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ORIGINAL RESEARCH PAPER

Characterization of ribose-5-phosphate isomerase of *Clostridium thermocellum* producing D-allose from D-psicose

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Abstract The *rpiB* gene, encoding ribose-5-phosphate isomerase (RpiB) from *Clostridium thermocellum*, was cloned and expressed in *Escherichia coli*. RpiB converted D-psicose into D-allose but it did not convert D-xylose, L-rhamnose, D-altrose or D-galactose. The production of D-allose by RpiB was maximal at pH 7.5 and 65°C for 30 min. The half-lives of the enzyme at 50°C and 65°C were 96 h and 4.7 h, respectively. Under stable conditions of pH 7.5 and 50°C, 165 g D-allose l^{-1} was produced without by-products from 500 g D-psicose l^{-1} after 6 h.

Keywords D-Allose · *Clostridium thermocellum* · Isomerization · D-Psicose · Ribose-5-phospate isomerase

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Introduction

D-Allose, one of the rare aldohexoses, has attracted much attention as an inhibitor for ischemia/reperfusion injury, segmented neutrophil production, and lowered platelet count (Hossain et al. 2003, 2004, 2006). Moreover, a combination use of D-allose as a potent immunosuppressant with a low dose of FK506 significantly increased the rate of allograft survival with less tissue damage (Hossain et al. 2000). The biological manufacture of D-allose has been studied using L-rhamnose isomerase (L-RI) from *Pseudomonas stutzeri*, which is the only reported enzyme (Bhuiyan et al. 1998; Leang et al. 2004a; Morimoto et al. 2006; Menavuvu et al. 2006). Therefore, the investigation and further development for other sources of enzymes to produce D-allose should be greatly needed.

Ribose-5-phosphate isomerase (EC 5.3.1.6, RpiB) forms a homodimer and catalyzes the conversion of D-ribose-5-phosphate to D-ribulose-5-phosphate in the branch of the pentose phosphate pathway (Zhang et al. 2003). Moreover, this enzyme can take part in D-allose metabolism (Kim et al. 1997; Poulsen et al. 1999). However, D-allose production by the enzyme has not yet been reported.

In this study, the ribose-5-phosphate isomerase B (*rpiB*) gene from *Clostridium thermocellum* was cloned and expressed in *E. coli*. Furthermore, the production of D-allose from D-psicose by RpiB was investigated.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The genomic DNA from *Clostridium thermocellum* was used as a source of *rpiB* gene. *E. coli* BL21(DE3) was used as a host. pBluescript II SK(+) (Stratagene, La Jolla, CA, USA) and pQE30 (Qiagen, Valencia, CA, USA) plasmids were used as cloning and expression vectors, respectively.

The recombinant *E. coli* for protein expression was cultivated in a Luria-Bertani (LB) medium containing 50 μg ampicillin ml^{-1} and 20 μg kanamycin ml^{-1} at 37°C with agitation at 200 rpm until the OD_{600} reached 0.5. IPTG was added to the culture medium at 0.1 mM to induce RpiB and then the culture was grown at 16°C for 16 h.

Gene cloning

The *rpiB* gene (450 bp) was amplified from the genomic DNA of *C. thermocellum* by PCR using *Pfu* DNA polymerase (Daemyung Science, Seoul, Korea). The sequence of the oligonucleotide primers used for gene cloning was based on the DNA sequence of the *C. thermocellum* RpiB gene (GenBank Accession Number, ZP 00503831). Forward (RpiB-F, 5′-CCCATGGAGGAAAGTATGAAAATTGG-3′) and reverse primers (RpiB-R, 5′-CCTGCAGATCAACGGATGATCCATAAC-3′) were designed to introduce the *Nco*I and *Pst*I restriction sites (underlined) for *rpiB* gene cloning. The primers were synthesized by Bioneer Co. (Daejon, Korea). The amplified DNA fragment obtained by PCR was purified and digested with both *Nco*I and *Pst*I endonucleases (Promega, Madison, WI, USA). The digested DNA fragment was extracted, and then inserted into the pBluescript II SK(+) plasmid digested with the same restriction enzymes. The resultant plasmid, pBCTrpiB, was digested with both *Nco*I and *Pst*I and the obtained fragment, contains *rpiB* gene, was treated by Klenow fragment (Takara, Shiga, Japan). Finally, the fragment was inserted into pQE30 plasmid digested to completion with *Sma*I and *Pst*I and then the pQCTrpiB plasmid was obtained. *E. coli* BL21(DE3) strain was transformed with the plasmid and plated on LB agar containing 50 μg ampicillin ml^{-1} and 20 μg kanamycin ml^{-1} to select transformants. The expression of the gene encoding ribose-5-phosphate isomerase was determined by both SDS-PAGE and the assay of enzyme activity.

Enzyme assay

The activity of RpiB was determined by measuring D-allose formation using D-psicose as a substrate. Unless otherwise stated, the reactions were carried out in a 50 mM Tris/HCl buffer (pH 7.5) containing 1% (w/v) D-psicose and 0.03 units enzyme ml^{-1} at 50°C for 30 min. For the investigation of substrate specificity, the reactions were performed with 1% (w/v) of each sugar for 16 h. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol D-allose per min at 50°C and pH 7.5.

Analytical methods

Protein concentrations were determined by a Bio-rad assay with bovine serum albumin as a standard. The concentrations of monosaccharides were analyzed by the cysteine-carbazole method (Dische and Borenfreund 1951) or HPLC with a refractive index detector (Shimadzu SCL-10A, Kyoto, Japan) using NH2P-50 4E (Showa Denko, Tokyo, Japan), BP-100 Ca^{2+} carbohydrate (Benson Ploymeric, Reno, NV, USA), and cosmosil (Nacalai Tesque., Kyoto, Japan) columns which were eluted at 30°C with 70% (v/v) acetonitrile at 0.8 ml min^{-1}, at 80°C with water at 0.4 ml min^{-1}, and at 35°C with 80% (v/v) acetonitrile at 1 ml min^{-1}, respectively.

Results and discussion

Gene cloning

The *rpiB* genes based on the reported DNA sequence of RpiB was cloned into the pQE30 plasmid to produce pQCTrpiB and expressed in *E. coli*. The expressed pQCTrpiB was detected using SDS-PAGE. The molecular mass of the enzyme was estimated to be approximately 17 kDa by SDS-PAGE (data not shown) and this was consistent with the calculated value of 16,950 Da based on the 149-residue amino acid and six histidine sequences.

Although RpiB from *C. thermocellum* and L-RI from *P. stutzeri* can produce D-allose, the low homology between two enzymes indicated that two enzymes were not well aligned.

Substrate specificity

RpiB was active with D-allose, D-psicose, and D-ribose-5-phosphate but not other sugars (see Table 1). However, L-RI from *Pseudomonas stutzeri* converted L-rhamnose, D-xylose, D-altrose, and D-galactose (Leang et al. 2004b). These results suggest that RpiB from *C. thermocellum* is different from L-RI from *P. stutzeri*. Although D-ribose-5-phosphate is a substrate for RpiB, this enzyme had higher activities for D-allose and D-psicose.

pH, temperature, and metal ions effects

D-Allose production by RpiB was maximal at pH 7.5, whereas optimum pH for D-allose production by L-RI was at pH 9.0 (Bhuiyan et al. 1997). The activity of RpiB was maximal at 65°C for 30 min, while that of L-RI was shown at 60°C (Bhuiyan et al. 1997). Thermal inactivation of RpiB followed first-order kinetics with half-lives ($t_{1/2}$) of 96 h, 18 h, 8.1 h, 4.7 h, 1.7 h, and 0.3 h at 50°C, 55°C, 60°C, 65°C, 70°C, and 75°C, respectively (Fig. 1). The results indicated RpiB from *C. thermocellum* has relatively unstable above 55°C. Therefore, the further reactions were performed at 50°C.

D-Allose-producing activity from D-psicose by RpiB was not activated by monovalent or divalent cations and not inhibited by EDTA because phosphosugar isomerases are metal-independent enzymes (Teplayakov et al. 1999). However, L-RI did not show any activity after its treatment with EDTA (Bhuiyan et al. 1997).

Table 1 Sugar conversion yield of ribose-5-phosphate isomerase of *C. thermocellum*

Sugars[a]	Conversion yield (%)	Product
D-Allose	69	D-Psicose
D-Psicose	33	D-Allose
D-Ribose 5-phosphate	9	D-Ribulose 5-phosphate

[a] No reactions with L-rhamnose, D-xylose, D-altrose, and D-galactose occurred

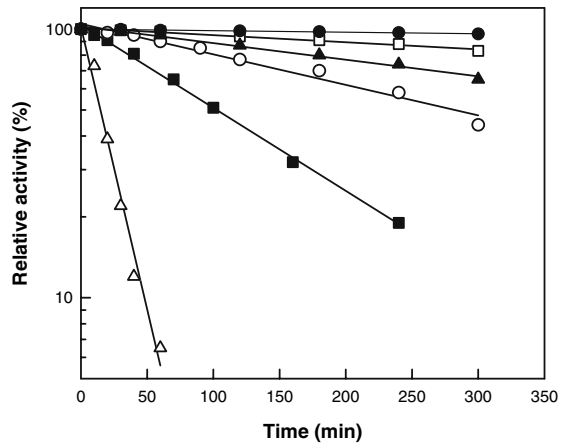

Fig. 1 Thermal inactivation of *C. thermocellum* RpiB for D-allose production at temperatures of 50°C (•), 55°C (□), 60°C (▲), 65°C (○), 70°C (■), and 75°C (Δ)

Bioconversion of D-psicose into D-allose

Using 500 g D-psicose l^{-1}, 165 g D-allose l^{-1} was obtained with RpiB after 6 h at 50°C and pH 7.5, corresponding to the conversion yield of 33% (Fig. 2). A similar conversion occurred using D-psicose at 1.25, 2.5, and 5 g l^{-1} with 2.8 units enzyme ml^{-1} for 6 h. Previously, the L-RI from *P. stutzeri* converted 100 g D-psicose l^{-1} into 25 g D-allose l^{-1} with 8 g D-altrose l^{-1} as a by-product

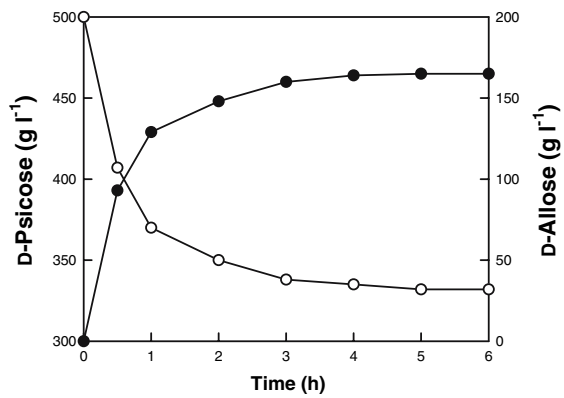

Fig. 2 Time course of D-allose production (•) from D-psicose (○) by *C. thermocellum* RpiB

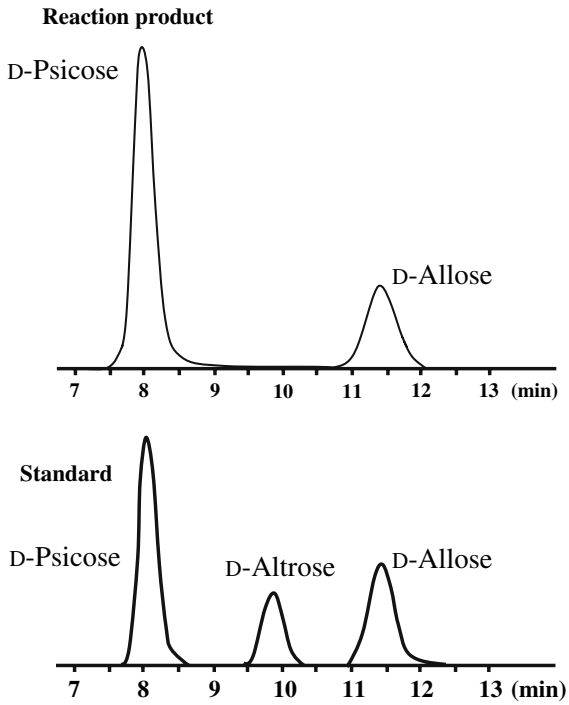

Fig. 3 HPLC analysis of the reaction product of *C. thermocellum* RpiB and standard compounds of D-psicose, D-altrose, and D-allose

(Menavuvu et al. 2006) and an immobilized L-RI produced 150 g D-allose l^{-1} from 500 g D-psicose l^{-1} as a by-product (Morimoto et al. 2006). The yield and concentration of D-allose obtained with RpiB of *C. thermocellum* were therefore higher than those with L-RI of *P. stutzeri* which, unfortunately, produced a significant amount of D-altrose as a by-product, which is a serious problem in the purification of D-allose. However, the RpiB of *C. thermocellum* used in this study did not produce D-altrose (Fig. 3).

The use of RpiB has advantages of higher yield and no by-product formation. Moreover, the metal-independent RpiB dose not require metal ions in the reaction, thus it is probably more suitable for diverse molecular evolution than metal-dependent L-RI. Therefore, RpiB of *C. thermocellum* is a more proper enzyme to produce D-allose from D-psicose than L-RI of *P. stutzeri*, which has been known to the only one enzyme for the D-allose production.

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