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Molecular Characterization of Alanine Racemase in the Kuruma Prawn *Marsupenaeus japonicus*†

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Aquatic crustaceans and some bivalve mollusks are known to contain copious amounts of free D-alanine in their tissues. For the first time in the animal kingdom, we have isolated a cDNA clone encoding alanine racemase from the muscle and hepatopancreas of the kuruma prawn *Marsupenaeus japonicus*. The recombinant enzyme expressed in *Escherichia coli* exhibited alanine racemase activity. The deduced amino-acid sequence showed only 23–31% identity to bacterial alanine racemases. However, the active site residues and some residues that interact with pyridoxal 5′-phosphate were also conserved in *M. japonicus* enzyme. There was higher alanine racemase mRNA expression in hepatopancreas than in muscle. In contrast, the D-alanine content in hepatopancreas was lower than that in muscle, suggesting that the physiological functions of free D-alanine may differ among tissues. These data suggest that the alanine racemase gene has been conserved from bacteria to invertebrates throughout a long evolutionary time scale.

Key words: alanine racemase, aquatic invertebrate, D-alanine, D-amino acid, *Marsupenaeus japonicus*.

Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; PLP, pyridoxal 5′-phosphate; RACE, rapid amplification of cDNA ends; RT, reverse transcription.

Alanine racemase (EC 5.1.1.1), a pyridoxal 5′-phosphate (PLP)-dependent enzyme, specifically catalyses the interconversion of D- and L-alanine. The racemization of alanine is important for bacterial survival since D-alanine is an essential component of the peptidoglycan layer of the cell wall structure. As this peptidoglycan biosynthetic pathway is unique to bacteria, alanine racemase is regarded as an attractive target for new antibacterial drugs. Thus, the bacterial alanine racemases have been investigated in detail and their catalytic mechanisms have been clarified with the aim of developing mechanism-based inactivators as antibacterial agents specifically targeting the enzyme (*1–4*). Under these circumstances, D-amino acids have long been considered not to exist in organisms other than eubacteria. Recently, however, free and peptide-bound D-amino acids as well as their biosynthetic enzymes have been found even in eukaryotes.

Cyclosporine A, which is produced by the fungus *Tolypocladium niveum*, contains D-alanine as a precursor and alanine racemase has been shown to biosynthesize D-alanine in *T. niveum* (*5*). In fission yeast *Schizosaccharomyces pombe*, a gene encoding a putative amino-acid racemase similar to bacterial alanine racemases has been confirmed to exist and has already been cloned and characterized (*6*).

Free D-amino acids have also been found to exist in many higher-order organisms and even in mammals. Mammalian brain contains high levels of free D-serine, an endogenous co-agonist of a glycine site on the *N*-methyl-D-aspartate (NMDA) receptor (*7–10*). A biosynthetic enzyme, serine racemase, has been purified from rat brain (*11*), and its cDNA has already been cloned from mouse (*12*) and human brain (*13*). D-Aspartate is another endogenous amino acid present in the nervous and neuronal endocrine tissues of mammals (*14–17*). Previous studies show that the levels of D-aspartate vary in a variety of organs during development (*18*, *19*). In rat testis, D-aspartate increases significantly in amount during sexual maturity and appears to accelerate the testosterone synthesis by stimulating the gene expression of steroidogenic acute regulatory protein in Leydig cells (*20*). The cDNA encoding aspartate racemase has previously only been cloned from a bivalve mollusk (*21*).

D-Alanine is also the third D-amino acid that is widely found in mammalian tissues. Its level in the rat anterior pituitary gland varies according to postnatal and circadian changes (*22*). Although the contents of D-amino acids are low in mammals (in the order of nanomoles per gram), some aquatic invertebrates such as marine crustacean and some molluskan species have been found to contain a large amount of free D-alanine, up to 100 μmol/g wet weight or more (*23*). The tissue levels of D- and L-alanine were both increased during high salinity acclimation of these invertebrates, indicating that D-alanine is a major osmolyte for intracellular isosmotic regulation in crustaceans (*24*, *25*) and

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†The nucleotide and deduced amino-acid sequences presented in this paper are available in the GenBank database under accession number AB097480.

Table 1. **DNA nucleotide sequences of primers used for cDNA cloning of *M. japonicus* alanine racemase.**

Designation	Sequence	Location[a]
Pm-F1	5′-GA(C/T)AT(A/T)GGITA(C/T)GACGG-3′	1339–1355
Pm-F2	5′-GA(A/G)GA(C/T)GA(A/G)TGGAT(A/C/T)GC(A/C/G/T)AA-3′	1372–1391
Pm-R1	5′-GG(G/A)CA(A/G/T)ATIC(T/G)IAC(A/G/T)ATCTT-3′	1678–1694
Pm-R2	5′-CT(A/C/G/T)AC(A/G/T)AT(T/C)TTICCGTT-3′	1684–1703
AUAP	5′-GGCCACGCGTCGACTAGTAC-3′	3′- or 5′-end
Mj-F1	5′-GGGTCGAGTATCCATTGATTCCATC-3′	1488–1512
Mj-F2	5′-CCGCCTCCCTGAACAACCTTTACCTG-3′	1518–1543
Mj-F3	5′-GCCACTACAGCGTGCGAGAGG-3′	29–50
Mj-R1	5′-GATGGAATCAATGGATACTCGACCC-3′	1488–1512
Mj-R2	5′-CTGTGCTAATCCGCCTAACCGCTCCG-3′	1443–1468
Mj-R3	5′-CGTCGACTCAAGCCATCAGACCATCC-3′	1402–1427
Mj-R4	5′-TTAACATTATCATAAAGTTCTCACTCCCG-3′	1734–1762

[a]Nucleotide numbers from the 5′-end of *M. japonicus* alanine racemase cDNA.

bivalves (*26–28*). However, the metabolism and accumulation mechanisms of D-alanine remain unknown in these invertebrates. Thus, structural and functional investigations of the biosynthetic enzyme would be useful to solve the physiological functions of D-alanine in animals.

Although alanine racemase has been purified to homogeneity from some invertebrate tissues in recent years (*29–32*), no report has been available on its nucleotide and amino-acid sequences. In a previous trial, however, we have succeeded in isolating the enzyme to homogeneity from the muscle of black tiger prawn *Penaeus monodon*, and we determined the partial amino-acid sequences of the purified enzyme, three of which showed homology to bacterial alanine racemases (*32*). Based on these sequences, we designed degenerate primers and employed a polymerase chain reaction (PCR)-based rapid amplification of cDNA ends (RACE) strategy to clone the cDNA of alanine racemase.

Here, for the first time in animals, we report the isolation of cDNA clones encoding alanine racemase from the muscle and hepatopancreas of the kuruma prawn *Marsupenaeus (Penaeus) japonicus* and the expression of its recombinant protein in *Escherichia coli*.

MATERIALS AND METHODS

Materials—Live specimens of the kuruma prawn *M. japonicus*, weighing 15–18 g, were obtained from a local prawn farm in Oita Prefecture, Japan. The animals were kept in a laboratory glass tank (60 l in volume) supplied with circulating natural seawater filtered and aerated at 15°C and were fed on commercial prawn pellets for at least a week prior to RNA isolation. The tail muscle and hepatopancreas of the prawn were dissected, immediately frozen in liquid nitrogen, and stored at −80°C until use.

cDNA Cloning of Alanine Racemase—Total RNA was extracted from the muscle and hepatopancreas of the prawn using Isogen solution (Nippon Gene, Tokyo, Japan). Poly(A)$^+$ RNA was isolated using an Oligotex-dT30<Super> (Takara Bio, Shiga, Japan). First strand cDNA was synthesized from approximately 80 ng of poly(A)$^+$ RNA using a 3′-RACE kit in accordance with the manufacturer's protocol (Invitrogen, Carlsbad, CA). Degenerate oligonucleotide primers were designed on the basis of partial amino-acid sequences of alanine racemase purified from the muscle of *P. monodon* (*32*). In order to minimize the degeneracy of these primers, two patterns of the sequences containing inosine were designed (Table 1). PCR amplifications were carried out for 4 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, 30 s of annealing at 45°C, and 45 s of extension at 72°C, using a DNA thermal cycler (GeneAmp PCR system 9,700; Applied Biosystems, Foster, CA). The last extension step at 72°C was extended for 5 min. Typically, 100 μl of reaction mixture contained 20 pmol of forward and reverse primers, 1 μl of first-strand cDNA as a template, 20 nmol of deoxynucleotide triphosphate (dNTP) mixture, 10 μl of 10× Ex Taq^{TM} buffer and 1 unit of Ex Taq^{TM} DNA polymerase (Takara Bio). After reamplification with the same combination of primers, a nested PCR was carried out using internal primers (Table 1). The 3′-site of the cDNA was amplified using the 3′-RACE system. The forward primer was designed from a partial cDNA sequence obtained from reverse transcription (RT)–PCR. The reverse primer used was an abridged universal amplification primer (AUAP). Subsequently, the 5′-site of the cDNA was amplified with three gene specific primers (Table 1) using a 5′-RACE system (Invitrogen). First-strand cDNA was synthesized with a primer Mj-R1, while PCR amplification was performed with primers AUAP and Mj-R2, followed by nested PCR with an internal primer Mj-R3. Amplified DNA fragments were subcloned into the plasmid vector pGEM-T Easy (Promega, Madison, WI) using *E. coli* strain JM109 (Promega) as a host bacterium. Sequencing was performed on both strands using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) after labelling the DNA with BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems).

Expression of M. japonicus Alanine Racemase in E. coli—The open reading frame region for the prawn alanine racemase was amplified by PCR using Pfu DNA polymerase (Stratagene, La Jolla, CA) with a forward primer 5′-GGTATTGAGGGTCGCATGACAGGAGAAAC GACAACG-3′ and a reverse primer 5′-AGAGGA GAGTTAGAGCCTCAACATGTGTATTGGAGGCTCAG-3′ and the full-length cDNA from *M. japonicus* as a template. The primers were designed to generate

products with vector cohesive overhangs which were shown in underline. The amplified product was ligated into a pET32 Xa/LIC vector (EMD Chemicals, San Diego, CA) according to the manufacturer's protocol. This vector is designed for the expression of the recombinant protein fused to thioredoxin, His-tag, and S-tag sequences upstream to the cloning site. The fusion tags have a molecular mass of 17.6 kDa. The recombinant plasmid was transformed into *E. coli* strain Rosetta(DE3) pLysS (EMD Chemicals). The transformed cells were cultured in Luria–Bertani media at 37°C ($OD_{600} = 0.5$), and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture media to induce the expression of prawn alanine racemase. The induction was carried out at 25°C overnight. Bacterial cells were suspended in 20 mM Tris–HCl, pH 8.0, after harvesting by centrifugation at 10,000 × *g* for 15 min at 4°C, and they were then disrupted through sonication with an ultrasonic homogenizer (Taitec, Saitama, Japan). The sonicated cell solution was centrifuged at 10,000 × *g* for 15 min at 4°C and the supernatant was used to determine alanine racemase activity as described below. The expression of induced alanine racemase was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) using a 10% polyacrylamide gel and stained with Coomassie brilliant blue R-250.

Preparation of Anti-Peptide Antibody for M. japonicus Alanine Racemase and Immunoblotting—An anti-peptide antibody that recognizes *M. japonicus* alanine racemase was prepared (Scrum, Tokyo, Japan). The antigen peptide (CSTGWSDGLSRRLSNG) was designed from deduced amino-acid sequence of *M. japonicus* alanine racemase, conjugated to keyhole limpet hemocyanin, and injected into rabbit (Scrum). The specific anti-peptide antiserum was purified using an affinity column binding the antigen peptide. Immunoblot analysis was performed with the purified anti-peptide antiserum. The supernatant of *E. coli* cells expressing the recombinant protein mentioned above was subjected to SDS–PAGE, and the proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Detection was carried out by chemiluminescence using Amersham ECL Plus western blotting detection reagents (GE Healthcare, Little Chalfont, UK).

Enzyme Assay—Alanine racemase activity was assayed by determining D- and L-alanine contents with high-performance liquid chromatography (HPLC). The reaction mixture contained 100 mM Tris–HCl buffer, pH 8.5, 200 mM D- or L-alanine, and enzyme solution. After incubation for 10 min at 37°C, an aliquot of the reaction mixture was deproteinized with 600 mM perchloric acid. Following centrifugation at 14,000 × *g* for 2 min, the supernatant was neutralized with 600 mM potassium bicarbonate and centrifuged as above. The resulting supernatant was injected into HPLC.

Alanine enantiomers were separated using an HPLC system (Jasco, Tokyo, Japan) equipped with a chiral column, Sumichiral OA-5000 (4.6 × 150 mm; Sumika Chemical Analysis Service, Osaka, Japan). As the mobile phase, 1 mM copper sulfate was used at a flow rate of 1 ml/min. At an ambient temperature, D- and L-alanine eluted from the column were monitored at 254 nm as alanine–copper complex. Enzyme activity was calculated from the increase of D- or L-alanine.

Determination of Free D- and L-Alanine Contents in the Muscle and Hepatopancreas of M. japonicus—To prepare the tissue extract, the muscle or hepatopancreas of *M. japonicus* was homogenized with a 10-fold excess of 8% perchloric acid. After centrifugation at 20,000 × *g* for 10 min at 4°C, the supernatant was neutralized with solid potassium bicarbonate and centrifuged again to eliminate the pellet. Amino acids including D- and L-alanine were derivatized with *o*-phthaldialdehyde (OPA) and *N-tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys) (*18*), and determined by the HPLC system (Jasco) using a reversed-phase Shim-pack CLC-ODS (250 × 4.6 mm I.D.; Shimadzu, Kyoto, Japan). A mobile phase A consisted of 50 mM phosphate buffer (pH 6.5), acetonitrile, and tetrahydrofuran (92:5:3) and a mobile phase B consisted of the same reagents (45:50:5). A linear gradient elution was carried out from 0% B to 73% B within 85 min at a flow-rate of 0.7 ml/min at 40°C. Eluates were monitored fluorimetrically at 344 and 443 nm for excitation and emission wavelength, respectively.

Real-Time PCR Analysis—Quantification of alanine racemase mRNA in the muscle and hepatopancreas of the prawn was carried out by two-step RT–PCR with a TaqMan probe. First strand cDNA was synthesized from 2 μg of total RNA using a High Capacity cDNA Archive kit with RT Primer mix (Applied Biosystems) according to manufacturer's instructions. Primers and probes were designed using Primer Express Software version 2.0 (Applied Biosystems). For alanine racemase, a specific primer pair, F(5′-GACCTCAGCCGTAGGAA TGG-3′)/R (5′-CCAATTGCCAGGAATCTCGTA-3′) and a TaqMan probe (5′-FAM-AGGAACCTTGGCGGCGCCA-3′) were designed, and for 18S rRNA, a specific primer set, F (5′-CGACGGAAAGGTGTCAAGCT-3′)/R (5′-ATGATCC TTCCGCAGGTTCA-3′), and a TaqMan probe (5′-FAM-TCGTAACAAGGTTTCCG-3′) were used (GeneBank Accession No. AF463512). Real-Time PCR was performed with an Mx3000P (Stratagene). Thermal cycling conditions consisted of the initial steps for 2 min at 50°C and then 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min. The PCR mixture consisted of 10 μM of each primer, 5 μM TaqMan probe or TaqMan universal PCR master mix (Applied Biosystems) in a final total volume of 20 μl.

The total RNA extracted from hepatopancreas was prepared as a standard. A calibration curve was constructed by measuring the levels of alanine racemase mRNA and 18S rRNA in a serial dilution of the standard samples. 18S rRNA served as an internal positive control and a normalizing reference for individual variation. The result was expressed as a relative gene expression level (alanine racemase/18S rRNA). Each value represents the mean ± SD obtained from triplicate determinations.

RESULTS

Isolation and Sequencing of cDNA Clones—PCR-based RACE methods involving both 3′- and 5′-RACE were used

to clone cDNA encoding the full-length alanine racemase from the muscle and hepatopancreas of *M. japonicus*. Several degenerate primers were designed for RT–PCR based on the partial amino-acid sequences of purified *P. monodon* alanine racemase (*32*). After various trials using a variety of combinations of primers, we finally succeeded in obtaining a cDNA fragment encoding alanine racemase by a forward primer, Pm-F1, and a reverse primer, Pm-R1 (Table 1). For initial amplification, PCR product was not detected on agarose gel after electrophoresis. Thus, reamplification was carried out under the same conditions with the same combination of primers. Subsequently, nested PCR was performed using the product of the second PCR as a template and with primers Pm-F2 and Pm-R2 and cDNA fragments of about 300 bp were cloned from both muscle and hepatopancreas. The nucleotide sequences of these clones were shown to be identical and the deduced amino-acid sequences closely correlated with the partial amino-acid sequences of alanine racemase isolated from *P. monodon*.

To obtain information on the nucleotide sequence of the entire coding region, 3′- and 5′-RACE were performed using gene-specific primers derived from the known sequence in the cloned fragment (Table 1). In 3′-RACE, PCR amplification was performed with primers Mj-F1 and AUAP followed by nested PCR with an internal primer Mj-F2. A fragment of 103 bp was obtained, sequenced, and was found to contain a 3′-site non-coding region with a putative polyadenylation signal, AATAA, and stop codon, TGA (Fig. 1), in addition to a coding region of 27 bp. PCR amplification of 5′-RACE was carried out with AUAP and Mj-R2 followed by nested PCR using an internal primer Mj-R3. This yielded a cDNA fragment of 1,371 bp that was found to contain a putative ATG start codon, a non-coding region of 459 bp, and a coding region of 912 bp. Altogether, the nucleotide sequence encoding *M. japonicus* alanine racemase was found to be composed of 1,798 bp, including a 5′-non-coding region of 459 bp and an open reading flame of 1,263 bp that was followed by 62 bp of 3′-non-coding region and poly $(A)^+$ tail.

To obtain a full-length clone, PCR amplification was also performed with primers Mj-F3 and Mj-R4 that were designed from 5′- and 3′-RACE products. However, electrophoresis of the PCR product revealed two distinct bands on the agarose gel both for muscle and hepatopancreas (Fig. 2). The sequence of the larger PCR product was identified to be a full-length cDNA sequence. On the other hand, the smaller one had a deleted region from 201 to 452 bp in the 5′-uncoding region. The missing region was close to the start codon and contained an estimated splice site around the 5′- and 3′-ends (Fig. 1).

Comparison of the Deduced Amino-Acid Sequence of M. japonicus Alanine Racemase with those of other Origins—The amino-acid sequence deduced from the nucleotide sequence encoding *M. japonicus* alanine racemase contained 421 residues with a predicted molecular weight of 45,770 for a subunit of the dimeric enzyme (*32*). This amino-acid sequence was almost identical to those of six partial peptide sequences previously obtained from the purified *P. monodon* enzyme (Fig. 3). However, some residues in the sequence were different from those in the peptide sequences of *P. monodon* enzyme. This may be the reason for the difficulty in cloning alanine racemase cDNA from *M. japonicus*. However, we succeeded in cloning the cDNA from *M. japonicus* because the primer sequences (Pm-F1, F2 and Pm-R1, R2) that were designed based on the *P. monodon* sequence were identical to the sequence from *M. japonicus*. The complete amino-acid sequence of *M. japonicus* alanine racemase showed 24%, 31%, 28% and 23% amino-acid identity to that of *Schizosaccharomyces pombe*, *Geobacillus stearothermophilus*, *Bacillus subtilis* Alr and Dadx, respectively.

In the studies of *G. stearothermophilus* alanine racemase by X-ray crystallography (*33*) and site-directed mutagenesis (*34*, *35*), it was shown that the two basic residues were essential for catalysis. Bound to PLP, lysine39 is proposed to remove the α-proton from D-alanine. Tyrosine265 is thought to remove α-hydrogen from L-alanine. The lysine residue and others around it are known to be well conserved in all bacterial alanine racemases. In *M. japonicus*, this region also showed similarity to yeast and bacterial enzymes (Fig. 3). Furthermore, X-ray crystallography of *G. stearothermophilus* alanine racemase also showed that tyrosine43, arginine136, serine204, arginine219 and tyrosine354 are involved in binding with PLP (*33*). With the exception of serine204, these residues are also conserved in *M. japonicus* alanine racemase.

Expression of M. japonicus Alanine Racemase in E. coli—The entire open reading frame of cDNA of *M. japonicus* alanine racemase was isolated using RT–PCR with primers Mj-Fs and Mj-Re. The cDNA was inserted into pET32 Xa/LIC vector and the plasmid was transformed into *E. coli* Rosetta(DE3)pLysS. *M. japonicus* alanine racemase was expressed in the presence of 1 mM IPTG. The induced recombinant protein was detected to have a molecular mass of about 62 kDa by SDS–PAGE (Fig. 4). This molecular mass was in agreement with the size of fusion protein of *M. japonicus* alanine racemase. A band corresponding to this molecular mass was also identified as alanine racemase using immunoblot analysis (Fig. 4).

The supernatant of the *E. coli* cells after induction of the enzyme with IPTG showed a significant level of alanine racemase activity (Fig. 5C). Although the supernatant of cells not induced with IPTG also showed a trace baseline activity (Fig. 5B), the activity of the induced cells was about 15-fold higher than that of non-induced cells. No activity was detected in the control clone only carrying the empty vector pET32 Xa/LIC (Fig. 5A).

mRNA Level of Alanine Racemase and D-Alanine Content in the Muscle and Hepatopancreas of M. japonicus—Following the isolation of a cDNA clone encoding alanine racemase both from the muscle and hepatopancreas of *M. japonicus*, we proceeded to quantify the alanine racemase mRNA in each tissue by performing real-time PCR using TaqMan probe. For this measurement, 18S rRNA was used as an internal reference value. To prepare the standard curve, first strand cDNA synthesized from hepatopancreas was used

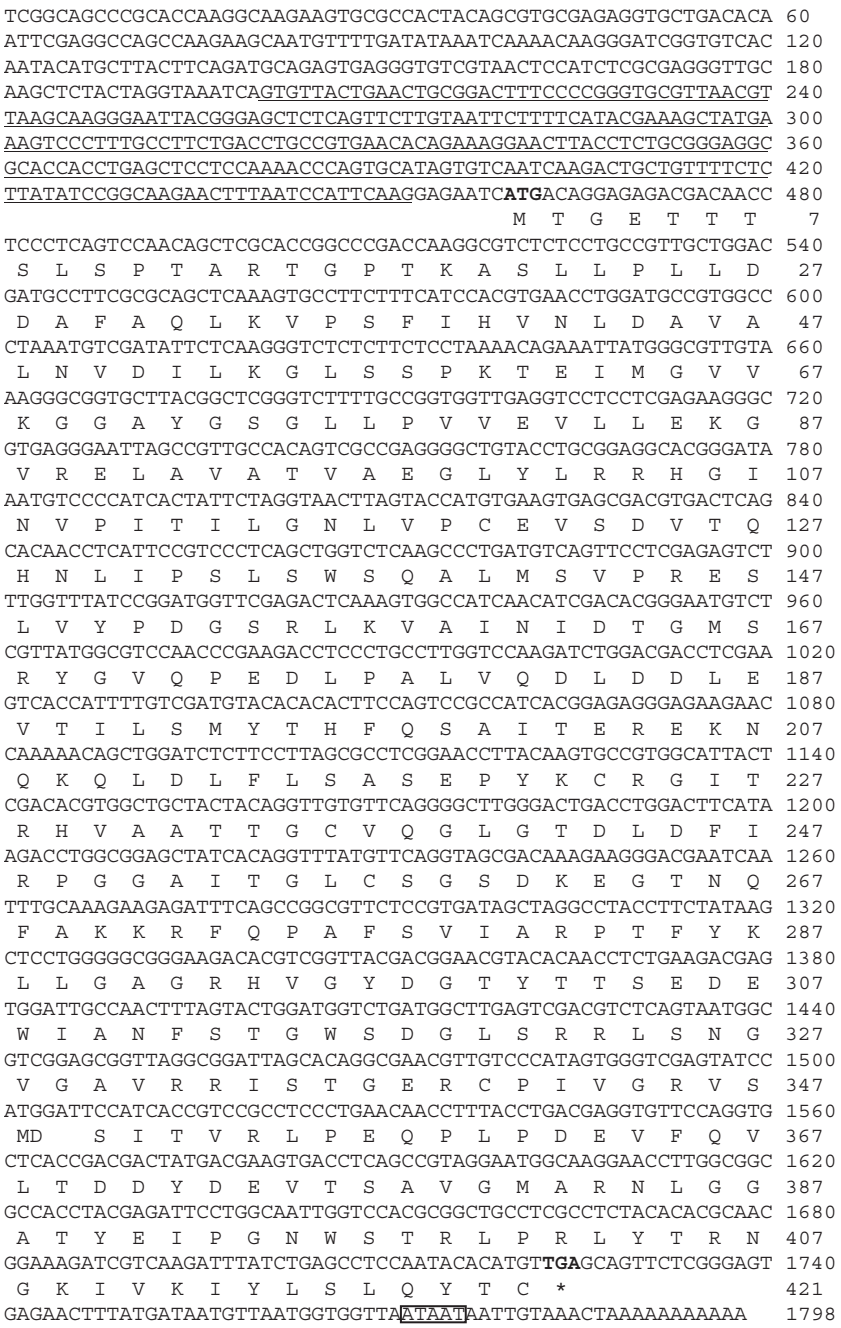

Fig. 1. **Nucleotide and deduced amino-acid sequences for cDNA clone encoding alanine racemase from the muscle and hepatopancreas of *M. japonicus*.** The initiation (ATG) and stop (TGA) codons are shown in bold. An asterisk also indicates the stop codon. The deduced amino-acid sequences of 421 residues are indicated in single-letter code below the nucleotide sequence. The putative polyadenylation signal was boxed. The deleted part found in the smaller PCR product when tried to isolate the entire coding regions was underlined.

to assay the target and control genes. Alanine racemase gene expression was normalized to 18S rRNA. From this, the expression level of alanine racemase mRNA in the hepatopancreas was found to be five times higher than the level in muscle (Fig. 6).

Furthermore, D- and L-alanine contents were determined in each tissue. D-Alanine content in muscle was found to be higher than that in hepatopancreas (Fig. 7). D-Alanine in muscle accounted for 43.7% of total alanine content, and the percentage in hepatopancreas was lower than that in muscle and calculated to be 36.5%.

DISCUSSION

This is the first report to describe the complete primary structure of alanine racemase derived from members of the animal kingdom. We cloned the alanine racemase

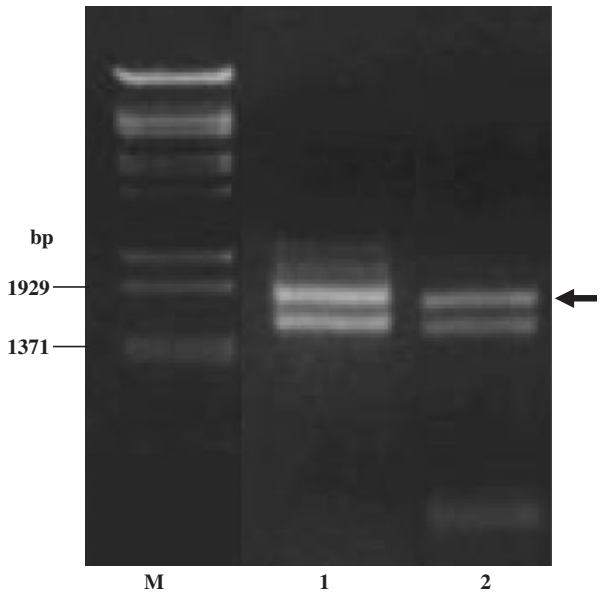

Fig. 2. **Full-length PCR products of *M. japonicus* alanine racemase.** First strand cDNA used as a template for PCR was constructed from 5 μg of total RNA from hepatopancreas (lane 1) and muscle (lane 2), respectively. RT–PCR was performed with primers Mj-F3/Mj-R4. Electrophoresis of the PCR products on an agarose gel showed two distinct bands. PCR product corresponding to the full-length alanine racemase is marked with an arrow.

gene from *M. japonicus* on the basis of partial amino-acid sequences of *P. monodon* because of the difficulty in obtaining and maintaining Southeast Asia-native *P. monodon* in Japan. *M. japonicus* had formerly been classified into the same genus as *P. monodon* and was also found to contain high activity of alanine racemase (*36*). The deduced amino-acid sequence of *M. japonicus* alanine racemase was similar to the partial sequences of *P. monodon* enzyme, suggesting a high homology in primary structure of the enzyme between these close relatives. As a consequence, we succeeded in isolating the alanine racemase gene both from the muscle and hepatopancreas of *M. japonicus*.

We have determined six peptide sequences of the purified enzyme from *P. monodon* (*32*). Of these sequences, only three peptides showed homology to bacterial alanine racemases. The comparison of the complete amino-acid sequence of *M. japonicus* also showed low similarity to bacterial enzyme sequences. However, the catalytic residues (lysine and tyrosine) in the bacterial enzyme as well as some residues that interact with PLP are also conserved in alanine racemase from *M. japonicus* (*33–35*). These data indicate that the alanine racemase gene is conserved from bacteria at least to aquatic crustaceans throughout a long evolutionary time scale, taking on novel functions that differ from those of bacterial alanine racemases.

On the other hand, some splice variants derived from alanine racemase-encoding mRNA were also obtained from the muscle and hepatopancreas of *M. japonicus* (Fig. 2). Some splice variants in the translational region as well as a partial deletion in the 5′-uncoding region were detected in the process of cDNA cloning (data not shown). These products may have arisen to regulate the process of gene transcription and expression. Furthermore, investigation is needed to determine if these alternative splice variants are translated to proteins or non-coding RNAs or immature mRNAs just before complete splicing.

As for bacterial alanine racemase, two distinct genes were reported in *Salmonella typhimurium*, *Alr* and *DadB* (*37*), and *E. coli*, *Alr* and *Dadx* (*38*). Although all of them retain the characteristic active site residues of alanine racemase, they have different physiological functions. The *DadB* alanine racemase is inductively formed and functions in the catabolism of L-alanine (*39*), while *Alr* provides D-alanine for the biosynthesis of peptidoglycans (*40*). The homology between *Alr* and *DadB* in *S. typhimurium* was shown to be 40%. Although this homology is rather high compared to those between alanine racemases of different bacterial species, these genes are known to map to two different regions on *S. typhimurium* chromosome (*40*). The sequence of *M. japonicus* alanine racemase was similar to *Alr* rather than *DadB* or *Dadx*.

As mentioned above, D-alanine in prokaryotes works as a central molecule in peptidoglycan assembly and cross-linking. Although the content of D-alanine in several mammalian tissues is extremely low, a relatively large amount of D-alanine was found in the anterior pituitary gland and pancreas of rat (*22*). In the anterior pituitary gland, D-alanine level is higher in the daytime than in the nighttime (*22*). D-Alanine is also localized to insulin secreting β-cells in pancreas (*41*). It has been hypothesized that D-alanine may participate in the release or biosyntheses of some hormones that are secreted from these tissues. However, the physiological functions and the origin of D-alanine remain unclear. It has further been proposed that D-alanine may be derived from the mammalian diet and intestinal bacteria since the mammalian biosynthetic enzyme of D-alanine has never been found. Thus, the analysis of the prawn alanine racemase may contribute to the discovery of the enzyme in mammalian tissues.

In *M. japonicus*, the same alanine racemase gene was detected in both muscle and hepatopancreas. The level of alanine racemase expression was five times higher in hepatopancreas than in muscle. This was also true of the alanine racemase activity (data not shown). On the other hand, the content of D-alanine was lower in hepatopancreas than in muscle. In the muscle of *M. japonicus*, D-alanine largely accumulates during high salinity acclimation, and D-alanine is regarded as one of the major osmolytes regulated intracellularly in relation to the level of salinity stress. Although the role of D-alanine in hepatopancreas remains unclear, it is possible that D-alanine and alanine racemase perform different specific functions in each tissue of the prawn. Alanine racemase in hepatopancreas may have tissue-characteristic functions such as the regulation of blood glucose level or some hormone secretion mechanisms, and D-alanine may be utilized in these functions without accumulation in the tissue. Furthermore, studies will be needed to determine the tissue-specific physiological functions of D-alanine.

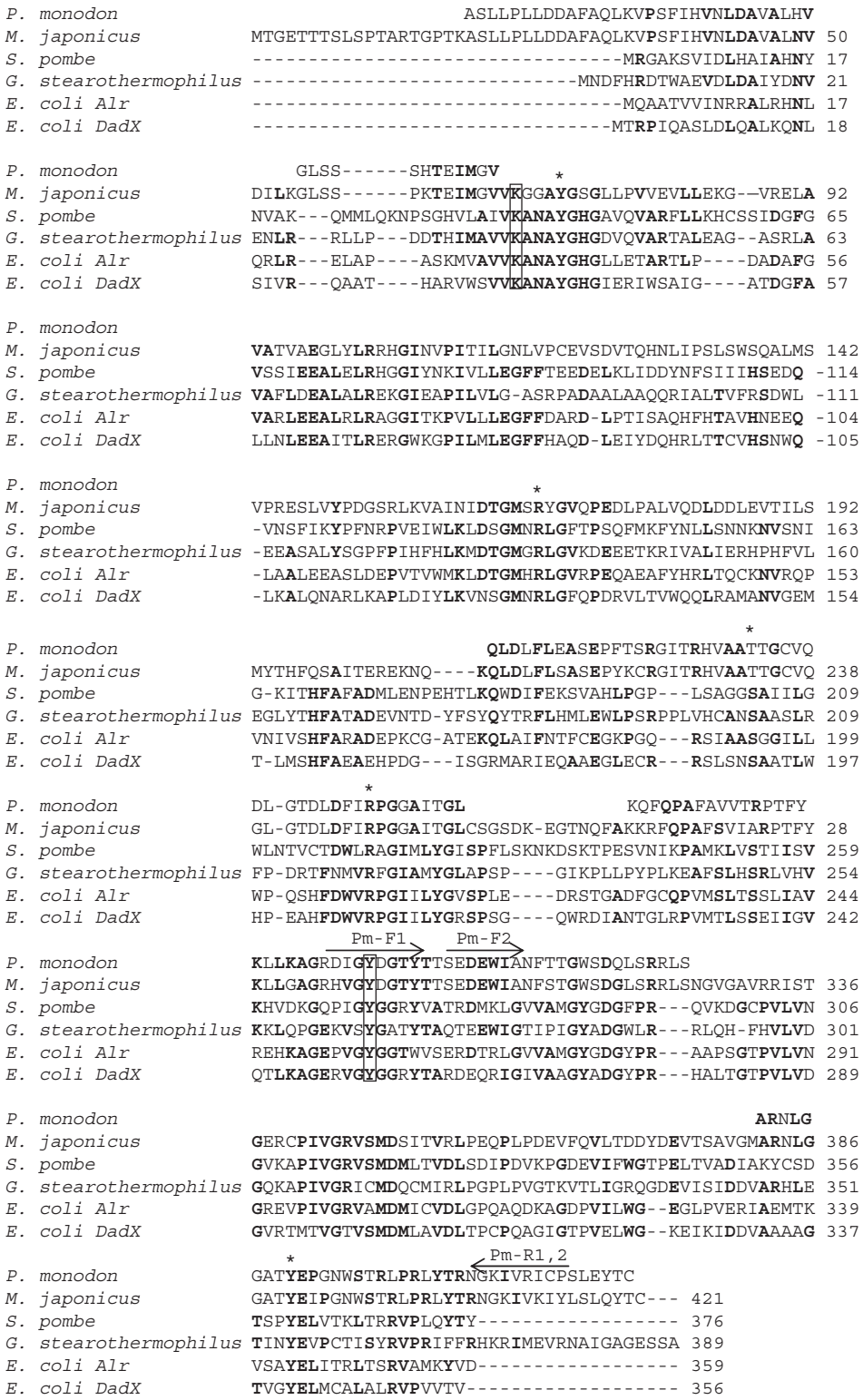

Fig. 3. **Comparison of amino-acid sequence of *M. japonicus* alanine racemase (GenBank accession no. AB097480) with partial amino-acid sequences of *P. monodon* alanine racemase, and those of *Schizosaccharomyces pombe* (GenBank accession no NP588518), *Geobacillus stearothermophilus* (GenBank accession no M19142), *E. coli* Alr (GenBank accession no NP756880), and *E. coli* DadX (GenBank accession no YP540389).** The alignment was generated with Clustal W (1.83). Conserved residues are shown in bold letters. The catalytic lysine and tyrosine residues were boxed. Asterisks represent the PLP-binding residues in *G. stearothermophilus*. The arrows showed the primer sequences that were constructed from the peptide sequences found in *P. monodon* enzyme to obtain the cDNA fragment from *M. japonicus* tissues.

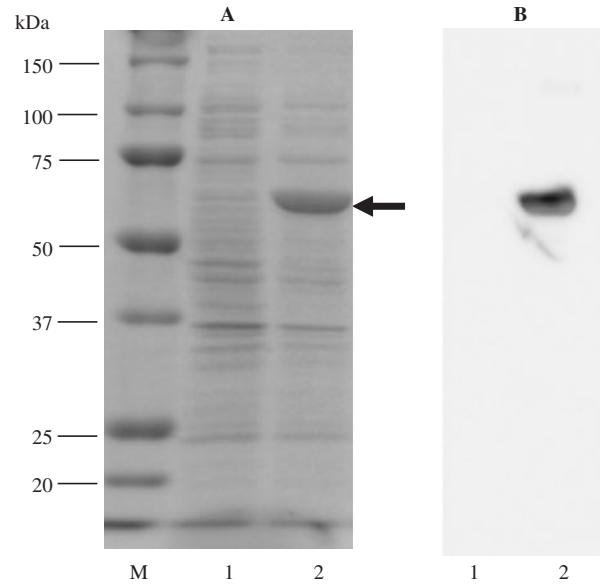

Fig. 4. **Expression of *M. japonicus* alanine racemase in *E. coli*.** SDS–PAGE (A) and immunoblotting (B) patterns of the recombinant alanine racemase were shown. Protein induction was performed with 1mM IPTG at 25°C for overnight. Lane 1, uninduced whole cell lysate; lane 2, induced whole cell lysate; M, molecular markers. The arrow indicates the induced recombinant alanine racemase.

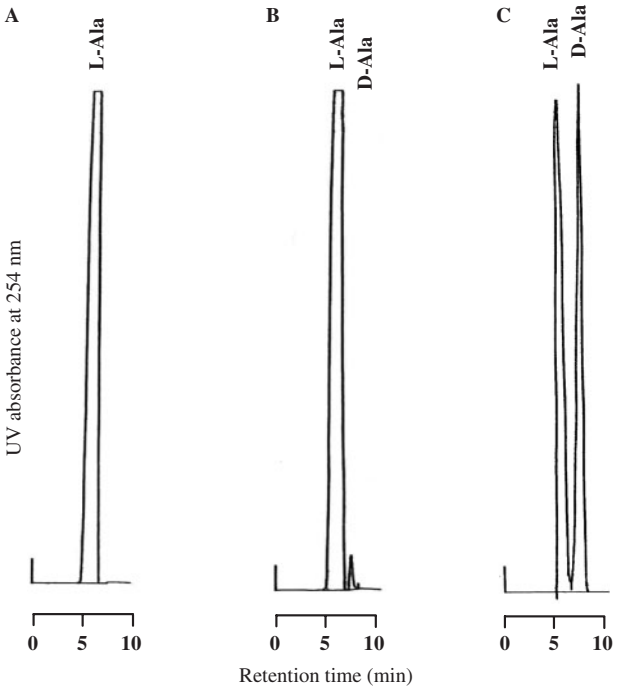

Fig. 5. **Alanine racemase activity of the recombinant protein.** Homogenate of *E. coli* cells transformed with the empty vector pET32 Xa/LIC was defined as the control (A). The uninduced (B) or induced (C) bacterial cells with 1 mM IPTG were disrupted through sonication. The supernatant fraction was used to determine alanine racemase activities. These chromatograms show the reaction from L to D direction. The reaction was carried out in 100 mM Tris–HCl buffer, pH 8.5, containing 200 mM L-Ala as the substrate for 20 min at 37°C.

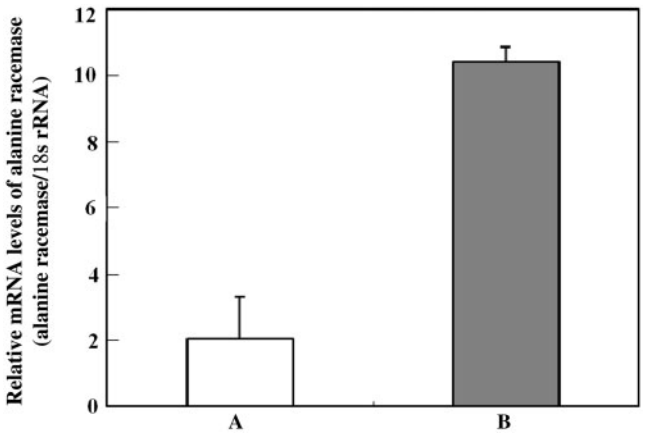

Fig. 6. **Quantification of mRNA expression of alanine racemase in the muscle (A, white bar) and hepatopancreas (B, grey bar) of *M. japonicus* using real-time PCR.** The quantities of alanine racemase transcripts were normalized to the 18S rRNA level. The ratio of the amount of the target to that of the reference within the same sample was considered the relative mRNA expression level. Bars indicate the mean and SD of three independent measurements.

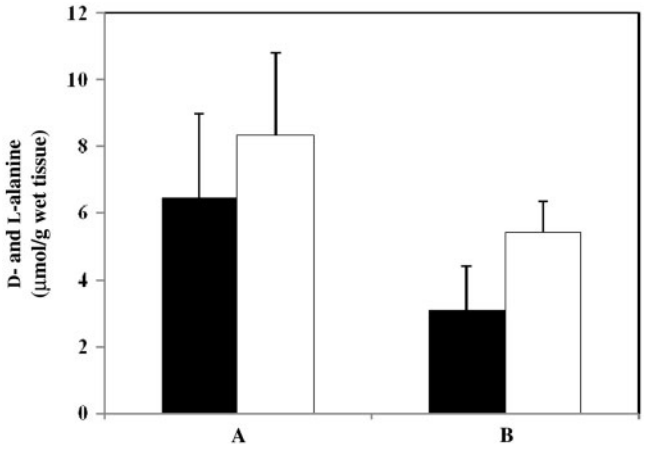

Fig. 7. **Contents of D- and L-alanine in the muscle (A) and hepatopancreas (B) of *M. japonicus*.** Closed column shows D-alanine and open column L-alanine. Values represent means and SD of five prawns.

In conclusion, we clarified that alanine racemase gene existed also in animal genome in addition to bacterial one. It appears that aquatic invertebrates may utilize D-alanine actively because they have a species-dependent copious amount of D-alanine in their tissues. To elucidate the functions of alanine racemase and D-alanine, further investigations are necessary. However, they might have previously unrecognized unique functions in animal kingdom. Thus, this study may be the first step toward solving the physiological functions of alanine racemase and D-alanine in animals.

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CONFLICT OF INTEREST

None declared.

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