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ORIGINAL ARTICLE

midD-encoded 'rhizomimosinase' from *Rhizobium* sp. strain TAL1145 is a C–N lyase that catabolizes L-mimosine into 3-hydroxy-4-pyridone, pyruvate and ammonia

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Abstract *Rhizobium* sp. strain TAL1145 catabolizes mimosine, which is a toxic non-protein amino acid present in *Leucaena leucocephala* (leucaena). The objective of this investigation was to study the biochemical and catalytic properties of the enzyme encoded by *midD*, one of the TAL1145 genes involved in mimosine degradation. The *midD*-encoded enzyme, MidD, was expressed in *Escherichia coli*, purified and used for biochemical and catalytic studies using mimosine as the substrate. The reaction products in the enzyme assay were analyzed by HPLC and mass spectrometry. MidD has a molecular mass of ~45 kDa and its catalytic activity was found to be optimal at 37 °C and pH 8.5. The major product formed in the reaction had the same retention time as that of synthetic 3-hydroxy-4-pyridone (3H4P). It was confirmed to be 3H4P by MS/MS analysis of the HPLC-purified product. The K_m, V_{max} and K_{cat} of MidD were 1.27×10^{-4} mol, 4.96×10^{-5} mol s^{-1} mg^{-1}, and 2,256.05 s^{-1}, respectively. Although MidD has sequence similarities with aminotransferases, it is not an aminotransferase because it does not require a keto acid as the co-substrate in the degradation reaction. It is a pyridoxal-5′-phosphate (PLP)-dependent enzyme and the addition of 50 μM hydroxylamine completely inhibited the reaction. However, the supplementation of the reaction with 0.1 μM PLP restored the catalytic activity of MidD in the reaction containing 50 μM hydroxylamine. The catalytic activity of MidD was found to be specific to mimosine, and the presence of its structural analogs including L-tyrosine, L-tryptophan and L-phenylalanine did not show any competitive inhibition. In addition to 3H4P, we also identified pyruvate and ammonia as other degradation products in equimolar quantities of the substrate used. The degradation of mimosine into a ring compound, 3H4P with the release of ammonia indicates that MidD of *Rhizobium* sp. strain TAL1145 is a C–N lyase.

Keywords MidD · *Rhizobium* · *Leucaena leucocephala* · Mimosine · 3-Hydroxy-4-pyridone · Rhizomimosinase · C–N lyase

Abbreviations

HPLC	High-performance liquid chromatography
PLP	Pyridoxal-5′-phosphate
3H4P	3-Hydroxy-4-pyridone
3,4-DHP	3,4-Dihydroxypyridine
2,3-DHP	2,3-Dihydroxypyridine
α-KG	α-Ketoglutarate
aq	Aqueous

Introduction

Leucaena leucocephala (leucaena) is a tropical tree-legume, which is considered as promising forage for livestock because its foliages are rich in protein, fiber, and minerals (Garcia et al. 1996). Although its foliages contain up to 18 % protein, they contain a toxic non-protein amino acid, mimosine, which limits the use of leucaena as a livestock feed (Soedarjo and Borthakur 1996). The mimosine content of leucaena foliage can be as high as 3 %

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on the dry weight basis. Mimosine is toxic because it chelates several bivalent metallic ions (Tang and Ling 1975) and also because it forms a stable complex with pyridoxal-5′-phosphate (PLP) (Lin et al. 1962). It inactivates a wide variety of enzymes by limiting their metallic-ion cofactors and PLP coenzyme. Mimosine toxicity in animals causes various physiological abnormalities, including enlarged thyroid glands (Hamilton et al. 1968), infertility (Joshi 1968), fetal deformities with uterine perforation (Dewreede and Wayman 1970), and hair loss (Crounse et al. 1962).

The toxic effects of mimosine on animals limit the use of leucaena as a nutritious animal feed, and therefore, it is important to develop leucaena plants with reduced mimosine content. Identification of efficient mimosine-catabolizing enzyme will be useful for developing leucaena plants with reduced mimosine content. Previously, seedling extract from *Mimosa pudica* was reported to degrade mimosine into serine (Suda 1960). Later on a C–N lyase enzyme was identified from leucaena seedling extract that converts mimosine into 3,4-dihydroxypyridone (3,4-DHP), pyruvic acid and ammonia (Smith and Fowden 1966). Some ruminants feeding on leucaena have been reported to contain microbes that degrade mimosine into 3H4P or 2,3-dihydroxypyridine (2,3-DHP) (Hegarty et al. 1979).

Leucaena forms nitrogen-fixing symbiosis with some *Rhizobium* strains including TAL1145 that can overcome mimosine toxicity by degrading it, and using the degradation products as a source of nutrients (Soedarjo et al. 1994). Complementation of non-mimosine-degrading strains, such as TAL182 and CIAT899 with a TAL1145 cosmid clone, pUHR181, containing five *mid* genes (*midABCD* and *midR*) resulted in the degradation of mimosine in the culture (Borthakur and Soedarjo 1999). The deduced amino acid sequences of *midA*, *midB* and *midC* were found to have homologies with substrate-binding periplasmic proteins, permeases and ATP-binding proteins, respectively of bacterial ABC transporters; therefore, these three genes may be involved in transport of mimosine to *Rhizobium*. The gene *midR* has homology with transcriptional activator proteins and has been shown to be involved in transcription of *mid* genes. The deduced amino acid sequence of *midD* was found to have high homology with various fold type-I PLP-dependent enzymes, including aminotransferases (up to 54 % homology) and C–S lyase (up to 50 % homology). Based on the higher homology with aminotransferases, the *midD*-encoded enzyme was previously suggested to be an aminotransferase involved in removal of the alanyl side chain of mimosine (Borthakur et al. 2003). However, there was no experimental evidence showing the catalytic action of the *midD*-encoded enzyme in mimosine catabolism. In addition, the homology of *midD* with both aminotransferase and C–S lyase raises the question if the *midD*-encoded protein is an aminotransferase or a lyase. An enzyme from leucaena seedling extract that has the ability to degrade mimosine was identified as a C–N lyase that degrades mimosine by breaking the C–N bond of mimosine (Fowden 1964). This finding along with the homology of *midD*-encoded enzyme with a C–S lyase, gives us a clue that the *midD*-encoded may be a C–N lyase instead of an aminotransferase. Therefore, the objectives of this study are to determine the catalytic role of the *midD*-encoded enzyme in mimosine catabolism, to identify whether the enzyme is an aminotransferase or a C–N lyase, and to determine its other biochemical properties.

Materials and methods

Construction of *midD*-expression plasmid

The bacterial strains, plasmids, and primers used in this study are described in Table 1. *Bam*HI restriction site was incorporated into *midD*-specific forward and reverse primers (*midD*-f, and *midD*-r). These primers were used to amplify the 1221-bp *midD* open reading frame (ORF) by PCR from the cosmid clone pUHR263, using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, MA, USA). The *midD* ORF with *Bam*HI restriction sites at the two ends was cloned into the *Bam*HI site of the expression vector pET-14b (Novagen, WI, USA) under the control of a T7 promoter, and introduced into *E. coli* JM109 (Promega, WI, USA). The resultant plasmid was verified by sequencing and named as pET-*midD*. Another plasmid, named pET-anti-*midD*, was also constructed by cloning *midD* in the antisense direction from the T7 promoter in pET14b.

Bacterial expression and purification of MidD

The expression constructs were transformed into *E. coli* strain BL21(DE3) pLysS (Promega). The transformed BL21 cells containing pET-*midD* were grown in LB and induced for the expression of *midD* by adding isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich, MO, USA) into the LB broth medium at mid-log phase ($OD_{600} = 0.6$) to a final concentration of 1 mM and incubating with shaking at 210 rpm at 37 °C for 1–10 h. To determine the optimum induction time, 200 µL induced cultures were collected at 1, 2, 4, 6, 8 and 10 h of induction. A non-induced culture of BL21(DE3)pLysS containing pET-*midD* and an induced culture of BL21(DE3)pLysS containing pET-anti-*midD* were used as negative controls. The induced and non-induced cultures were harvested by centrifugation at 4000 rpm for 10 min at 4 °C. To make total soluble proteins, cell pellets of harvested samples

Table 1 Bacterial strains, plasmids, and primers

Bacterial strains/ plasmids/primers	Description or sequence (5′–3′)	Source/reference
E. coli strains		
JM109	Host strain used for plasmid transformation	Promega
BL21(DE3)pLysS	Host strain used for expression of *midD*	Promega
Plasmids		
pUHR263	Cosmid clone isolated from the genomic library of *Rhizobium* sp. strain TAL1145, contains *mid* genes	(Fox and Borthakur 2001)
pET-14b	Protein expression vector with a T7 promoter and an N-terminal His-tag sequence	Novagen
pET-*midD*	pET-14b vector with an in-frame *midD* ORF in sense orientation at its *Bam*HI site	This study
pET-anti-*midD*	pET-14b vector with *midD* ORF in antisense orientation at its *Bam*HI site	This study
Primers		
midD-f	CCTAA*GGATCC*G**ATGCACGATTTGCACC**[a,b]	This study
midD-r	ACTTT*GGATCC*AT**TCATCCTTCGTGGCTGGACAA**[a,b]	This study
T7-P	TAATACGACTCACTATAGGG	This study
T7-T	GGGTTATGCTAGTTATTGCT	This study

[a] Italicized texts indicate the *Bam*HI restriction site

[b] Bold texts in primer sequence show the homologous sequence to *midD* ORF

were re-suspended in a SDS-PAGE loading buffer followed by heating at 95 °C for 5 min. MidD protein was purified from the cell pellets of the induced samples of *E. coli* containing *midD* using the MagneHis^{TM} Protein Purification System (Promega) according to the manufacturer's instructions. Total soluble proteins and purified recombinant protein were analyzed through SDS-PAGE.

Mimosine degradation assay

In vitro enzymatic assay was performed to study the effect of MidD on mimosine catabolism by incubating the purified MidD enzyme with 1 mM mimosine as the substrate in 0.1 M Tris–HCl. The final pH of the reaction mixtures was set at 7.5. To test whether MidD is an aminotransferase or not, we carried out the enzymatic reactions in the presence and absence of 20 μM PLP and 1 mM α-ketoglutarate (α-KG). PLP and α-KG are the key requirements for the aminotransferases and are used as coenzyme and cosubstrate, respectively. For each 1,000 μL reaction, 0.016 mg of the purified enzyme was used and the reaction was incubated at 37 °C for 1 h unless otherwise stated. Each reaction type was performed in three replications and the reaction mixtures were analyzed for the amount of the product formed through high-performance liquid chromatography (HPLC) using a C_{18} column (4.6 × 250 mm; Dionex acclaim 120). To determine whether MidD enzyme is PLP-dependent or not, enzymatic assay was performed by supplementing the reaction buffer with hydroxylamine, an inhibitor of PLP-dependent enzymes. The hydroxylamine was added at a concentration range of 0.01–50 mM to the reaction mixture containing the enzyme and incubated for 5 min at room temperature followed by addition of the substrate. In a separate set of reactions, the reaction mixture containing 50 μM hydroxylamine and the enzyme were supplemented with 0.1–20 μM PLP after 5 min of incubation to restore the enzyme activity if it was inhibited by hydroxylamine. The concentrations of inhibitor and cofactor followed a similar trend as used by El-Sayed (2011). For quantitative estimation of the product formed, different concentrations of chemically synthesized 3-hydroxy-4-pyridone (synthetic 3H4P) were used as standards. The synthetic 3H4P was kindly provided by Dr. Behrman, Ohio State University, Columbus, OH. An isocratic solvent system of 0.02 M o-phosphoric acid with a flow rate of 1 mL min^{-1}, and an UV detection photodiode array (200–400 nm) were used for HPLC analysis. The peak area of the product (3H4P) formed was obtained from HPLC chromatogram of the reactions and the amount of product formed was quantified from the slopes of standard curve formed by plotting the peak area of known concentrations of synthetic 3H4P.

Characterization of *midD*-encoded enzyme

The characterization of *midD*-encoded enzyme was performed at 37 °C in 0.1 M Tris–HCl buffer for 1 h unless otherwise stated. For the termination of MidD-catalyzed reactions, the enzyme was inactivated by heating the reaction mixture at 100 °C for 3 min. The optimum pH of the enzyme was studied by conducting mimosine degradation assay at different pHs ranging from pH 3 to 12. Similarly, the optimum temperature for the enzyme was studied by conducting the mimosine degradation assay at

different temperatures including 4, 22, 30, 37, 45, 55, and 65 °C. The subsequent enzymatic assays were performed at the optimum pH and temperature unless otherwise stated. The enzyme was also studied for its thermal stability by pre-incubating the enzyme reaction mixture in 0.1 M Tris–HCl buffer lacking mimosine at different temperatures (4, 37, 40, 50, 55, 60, 65, and 70 °C) for 30 min before performing the actual mimosine degradation assay. The reaction mixtures in the enzyme characterization study were analyzed through HPLC for quantification of the mimosine degradation product. The aromatic amino acids L-tyrosine, L-tryptophan, and L-phenylalanine, which are structural analogs of mimosine, were tested as possible competitive inhibitors of MidD in mimosine degradation. Each aromatic amino acid was mixed with mimosine in the ratios of 1:1, 2:1, and 3:1 and the resultant mixtures were studied for mimosine degradation using MidD enzyme, and the product formed was quantified using HPLC.

Kinetic study of *midD*-encoded enzyme

All experiments to study kinetic properties of MidD were performed at 37 °C and an optimum pH of 8.5 (see results). The rate of reaction for different substrate concentrations (0.05, 0.10, 0.15, 0.25, 0.35, 0.50 mM) was calculated at 0, 2, 3, 5, 10, 20 and 30 min. The initial velocities of the enzyme catalysis were measured as slopes formed by quantifying the amount of product formed at 0 and 2 min of reaction time as the reaction was linear between 0 and 2 min. The values of initial velocities and substrate concentrations were then used to determine kinetic constants V_{max}, and K_m using Lineweaver–Burk plot according to the standard procedure. The mole of enzyme active sites per mg of the enzyme ([Et]) was calculated assuming that there is only one active site per enzyme molecule. Turnover number (K_{cat}) of the enzyme was also determined using V_{max} and [Et].

Mass spectrometry of the degradation product

The characterization of MidD-catalyzed degradation product of mimosine was performed using AB/MDS-Sciex ESI–MS API 3000 triple quadrupole mass spectrometer (Ontario, Canada). The ESI–MS system was calibrated manually in positive mode with PPG 3000 (AB/MDS-Sciex) to achieve <5-ppm mass accuracy, as per manufacturer's protocol. The product of mimosine degradation was purified via HPLC from MidD-catalyzed reaction. The HPLC-purified sample and reference material (synthetic 3H4P) were freeze-dried and individually dissolved in carrier solvent (50/50 % v/v of a 0.09 % v/v aq. Formic acid and 90/10 % v/v CH_3CN/0.09 % v/v aq. Formic acid). Samples were delivered to the atmospheric pressure ionization (API) source of the mass spectrometer using a continuous flow of 5–10 μL/min provided by a microsyringe infusion pump (Harvard Apparatus, MA, USA).

MS/MS was performed with N_2 bombardment confined to quadrupole-2 (Q-2) with a collision cell gas thickness of 3×10^{14} atoms cm^{-2} and a collision energy (Q-0 to Q-2 rod offset voltage) typically set at ~20–40 eV. The resulting MS/MS (daughter ion) spectra were obtained by scanning quadrupole-3 (Q-3) from *m/z* 10–150 in 0.6 s with a step size of 0.1 Da. ESI–MS data analysis was assisted with the use of Mac BioSpec v1.01 (PE Sciex, Ontario, Canada).

Identification of products of mimosine degradation besides 3H4P

To identify additional products, other than 3H4P, of the mimosine degradation by MidD, the reaction products were analyzed for the presence of alanine, serine, pyruvate, and ammonia. For detection of alanine and serine, the reaction mixtures were derivatized using *o*-phthalaldehyde (OPA) and β-mercaptoethanol that react with primary amines in basic medium (pH 9–11). Considering that the 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), present in Tris buffer, is a primary amine that may also react with OPA, the buffer for enzymatic catalysis was changed to 40 mM sodium phosphate. Mimosine (1 mM) was used as the substrate and the reaction was performed at 37 °C and pH 8.5 for 1 h. To verify if the sodium phosphate buffer does not interfere with the mimosine degradation reaction, the reaction products in 40 mM sodium phosphate buffer were first analyzed for the presence of 3H4P using a C_{18} column as described in the previous section. Solutions of alanine and serine, 1 mM each, were also prepared in the 40 mM sodium phosphate buffer for use as controls. The pH of the reaction products as well as amino acid controls was adjusted to 10 before performing the OPA derivatization. OPA was used at a final concentration of 5 mM for the pre-column derivatization of controls and reaction samples, and the derivatization reactions were continued for 5 min at room temperature before analyzing by HPLC. The Kinetex C_{18} column (2.6 μ, 100 × 4.6 mm) was used in a gradient system in which mobile phase consists of 40 mM potassium phosphate, pH 7.8 (buffer A) and 50/50 methanol/acetonitrile (buffer B). The run time was 20 min with 3–60 % buffer B and the flow rate was 1.5 ml/min. The detection of the product was performed at 338 nm using UV detection photodiode array (200–400 nm). Pyruvate and ammonia in the reaction were detected using a pyruvate assay kit (Biovision, CA, USA) and an ammonia assay kit (Sigma-Aldrich, MO, USA), respectively, according to the manufacturers' instructions.

For mass balance between products and reactants, the reaction was performed to achieve the catalysis of the

substrate to near completion. This was done using lower substrate concentration (500 µM mimosine) with the excess of the enzyme (0.2 mg) in 40 mM phosphate buffer. The final pH of the reaction was 8.5 and the reaction was incubated for 1 h at 37 °C followed by identification and quantification of the degradation products. The standard curves for pyruvate, ammonia, and 3H4P were plotted, and the products in MidD-catalyzed reaction were quantified using the line equation from the respective standard curve.

Prediction of catalytic and PLP-binding site of MidD

To predict the catalytic and PLP-binding sites of MidD, blastp search of MidD was performed using pdb database as the reference. The deduced amino acid sequences of enzymes that showed high homology with MidD were aligned with the deduced amino acid sequence of MidD using the ClustalW2 multiple sequence alignment program and the conserved residues were analyzed.

Results

Expression of *midD*-encoded protein in *E. coli* and its purification

To express *midD*-encoded protein, an expression plasmid pET-*midD* was constructed in which the 1,221-bp *midD* ORF was cloned downstream of a polyhistidine-tag under the control of the T7 promoter (Fig. 1a). The same insert cloned in the anti-sense direction from the T7 promoter served as the negative control. SDS-PAGE analysis of total soluble proteins from the IPTG-induced culture exhibited a major band of size ~45 kDa. The corresponding band was absent in proteins from the induced cells containing the antisense construct or from the non-induced cells containing the sense construct. The polyhistidine-tagged protein was purified from the induced culture and it appeared as a single band with a molecular mass of ~45 kDa (Fig. 1b). The optimum induction time for expression of MidD protein in *E. coli* was found to be 6 h from the induction experiment (Fig. 1c). The cultures were induced for 6 h for subsequent purification of MidD protein for enzymatic assays.

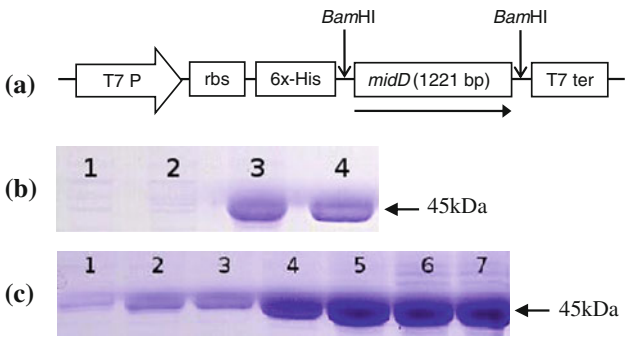

Fig. 1 Construction of *midD* expression plasmid and its expression in *E. coli*. **a** *midD* expression plasmid was constructed by cloning *midD* ORF into the expression vector pET-14b in the sense orientation at *Bam*HI restriction site of the vector. **b** Expression of *midD*-encoded enzyme in *E. coli*. *Lane 1* total soluble protein from 4 h induced *E. coli* containing pET-anti-*midD*; *lane 2* total soluble protein from non-induced *E. coli* containing pET-*midD*; *lane 3* total soluble protein from 4 h induced *E. coli* containing pET-*midD*; *lane 4* purified *midD*-encoded enzyme from the 4 h induced *E. coli* containing pET-*midD*. **c** Expression of MidD protein in *E. coli* at different induction time. *Lane 1–7*, total soluble protein from *E. coli* containing pET-*midD* induced for 0, 1, 2, 4, 6, 8 and 10 h, respectively

Mimosine degradation analysis

To study the possible role of *midD*-encoded enzyme on the catabolism of mimosine, in vitro mimosine degradation assay was performed using mimosine as the substrate with the purified MidD enzyme. The chromatograms of the mimosine and the synthetic 3H4P showed retention times of 3.2 and 5.2 min, respectively (Fig. 2a). In the control reaction, where heat-inactivated enzyme was added to the reaction mixture, no reduction in the amount of substrate was observed.

However, the reactions containing the purified MidD showed significant reduction in the amount of mimosine and the resultant product had the same retention time as that of 3H4P (Fig. 2b). MidD caused mimosine degradation irrespective of the presence or absence of both PLP and α-KG in the reaction. Because the enzyme did not require α-KG as the cosusbstrate it is clear that the enzyme is not an aminotransferase and hence it is likely that MidD is a C–N lyase. However, lyases are known to be PLP-dependent enzymes, and therefore, to be considered as a lyase, MidD should also be a PLP-dependent enzyme. In contrast, MidD-catalyzed degradation of mimosine took place even in the absence of exogenously added PLP in the buffer. Although it suggests that the enzyme purified from *E. coli* did not require PLP as a cofactor, it is still possible that the recombinant enzyme made in *E. coli* carried bound PLP. To determine if the purified enzyme from *E. coli* carried bound PLP and thereby performed its catalytic activity even in the absence of any additional exogenous PLP, hydroxylamine was added to the reaction at a final concentration of 0.01–50 mM. Hydroxylamine at a final concentration of 50 µM completely inhibited MidD catalytic activity (Fig. 2b). However, the supplementation of the reaction with 0.1 µM PLP restored the catalytic activity of MidD, indicating that it is a PLP-dependent enzyme and the purified enzyme carried bound PLP from *E. coli*. This results shows that PLP serves as a cofactor in MidD catalyzed reaction.

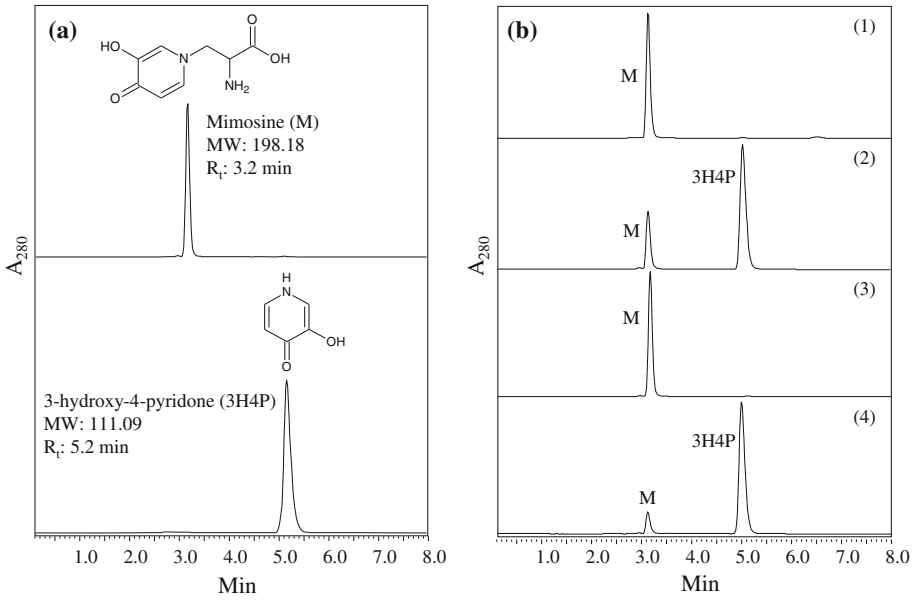

Fig. 2 HPLC chromatograms of mimosine and 3H4P standards, and MidD-catalyzed reaction mixtures. **a** HPLC chromatograms of mimosine and 3H4P standards had a retention time 3.2 and 5.2 min, respectively. **b** HPLC chromatograms of MidD-catalyzed reaction mixtures: (*1*) reaction mixture in which heat-inactivated MidD was added showed a single peak of unutilized substrate, mimosine; (*2*) reaction catalyzed by functionally active MidD in the absence of exogenously added α-KG and PLP exhibited a small peak of the residual substrate, mimosine, and a larger peak of a degradation product with the same retention time as that of 3H4P; (*3*) reaction catalyzed by functionally active MidD in the presence of a potent inhibitor of PLP-dependent enzymes, hydroxylamine (50 μM) had only one peak of unutilized substrate; (*4*) functionally active MidD-catalyzed reaction in the presence of inhibitor, 50 μM hydroxylamine, restored the enzyme activity when supplemented with 0.1 μM PLP and showed a small peak of residual mimosine and a larger peak of its degradation product with the same retention time as that of 3H4P

Biochemical characterization of MidD

The optimum temperature and pH for MidD were determined by quantifying the product of mimosine catalysis by the enzyme at different temperatures and pHs. The catalytic activity of the enzyme was calculated as the concentration of product formed [*P*], in 1 h. The optimum temperature for the enzyme was found to be 37 °C, since the highest catalytic activity was observed at this temperature (Fig. 3a). Sharp decline in the enzyme catalytic

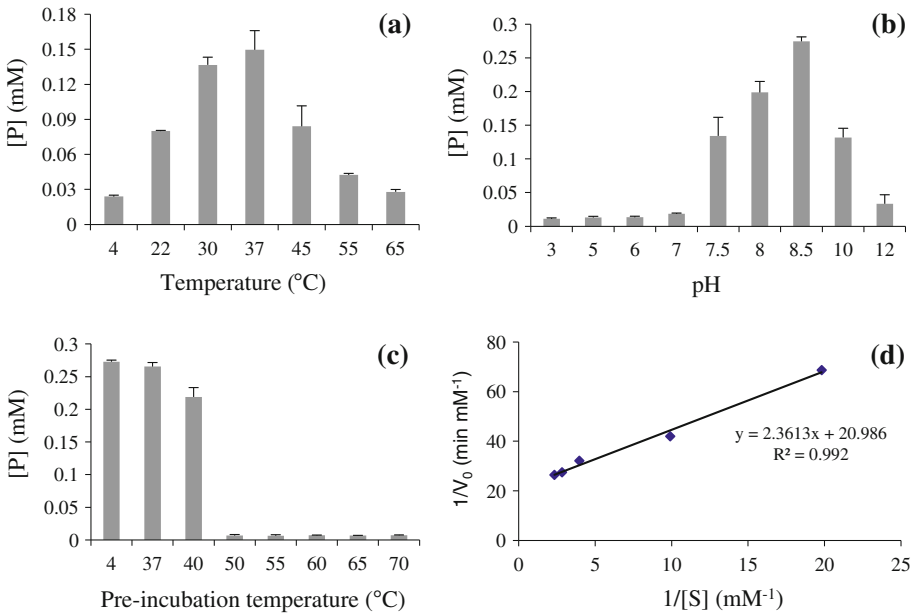

Fig. 3 Biochemical characterization of MidD and determination of its kinetic parameters. Catalytic activity of MidD measured as the product formed [*P*] (mM) at **a** different temperatures (°C); **b** different pHs; and **c** different pre-incubation temperatures (°C). **d** Estimation of kinetic parameters of MidD obtained by plotting Lineweaver–Burk plot of initial velocities at different substrate concentrations

activity was observed at temperature 45 °C or higher and below 22 °C. The enzyme showed activities at a pH range from 7.5 to 10.0, with the optimum pH of 8.5 (Fig. 3b). The thermal stability of the enzyme was determined by pre-incubating the reaction mixture in the absence of the substrate, mimosine for 30 min at different temperatures and then adding mimosine to start the reaction. The percent enzyme activity for different pre-incubation temperatures was measured with reference to the enzyme activity at pre-incubation temperature of 4 °C. The enzyme activity at 37 °C pre-incubation temperature was found to be 97.3 % of that of the reference, and it was reduced to 80.2 % when the enzyme was pre-incubated at 40 °C. A sharp decline in the enzyme activity was observed at pre-incubation temperatures of 50 °C or higher (Fig. 3c). This shows that the enzyme is fairly stable up to 40 °C.

The kinetic parameters of MidD were measured at the optimum temperature (37 °C) and pH (8.5). The rate of reaction for different substrate concentrations ranging from 0.05 to 0.50 mM was found to be linear from 0 to 2 min (data not shown). Initial velocities of the enzyme for each substrate concentration were calculated as the slope of product formed at 0 and 2 min. The enzyme followed the typical Michaelis–Menten kinetics. The apparent K_m and V_{max} values for MidD were experimentally determined from linear regressions by plotting Lineweaver–Burk plot (Fig. 3d) and were found to be 1.274×10^{-4} mol and 4.9633×10^{-5} mol s^{-1} mg^{-1}, respectively. Assuming one active site per enzyme molecule, the total enzyme concentration [E_t], was calculated to be 0.22×10^{-7} mol mg^{-1}. Turnover number (K_{cat}) of MidD was determined to be 2,256.05 s^{-1}.

Possible competitive inhibition of mimosine catalysis was studied by adding the aromatic amino acids L-tyrosine, L-tryptophan, and L-phenylalanine separately to the reaction mixture containing mimosine. Despite the structural similarities to mimosine, the presence of these amino acids at onefold, twofold, or threefold concentration of mimosine did not affect the amount of product formed (Fig. 4), suggesting that these aromatic amino acids are not competitive inhibitors of mimosine catalysis by MidD.

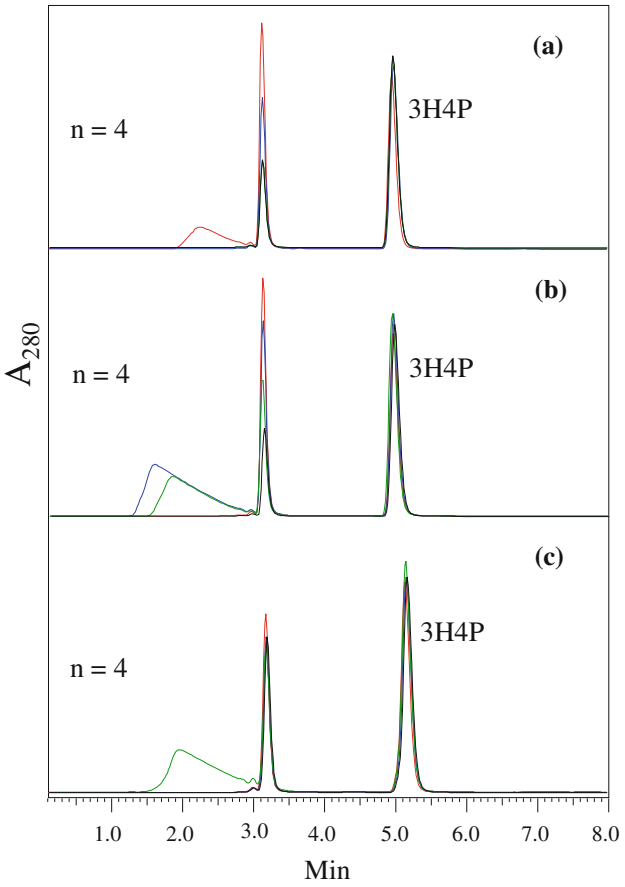

Fig. 4 HPLC chromatograms obtained in mimosine degradation assays in both presence and absence of structural analogs of mimosine, including **a** L-tyrosine; **b** L-phenylalanine; and **c** L-tryptophan. Each of these structural analogs was used in three different concentrations (1 mM, 2 mM and 3 mM) in separate reactions with 1 mM mimosine. The reaction in the absence of structural analog served as the control. The product formed in control and test reactions showed no difference in the amount of product formed. This shows that none of the structural analogs competitively inhibits MidD-catalyzed reaction. $n = 4$ represents 4 reactions; n1–n3: structural analog at concentrations 1, 2 and 3 mM, respectively; n4: reaction without structural analog. Each of the reaction had 1 mM of the substrate

Characterization of the degradation product of mimosine by MidD

The chromatogram of the product of mimosine degradation by MidD was identical to that of synthetic 3H4P standard. This suggests that mimosine is converted to 3H4P by MidD. To confirm this correlation, its induced fragmentation pattern obtained through MS/MS of the parent ion (*m/z* 112.2) was compared with that of synthetic 3H4P (*m/z* 112.2). The resulting fragmentation patterns of synthetic 3H4P (reference sample) and mimosine-degradation product (test sample) were found to be identical, generating common internal structural fragments of *m/z* 94.0 ($[M–H_2O]^+$), 65.9 ($[M–H_2O–CO]^+$), 56.0, and 39.1 amu (Fig. 5a, b). This confirms that the degradation product of mimosine by MidD is indeed 3H4P.

Other products of mimosine degradation

Mimosine is an aromatic compound with 8 carbons and 2 nitrogens. Identification of 3H4P as the major degradation product of mimosine accounts for only 5 carbons and one nitrogen. Therefore, MidD-catalyzed mimosine degradation must form other products that should account for the remaining three carbons and one nitrogen. Previous studies

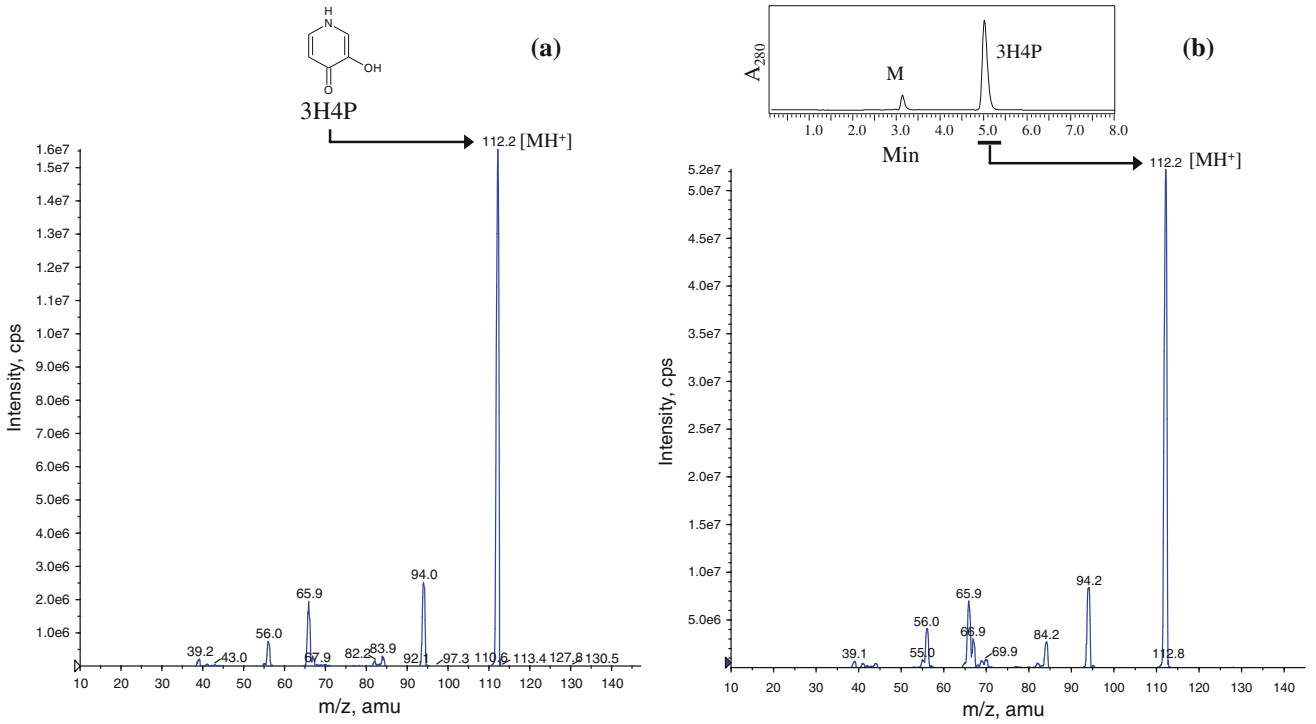

Fig. 5 MS/MS spectra of **a** the synthetic 3H4P (reference sample) and **b** the HPLC-purified mimosine-degradation product formed in MidD-catalyzed reaction (test sample)

with mimosine-degrading enzymes from *Mimosa pudica* (Suda 1960) and leucaena (Smith and Fowden 1966) showed serine and pyruvate, respectively as the degradation products. Therefore, we tested MidD-catalyzed reactions for the presence of serine or pyruvate. We also tested the reactions for the presence of alanine as it is also a 3-carbon amino acid with structure similar to serine. Neither serine nor alanine appeared in the HPLC chromatograms of MidD-catalyzed reactions (Supplementary material, Fig. S1). However, we successfully detected pyruvate and ammonia spectrophotometrically in MidD-catalyzed reactions as mimosine degradation products and quantified the products (Fig. 6). The

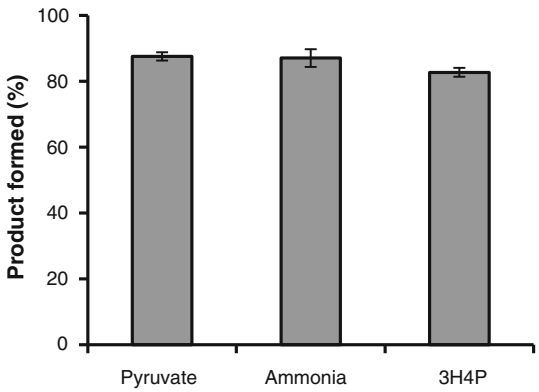

Fig. 6 The percent of product formed with respect to the substrate used. The *error bar* represents the standard deviation of three replicates

amounts of pyruvate, ammonia, and 3H4P produced in the mimosine degradation reaction were found to be 87.5, 87.0, and 82.7 %, respectively, of mimosine, indicating that these products are formed in equimolar quantities as that of the substrate. Therefore, the completely balanced reaction of MidD-catalyzed mimosine-degradation can be represented as follows:

$$C_8H_{10}N_2O_4(\text{mimosine}) + H_2O \rightarrow C_5H_5NO_2(3H4P) + C_3H_4O_3(\text{pyruvate}) + NH_3$$

Prediction of catalytic and PLP-binding site of MidD

The blastp analysis of MidD using pdb as reference database showed homology of MidD with β C–S lyase (gi|392935441), MetC (gi|317455161) and cystalysin (gi|9955069) from *Streptococcus anginosus*, *Streptococcus mutans* and *Treponema denticola*, respectively. The sequence alignment showed presence of the active site residues and PLP-binding lysine in MidD corresponding to these residues in cystalysin of *T. denticola* (Fig. 7).

Discussion

In this report, we studied mimosine degradation by *midD*-encoded enzyme of the root nodule bacterium *Rhizobium* sp. strain TAL1145 that forms nitrogen-fixing nodules on *Leucaena*. Mimosine degradation is a mechanism for

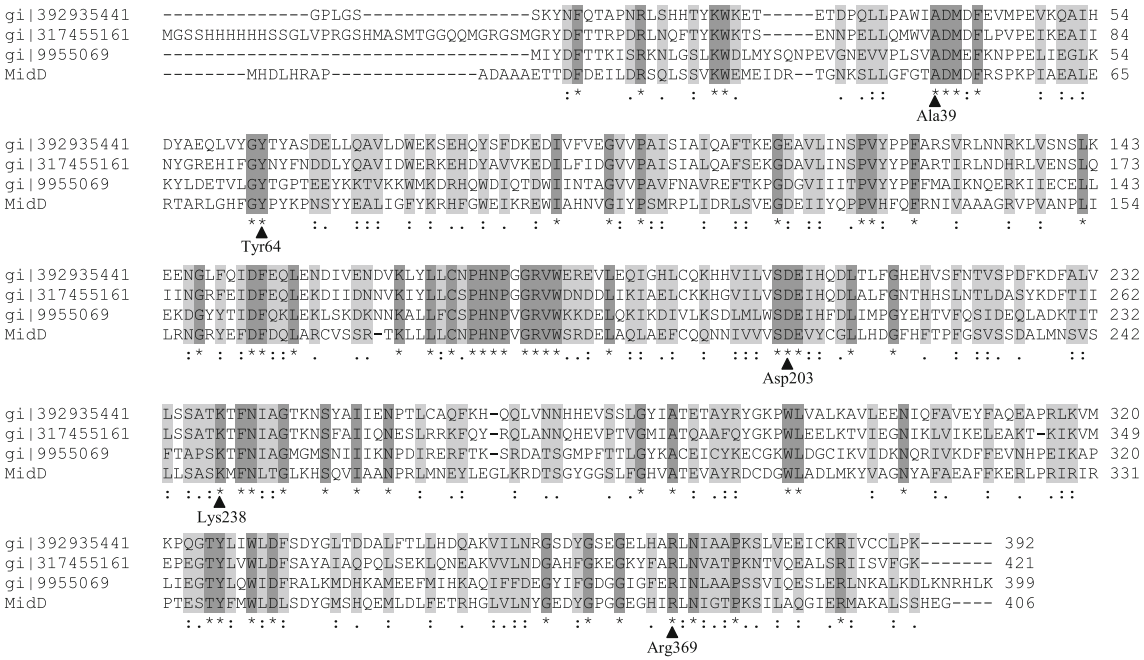

Fig. 7 Sequence alignment of MidD with β C–S lyase (gil392935441), MetC (gil317455161), and cystalysin (gil9955069) from *Streptococcus anginosus, Streptococcus mutans and Treponema denticola*, respectively. Asterisks represent identical residues and single or double dots symbolize similar residues. The catalytic residues of cystalysin, which are conserved in the other three lyases are indicated by '*filled triangle*' and the three letter code of amino acid followed by the position of the residue. The alignment was performed using the ClustalW2 multiple sequence alignment program

detoxification as well as acquiring additional source of nutrients by the bacterium. Although *Rhizobium* bacteroids are provided with malate as the source of carbon by the plant (McKay et al. 1988), in the *Leucaena* nodules, mimosine may also be used as a source of carbon by the *Rhizobium* bacteroids (Borthakur et al. 2003). We have established that the *Rhizobium* enzyme MidD degrades mimosine into 3H4P. MS/MS analysis using synthetic 3H4P and the purified product of mimosine degradation, which had identical chromatogram as 3H4P, showed that both have identical fragmentation patterns confirming that the purified compound was 3H4P. A plant enzyme that degrades mimosine into 3H4P is also known to be present in the foliage of *Leucaena* (Smith and Fowden 1966; Tangendjaja et al. 1986). Because the mimosine-degrading enzyme from *Leucaena* has been named as mimosinase, we used the name 'rhizomimosinase' to distinguish the *Rhizobium* enzyme from the *Leucaena* enzyme with similar function.

Previously, based on the sequence similarity, rhizomimosinase was referred to as mimosine aminotransferase (Borthakur et al. 2003). However, in this study, we have shown that rhizomimosinase is not an aminotransferase because it does not require a keto acid as the co-substrate for accepting the amino group from mimosine. Although, no exogenous PLP was needed for the catalytic activity of the recombinant enzyme purified from *E. coli*, the presence of hydroxylamine completely inhibited the reaction, whereas, the addition of exogenous PLP overcame the inhibitory effect of hydroxylamine and restored the catalytic activity of rhizomimosinase, suggesting that it is a PLP-dependent enzyme. Besides 3H4P, we also identified pyruvate and ammonia as the products of rhizomimosinase-catalyzed mimosine degradation. In addition, the production of ammonia with the aromatic compound 3H4P as mimosine-degradation products establishes that MidD is a C–N ammonia lyase as the release of ammonia with the formation of a double bond or a ring compound is the key characteristic of C–N ammonia lyases. Based on these results, MidD can be classified in the enzyme category with the Enzyme Commission number EC4.3.1.

Among the lyases that showed homology with rhizomimosinase, the catalytic and PLP-binding sites of cystalysin from *T. denticola* have been studied in detail (Krupka et al. 2000). Although cystalysin is a C–S lyase, and rhizomimosinase is a C–N lyase, both enzymes produce pyruvate and ammonia and share many conserved residues. The Arg369, Lys238, Asp203, Tyr64 and Ala39 of cystalysin, which are conserved in all four lyases studied and had been shown to be involved in the catalytic site in cystalysin, correspond to Arg380, Lys248, Asp213, Tyr75, and Ala50 in rhizomimosinase, respectively. The Lys238 of cystalysin is known to interact with PLP forming an internal aldimine (Krupka et al. 2000). The conserved Arg

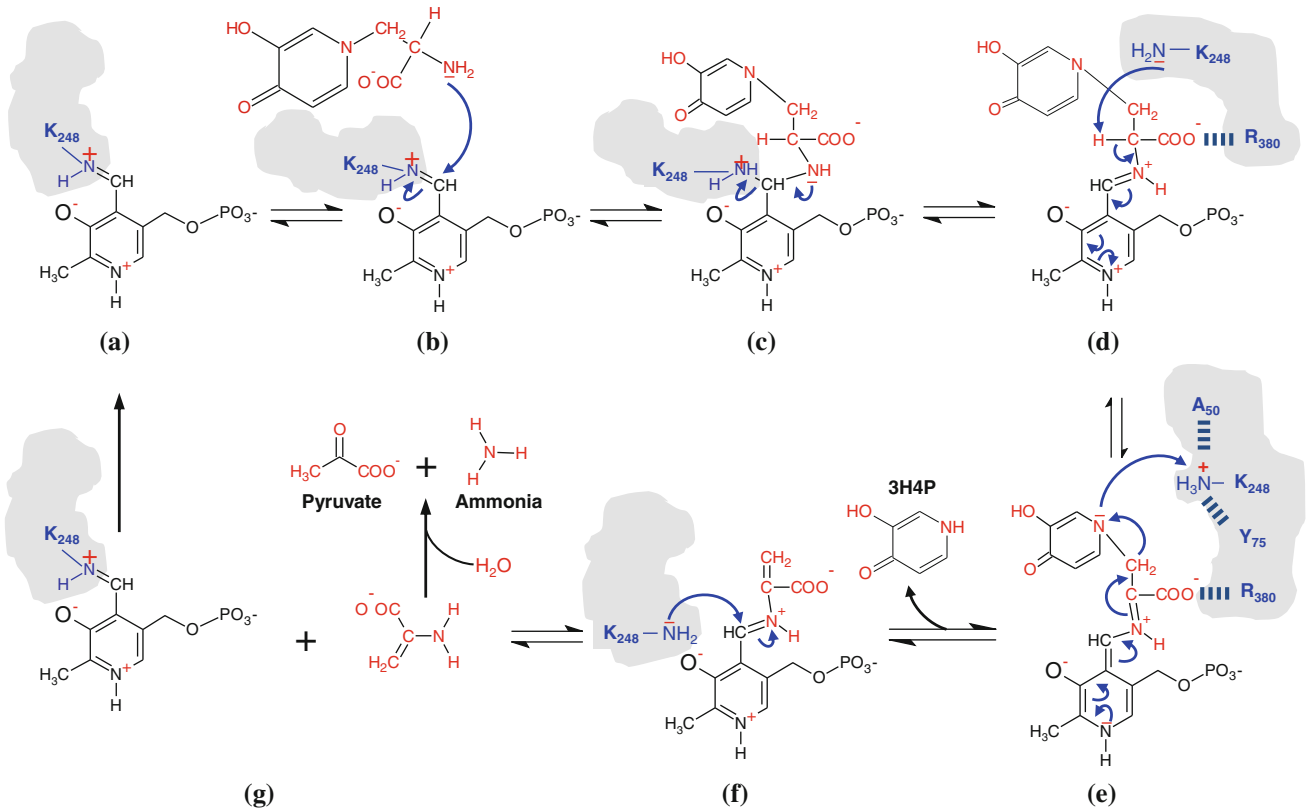

Fig. 8 Proposed molecular mechanism of mimosine degradation catalyzed by rhizomimosinase. The rhizomimosinase enzyme is indicated as a shaded area, and the substrate and products are shown in *red*. The proposed mechanism is based on the high homology and conserved catalytic residues between rhizomimosinase and cystalysin. Lys248 of rhizomimosinase binds with PLP forming internal aldimine (**a**). Nucleophilic attack on C4 of internal aldimine by deprotonated amino group of mimosine (**b**) produces geminal substrate diamine (**c**). The amino group of Lys248 is detached from C4 of geminal substrate diamine, forming external substrate diamine (**d**). Nucleophilic attack by the amino group of Lys248 of rhizomimosinase on the H of Cα of mimosine followed by electrophilic aromatic substitution of the attached PLP results in quinonoid intermediate (**e**). Electron transfer from the amino group of Lys248 creates positive charge on the amino group of Lys248. Interactions of Ala50, Tyr75 and Lys248 of rhizomimosinase with the quinonoid intermediate cleave the C–N bond of mimosine. This results in the release of 3H4P as the first degradation product and formation of PLP-aminoacrylate intermediate (**f**). Reverse transaldimination reaction results in the formation of internal aldimine and 2-aminoprop-2-enoate; the later is hydrolyzed to form ammonia and pyruvate as additional degradation products of mimosine (**g**) (color figure online)

corresponding to Arg369 of cystalysin has been suggested to be the docking site for α-carboxylate of substrate (Mehta et al. 1993; Krupka et al. 2000). Lys238, Tyr64 and Ala39 have been reported to be involved in C–S cleavage in cystalysin (Krupka et al. 2000). Similarly, Asp203 has been shown to be involved in charge stabilization of pyridoxal system during the cystalysin reaction. Based on the high homology and conserved residues at the catalytic and PLP-binding sites between cystalysin and rhizomimosinase as described above, it is likely that the C–N bond of mimosine is cleaved by rhizomimosinase in a similar way as for the C–S bond of cysteine by cystalysin (Fig. 8). In our experiment, the purified recombinant rhizomimosinase formed a complex with PLP in *E. coli*. The amino group of mimosine may be deprotonated under the optimum pH (8.0–8.5) to perform a nucleophilic attack on C4 of the enzyme-bound PLP, which results in the formation of a Schiff base (mimosine-PLP complex). Lys248, Tyr75 and Ala50 in the rhizomimosinase catalytic site may be involved in C–N bond cleavage of mimosine of the mimosine-PLP complex forming 3H4P. After the release of 3H4P, reverse transaldimination reaction may take place resulting in the formation of 2-aminoprop-2-enoate, which can be hydrolyzed to form ammonia and pyruvate outside the catalytic site of rhizomimosinase.

Rhizomimosinase is found to be specific for mimosine and the presence of its structural analogs, L-tyrosine, L-tryptophan, and L-phenylalanine did not affect the rhizomimosinase's efficiency for mimosine degradation. The optimum pH for rhizomimosinase (8.5) is higher than the physiological pH for *Rhizobium* (6.8) or plant cytoplasm (7.4–7.5) (Gout et al. 1992). The activity of the rhizomimosinase is highly reduced below pH 7.5 or above pH 10.0. Although *Rhizobium* strains grow best at pH 6.8, some *Rhizobium* enzymes, such as xylitol dehydrogenase, malate dehydrogenase and aspartate aminotransferase, are

known to have an optimal pH of 8.0 or higher (Waters et al. 1985; Alfano and Kahn 1993; Tiwari et al. 2010).

This study is the first experimental evidence that shows the catalytic action of rhizomimosinase in mimosine catabolism. It will be useful in developing transgenic *Leucaena* with reduced mimosine content by expressing *midD* gene in *Leucaena*.

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Conflict of interest The authors declare that they have no conflict of interest.

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