

Nylon Oligomer Degradation Gene, *nylC*, on Plasmid pOAD2 from a *Flavobacterium* Strain Encodes Endo-Type 6-Aminohexanoate Oligomer Hydrolase: Purification and Characterization of the *nylC* Gene Product

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A new type of nylon oligomer degradation enzyme (EIII) was purified from an *Escherichia coli* clone harboring the EIII gene (*nylC*). This enzyme hydrolyzed the linear trimer, tetramer, and pentamer of 6-aminohexanoate by an endo-type reaction, and this specificity is different from that of the EI (*nylA* gene product) and EII (*nylB* gene product). Amino acid sequencing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified EIII demonstrated that the enzyme is made of two polypeptide chains arising from an internal cleavage between amino acid residues 266 and 267.

Enzymes responsible for the degradation of man-made compounds are not only interesting in terms of the enzyme evolution but also important for the biological removal or detoxification of the synthetic compounds released into the natural environment. We have revealed that two enzymes, 6-aminohexanoate cyclic dimer hydrolase (EI) (4) and 6-aminohexanoate dimer hydrolase (EII) (5) are responsible for the degradation of 6-aminohexanoate cyclic dimer (AC2), a by-product of nylon manufacture, and that the EI (*nylA*) and EII (*nylB*) genes are encoded in pOAD2, one plasmid of the three harbored in *Flavobacterium* sp. strain KI72 (11, 12). The EI enzyme is composed of 493 amino acids (14) and is specifically active towards 6-aminohexanoate cyclic dimer (4). The EII enzyme is composed of 392 amino acid residues (10, 13) and digests 6-aminohexanoate oligomers with a degree of polymerization of 2 to 20 by exo-type reactions (5).

Recently, we found a new nylon oligomer-degrading gene (*nylC*) on pOAD2. Cloning and sequencing analysis of the gene have shown that *nylC* was found at positions 4.2 to 5.3 kb on the pOAD2 map in the same orientation with the *nylB* gene. The *nylC* gene codes for 355 amino acids with an open reading frame of 1,065 bp beginning at ATG and terminating at TAG (8). In this article, we purified the EIII enzyme from *Escherichia coli* KP3998 harboring pULC4 (a hybrid plasmid which consists of expression vector pKP1500 [7] and the 1.2-kb *Bgl*III-*Bam*HI fragment of pOAD21 encoding the *nylC* gene [8]) to homogeneity and characterized the enzyme.

The cells grown to 2×10^9 /ml at 37°C in 8 liters of LB medium containing 0.1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) were suspended in 200 ml of buffer A (20 mM potassium phosphate [pH 7.2] containing 15% glycerol) and disrupted with a French press (500 kg/cm²). The supernatant (205 ml) obtained by centrifugation (10,000 \times g, 10 min) was used as the crude enzyme solution. The crude EIII enzyme precipitated with 35% saturation of ammonium sulfate was dissolved in 40 ml of buffer A and dialyzed against the same buffer. The sample (48 ml) was applied to a DEAE-Sephadex

A-50 column (4 by 50 cm) equilibrated with buffer A. Washing the column with 500 ml of buffer A containing 0.2 M NaCl and eluting the enzyme with a linear gradient of 0.2 to 0.8 M NaCl (total volume of 2 liters) followed. The active fractions (56 ml) were concentrated by ultrafiltration, and the concentrated sample (4 ml) was reapplied to a DEAE-Sephadex A-50 column (2 by 20 cm) equilibrated with buffer A. The column was then washed with 100 ml of buffer A, and finally, the purified enzyme was eluted out by the same linear gradient of NaCl as that described above in a total volume of 300 ml.

Proteins were measured by the A_{280} . Concentrations of the EIII enzyme were calculated by assuming that $A_{280}^{1\%}$ is 20. To measure the EIII enzyme activities, the enzyme was incubated at 30°C with 2.5 mg of unfractionated nylon oligomers, nylon oligomer mixture type 2 (Nom2), per ml (8) in buffer A. Free amino groups liberated by hydrolysis were measured by the trinitrobenzene sulfonic acid method (3, 4). One unit of the enzyme was defined as the amount of enzyme which generates one micromole of amino group in 1 min.

The EIII enzyme was purified 95-fold from the cell extracts with a yield of 15% (Table 1). The purified enzyme gave a single band having a relative mobility (R_f) of 0.60 on nondenatured polyacrylamide gel electrophoresis (nondenatured PAGE; Fig. 1A) and a single peak on high-performance gel filtration chromatography (data not shown), indicating that the enzyme is homogeneous. This enzyme preparation was the one used for further study.

Amino acid sequencing of the N-terminal region of the

TABLE 1. Purification of endo-type 6-aminohexanoate-oligomer hydrolase from *E. coli* KP3998(pULC4)

Purification step	Protein (mg)	Activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Cell extract	15,000	370	0.025	100	1
Ammonium sulfate precipitation	1,700	360	0.21	97	8.4
DEAE-Sephadex A-50 (first)	70	150	2.1	41	84
DEAE-Sephadex A-50 (second)	25	57	2.3	15	92

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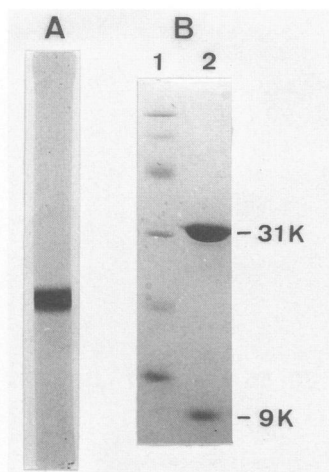


FIG. 1. PAGE of the EIII enzyme. The EIII enzyme purified from *E. coli*(pULC4) was analyzed by nondenatured 7% PAGE (1) (A) or by SDS-15% PAGE (6) (B). Lanes: 1, M_r standard proteins including phosphorylase *b* (97,400; $R_f = 0.1$), bovine serum albumin (67,000; $R_f = 0.16$), ovalbumin (43,000; $R_f = 0.25$), carbonic anhydrase (30,000; $R_f = 0.42$), trypsin inhibitor (20,100; $R_f = 0.62$), and α -lactalbumin (14,400; $R_f = 0.82$); 2, purified EIII enzyme.

enzyme gave a mixture of two polypeptides for each sequencing cycle. One is that of Met-Asn-Thr-Thr-Pro-Val-, which is identical to the sequence starting from the initiation codon deduced from its nucleotide sequence, while the other is of Thr-Thr-Ile-Ser-Ala-, which corresponds to the sequence starting from position 267 of the deduced open reading frame. On the basis of the deduced sequence, the M_r s of polypeptides I (amino acid positions 1 to 266) and II (amino acid positions 267 to 355) were calculated to be 27,408 and 9,513, respectively (8). These results are congruent with the M_r s of the two polypeptides identified by sodium dodecyl sulfate (SDS)-PAGE, i.e., 31,000 and 9,000 (Fig. 1B). These results suggest that the two polypeptides (polypeptides I and II) were generated by specific cleavage between Asn-266 and Thr-267 in the EIII polypeptide, although it is not known when such specific cleavage occurred.

When the purified EIII enzyme was incubated with unfractionated nylon oligomers (Nom2) (8), linear oligomers of 6-aminohexanoate such as the tetramer (A4) and the pentamer (A5) were initially produced and subsequently converted to smaller oligomers such as the dimer (A2) (Fig. 2). From the synthetic A4, the EIII enzyme produced A2 as the sole reaction product (Fig. 3A). This result is quite different from that of the EII enzyme, which hydrolyzes the 6-aminohexanoate-oligomer from its amino terminus (5), thereby producing 6-aminohexanoate and A3 from A4 at the initial reaction stage. When similar enzymatic reactions were done with A5 as a substrate, almost equal amounts of A2 and A3 were formed initially with the EIII enzyme, whereas A4 and A1 were detected with the EII enzyme (Fig. 3B). In addition, when the EIII enzyme was incubated with the *N*-carbobenzoxy-A4 (Z-A4), A2 and A3 were initially produced; A1 production, which was detected only after 22 h of reaction (data not shown), followed. This indicates that the enzyme predominantly cleaved amide bonds located at the second

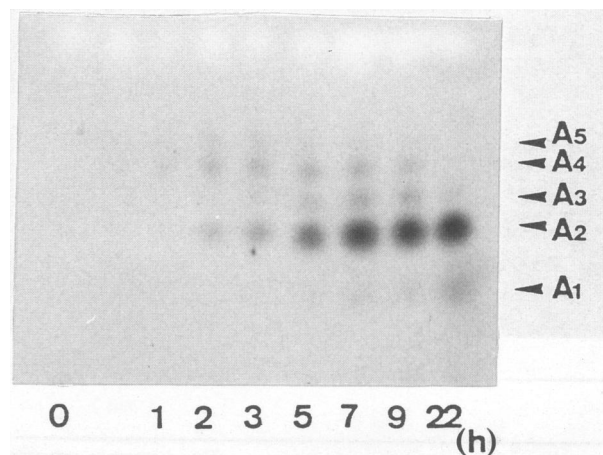


FIG. 2. Thin-layer chromatography for the detection of nylon oligomer-degrading activity of the EIII enzyme. The purified EIII enzyme from *E. coli*(pULC4) (3 μ g/ml) was incubated with unfractionated nylon oligomers (Nom2) (8) in buffer A (see text), and 2 μ l of the reaction mixture was sequentially spotted on a thin-layer silica gel plate (60F₂₅₄; Merck Co. Ltd.) at various times as indicated below the plate. After the plate was developed with solvent II (*n*-propanol, water, ethyl acetate, ammonia [24:12:4:1]) and dried, reaction products were detected with ninhydrin (0.2% in *n*-butanol) (2, 8). A1, A2, A3, A4, and A5 represent the monomer, dimer, trimer, tetramer, and pentamer of 6-aminohexanoate, respectively.

and third positions from the carbobenzoxy group. Therefore, the results mentioned above reveal that the EIII enzyme catalyzes the endo-type mode of reaction.

Table 2 shows the specific activities of the purified EIII enzyme towards various 6-aminohexanoate-oligomers. This enzyme favored a longer chain of linear oligomer digesting the internal amide bonds preferentially but had no activity toward the cyclic dimer and caprolactam. The activity of the enzyme towards natural peptides was also tested by using 77 kinds of dipeptides. However, the EIII enzyme, at a concentration of 0.9 mg/ml, showed no activity towards any of

TABLE 2. Specific activity of the EIII enzyme toward various nylon oligomers^a

Substrate	Sp act (mol of NH ₂ liberated/mol/s)
6-Aminohexanoate	
Dimer	2.7×10^{-4}
Trimer	0.067
Tetramer	0.26
Pentamer	0.29
Caprolactam	$<1.0 \times 10^{-4}$
6-Aminohexanoate cyclic dimer	$<1.0 \times 10^{-4}$

^a The EIII enzyme used was the sample at the stage of second DEAE-Sephadex column chromatography. Enzyme reactions were performed with 1 mM of substrate in buffer A (see text), and increases in the amino group were analyzed by the trinitrobenzene sulfonic acid method (4, 5). The 6-aminohexanoate dimer, trimer, tetramer, and pentamer were chemically synthesized in our laboratory by mixed-anhydride methods (3). Caprolactam was obtained from Nacalai Tesque Co. The 6-aminohexanoate cyclic dimer was purified from a nylon oligomer mixture obtained from Toyobo Co. by recrystallization (4). Molar absorption coefficients of the enzymes were calculated from the content of Tyr, Trp, and Phe in the deduced amino acid sequence, and specific activities were expressed as turnover number per subunit.

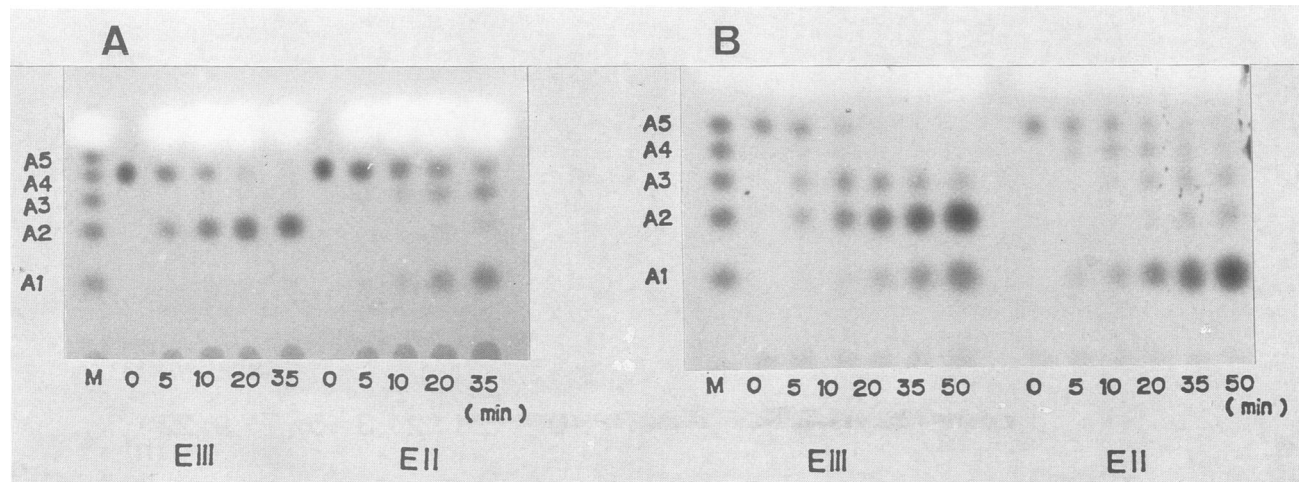


FIG. 3. Hydrolysis of 6-aminohexanoate tetramer (A4) and pentamer (A5) by the EIII enzyme. The purified EIII enzyme (30 $\mu\text{g/ml}$) was incubated at 30°C with 10 mM of A4 (A) or A5 (B) in buffer A (see text). To compare the pattern of the degradation, the EII enzyme (30 $\mu\text{g/ml}$), which was purified from *E. coli* 294 harboring pNL4 (9) and stored at -80°C, was incubated with the substrates under the same condition. The reaction mixtures were sequentially spotted on a thin-layer silica gel plate at the time corresponding to that shown below the plate, developed with solvent II, and detected with ninhydrin (see Fig. 2 legend).

the natural peptides tested to date even after 18 h of reaction. Moreover, the enzyme had no detectable activity towards a tetrapeptide (Gly-Asn-Thr-Thr) which is identical to the sequence appearing at the cleavage site mentioned earlier. Thus, the hydrolytic activity of the EIII enzyme seems to be highly specific to 6-aminohexanoate-oligomers.

The present study revealed that the EIII enzyme is a new amide hydrolase which digests highly polymerized 6-aminohexanoate-oligomer by an endo-type reaction. This enzyme is considered to be classified in EC.3.5.-.-. by the rules of enzyme nomenclature.

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REFERENCES

- Gabriel, O. 1971. Analytical disc gel electrophoresis. *Methods Enzymol.* **22**:565-578.
- Kanagawa, K., M. Oishi, S. Negoro, I. Urabe, and H. Okada. 1993. Characterization of the 6-aminohexanoate-dimer hydrolase from *Pseudomonas* sp. NK87. *J. Gen. Microbiol.* **139**:787-795.
- Kinoshita, S., S. Kageyama, K. Iba, Y. Yamada, and H. Okada. 1975. Utilization of a cyclic dimer and linear oligomers of ϵ -aminocaproic acid by *Achromobacter guttatus* KI72. *Agric. Biol. Chem.* **39**:1219-1223.
- Kinoshita, S., S. Negoro, M. Muramatsu, V. S. Bisaria, S. Sawada, and H. Okada. 1977. 6-Aminohexanoic acid cyclic dimer hydrolase: a new cyclic amide hydrolase produced by *Achromobacter guttatus* KI72. *Eur. J. Biochem.* **80**:489-495.
- Kinoshita, S., T. Terada, T. Taniguchi, Y. Takene, S. Masuda, N. Matsunaga, and H. Okada. 1981. Purification and characterization of 6-aminohexanoic acid oligomer hydrolase of *Flavobacterium* sp. KI72. *Eur. J. Biochem.* **116**:547-551.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Miki, T., T. Yasukochi, H. Nagatani, M. Furuno, T. Orita, H. Yamada, T. Imoto, and T. Horiuchi. 1987. Construction of a plasmid vector for the regulatable high level expression of eukaryotic genes in *Escherichia coli*: an application to overproduction of chicken lysozyme. *Protein Eng.* **1**:327-332.
- Negoro, S., S. Kakudo, I. Urabe, and H. Okada. 1992. A new nylon oligomer degradation gene (*nylC*) on plasmid pOAD2 of *Flavobacterium* sp. *J. Bacteriol.* **174**:7948-7953.
- Negoro, S., T. Mitamura, K. Oka, K. Kanagawa, and H. Okada. 1989. Determination of the active-site serine of 6-aminohexanoate-dimer hydrolase. *Eur. J. Biochem.* **185**:521-524.
- Negoro, S., S. Nakamura, H. Kimura, K. Fujiyama, Y. Zhang, N. Kanzaki, and H. Okada. 1984. Construction of hybrid genes of 6-aminohexanoic acid-oligomer hydrolase and its analogous enzyme: estimation of the intramolecular regions important for the enzyme evolution. *J. Biol. Chem.* **259**:13648-13651.
- Negoro, S., H. Shinagawa, A. Nakata, S. Kinoshita, T. Hatozaki, and H. Okada. 1980. Plasmid control of 6-aminohexanoic acid cyclic dimer degradation enzymes of *Flavobacterium* sp. KI72. *J. Bacteriol.* **143**:238-245.
- Negoro, S., T. Taniguchi, M. Kanaoka, H. Kimura, and H. Okada. 1983. Plasmid-determined enzymatic degradation of nylon oligomers. *J. Bacteriol.* **155**:22-31.
- Okada, H., S. Negoro, H. Kimura, and S. Nakamura. 1983. Evolutionary adaptation of plasmid-encoded enzymes for degrading nylon oligomers. *Nature (London)* **306**:203-206.
- Tsuchiya, K., S. Fukuyama, N. Kanzaki, K. Kanagawa, S. Negoro, and H. Okada. 1989. High homology between 6-aminohexanoate-cyclic-dimer hydrolases of *Flavobacterium* and *Pseudomonas* strains. *J. Bacteriol.* **171**:3187-3191.