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Purification and characterization of alanine racemase from hepatopancreas of black-tiger prawn, *Penaeus monodon*

Takuma Uo, Momoko Ueda, Tozo Nishiyama, Tohru Yoshimura, Nobuyoshi Esaki*

Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

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Abstract

Alanine racemase has been purified to homogeneity from the hepatopancreas of the black tiger prawn, *Panaeus mondon*. The enzyme depends on pyridoxal 5′-phosphate and consists of two subunits with an identical molecular weight of 41,000. V_{max} and K_m values for L-alanine are 460 μmol/min/mg and 50 mM, and those for D-alanine are 94 μmol/min/mg and 24 mM, respectively. The enzyme is highly specific toward alanine. Among other amino acids examined, only serine served as a substrate: L-serine was racemized at a rate of approximately 0.5% of that of L-alanine. The prawn enzyme is immunochemically distinguishable from the enzymes of *Bacillus stearothermophilus* and *Schizosaccharomyces pombe*, which resemble each other. The prawn enzyme is activated and stabilized by the presence of monovalent anions including chloride. This is consistent with the previous hypothesis (e.g. E. Fujita, E. Okuma, H. Abe, Comp. Biochem. Physiol. 116A (1997) 83–87) that D-alanine serves as an osmoregulator in marine and euryhaline animals.

Keywords: D-Alanine; Alanine racemase; Prawn; Osmoregulation; Pyridoxal 5′-phosphate

1. Introduction

D-Alanine is indispensable for eubacteria as a component of the peptidoglycans of their cell walls [1,2]. Although D-alanine was found as a component of opioid-like peptides [3,4] and antibiotics such as cyclosporin A [5], little attention has been paid to the physiological function of D-alanine in eukaryotes. However, recent advances in analytical techniques have enabled the demonstration of D-alanine in various animals including mammals [6–8]. It is proposed that D-alanine is involved in the osmotic stress response in several marine and euryhaline invertebrates [9–14]. These animals contain high concentrations of free amino acids, which probably serve as the intracellular pool of osmolytes in the event of osmotic stress. D-Alanine in this pool probably serves as a metabolic reserve or a regulatory factor [10–13]. It was found that crayfish in fresh water accumulates free D-alanine in muscle by acclimation to seawater: its concentration is elevated more than 30-fold by the acclimation. This is consistent with the above hypothesis [13]. Alanine racemase activity was detected in the homogenates of the tissues of several marine invertebrates, suggesting that the enzyme is responsible for the synthesis of D-alanine [10,14]. However, the alanine racemase of animals has not been purified, and its enzymological properties are poorly understood.

* Corresponding author. Tel.: +81-774-38-3240; fax: +81-774-38-3248.
E-mail address: esaki@scl.kyoto-u.ac.jp (N. Esaki).

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To understand the physiological role of D-alanine, detailed studies of the enzyme involved in its biosynthesis are required. In this paper, we describe the purification and characterization of alanine racemase from the hepatopancreas of the black tiger prawn, *Penaeus monodon*. We also provide evidence that monovalent anions are involved in the regulation of the enzyme.

2. Materials and methods

2.1. Materials

Frozen hepatopancreas of black-tiger prawns were provided by Dr. T. Kohriyama of Nippon Suisan Co. Ltd., Tokyo, Japan. L-Alanine dehydrogenase (EC 1.4.1.1.) of *Bacillus stearothermophilus* was obtained from Unitika Ltd (Osaka, Japan). D-Amino acid oxidase (EC 1.4.3.3) from pig kidney and L-lactate dehydrogenase (EC 1.1.1.27) from pig heart were purchased from Boehringer Mannheim (Germany). Alanine racemases of *B. stearothermophilus* [15] and the fission yeast, *Schizosaccharomyces pombe* [16] were purified from recombinant *Escherichia coli* cells. Antiserum against the enzyme of *B. stearothermophilus* was prepared as described previously [15]. The preparation of the antiserum against the *S. pombe* enzyme is described elsewhere. Butyl-TOYOPEARL was from Tosoh (Tokyo, Japan). DEAE-Sephacel, Q-Sepharose HP, Superose 12 HR 10/30, and Protein G-Sepharose 4 Fast Flow were from Pharmacia (Uppsala, Sweden). Silver Stain Kit was from Bio-Rad (USA). All other chemicals used were of analytical grade.

2.2. Purification of alanine racemases

Alanine racemase of black-tiger prawn was purified as described below. All procedures were carried out at 4°C. The standard buffer used throughout the purification was 20 mM Tris[hydroxymethyl]aminomethane (Tris)–HCl buffer (pH 7.8).

2.2.1. Preparation of crude extract

Hepatopancreas of black-tiger prawns (about 350 g) were homogenized with 5 volumes of a standard buffer containing 50 mM KCl in a Potter–Elvenhjem glass equipped with a motor-driven Teflon pestle (3000 rpm). The homogenate was centrifuged at 20,000 × *g* for 20 min. The supernatant solution was dialyzed against the standard buffer, followed by centrifugation.

2.2.2. DEAE-Sephacel column chromatography

The supernatant solution was loaded onto a DEAE-Sephacel column (500 ml) equilibrated with the standard buffer. After washing with 1000 ml of the standard buffer, the enzyme was eluted with a linear gradient of 0–400 mM KCl in the standard buffer. The active fractions were combined and diluted to 2-fold with the standard buffer, followed by centrifugation.

2.2.3. Q-Sepharose HP column chromatography

The supernatant solution was loaded onto a Q-Sepharose HP column (200 ml) equilibrated with the standard buffer. The column was washed with 500 ml of the standard buffer containing 200 mM KCl. The enzyme was eluted with a linear gradient of 200–400 mM KCl in the standard buffer. The active fractions were collected and brought to 30% saturation with ammonium sulfate. The solution was stirred for 1 h, followed by centrifugation.

2.2.4. Phenyl-Sepharose column chromatography

The supernatant solution was loaded onto a Phenyl-Sepharose column (80 ml). After the column was washed with 400 ml of the standard buffer 10%-saturated with ammonium sulfate, the enzyme was eluted with a linear gradient of 10–0% saturation of ammonium sulfate in the standard buffer. The active fractions were pooled and diluted to 2-fold with the standard buffer 60%-saturated with ammonium sulfate. The solution was stirred for 1 h, followed by centrifugation.

2.2.5. Butyl-TOYOPEARL column chromatography

The supernatant solution was loaded onto a Butyl-TOYOPEARL column (4 ml) equilibrated with the standard buffer 30%-saturated with ammonium sulfate. After the column was washed with 20 ml of the same buffer, the enzyme was eluted with a linear gradient of 30–10% saturation of ammonium sulfate in the standard buffer. The active fractions were pooled

and dialyzed against the standard buffer. After centrifugation, the supernatant solution was concentrated with Centricon-30 (Amicon, USA).

2.2.6. First Superose-12 HR 10/30 column chromatography

The enzyme was loaded onto a Superose-12 HR 10/30 column equilibrated with the standard buffer containing 100 mM KCl and eluted at 0.25 ml/min. The active fractions were pooled and concentrated.

2.2.7. Second Superose-12 HR 10/30 column chromatography

This step was performed under the same conditions described in Section 2.2.6.

2.2.8. Preparative native-PAGE

An enzyme solution containing 3.75% (v/v) glycerol was subjected to a preparative polyacrylamide gel electrophoresis (PAGE) (pH 8.8) with 6% (w/v) acrylamide gel. Bromophenol blue containing 3.75% (v/v) glycerol was used as a top marker. The electrophoresis was performed at 4°C for 3 h. A portion of the gel containing the enzyme, visualized by active staining as described below, was cut into 2 mm pieces. The pieces were homogenized with a small volume of the standard buffer. The supernatant solution obtained by centrifugation at 12,000 × g for 10 min was dialyzed against the standard buffer and stored at 4°C. The homogeneity of the final preparation was confirmed by sodium dodecyl sulfate (SDS)–PAGE with silver staining (Fig. 1). The enzyme was stable without loss of activity during storage at 4°C for at least 1 month.

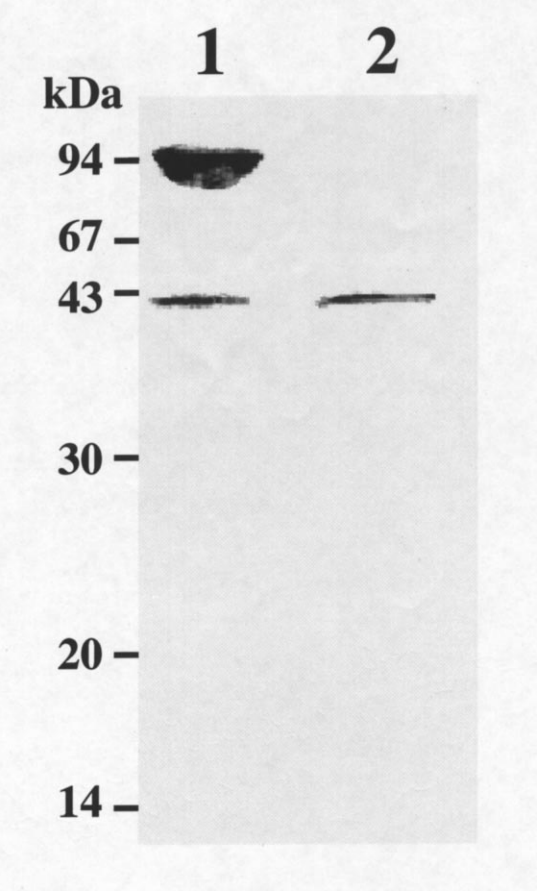

Fig. 1. SDS–PAGE of the purified alanine racemase of black-tiger prawn. The preparations after Step 7 (1 μg, lane 1) and Step 8 (0.5 μg, lane 2) were subjected to SDS–PAGE and detected by silver staining.

2.3. Active staining

Alanine racemase in the native-PAGE gel was visualized with the reduced *p*-iodonitrotetrazolium produced as a result of electron transfer from NADH. The native-PAGE gel containing alanine racemase was washed with 200 mM Tris–HCl (pH 8.0) and incubated in the dark at 37°C in 200 mM Tris–HCl (pH 8.0) buffer containing 50 mM KCl, 50 mM D-alanine, 0.625 mM NAD^+, 0.064 mM 1-methoxy-5-methylphenazium, and 0.24 mM *p*-iodonitrotetrazolium. After appearance of the red color of formazan, the reaction was terminated by immersion of the gel in distilled water.

2.4. Assay of alanine racemase

The formation of L-alanine from D-alanine catalyzed by the alanine racemases of *B. stearothermophilus* and *S. pombe* was determined with the reaction mixture (1 ml) containing 100 mM CHES buffer (pH 9.0), 0.15 unit of L-alanine dehydrogenase, 30 mM D-alanine, 2.5 mM NAD^+, and the enzyme. Increase in absorbance at 340 nm owing to the formation of NADH was monitored at 37°C. The enzyme from the black-tiger prawn was assayed under similar conditions except that the reaction mixture contained 50 mM D-alanine, 50 mM KCl, and 20–100 ng of enzyme. The formation of D-alanine from L-alanine

catalyzed by the prawn enzyme was assayed in a reaction mixture containing 100 mM CHES buffer (pH 9.0), 50 mM KCl, 0.75 unit of D-amino acid oxidase, 5.5 units of L-lactate dehydrogenase, 0.16 mM NADH, various concentrations of L-alanine, and enzyme in a final volume of 1 ml at 37°C. The decrease in absorbance at 340 nm was monitored. One unit of alanine racemase is defined as the amount of enzyme that catalyzes the formation of 1 μmol of L- (or D-) alanine per minute.

The enzymatic activities toward various L-amino acids were assayed with a reaction mixture containing 100 mM CHES buffer (pH 9.0), 50 mM KCl, 50 mM each L-amino acid, and enzyme in a final volume of 50 μl. The reaction was carried out for 30 min at 37°C. The amount of the corresponding D-amino acid formed was determined by HPLC after derivatization of the amino acid to a diastereomer as described previously [17]. Protein was assayed according to the method of Bradford with bovine serum albumin as a standard [18].

2.5. Immunoprecipitations

In order to avoid non-specific binding to Protein G-Sepharose (Pharmacia-LKB, Sweden), alanine racemase (25 ng) was preincubated with an equal volume (10 ml) of the resin at 4°C overnight prior to the reaction. After centrifugation, the supernatant was incubated for 1 h at 4°C with 2, 4, 6, and 10 μl of antiserum. Protein G-Sepharose (10 ml) was added to the reaction mixture, followed by incubation for 1 h at 4°C. After centrifugation, the supernatant was subjected to the alanine racemase assay. The precipitation was washed five times with 100 volumes of potassium phosphate buffer (pH 7.2) and subjected to SDS–PAGE. Alanine racemase in the immune complex was detected by Western blotting.

3. Results

3.1. Purification

We purified the alanine racemase of black-tiger prawn by using DEAE-Sephacel, Q-Sepharose HP, Phenyl-Sepharose, Butyl-TOYOPEARL, and two Superose 12 column chromatographies, followed by a preparative native-PAGE (Table 1). The final preparation gave a single band on SDS–PAGE (Fig. 1, lane 2). The prawn enzyme was purified to near homogeneity at 2856-fold with a yield of 0.29%. The specific activity of the purified enzyme was 65.7 (units/mg). The molecular masses of the enzyme obtained by SDS–PAGE and gel filtration with a calibrated column of Superose 12 were 41 and 80 kDa, respectively. This suggests that the enzyme is a homodimer.

Upon column chromatographies, alanine racemase was eluted in a single fraction. Although we can not rule out the possibility that the prawn contains multiple subforms (or isozymes) of alanine racemase in the same manner as Salmonella typhimurium, the purified

Table 1
Purification of alanine racemase from the hepatopancreas of black-tiger prawn

Step	Total protein (mg)	Specific activities (units/mg)	Total activities (units)	Purification fold	Yield (%)
(1) Crude extract	7200	0.023	170	1	100
(2) DEAE-Sephacel	2700	0.056	150	2.40	90
(3) Q-Sepharose HP	130	0.44	55	19.0	33
(4) Phenyl-Sepharose	6.5	1.4	9.1	61.0	5.4
(5) Butyl-TOYOPEARL	2.4	3.2	7.7	139	4.6
(6) First Superose-12 HR 10/30	0.33	7.2	2.3	304	1.4
(7) Second Superose-12 HR 10/30	0.050	14.0[a]	0.70	609	0.42
(8) Preparative native-PAGE[b]	0.0030	66.0[a]	0.20	2860	0.29[c]

[a] These values were obtained with 50 mM D-alanine as substrates. With 100 mM D-alanine, the specific activities of the preparation after Step 7 and 8 were 20 and 94 (units/mg), respectively.

[b] The enzyme (20 μg) obtained at Step 7 was subjected to preparative native-PAGE.

[c] The expected value when all the protein after Step7 is subjected to the preparative native-PAGE.

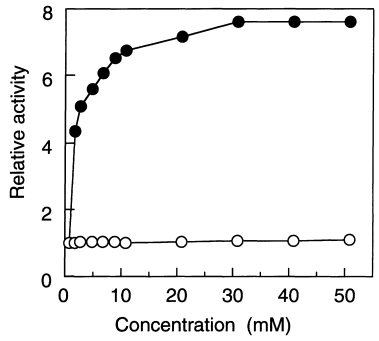

Fig. 2. Effect of KCl and K_2SO_4 on the prawn alanine racemase. The rates of the reaction from D- to L-alanine were measured at 37°C in the presence of various concentrations of KCl (closed circles) or K_2SO_4 (open circles). Relative activities to that without salt were plotted.

enzyme is probably the major form (or isozyme) in the prawn.

3.2. Substrate specificity

The enzyme acts exclusively toward alanine. Serine is the only exception; L-serine is racemized at a rate of about 0.5% of that of L-alanine. The following amino acids are inert: L-valine, L-leucine, L-isoleucine, L-methionine, L-tryptophan, L-phenylalanine, L-threonine, L-asparagine, L-glutamine, L-aspartate, L-glutamate, L-arginine, L-2-aminobutyrate, L-norvaline, L-norleucine and L-homoserine. The enzyme showed maximum activities toward D- and L-alanine at pH 10.0 and 9.0, respectively (D-alanine, Fig. 2; L-alanine, data not shown).

Plots of the specific activities of the enzyme against various concentrations of D- or L-alanine in the presence of 50 mM KCl gave hyperbolic curves (data not shown). Apparent K_m and V_{max} values were estimated: L-alanine, $K_m = 150$ mM, $V_{max} = 460$ μmol/min/mg; D-alanine, $K_m = 24$ mM, $V_{max} = 94$ μmol/min/mg. Thus, V_{max}/K_m values for the conversion from D- to L-alanine and from L- to D-alanine were similar to each other: 3.0 and 3.9 μmol/min/mg/mM, respectively. This is consistent with the theory of enzymatic racemization demonstrated by Briggs and Haldane [19].

3.3. Cofactor

All amino acid racemases acting on neutral amino acids so far studied require pyridoxal 5′-phosphate (PLP) for the catalysis. The prawn enzyme was completely inactivated by incubation with 10 mM sodium borohydride, a common inactivator for PLP enzymes [20]. Approximately 90% of the enzyme activity was lost by incubation with 30 mM hydroxylamine, a common inhibitor for PLP enzymes. The prawn enzyme was also inactivated by either β-chloro-L- or D-alanine, a common mechanism-based inactivator for bacterial alanine racemases (Table 2) [21–23]. These results suggest that the prawn enzyme depends on PLP.

3.4. Effect of monovalent anions

Abe and his colleagues have reported that alanine racemase activity in the homogenate of the crayfish with seawater acclimation was 1.5 to 2.2 times higher

Table 2
Inactivation of alanine racemases of the black-tiger prawn and *B. stearothermophilus* with β-Cl-D- and L-alanine[a,b]

	Residual activity (%)			
	Black-tiger prawn enzyme		*B. stearothermophilus* enzyme	
Concentration (mM)	β-Cl-L-Ala	β-Cl-D-Ala	β-Cl-L-Ala	β-Cl-D-Ala
0.1	12	100	50	13
1.0	5.0	54	0.05	0.03
10	N.D.[c]	4.0	N.D.	N.D.

[a] The prawn enzyme (2–8 ng) or the *B. stearothermophilus* enzyme (1–5 ng) was incubated at 37°C for 30 min in 100 mM potassium phosphate buffer (pH 7.2) containing 50 mM KCl and the indicated concentrations of β-Cl-D- or L-alanine in a final volume of 50 μl.

[b] A 40 μl aliquot was subjected to the racemase assay in the D- to L-direction as described in text. The ratio of the residual to the initial activity was calculated.

[c] Not detectable.

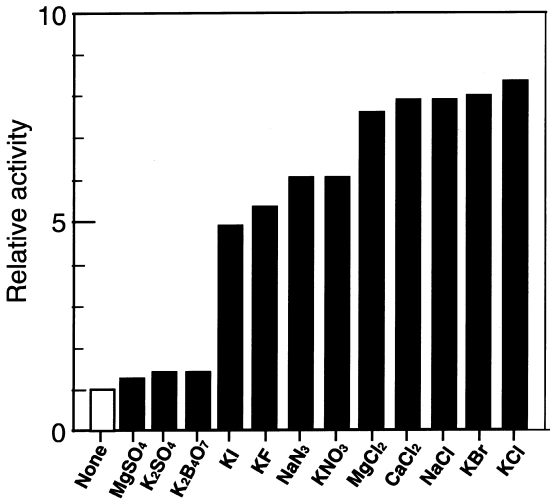

Fig. 3. Effect of various salts on alanine racemase activity. The rates of the reaction from D- to L-alanine catalyzed by the prawn enzyme in the presence of various salts were measured at 37°C. Concentrations of salts were 25 mM for $MgCl_2$ and $CaCl_2$ and 50 mM for others. Relative activities to that without salt are shown.

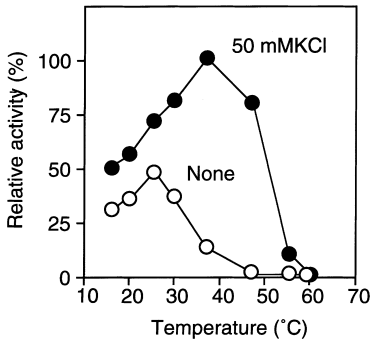

Fig. 4. Effect of temperature on alanine racemase activity in the presence or absence of KCl. The rates of the reaction from D- to L-alanine catalyzed by the prawn enzyme with or without 50 mM KCl were measured at the indicated temperatures. Relative activities to that at 37°C with 50 mM KCl were plotted.

than that without acclimation [13]. However, the activity of the homogenate of black-tiger prawn was not enhanced by the addition of NaCl or KCl to the assay mixture [14]. We examined the effect of salts on the enzyme purified from black-tiger prawn. The enzyme was freed from potassium and chloride ions by dialysis against 5,000 volumes of 20 mM Tris–sulfate buffer (pH 7.8) and reacted with D-alanine in the presence of various concentrations of KCl or K_2SO_4. As shown in Fig. 3, the rate of conversion from D- to L-alanine catalyzed by the prawn enzyme increased with the increase in KCl concentration. In contrast, K_2SO_4 had no effect, suggesting that the effect of KCl is not due to the increase in ionic strength. The effects of other salts were examined (Fig. 4). The reaction rate was enhanced by salts of monovalent anions such as chloride, bromide, nitrate, azide, fluoride, and iodide. In contrast, salts consisting of same cations and multivalent anions such as sulfate and borate had little effect.

Fig. 5 shows the effect of temperatures on enzyme activity in the presence or absence of KCl. The enzyme showed apparent maximum activities at 37 or 25°C in the presence or absence of KCl, respectively. The result suggests that the enzyme is not only activated but also stabilized by the presence of KCl, which was confirmed by the following experiments. The enzyme was incubated at 37°C for 30 min with or without 50 mM KCl at pH 9.0 and then subjected to the racemase assay at 37°C in the presence of 50 mM KCl. The activity of the enzyme preincubated with KCl was 1.7-fold higher than that without KCl.

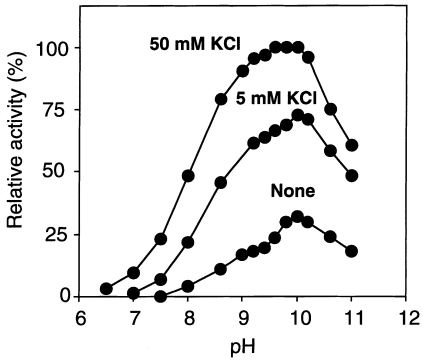

Fig. 5. Effect of KCl on the pH-profiles of the prawn alanine racemase. The rates of the reaction from D- to L-alanine catalyzed by the prawn enzyme were assayed in the presence of 0, 5, or 50 mM KCl. The reaction was carried out as described in the text except that 100 mM 2-[*N*-Cyclohexylamino]-ethanesulfonic acid (CHES)-NaOH buffer was replaced by 50 mM 2-[*N*-Morphilino]ethanesulfonic acid (MES)-N, N-bis[2-Hydroxyethyl]glycine (Bicine)-3-[Cyclohexylamino]-1-propanesulfonic acid (CAPS)-NaOH buffer. Relative activities to that at pH 10.0 with 50 mM KCl were plotted.

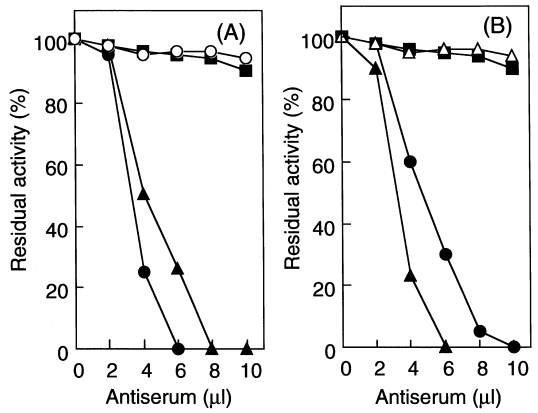

Fig. 6. Immunochemical titration of alanine racemases from black-tiger prawn, *B. stearothermophilus*, and *S. pombe* with the antiserum against the *B. stearothermophilus* or *S. pombe* enzymes. Alanine racemase from the black-tiger prawn (closed squares), *B. stearothermophilus* (closed circles) or *S. pombe* (closed triangles) reacted with the antiserum against the *B. stearothermophilus* (A) or *S. pombe* (B) enzymes. Open circles and open triangles indicate the residual activities of *B. stearothermophilus* and *S. pombe* enzymes after incubation with the preimmune antiserum, respectively.

K_m values for D- and L-alanine were not affected by the presence of KCl (data not shown). Monovalent anions probably increase the maximum velocity of alanine racemization but do not affect the affinity between the enzyme and substrates.

The pH dependence of alanine racemization was also studied at saturating and subsaturating KCl concentrations (Fig. 6). The pH profiles for the prawn enzyme with and without KCl were similar to each other except that the activity at neutral pHs was not detectable without KCl.

3.5. Immunochemical study

Reactivities of the prawn enzyme with the antisera against the alanine racemases of *B. stearothermophilus* and *S. pombe* were examined by immunoprecipitation. As shown in Fig. 2, each antiserum inactivated both microbial enzymes. The reaction with the antibodies was confirmed by Western blot analysis (data not shown). In contrast, neither antisera showed an effect on the prawn enzyme. Therefore, the prawn enzyme differs immunochemically from those of *B. stearothermophilus* and *S. pombe*.

4. Discussion

It is suggested that D-Alanine serves as an osmoregulator in marine and euryhaline invertebrates. It is notable that monovalent anions, including chloride, show activation and stabilizing effects on the purified alanine racemase of the black-tiger prawn. Abe and his colleagues reported that neither NaCl nor KCl showed any effects on alanine racemase activity of the homogenate of black-tiger prawn [14]. The discrepancy is probably due to the fact that they assayed the enzyme in 70 mM Tris–HCl buffer. Under such conditions, the enzyme is fully activated by the chloride in Tris–HCl. Our results shown in Fig. 6 indicate that activation by chloride is intense between 0–20 mM. Taking the chloride concentration inside the cells (5–15 mM) into account, the activation effect probably contributes to the regulation of D-alanine concentration under osmotic stress.

Activation of the enzyme with monovalent anions has also been observed with mammalian and bacterial α-amylases [24–26]. On the basis of X-ray crystallography and kinetic analysis, it is believed that monovalent anions are bound with the specific site of α-amylase and cause a p*K*a shift of catalytic residues of the enzyme. This causes a remarkable change in the pH profile of the enzyme. In contrast, the pH profile of the prawn enzyme was not affected by the addition of KCl (Fig. 6). Further work is required to clarify the activation mechanism of the prawn enzyme.

In addition to the prawn enzyme, two eukaryotic alanine racemases have been purified from fission yeast, *S. pombe* [16] and the fungus, *Trypocladium niveum*, which produces cyclosporin A [27]. The fungal enzyme differs from the bacterial ones in the subunit structure: it consists of four subunits with an identical molecular weight of 37,000 [27]. The primary structure of the fungal enzyme has not been elucidated. The amino acid sequence of the *S. pombe* enzyme, which has been registered in the database (GenBank accession No. SPC965), shows about 30% homology with those of the bacterial enzymes. This is consistent with the immunochemical similarity between the *S. pombe* and *B. stearothermophilus* enzymes (Fig. 2). The prawn enzyme is similar to the bacterial enzymes in molecular weight and subunit structure but differs immunochemically from the enzymes of *S. pombe* and *B. stearothermophilus*.

Recently, we partially purified the PLP-dependent serine racemase from the silkworm, *Bombyx mori* [17]. The enzyme shows similar properties to the rat brain serine racemase in pH optimum, substrate specificity, and behaviors upon chromatographies [17,28]. Part of the sequence of the rat enzyme has been clarified by Wolosker et al. [28]. They have described that the sequence shows no similarity with those of any other known proteins. In contrast, a new bacterial serine racemase, a membrane-bound enzyme from vancomycin-resistant *Enterococcus gallinarum*, contains a similar sequence highly conserved among the bacterial alanine racemases [29]. Though only limited information is available for the structure of the animal enzymes, the results so far obtained suggest structural divergence between the PLP-dependent amino acid racemases of animals and those of microorganisms.

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References

[1] C.T. Walsh, J. Biol. Chem. 264 (1989) 2393.
[2] K. Soda, N. Esaki, Pure Appl. Chem. 66 (1994) 709.
[3] G. Kreil, J. Biol. Chem. 269 (1994) 10967.
[4] L.H. Lazarus, S.D. Bryant, P.S. Cooper, S. Salvadori, Prog. Neurobiol. 57 (1999) 377.
[5] R. Traber, H. Hofmann, H. Kobel, J. Antibiot. 42 (1989) 591.
[6] A. Hashimoto, T. Nishikawa, R. Konno, A. Niwa, Y. Yasumura, T. Oka, K. Takahashi, Neurosci. Lett. 152 (1993) 33.
[7] Y. Nagata, K. Yamamoto, T. Shimojo, R. Konno, Y. Yasumura, T. Akino, Biochim. Biophys. Acta 1115 (1992) 208.
[8] A. Hashimoto, A. Oka, Prog. Neurobiol. 52 (1997) 325.
[9] A. D'Aniello, A. Giuditta, Comp. Biochem. Physiol. 60B (1980) 319.
[10] O. Matsushima, H. Katayama, K. Yamada, Y. Kado, Mar. Biol. Lett. 5 (1984) 217.
[11] H. Felbeck, S. Wiley, Biol. Bull. 173 (1987) 252.
[12] O. Matsushima, Y.S. Hayashi, Comp. Biochem. Physiol. 102A (1992) 465.
[13] E. Okuma, H. Abe, Comp. Biochem. Physiol. 109A (1994) 191.
[14] E. Fujita, E. Okuma, H. Abe, Comp. Biochem. Physiol. 116A (1997) 83.
[15] K. Inagaki, K. Tanizawa, B. Badet, C.T. Walsh, H. Tanaka, K. Soda, Biochemistry 25 (1986) 3268.
[16] T. Uo, T. Yoshimura, K. Takegawa, N. Tanaka, N. Esaki, Nippon Nougeikagaku Kaishi 73 (1999) 55 in Japanese.
[17] T. Uo, T. Yoshimura, S. Shimizu, N. Esaki, Biochem. Biophys. Res. Commun. 246 (1998) 31.
[18] M.M. Bradford, Anal. Chem. 72 (1976) 248.
[19] G.E. Briggs, J.B.S. Haldane, Biochem. J. 19 (1925) 338.
[20] P.D. van Poelje, E.E. Snell, Annu. Rev. Biochem. 59 (1990) 29.
[21] B. Badet, D. Roise, C.T. Walsh, Biochemistry 23 (1984) 5188.
[22] N. Esaki, C.T. Walsh, Biochemistry 25 (1986) 3261.
[23] H. Ueno, J.J. Likos, D.E. Metzler, Biochemistry 21 (1982) 4387.
[24] A. Levitzki, M. Ster, Eur. J. Biochem. 41 (1974) 171.
[25] G. Feller, O. Bussy, C. Houssier, C. Gerday, J. Biol. Chem. 271 (1996) 23836.
[26] N. Aghajari, G. Feller, C. Gerday, R. Haser, Protein. Sci. 7 (1998) 564.
[27] K. Hoffmann, E. Schneider-Scherzer, H. Kleinkauf, R. Zocher, J. Biol. Chem. 269 (1994) 12710.
[28] H. Wolosker, K.N. Sheth, M. Takahashi, J.P. Mothet, R.O. Brady Jr., C.D. Ferris, S.H. Snyder, Proc. Natl. Acad. Sci. USA 96 (1999) 721.
[29] C.A. Arias, M. Martin-Martinez, T.L. Blundell, M. Arthur, P. Courvalin, P.E. Reynolds, Mol. Microbiol. 31 (1999) 1653.