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First characterization of an archaeal amino acid racemase with broad substrate specificity from the hyperthermophile *Pyrococcus horikoshii* OT-3

Short title: First characterization of archaeal amino acid racemase

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Abstract

A novel amino acid racemase with broad substrate specificity was recently isolated from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT-3. Characterization of this enzyme has been difficult, however, because the recombinant enzyme is produced mainly as an inclusion body in *Escherichia coli*. In this study, expression of the recombinant protein into the soluble fraction was markedly improved by co-expression with chaperone molecules. The purified enzyme retained its full activity after incubation at 80°C for at least 2 h in buffer (pH 7-10), making this enzyme the most thermostable amino acid racemase so far known. Besides the nine amino acids containing hydrophobic and aromatic amino acids previously reported (Kawakami et al., Amino acids, 47, 1579-1587, 2015), the enzyme exhibited substantial activity toward Thr (about 42% of relative activity toward Phe) and showed no activity toward Arg, His, Gln, and Asn. The substrate specificity of this enzyme thus differs markedly from those of other known amino acid racemases. In particular, the high reaction rate with Trp and Tyr, in addition to Leu, Met and Phe as substrates is a noteworthy feature of this enzyme. The high reactivity toward Trp and Tyr, as well as extremely high thermostability, is likely a major advantage of using BAR for biochemical conversion of these aromatic amino acids.

Introduction

Amino acid racemase (moderately substrate-specific amino acid racemase, EC 5.1.1.10, AAR) catalyzes the racemization of amino acids using pyridoxal 5'-phosphate (PLP) as a coenzyme and is able to utilize several substrates, unlike highly substrate-specific amino acid racemases such as Ala racemase and Glu racemase (1-3). To date, several AARs have been found and characterized. The enzymes from *Pseudomonas* strains mainly catalyze racemization of Lys, Arg, and Gln, but exhibit much less reactivity toward hydrophobic and aromatic amino acids (4-8). On the other hand, AAR from *Lactobacillus buchneri* JCM1115, which was recently isolated and characterized as an Ile 2-epimerase, shows high reactivity toward branched chain amino acids such as Ile and Leu, but no reactivity toward Lys or Arg (9). All of the previously identified AARs show little (or no) reactivity toward Trp and Tyr (4-9).

D-Amino acids are widely distributed in microbes, plants, and animals and are also contained in fermented foods such as wine and dairy products (10-12). Along with the understandings for the distribution in nature and the importance in various physiological processes concerning D-amino acids, enantioselective assay using HPLC and spectrophotometric assay using enzymes have developed for the determination of D-amino acids (13,14) and various enzymes such as hydrolases, oxidoreductases, and aminotransferases have used for the synthesis of D-amino acids (10). Besides these enzymes, AARs have high potential for biochemical conversion of chiral amino acids; for example, for synthesis of DL-Trp from L-Trp using AAR from *P. putida* IFO12996 and for D-aspartate spectrophotometric assay using the coupling reaction of Asp racemase and L-Asp dehydrogenase (5,15).

We recently demonstrated the growth of *P. horikoshii* on the medium containing

D-amino acids, detected AAR activity in the crude extract, identified the enzyme gene (PH0138, the gene information is available in the KEGG, Kyoto Encyclopedia of Genes and Genomes database), and determined the substrate specificity of the recombinant enzyme (16). Until then, no AAR had been found in an archaeon, other than highly substrate-specific amino acid racemases such as Asp racemase and Ser racemase in *P. horikoshii* and *Pyrobaculum islandicum* DSM4184, respectively (17-19). Results from spectrophotometric assays and preliminary analyses using ultra-performance liquid chromatography (UPLC) demonstrated that the enzyme is active mostly toward Met, Leu, Phe, Ala, Ser, Ile, Val, Trp, and Tyr, indicating it is a novel broad substrate specificity amino acid racemase (BAR). However, reactivity toward other amino acids (especially Arg) and the kinetic parameters for substrates remained unknown. These analyses may provide critical information that will facilitate a better understanding of the physiological role of this enzyme on growth in the medium containing D-amino acids and may be useful for further development of practical applications. Because the recombinant BAR was produced mainly as an inclusion body in *E. coli* cells, we worked to improve the expression system and succeeded in increasing the amount of enzyme present in the supernatant fraction, which made it possible to determine its biochemical properties. Here we report the first characterization of a novel BAR from an archaeon.

Materials and methods

Materials

Chaperone plasmids were purchased from Takara (Kyoto, Japan). *o*-Phtalaldehyde (OPA)

and *N-tert*-butyloxycarbonyl-L-cystein (NBC) were from Wako (Osaka, Japan) and Sigma-Aldrich (Tokyo, Japan), respectively. D- and L-amino acids were from Wako and Tokyo Chemical Industry (Tokyo). All other chemicals were of reagent grade.

Construction of expression plasmid for re-annotated BAR gene

In the previous report, we constructed the expression plasmid for PH0138 gene based on the information from KEGG database (16). The *P. horikoshii* genome DNA sequence was first opened in 1998 and the "PH0138" gene was annotated as an ORF for 4-aminobutyrate aminotransferase (20). After that, the genome information of *P. horikoshii* was totally re-annotated in 2007 based on more accurate estimation of ORF length. As the result, the "PH0138" gene was re-annotated as PH0138.1 gene whose start codon was positioned 9 bp downstream of the original gene (http://www.bio.nite.go.jp/dogan/project/view/OT3). We therefore reconstructed the expression plasmid for PH0138.1 gene. The expression plasmid pET11a/PH0138.1 was constructed using pET11a/PH0138 (16) as the template for PCR. The oligonucleotide primers used to amplify the gene fragment were 5'-CTC**CATATG**ACCAAGTGGGATGAAATTAG-3', which contains a unique *Nde*I restriction site (bold) overlapping the 5' initiation codon, and 5'-T**AGATCT**TCACCATGCCCGTAAGAATTTC-3', which contains a unique *Bgl*II restriction site (bold) proximal to the 3' end of the termination codon. The amplified 1.4-kb fragment was digested with *Nde*I and *Bgl*II and ligated with the expression vector pET11a (Novagen, Tokyo) previously linearized with *Nde*I and *Bam*HI to generate pET11a/PH0138.1.

Co-expression with chaperone plasmids and purification of the recombinant enzyme

Escherichia coli BL21 (DE3) competent cells (Stratagene, Tokyo) harboring chaperone plasmids (pG-KJE8, pGro7, pKJE7, pG-Tf2, or pTf16) were constructed according to the manufacturer's instructions, and the resultant competent cells were further transformed with pET11a/PH0138.1. Transformants harboring each chaperone plasmid were separately cultivated in LB medium containing 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, and appropriate inducers (0.5 mg/ml L-arabinose for *ara*B and/or 5 ng/ml tetracycline for *Pzt*-1) for 9 h at 37°C. Isopropyl-β-D-thiogalactopyranoside was then added to the medium to a final concentration of 0.5 mM, and cultivation was continued for an additional 4 h. The cells were then harvested by centrifugation and stored at -20°C until use.

For purification of the recombinant enzyme, the cells were suspended in 10 mM Tris/HCl (pH 8.0) and disrupted by sonication. After centrifugation, the crude extract was heat-treated at 90°C for 20 min in the presence of 0.1 M citrate (pH 5.5), cooled on ice, and centrifuged to remove precipitants. Ammonium sulfate was added to the resultant enzyme solution up to 40%-saturation, after which the enzyme solution was applied to an Ether-Toyopearl (Tosoh, Tokyo) column previously equilibrated with 10 mM Tris/HCl (pH 8.0) supplemented with 40%-saturated ammonium sulfate, and the flow-through that showed BAR activity was applied to a Butyl-Toyopearl (Tosoh) column previously equilibrated with 10 mM Tris/HCl (pH 8.0) supplemented with 40%-saturated ammonium sulfate. The column was then washed with the same buffer, and the enzyme was eluted using a 40% to 0% gradient of ammonium sulfate. Active fractions were pooled and dialyzed with 10 mM Tris/HCl (pH 8.0). The enzyme solution was finally applied to a DEAE cellurofine column previously equilibrated with 10 mM Tris/HCl (pH 8.0), washed with the same buffer, eluted using a 0 to 0.5 M NaCl gradient in the buffer. Active fractions were pooled, dialyzed with 10 mM potassium phosphate (pH 7.0) and used for characterization.

During the purification process, BAR activity was assayed using a spectrophotometric method, as described previously (16). L-Met served as the substrate and protein concentrations were determined using the Bradford method, with bovine serum albumin serving as the standard (21).

Analysis of D- and L-amino acids using UPLC system

To analyze the D- and L-amino acids produced through the enzyme reaction, amino acids were derivatized with OPA and NBC (22). To prepare the derivatization reagent, 10 mg of OPA and 10 mg of NBC were dissolved in 1 ml of methanol, after which the solution was mixed with 2 ml of 0.4 M borate buffer (pH 9.25). The diastereoisomeric derivatives of amino acids were prepared by incubating a mixture of amino acid sample (50 µl) and derivatization reagent (50 µl) for 2 min at 20°C, after which an aliquot (1 µl) of the reaction mixture was applied to an X-pressPak V-C18 column (2.0 mm by 50 mm, Jasco, Tokyo) previously equilibrated with 75% eluent A (3.5 mM citrate [pH 5.8]) and 25% eluent B (3.5 mM citrate [pH 5.8] in 60% ethanol). The elution was operated at 0.3 ml/min at 40°C, and the gradient program was operated as follows: 25% B for 0.5 min, 25% to 70% B for 11 min, 70% B for 1 min, and 70% to 25% B for 0.5 min. The eluted diastereoisomeric amino acid derivatives were monitored using a fluorescence detector. The excitation and emission wavelengths were 345 nm and 455 nm, respectively. This analysis was performed using an UPLC system (EXTREMA, Jasco, Tokyo) equipped with an autosampler (AS-4250), binary pump (PU-4285), column oven (CO-4062), and fluorescence detector (FP-4020), and the data were processed using ChromNAV ver. 2. The present method based on derivatization with OPA and NBC cannot be applied to the detection of Cys, Lys and Pro (22). In addition, derivatives of DL-Asp and DL-Glu are not separated by this elution system.

Enzyme assay

To assess BAR activity, a reaction mixture (100 µl) composed of 100 mM HEPES (pH 7.0), 10 mM D- or L-amino acid, 0.04 mM PLP, and 1 µg of enzyme was used for the standard assay. After incubation at 80°C for 15 min, the reaction mixture was rapidly cooled, and the reaction was stopped by addition of 25 µl of 30% trichloroacetic acid. After incubation for 5 min at room temperature, the reaction mixture was centrifuged (15,000 rpm for 10 min) and an aliquot (100 µl) of the supernatant was neutralized by addition of 60 µl of 0.6 M NaOH. The resultant solution was used as the amino acid sample for derivatization.

To characterize the pH dependency of the activity, BAR activity toward L-Phe was assayed at 60°C under various buffer conditions (100 mM each of acetate [pH 4.0, 5.0, and 6.0], potassium phosphate [pH 6.0, 6.5, 7.0, 7.5, and 8.0], HEPES [pH 6.5, 7.0, 7.5, 8.0, and 8.5], and CHES [pH 8.5, 9.0, 9.5, and 10.0]). To characterize the temperature dependency of the activity, BAR activity toward L-Phe was assayed at temperatures between 50°C and 95°C. To investigate the pH stability of BAR, the 0.2 mg/ml enzyme solution (100 µl) was incubated at 80°C for 2 h in various buffers (at final concentration of 100 mM), after which the residual activity toward L-Phe was assayed at 60°C. The buffers used for the assay were acetate (pH 4.0, 5.0, and 6.0), HEPES (pH 7.0 and 8.0), CHES (pH 9.0 and 10.0), and phosphate (pH 11.0).

To determine the kinetic parameters, the initial velocity toward various substrates was determined by varying the concentration of each substrate (2 to 50 mM for Ala, 2 to 20 mM for Val, Ile, Leu, Met, and Phe, 0.4 to 4 mM for Tyr, and 0.8 to 8 mM for Trp) (n=3). Reaction rates were independently calculated and apparent V_{max} and K_m values and standard errors were determined with Prism 5.0 (GraphPad software) using a non-linear regression

model.

Determination of subunit structure

SDS-PAGE was carried out using the method of Laemmli (23). Myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase B (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa) were used as molecular mass standards (BIO-RAD, Tokyo). The subunit molecular mass of BAR was estimated from standard curve made with the molecular weight marker protein (relative mobility vs log of molecular mass of standards).

The native molecular mass was determined by gel filtration chromatography using a Protein WS-804F column (8 mm × 300 mm, Shodex, Tokyo) with the flow rate of 1.0 ml/min. Apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa) were used as molecular mass standards (Sigma-Aldrich).

Results

Co-expression with chaperone plasmids in *E. coli*

We previously observed that recombinant BAR is mainly produced as an inclusion body, though a small portion was present in the soluble fraction. In an effort to increase the soluble fraction, we induced at low temperature, changed the expression plasmid (pCold plasmid), and induced a stress response using ethanol or antibiotics (24,25). However, none of these

manipulations affected the amount of the BAR soluble fraction (data not shown). We had more success in the present study where we co-expressed BAR with chaperones (26,27). SDS-PAGE analysis of BAR expression using chaperone systems indicated that a co-expression system using pGro7, pG-Tf2, or pTf16 decreased the amount of the inclusion body as compared expression without a chaperone (Fig. 1A). In addition, increases in the amount of BAR in the soluble fraction were observed with pG-KJE8, pG-Tf2, and pTf16, which supply the chaperone proteins DnaK-DnaJ-GrpE & GroEL-GroES, GroEL-GroES & Tf, and Tf, respectively (Fig. 1B). The specific activities of BAR in the soluble fractions with pG-KJE8 and pTf16 were respectively about 9.0 times and 7.5 times higher than without a chaperone (Fig. 1C). We therefore employed a co-expression system using pG-KJE8, which enabled us to obtain about 2.16 mg of the purified BAR from *E. coli* cells cultured in 0.8 L of medium. By contrast, only 0.73 mg of purified BAR was obtained from cells under the same conditions without chaperone.

Molecular mass and subunit structure of BAR

SDS-PAGE showed the subunit molecular mass of purified BAR to be about 55.8 kDa, which is approximately consistent with the molecular mass (52.1 kDa) calculated from the amino acid sequence. The native molecular mass of about 91 kDa, determined using gel filtration chromatography, suggests the native enzyme is a homodimer.

Effects of pH and temperature on BAR activity and stability

To optimize the reaction conditions for UPLC assays, we first determined the effects of pH and temperature on BAR activity using L-Phe as the substrate. At a temperature of 60°C, the highest activity was detected at around pH 6.5 - 7.5 (Fig. 2A). On the other hand, we

observed temperature-dependent increase of the activity from 50°C to 95°C (Fig. 2B), and the relative activities at 50°C and 80°C were 12% and 70%, respectively, as compared with that at 95°C. When the enzyme was incubated at 80°C for 2 h under various pH conditions, BAR showed no loss of activity at pHs ranging from 7 to 10 (Fig. 2C). At pH 7.0, the enzyme retained its full activity, even after incubation at 80°C for 24 h (data not shown). We therefore selected 80°C and pH 7.0 as a favorable temperature and pH for the standard assay.

Substrate specificity of BAR

We previously confirmed that the enzyme exhibited racemase activity toward Met, Leu, Phe, Ile, Val, Ala, Trp, Tyr, and Ser, and Pro, Asp, and Glu are inert as substrates (16).We thus determined the reactivity of the enzyme toward Thr, Arg, His, Asn, and Gln in this study. After the enzyme was incubated for 2 h at 80°C in reaction mixtures containing the respective L-forms of these amino acids (substrate concentration: 10 mM), peak for the D-form of Thr was detectable using UPLC, whereas the D-forms of Arg, His, Asn, and Gln were not quantitatively detected. Similar results were obtained for the reverse reaction. Thus, BAR predominantly shows activity toward hydrophobic and aromatic amino acids.

Kinetic analysis of hydrophobic amino acids

To determine the kinetic parameters of BAR activity toward Ala, Val, Ile, Leu, Met, Phe, Tyr, and Trp, initial velocity analyses for both D- and L-amino acid formation were performed. The curve of initial velocity at each substrate concentration fitted well into a non-linear regression model. The calculated V_{max} and K_m values are shown in Table 1. The highest V_{max} values were observed for L-Phe (59.9 μmol/min/mg) and D-Met (65.7 μmol/min/mg) in D- and L-amino acid forming reactions, respectively. The V_{max} values obtained with Ala, Val, Ile,

Leu, and Met in D-amino acid forming reactions were lower than in L-amino acid forming reactions. By contrast, the V_{max} values for Phe, Tyr, and Trp in D-amino acid forming reactions were higher than in L-amino acid forming reaction. K_m values for L- and D-Ala (56.0 and 35.6 mM, respectively) were substantially higher than those of other substrates (2 to 10 mM).

Discussion

In an earlier report, we identified an enzyme functioning in D-amino acid metabolism in the hyperthermophilic archaeon *P. horikoshii* OT-3 as an amino acid racemase with broad substrate specificity, BAR (16). This was the first observation of an archaeal BAR. In the present study, we determined the biochemical properties of this enzyme.

In general, hyperthermophilic enzymes have a high potential for utilization as catalytic elements in biosensors and bioreactors. However, heterologous expression of the enzyme gene using *E. coli* is necessary to obtain these enzymes in large quantities at low cost. These recombinant proteins are sometimes expressed in the insoluble fraction as an inclusion body, and this was the case with BAR. Co-expression with a chaperone is an effective means of preventing expression in the insoluble fraction (28-30). In the present study, we succeeded in increasing the soluble expression of BAR using pG-KJE8 plasmid, which suggests this may be an effective approach to heterologous expression of hyperthermophilic enzymes that tend to form inclusion bodies.

Up to now, AARs from *Pseudomonas* strains have been extensively investigated, and their substrate specificities are well examined. AARs from *P. putida* ATCC17642, IFO12996, KT2440 and SCRC-744, as well as *P. taetrolens* NBRC3460 all utilize Lys, Arg and Gln as

their prefer substrates, but show little (or no) reactivity toward hydrophobic and aromatic amino acids (4-8) (Table 2). By contrast, *P. horikoshii* BAR exhibited high activity toward Leu, Phe, and Met, but not toward Arg or Gln. The BAR and *Pseudomonas* enzymes share similar dimeric structures, but the primary structure of BAR does not show homology with those of the *Pseudomonas* enzymes. Whereas BAR is a fold-type I PLP-dependent enzyme, *Pseudomonas* enzymes belong to the fold-type III group (16,31). On the other hand, the AAR from *L. buchneri* JCM1115 (characterized as Ile 2-epimerase), which is also a fold-type I enzyme, shows about 40% amino acid sequence identity with *P. horikoshii* BAR, but has a tetrameric structure (9). *L. buchneri* AAR exhibits high reactivity toward branched chain amino acids such as Ile and Leu as substrates, but not Trp, Lys, or Arg (9) (Table 2). Notably, all of the *Pseudomonas* and *L. buchneri* AARs show little (or no) reactivity toward Trp and Tyr. For example, AARs from *P. putida* IFO12996 and SCRC-744 exhibit reactivity toward Trp, but the relative activity is less than 1% compared to their most preferred substrates (Lys and Gln, respectively). By contrast, *P. horikoshii* BAR exhibits much higher relative activities of 64 and 27% toward L-Tyr and L-Trp, respectively. These results indicate that *P. horikoshii* BAR differs markedly from previously studied AARs. All of the previously identified AARs were derived from mesophiles, whereas BAR is from hyperthermophile. Hyperthermophilic enzymes are generally much more stable at high temperature and broad pH range than mesophilic ones. Ser racemase from hyperthermophile, *P. islandicum*, has so far been reported as the most thermostable racemase; the remaining activity is 67.1% after incubation at 75 °C for 8 h (19). As BAR from *P. horikoshii* shows full activity after incubation at 80 °C for 24 h, the enzyme is the most thermostable amino acid racemase so far known. The high reactivity toward Trp and Tyr, as well as extremely high thermostability, is likely a major advantage of using BAR for biochemical conversion of these aromatic amino acids. It is

known that the k_{cat}/K_m value for an L-amino acid often equals that for the corresponding D-amino acid in many racemase reactions because the equilibrium constant (K_{eq}) of racemization is 1 in the Haldane equation (9). In the case of BAR, however, the k_{cat}/K_m value for the L-amino acid did not coincide with that for D-amino acid. On the UPLC analysis, when substrate concentration of more than 20 mM was used, detection of the small amount of amino acid produced by initial reaction is not easy because the derivatized L-amino acid was contiguously eluted with the derivatized D-amino acid. Thus, the substrate concentrations of less than 20 mM were mainly used for the kinetic study. Restriction of higher concentration of substrate might affect the k_{cat}/K_m values in both directions. On the other hand, it is known that k_{cat}/K_m values for both directions are not equal in several amino acid racemases. In the case of alanine racemase from *P. putida* KT2440, the k_{cat}/K_m value in D to L direction for Lys was 4-fold higher than that in the reverse direction (7). In the case of Ile 2-epimerase from *L. buchneri*, the k_{cat}/K_m value in D to L direction for Ile was 0.7-fold higher than that in the reverse direction (9). In the case of BAR, k_{cat}/K_m values in D to L direction for each substrate, except for Ala were 0.5 to 1.8-fold higher than those in the reverse direction. In this regard, BAR has features similar to other amino acid racemases.

We previously pointed out that BAR is responsible for the growth of *P. horikoshii* in medium containing D-isomers of Met, *allo*-Ile, Leu, Val, Phe, Tyr, or Trp (16). As shown in Table 1, *P. horikoshii* BAR exhibits high reactivity against all of these amino acids. This observation strongly supports our prospect about physiological role of BAR; the enzyme is responsible for the utilization of D-amino acids for growth of *P. horikoshii*. When we examined growth of *P. horikoshii* with several D-amino acids in the previous study, we found that *P. horikoshii* exhibits normal growth in D-Arg containing medium (16). As mentioned above, D-Arg is inert as the substrate for BAR, suggesting that the BAR is not related to the

growth with D-Arg. Our next focus is to identify the enzyme responsible for D-Arg metabolism in *P. horikoshii*.

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Table 1 Kinetic parameters of BAR

D-Amino acid forming reaction				L-Amino acid forming reaction			
L-Form	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (mM)	k_{cat}/K_m (sec^{-1}·mM^{-1})	D-Form	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (mM)	k_{cat}/K_m (sec^{-1}·mM^{-1})
Ala	5.88±0.584	56.0±9.02	0.159±0.0301	Ala	17.6±0.782	35.6±2.44	0.750±0.0703
Val	4.42±0.163	3.41±0.414	1.97±0.249	Val	17.3±0.869	9.82±1.07	2.67±0.320
Ile	14.3±0.264	3.94±0.225	5.50±0.330	*allo*-Ile	25.9±1.23	9.54±0.991	4.12±0.470
Leu	38.9±0.849	4.95±0.304	11.9±0.776	Leu	50.9±0.728	4.19±0.182	18.4±0.848
Met	23.3±0.358	4.28±0.197	8.26±0.400	Met	65.7±1.44	7.86±0.405	12.7±0.709
Phe	59.9±1.56	8.23±0.495	11.0±0.388	Phe	36.4±0.547	2.78±0.150	19.9±1.11
Tyr	16.8±1.27	2.23±0.349	11.4±1.98	Tyr	10.4±0.639	2.77±0.324	5.69±0.752
Trp	9.23±0.522	5.84±0.619	2.39±0.288	Trp	1.45±0.0504	1.68±0.176	1.31±0.144

Table 2 Relative activity of BAR and the known AARs toward L-amino acids.

	P. horikoshii OT-3*	*L. buchneri* JCM1115 (9)	*P. taetorolens* NBRC3460 (6)	*P. putida* SCRC-744 (8)**	*P. putida* IFO12996 (5)	*P. putida* ATCC17642 (4)	*P. putida* KT2440 (7)
Ala	5	3	12	15	20	6	1
Val	13	48	-	-	<1	-	-
Ile	48	100	-	-	<1	-	-
Leu	86	30	2	13	3	10	<1
Met	64	21	12	43	14	66	6
Phe	100	24	<1	1	<1	-	-
Tyr	64	-	-	<1	-	-	-
Trp	27	-	-	<1	<1	-	-
Thr	42	-	-	-	<1	3	-
Ser	10	6	-	31	20	10	2
Lys	-	-	100	22	100	100	100
Arg	-	-	95	21	65	79	11
Gln	-	-	-	100	-	54	3
Asn	-	-	-	6	<1	12	<1
Cys	-	-	-	6	14	-	-
His	-	-	-	5	2	6	<1
Glu	-	-	-	-	-	-	-
Asp	-	-	-	-	<1	-	-
Pro	-	-	-	-	<1	-	-

( ): Reference No. cited. *The values for BAR are relative activity at the substrate concentration of 10 mM except for 2.5 mM of Tyr.
**The values for *P. putida* SCRC-744 AAR are relative activity toward D-amino acid.

Figure legends

FIG. 1. SDS-PAGE analysis of BAR expression using chaperone systems and the specific activities of the enzyme in the soluble fractions. Lanes represent the following: M, markers; 0, recombinant *E. coli* without chaperone; 1 to 5, recombinant *E. coli* with pG-KJE8, pGro7, pKJE7, pG-Tf2, and pTf16, respectively.

(A) Cultivated *E. coli* cells were disrupted by sonication, after which the cell debris was suspended in TE buffer containing 4% Triton X-100, incubated for 30 min at 37°C, and centrifuged. After washing with TE, the precipitant was used as the insoluble fraction for SDS-PAGE.

(B) Crude extract containing 0.1 M citrate (pH 5.5) was heated at 90°C for 20 min and cooled on ice. After centrifugation, the supernatant was used as the soluble fraction for SDS-PAGE.

(C) Specific activities of BAR in the soluble fractions after heat treatment. The activities were measured in spectrophotometric assays using L-Met as the substrate.

FIG. 2. Effects of pH (A) and temperature (B) on BAR activity and the pH stability (C) of the purified BAR.

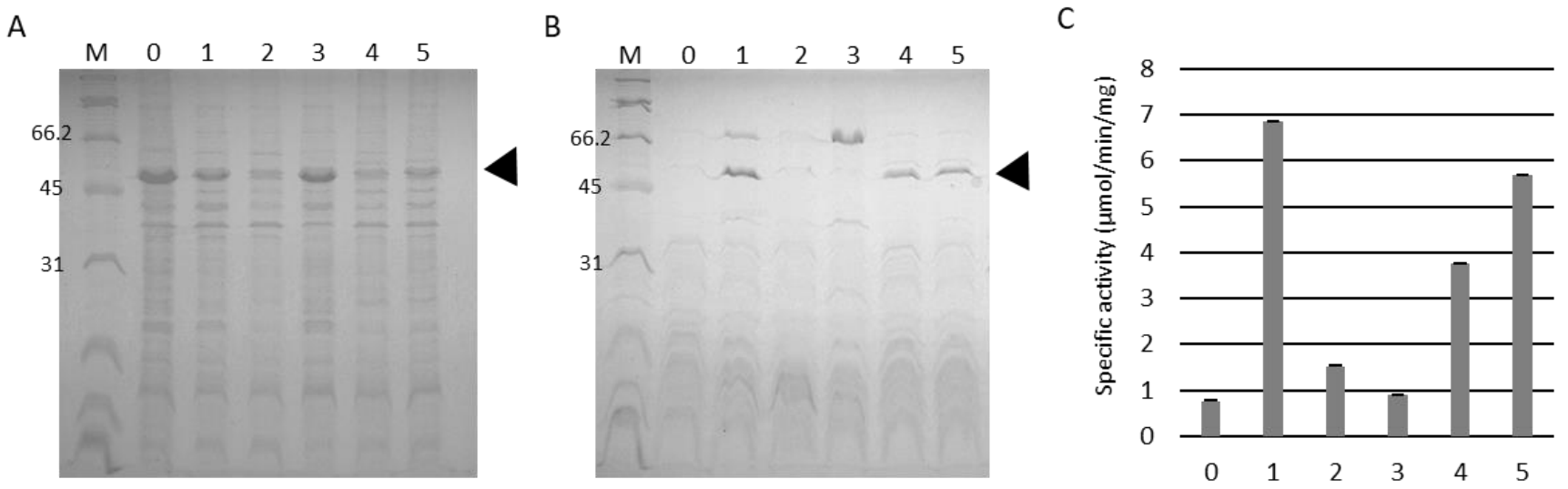

Fig. 1 Kawakami et al.

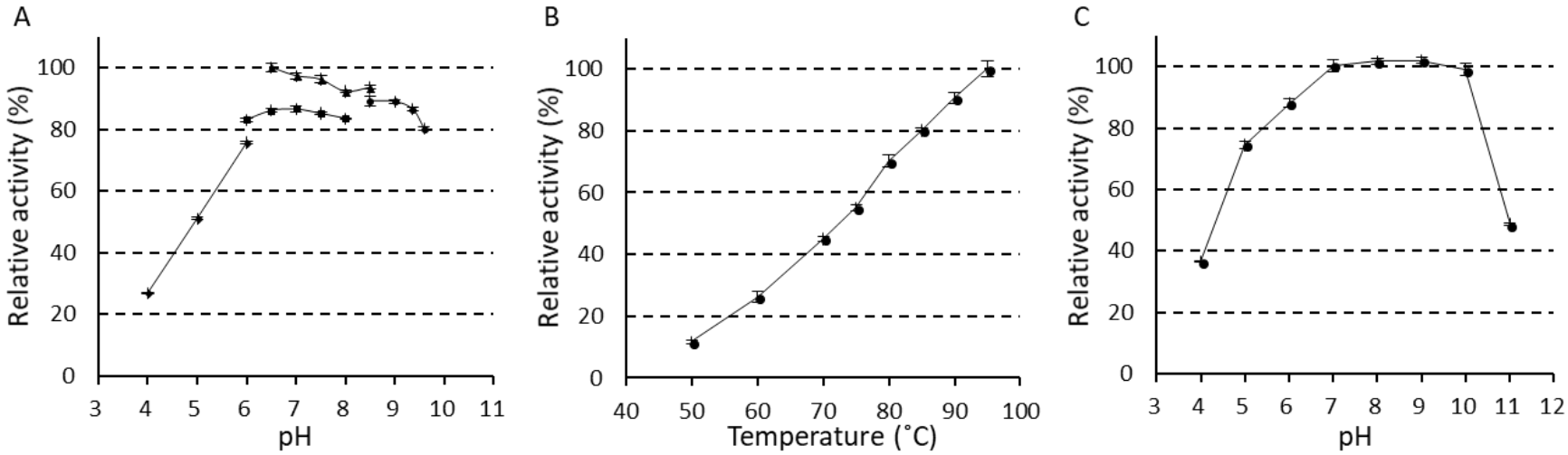

Fig. 2 Kawakami et al.

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