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Insight into the Substrate Specificity of *Lactobacillus paracasei* Aspartate Ammonia-Lyase

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Abstract: Aspartate ammonia-lyase (AAL) catalyzes the reversible conversion reactions of aspartate to fumaric acid and ammonia. In this work, *Lactobacillus paracasei LpAAL* gene was heterologously expressed in *Escherichia coli*. As well as a recombinant His-tagged LpAAL protein, a maltose-binding protein (MBP) fused LpAAL protein was used to enhance its protein solubility and expression level. Both recombinant proteins showed broad substrate specificity, catalyzing aspartic acid, fumaric acid, phenylalanine, and tyrosine to produce fumaric acid, aspartic acid, *trans*-cinnamic acid, and *p*-coumaric acid, respectively. The optimum reaction pH and temperature of LpAAL protein for four substrates were measured at 8.0 and 40 °C, respectively. The K_m values of LpAAL protein for aspartic acid, fumaric acid, phenylalanine, and tyrosine as substrates were 5.7, 8.5, 4.4, and 1.2 mM, respectively. The k_{cat} values of LpAAL protein for aspartic acid, fumaric acid, phenylalanine, and tyrosine as substrates were 6.7, 0.45, 4.96, and 0.02 s^{-1}, respectively. Therefore, aspartic acid, fumaric acid, phenylalanine, and tyrosine are *bona fide* substrates for LpAAL enzyme.

Keywords: aspartate ammonia-lyase; *Lactobacillus paracasei*; maltose-binding protein; phenylalanine ammonia-lyase; substrate specificity; tyrosine ammonia lyase

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1. Introduction

Aspartate ammonia-lyase (AAL, EC 4.3.1.1), which catalyzes the reversible non-oxidative deamination of L-aspartic acid to fumaric acid (Figure 1), is an important part of the link between amino acid metabolism and organic acid metabolism [1]. AAL enzyme activity is widely present in various microorganisms and has been purified from thermophilic bacteria, *Escherichia coli*, *Pseudomonas fluorescent*, *Bacillus subtilis*, and *Bacillus thermophilus* [2–5]. *Escherichia coli* AAL (eAAL) protein has been studied extensively; the eAAL protein is composed of 477 amino acids with a molecular weight of approximately 52 kDa, which is a tetrameric enzyme consisting of four identical subunits [2,3]. Its crystal structure has been elucidated, and it has been shown that the catalysis reaction must be carried out under Mg^{2+} and alkaline conditions [2,6,7]. The bAAL protein purified from *Bacillus thermophilus* is similar in molecular weight to other AAL proteins from mesophilic microorganisms and is a homotetrameric protein, but shows different biochemical properties with eAAL protein [8]. One of the most obvious features is that the bAAL protein is structurally more stable under high temperature conditions, and the catalytic reaction also requires the participation of Mg^{2+} under alkaline conditions [8]. Previous studies have shown that AAL proteins only accept L-aspartic acid as a substrate to catalyze the formation of fumaric acid and ammonia under deamination reaction, and lack the activity of catalyzing other L-amino acids, other unsaturated acids, or D-aspartic acid [9,10]. In other words, L-aspartic acid is a very specific substrate for AAL enzymes [9,10]. However, it is later shown that the AAL proteins are capable of catalyzing other substrates. The AAL protein purified from *Pseudomonas aeruginosa* not only catalyzes the reverse reaction of

L-aspartic acid to generate fumaric acid and ammonia, but also catalyzes L- phenylalanine to form *trans*-cinnamic acid [11].

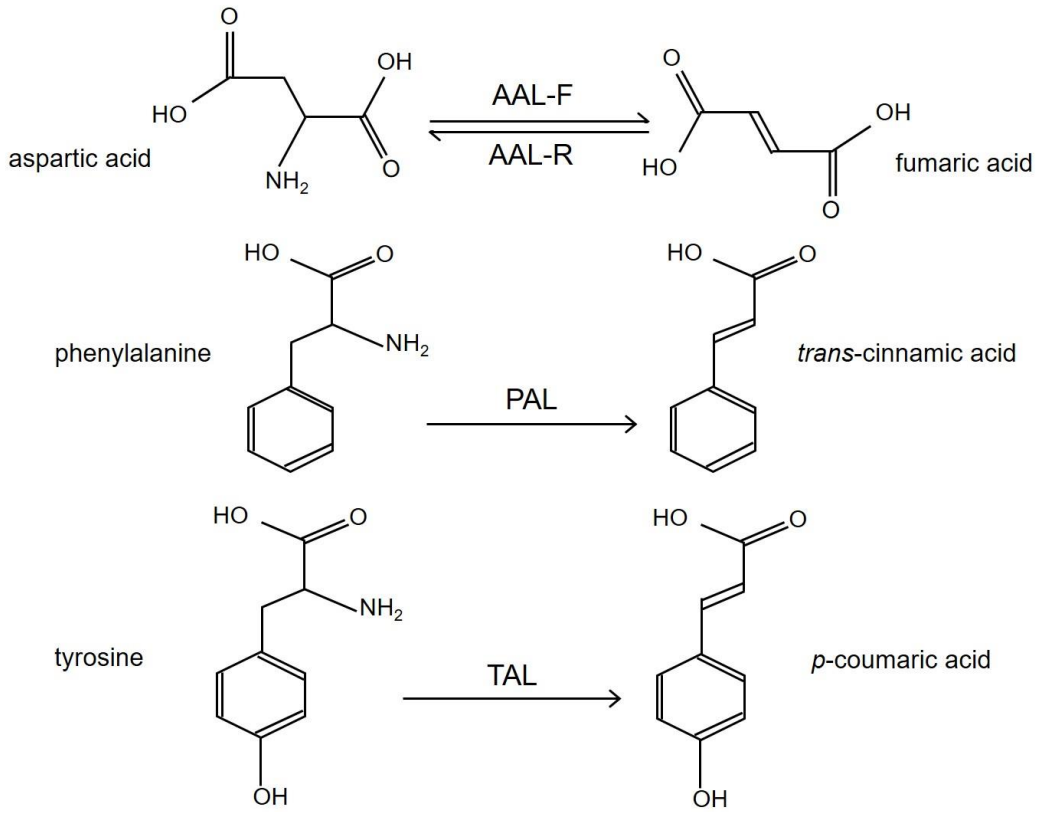

Figure 1. The enzyme reactions catalyzed by the LpAAL protein. AAL-F: aspartate ammonia-lyase forward reaction; AAL-R: aspartate ammonia-lyase reverse reaction; PAL: phenylalanine ammonia-lyase; TAL: tyrosine ammonia-lyase.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) can utilize L-phenylalanine as a substrate to generate *trans*-cinnamic acid and ammonia under non-oxidative deamination reaction [12,13]. Its product, *trans*-cinnamic acid, is then functioned as an important intermediate in the plant phenylpropanoid pathway, being utilized as precursors for thousands of compounds, such as tannins, flavonoids, lignin, etc. [12,13]. PAL proteins from dicot plants are reported to exhibit high specificity to its substrate phenylalanine, whereas, in our previous studies, that monocot plant *Bambusa oldhamii* phenylalanine ammonia-lyase 4 protein (BoPAL4, EC 4.3.1.25) is a dual functional phenylalanine-tyrosine ammonia-lyase (PTAL) enzyme, catalyzing *trans*-cinnamic acid and *p*-coumaric acid production from L-phenylalanine and L-tyrosine via its PAL and tyrosine ammonia-lyase (TAL) activities [13].

Lactobacillus paracasei is a Gram-positive, heterotrophic *Lactobacillus*, distributed in a variety of media, including many fermented dairy products, vegetables, and the human gastrointestinal tract [14,15]. Because of its industrial value and health-promoting potential, *Lactobacillus paracasei* has been extensively studied and used as probiotic [16] and is believed to have various health-promoting properties for humans, including antitumor, anti-inflammatory, and biologics such as microbiological modulation intestinal bacteria [14]. Although AAL protein function was studied in *Lactobacilli* [17,18], our understanding of AAL enzyme in this Genus is still very limited. In addition, *Pseudomonas aeruginosa* AAL protein is reported to have PAL enzymatic activity in 2017 [11], which aroused our interest in the hypothesis that AAL may have similar substrate specificity as bamboo PALs [12,13]. In this study, we further examined and proved that *Lactobacillus paracasei* AAL (LpAAL) has both PAL and TAL activities by using the BoPAL4 protein as a control group to comprehensively study the substrate specificity in the LpAAL protein.

2. Materials and Methods

2.1. Chemicals

L-aspartic acid, fumaric acid, L-phenylalanine, L-tyrosine, *trans*-cinnamic acid, and *p*-coumaric acid were obtained from MilliporeSigma (Burlington, MA, USA). Reagents for protein electrophoresis and molecular biology manipulations were described in previous publications [12,13,19,20].

2.2. Construction of the Expression Vectors

The full-length *LpAAL* gene was chemically synthetic by MDBio, Inc. (New Taipei City, Tai wan) and then subcloned into pET28a plasmid for making pET28a-LpAAL plasmid (Table 1). The *LpAAL* gene fragment was amplified from pET28a-LpAAL plasmid by a PCR reaction and then subcloned into pMAL-c2x plasmid for making a maltose-binding protein fused LpAAL protein (Table 1).

Table 1. Expression plasmids used for recombinant proteins expressions in *Escherichia coli*.

Plasmids	Relevant Characteristics	Source/Reference
pET28a	*E. coli* expression vector with N-terminal His_6-tag fusion	Invitrogen
pET28a-LpAAL	*LpAAL* coding sequence inserted into pET28b	This study
pMAL-c2x	*E. coli* expression vector with N-terminal MBP fusion	New England Biolab
pMAL-LpAAL	*LpAAL* coding sequence inserted into pMAL-c2x	This study
pTrcHis-BoPAL4	*BoPAL4* coding sequence inserted into pTrcHisA	[13]

2.3. E. coli Strains and Protein Expression Conditions

E. coli Top10 strain was used for recombinant BoPAL4 protein expression [13]. *E. coli* DH5α and BL21(DE3) strains were used for plasmid storage and for LpAAL and MBP-LpAAL expression, respectively. *E. coli* BL21(DE3) cells carrying the expression plasmids (Table 1) were grown at 30 °C in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl, and pH 7.0) supplemented with 100 mg/mL ampicillin or 50 mg/mL *kanamycin*. The expression of LpAAL and MBP-LpAAL proteins was induced at 30 °C with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 h. Cells were centrifuged at 6000× *g* for 10 min, and pellets were stored at −20 °C freezer before use.

2.4. Preparations of LpAAL and MBP-LpAAL Enzymes

E. coli cells were re-dissolved with 20 mL of 1× lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM imidazole, 100 mM NaCl, 1 mM PMSF) and disrupted by sonication using a Branson cell disruptor [19,20]. Cell lysates with LpAAL protein or MBP-LpAAL protein were placed in a Ni-NTA column for affinity column chromatography, and finally eluted with buffer containing 250 imidazole.

2.5. SDS-Polyacrylamide Gel Electrophoresis

Purity of target proteins were analyzed by polyacrylamide gel electrophoresis (PAGE) using a Mini-PROTEAN Tetra Cell system (Bio-Rad, Hercules, CA, USA). Protein samples on gels were stained and decolorized with Coomassie Brilliant Blue R-250 and 20% methanol. The image of the gel was captured by a Gel Doc XR+ Imaging System (Bio-Rad, Hercules, CA, USA).

2.6. AAL-F Activity Assay

The aspartate ammonia-lyase forward reaction activity (AAL-F) was assayed by measuring the production of fumaric acid as the absorbance increased at 240 nm [21] using

fumaric acid as the standard. The reaction mixture for the AAL-F assay contained 50 mM Tris−HCl, pH 8.0, 11 mM fumaric acid, and 50 µL of enzyme solution in a total volume of 1.0 mL. The reaction mixture was incubated at 40 °C for 3 min. AAL-F activity unit was defined as nanomole of fumaric acid formatted per minute.

2.7. AAL-R Activity Assay

The aspartate ammonia-lyase reverse reaction activity (AAL-R) was assayed by measuring the production of L-aspartic acid as the absorbance increased at 293 nm [22] using aspartic acid as the standard. The reaction mixture for the AAL-R assay contained 50 mM Tris−HCl, pH 8.0, 40 mM L-aspartic acid, and 50 µL of enzyme solution in a total volume of 1.0 mL. The reaction mixture was incubated at 40 °C for 6 min. AAL-R activity unit was defined as nanomole of aspartic acid formed per minute.

2.8. PAL Activity Assay

The PAL activity was assayed by measuring the production of *trans*-cinnamic acid as the absorbance increased at 290 nm [12] using *trans*-cinnamic acid as the standard. The reaction mixture for the PAL assay contained 50 mM Tris−HCl, pH 8.0, 40 mM L-phenylalanine, and 50 µL of enzyme solution in a total volume of 1.0 mL. The reaction mixture was incubated at 40 °C for 6 min. PAL activity was defined as nanomole of *trans*-cinnamic acid formed per minute.

2.9. TAL Activity Assay

The TAL activity was assayed by measuring the production of *p*-coumaric acid as the absorbance increased at 310 nm [12] using *p*-coumaric acid as the standard. The reaction mixture for the AAL-R assay contained 50 mM Tris−HCl, pH 8.0, 40 mM L-tyrosine, and 50 µL of enzyme solution in a total volume of 1.0 mL. The reaction mixture was incubated at 40 °C for 6 min. TAL activity was defined as nanomole of *p*-coumaric acid formed per minute.

2.10. Enzyme Biochemical Properties and Kinetics

To measure the optimum reaction temperature, activities' assays were conducted at standard reaction condition in a range of temperatures from 30 to 70 °C. To measure the optimum reaction pH, activities' assays were conducted at standard reaction condition in a range of pH from 5.0 to 9.0. To determine the kinetic parameter of AAL-F, AAL-R, PAL, and TAL, a range of concentration of L-aspartic acid, fumaric acid, L-phenylalanine, and L-tyrosine were varied from 0~20 mM, from 0~11 mM, from 0~12 mM, and from 0~2 mM, respectively. K_m and k_{cat} values were calculated from substrate saturation curves [12,13,19,20].

3. Results

3.1. Analysis of the LpAAL Gene

The *LpAAL* gene (GenBank Accession No. EEQ60018) has an open-reading frame (ORF) of 1395 bp and can be deduced into a 464 amino acid or a 50 kDa polypeptide. The three-dimensional structure of the AAL protein monomer subunit is dominated by the α-helix, and, as a homotetramer, it contains a total of four fusion active sites, and each active site consists of three monomer sequences identified to be conserved, in which about 10 rings (SS-loop) are formed by amino acid residues with the characteristic sequence GSSxxPxKxN [2,21,23]. The sequence of the LpAAL SS-loop is G_{308}SSIMPGKVN_{317}.

3.2. Expression and Purification of Recombinant LpAAL and MBP-LpAAL Proteins in Escherichia coli

Initially, full length *LpAAL* gene was inserted in pET28a plasmid for expressing an N-terminal His-tagged LpAAL recombinant protein (Figure 2A). The solubility of recombinant LpAAL protein was low, and most of the LpAAL proteins became inclusion bodies.

This result was similar to the expression of *Entamoeba histolytica* aspartate ammonia-lyase protein [24]. Therefore, we constructed another expression vector for expressing a maltose-binding protein (MBP)-tagged LpAAL with C-terminal His-tag for facilitating purification (Figure 2A) and stepped forward the induction conditions, including reduced induction temperature, prolonged induction time, and decreased IPTG concentration, to overcome the scenario of insoluble LpAAL proteins. The results showed that the recombinant proteins of LpAAL and MBP-LpAAL purified with Ni-resin resin were separated SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining (Figure 2B). Both recombinant proteins, LpAAL and MBP-LpAAL, were migrated to their anticipated positions, and molecular weights were approximately 50 and 92.5 kDa, respectively. Therefore, *E. coli* expression system is capable of synthesizing LpAAL and MBP-LpAAL recombinant proteins [25].

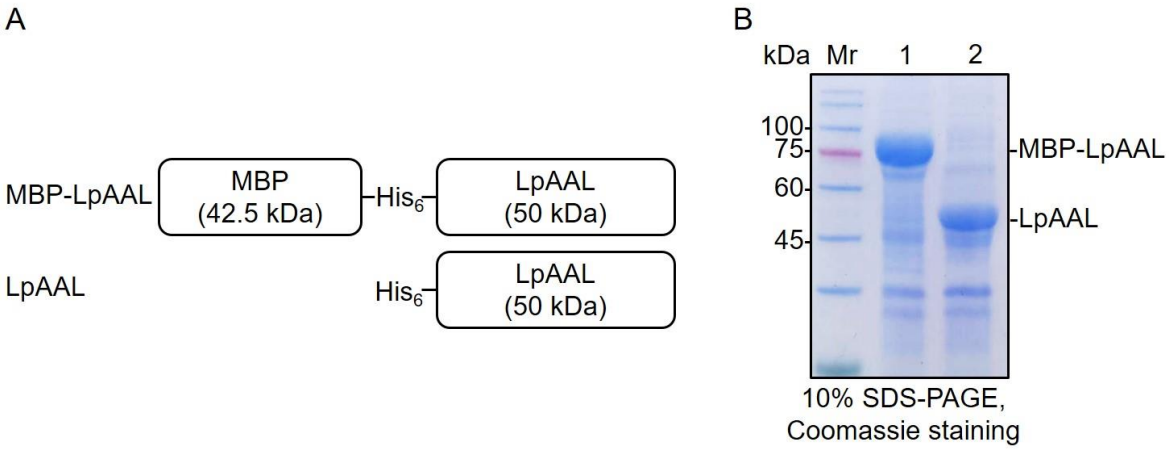

Figure 2. Preparation of recombinant MBP-LpAAL and LpAAL proteins. (**A**) LpAAL protein and MBP-LpAAL fusion protein were expressed in *Escherichia coli*. (**B**) Recombinant proteins were purified using Ni-NTA affinity column and separated using 10% SDS–PAGE, and then stained with Coomassie Blue dye. Mr, molecular weight SDS–PAGE marker; lane 1, MBP-LpAAL; lane 2, LpAAL. MBP: maltose-binding protein.

3.3. Optimum Temperature of AAL and AAL-R Activities of LpAAL and MBP-LpAAL Proteins

A previous study had shown that two different AAL proteins purified from *Aeromonas medium* and *Pseudomonas aeruginosa* had optimal reaction temperatures of 35 °C and 40 °C [7,11]. Singh and Yadav further showed that AAL protein was more stable between 25 and 40 °C, after which it may lose AAL activity, presumably due to denaturation at higher temperatures [7,11]. Recombinant LpAAL and MBP-LpAAL proteins purified from *E. coli* were both active enzymes. Our results showed that the optimal reaction temperatures of AAL-F and AAL-R activities of LpAAL enzyme were both 40 °C (Figure 3A), while the optimal reaction temperatures of AAL-F and AAL-R activities of MBP-LpAAL enzyme are 45 °C and 35 °C, respectively (Figure 3B). Taken together, the optimal reaction temperature of LpAAL and MBP-LpAAL recombinant proteins were comparable with other microbial AAL proteins.

3.4. Optimum pH for AAL-F and AAL-R Activities of LpAAL and MBP-LpAAL Proteins

Normally, AAL enzymes are used to react in alkaline conditions, where the optimum pH is between 7.0 and 9.0 [23]. The optimal pH of the AAL enzyme purified from *Escherichia coli* and *Pseudomonas aeruginosa* were 8.0 and 8.5, respectively [11,21]. Our result showed that the optimal reaction pH of both AAL-F and AAL-R activities of LpAAL enzyme was 8.0 (Figure 4A), while the optimal pHs of AAL-F and AAL-R activities of MBP-LpAAL enzyme were 8.5 and 8.0, respectively (Figure 4B). As a result, the optimal reaction pH of LpAAL and MBP-LpAAL proteins was comparable with other microbial AAL proteins.

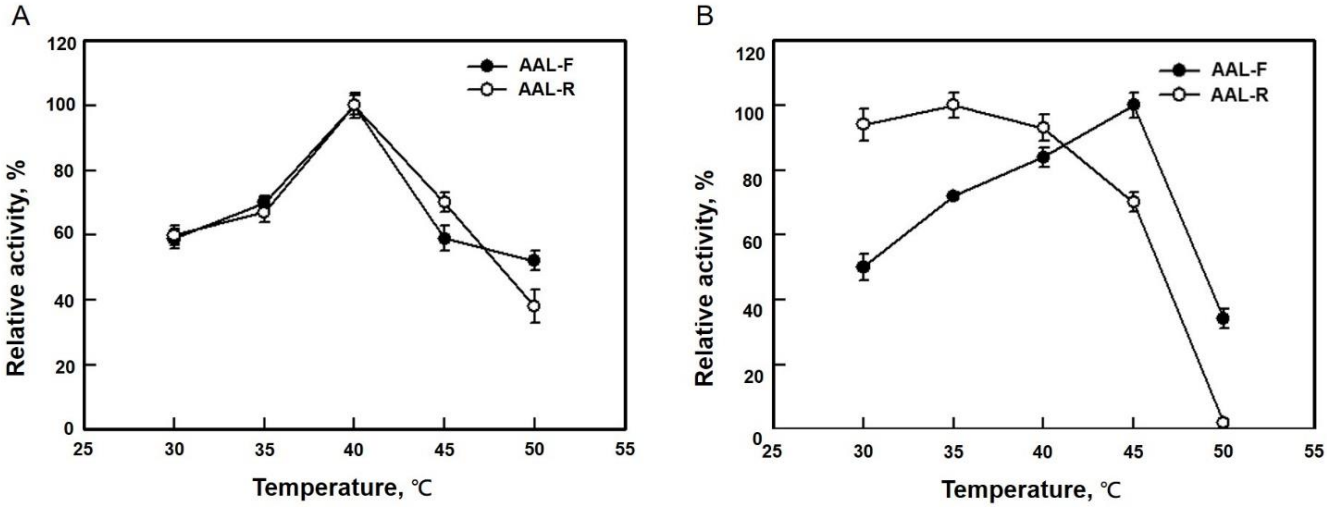

Figure 3. Optimum reaction temperature of AAL-F and AAL-R activities of LpAAL (**A**) and MBP-LpAAL (**B**) enzymes. Activities were measured under standard assay conditions in a range of temperatures from 30 to 50 °C. All experiments were performed in triplicate and expressed as average ± standard deviation (S.D., error bars). The average of maximum activity was normalized as 100%.

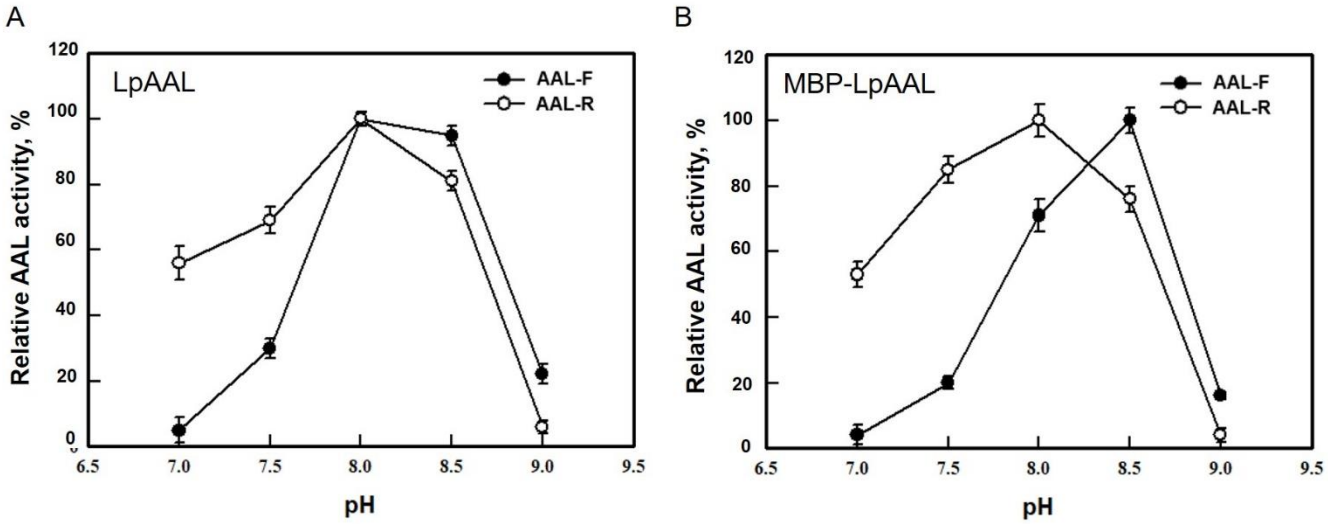

Figure 4. Optimum reaction pH of AAL-F and AAL-R activities of LpAAL (**A**) and MBP-LpAAL (**B**) enzymes. Activities were measured under standard assay conditions in a range of pH from 7.0 to 9.0. All experiments were performed in triplicate and expressed as average ± standard deviation (S.D., error bars). The average of maximum activity was normalized as 100%.

3.5. Kinetic Parameters for AAL-F and AAL-R Activities of LpAAL and MBP-LpAAL Proteins

The kinetics parameters of the LpAAL and MBP-LpAAL enzymes were studied and the K_m values of the enzymes were calculated using saturation curves (Figure 5) and the Michaelis–Menten equation. After calculation, the result showed that the K_m values of LpAAL and MBP-LpAAL proteins using aspartic acid as a substrate were 5.7 (Figure 5A) and 5.2 mM (Figure 5B) at pH 8.0, respectively, and were similar to that of *Pseudomonas fluorescens* AAL (K_m = 5.1 mM) [21]. The K_m values of LpAAL and MBP-LpAAL proteins using fumaric acid as a substrate were both 8.50 mM at pH 8.0 (Figure 5C,D), which is significantly different from the study showing the K_m value of the *Pseudomonas aeruginosa* AAL enzyme using fumaric acid as a substrate to be 21,234 mM [11].

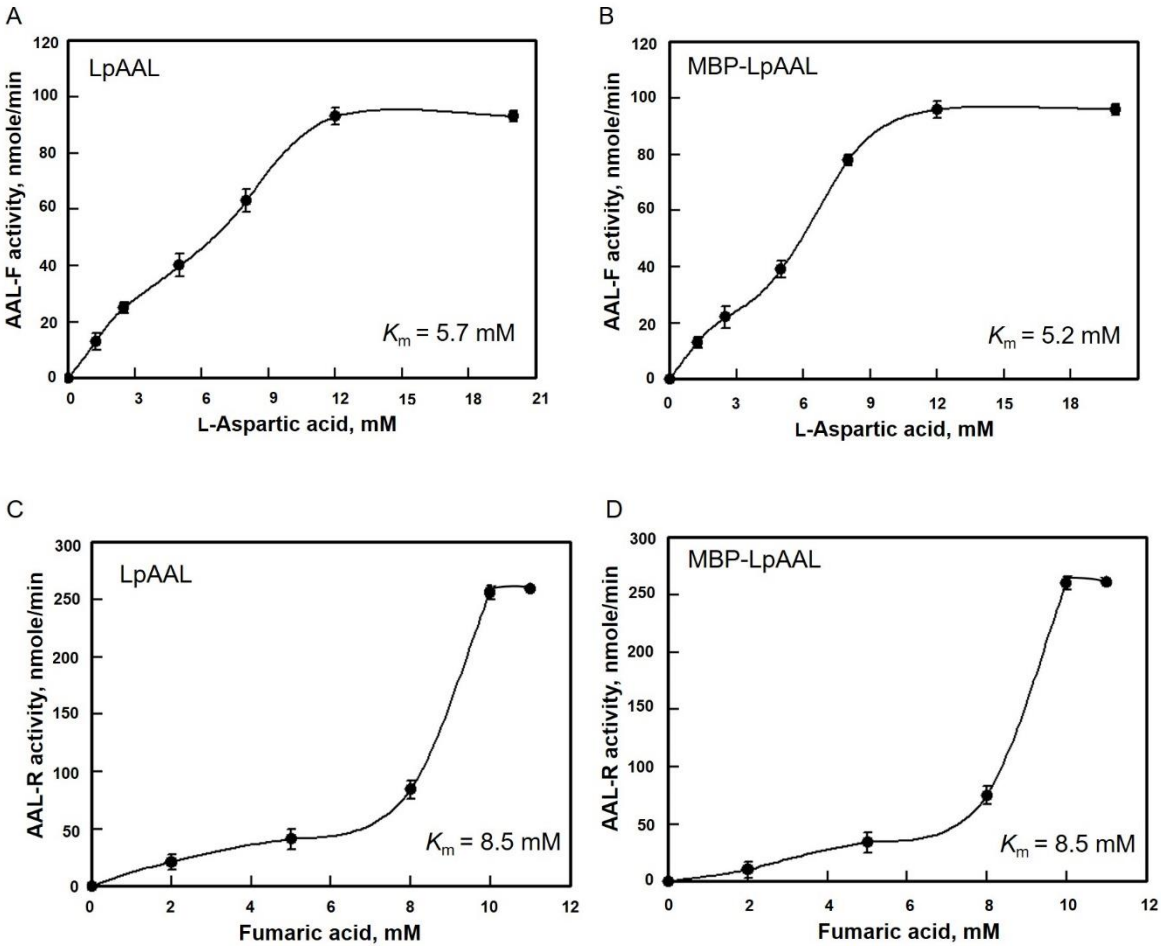

Figure 5. Kinetic parameters of LpAAL and MBP-LpAAL with L-aspartic acid and fumaric acid as substrate. (**A**), to determine LpAAL kinetic parameters using L-aspartic acid as substrate, substrate saturation curve. (**B**), to determine MBP-LpAAL kinetic parameters using L-aspartic acid as substrate, substrate saturation curve. (**C**), to determine LpAAL kinetic parameters using fumaric acid as substrate, substrate saturation curve. (**D**), to determine MBP-LpAAL kinetic parameters using fumaric acid as substrate, substrate saturation curve. All experiments were performed in triplicate and expressed as average ± standard deviation (S.D., error bars). The average of maximum activity was normalized as 100%.

The V_{max} values were obtained from double reciprocal plots derived from substrate saturation curve (Figure 5, Table 1). The V_{max} values were divided by protein amounts (nmole) to obtain the k_{cat} values. The k_{cat} values of LpAAL for L-aspartic acid and fumaric acid were estimated as 6.70 and 0.45 s^{-1}, respectively. Moreover, the k_{cat} values of MBP-LpAAL for L-aspartic acid and fumaric acid were estimated as 13.1 and 37.6 s^{-1}, respectively (Table 2). In conclusion, both recombinant LpAAL and MBP-LpAAL proteins were undoubtedly functional AAL enzymes, and LpAAL and MBP-LpAAL enzyme activities were comparable.

Table 2. Comparison of biochemical properties and kinetic parameters of AAL activities in various AAL proteins.

Enzyme	Substrate	Specific Activity (U/mg)	Optimum pH	Optimum Temp (°C)	V_{max} (nmole/min)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} / K_m (s^{-1} mM)	Ref.
LpAAL	L-Asp	7.7×10^7	8.0	40	93	6.7	5.70	1.18	This study
	fumaric acid	6.09	8.0	40	257	0.45	8.50	0.05	

Table 2. *Cont.*

Enzyme	Substrate	Specific Activity (U/mg)	Optimum pH	Optimum Temp (°C)	V_{max} (nmole/min)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} / K_m (s^{-1} mM)	Ref.
MBP-LpAAL	L-Asp	8.3×10^7	8.5	45	97	13.1	5.20	5.20	This study
	fumaric acid	5.73	8.0	35	267	37.64	8.50	8.5	
pAAL [1]	L-Asp	—	—	—	—	4.91	3×10^{-4}	—	[11]
BAAL [2]	L-Asp	—	—	—	287 (mM/h)	—	213	—	[26]
pfAAL [3]	L-Asp	—	8.8	—	—	130	5.1	25	[21]

[1] pAAL: Pseudomonas aeruginosa PAO1; [2] BAAL: Bacillus sp. YM55-1; [3] pfAAL: Pseudomonas fluorescens R124.

3.6. Optimum Temperature and pH between PAL and TAL for LpAAL and MBP-LpAAL

Recently, Patel et al. reported that *Pseudomonas aeruginosa* AAL protein can utilize phenylalanine as a substrate to yield cinnamic acid [11]. To investigate whether LpAAL/MBP-LpAAL proteins can also use L-phenylalanine or can further use L-tyrosine as a substrate, the optimum temperature of PAL and TAL activities were determined to between 30~50 °C. The optimal PAL and TAL activities of LpAAL protein were both at 40 °C (Figure 6A), similar to the optimal temperature of MBP-LpAAL (Figure 6B). However, the optimal temperatures of BoPAL4 protein for PAL (50 °C) and TAL activities (60 °C) were significantly higher than that of LpAAL and MBP-LpAAL proteins (Figure 6A,B).

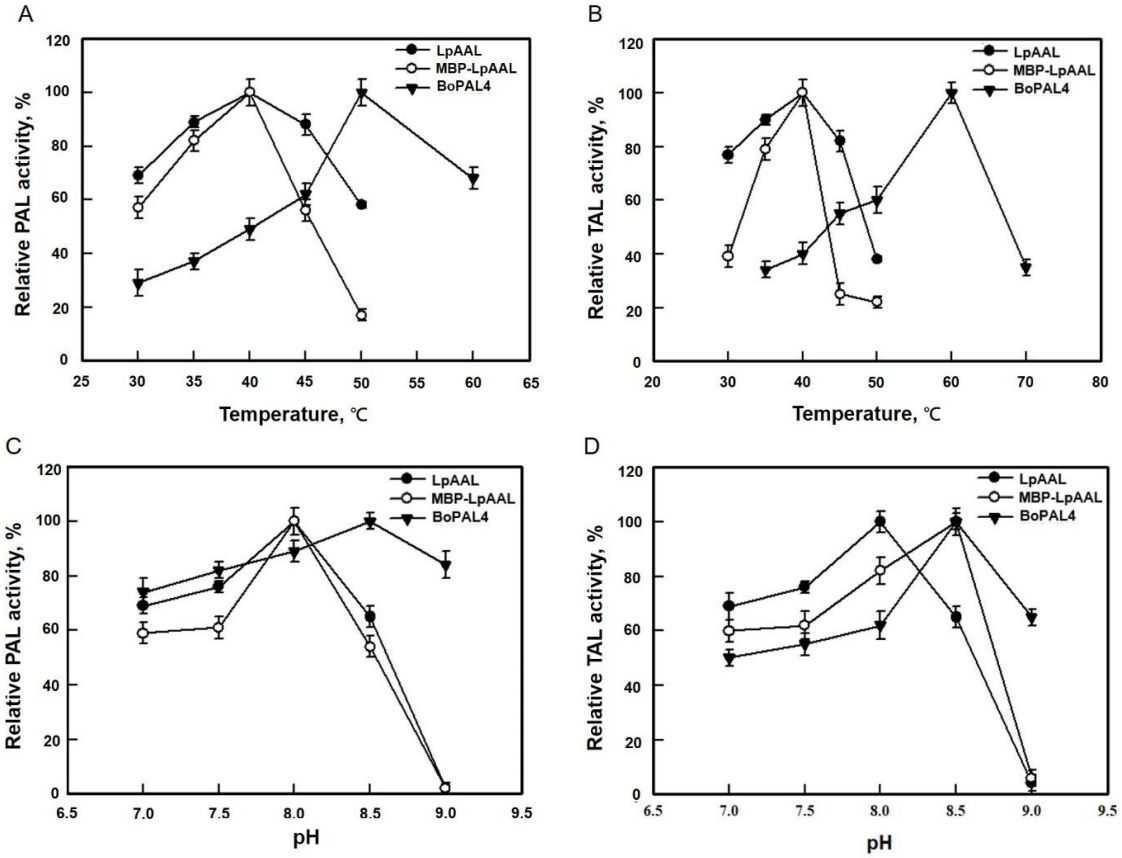

Figure 6. Optimum reaction temperature and pH of PAL and TAL activities of LpAAL, MBP-LpAAL and BoPAL4 enzymes. (**A**) PAL activities were measured under standard assay conditions in a range

of temperatures from 30 to 60 °C. (**B**) TAL activities were measured under standard assay conditions in a range of temperatures from 30 to 60 °C. (**C**) PAL activities were measured under standard assay conditions in a range of pH from 7.0 to 9.0. (**D**) TAL activities were measured under standard assay conditions in a range of pH from 7.0 to 9.0. All experiments were performed in triplicate and expressed as average ± standard deviation (S.D., error bars). The average of maximum activity was normalized as 100%.

Accordingly, the optimum pH for PAL and TAL activities were determined between 7.0 and 9.0, and the optimum pH for PAL and TAL activities of LpAAL protein were both 8.0 (Figure 6C), which is similar to the optimum pH for MBP-LpAAL protein (Figure 6D). In addition, the optimum pH of BoPAL4 protein for both PAL and TAL activities was 8.5, which was comparable with that of LpAAL and MBP-LpAAL proteins (Figure 6C,D). LpAAL and MBP-LpAAL proteins exhibited better PAL/TAL relative activities at lower temperature and pH than that of BoPAL4 protein. Taken together, LpAAL and MBP-LpAAL proteins were able to produce cinnamic acid and *p*-coumaric acid through their PAL and TAL activities.

3.7. Kinetics Parameters for LpAAL and MBP-LpAAL with L-Phenylalanine and L-Tyrosine as Substrates

The K_m of LpAAL protein with L-phenylalanine and L-tyrosine as substrates were 4.43 and 1.17 mM, respectively, and the K_m of MBP-LpAAL protein of L-phenylalanine and L-tyrosine as substrates were 4.07 and 1.10 mM, respectively (Table 3). This was not similar to the result previously reported by Patel et al. with a K_m value with L-phenylalanine of 1.7 mM for AAL [11]. In addition, the k_{cat} of LpAAL enzyme with L-phenylalanine and L-tyrosine were 0.45 and 0.02 s^{-1} respectively, and the k_{cat} of MBP-LpAAL enzyme based on L-phenylalanine and L-tyrosine were 0.84, 0.04, and s^{-1}, respectively. By comparing with the kinetic parameters of BoPAL4 protein, LpAAL and MBP-LpAAL proteins were truly TAL and PAL enzymes in spite of showing lower catalytic capability than that of BoPAL4 protein (Table 3).

Table 3. Comparison of biochemical properties and kinetic parameters of PAL and TAL activities in LpAAL, MBP-LpAAL, and BoPAL4 proteins.

Proteins	Substrate	Optimum pH	Optimum Temp (°C)	k_{cat} (s^{-1})	K_m (mM)	Ref.
LpAAL	L-Phe	8.0	40	0.45	4.43	This study
	L-Tyr	8.0	40	0.02	1.17	
MBP-LpAAL	L-Phe	8.0	40	0.84	4.07	This study
	L-Tyr	8.5	40	0.04	1.10	
BoPAL4	L-Phe	9.0	50	1.14	2.10	[13]
	L-Tyr	8.5	60	0.18	0.10	

3.8. Comparison of Specific Activities in LpAAL, MBP-LpAAL, and BoPAL4 Proteins

Besides enzyme kinetic parameters, the specific activities of AAL-F, AAL-R, PAL, and TAL were compared (Figure 7). The specific AAL-F activity of LpAAL protein was similar to that of MBP-LpAAL protein (Figure 7A). The specific AAL-F activities of both recombinant proteins were slightly higher (1.3-fold) than that of the specific AAL-R activities (Figure 7A). Accordingly, BoPAL4 protein was used as control to evaluate the PAL and TAL activities of LpAAL and MBP-LpAAL proteins. The results showed that the PAL (Figure 7B) and TAL (Figure 7C) activities of LpAAL and MBP-LpAAL proteins were about one-half and one-sixth of BoPAL4 protein. Although the PAL and TAL activities of LpAAL and MBP-LpAAL proteins were lower than that of BoPAL4, phenylalanine and tyrosine were *bona fide* substrates for both LpAAL and MBP-LpAAL proteins.

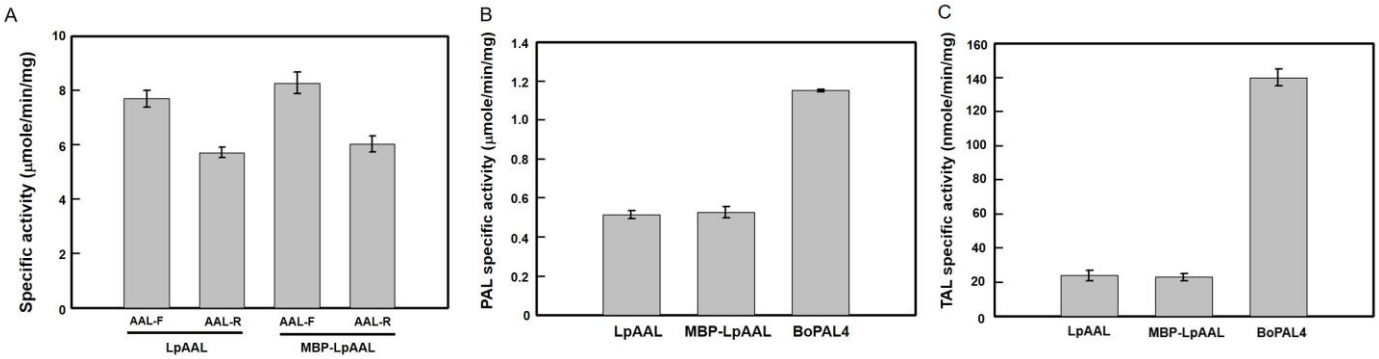

Figure 7. Comparison of specific activities in LpAAL, MBP-LpAAL, and BoPAL4. (**A**) Specific AAL-F and AAL-R activities in LpAAL and MBP-LpAAL were measured under standard assay condition. (**B**) Specific PAL activities in LpAAL, MBP-LpAAL and BoPAL4 were measured under standard assay condition. (**C**) Specific TAL activities in LpAAL, MBP-LpAAL and BoPAL4 were measured under standard assay condition. All experiments were performed in triplicate and expressed as average ± standard deviation (S.D., error bars).

4. Discussion

In this study, we proved that *Lactobacillus paracasei* aspartate ammonia-lyase, LpAAL, has broad substrate specificity and can be used to synthesize at least four products from four substrates. LpAAL protein is a multifunctional enzyme that is active under alkaline and mesophilic conditions, catalyzing the nonoxidative deamination of L-aspartic acid, fumaric acid, L-phenylalanine, and L-tyrosine to yield fumaric acid, L-aspartic acid, *trans*-cinnamic acid, and *p*-coumaric acid. Recombinant LpAAL expressed in *E. coli* was prone to form inclusion bodies, and MBP can significantly increase its solubility without affecting enzymatic activities. In Figure 7A, when the same protein amount of LpAAL and MBP-LpAAL enzymes were subjected to the same reaction, there was no significant difference in the specific activity of both proteins. The lower mole ratio of MBP-LpAAL protein compared to LpAAL could catalyze substrates more efficiently, presumably due to MBP can stabilize LpAAL protein structure. Most of the AAL enzymes were highly specific for using aspartic acid as the substrate, but a few other substrate-catalyzing cases were also observed, including an AAL protein from *Pseudomonas aeruginosa* with PAL activity [11,21]. In this study, LpAAL and MBP-LpAAL proteins were comprehensively elevated to have four enzyme activities, namely AAL-F, AAL-R, PAL, and TAL activities. To our knowledge, our result is the first finding that AAL enzyme can also function as TAL enzyme using a dual function PAL and TAL enzyme, BoPAL4, as the control protein. In the future, other putative aromatic substrates can be tested to figure out if they could be utilized as substrate by LpAAL protein.

Fumaric acid can be used as a medicine for skin diseases [27,28]; L-aspartic acid is used as a raw material for the production of aspartame [29]; *trans*-cinnamic acid has the protective effects in the nervous system [30] and cardiovascular system [31]; and *p*-coumaric acid exhibits antimicrobial and anti-inflammatory effects [32]. Immobilization of microbial AAL proteins has been applied for L-aspartate synthesis [21,26]. All four metabolites of LpAAL enzyme are applied in food, cosmetic, and medical industries. Therefore, LpAAL protein can also be immobilized on electrospun nanofibers to further characterize its recyclability, storage, and kinetic properties.

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Abbreviations

AAL	aspartate ammonia-lyase
AAL-F	aspartate ammonia-lyase forward reaction
AAL-R	aspartate ammonia-lyase reverse reaction
bAAL	B: *Bacillus* sp. YM55-1 aspartate ammonia-lyase
BoPAL4	*Bambusa oldhamii* phenylalanine ammonia-lyase 4
eAAL	e: *E. coli* aspartate ammonia-lyase
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
LpAAL	Lp: *Lactobacillus paracasei* aspartate ammonia-lyase
MBP	maltose-binding protein
pAAL	p: *Pseudomonas aeruginosa* PAO1 aspartate ammonia-lyase
PAL	phenylalanine ammonia-lyase
pfAAL	pf: *Pseudomonas fluorescens* R124 aspartate ammonia-lyase
PTAL	phenylalanine-tyrosine ammonia-lyase
TAL	tyrosine ammonia-lyase

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