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MINI-REVIEW

Poly(aspartic acid) (PAA) hydrolases and PAA biodegradation: current knowledge and impact on applications

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Abstract Thermally synthesized poly(aspartic acid) (tPAA) is a bio-based, biocompatible, biodegradable, and water-soluble polymer that has a high proportion of β-Asp units and equivalent moles of D- and L-Asp units. Poly(aspartic acid) (PAA) hydrolase-1 and hydrolase-2 are tPAA biodegradation enzymes purified from Gram-negative bacteria. PAA hydrolase-1 selectively cleaves amide bonds between β-Asp units via an endo-type process, whereas PAA hydrolase-2 catalyzes the exo-type hydrolysis of the products of tPAA hydrolysis by PAA hydrolase-1. The novel reactivity of PAA hydrolase-1 makes it a good candidate for a biocatalyst in β-peptide synthesis. This mini-review gives an overview of PAA hydrolases with emphasis on their biochemical and functional properties, in particular, PAA hydrolase-1. Functionally related enzymes, such as poly(*R*-3-hydroxybutyrate) depolymerases and β-aminopeptidases, are compared to PAA hydrolases. This mini-review also provides findings that offer an insight into the catalytic mechanisms of PAA hydrolase-1 from *Pedobacter* sp. KP-2.

Keywords Poly(aspartic acid) · Poly(aspartic acid) hydrolase · β-Amide linkage · Stereoselectivity · Enzyme-catalyzed polymerization

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Introduction

Poly(aspartic acid) (PAA) is a bio-based, biocompatible, biodegradable, and eco-friendly alternative to conventional non-biodegradable polycarboxylates, such as poly(acrylate). Studies of PAA have focused on the development of synthetic methods, structure analyses, and applications (Low et al. 1996; Freeman et al. 1996; Tang and Wheeler 2001; Ross et al. 2001; Joentgen et al. 2003; Thombre and Sarwade 2005). The thermal synthesis of PAA has been well studied, and the resultant PAA (tPAA) contains a high proportion of β-Asp units (70 %), D-Asp units (50 %), branched units, and irregular end groups as shown in Fig. 1 (Pivcova et al. 1981, 1982; Wolk et al. 1994; Matsubara et al. 1998; Nakato et al. 1998). To effectively demonstrate the functions of polymer materials, it is important that their structure-function relationship be well understood to enable application to the design of materials showing the desired functions. tPAA biodegradability is one of the most vital functions that should be taken into account in practical use. The semicontinuous activated sludge (SCAS), mini-continuous activated sludge (Mini-CAS), and modified sturm (CO_2 production) tests have been used to assess tPAA biodegradability in the natural environment (Freeman et al. 1996; Tang and Wheeler 2001). However, despite the direct responsibilities of microorganisms and enzymes in the biodegradation and lifetime control of tPAA, there have been no reports of tPAA biodegradation by isolated microorganisms and enzymes until our work. To elucidate tPAA biodegradation, our research group isolated two tPAA-degrading bacteria, *Sphingomonas* sp. KT-1 and *Pedobacter* sp. KP-2, from river water and investigated their tPAA biodegradation behaviors (Tabata et al. 1999, 2000). Furthermore, two isolated enzymes, PAA hydrolase-1 and hydrolase-2, which were surmised to participate in tPAA biodegradation, were characterized and their catalytic mechanisms were examined (Tabata

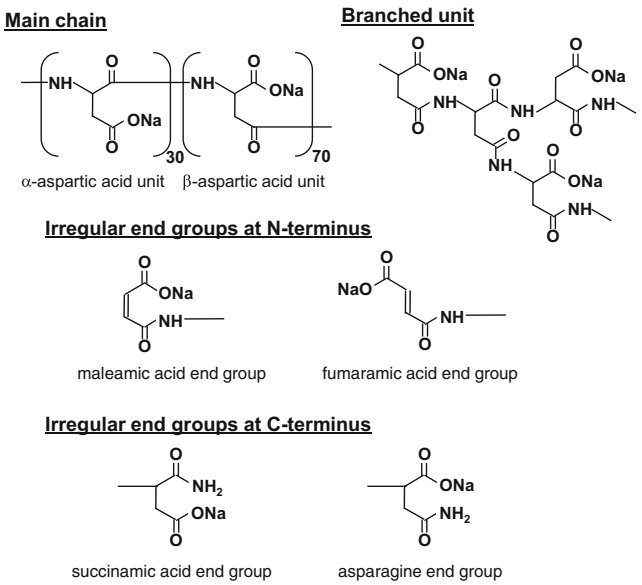

Fig. 1 Structures of monomeric units, branched units, and irregular end groups of tPAA polymer

et al. 2001; Hiraishi et al. 2003a, 2003b, 2004, 2009, 2015; Hiraishi and Maeda 2011).

As polymer structure control generally results in high functionalization and performance improvement, the creation of structure-controlled PAA is one of the most preferred ways to maximize the potential of PAA as a functional material. Recently, β-peptides have attracted interest as functional materials that exhibit the functions of α-peptides and metabolic stability. In green polymer chemistry, an active research area is the use of purified enzymes for the enzyme-catalyzed synthesis of polypeptides, by taking advantage of their substrate specificities. From the standpoint of eco-friendly and low-cost synthesis of structure-controlled PAA, enzymatic synthesis is expected to become a highly significant synthetic process. Thus, as one of the attractive applications of PAA hydrolases, we performed the enzyme-mediated synthesis of β-linked PAA (β-PAA), which is composed of only β-linkages and belongs to β-peptides, using the unique substrate recognition ability of PAA hydrolase-1 ($PahZ1_{KP-2}$) from *Pedobacter* sp. KP-2 (Hiraishi et al. 2011).

This mini-review presents an overview of the biochemical and functional properties of PAA hydrolases. It also focuses on the enzymatic synthesis of structure-controlled β-PAA by taking advantage of the substrate specificity of $PahZ1_{KP-2}$.

PAA hydrolases from *Sphingomonas* sp. KT-1

Tabata et al. (1999, 2000) isolated two bacteria, *Sphingomonas* sp. KT-1 and *Pedobacter* sp. KP-2, as tPAA-degrading and -assimilating microorganisms from river water. Until now, only *Sphingomonas* sp. KT-1 and *Pedobacter* sp. KP-2 have been isolated as microorganisms producing PAA hydrolases. tPAA biodegradation by *Sphingomonas* sp. KT-1 proceeded endogenously, and tPAA was completely degraded into Asp monomers by its soluble fraction. PAA hydrolase-1 ($PahZ1_{KT-1}$) purified from the soluble fraction of *Sphingomonas* sp. KT-1 was the first enzyme related to tPAA metabolism (Tabata et al. 2001). The biochemical, structural, and genetic properties of $PahZ1_{KT-1}$ are listed in Table 1. The $PahZ1_{KT-1}$ gene encodes a signal sequence of 35 amino acids, indicating that $PahZ1_{KT-1}$ is located in the periplasmic space (Hiraishi et al. 2003a). SDS-PAGE of the enzyme purified from the wild strain revealed that the molecular weight of the mature enzyme is approximately 30 kDa, in agreement with that of the amino acid sequence deduced from $PahZ1_{KT-1}$ gene. Sensitivity to inhibitors and mutagenesis study indicated that $PahZ1_{KT-1}$ is a Ser-type hydrolase having a Ser141 residue in the lipase box (Gly-Xaa-Ser-Xaa-Gly) as the catalytic center. Nuclear magnetic resonance (NMR) and gel permeation chromatography (GPC) analyses of the products of tPAA hydrolysis by $PahZ1_{KT-1}$ indicated that the enzyme selectively cleaves the amide bonds between β-Asp units in tPAA to generate oligo(aspartic acid)s (OAA) having molecular weights of a few thousand Daltons via an endo-type process.

PAA hydrolase-2 ($PahZ2_{KT-1}$) was purified as a second enzyme that likely participated in the tPAA biodegradation by *Sphingomonas* sp. KT-1 (Hiraishi et al. 2003b). Table 1 shows several properties of $PahZ2_{KT-1}$. The predicted polypeptide encodes a preprotein of 425 amino acids containing the first 21 amino acids as the signal peptide, indicating that $PahZ2_{KT-1}$ is located in the periplasm of cells. The molecular mass of the deduced amino acids of $PahZ2_{KT-1}$ (42,584 Da) agreed well with the value (42 kDa) determined by SDS-PAGE of the purified protein from the wild strain. $PahZ2_{KT-1}$ exhibited limited activity for tPAA but was able to hydrolyze OAA. From a viewpoint of the terminal structures, tPAA has the irregular end groups (Fig. 1), while OAA has a freshly generated end groups. $PahZ2_{KT-1}$ was also able to hydrolyze high-molecular-weight α-poly(L-Asp), which does not have the irregular end groups, to yield Asp monomers. These findings suggest that the irregular end groups of tPAA disturb the exo-mode hydrolysis by $PahZ2_{KT-1}$. The deduced amino acid sequence of $PahZ2_{KT-1}$ showed similarities to that of carboxypeptidase G2 that hydrolyzed the C-terminal glutamate moiety of folic acid, thereby supporting the abovementioned exo-mode hydrolysis by $PahZ2_{KT-1}$. The deduced amino acid sequence also showed similarities to that of a putative peptidase belonging to the metallopeptidase M20/M25/M40 family in *Caulobacter crescentus* CB15 (63.4 % identity in 413 aa), suggesting that $PahZ2_{KT-1}$ contained metal ion(s) within its active site, in agreement with the fact that the enzyme was inhibited by EDTA.

Table 1 tPAA- and β-peptide-degrading microorganisms and characteristics of their hydrolases

Poly(amino acid), microorganism and enzyme	Enzyme characteristics									
	Number of amino acids	Signal sequence (aa)	Active form	Subunits (aa)	Temperature (°C)		pH optimum	Inhibitor	Degradation type	References
					Optimum	Stability				
tPAA										
Sphingomonas sp. KT-1 ($PahZ1_{KT-1}$)	314	35	n.d.	36–314	40	40	10	DFP, PMSF	Endo type (β-amide linkage hydrolysis)	K. Tabata et al. 2001 T. Hiraishi et al. 2003a T. Hiraishi et al. 2004
Sphingomonas sp. KT-1 ($PahZ2_{KT-1}$)	425	21	n.d.	22–425	55	50	7	EDTA, DFP, PMSF	Exo type	T. Hiraishi et al. 2003b T. Hiraishi et al. 2004
Pedobacter sp. KP-2 ($PahZ1_{KP-2}$)	306	41	Monomer	42–306	40	40	7.5	DFP, PMSF	Endo type [β-amide linkage hydrolysis between (L-Asp)-(D-Asp)]	T. Hiraishi et al. 2009 T. Hiraishi et al. 2015
β-Peptides										
Ochrobactrum anthropi LMG7991 (DmpA)	375	none	$(\alpha\beta)_4$	α: 1-249 β: 250-375	n.d.	55	7.5-8.5	n.i.	Exo type (removal of L-α- and L-β-amino acid at N-terminus)	Fanuel et al. 1999 Heck et al. 2006
Pseudomonas sp. MCI3434 (Ps BapA)	366	none	$(\alpha\beta)_4$	α: 1-238 β: 239-366	60	55	9-10	*p*-chloromercuribenzoate, *N*-ethylmaleimide, dithiothreitol, $HgCl_2$, $ZnSO_4$, $ZnCl_2$, $AgNO_3$, $CdCl_2$	Exo type (removal of L-β-amino acid at N-terminus)	Komeda and Asano 2005
Sphingosinicella xenopeptidilytica 3-2W4 (3-2W4 BapA)	402	29	$(\alpha\beta)_4$	α: 30-278 β: 279-402	n.d.	70	8-9	Pefabloc SC	Exo type (removal of L-β-amino acid at N-terminus)	Geueke et al. 2005 Geueke et al. 2006
Sphingosinicella microcystinivorans Y2 (Y2 BapA)	399	26	$(\alpha\beta)_4$	α: 27-275 β: 276-399	n.d.	60	10	Pefabloc SC	Exo type (removal of L-β-amino acid at N-terminus)	Geueke et al. 2006
Pseudomonas aeruginosa PAO1(BapF)	366	none	n.d.	α: 1-236 β: 237-366	37	55	5.5	n.d.	Exo type (removal of L-β-amino acid at N-terminus)	Fuchs et al. 2011

n.d. not determined, *DFP* diisopropyl fluorophosphates, *PMSF* phenylmethylsulfonyl fluoride, *EDTA* ethylenediaminetetraacetic acid, *n.i.* not inhibited by antipain, aprotinin, bestatin, chymostatin, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), EDTA, leupeptin, pefabloc SC, and 1,10-phenanthroline

Substrate specificities of PahZ1$_{KT-1}$ and PahZ2$_{KT-1}$

Research of the enzymatic hydrolysis of well-defined oligomer substrates, including α- and β-tetra(L-Asp)s, demonstrated that PahZ1$_{KT-1}$ is capable of hydrolyzing oligomers not smaller than trimer composed of β-linkages (Hiraishi et al. 2004). The hydrolysis of well-defined oligomers by PahZ2$_{KT-1}$ indicated that this enzyme can hydrolyze both β- and α-linked Asp oligomers via an exo-type process and probably cleaves the amide bond at the C-terminus (Hiraishi et al. 2004).

Based on the biological, genetic, and functional characterization of PahZ1$_{KT-1}$ and PahZ2$_{KT-1}$, the following mechanisms for the microbial degradation of tPAA by *Sphingomonas* sp. KT-1 are proposed:

1. Not high-molecular-weight but low-molecular-weight tPAA is internalized into the cell.
2. The internalized polymer is hydrolyzed by PahZ1$_{KT-1}$ via an endo-type process to generate OAA in the periplasmic space.
3. The resultant OAA is subsequently hydrolyzed into Asp monomers via an exo-type process by PahZ2$_{KT-1}$ that is possibly located in the periplasm fraction.
4. The resultant monomers are utilized in the Asp metabolic process.

PAA hydrolase-1 from *Pedobacter* sp. KP-2

PAA hydrolase-1 (PahZ1$_{KP-2}$) was purified from the soluble fraction of *Pedobacter* sp. KP-2 and found to be localized in the periplasm fraction (Hiraishi et al. 2009). The properties of PahZ1$_{KP-2}$ are listed in Table 1. Gene analysis suggested that this enzyme contains a signal peptide sequence (41 aa), supporting its periplasmic localization in the cell. The molecular weight of the mature enzyme deduced from its gene was 30,274 Da, in accord with that determined by SDS-PAGE of the purified enzyme from wild strain. The relative molecular mass of PahZ1$_{KP-2}$ as estimated by gel filtration was around 31 kDa, suggesting that PahZ1$_{KP-2}$ is a monomeric enzyme. The enzyme exhibited a temperature optimum of 40 °C, and was, like PahZ1$_{KT-1}$, inhibited by diisopropyl fluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF). This sensitivity to the two inhibitors suggested that PahZ1$_{KP-2}$ is also a Ser-type hydrolase; this is strongly supported by the presence of a lipase box containing Ser125 in its deduced amino acid sequence and the complete loss of activity upon amino acid substitution of Ser125 with Ala.

Substrate specificity of PahZ1$_{KP-2}$

GPC analysis of the products of hydrolysis of tPAA by PahZ1$_{KP-2}$ demonstrated that products with molecular weights of approximately 4000 Da were accumulated, indicating that this enzyme selectively hydrolyzes a portion of the amide linkages in certain tPAA sequences to yield OAA (Hiraishi et al. 2009). ^{1}H and ^{13}C NMR analyses indicated that this enzyme specifically, but not completely, cleaves the amide bond between β-Asp units in tPAA via an endo-type process.

As stated earlier, as the tPAA molecule contains equivalent moles of D- and L-Asp units, uncommonly occurring sequences in nature [(D-Asp)-(D-Asp), (D-Asp)-(L-Asp), (L-Asp)-(D-Asp)] in addition to the (L-Asp)-(L-Asp) sequence may be formed, which may affect the cleavage of the β-β amide bonds in tPAA by PahZ1$_{KP-2}$. To reveal the effects of the sequences of L- and D-Asp units on tPAA hydrolysis by PahZ1$_{KP-2}$, we performed the hydrolysis of β-tri(Asp)s having all possible combinations of L- and D-Asp units by PahZ1$_{KP-2}$ (Hiraishi et al. 2015). The results provided the following information of its substrate recognition mechanism (Fig. 2). The substrate-binding site of PahZ1$_{KP-2}$ is composed of at least four subsites (subsites 2, 1, −1, and −2). When Asp units occupy three of the four subsites, amide bond cleavage occurs between subsites 1 and −1. Subsite 1 can recognize only the L-Asp unit, whereas the other subsites can recognize both L- and D-Asp units. PahZ1$_{KP-2}$ cleaves the amide bond at the carboxyl part of the β-L-Asp unit in stereoisomeric β-tri(Asp)s. Among the dimer sequences, the (L-Asp)-(D-Asp) sequence is the most acceptable to the two central subsites.

Functionally and structurally related enzymes to PahZ1$_{KT-1}$ and PahZ1$_{KP-2}$

Figure 3 shows the multi-alignment of the deduced amino acids of matured PahZ1$_{KT-1}$ and PahZ1$_{KP-2}$ with those of PHB depolymerases using ClustalW2 (GENETYX software). BLAST analysis revealed that the deduced amino acid sequence of matured PahZ1$_{KP-2}$ had similarity to that of PahZ1$_{KT-1}$ (39 % identity in 264 aa) (Hiraishi et al. 2009). Based on the ESTHER database (http://bioweb.ensam.inra.fr/esther), PahZ1$_{KT-1}$ and PahZ1$_{KP-2}$ are classified into α/β-hydrolase_5 family, which have an α/β-hydrolase fold in their structures and contain putative PHB depolymerase (LpqC) from *Bordetella parapertussis*. Alignment of the deduced amino acid sequences of PahZ1$_{KT-1}$ and PahZ1$_{KP-2}$ demonstrated that the residues possibly composing their catalytic triads, in which the Ser residue formed together with Asp and His residues, were highly conserved (Fig. 3).

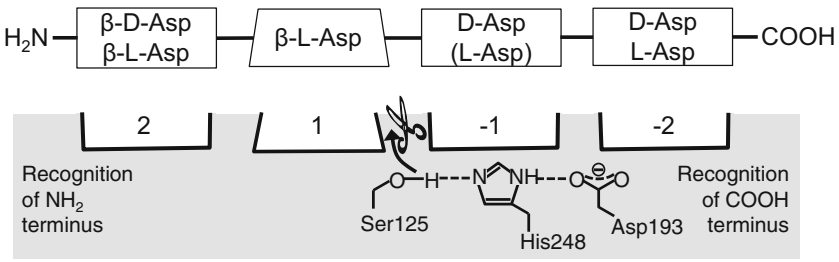

Fig. 2 Schematic model of substrate recognition site of $PahZ1_{KP-2}$

Previous sequence analyses unveiled the similarity of the amino acid sequence of $PahZ1_{KT-1}$ to those of PHB depolymerases from *Alcaligenes faecalis* AE122 ($PhaZ_{AfaAE122}$) (26.5 % identity, 257 aa) and *Pseudomonas lemoignei* ($PhaZ2_{Ple}$) (25.8 % identity, 244 aa) (Hiraishi et al. 2003a). As shown in Fig. 3, the proposed active site residues are conserved in $PahZ1_{KT-1}$, $PahZ1_{KP-2}$, $PhaZ_{AfaAE122}$, and $PhaZ2_{Ple}$. PHB depolymerases are monomer enzymes having an α/β-hydrolase fold and cleave β-ester bonds in PHB via an endo-type process (Jendrossek and Handrick 2002; Hisano et al. 2006; Wakadkar et al. 2010). Early hydrolysis studies of oligo(3-hydroxybutyrate)s having well-defined sequences yielded the following information of the substrate recognition sites of PHB depolymerases: (i) the active site has four subsites (2, 1, −1, and −2), three of which should be occupied by monomer units for cleavage to occur, and (ii) for the hydrolysis to proceed, subsites 1 and −1 should be occupied by the *R*-3-hydroxybutyrate (R3HB) unit, whereas the other two

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PahZ1KT-1      1 APAAASKGKAAALPDLKPGAGSFLFTGWAGKPLKVHYYAPDKIT---ETTRILFVIHGAG  57
PahZ1KP-2      1 ---DEGVG-EFIYQDYKP---------LDNKPIKVRYYNPGKN-----DAQVLFIMHGNG  42
PhaZAfaAE122   1 ----------GAWQNNLS----------LGGFNKVHLYTPDGDSPVGNGKALLIVLHGCT  40
PhaZ2Ple       1 ---------ATQVTGFGS----------NPGNLLMYKHVPSSMP---ANAPLVIAMHGCT  38

PahZ1KT-1     58 RNADGYRDAWIPYAKEGQ-YIVLTPEYSMADFPT-SLTYN-VGHIVDEAGNPRPREEWSF 114
PahZ1KP-2     43 RNAEGYFKAMLKHAQQHN-VLLVVPEFDEQQFS--SREYH-QGGILDKQSKLRPREDWTF  98
PhaZAfaAE122  41 QSIDAYKTANLEVAAEEYGMVVAVPDMNKAGFS--CWSYW-QGTKSRSAGDYKNLINLAN  97
PhaZ2Ple      39 QSASAYEATGWTQLANTYKFYVVYPEQQSSNNQNKCFNWFEPGDIARGQGEALSIKQMVD  98

PahZ1KT-1    115 ASIEPMFDQVRKATGSKVPTYAIYGHSAGGQFVHRFVELWPDARYS-RAVAA--NAGWYT 171
PahZ1KP-2     99 SIIEPLFDYVKKLTGNTSAGYMLYGFSAGSQFVHRFLMFNPENRVT-RAIAG--SAGTYT 155
PhaZAfaAE122  98 TLSG------DAARGIDPNQVYIAGLSSGASFANTTACLAPDVFAG-VGVSAGPSVGTSS 150
PhaZ2Ple      99 KMKA--------DHSIDTNRVYVTGLSAGGYMVNVMLATYPDVFAGGAPFSGGPYNCATS 150
                                  lipase box
PahZ1KT-1    172 MPDLAIKYPYGLKDAPTDAAG--LKATL-----EKPLTILLGTADTDVNHHQLSRTPEAM 224
PahZ1KP-2    156 MPDYNIDYSYGLKNVNLPQKN--LNKFF-----AKNLMVIVGDADTVLSRTDLVKTPAAN 208
PhaZAfaAE122 151 SGAIGTCEQADVESRCRDLGGGYQSAFD-----TQVASIAHGDADTTVDTCYNRQNAEGM 205
PhaZ2Ple     151 MTNAFTCMSPGVDKTPAAWGDLARGGYSGYTGRKPIVSIWHGDADYTVKQSNQVEEVEQW 210

PahZ1KT-1    225 T--QGVHRLARG-EFFYAYGRKVAHELNAKFAWKLDYAPDIAHSNTGMS-------QYAQ 274
PahZ1KP-2    209 Q--QGRDRVERG-QTFFNRSKAIAEQLKTPFNWKFQLIPHVGHSQGEMA-------GPVA 258
PhaZAfaAE122 206 AGLYGVSEVAGS-TVINADGGSAEEFLWQDGRVSMLWFHGLDHSWSGGQ-------GASG 257
PhaZ2Ple     211 TNYHGIDQTADVSDTVAGFPHKVYKDASGNALVETYTITGMGHGTPVDPGTGSLQCGTAG 270

PahZ1KT-1    275 KLVWE----------------------                               279
PahZ1KP-2    259 KLLFEDK--------------------                               265
PhaZAfaAE122 258 SYVSGASINYARYLGGFFAEHNARIDR                               284
PhaZ2Ple     271 AYILDVNICSSYYVAKFWGLIGGSGTT                               297
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Fig. 3 Multi-alignment of putative amino acids of matured PAA hydrolases-1 with those of matured PHB depolymerases using ClustalW2 (GENETYX software). Sequences of PAA hydrolases-1 from *Sphingomonas* sp. KT-1 ($PahZ1_{KT-1}$) and *Pedobacter* sp. KP-2 ($PahZ1_{KP-2}$) and PHB depolymerases from *Alcaligenes faecalis* AE122 ($PhaZ_{AfaAE122}$) and *Pseudomonas lemoignei* ($PhaZ2_{Ple}$) are shown. Identical and conserved amino acids are marked in *black* and *gray*, respectively. *Box* indicates lipase box. Proposed active site residues are marked by *closed circles*

subsites may accept both R3HB and *S*-3-hydroxybutyrate units (Bachmann and Seebach 1999; Hiraishi et al. 2000; Scherer et al. 2000).

Aside from the PAA-hydrolyzing enzymes ($PahZ1_{KT-1}$, $PahZ2_{KT-1}$, and $PahZ1_{KP-2}$), five β-aminopeptidases (three BapA enzymes, one BapF enzyme, and one DmpA enzyme) are known to hydrolyze short β-peptides and β-amino-acid-containing peptides (Geueke et al. 2005, 2006; Heck et al. 2006, 2007, 2012; Geueke and Kohler 2007; Fuchs et al. 2011). Their properties are listed in Table 1. They remove the β-amino acid unit from the N-terminus of oligopeptides, amides, and esters via an exo-type process. BapA enzymes from *Sphingosinicella xenopeptidilytica* 3-2W4 (3-2W4 BapA) and *Sphingosinicella microcystinivorans* Y2 (Y2 BapA) hydrolyze β-dipeptides, as well as β-tripeptides, and prefer those with the L-configuration of the N-terminal unit (Geueke et al. 2005, 2006). DmpA from *Ochrobactrum anthropi* LMG7991 hydrolyzes both α- and β-peptides, but the rate of hydrolysis of α-peptides is lower than that of β-peptides (Heck et al. 2006). In the MEROPS database (Rawlings et al. 2010), β-aminopeptidases are classified into peptidase family P1 that includes aminopeptidases and self-processing proteins.

Figure 4 shows a phylogenetic tree on the basis of amino acid sequences showing relationships among PAA hydrolases-1 ($PahZ1_{KT-1}$ and $PahZ1_{KP-2}$), PHB depolymerases ($PhaZ_{AfaAE122}$ and $PhaZ2_{Ple}$), and β-aminopeptidases [Y2 BapA, Ps BapA (BapA from *Pseudomonas* sp. MCI3434), 3-2W4 BapA, BapF (BapF from *Pseudomonas aeruginosa* PAO1), and DmpA]. The analysis suggests that $PahZ1_{KT-1}$ and $PahZ1_{KP-2}$ are related to $PhaZ_{AfaAE122}$ and $PhaZ2_{Ple}$, but not to β-aminopeptidases. Thus, based on these functional and structural findings of PAA hydrolases-1, β-aminopeptidases, and PHB depolymerases, it is assumed that PAA hydrolases-1 share a common ancestor with PHB depolymerases rather than β-aminopeptidases.

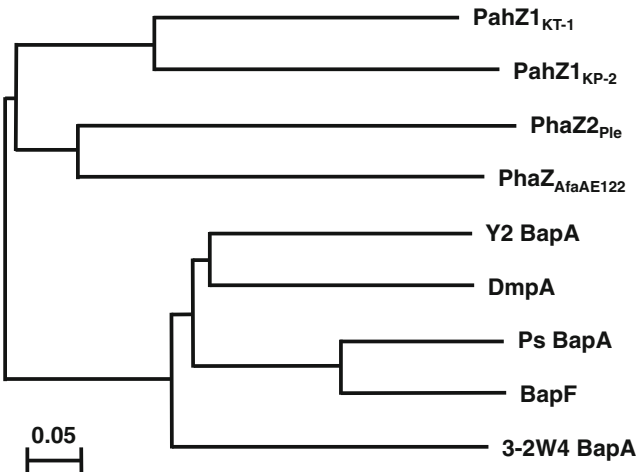

Fig. 4 Neighbor-joining tree showing phylogenetic relationships among PAA hydrolases-1, PHB depolymerases, and β-aminopeptidases. The *scale bar* represents the expected number of substitutions per amino acid position

Application of PAA hydrolases: $PahZ1_{KP-2}$-catalyzed synthesis of β-PAA

Generally, the substrate specificity of the enzymes for polymer hydrolysis is tightly linked to that for polymer synthesis. Proteases specifically recognizing α-linked polypeptides are the most commonly used for the enzyme-catalyzed synthesis of poly(amino acid)s, and the resultant poly(amino acid)s are composed of α-amino acid units (Aso et al. 1988; Uemura et al. 1990; Matsumura et al. 1999; Uyama et al. 2002; Soeda et al. 2003; Li et al. 2006, 2008). Due to the novel substrate specificity of PahZ1 enzymes, their application to enzyme-catalyzed polymerization may result in the synthesis of β-linked PAA (β-PAA), which may possess such unexpected properties as high metabolic stability, in keeping with the advantages of α-peptides (Seebach et al. 2004; Seebach and Gardiner 2008). Therefore, PahZ1 enzymes could be used in enzyme-mediated polymerization as one of the more active uses of the enzymes by taking advantage of their substrate specificities.

We reported the first enzyme-catalyzed synthesis of β-PAA using $PahZ1_{KP-2}$ (Hiraishi et al. 2011). β-PAA synthesis from diethyl L-aspartate substrate was accomplished by using $PahZ1_{KP-2}$ modified with poly(ethylene glycol) (PEG). Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis demonstrated that the synthesized polymer produced pairs of peaks having regular peak-to-peak intervals of m/z = 143, which corresponded to the molecular mass of repeat units. The synthesized polymer was observed in the range of m/z = 750–2500 (polymerization degree 5–17), and the most abundant peaks were found at 9 to 11 degrees of polymerization. Moreover, the mass difference between each pair of major and minor peaks was 28, which was equal to that between $–OCH_2CH_3$ and –OH, indicating that the product is a mixture of ethyl ester and free carboxyl groups at the C-terminus. ^{1}H NMR analysis revealed that synthesized polymer was composed of only β-amide linkages. Available data led us to conclude that the modification of $PahZ1_{KP-2}$ with PEG improved dispersibility in organic solvents and the resultant PEG-modified enzyme was useful for the synthesis of β-PAA due to its unique substrate specificity.

Conclusions

In this mini-review, the biochemical and genetic characteristics of PAA hydrolases ($PahZ1_{KT-1}$, $PahZ2_{KT-1}$, and $PahZ1_{KP-2}$) as well as one of their applications, namely, the enzymatic

synthesis of structure-controlled β-PAA, were described. To obtain information regarding tPAA biodegradation, tPAA-degrading and tPAA-assimilating microorganisms were isolated from river water and their tPAA biodegradation behaviors were examined. Two PAA-hydrolyzing enzymes, $PahZ1_{KT-1}$ and $PahZ2_{KT-1}$, were purified from *Sphingomonas* sp. KT-1, whereas $PahZ1_{KP-2}$ was purified from *Pedobacter* sp. KP-2. Among these enzymes, $PahZ1_{KT-1}$ and $PahZ1_{KP-2}$, which showed similarities in their deduced amino acid sequences, caught our interest because they possessed unique substrate recognition properties, that is, these enzymes were able to specifically, but not completely, hydrolyze the amide bond between β-Asp units. Taking into consideration that β-Asp units accounted for 70 % of the total units in tPAA, we assumed that PahZ1 enzymes would play a vital role in tPAA biodegradation in the natural environment. However, the existence of other hydrolase(s), including $PahZ2_{KT-1}$, may be important for the complete tPAA biodegradation in the natural environment because of the incomplete tPAA degradation by PahZ1 enzymes.

The β-amino acid derived substructures are found as part of a wide variety of bioactive secondary metabolites, such as coenzyme A, L-carnosine, taxol, microcystin-LR, and bestatin, in bacteria, fungi, and plants (Heck et al. 2012). Moreover, β-L-Asp and β-D-Asp units are found in various proteins, including αA- and αB-crystallins, β-amyloid protein, and elastin, from diverse tissues of elderly individuals (Fujii et al. 2011). As there are no known natural peptides that are solely composed of β-amino acid units, compounds containing β-amino acid derived substructures and mixed α,β-peptides produced in nature may serve as physiological substrates for PahZ1 and PahZ2 enzymes as well as DmpA and BapA ones.

In green polymer chemistry, the isolated enzymes have seen increasing applications as catalysts for biopolymer synthesis in vitro. In vitro enzymatic polymerization offers many advantages, such as easier control of polymer structure and monomer reactivity than conventional chemical methods, as well as the enzymes themselves in part of a sustainable system. Novel enzymes capable of cleaving peptides containing β-amino acids, such as $PahZ1_{KT-1}$ and $PahZ1_{KP-2}$, could be useful biocatalysts for β-peptide production because of the close relationship between their substrate specificities for polymer hydrolysis and synthesis. Based on this concept, in addition to our work, there have been attempts to synthesize β-peptides in the presence of an enzyme (Heck et al. 2007), and in those cases, exo-type BapA and DmpA enzymes were used. In the future, custom-made enzymes generated by evolutionary engineering may affect the creation of high-performance β-peptides in improved in vitro systems. The development of such enzymes is expected to catalyze dramatic breakthroughs in the industrial, pharmaceutical, and agricultural fields.

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Compliance with ethical standards

Conflict of interest I declare no conflict of interest.

References

Aso K, Uemura T, Shiokawa Y (1988) Protease-catalyzed synthesis of oligo-L-glutamic acid from L-glutamic acid diethyl ester. Agric Biol Chem 52:2443–2449

Bachmann BM, Seebach D (1999) Investigation of the enzymatic cleavage of diastereomeric oligo(3-hydroxybutanoates) containing two to eight HB units. A model for the stereoselectivity of PHB depolymerase from *Alcaligenes faecalis* T1. Macromolecules 32: 1777–1784

Fanuel L, Goffin C, Cheggour A, Devreese B, Van Driessche G, Joris B, Van Beeumen J, Frere JM (1999) The DmpA aminopeptidase from *Ochrobactrum anthropi* LMG7991 is the prototype of a new terminal nucleophile hydrolase family. Biochem J 341:147–155

Freeman MB, Paik YH, Swift G, Wilczynski R, Wolk SK, Yocom KM (1996) Biodegradability of polycarboxylates: structure-activity studies. In: Ottenbrite RM, Huang SJ, Park K (eds) Hydrogels and biodegradable polymers for bioapplications. ACS Symposium Series, American Chemical Society, Washington DC, pp. 118–136

Fuchs V, Jaeger KE, Wilhelm S, Rosenau F (2011) The BapF protein from *Pseudomonas aeruginosa* is a β-peptidyl aminopeptidase. World J Microbiol Biotechnol 27:713–718

Fujii N, Kaji Y, Fujii N (2011) D-amino acids in aged proteins: analysis and biological relevance. J Chromatogr B 879:3141–3147

Geueke B, Namoto K, Seebach D, Kohler HPE (2005) A novel β-peptidyl aminopeptidase (BapA) from strain 3-2W4 cleaves peptide bonds of synthetic β-tri and β-dipeptides. J Bacteriol 187:5910–5917

Geueke B, Heck T, Limbach M, Seebach D, Kohler HPE (2006) Bacterial β-peptidyl aminopeptidases with unique substrate specificities for β- and mixed β/α-oligopeptides. Febs j 273:5261–5272

Geueke B, Kohler HPE (2007) Bacterial β-peptidyl aminopeptidases: on the hydrolytic degradation of β-peptides. Appl Microbiol Biotechnol 74:1197–1204

Heck T, Limbach M, Geueke B, Zacharas M, Gardiner J, Kohler HPE, Seebach D (2006) Enzymatic degradation of β- and mixed α,β-oligopeptides. Chem Biodivers 3:1325–1348

Heck T, Kohler HPE, Limbach M, Flcgel O, Seebach D, Geueke B (2007) Enzyme-catalyzed formation of β-peptides: β-peptidyl aminopeptidases BapA and DmpA acting as β-peptide-synthesizing enzymes. Chem Biodivers 4:2016–2030

Heck T, Geueke B, Kohler HPE (2012) Bacterial β-aminopeptidases: structural insights and applications for biocatalysts. Chem Biodivers 9:2388–2409

Hiraishi T, Ohura T, Ito S, Kasuya K, Doi Y (2000) Function of the catalytic domain of poly(3-hydroxybutyrate) depolymerase from *Pseudomonas stutzeri*. Biomacromolecules 1:320–324

Hiraishi T, Kajiyama M, Tabata K, Yamato I, Doi Y (2003a) Genetic analysis and characterization of poly(aspartic acid) hydrolase-1 from *Sphingomonas* sp. KT-1. Biomacromolecules 4:80–86

Hiraishi T, Kajiyama M, Tabata K, Abe H, Yamato I, Doi Y (2003b) Biochemical and molecular characterization of poly(aspartic acid) hydrolase-2 from *Sphingomonas* sp. KT-1. Biomacromolecules 4: 1285–1292

Hiraishi T, Kajiyama M, Yamato I, Doi Y (2004) Enzymatic hydrolysis of α- and β-oligo(L-aspartic acid)s by poly(aspartic acid) hydrolases-1 and 2 from *Sphingomonas* sp. KT-1. Macromol Biosci 4:330–339

Hiraishi T, Masuda E, Kanayama N, Nagata M, Doi Y, Abe H, Maeda M (2009) Cloning of poly(aspartic acid) (PAA) hydrolase-1 gene from *Pedobacter* sp. KP-2 and hydrolysis of thermally synthesized PAA by its gene product. Macromol Biosci 9:10–19

Hiraishi T, Maeda M (2011) Poly(aspartate) hydrolases: biochemical properties and applications. Appl Microbiol Biotechnol 91:895–903

Hiraishi T, Masuda E, Miyamoto D, Kanayama N, Abe H, Maeda M (2011) Enzymatic synthesis of poly(α-ethyl β-aspartate) by poly(-ethylene glycol) modified poly(aspartate) hydrolase-1. Macromol Biosci 11:187–191

Hiraishi T, Abe H, Maeda M (2015) Substrate stereoselectivity of poly(Asp) hydrolase-1 capable of cleaving β-amide bonds as revealed by investigation of enzymatic hydrolysis of stereoisomeric β-tri(Asp)s. AMB Express 5:31

Hisano T, Kasuya K, Tezuka Y, Ishii N, Kobayashi T, Shiraki M, Oroudjev E, Hansma H, Iwata T, Doi Y, Saito T, Miki K (2006) The crystal structure of polyhydroxybutyrate depolymerase from *Penicillium funiculosum* provides insights into the recognition and degradation of biopolyesters. J Mol Biol 356:993–1004

Jendrossek D, Handrick R (2002) Microbial degradation of polyhydroxyalkanoates. Annu Rev Microbiol 56:403–432

Joentgen W, Müller N, Mitschker A, Schmidt H (2003) Polyaspartic acids: polyamides and complex proteinaceous materials I. In: Fahnestock SR, Steinbüchel A (eds) Biopolymers 7. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, pp. 175–199

Komeda H, Asano Y (2005) A DmpA-homologous protein from *Pseudomonas* sp. is a dipeptidase specific for β-alanyl dipeptides. Febs j 272:3075–3084

Li G, Vaidya A, Viswanathan K, Cui J, Xie W, Gao W, Gross RA (2006) Rapid regioselective oligomerization of L-glutamic acid diethyl ester catalyzed by papain. Macromolecules 39:7915–7921

Li G, Raman VK, Xie W, Gross RA (2008) Protease-catalyzed co-oligomerizations of L-leucine ethyl ester with L-glutamic acid diethyl ester: sequence and chain length distributions. Macromolecules 41: 7003–7012

Low KC, Wheeler AP, Koskan LP (1996) Commercial poly(aspartic acid) and its uses. In: Glass J (ed) Hydrophilic polymers Adv chem. American Chemical Society, Washington DC, pp. 99–111

Matsubara K, Nakato T, Tomida M (1998) End group and irregular structure analysis in thermally prepared sodium polyaspartate by ^{1}H and ^{13}C NMR spectroscopy. Macromolecules 31:1466–1472

Matsumura S, Tsushima Y, Otozawa N, Murakami S, Toshima K, Swift G (1999) Enzyme-catalyzed polymerization of L-aspartate. Macromol Rapid Commun 20:7–11

Nakato T, Yoshitake M, Matsubara K, Tomida M, Kakuchi T (1998) Relationships between structure and properties of poly(aspartic acid)s. Macromolecules 31:2107–2113

Pivcova H, Saudek V, Drobnik J, Vlasak J (1981) NMR study of poly(-aspartic acid). I. α- and β-peptide bonds in poly(aspartic acid) prepared by thermal polycondensation. Biopolymers 20:1605–1614

Pivcova H, Saudek V, Drobnik J (1982) 13C n.m.r. study of the structure of poly(aspartic acid). Polymer 23:1237–1241

Rawlings ND, Barrett AJ, Bateman A (2010) MEROPS: the peptidase database. Nucleic Acids Res 38:D227–D233

Ross RJ, Mazo GY, Mazo J (2001) New methods in the synthesis of thermal poly(aspartates). In: Gross R, Scholz C (eds) Biopolymers from polysaccharides and agroproteins. ACS Symposium Series, American Chemical Society, Washington DC, pp. 172–181

Scherer TM, Fuller RC, Goodwin S, Lenz RW (2000) Enzymatic hydrolysis of oligomeric models of poly-3-hydroxybutyrate. Biomacromolecules 1:577–583

Seebach D, Beck AK, Bierbaum DJ (2004) The world of β- and γ-peptides comprised of homologated proteinogenic amino acids and other components. Chem Biodiver 1:1111–1239

Seebach D, Gardiner J (2008) β-peptidic peptidomimetics. Acc Chem Res 41:1366–1375

Soeda Y, Toshima K, Matsumura S (2003) Sustainable enzymatic preparation of polyaspartate using a bacterial protease. Biomacromolecules 4:196–203

Tabata K, Kasuya K, Abe H, Masuda K, Doi Y (1999) Poly(aspartic acid) degradation by a *Sphingomonas* sp. isolated from freshwater. App Environ Microbiol 65:4268–4270

Tabata K, Abe H, Doi Y (2000) Microbial degradation of poly(aspartic acid) by two isolated strains of *Pedobacter* sp. and *Sphingomonas* sp. Biomacromolecules 1:157–161

Tabata K, Kajiyama M, Hiraishi T, Abe H, Yamato I, Doi Y (2001) Purification and characterization of poly(aspartic acid) hydrolase from *Sphingomonas* sp. KT-1. Biomacromolecules 2:1155–1160

Tang Y, Wheeler AP (2001) Environmental factors that influence biodegradation of thermal poly(aspartate). In: Gross R, Scholz C (eds) Biopolymers from polysaccharides and agroproteins. ACS Symposium Series, American Chemical Society, Washington DC, pp. 157–171

Thombre SM, Sarwade BD (2005) Synthesis and biodegradability of polyaspartic acid: a critical review. J Macromol Sci, Part A: Pure Appl Chem 42:1299–1315

Uemura T, Fujimori M, Lee HH, Ikeda S, Aso K (1990) Polyethylene glycol-modified papain catalyzed oligopeptide synthesis from the esters of L-aspartic and L-glutamic acids in benzene. Agric Biol Chem 54:2277–2281

Uyama H, Fukuoka T, Komatsu I, Watanabe T, Kobayashi S (2002) Protease-catalyzed regioselective polymerization and copolymerization of glutamic acid diethyl ester. Biomacromolecules 3:318–323

Wakadkar S, Hermawan S, Jendrossek D, Papageorgiou AC (2010) The structure of PhaZ7 at atomic (1.2 Å) resolution reveals details of the active site and suggests a substrate-binding mode. Acta Cryst F66: 648–654

Wolk SK, Swift G, Paik YH, Yocom KM, Smith RL, Simon ES (1994) One- and two-dimensional nuclear magnetic resonance characterization of poly(aspartic acid) prepared by thermal polymerization of L-aspartic acid. Macromolecules 27:7613–7620