII enzymes is 35% including gaps (Fig. 3). This value is much less than that between P-EI and F-EI (99%) (Tsuchiya *et al.*, 1989).

Purification of P-EII

P-EII was initially purified from a 20 litre culture of *Pseudomonas* sp. NK87 (Table 1a), but the amount of the enzyme was insufficient for characterization of the enzyme. Therefore, we constructed plasmid pNK3 in which the P-*nylB* gene (Kanagawa *et al.*, 1989) was integrated into the *E. coli* expression vector pKP1500 (Fig. 1a). The P-EII enzyme was purified 25-fold from *E. coli* KP3998(pNK3) at a yield of 19% (Table 1b). The

Fig. 3. Sequence alignment of P-EII and F-EII. Amino acids are shown in one-letter code. The sequences are numbered from the N-termini. Alignment of the sequences was done by the method of Dayhoff *et al.* (1978). Identical amino acids between P-EII and F-EII are marked by asterisks. The active-site serine of F-EII enzyme (Ser112) is marked by a star.

final specific activity of the enzyme from the *E. coli* clone [4.0 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ (U mg^{-1})] was slightly higher than that from parental strain NK87 (2.7 U mg^{-1}) (Table 1). The enzyme from *E. coli* clone was judged to be homogeneous, yielding a single protein band on SDS-PAGE (Fig. 4). This enzyme preparation was used for characterization of the P-EII enzyme.

Characterization of P-EII and comparison with F-EII

The M_r of P-EII was estimated to be 76000 by HPLC using a TSK-GEL G3000SW column. The M_r of the subunit of the P-EII enzyme estimated by SDS-PAGE was 42000. This value agrees well with that calculated for the ORF of the P-*nylB* gene (M_r 43383) (Fig. 2). From these results, P-EII was estimated to be a homodimer enzyme like F-EII (Kinoshita *et al.*, 1981).

The purified P-EII enzyme had nearly equal activities against various oligomers of 6-aminohexanoate, from the dimer to the pentamer (AL2-AL5) (Table 2). It had no significant activities against 56 different dipeptides (see Methods). When AL4 was used as a substrate for the

Fig. 2. Nucleotide sequence of P-*nylB* and deduced amino acid sequence of P-EII. The predicted amino acid sequence of the P-EII enzyme, which starts from position 611 (frame 2) is indicated below the nucleotide sequence in one-letter code. Putative translational initiation and termination codons and a putative ribosome-binding site (Shine-Dalgarno sequence) are boxed. Amino acid sequences of two unknown ORFs (frames 1 and 3) located upstream of the P-*nylB* gene are also shown. Palindromic sequences are indicated by arrows.

Table 1. Purification of P-EII from (a) *Pseudomonas* sp. NK87 and (b) *E. coli* KP3998(pNK3)

Purification step	Protein (mg)	Activity (U)	Sp. act. (U mg ⁻¹)	Yield (%)	Purification (-fold)
(a)					
Crude extract	14000	167	0.0058	100	1
DEAE-Sephadex A-50 (1st)	1580	75.9	0.048	45	8.2
DEAE-Sephadex A-50 (2nd)	175	50.8	0.29	30	50
Toyopearl HW55	94.9	41.1	0.43	24.5	75
DEAE-Toyopearl 650S	38.0	25.1	0.66	15.1	114
Preparative electrophoresis	1.5	4.2	2.7	2.5	460
(b)					
Cell extract	5100	790	0.16	100	1
Ammonium sulphate precipitation	420	560	1.3	71	8.6
DEAE-Sephacrose CL-6B	150	420	2.8	54	18
Q-Sephacrose	37	150	4.0	19	25

Fig. 4. SDS-PAGE of P-EII enzyme from *E. coli* KP3998(pNK3) at each purification stage. Lane M, M_r markers [from the top, phosphorylase *b* (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000) and soybean trypsin inhibitor (20100)]; lane 1, cell extract of *E. coli* KP3998(pNK3); lane 2, supernatant fraction after precipitation with ammonium sulphate (30% saturated); lane 3, fraction of cell extract precipitated by ammonium sulphate (30% saturated); lane 4, partially purified P-EII after DEAE-Sephacrose CL-6B column chromatography; lane 5, purified P-EII after Q-Sephacrose Fast Flow chromatography.

P-EII enzyme, 6-aminohexanoate and AL3 were produced as major products, and AL3 was subsequently subjected to hydrolysis (Fig. 5). Moreover, the enzyme had no significant activity toward Z-AL2 (Table 2). These results suggest that the P-EII enzyme digests linear oligomers of 6-aminohexanoate exogenously from the amino-terminus as a monomeric unit. This characteristic

Table 2. Substrate specificity of P-EII

Enzyme assays were done at 30 °C in buffer A containing 1.5 mM of each substrate (see Methods for details).

Substrate	Relative activity (%)
6-Aminohexanoate-dimer (AL2)	100
6-Aminohexanoate-trimer (AL3)	98
6-Aminohexanoate-tetramer (AL4)	115
6-Aminohexanoate-pentamer (AL5)	82
6-Aminohexanoate-cyclic dimer (AC2)	< 0.1
N-Carbobenzoxymethyl-6-aminohexanoate-dimer (Z-AL2)	< 0.1

is similar to that defined for the F-EII enzyme (Kinoshita *et al.*, 1981).

Initial rates of the enzyme reaction were measured at 30 °C by using various concentrations of AL2 (0.2–2.0 mM). From the Lineweaver–Burk plot, the K_m was estimated to be 0.6 mM; the k_{cat} obtained by using the M_r of the native enzyme of 84000 was 9.2 s⁻¹. The K_m and k_{cat} values of the F-EII enzyme were 15 mM and 19 s⁻¹, respectively (Fujiyama *et al.*, 1991). These results indicate that the P-EII enzyme has a higher affinity for AL2 than does the F-EII enzyme.

The optimum pH for the hydrolysis of AL2 was 7.5. The enzyme was stable only at around neutral pH (between pH 6.8 and 8.5). The optimal temperature for hydrolase activity was 48 °C in the 10 min of the enzyme reaction. To examine the thermostability of the enzyme, the enzyme in buffer A was incubated at various temperatures for 30 min, then the residual activities were assayed at 30 °C and compared with the untreated sample. The enzyme was stable up to 45 °C.

Fig. 5. Thin-layer chromatography for detection of hydrolysates from 6-aminohexanoate-tetramer (AL4) by P-EII enzyme reaction. The P-EII enzyme from *E. coli* KP3998(pNK3) (at the stage of Q-Sepharose Fast Flow chromatography) ($78 \mu\text{g ml}^{-1}$) was incubated at 30°C with 10 mM -6-aminohexanoate tetramer in buffer A. Aliquots ($0.3 \mu\text{l}$) of the reaction mixture were sequentially spotted on the thin-layer plate at the time shown below the plate, and developed with *n*-propanol/water/ethyl acetate/aqueous ammonia (24:12:4:1, by vol.) Reaction products were detected with ninhydrin. AL1, AL2, AL3 and AL4 indicate authentic 6-aminohexanoate, 6-aminohexanoate-dimer, -trimer and -tetramer, respectively.

Fig. 6. Inhibition of EII activity by DFP and EDTA. The purified EII enzymes (0.1 ml) ($78 \mu\text{g ml}^{-1}$ for P-EII, 0.63 mg ml^{-1} for F-EII) were mixed with an inhibitor (0.1 ml), and the mixtures were preincubated at 30°C for 1 h before the enzyme assay. Activity was expressed as a percentage of the activity without an inhibitor.

Table 3. Comparison of F-EII and P-EII

Characteristic	F-EII*	P-EII
M_r	42000 (dimer)	42000 (dimer)
Encoded on	pOAD2	pNAD6
K_m } (towards AL2) k_{cat}	15 mM 19 s^{-1}	0.6 mM 9.2 s^{-1}
Optimum pH	9.0	7.5
Optimum temp.	40°C	48°C

* The results for the F-EII enzyme were described previously (Kinoshita *et al.*, 1981; Fujiyama *et al.*, 1991).

The addition of 10 mM -EDTA to the reaction mixture had no effect on the enzyme activity (Fig. 6), suggesting that the enzyme does not require divalent cations. Addition of 1 mM -diisopropyl fluorophosphate (DFP),

however, inhibited approximately 80% of the initial activity, implying that a serine residue plays an important role in the catalytic function of the enzyme. Similar results were observed for the F-EII enzyme (Kinoshita *et al.*, 1981) (Negoro *et al.*, 1989). Basic characteristics of the P-EII and F-EII enzymes are summarized in Table 3.

Discussion

The *F-nylB* and *P-nylB* genes possessed 35% similarity (after alignment and insertion of gaps into both sequences) with respect to the amino acid sequences in the whole coding regions. This suggests that these genes have diverged from a common antecedent. The F-EII and P-EII enzymes exhibited similar basic enzymic characteristics; for example, their activities showed similar DFP inhibition profiles (Fig. 6). We have previously shown that radioactive DFP specifically binds to Ser-112 among 26 Ser residues in the F-EII enzyme and that changing Ser-112 to Ala by site-directed mutagenesis resulted in a drastic decrease of the enzyme activity to the detection threshold (Negoro *et al.*, 1989). These results suggest that one of the active-site residues of the F-EII is Ser-112.

The amino acid corresponding to Ser-112 in the F-EII enzyme is aligned with Ser-115 in the P-EII enzyme (Fig. 3). However, Ser-112 of F-EII is coded by AGC (Okada *et al.*, 1983), while Ser-115 of P-EII is coded by TCG (Fig. 2). When an AGY-type Ser codon ($Y = \text{T, C}$) is changed to a TCN-type Ser codon ($N = \text{A, G, C, T}$), or a TCN codon is changed to an AGY codon, intermediate codons such as TGG (Trp), TGY (Cys) or ACN (Thr) would be generated, in which a single nucleotide substitution occurred at the first or second base in the Ser codons. Although the possibility remains that the conversion of the AGY to TCN codons occurred in one

step by error-prone repair (Echols & Goodman, 1991), an alternative explanation is that the Ser residues in at least either one of the EII enzymes were not conserved constantly through evolution. Thus the Ser might be changed once to other amino acids by a single base replacement in the codon and then the altered amino acids would be returned to Ser by a successive base replacement, if the smallest number of the base substitutions occurred in the codon. If this were the case, the following two possibilities could be speculated: (i) micro-organisms possess some mechanisms to maintain genes whose products are useless, even if the function of the gene product is lost; (ii) Ser-112 of the F-EII enzyme and Ser-115 of the P-EII enzyme were not involved in enzyme activity. Further study to identify the active-site residue in the EII enzymes is required.

Analysis of the upstream portion of the P-EII coding region revealed that there are two ORFs which start from the 5'-proximal unsequenced region of the P-*nylB* gene and end at 18 bp upstream (frame 1) or 25 bp upstream (frame 3) of the putative Shine-Dalgarno sequence of P-*nylB* (Fig. 2). The ORF in frame 1 is expected to be efficiently expressed in *Pseudomonas* cells, since its codon usage agreed well with the codon preference of *Pseudomonas* strains. The F-*nylB* gene borne on pOAD2 of *Flavobacterium* strain KI72 is expressed from a promoter located 6 kb upstream of the gene in *E. coli* (Okazaki *et al.*, 1988; Zhou *et al.*, 1990). These results suggest that the P-*nylB* gene might be expressed as a part of an operon, although the possibility remains that the P-*nylB* gene has its own promoter.

No significant sequence similarity was observed between the F-EI and F-EII enzymes or between the F-EI and P-EII enzymes. This suggests that the *nylA* family and *nylB* family evolved independently. In contrast, alignment of the F-EII and P-EII sequences has shown that the similarities are 53% in the nucleotide sequence and 35% in the amino acid sequence. These values are much lower than those observed for the F-EI and P-EI sequences (99%). This suggests that the two *nylB* genes diverged from their common antecedent much less recently than the *nylA* genes diverged from each other.

EI and EII catalyse reactions in the sequential pathways for metabolism of 6-aminohexanoate-cyclic dimer. It is reasonable to assume that an ancestral *nylB* gene, which encoded an enzyme reaction closer to the central metabolic pathways than the *nylA* gene, may have spread among micro-organisms earlier on during the evolutionary process. While the ancestral *nylB* gene has evolved to give the present F-*nylB* and P-*nylB* genes, micro-organisms having enzyme activities against 6-aminohexanoate-dimer seem to have acquired the new ability for cyclic-dimer metabolism by incorporating a more recently evolved *nylA* gene.

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