ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 17 (2002) 223–233

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Application of a thermostable glutamate racemase from *Bacillus* sp. SK-1 for the production of D-phenylalanine in a multi-enzyme system

Hee-Sung Bae [a], Seung-Pyo Hong [b], Seung-Goo Lee [a], Mi-Sun Kwak [a], Nobuyoshi Esaki [c], Moon-Hee Sung [a,b,*]

[a] *Microbial Genomics Laboratory, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 52 Oun-dong, Yusong, Taejon, South Korea*

[b] *BioLeaders Crop. Bio-Venture Center, KRIBB, Yusong, Taejon, South Korea*

[c] *Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611-0011, Japan*

Received 6 December 2001; received in revised form 21 January 2002; accepted 21 January 2002

Abstract

A gene encoding glutamate racemase (GluRA) was found in a thermophilic *Bacillus* strain named SK-1. The gene was cloned and expressed in *Escherichia coli* WM335, a D-glutamate auxotroph. It consists of 792 bp with a start codon, TTG. The amino acid sequence deduced from the gene indicates that the GluRA has two cysteines and their surrounding regions are well conserved. The GluRA produced in the recombinant *E. coli* was purified to homogeneity by heat-treatment and Resource Q and Phenyl sepharose column chromatographies. The enzyme, which was determined to be a monomeric protein with a molecular weight of 29,000, did not require a cofactor such as pyridoxal 5′-phosphate, nicotinamide, or flavin for its activity. The enzyme was stable after incubation at 55 °C and retained 60% of its original activity after incubation at 60 °C. It was found to be stable in the region of pH 6.0–11.5. The thermostable GluRA was used as a catalyst in a multi-enzyme system composed of four enzyme reactions for the production of D-phenylalanine. By running the multi-enzyme system for 35 h, 58 g l^{-1} of D-phenylalanine was produced with 100% of optical purity from equimolar amount of phenylpyruvate.

Keywords: Glutamate racemase; Thermostability; *Bacillus* sp. SK-1; Overproduction; Molecular cloning

1. Introduction

Glutamate racemase (GluRA, EC 5.1.1.3) catalyzes the racemization of L- and D-glutamate and plays an important role in the biosynthesis of D-glutamate, which is an essential component of the peptidoglycan layer in a bacterial cell wall. The enzyme has been found in various microorganisms, including lactic acid bacteria [1–4], *Escherichia coli* [5–7], *Staphylococcus haemolyticus* [8], *Bacillus pumilus* [9], *Brevibacterium lactofermentum* [10], and *Aquifex pyrophilus* [11]. Previous studies showed that GluRA requires neither a cofactor such as pyridoxal 5′-phosphate (PLP) nor metal ions for its activity [3,12] but contains two cysteine residues at the active site [13].

* Corresponding author. Tel: +82-42-860-4372; fax: +82-42-860-4595.
E-mail address: smoonhee@mail.kribb.re.kr (M.-H. Sung).

1381-1177/02/$ – see front matter
PII: S1381-1177(02)00011-5

The sequences around the cysteine residues are well conserved [4].

Since D-amino acids are useful raw materials in the pharmaceutical field for the synthesis of antibiotics, bioactive peptides, and other physiologically active compounds, particular attention has been paid to the enzymatic production of D-amino acids. Recently, we studied the production of aromatic D-amino acids in a multi-enzyme system, which is composed of four enzyme reactions catalyzed by GluRA, D-amino acid amino transferase (D-AAT), formate dehydrogenase (FDH), and glutamate dehydrogenase (GDH) [14]. As reported previously, D-phenylalanine and D-tyrosine with 100% optical purity were produced in the enzyme system at an initial production rate for 8–13 h when the corresponding substrate, phenylpyruvate or hydroxyphenylpyruvate, respectively, was repeatedly fed in a low level (50 mM). However, the production rate was gradually decreased after 8–13 h due to the instability of GluRA. Thus, we planned to study the occurrence of new GluRAs in microorganisms in order to improve the multi-enzyme system. In series of our study, we found that a thermophilic *Bacillus* sp. SK-1 isolated from a soil sample has thermostable GluRA activity. We here report the molecular cloning, overproduction, and application of the thermostable GluRA for the production of D-phenylalanine in the multi-enzyme system.

2. Experimental

2.1. Bacterial strains and plasmid

The thermophilic *Bacillus* sp. SK-1 used as a source of the GluRA gene in this study was isolated from a soil sample. *E. coli* WM335, a D-glutamate auxotroph [15], was used as a recipient strain for the cloning of the GluRA gene. Plasmid pUC118 (Takara Shuzu Co., Japan) was used as the cloning and expression vector for the GluRA gene.

2.2. Media and culture conditions

The medium for the culture of *Bacillus* sp. SK-1 consisted of 15 g polypeptone, 2 g yeast extract, 2 g meat extract, 2 g glycerol, 2 g K_2HPO_4, 2 g KH_2PO_4, and 0.26 g NH_4Cl in 1 l of distilled water. The pH of the medium was 7.0. *Bacillus* sp. SK-1 was aerobically cultivated at 60 °C. *E. coli* WM335 was aerobically cultivated in Luria–Bertani's (LB) medium containing ampicillin (100 μg ml^{-1}) and D-glutamate (100 μg ml^{-1}) at 37 °C.

2.3. Cloning of the GluRA gene of Bacillus sp. SK-1 and sequence analysis

The chromosomal DNA from *Bacillus* sp. SK-1 was isolated as described by Saito and Miura [16] and partially digested with 0.02 U of *Sau*3AI at 37 °C for 10 min. Fragments of 3–10 kb were isolated by sucrose gradient (5–40%, w/v) centrifugation at 25,000 rpm for 20 h and then ligated into the *Bam*HI site of pUC118 at 12 °C for 12 h with T4 ligase. *E. coli* WM335 was directly transformed with the ligation mixture by electroporation. The transformants were selected on LB plates containing ampicillin (100 μg ml^{-1}) in the absence of added D-glutamate.

The cloned nucleotides of the GluRA gene were sequenced by the dideoxy chain termination method of Sanger et al. [17] with Sequenase ver.2.0 and α-^{32}P (Amersham, CA). The sequences obtained were analyzed by the GENETIX program (Software Development Co., Japan).

2.4. Purification of GluRA

The recombinant *E. coli* WM335 cells carrying the plasmid pGSK23, which contains the GluRA gene of *Bacillus* sp. SK-1, were cultivated overnight in 3 l of LB broth at 37 °C and harvested by centrifugation. The cells (wet weight, 10 g) were washed twice with 0.85% NaCl, suspended in a 50 mM Tris–HCl buffer (pH 7.5) containing 2-mercaptoethanol (0.2%, v/v) and 0.1 mM phenylmethylsulfonyl fluoride, and then disrupted with a Branson sonicator (Branson Ultrasonics Co., CT). Cell debris and intact cells were removed by centrifugation, and the clear supernatant was used as a crude extract.

2.4.1. Heat treatment

The crude extract was kept at 55 °C for 30 min to remove heat-labile *E. coli* proteins, which had been aggregated during incubation, by centrifugation at 10,000 × *g* for 30 min.

2.4.2. Resource Q column-fast protein liquid chromatography (FPLC)

The heat-treated enzyme solution was applied to a Resource Q column with a 6 ml bed volume (Pharmacia, Sweden) equipped in a Pharmacia FPLC. The column was equilibrated with a 20 mM Tris–HCl buffer (pH 8.0), and the enzyme was eluted with a linear gradient of potassium chloride concentrations (0–0.5 M) in the same buffer at a flow rate of 6 ml min^{-1}. The active fractions were collected and dialyzed against a 50 mM potassium phosphate buffer (pH 7.2).

2.4.3. Phenyl sepharose column-FPLC

The dialyzed enzyme solution was purified with a Phenyl sepharose column (bed volume 20 ml, Pharmacia, Sweden) in FPLC. The elution was done with a linear gradient of 1.7–0.0 M ammonium sulfate in a 50 mM potassium phosphate buffer (pH 7.2) at a flow rate of 5 ml min^{-1}. The active fractions were collected and concentrated by ultrafiltration.

2.5. Molecular weight determination

The molecular weight of the purified GluRA was determined by gel filtration on a Superose 12 HR 10/30 column (Pharmacia, Sweden) with a 0.5 M potassium phosphate buffer (pH 7.5) as the mobile phase. The molecular weight was also determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide.

2.6. Production of D-phenylalanine in a multi-enzyme system

The production of D-phenylalanine was examined in a multi-enzyme system composed of four enzyme reactions, which are catalyzed by GluRA, D-AAT, GDH, and FDH (Fig. 1). D-Phenylalanine was produced from phenylpyruvate by a D-AAT reaction with the consumption of D-glutamate, which is

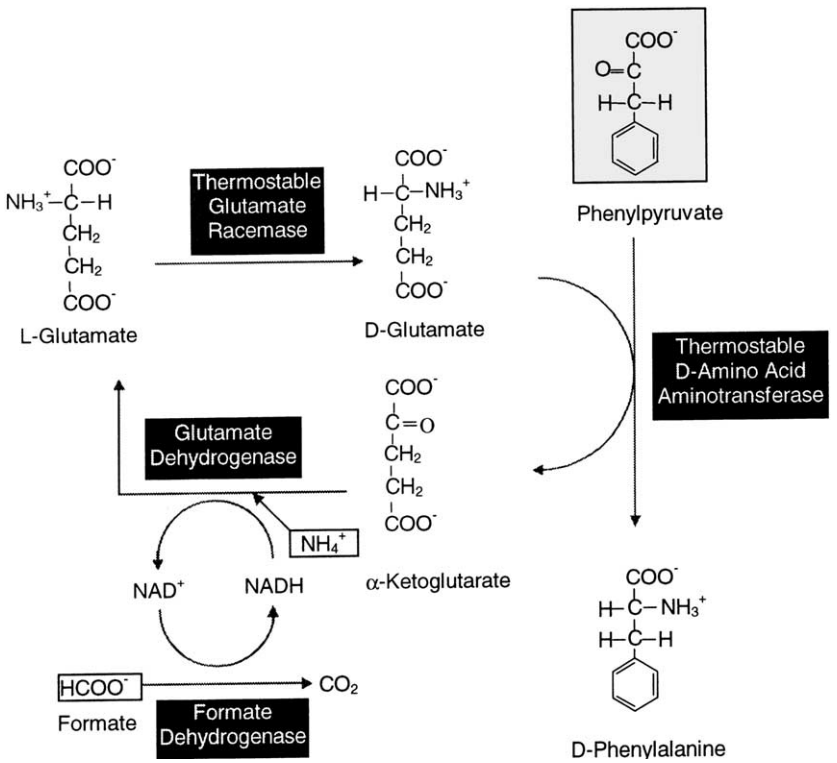

Fig. 1. A multi-enzyme system for the production of D-phenylalanine from phenylpyruvate.

continuously generated by the coupled reaction of GDH and GluRA from phenylpyruvate, NADH and ammonia. NADH is also generated by the FDH reaction from formate. Unless stated otherwise, the standard reaction mixture contained 10 mM L-glutamate, 1 mM NAD^+, 50 μM pyridoxyal 5′-phosphate (PLP), 100 mM Tris–HCl buffer (pH 8.5), 1 mM ammonium formate, an appropriate amount of phenylpyruvate, and four enzymes, GluRA, D-AAT, GDH, and FDH. The ratio for four enzyme was adjusted as follows: GluRA:D-AAT:GDH:FDH=1:5:10:1 in units each enzyme activity. The reaction was started by the addition of phenylpyruvate. One hundred microliters aliquot of the reaction mixture was sampled after the appropriate period of incubation, and then supplemented with 5 μl of 12N HCl to stop the reaction.

2.7. Enzyme and protein assay

Crude, partially purified, and purified enzyme solutions were prepared as described. The GluRA activity was assayed by measuring L-glutamate formed from the D-counterpart by HPLC with a C_{18} column and a fluorescence detector (excitation at 342 nm and emission at 452 nm). A mixture solution of an acetate buffer (50 mM, pH 6.8) and methanol (90:10 (v:v)) was used as a running buffer for HPLC analysis at the flow rate of 1 ml min^{-1}. The assay mixture (1 ml) containing 10 μmol D-glutamate, a 100 μmol Tris–HCl buffer (pH 8.5), and enzyme was incubated at 55 °C. The enzyme reaction was terminated by adding 5 μl 6 N HCl and centrifuged to obtain a clear supernatant. The supernatant solution was incubated with 10 volumes of an *o*-phthalaldehyde solution containing 10 mM *o*-phthalaldehyde and 20 mM *N*-acetyl-L-cysteine in a borate buffer (pH 9.5) at room temperature for 2–5 min. Ten microliters of an *o*-phthalaldehyde-tagging sample was loaded to HPLC to assay L- and D-glutamate in the reaction mixture.

One unit of enzyme was defined as the amount of enzyme catalyzing the formation of 1 μmol of product per min under the conditions used. Specific activity was expressed as units per mg of protein. Protein was determined by the method of Bradford [18] with bovine serum albumin as a standard protein.

3. Results and discussion

3.1. Cloning of the GluRA gene of Bacillus sp. SK-1

The chromosomal DNA of *Bacillus* sp. SK-1 was partially digested with *Sau*3AI and ligated into a *Bam*HI site of pUC118. *E. coli* WM335, a D-glutamate auxotroph [15], was transformed by the ligation mixture, and the transformants that grew on an LB medium containing ampicillin (100 μg ml^{-1}) in the absence of added D-glutamate were selected. A total of 35 D-glutamate-independent, ampicillin-resistant colonies were obtained. GluRA and D-AAT activities were assayed in the crude extracts of the colonies. Of the colonies, 30 exhibited GluRA activity, and another five exhibited D-AAT activity. From one of the colonies with GluRA activity, a plasmid containing a 3-kb insert was isolated, which was named pGSK23 (Fig. 2). The size of the recombinant plasmid pGSK23 was reduced to 1.8 kb with *Hin*d III, and the resulting plasmid was named pGSK231 (Fig. 2).

It is notable that the activities of both GluRA and D-AAT were detected in the transformed *E. coli* WM335 cells. The results indicate that *Bacillus* sp. SK-1 contains both GluRA and D-AAT genes. Earlier studies have indicated that *Bacillus* strains synthesize D-glutamate by D-AAT [19,20] and lactic acid bacteria by GluRA [3,21,22]. Thus, it is interesting to note that *Bacillus* sp. SK-1 contains both of the distinct genes in the same manner as *S. haemolyticus* [8].

3.2. DNA sequence and primary structure of Bacillus GluRA

The DNA sequence of the GluRA gene in the plasmid pGSK231 was analyzed (Fig. 3). The sequences show the predicted start codon, TTG, the SD sequence (GAGG) in 12 bases up-stream from the start codon, and typical promoter sites such as −10 (AAAACT) and −35 (TTGTCT). The open reading frame (ORF) consists of 792 bases encoding a protein of 264 amino acids. To confirm the start codon, the first 20 N-terminal amino acid sequence of the recombinant GluRA gene was determined by automated Edman degradation. The analyzed amino acid sequences are very consistent with those deduced from the DNA sequence and indicate that the first TTG codon serves as a start codon of GluRA.

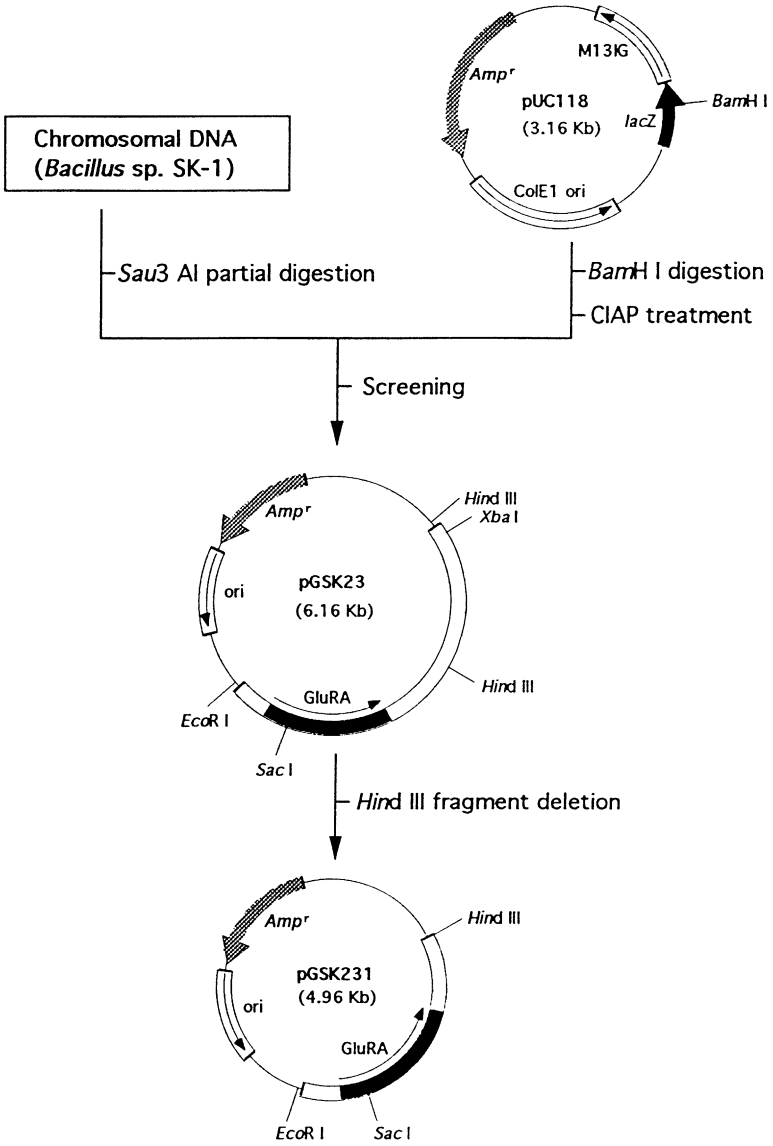

Fig. 2. Cloning of the glutamate racemase gene from a thermophilic *Bacillus* sp. SK-1. The closed bars indicate the structural gene of the thermostable glutamate racemase.

The amino acid sequence deduced from the SK-1 GluRA gene was compared with those found in other bacteria (Fig. 4). SK-1 GluRA shows 59.6% homology to that of *B. pumilis* [9], 50.8% to that of *S. haemolyticus* [8], 43.0% to that of *A. pyrophilus* [11], 37.0% to that of *Lactobacillus fermentum* [3], and 31.8% to that of *E. coli* [7]. The homologous regions were clustered, and two cysteine residues (Cys72 and Cys183) were found in these clusters, which is a common feature reported in other studies [4].

3.3. Expression and purification of glura in recombinant Escherichia coli

The recombinant *E. coli* cells containing pGSK23 revealed 0.025 U mg^{-1} of GluRA activity in crude

```
   1  TCAACACAGGGAAACAGCTATGACCCATGGATTACGGATTTCGAGTTTCGGTACCCCGGGGATTCGGCCACGAACTGAACGGGTTGAACG   91
  91  CCTTTCTTCTTCTTCGAAAAAGGGAAACGAATGGTCGAGGAAGTGATAAAAAGCGTCAACATGATTTGGTACAAGTGTTGCAAAATTTTT  181
 181  CTAATCAAGAAATTGTCTTTTTAGAAAATACTTACGAAAACTACATCAAGAAATGAAAAAAAGAATGAGGCGGATCAATTGGAAAGAGCA  271
                 -35                  -10                  SD        M  E  R  A
 271  ATTGGTGTTATTGACTCAGGAGTTGGTGGATTAACTGTTGCAAAAGAAATTATGCGACAGTTACCAAAAGAACGGATTGTTTATCTTGGG  361
      I  G  V  I  D  S  G  V  G  G  L  T  V  A  K  E  I  M  R  Q  L  P  K  E  R  I  V  Y  L  G
 361  GATACAGCTCGTTGCCCATATGGGCCACGCCCTAAAGAGGAAATTCGTCAATTTACATGGGAAATGACGAATTATTTATTGCGGTATAAT  451
      D  T  A  R  C  P  Y  G  P  R  P  K  E  E  I  R  Q  F  T  W  E  M  T  N  Y  L  L  R  Y  N
 451  ATTAAAATGCTTGTTATTGCTTGTAATACGGCAACAGCTGTCGTATTAGACGAAATTCGGGAACAGCTCGATATTCCGGTATTAGGAGTG  541
      I  K  M  L  V  I  A  C  N  T  A  T  A  V  V  L  D  E  I  R  E  Q  L  D  I  P  V  L  G  V
 541  ATACATCCTGGAGCTCGCGCGGCGTTAAAAGCGACGAAAAACGGGCATATCGGTGTGATTGGTACAATTGGTACAGTAAAAAGCGGTGCG  631
      I  H  P  G  A  R  A  A  L  K  A  T  K  N  G  H  I  G  V  I  G  T  I  G  T  V  K  S  G  A
 631  TATGAAAAGGCGCTAAAATCCATTAACCATCGAGTTCGTGTAGAAAGTTTAGCATGTCCAAAGTTTGTCCCGCTTGTAGAGAGCGGAAAC  721
      Y  E  K  A  L  K  S  I  N  H  R  V  R  V  E  S  L  A  C  P  K  F  V  P  L  V  E  S  G  N
 721  TTCGAAGGAGAGGAAGCGAGAAAGATAGTCGCTGAATCGCTTGAACCGTTTAAATCGAGCAATATGGATGTCCTTATTTTAGGATGCACT  811
      F  E  G  E  E  A  R  K  I  V  A  E  S  L  E  P  F  K  S  S  N  M  D  V  L  I  L  G  C  T
 811  CACTATCCTTTGTTAAGTCCTCTTATTAAAGAATATATGGGAAAACGCGTAAAACTAATTTGTTCCGGTGATGAAACCGCACGTGAAGTT  901
      H  Y  P  L  L  S  P  L  I  K  E  Y  M  G  K  R  V  K  L  I  C  S  G  D  E  T  A  R  E  V
 901  AGTGCGATTTTGCATCATAGCCATCTTCTTTATACAGGGCAACGAGAGGCTGAACATTTATTCTTTACGACAGGATCAAAGGAGCTATTC  991
      S  A  I  L  H  H  S  H  L  L  Y  T  G  Q  R  E  A  E  H  L  F  F  T  T  G  S  K  E  L  F
 991  CAAAAAATTGCTTCCAAATGGTTTGGAAAGCCAATTGAAAATGTTCAAACGATTGAATTATAAAAAAATCCTTTAGCGTAAAAGCTAAAG 1081
      Q  K  I  A  S  K  W  F  G  K  P  I  E  N  V  Q  T  I  E  L
1081  GATTTTTTTATTCTATTTTCAAAAAAGGCTCGTATACATAGATAGTAGTACAAACTATGAGTAGGAGGGAAAATTCATGTTTCATCGAGG 1171
1171  AACGTGGAAACTAGCTGCATCCGTTGTTACATCGCTGTTATTACTTAGTGGTTGTGGGTTATTTGGAAAGGATGAAGCTGTTAAAGAAAT 1261
1261  TGACCCGCCACAGGATGTGAGCTATTTAAAAGATGGGCAATCATTACAAGAAACAACTGGAAAACAAGGGGGAGAAAAAGACGAGAAAAA 1351
1351  AGCCACCGAAACAGTAAAACGCGAGCTTTACTTAATTGATAAAAATGGATTTGTCGTGCCGCAGACCGTAGAAACTGCCGAAGACAGATG 1441
1441  CTGTGGCAAAGCAAGTGCTCGAATATTTGGTGGAAAGATGGCCCTGTTTCGGAAATTGTTGCC  1503
```

Fig. 3. Nucleotide sequence of the gene encoding *Bacillus* sp. SK-1 glutamate racemase. The nucleotide numbering begins at the start codon, TTG. The deduced amino acid sequence is shown beneath the corresponding codons. The underlined amino acid sequence was determined.

extract (Table 1). Addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture did not affect the activity level of this enzyme, indicating that the enzyme was produced constitutively in the recombinant cells due to the functional promoter driven from *Bacillus* sp. SK-1. The production of GluRA in the recombinant *E. coli* cells was also indicated by the appearance of a thick protein band at the position corresponding to molecular weight 29,000 on SDS-PAGE (Fig. 5). Interestingly, neither GluRA activity nor a notable protein band at molecular weight 29,000 on SDS-PAGE was detected in the crude extract of *Bacillus* sp. SK-1, indicating that GluRA is not at all expressed or expressed at an extremely low level. In contrast, the crude extract of *Bacillus* sp. SK-1 showed 0.02 U mg^{-1} of D-AAT activity throughout the growth stages, suggesting that strain SK-1 would obtain D-glutamate via a route involving D-AAT activity.

GluRA expressed in the recombinant *E. coli* was purified from crude extract in three steps that included a heat treatment and two chromatographic procedures (Table 1 and Fig. 5). The heat treatment increased

```
Aquifex aerolicus            1:M-------------K-IGI-----------FDSGAGGMTVLKAIREAYPNVDVVYLGDTA 35
Bacillus pumilus             1:MLD-----------QPIGV-----------IDSGVGGLTVAKEIMRQLPKEKIIYVGDTK 38
Escherichia coli             1:MRQSMATKLQDGNTPCLAATPSEPRPTVLVFDSGVGGLSVYDEIRHLLPDLHYIYAFDNV 60
Lactobacillus fermentum      1:MDN-----------RPIGV-----------MDSGLGGLSVVRVIQQKLPNEEVIFVGDQG 38
Staphylococcus haemolyticus  1:MNK-----------PI-GV-----------IDSGVGGLTVAKEIMRQLPNETIYYLGDIA 37
Bacillus sp. SK-1            1:MER------------AIGV-----------IDSGVGGLTVAKEIMRQLPKERIVYLGDTA 37
                               *               ...            ***.**..* ..*   .* .   . .*

Aquifex aerolicus           36:RVPYGIRSKETIVRYSKECA-NFLK-DKGVDLLVVA-CNTASAYALEELKREFPFPVFGV 92
Bacillus pumilus            39:RCPYGPRKEEEVLHYTWEMA-HYLL-KHHHIKMLLIACNTATAIALDEIKATLDIPVIGV 96
Escherichia coli            61:AFPYGEKSEAFIVERVVAIVTA-VQERYPLALAVVA-CNTASTVSLPALREKFDFPVVGV 118
Lactobacillus fermentum     39:HFPYGTKDQAEVRQLALSIG-AFLL-KHDVKMMVVA-CNTATAAALPALQAALPIPVIGV 95
Staphylococcus haemolyticus 38:RCPYGPRPGDEVKQFTTQLA-NKLM-QFDIK-MLVIACNTATAVALEHLQQMLPIPVIGV 94
Bacillus sp. SK-1           38:RCPYGPRPKEEIRQFTWEMT-NYLL-RYNI-KMLVIACNTATAVVLDEIREQLDIPVLGV 94
                               . *** .   .          .       . .  ****.. .*  .   . .** **

Aquifex aerolicus           93:IEPGVKEAL-RKSKTKRIGVIGTQATIKSGAYQEALK-RA--GAEV--YSKACP-LFVPL 145
Bacillus pumilus            97:IQPGARTAI-KVTNNQHIGVIGTINTIKSEAYKEALL-SLKAGLTV--QSLAC--PLLVP 150
Escherichia coli           119:VP-AIKPAARLTAN-GIVGLLATRGTVKRS-YTHELIARFANECQIEMLGSAEMV-EL-A 173
Lactobacillus fermentum     96:IEPGARAAL-AQDKKGPIGVIATTATTTAGAYPATIE-RLAPGTPV--IAKATQPM-VEI 150
Staphylococcus haemolyticus 95:IEPGSRTAI-MTTKNQNVLILGTEGTIKSEAYRHHIK-HINPNVHV----LWCGLPGFVP 148
Bacillus sp. SK-1           95:IHPGARAAL-KATKNGHIGVIGTIGTVKSGAYEKALK-SINHRVRV--ESLAC--PKFVP 148
                               . .. . *     .   .....*  * .. .*   .         .     ..

Aquifex aerolicus          146:---VEEGMIEGEIPKKVVEHYLKDFKGKV-D--TLI-LGCTHYPLLKREIQNFLEGVNV- 197
Bacillus pumilus           151:F--VESGTFLDQTAEAVVKDS-LEPMKET-GIDTLI-LGCTHYPILKEP-IQRFMGSDVS 204
Escherichia coli           174:EAKLH-GE--DVSLDA--LKRILRPWLRMKEPPDTVVLGCTHFPLLQEELLQVLPEGTRL 228
Lactobacillus fermentum    151:---VEHGQTGTAKAQEVVSEQLMTFKEHP-VK-TLI-MGCTHFPFLAPEISKAVGPTVAL 204
Staphylococcus haemolyticus 149:L--VEQMRYDDPTITSIVIHQTLKQWRNT-DADTII-LGCTHYPLLYKPINDY-FGGEKK 203
Bacillus sp. SK-1          149:L--VESGNFEGEEARKIVAES-LEPFKSS-NMDVLI-LGCTHYPLLS-PLIKEYMGKRVK 202
                                  .. .        .   .          ... .****.*.*        .

Aquifex aerolicus          198:IDSSRATAK-SLKDFVKNE---GSGSL-----ELYFTDRSQN-LERLIK-L-ILGE-EVE 244
Bacillus pumilus           205:IISSGDETA-REASTILSYKGLLNTSKETPVHTFYTTGQQQN-FQNIARDW-FGYL-PGK 260
Escherichia coli           229:VDSGAAI-ARRTAW-LLEHEAPDAKSADANIAFCMAMTPGAEQLLPVL-QRYG-FETLE- 283
Lactobacillus fermentum    205:VDPAKETVA-TAKSWLEQHQAMGNHAHPNY--HLYSTGNLPD-LRAGVNKW-LLSG-HFD 258
Staphylococcus haemolyticus 204:VISSGLETA-REVSALLTFSNEHASYTQHPEHRFFATGDTVH-IKNIILQW-LKLD-VEV 259
Bacillus sp. SK-1          203:LICSGDETA-REVSAILHHSHLLYTGQREAEHLFFTTGSKEL-FQKIASKW-FGKP-IEN 258
                                 ..    . .  .  .                    ..           .

Aquifex aerolicus          245:PKITSEVF-VL-                                          254
Bacillus pumilus           261:VETVSLEHIYQQ                                         272
Escherichia coli           284:-KLAVLG-----                                         289
Lactobacillus fermentum    259:LGTAQ-IEEGD-                                         268
Staphylococcus haemolyticus 260:ERIS-VDE----                                        266
Bacillus sp. SK-1          259:VQTIEL------                                         264
```

Fig. 4. Comparison of deduced amino acid sequences of *Bacillus* sp. SK-1 glutamate racemase with those of glutamate racemases from *A. aerolicus*, *B. pumilus*, *E. coli*, *L. fermentum* and *S. haemolyticus*. Identical regions containing the essential cysteine residues were indicated in the boxes. Other identical amino acid sequences were indicated by asterisks.

Table 1
Purification of thermostable glutamate racemase from *E. coli* WM335 carrying pGSK23

Step	Total protein (mg)	Total activity (unit)	Specific activity ($U\,mg^{-1}$)	Yield (%)
Cell extract	1200	30.0	0.025	100
Heat treatment	252	25.0	0.100	83
Resource Q	37	13.7	0.370	46
Phenyl sepharose	29	12.2	0.420	41

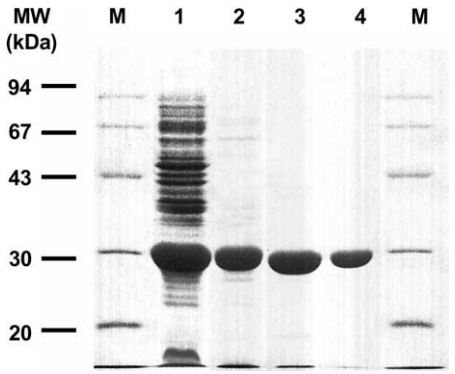

Fig. 5. SDS-PAGE analysis of *Bacillus* sp. SK-1 glutamate racemase at different stages of purification. Lane M, molecular weight markers; lane 1, crude extract of *E. coli* WM335 harboring plasmid pGSK23; lane 2, soluble fraction of crude extract after heat treatment at 55 °C for 30 min; lane 3, the partial purified enzyme after Resource Q column chromatography; lane 4, the purified glutamate racemase after phenyl sepharose column chromatography.

the purity by four-fold by removing the thermolabile *E. coli* proteins (Table 1). The homogeneous enzyme was obtained by further purifying the heat-treated enzyme solution with a resource Q column chromatography and a phenyl sepharose column chromatography. From 10 g of wet *E. coli* cells, we obtained 29 mg of a homogeneous enzyme with an overall yield of 41% (Table 1). The specific activity of the purified GluRA was 0.42 U mg^{-1}. On the basis of the specific activity of the purified GluRA compared with the activity in a crude cell extract, GluRA produced in the recombinant *E. coli* was calculated to be 10% of the total soluble cell protein.

3.4. Physicochemical properties of GluRA

As shown in Fig. 5 (lane 4), the homogeneously purified GluRA revealed a molecular weight of 29,000 on SDS-PAGE, which is quite consistent with the value calculated from the deduced amino acid sequence (29,386). On a gel filtration chromatography in the absence of denaturants, the enzyme also showed a molecular weight of 29,900 indicating that the GluRA is a monomeric protein.

The GluRA did not lose its activity after dialysis against a 50 mM potassium phosphate buffer (pH 7.2). The addition of pyridoxal 5′-phosphate (PLP), nicotinamides, or flavins up to 1 mM did not affect the enzyme activity. The spectrophotometric analysis of the purified enzyme did not show a characteristic absorption between 330 and 420 nm. These results suggest that the enzyme does not require PLP or other cofactors for its activity.

The catalytic constants of the enzyme were measured at 55 °C using D-glutamate or L-glutamate as a substrate. In the conversion of D-glutamate to L-glutamate, the K_m, V_{max}, and k_{cat} values were determined as 1.07 mM, 0.13 mM min^{-1}, and 0.36 s^{-1}, respectively. In the conversion of L-glutamate to D-glutamate, the K_m, V_{max}, and k_{cat} values were determined as 0.59 mM, 0.59 mM min^{-1}, and 1.50 s^{-1}, respectively.

3.5. Effects of pH and temperature on stability

To determine the thermostability of *Bacillus* sp. SK-1 GluRA, the purified enzyme was incubated at various temperatures for 30 min, and the remaining activity was measured. As shown in Fig. 6A, the enzyme was fully stable after incubation at 30–55 °C and maintained 60% of its original activity after incubation at 60 °C. However, more than 90% of the original activity was lost after incubation at 65–70 °C. The stability of the enzyme against pH was also determined by measuring the residual activity after incubation of the enzyme at pH 4.0–11.5 and 55 °C for 60 min. The enzyme maintained above 95% of its original activity after incubation at pH 6.0–11.5 (Fig. 6B). When the enzyme was incubated at pH 4 and 5, it lost 30 and 12% of the original activity, respectively.

Recently, a thermostable GluRA from a hyperthermophilic bacterium, *Aquifex pyrophilus*, was studied [11]. The *A. pyrophilus* GluRA was stable after incubation at 60 °C and retained 50% of the original activity after incubation at 85 °C in the presence of 1 M sodium phosphate. The thermostability of the enzyme is higher than that of *Bacillus* SK-1. On the other hand, *A. pyrophilus* GluRA has a 0.25 s^{-1} conversion rate (*k*cat) from L-glutamate to D-glutamate or from D- to L-glutamate. The *k*cat value is lower than those of *Bacillus* SK-1 GluRA (0.36 s^{-1} for the conversion from D- to L-glutamate, and 1.5 s^{-1} for the conversion from D- to L-glutamate).

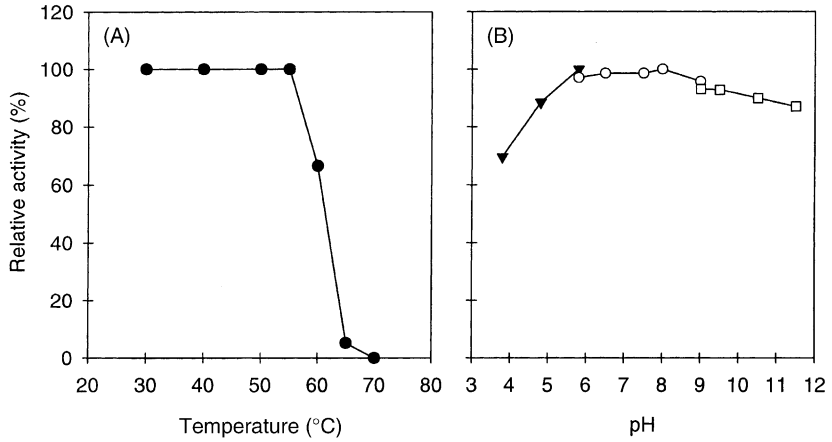

Fig. 6. Effect of temperature (A) and pH (B) on the stability of *Bacillus* sp. SK-1 glutamate racemase. The enzyme was incubated at a different temperature in a 50 mM Tris–HCl buffer (pH 7.5) for 30 min. The enzyme was also incubated at 55 °C for 60 min in buffers (50 mM) with a different pH, an acetate buffer (▼), a Tris–HCl buffer (○), and a borate buffer (□).

3.6. Production of D-phenylalanine in a multi-enzyme system

The *Bacillus* SK-1 GluRA was employed into the multi-enzyme system composed of four enzyme reactions (Fig. 1) for the production of D-phenylalanine. Fig. 7 shows the conversion of phenylpyruvate to D-phenylalanine in the multi-enzyme system. Phenylpyruvate was intermittently fed into 10 ml standard reaction mixture in order to keep its concentration below 50 mM at 37 °C. The D-phenylalanine production was compared with the result obtained

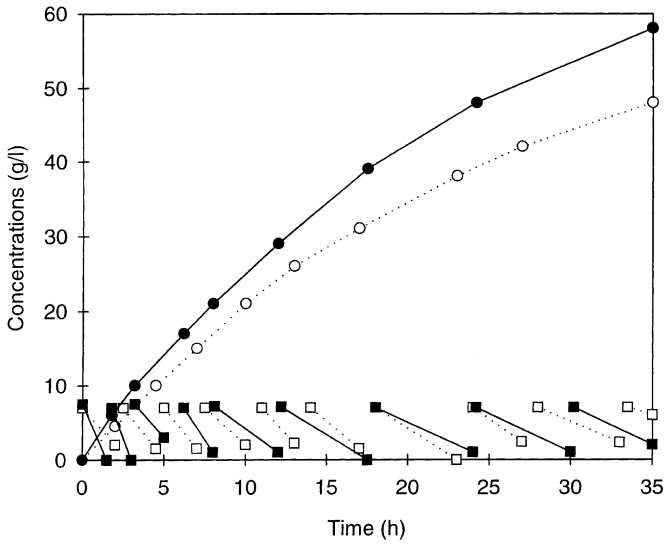

Fig. 7. Production of D-phenylalanine (●, ○) by successive feeding of phenylpyruvate (■, □) in the multi-enzyme system with *Bacillus* SK-1 GluRA (●, ■) or *E. coli* GluRA (○, □). The result from the multi-enzyme system with *E. coli* GluRA was adopted from [14]. The initial ammonium formate concentration was 1.0 M and the phenylpyruvate was intermittently added as described in text.

previously from the multi-enzyme system with *E. coli* GluRA [14]. By running the multi-enzyme system for 35 h, 58 g l^{-1} of D-phenylalanine was produced from the equimolar amount of phenylpyruvate supplemented. This productivity is higher than that in the multi-enzyme system with *E. coli* GluRA (48 g l^{-1}), which is due to an increased enzyme amount as indicated by the more rapid initial conversion rate. It is notable that the initial production rate of D-phenylalanine gradually decreased as the incubation time increased, which suggests that an enzyme(s) was inactivated during the operation of the multi-enzyme system. To determine the stability of four enzymes used at 37 °C, each enzyme was incubated at 37 °C for 10 h and the remaining activity was determined. GDH lost its activity more than 50% after 10 h incubation at 37 °C whereas other three enzymes, GluRA, D-AAT, and FDH retained their activities more than 85%. The result indicates that the decrease of D-phenylalanine production rate found in the multi-enzyme system is attributable to the instability of GDH.

4. Conclusion

We found a GluRA gene in a thermophilic *Bacillus* species SK-1. The gene was over-expressed in recombinant *E. coli* up to 10% of the total soluble protein of the cells although its expression was not demonstrated in stain SK-1. The gene product, GluRA, has thermostability after incubation at 55 °C and pH stability in the range of 6.0–11.5 (Fig. 6). Thermostability makes it possible to obtain highly purified GluRA from *E. coli* proteins with a simple heat-treatment procedure. The *Bacillus* SK-1 GluRA was employed in the multi-enzyme system for the production of D-phenylalanine at 37 °C. After running the multi-enzyme system for 35 h, 58 g l^{-1} of D-phenylalanine was produced from an equimolar amount of phenylpyruvate. During the operation of the multi-enzyme system, D-phenylalanine production rate gradually decreased because of the inactivation of GDH. Of four enzymes used in the multi-enzyme system, three enzymes, the thermostable *Bacillus* SK-1 GluRA, the thermostable D-AAT from a thermophilic *Bacillus* sp. YM-1 [23] and FDH from *Candida boidinii* (Boehringer Mannheim, Germany), showed a high stability at 37 °C while GDH from bovine liver (Boehringer Mannheim, Germany) showed a relatively low stability. Thus, instability of GDH is a critical point for the production of D-amino acids such as D-phenylalanine in the multi-enzyme system. Our next strategy for improving the multi-enzyme system is to replace GDH with a new thermostable GDH. Recently, we have found a gene encoding GDH in a commensal thermophile, *Symbiobacterium toebii*, which was isolated from a soil sample [24]. Studies on the production of the thermostable GDH and its application in the multi-enzyme system will be carried out.

Acknowledgements

This work was supported by National Research Laboratory (NRL) program grant NLM0020123 from the Ministry of Science and Technology of Korea.

References

[1] N. Nakajima, K. Tanizawa, H. Tanaka, K. Soda, J. Biotechnol. 8 (1988) 243.
[2] S.-Y. Choi, N. Esaki, T. Yoshimura, K. Soda, Protein Expres Purif. 2 (1991) 90.
[3] K.A. Gallo, J.R. Knowles, Biochemistry 32 (1993) 3981.
[4] M. Yagasaki, K. Iwata, S. Ishino, M. Azuma, A. Ozaki, Biosci. Biotechnol. Biochem. 59 (1995) 610.
[5] P. Doublet, J. van Hejienoort, D. Mengin-Lecreulx, J. Bacteriol. 174 (1992) 5772.
[6] T.J. Dougherty, J.A. Thanassi, M.J. Pucci, J. Bacteriol. 175 (1993) 111.
[7] T. Yoshimura, M. Ashiuchi, N. Esaki, C. Kobatake, S.-Y. Choi, K. Soda, J. Biol. Chem. 268 (1993) 24242.
[8] M.J. Pucci, J.A. Thanassi, H.T. Ho, P.J. Falk, T.J. Dougherty, J. Bacteriol. 177 (1995) 336.
[9] L. Liu, T. Yoshimura, K. Endo, N. Esaki, K. Soda, J. Biochem. 121 (1997) 1155.
[10] K.C. Malathi, M. Wachi, K. Nagai, FEMS Microbiol. Lett. 175 (1999) 193.
[11] S.S. Kim, I.-G. Choi, S.-H. Kim, Y.-G. Yu, Extremophiles 3 (1999) 175.
[12] M. Yagasaki, A. Ozaki, Y. Hasimoto, Biosci. Biotech. Biochem. 57 (1993) 1499.
[13] K.A. Gallo, M.E. Tanner, J.R. Knowles, Biochemistry 32 (1993) 3991.
[14] H.-S. Bae, S.-G. Lee, S.-P. Hong, M.-S. Kwak, N. Esaki, K. Soda, M.-H. Sung, J. Mol. Catal. B-Enzyme 6 (1999) 241.
[15] E.J.J. Lugtenberg, H.J.W. Wijsman, D. van Zaane, J. Bacteriol. 114 (1973) 499.

[16] H. Saito, K. Miura, Biochim. Biophys. Acta 72 (1963) 619.
[17] F. Sanger, S. Nicklen, A.R. Coulson, Proc. Natl. Acad. Sci. U.S.A. 74 (1977) 5463.
[18] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[19] C.B. Thorne, D.M. Molnar, J. Bacteriol. 70 (1955) 420.
[20] H.K. Kuramitsu, J.E. Snoke, Biochim. Biophys. Acta 62 (1962) 114.
[21] N. Nakajima, K. Tanizawa, H. Tanaka, K. Soda, Agric. Biol. Chem. 50 (1986) 2823.
[22] S.-Y. Choi, N. Esaki, T. Yoshimura, K. Soda, J. Boichem. 112 (1992) 139.
[23] K. Tanizawa, Y. Masu, S. Asano, H. Tanaka, K. Soda, in: T. Korpela, P. Christen (Eds.), Biochemistry of Vitamin B6, Birkhauser, Basel, 1987, p. 43.
[24] S.-P. Hong, J.-H. Park, Y.-S. Kim, H.-J. Hwang, S.-K. Rhee, S.-G. Lee, N. Esaki, M.-H. Sung, J. Microbiol. Biotechnol. 10 (2000) 405.