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Article

Biochemical Characteristics of an Alanine Racemase from *Xanthomonas oryzae* pv. oryzae

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A gene encoding a putative alanine racemase in *Xanthomonas. oryzae* pv. oryzae was cloned, expressed and characterized. Expression of the cloned gene was performed in *Escherichia coli* BL21(DE3)pLys using a pET-21(a) vector harbouring 6× histidine tag. Purification of the recombinant alanine racemase by affinity chromatography resulted in major one band by sodium dodecyl sulfate polyacryl amide gel electrophoresis analysis, showing about 45 kDa of molecular weight. The alanine racemase gene, cloned in this experiment, appears to be constitutively expressed in *X. oryzae*, as analyzed by reverse transcriptase polymerase chain reaction. The enzyme was the most active toward L-alanine and secondly D-alanine, showing a racemic reaction, thus the enzyme is considered as an alanine racemase. The enzyme was considerably activated by addition of pyridoxal-5-phosphate (PLP), showing that 75% increase in activity was observed at 0.3 mM, compared with control. D-Cysteine as well as L-cysteine significantly inhibited the enzyme activity. The inhibitions by cysteines were more prominent in the absence of PLP, showing 9 and 5% of control activity at 2 mM of addition, respectively. The enzyme was the most active at pH 8.0 and more stable at alkaline pHs than acidic pH condition.

Key words: alanine racemase, characteristics, *Xanthomonas oryzae*

Alanine racemase (EC 5.1.1.1) catalyzes the interconversion of L-alanine and D-alanine and is one of the pyridoxal-5-phosphate (PLP) dependent enzymes. Although L-amino acids are predominant in living organisms, D-amino acids including D-alanine are also distributed ubiquitously in microbes. D-Alanine is required for the biosynthesis of peptidoglycan layer which is necessary for the cell membrane of prokaryotes. Thus the alanine racemase is considered as one of essential enzymes in prokaryotes. Almost all bacteria contain alanine racemases, but the enzyme is generally absent in higher eukaryotes with only some exceptions. In eukaryotes, alanine racemase is involved in D-alanine metabolism in yeast, *Schizosaccharomyces pombe* [Uo *et al*., 2001a; 2001b], osmotic regulation in a freshwater crayfish, *Procambarus clarkia* [Shibata *et al*., 2000] or brackish-water mollusk, *Corbicula japonica* [Nomura *et al*., 2001]. Biochemical characterization of the enzyme has shown that eukaryotic alanine racemases are distinct from those of microbial enzymes in quaternary structure, pH-dependency, PLP requirement, and kinetic properties. Alanine racemase is encodes by *alr* gene which is constitutively expressed in the microbes [Strych *et al*., 2000; Saito *et al*., 2007; Zhang *et al*., 2007]. Another gene of alanine racemase, *dadx*, is induced by the presence of high concentration of L-alanine and also involved in the interconversion of alanine [Lobocka *et al*., 1994; Zhang *et al*., 2007].

By reason of unique presence in bacteria and of its biochemical role, alanine racemase has often been proposed as an attractive target protein for the development of new antimicrobial agents [Badet and Walsh, 1985; Silverman, 1988]. Some substances which inhibit the biosynthesis of peptidoglycan have been known and these materials include D-cysteine, D-cycloserine (4-amino-3-isoxazolidinone) and D-carbamyl-D-serine. The target of inhibition was known to be alanine racemase [Preston and Douchit, 1984; Hoffmann *et al*., 1994; Noda *et al*., 2004]. D-Cycloserine is general inhibitor for PLP-dependent enzymes including alanine racemase. Alanine analogues including β,β,β,-trifluoroalanine [Faraci and Walsh, 1989], alanine phosphonate [Copie *et al*., 1988], 1-amino-cyclo propane phosphonate [Erion and Walsh, 1987], and β-chloro and β-fluoalanine [Wang and Walsh, 1978] were known to inhibit the activity of alanine racemase. Contrary to the finding that D-cysteine inhibits an alanine racemase, another finding showed that an alanine racemase from *E. coli* was not inhibited by D-cysteine [Lambert and Neuhaus, 1972]. Enzyme activity of an alanine racemase from Lactobacillus was enhanced by a

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sulfhydryl compound, glutathione [Johnston, 1969]. Enzyme activity of an alanine racemase from *Tolypocladium niveum* was reported to be increased by thiol reducing agents such as glutathione, mercaptoethanol or dithiothreitol [Hoffmann *et al*., 1994]. In the case of alanine racemase from *Lactobacillus fermenti,* the enzyme activity was increased by addition of β-mercaptoethanol or glutathione but the activity decreased by dithiothreitol [Johnston, 1969]. Such controversial results necessitate further research of alanine racemase, especially in the part of active site and catalytic mechanism.

Alanine racemase is also valuable in the development of a process for the production of D-amino acid, which is widely used in the field of agro-chemical or drug materials [Kuniki *et al*., 2007]. In this regard, a gene encoding a putative alanine racemase was isolated from *X. oryzae* based on the data in NCBI gene bank and expressed in *E. coli*. This paper describes the expression of a putative alanine racemase gene of *X. oryzae* and confirmation and characterization of the alanine racemase.

Materials and Methods

Bacterial strains and culture media. *X. oryzae* pv. *oryzae* (ATCC 35393) was obtained from Korean Agricultural Culture Collection. The cells were cultured using Luria broth medium (LB) supplemented with sucrose (5 g/L), yeast extract (10 g/L), and peptone (5 g/L) [Sambrook and Russel, 2001]. *E. coli* DH5α was used as a cloning host for the gene encoding a putative alanine racemase of *X. oryzae*. *E. coli* BL21(DE3)pLys, comprising the gene for T7 RNA polymerase under the control of *lac* promoter, was used for the expression of an alanine racemase gene. These *E. coli* strains were stock-cultured and generally grown using LB medium which was supplemented with appropriate concentration of antibiotics.

Gene cloning. For the purpose of isolating an alanine racemase gene, *X. oryzae* cells were cultured using the medium for 24 h. General DNA manipulations including preparation of total genomic DNA were performed according to routine methods [Sambrook and Russel, 2001]. A putative alanine racemase gene (NCBI accession number, NC 006834; GI, 58579623) was amplified by PCR technique using a sense primer, 5'-atgcgt cctgcgcaagcgtcgatc-3' and an antisense primer, 5'-ccgcgctgcaac ctcgcccactgc-3. Secondary PCR was intended to attach *Nde*I adaptor site using the first PCR product as a template DNA. The primer of this PCR was as follows: a sense primer, 5'-catatg tgtcctaggtcatggtggttt-3' (underlined catatg is for *Nde*I adaptor site) and the same antisense primer as that for the first PCR. The amplified gene was designed to elimninate a stop codon for the subsequent preparation of His6-tagged recombinant protein. The PCR product (ca. 1.1 kb) was isolated and ligated into a pGEM-T Easy vector (Promega, Sanfrancisco, CA). The ligation mixture was transformed into *E. coli* DH5α cells by a heat-shock method, then inoculated in the LB agar medium supplemented with ampicillin (100 μg/mL), isopropyl β-D-thio-galacto pyranoside (IPTG; 0.5 mM), and 5-bromo-4-chloro-3-indolyl β-D-galacto-pyranoside (75 μg/mL). After culture at 37°C for 24 h, desired colonies were further cultured in to a 5 mL LB medium containing ampicillin, followed by a plasmid isolation. The evident insertion of a desired gene was confirmed by both PCR technique and DNA sequence analysis. DNA was dideoxy-cycle-sequenced with fluorescent terminators (Big Dye, Applied Biosystems, Foster City, CA) in an Applied Biosystems ABI Prism 377 automated DNA sequencer.

Plasmid construction. The pGEM-T Easy vector inserted with the putative alanine racemase gene was double digested by *Nde*I and *Sal*I restriction enzymes. The digested gene was inserted into a pET-21(a) vector which was also digested by the same enzymes. The constructed pET-21(a) vector harboring the gene was named pET-21-*ala* and transformed into *E. coli* DH5α cells. After incubation of the cells in LB-agar medium containing ampicillin (100 μg/mL) for 18 h, the desired colony harbouring the pET-21-*ala* was further cultured in 5 mL LB liquid medium containing ampicillin, followed by isolation of pET-21-*ala*. Insertion of the gene into the pET-21(a) was confirmed by both double digestion with *Nde*I and *Sal*I and DNA sequence method as described.

Expression and preparation of cell-free extract. The pET-21-*ala* was transformed into *E. coli* BL21(DE3)pLys cells in order to express the gene inserted. The transformed cells were inoculated in LB-agar medium containing ampicillin (100 μg/mL). After culture for 18 h, the desired colony harboring the pET-21-*ala* was further cultured in 5 mL LB medium supplemented with ampicillin. The incubation was continued with shaking at 37°C until the A_{600nm} reached 0.4, and the cells were inoculated in to 200 mL of the same medium to obtain sufficient quantity of alanine racemase. After further incubation for 4 h, the induction was performed by adding IPTG to a final concentration of 1.0 mM. An additional culture was carried out for 8 h with slow shaking (50 rpm) at 25°C. The cells were harvested by centrifugation and washed with buffer A (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 5% glycerol). The cells suspended in buffer A were disrupted by sonication on ice, followed by centrifugtion at 10,000 g for 5 min for clarification.

Affinity purification of the His6-tagged alanine racemase. The cell-free extract suspended in 50 mL of the buffer A was loaded directly into a His-Bind Resin column (1 cm × 5 cm) previously charged with Ni(II) ion and equilibrated with the same buffer. The column was washed with the same buffer, then protein was eluted with an elution buffer (50 mM Tris-HCl pH 7.5, 5% glycerol, and a linear gradient of 0–500 mM imidazole) at a flow rate of 0.5 mL/min. The fraction containing an alanine

racemase was traced by assaying the enzyme activity with each fraction. The fractions pooled with alanine racemase were then collected and dialyzed with buffer A in order to eliminate excessive imidazole. The active fraction was used for analysis of sodium dodecyl sulfate polyacryl amide gel electrophoresis (SDS-PAGE) and characterization study of the enzyme.

Assay of alanine racemase. The alanine racemase activity was measured by the colorimetric method of Shibata *et al*. [2000] with minor modifications. In brief, the reaction of converting L-alanine to D-alanine was performed in a 0.5 mL mixture containing 10 mM L-alanine, 100 mM Tris-HCl (pH 8.0), 0.2 mM NADH, 0.2 U of D-amino acid oxidase (from porcine kidney, Sigma, St. Louis, MO), and 2.5 U of L-lactate dehydrogenase (from *Lactobacillus leichmanii*, Sigma). The reaction was started by addition of 50 μL enzyme preparation. After incubation at 37°C for 30 min, the enzyme activity was tested spectrophotometrically by measuring decrease in absorbance 340 nm. For the assay of reaction converting D-alanine to L-alanine, reaction mixture was consisted of 0.2 U L-alanine dehydrogenase (from *Bacillus subtilis*, Sigma,), 20 mM D-alanine, and 2.5 mM NAD^+. The enzyme activity of this direction was tested by measuring increase in absorbance 340 nm. One unit of enzyme activity was defined as the amount of L or D-alanine (μmol) produced per min per mg protein.

Test of substrate specificity Substrate specificity of the racemase was based upon measuring the amount of L- or D-amino acid produced by the enzymatic reaction. Racemase activity converting L-amino acid to D-amino acid was measured using different L-amino acids as substrate. In this case, D-amino acids produced by the enzyme was measured by the same method of alanine racemase assay, since detection of D-amino acid is based upon the use of D-amino acid oxidase by the same way. Detection of L-amino acid produced by the enzymatic reaction was performed using L-amino acid oxidase (from *Crotalus adamanteus*, Sigma) in place of D-amino acid oxidase. In general, L- or D-amino acids can be racemized, each other, by heat treatment [Liardon and Hurrel, 1983] as well as by the reaction of racemase. Therefore all amino acids used in this experiment were filter-sterilized in place of heat-mediated sterilization and freshly prepared just before use.

Inhibition of alanine racemase by amino acids. The alanine racemase activity was measured for the reaction converting L-alanine to D-alanine and *vice verse* in the presence of L- or D-amino acids as potential inhibitor. The enzymatic reaction and measurement of activity were performed as described above under standard assay condition. Inhibition of alanine racemase activity was assessed by measuring the residual activity of alanine racemase.

Effect of pH on the activity and stability. The effect of different pH on the enzyme activity was determined under the standard assay method, with exception of buffer system. The enzymatic reactions at pH 4.0–5.5, 5.5–7.0, and 7.0–9.0 were performed with 50 mM citrate phosphate, potassium phosphate, and Tris-HCl buffer, respectively. For the test of pH stability, the enzyme fraction (50 μL) was distributed into different buffers (200 μL) showing different pH values ranging from 3.0 to 10.0: citrate phosphate (pH 3.0 to 5.0), potassium phosphate (pH 6.0 to 7.0), and Tris-HCl buffer (pH 8.0 to 10.0). After keeping for 4 h at room temperature (about 25°C), the aliquots were removed, and the alanine racemase activity was measured under the standard assay condition.

Other analytical methods. Total RNA was extracted from *X. oryzae* using a commercial kit (Promega). An expression of alanine racemase gene in *X. oryzae* was confirmed by reverse transcriptase (RT)-PCR. Protein concentration was determined according to the method of Bradford [1976] using bovine serum albumin as a standard protein. SDS-PAGE was carried out as described by Laemmli [1970] using 5% stacking gel and 12.5% resolving gel, followed by staining of the proteins with Coomassie Brilliant Blue R-250. Unless otherwise mentioned, all of the data were represented as mean values of three independent experiments.

Results and Discussion

Expression of alanine racemase in *E.coli* and affinity purification. To obtain a sufficient quantity of the alanine racemase, an attempt was tried to express the enzyme in *E. coli* BL21(DE3)pLys harboring the pET-*ala*. A routine culture of the cell at 37°C and 200 rpm resulted in a formation of inclusion body, thus it was difficult to obtain a soluble form of alanine racemase which shows a considerable activity. Crude extracts were prepared by mechanical disruption, sonication, cell lysis using commercial kit, or combination of each method. In any case, formation of inclusion body was evident under the basis of very low enzyme activity and the absence of the expected protein band on SDS-PAGE of the crude extract prepared (detailed data not shown). In order to avoid a formation of inclusion body which frequently occurs through over-expression mediated by T7 promoter in *E. coli*, the cells were cultured under slow growth condition by adjusting the incubation temperature at 25°C and keeping an agitation rate below 50 rpm. Under this condition, the alanine racemase gene was over-expressed with a soluble form. The cells were collected every 2 h until 12 h and the enzyme activity measured. When the bacterial sample was collected after 8 h, the specific activity was the highest, 5.4 U/mg protein (detailed data not shown). Therefore the content of an alanine racemase accumulated in the cell may be relatively the most under this condition of expression, thereby rendering the protein purification more

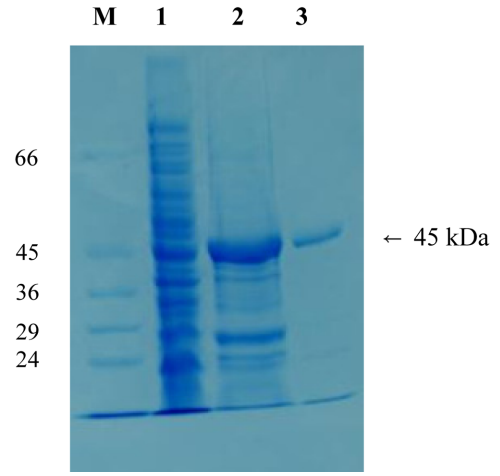

Fig. 1. SDS-PAGE analysis of a purified alanine racemase which was cloned from *X. oryzae* and expressed in *E. coli*. Lane M, molecular mass markers; lane 1, crude extract from *E. coli* BL21(DE3)pLys without insert gene (*i.e.*, vector control); lane 2, crude extract from *E. coli* cell transformed with pET-21-*alr*; lane 3, an alanine racemase purified by affinity chromatography using nickel charged His-bind resin.

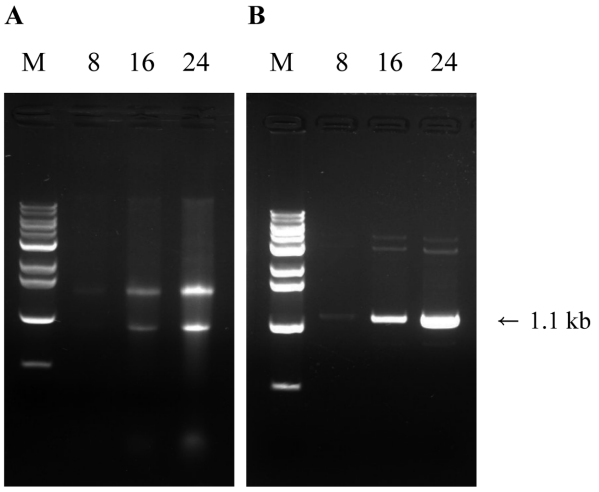

Fig. 2. Extraction of total RNA from *X. oryzae* (A) and reverse transcriptase PCR using the total RNA (B). Total RNAs were extracted at an interval of 8 h during the culture of bacteria. An expression of alanine racemase gene in *X. oryzae* was confirmed using a reverse transcriptase PCR. The PCR was performed by a reverse transcriptase using the total RNA as a template. M, marker; 8, 16 and 24, each time (h) of total RNA extraction.

facile. Purification of the alanine racemase was performed by affinity chromatography owing to six histidine residues of the enzyme. A SDS-PAGE analysis was performed with the active fraction obtained from the affinity chromatography, resulting in one major band with a molecular mass of about 45 kDa (Fig. 1). Affinity purification using His-tag endows other additive advantage that intrinsic alanine racemase in *E.coli* could be passed without binding to the affinity column.

The molecular weight shows a similar range to the enzyme from other sources such as 41 kDa from *Penaeus monodon* [Uo *et al*., 2001a; 2001b], 45 kDa from *Acetobacter aceti* [Francois and Kappock, 2007], and 50 kDa from *Bifidobacterium bifidum* [Yamashita *et al*., 2003]. However, the molecular weights from *Corynebacterium glutamicum* or *Chlamydomonas reinhardtii* [Oikawa *et al*., 2006; Nishimura *et al*., 2007] were 78 and 72 kDa, respectively, showing a much difference from our result.

Table 1. Substrate specificity of the purified alanine racemase, which was cloned from *X.oryzae* and expressed in *E. coli*. The enzyme activities which converts L-amino acid to D-amino acid and *vice versa*, were measured

Substrate	Enzyme activity (U/mg protein)	
	L-Amino acid→ D-Amino acid reaction	D-Amino acid→ L-Amino acid reaction
Alanine	11.5	9.5
Serine	3.7	2.1
Lysine	<0.1	<0.1
Arginine	<0.1	<0.1
Leucine	<0.1	0.4
Asparagine	<0.1	<0.1
Methionine	<0.1	<0.1
Glutamine	0.9	<0.1
Cysteine	<0.1	<0.1
Valine	<0.1	<0.1
Glutamic acid	<0.1	0.3
Proline	0.7	0.5
Tyrosine	1.5	1.2
Tryptophan	1.4	<0.1
Phenylalanine	<0.1	0.7
Histidine	1.9	1.2
Glycine	0.6	1.4

Constitute expression of the alanine racemase gene in *X. oryzae*. If the cloned gene is not constitutively expressed in *X. oryzae* cell, the researches such as biochemical characteristics and inhibition test of alanine racemase would be less valuable. In this regard, an expression of the cloned gene in *X. oryzae* was tested by a reverse transcriptase PCR technique (Fig. 2). Total RNAs were extracted from the cells ranging from logarithmic to stationary phase and analyzed for the gene expression. The primers used for this PCR technique were the same as those for the cloning of alanine racemase gene. The result of PCR product represents a gradual increase of 1.1 kb along with the incubation time, which corresponds to the cloned gene. Therefore, this result indicates that the cloned gene might be constitutively expressed in *X. oryzae* cell.

Substrate specificity of purified alanine racemase. The purified alanine racemase was assayed using different L or D-amino acids as substrate and interconversions between L- and D-amino acids were measured (Table 2). The highest activity as measured by the production of D-amino acid was observed with the enzymatic incubation using L-alanine as a substrate, showing the activity of 11.5 U/mg protein. The highest reaction of D- to L-amino acid production was also occurred by the enzymatic

Table 2. Eventual inhibition of the alanine racemase activity, which was cloned from *X. oryzae* and expressed in *E. coli*. The enzyme activities which converts L-amino acid to D-amino acid or *vice versa*, were measured. Concentration of each inhibitor was 10 mM of amino acid

	Enzyme activity (U/mg protein)		
Inhibitor	L-Amino acid→ D-Amino acid reaction	Inhibitor	L-Amino acid→ D-Amino acid reaction
Control	12.4	Control	9.8
L-Ala	14.5	D-Ala	10.1
L-Ser	10.2	D-Ser	7.8
L-Lys	7.5	D-Lys	6.4
L-Arg	10.4	D-Arg	10.2
L-Leu	12.1	D-Leu	11.4
L-Asn	13.1	D-Asn	10.5
L-Met	10.7	D-Met	8.5
L-Gln	9.5	D-Gln	7.9
L-Cys	2.5	D-Cys	2.4
L-Val	13.1	D-Val	12.3
L-Glu	11.4	D-Glu	7.9
L-Pro	10.8	D-Pro	7.8
L-Tyr	10.6	D-Tyr	10.2
L-Trp	9.4	D-Trp	10.7
L-Phe	8.4	D-Phe	8.8
L-His	10.5	D-His	10.2
Gly	11.3	Gly	9.8

reaction with D-alanine as a substrate, but to a lesser extent than the conversion rate of L- to D-alanine reaction. Productions of L- or D-serine were also observed but the activities were less compared with the production of L- or D-alanine. With other amino acids, essentially no detectable activities of racemase were found in any directions of L- or D-amino acid production.

Taken together, the gene annotated as a putative alanine racemase in NCBI data bank is considered as a gene coding for an evident alanine racemase under the basis of data that represents a substrate specificity as well as a racemic reaction. Similar to our result, alanine racemases from *Penaeus monodon* [Uo *et al*., 2001a; 2001b] or *B. bifidum* [Yamashita *et al*., 2003] showed some activity with L-serine as well as L-alanine. However, the enzyme from *Corynebacterium glutamicum* did not show any enzyme activity with L-serine [Oikawa *et al*., 2006]. Thus, it would be necessary to further research whether alanine racemase is involved in the biosynthesis of D-serine in bacterial cells.

Effect of PLP on the enzyme activity The enzyme activity of alanine racemase was measured in the presence of PLP (Fig. 3). In the absence of PLP, the enzyme activity was 11.2 U/mg protein. Addition of PLP up to about 0.3 mM gradually enhanced the enzyme activity, showing 172% activity of control at 0.3 mM.

Up to now, it has been shown that many of alanine racemase need PLP as a cofactor [Francois and Kappock, 2007]. However, an alanine racemase from *Bacillus anthracis* [Kanodia *et al*., 2008] showed considerable activity without PLP and the activity increased by addition of PLP [Kanodia *et al*., 2008]. The alanine racemase, in this experiment, is similar to the alanine racemase from *B.anthracis* in the light of PLP dependency. On the analogy of our result and the previous findings, PLP, a cofactor might be weakly bound to the alanine racemase by non-covalent binding. Thus it is speculated that PLP bound to the enzyme might be gradually lost during protein purification procedure. Therefore supplement of PLP would be necessary for the full activation of the enzyme.

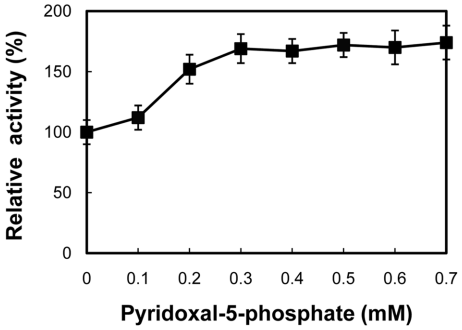

Fig. 3. Effect of PLP on the alanine racemase activity from *X.oryzae*. The purified alanine racemase was assayed in the presence or absence of PLP. The activity without PLP was considered as 100% activity which was equivalent to 11.2 unit/mg protein.

Eventual inhibition of alanine racemase activity by amino acids. An inhibitory effect of amino acids on the alanine racemase was performed by measuring the enzymatic activity converting L-alanine to D-alanine or *vice verse* in the presence of different amino acids (10 mM) as inhibitor (Table 2). Among the amino acids tested, L- or D-cysteine considerably inhibited the alanine racemase activity to the extent of 79 and 76 % inhibition, respectively. Secondly, L- or D-lysine also inhibited the enzyme activities, showing 40 and 35% inhibition, respectively. Other amino acids appears to less inhibit the enzyme or do not affect the enzyme activity at all. Therefore the inhibitory effect by L- or D-cysteine was further examined in detail by measuring the activity in the presence or absence of PLP, a general cofactor of alanine racemase (Fig. 3). In the presence of PLP, the enzyme activity was inhibited by L- or D-cysteine, showing 65 and 67% activities of control at 2 mM addition (*i.e*, 35 and 37% inhibition), respectively. In the absence of PLP, the activities also much decreased and the extent of decrease appears to be more significant than that in the presence of PLP.

Similar to our result, it has been reported that enzyme activities of alanine racemase from *Bacillus cereus* [Preston and Douthit, 1984], *Lactobacillus fermenti* [Johnston, 1969], or *Tolypocladium niveum* [Hoffmann *et al*., 1994] were inhibited by D-cysteine. But the enzyme from *E.coli* was not inhibited by D-cysteine [Lambert and Neuhaus, 1972]. In general, cysteine is neither a substrate of the alanine racemase nor an analogue of

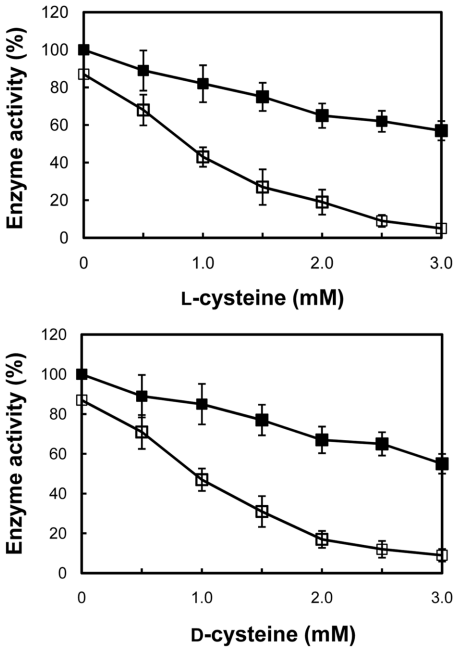

Fig. 4. Influence of L- or D-cysteine on the activity of the alanine racemase. Inhibitory effects of L- or D-cysteine on the alanine racemase activity were assayed in the presence (1 mM, -■-) or absence (-□-) of PLP. One hundred percent activity was equivalent to 10.6 U/mg protein.

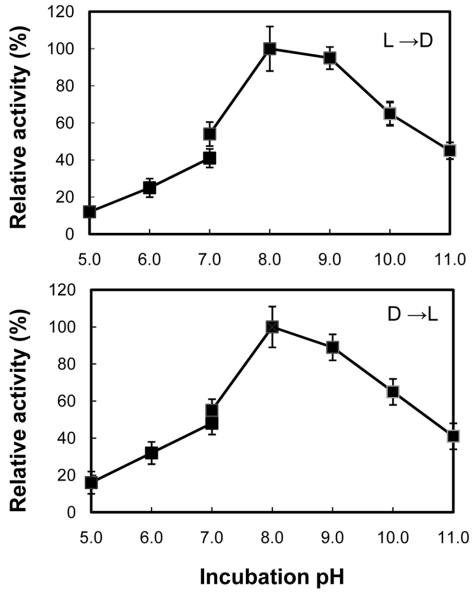

Fig. 5. pH effect on the alanine racemase activity of *X.oryzae*. The reaction mixture consisted of potassium phosphate for pH 5.0–7.0 and Tris-Cl for pH 7.0–11.0. Maximum activities were considered as 100%, equivalent to 12.0 and 10.9 U/mg protein (for L→D and D × L reaction, respectively).

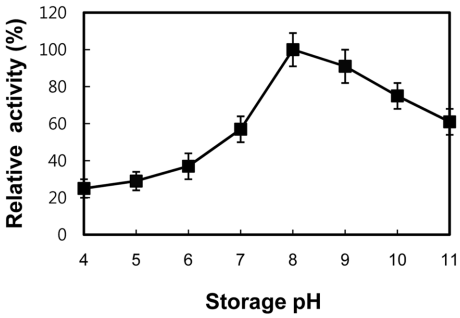

Fig. 6. pH stability of the alanine racemase activity from *X. oryzae*. The alanine racemase was suspended in different buffers showing pH ranges from 4.0 to 11.0; 50 mM citrate phosphate buffer for pH 4.0-5.0, potassium phosphate buffer for pH 6.0–7.0, and Tris-Cl buffer for pH 8.0–11.0. After 4 h incubation, an aliquot was withdrawn and the alanine racemase activity was measured under standard assay condition. Maximum activity at pH 8.0 was considered as 100% activity equivalent to 12.3 U/mg protein. Each point represents the average of three experiments.

the substrate. But the enzyme activity is often inhibited by D- or L-cysteine, more or less. It has been well known that cysteine binds to PLP not only *in vivo* [Buell and Hansen, 1960] but also *in vitro* [Mackay, 1962] without aid of enzymatic reaction. Under the basis of these findings, D- or L-cysteine might bind to PLP of the alanine racemase, thereby appears to inhibit the enzymatic activity. Similar to our result, D- or L-cysteine inhibited the activity of serine racemase from mouse, which is also one of PLP-dependent enzyme [Panizzutti *et al*., 2001]. Cysteine is considered as an analogue of serine in that these amino acids carry an alcohol group. Thus this group could be involved in the binding of alanine racemase.

Optimum pH and pH stability Optimum pH of the alanine racemase was assayed by measuring the enzyme activities which converts L-alanine to D-alanine or *vice versa* (Fig. 5). In the reaction of L-alanine to D-alanine production, optimum pH was observed around pH 8.0. The opposite reaction, D-alanine to L-alanine, was also the most reactive at pH 8.0. In both reactions, the enzyme aivivities gradually decreased at pH above 9.0 or acidic pH. The optimum pH is similar to those of *E. coli*, *B. bifidum, A. aceti,* and *Bacillus pseudofirmus* OF4 [Lambert and Neuhaus, 1972; Yamashita *et al*., 2003; Ju *et al*., 2005; Francois and Kappock, 2007]. pH stability of the alanine racemase was measured at the pH values ranging from pH 4.0 to 11.0. After storage for 4 h at different pH values, pH stability was assessed by measuring the residual activities (Fig. 6). When the enzyme was treated at pH 8.0, the stability appears to be the most satisfactory. The stability of enzyme drastically decreased especially under acidic pH condition, showing only 27% of maximum activity by treatment of pH 5.0. Overally the enzyme appears to be more stable at weak alkaline pH than acidic pH.

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