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Research Article

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Expression and characterization of recombinant L-amino acid deaminase of *Proteus mirabilis* isolated from acute pyelonephritis patients

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ABSTRACT

To investigate function and enzymatic characteristics of L-amino acid deaminase of Proteus mirabilis from acute pyelonephritis patients, L-amino acid deaminase encoding genes (pmzd1 and pmzd2) were cloned from P. mirabilis ZD1 and P. mirabilis ZD2 and nucleotide as well as amino acid sequences were analyzed. Prokaryotic expression system was established to express recombinant PMZD1 and PMZD2 and enzymatic characteristics were analyzed. Growth promotion tests were performed to establish the iron chelation and growth promotion effect of recombinant enzymes products. Results showed that recombinant PMZD1 and PMZD2 exhibited function of second type of L-amino acid deaminase but different in enzymatic characteristics, including affinity to substrates, reaction speed as well as optimal reaction temperature and pH, probably due to different amino acid sequences. Growth promotion tests indicated that products of the recombinant enzymes had growth promotion activities to P. mirabilis but exhibited no difference between the two enzymes.

Key words: *Proteus mirabilis*, L-amino acid deaminase, enzymatic characteristics, growth promotion

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INTRODUCTION

Proteus mirabilis, a motile Gram-negative bacterium within the Enterobacteriaceae, undergoes dramatic morphological changes in response to growth on solid surfaces [1-3]. It is commonly found in soil, water, mud, intestinal tract and on skin of human and animals. *P. mirabilis* is a frequent etiological agent especially in catheterized patients or individuals with structural abnormalities of the urinary tract and it can cause serious complications including acute pyelonephritis, bladder and kidney stones, and bacteremia [1, 2]. In addition, *P. mirabilis* has been reported as one of the causative agents of human pneumonia and other lung infection conditions, endotoxin-induced sepsis and central nervous system infection [3-5].

It is known that all members of the genus *Proteus* have L-amino acid deaminases [6]. In addition, it is reported that *P. mirabilis* contain two types of amino acid deaminases [6-8]. One type deaminated a wide range of aliphatic and aromatic amino acids, whereas the other deaminated only a small range of basic amino acids [6-8]. Massad *et al.* cloned *pma* from *P. mirabilis* KCTC and the Pma could catalyze such aromatic amino acids as L-phenylalanine, L-leucine, L-aspartic acid, L- methionine to corresponding α-keto acids, so it belonged to the first type of amino acid deaminase [9]. Then Baek *et al.* discovered *pm1* from the same strain and the Pm1 could deaminate only a narrow range of L-amino acids, especially basic molecules including L-arginine and L-histidine, indicating that it belong to the second type of amino acid deaminase [10, 11].

Iron acquisition is required for bacterial survival and siderophores are essential for iron nutrition in virtually all microorganisms [12]. Since *P. mirabilis* infects the iron-limited environment of the urinary tract, it is hypothesized

that a siderophore is necessary to capture iron for its survival [13]. However, *P. mirabilis* does not produce any of such traditional siderophores as enterobactin, aerobactin, or the ferroxamine-type siderophores) [13-15]. As compensation, it produces α-keto acids generated from amino acid deaminases as siderophore, which are capable of binding iron [16, 17].

In the present work, the second type of amino acid deaminase, PMZD1 and PMZD2, encoding genes (*pmzd1* and *pmzd2*) were cloned from strains isolated from acute pyelonephritis patients. And the enzymatic characteristics were studied and compared, in addition, growth promoting effect of α-keto acids produced by the enzymes was also determined.

EXPERIMENTAL SECTION

Strains and plasmids

Escherichia coli DH5α and *E. coli* BL21(DE3) stored in our lab were used as hosts for cloning and expression, respectively. *P. mirabilis* ZD1 and *P. mirabilis* ZD2 were isolated from acute pyelonephritis patients. pMD19-T simple vector [TAKARA Biotechnology(Dalian) Co., Ltd.] and pET-42a (laboratory stock) were used as cloning and expression vector, respectively.

Media

E. coli and *P. mirabilis* were grown at 37℃ in Luria-Bertani medium [LB, 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl]. Kanamycin (50 μg/ml) or ampicillin (100 μg/ml) was added for strain selection.

Primers

Primers used were listed in Table 1.

Table 1 Primers

Primers	Sequence (5'-3')
PM-1	ATGGCAATAAGTAGAAGAAAATTTATTCTT
PM-2	TTAGAAACGATACAGACTAAATGGTTTG
PM-3	CGAGAAGGGCGTTTTGTTCC
PM-4	GGCCATATGGCAATAAGTAGAAGACGAGAAGGGCGTTTTGTTCC (*Nde* Ⅰ)
PM-5	GGCCTCGAGGAAACGATACAGACTAAATGGTTTGG (*Xho* Ⅰ)

Cloning of *pmzd1* and *pmzd2*

Pmzd1 and pmzd2 were amplified cloned from *P. mirabilis* ZD1 and *P. mirabilis* ZD2 using the primers PM-1 and PM-2. The PCR product was cloned into pMD19-T simple vector and was sequenced by GENEWIZ Biotechnology Co., Ltd.

Amplification of *pmzd1* and *pmzd2* genes with deletion of N-terminal transmembrane region and expression in *E. coli* BL21 (DE3)

As reported, second type of amino acid deaminase is a membrane-bound protein, PMZD1 and PMZD2 were predicted contained the same region. To achieve its soluble and high-level expression, the two genes with deletion of N-terminal transmembrane region (from 21 to 87th nucleotide) was first amplified by PCR using primes PM-3 and PM-5 (88-1416bp) from *P. mirabilis* ZD1 and *P. mirabilis* ZD2 and then was amplified using PM-5 and PM-4 from the above generated fragments, named *pmzdΔTR1* and *pmzdΔTR2* - respectively.

PmzdΔTR1 and *pmzdΔTR2* were digested by *Nde* Ⅰ and *Xho* Ⅰand were subcloned into the corresponding restriction sited of pET-42a, resulting in pET-pmzd1 and pET-pmzd2, respectively. Then the recombinant plasmids were transformed to *E. coli* BL21(DE3), generating BL-pmzd1 and BL-pmzd2, respectively. The strains could express the PMZD1 and PMZD1 protein with a 6× histidine tag fused to the C-terminal.

BL-pmzd1 and BL-pmzd2 was cultured in 20 mL of LB medium supplemented with kanamycin (50 μg/mL) in 100 mL flask on a shaker (200 rpm) at 37℃. After cultivation for 12 h, 1 mL of seed culture was inoculated into 100 mL of the same LB medium in 500 mL flask and was culture in the same condition until OD_{600} reached 0.8. Then isopropyl β-D-1-Thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mmol/L followed by incubation for another 4h. Induced cells were harvested and disrupted by sonication. Cell lysates were separated into soluble and insoluble fractions by centrifugation at 10,000×*g* for 30 min. To confirm the expression of PMZD1 and PMZD2, SDS-PAGE was performed.

The 6×His tagged PMZD1 and PMZD2 were purified using Ni^{2+}-NTA affinity column. And the enzyme

concentration was determined by the BCA assay kit (Bio-Rad, USA).

Amino acid deaminase assay

Amino acid deaminase activity was determined by the method of Massad *et al*. [9]. Briefly, 200μg of protein was added to 50 mmol/L Tris-HCl (pH 7.0, containing 10 mmol/L substrate, 0.5 mL total volume). 500 μL ferric chloride solution (1%, w/v) was added after incubation at 37□ for 10 min and incubation was continued for 10 min. The decrease of substrate was determined by amino acids analyzer (Sykam, Germany). Amino acid deaminase activity was defined as micromoles substrate decrease per min per mg of protein.

The optimum temperature and pH was determined by measuring the amino acid deaminase activity at different temperature and pH using L-histidine as substrate as described above.

Growth promotion test

2 μmmol of L- histidine, L- arginine, L-phenylalanine, L-cysteine and L- serine was added to 50 mmol/L Tris-HCl (pH 7.0, containing 400μg of enzyme, 1 mL total volume). After incubation as described above for 60 min, the system was sterilized by filtration and was added to LB medium containing 0.04g/L 2, 2'-dipyridyl [6, 16].10% (v/v) of *P. mirabilis* ZD1 or *P. mirabilis* ZD2 seed culture was inoculated into the above medium and OD_{600} was detected after 24 h of culture at 37□.

RESULTS AND DISCUSSION

Cloning and sequence analysis of *pmzd1* and *pmzd2*

Pmzd1 and *pmzd2* were successfully cloned from *P. mirabilis* ZD1 and *P. mirabilis* ZD2 with the molecular weight similar to that of *pm1* from *P. mirabilis* KCTC 2566 (data not shown), respectively [11]. Considering that previous study have cloned the first amino acid deaminase encoding genes from the two strains (data not shown), it could be concluded that both *P. mirabilis* ZD1 and *P. mirabilis* ZD2 possess two type of amino acid deaminases.

Sequencing analysis showed that the open reading frame of *pmzd1* and *pmzd2* were both 1416 bp in length and encoded 472-amino acid protein with an estimated molecular weight of 51,500 Da and 51,539 Da, respectively. And the two genes exhibited 98.23% (1391/1416) and 99.65% (1411/1416) similarity with nucleotides of *pm1* from *P. mirabilis* KCTC 2566, respectively. Amino acid sequence of PMZD2 was identical to that of Pm1, while that of PMZD1 differed in 8 amino acid residues, Asp-42, Gly-55, Val-82, His-97, Ser-150, Thr-306, Ala-318 and Ser-355, with respect to the corresponding Gly, Asp, Ile, Tyr, Ser, Ala, Val and Ala residues of Pm1. The above results indicated that PMZD1 from *P. mirabilis* might exhibit different enzymatic characteristics from Pm1.

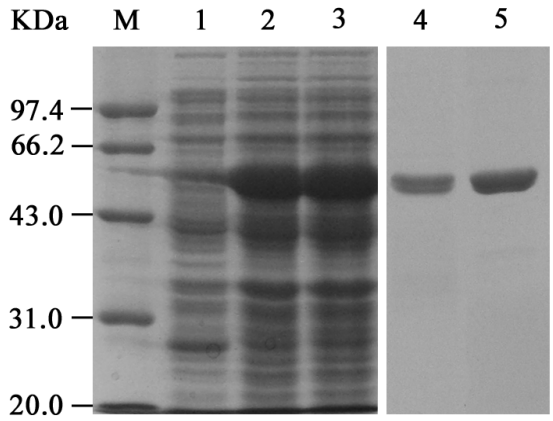

Fig. 1 SDS-PAGE analysis of recombinant PMZD1 and PMZD2 expression in *E. coli* BL21(DE3)

M, marker; Lane1, E. coli BL21(DE3) harboring pET42a; Land 2 and 3, E. coli BL21(DE3) harboring pET-pmzd1 or pET-pmzd2; Land 4 and 5, purified recombinant PMZD1 and PMZD2.

Expression of *pmzdΔTR1* and *pmzdΔTR2* in *E. coli* BL21(DE3)

It is reported that unlike eukaryotic and other prokaryotic L-amino acid deaminases, L-amino acid deaminases from *Proteus* species are membrane-bound [9, 18-19]. Transmembrane helices of PMZD1 and PMZD2 located between residues 7 and 29, with 97.53% and 97.58% probability were predicted by the on-line program (TMHMM Server v.2.0, http://www.cbs.dtu.dk/services/TMHMM-2.0/, data not shown), which is similar to Pm1 [11]. And therefore to study the characteristics of PMZD2 and to achieve its soluble and high-level expression, the gene without transmembrane region was expressed in *E. coli* BL21(DE3) with the plasmid pET42a. Since PMZD2 and Pm1 had

the same amino acid sequence, it was expressed for comparison.

The expression of *pmzdΔTR1* and *pmzdΔTR2* was analyzed by SDS-PAGE. Obvious bands were observed at approximately 50 kDa, corresponding to the expected size (448 amino acids of PMZD1 or PMZD2 without transmembrane region plus 6×His), in lysates of cells harboring pET-pmzd1 or pET-pmzd2 but not in those of pET42a harboring cells, indicating the success expression of *pmzdΔTR1* and *pmzdΔTR2*. Recombinant PMZD1 and PMZD2 was purified (Fig. 1) and the concentration was 2.7 mg/L and 3.1 mg/L, respectively.

Enzymatic characterization of PMZD1 and PMZD2

As described above, PMZD1 and PMZD2 exhibited high similarity with Pm1 belonging to the second type of amino acid deaminases [11]. In the present study, it is found that PMZD1 and PMZD2 effectively deaminated basic amino acids such as L-histidine and L-arginine, thus these two genes were placed among enzymes of the second type (Table 2). Notably, PMZD1 showed a somewhat higher conversion rate of L-histidine, L-arginine and L-phenylalanine than PMZD2. As is known, urocanate formed by histidinase or histidine ammonia-lyase after elimination of ammonium is a natural photoprotectant against UV radiation-induced DNA damage [20], so PMZD1 might more suitable to be explored in industrial production of urocanate. Notably, PMZD1 also exhibited significant activity of deaminating L-cysteine to produces 3-mercaptopyruvic acid that was studied as a potential treatment for cyanide poisoning [21].

Table 2 Conversion rate of 20 L-amino acids by recombinant enzymes

L-amino acids	Conversion rate (%)		L-amino acids	Conversion rate (%)	
	PMZD1	PMZD2		PMZD1	PMZD2
L-Ala	15.9	16.0	L-Leu	2.1	3.2
L-Arg	58.1	43.2	L-Lys	9.6	4.7
L-Asn	6.7	4.2	L-Met	4.5	3.3
L-Asp	3.7	4.6	L-Phe	52.1	44.3
L-Cys	31.2	11.2	L-Pro	10.8	11.3
L-Gln	2.3	4.2	L-Ser	18.9	4.5
L-Glu	9.7	11.8	L-Thr	11.7	9.5
L-Gly	5.3	3.6	L-Trp	8.9	9.7
L-His	90.1	81.2	L-Tyr	5.7	4.2
L-Ile	2.1	5.4	L-Val	4.2	3.4

Purified PMZD1 and PMZD2 were used to examine the kinetics of activity. The Km value for histidine was 10.72 mmol/L and 15.34 mmol/L, and the Vmax was 207.19 μmol/min/mg and 186.97 μmol/min/mg, respectively (Fig. 2). The result indicated that PMZD1 had a higher deamination activity and substrate specificities when histidine was used as substrate.

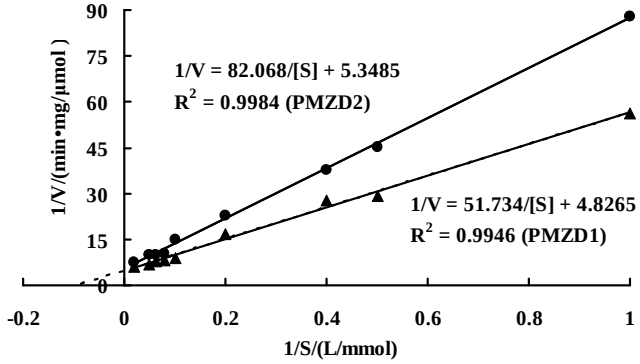

Fig. 2 Hyperbolic kinetics of recombinant enzymes

The optimal temperature (at the range of 10℃-70℃) and pH (at the range of 3.0-10.0) of PMZD1 and PMZD2 were determined. Results showed that PMZD1 and PMZD2 had an optimal temperature of 40℃ and 45℃ (Fig. 3), and had an optimal pH of 7.0 and 8.0 (Fig. 4). The differences in above enzymatic characteristic of PMZD1 and PMZD2 may be due to the divergence in amino acid sequence of two enzymes.

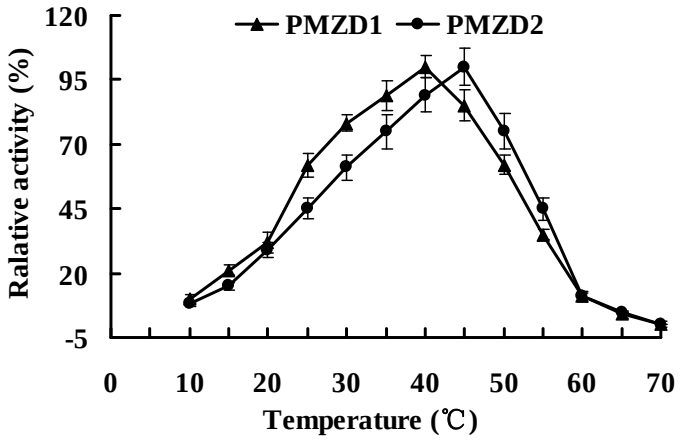

Fig. 3 Effect of temperature on recombinant enzyme activity

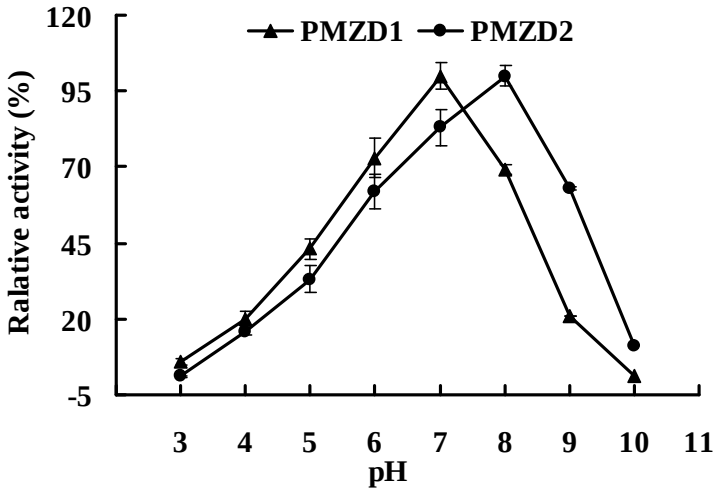

Fig. 4 Effect of pH on recombinant enzyme activity

Effect of products by PMZD1 and PMZD2 on *P. mirabilis* growth

As it has been reported, *P. mirabilis* depend on α-keto acids produced by amino acid deaminase as siderophore to chelate iron and transport it back into the cell in iron-limited environment of the urinary tract [12-14]. To determined effect of products by PMZD1 and PMZD2 on *P. mirabilis* growth, growth promotion tests were performed. Result showed that reaction products by PMZD1 and PMZD2 really promoted cell growth but the two enzymes exhibited no difference in promotion effect (Table 3).

Table 3 Promotion effect by comples of recombinant enzyme chelating iron

Strains	Biomass (OD_{600})		
	PMZD1	PMZD2	Control*
P. mirabilis ZD1	2.23±0.18	2.30±0.13	-**
P. mirabilis ZD2	2.40±0.23	2.32±0.11	-

** The control is reaction system without any enzyme.*
*** Significant growth had not been detected.*

CONCLUSION

Pmzd1 and *pmzd2* were successfully cloned from *P. mirabilis* ZD1 and *P. mirabilis* ZD2. PMZD1 and PMZD2 had function of second type of L-amino acid deaminase but different enzymatic characteristics due to the different amino acid sequences. Products generated by two enzymes could promote *P. mirabilis* growth but exhibited no different promotion effect.

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