# Microbial and enzymatic hydrolysis of poly(aspartic acid)

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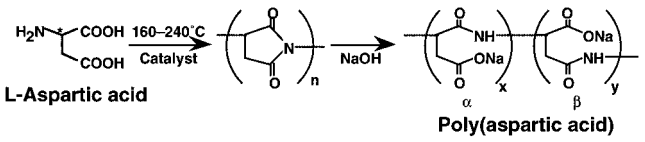
The microbial and enzymatic hydrolysis of poly(aspartic acid) (PAA) has been reviewed. Two PAA-degrading bacteria (*Pedobacter* sp. KP-2 and *Sphingomonas* sp. KT-1) were isolated from flesh river water. *Pedobacter* sp. KP-2 hydrolyzed PAA of high molecular weights over 5000. *Sphingomonas* sp. KT-1 hydrolyzed only PAA of low molecular weights (<5000), while the cell extract could hydrolyze high-molecular-weights PAA to yield aspartic acid monomer. PAA hydrolase was purified from the cell extract of *Sphingomonas* sp. KT-1 and characterized. The molecular cloning results indicate that the structure of this enzyme is similar to those of PHB depolymerases and conserves the lipase box as an active center. The results of NMR and GPC analyses showed that this enzyme hydrolyzed the amide bond between $\beta$ aspartic acid units in PAA to yield aspartic acid oligomers.

## Introduction

Poly(aspartic acid) (PAA), belonging to the family of synthetic polypeptides, is a biodegradable water-soluble polymer. PAA polymers have been attracted as environmentally degradable water-soluble materials to be used as dispersants, detergent builders, and in biomedical applications.[1)]

PAA is produced by thermal polymerization of L-aspartic acid. Polysuccinimide (PSI) is synthesized as initial product and PAA is produced by hydrolysis of PSI[2)] as shown in Scheme 1. The thermal polymerization of L-aspartic acid with or without catalyst leads to the formation of a mixture of L- and D-succinimide units, and the resulting PAA after hydrolysis of PSI is composed of 70% of $\beta$-amide and 30% of $\alpha$-amide units.[2)] Pivcová *et al.*[3)] investigated the sequential structure of amide units in thermally synthesized PAA by $^{13}$C NMR analysis and concluded that the $\alpha$- and $\beta$-amide units are randomly distributed in the PAA sequence. The thermal polymerization of L-aspartic acid in the absence of catalyst gives low molecular weight PAA below 10000, while high molecular weight PAA of 10000–90000 can be produced in the presence of phosphoric acid as a catalyst.

The biodegradability of thermally synthesized PAA has been extensively studied by many scientists. Alford *et al.*[4)] prepared PAA polymers by the thermal polymerization of $^{14}$C-labeled L-aspartic acid in the absence of catalyst and evaluated the biodegradability of $^{14}$C-labeled PAA in activated sludge. A portion of PAA polymers remained undegraded in the sludge. They have suggested that some unusual structures in PAA such as $\beta$-amide units and D-aspartic acid units may limit the biodegradation of thermally synthesized PAA. Recently, Nakato *et al.*[5)] prepared several PAA polymers, such as poly($\alpha$-L-aspartic acid), poly($\alpha$-D-aspartic acid), poly($\beta$-L-aspartic acid), and poly($\alpha$,$\beta$-D,L-aspartic acid)s, and investigated the structural effects on biodegradability of PAA in activated sludge. Both the chirality of monomeric units and amide bond structures in PAA did not affect the biodegradability of PAA, while the biodegradability of PAA decreased with an increase in the amount of irregular end groups in PAA.




Scheme 1. Synthesis of poly(aspartic acid).

The biodegradation of PAA chains must be caused by some bacteria in the natural environment. However, there has been no report on the isolation of PAA-degrading bacteria from environments and the enzymatic hydrolysis of PAA. In this paper, we reported the isolation and characterization of PAA-degrading bacteria from river water and the enzymatic hydrolysis of PAA with purified PAA hydrolase.

## Isolation of poly(aspartic acid)-degrading microorganisms

The biodegradabilities of PAA samples in river water (Arakawa river, Saitama, Japan) were measured by biochemical oxygen demand (BOD) assay and by gel permeation chromatography (GPC) analysis. Two types of poly(aspartic acid) samples, PAA-T (Mn: 2100, Mw: 4500) and PAA-P (Mn: 7500, Mw: 20000), were used for biodegradation test. The BOD-biodegradabilities of PAA-P and PAA-T in river water at 25°C increased with time to reach about 78% and 70% within 15 days, respectively. PAA-P sample was completely hydrolyzed within 12 days and no product was detected by GPC. In contrast, high-molecular-weight fractions over 1000 of PAA-T sample were completely degraded within 12 days, while low-molecular-weight fractions below 1000 were slowly degraded and a portion remained undegraded after the test of 28 days. These results indicate that PAA-degrading microorganisms are present in river water.

Two PAA-degrading bacteria were isolated from river water by an enrichment culture in the mineral medium containing

0.15% PAA-P and PAA-T as a substrate, respectively.[6, 7] One bacterium capable of degrading PAA-T was designed KT-1 and identified as a member of the genus *Sphingomonas* belonging in the alpha subclass of *Proteobacteria*. The other one, which degraded PAA-P, was designated KP-2 and identified as a member of *Pedobacter*, which belongs in *Cytophaga-Flavobacterium-Bacteriods-Sphingobacterium* group.

### Microbial degradation of poly(aspartic acid)

Strain KT-1 or KP-2 was incubated in the mineral medium containing 0.15% PAA-P as a carbon source, and the time-dependent change in molecular weights of PAA-P was analyzed by GPC (Fig. 1). Strain KT-1 hydrolyzed only a small portion of PAA polymers in the range of low-molecular-weights below 5000 (Fig. 1 (a)). In the case of Strain KP-2, the amount of PAA-P in the medium decreased with incubation time, and an original GPC peak of PAA-P disappeared after 20 days (Fig. 1 (b)). During the course of incubation, low molecular products of 250-5000 were accumulated and their amounts increased with time. Fig. 1 (c) shows the GPC profiles of PAA-P during the course of mixed culture with *Sphingomonas* sp. KT-1 and *Pedobacter* sp. KP-2. The amount of PAA-P polymer in the medium decreased with incubation time and no product was accumulated. After 12 days, all of PAA-P polymers were degraded. Thus, a mixed culturing of *Pedobacter* sp. KP-2 with *Sphingomonas* sp. KT-1 resulted in a complete degradation of PAA-P sample with high-molecular-weights.

### Purification and characterization of poly(aspartic acid) hydrolase from *Sphingomonas* sp. KT-1

*Sphingomonas* sp. KT-1 hardly degraded PAA polymers in the range of high-molecular-weights over 5000, while the cell extract of strain KT-1 could hydrolyze PAA to yield aspartic acid. This result indicates that *Sphingomonas* sp. KT-1 has some enzymes capable of hydrolyzing unusual amide linkages such as $\beta$-amide linkage in the cell. Therefore, we purified PAA degrading enzyme from cell free extract and characterized its properties.[8] As listed in Table 1, PAA hydrolase was purified 78-fold, recovering 12% of the activity present in the initial soluble fraction after purification with Mono S column. SDS-PAGE analysis of combined fractions containing PAA hydrolase activity, eluted from the Mono S column, revealed a single polypeptide band at 30 kDa. The N-terminal amino acid sequence of PAA hydrolase was determined as APAAASKGKA AALPDLKPGA GSFLFTG by automated Edman degradation. The result of a BLASTP search has suggested that the N-terminal amino acid sequence of purified PAA hydrolase has no similarity to those of other proteins. The effects of protease inhibitors on PAA-degrading activity of purified PAA hydrolase were investigated. The PAA degrading activity was significantly inhibited by the addition of diisopropyl fluorophosphates (DFP) or phenylmethane sulfonyl fluoride (PMSF), suggesting that the PAA hydrolase from *Sphingomonas* sp. KT-1 is a serine-type hydrolase.

### Molecular cloning of poly(aspartic acid) hydrolase from *Sphingomonas* sp. KT-1

For detail information of PAA hydrolase, the PAA hydrolase gene from *Sphingomonas* sp. KT-1 was cloned and sequenced. As the result, the PAA hydrolase gene was constructed with 942 bp, revealing that the open reading frame starts from the putative initiation codon ATG at nucleotide 1, located 10 bp downstream of the putative ribosomal binding sequence. The encoded polypeptide is a preprotein of 314 amino acids with a predict molecular weight of 34087 Da. Compared with the mature PAA hydrolase from *Sphingomonas* sp. KT-1, the 35 amino acid polypeptide from N-terminal is signal peptide.

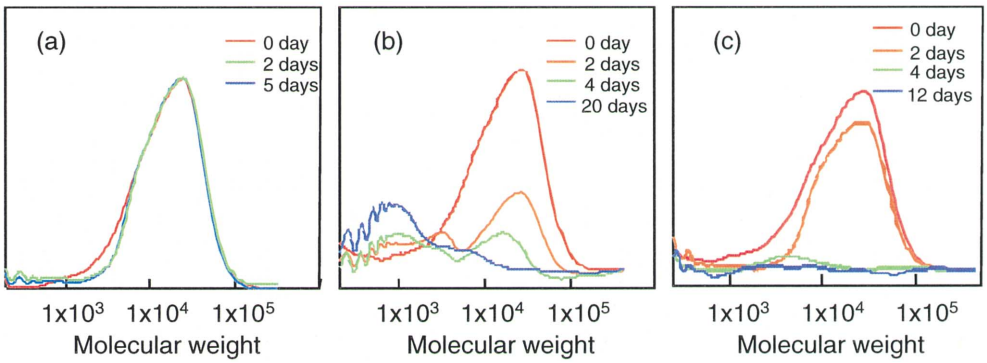



Fig. 1. Time-dependent changes in molecular weights of PAA-P in culture media incubated with PAA-degrading bacteria at 25°C. (a) *Sphingomonas* sp. KT-1, (b) *Pedobacter* sp. KP-2, and (c) Coculture of *Sphingomonas* sp. KT-1 and *Pedobacter* sp. KP-2.

Table 1. Purification of PAA hydrolase from *Sphingomonas* sp. KT-1

| Step | Total activity Units | Total protein (mg) | Specific activity (Units/mg) | Yieid (%) |
|---|---|---|---|---|
| Soluble fraction | 5300 | 3100 | 1.8 | 100 |
| Sp sepharose HP | 720 | 100 | 72 | 14 |
| Hydroxyapatite | 820 | 6.4 | 130 | 15 |
| Mono S | 640 | 4.6 | 140 | 12 |

The molecular weight deduced from PAA hydrolase gene was 30812 Da, which was in agreement with the value determined by SDS-PAGE.

The deduced amino acid sequence of PAA hydrolase was searched for homology by using FASTA. The result of FASTA showed that the amino acid sequence of PAA hydrolase was similar to those of poly(3-hydroxybutyrate) (PHB) depolymerases from *Alcaligenes faecalis* AE122 (26.5% identity, 257 aa) and from *Pseudomonas lemoignei* (25.8% identity, 244 aa), and conserved the lipase box (Ser176) which functions as an active center in well-known serine hydrolase.

## Enzymatic hydrolysis of poly(aspartic acid)

The hydrolysis of PAA by purified PAA hydrolase was examined by GPC. The time-dependent changes in GPC profile of PAA by treatment with purified PAA hydrolase is shown in Fig. 2. The molecular weight of PAA sample before hydrolysis was distributed from 1000 to 150000, and the number-average and weight-average molecular weights (Mn and Mw) were 7500 and 20000, respectively. The peak value of products shifted toward low-molecular-weights with time and corresponded to the oligomers of 5 to 6 aspartyl residues after 24 h. The production of aspartic acid was hardly detected during the course of reaction. These results suggest that the purified PAA hydrolase hydrolyzes a portion of amid linkages in PAA to yield oligomers of aspartic acid.

To investigate the mechanism of PAA hydrolysis with the purified enzyme, we carried out the characterization of hydrolyzed products of PAA polymer by using NMR spectroscopy. Before hydrolysis, the mole ratio of $\alpha$-unit to $\beta$-unit calculated from the peak areas was 30 to 70. The fraction of $\beta$-monomeric units in main chain was decreased from 70 mol% to 44 mol% after enzymatic hydrolysis for 24 h, while the fraction (30 mol%) of $\alpha$-monomeric unit in main chain remained almost unchanged during the course of enzymatic hydrolysis.

Since both $\alpha$- and $\beta$-units are connecting randomly in a polymer chains, the four types of sequential diads are present in PAA polymer prepared by thermal polycondensation. The $^{13}$C resonances of carbonyl carbon in amide linkages are resolved into three peaks due to diad sequences as shown in

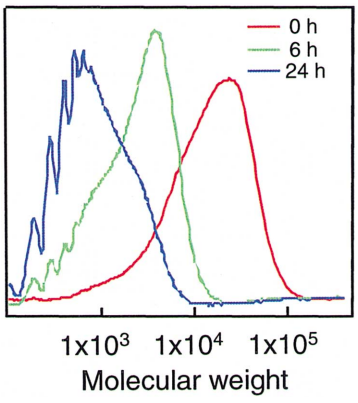



Fig. 2. Time-dependent changes in molecular weights of PAA-P treated with purified PAA hydrolase at 30°C and pH 8.0.

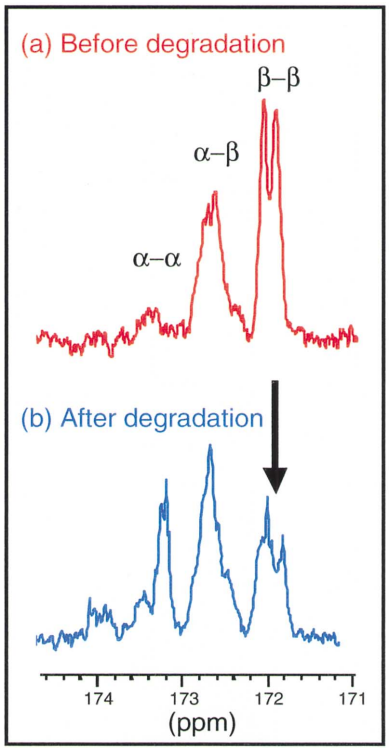



Fig. 3. $^{13}$C NMR spectra of carbonyl carbon in PAA and degraded products in $D_2O$. (a) PAA and (b) degraded products after 24 h of enzymatic hydrolysis.

Fig. 3. Before hydrolysis, the ratios of these peak areas were 1: 4: 5 in the spectrum of Fig. 3 (a). In contrast, the ratios of three peak areas were 1: 4: 3 in the spectrum of products after 24 h of enzymatic hydrolysis of PAA (Fig. 3 (b)). Thus, the peak area of amide carbonyl carbon of $\beta - \beta$ diad sequence was only reduced during the enzymatic hydrolysis of PAA with purified enzyme. These results indicate that the purified PAA hydrolase is an endo-type hydrolase and hydrolyzes selectively the amide linkage of $\beta - \beta$ diad sequence in PAA chain.

Taking the molecular structure of PAA and PHB into consideration, the amide linkage of $\beta - \beta$ diad sequences in PAA are similar to the ester linkage in PHB. In addition, the homology of amino acid sequences between PAA hydrolase and PHB depolymerases have suggested that the structure of PAA hydrolase is similar to those of PHB depolymerases from *A. faecalis* AE122 and *P. lemoignei*. These results suggest that the hydrolysis mechanism of PAA by PAA hydrolase may resemble to that of PHB by PHB depolymerases.

## References


1) S. Roweton, S. J. Huang, and G. Swift: J. Environ. Polym. Degrad. **5**, 175 (1997).
2) H. Pivcovà, V. Saudek, J. Dronbnik, and J. Vlasak: Biopolymer **20**, 1605 (1980).
3) H. Pivcovà, V. Saudek, and H. Dronbnik: Polymer **23**, 1237 (1982).
4) D. D. Alford, A. P. Wheeler, and C. A. Pettigrew: J. Environ. Polym. Degrad. **2**, 225 (1994).
5) T. Nakato, M. Yoshitake, K. Matsubara, M. Tomida, and T. Kakuchi: Macromolecules **31**, 2107 (1998).

6) K. Tabata, K. Kasuya, H. Abe, K. Masuda, and Y. Doi: Appl. Environ. Microbiol. **65**, 4268 (1999).
7) K. Tabata, H. Abe, and Y. Doi: Biomacromolecules **1**, 157 (2000).
8) K. Tabata, M. Kajiyama, T. Hiraishi, H. Abe, I. Yamato, and Y. Doi: Biomacromolecules, accepted for publication.