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RESEARCH PAPER

Characterization of a Recombinant L-Rhamnose Isomerase from *Bacillus subtilis* and Its Application on Production of L-Lyxose and L-Mannose

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Abstract The gene of an L-rhamnose isomerase (RhaA) from *Bacillus subtilis* was cloned to the pET28a(+) and then expressed in the *E. coli* ER2566. The expressed enzyme was purified with a specific activity of 3.58 U/mg by His-Trap affinity chromatography. The recombinant enzyme existed as a 194 kDa tetramer and the maximal activity was observed at pH 8.0 and 60ºC. The RhaA displayed activity for L-rhamnose, L-lyxose, L-mannose, D-allose, D-gulose, D-ribose, and L-talose, among all aldopentoses and aldohexoses and it showed enzyme activity for L-form monosaccharides such as L-rhamnose, L-lyxose, L-mannose, and L-talose. The catalytic efficiency (k_{cat}/K_m) of the recombinant enzyme for L-rhamnose, L-lyxose, and L-mannose were 7,460, 1,013, and 258 M/sec. When L-xylulose 100 g/L and L-fructose 100 g/L were used as substrates, the optimum concentrations of RpiB were determined with 6 and 15 U/mL, respectively. The L-lyxose 40 g/L was produced from L-xylulose 100 g/L by the enzyme during 60 min, while L-mannose 25 g/L was produced from L-fructose 100 g/L for 80 min. The results suggest that RhaA from *B. subtilis* is a potential producer of L-form monosaccharides.

Keywords: *Bacillus subtilis*, characterization, L-rhamnose isomerase, substrate specificity, L-lyxose, L-mannose

1. Introduction

Functional monosaccharides have recently gained increasing attention due to their possible applications as low-calorie sweeteners, bulking agents, antioxidants, inhibitors of microbial growth and glycosidases, nucleoside analogues, and immunosuppressants [1-8].

Generally, functional monosaccharides have been mainly produced by chemical synthesis [9-11]. However, the chemical processes have many disadvantages, including low overall yield, many reaction steps, complex purification steps, and by-product formation. Because of them, the biotransformation of functional monosaccharides by enzymes have been recently attracted much attention as one of alternative production methods for functional monosaccharides to overcome disadvantages of chemical production.

Among various enzymes such as sugar isomerase, sugar epimerase, and phosphate-sugar isomerase, sugar isomerases with broad specificities have been especially paid attention for the conversion of monosaccharides because they have enzyme activity for various types of monosaccharides [12,13].

The L-rhamnose isomerase (RhaA, EC 5.3.1.14), reversibly catalyzes the isomerization of L-rhamnose to L-rhamnulose, is one of the sugar isomerases and have been researched to produce functional monosaccharides [14-17].

To date, RhaAs from *Escherichia coli*, *Pseudomonas stutzeri*, *Aeribacillus pallidus,* and *Thermotoga maritime* have been characterized the substrate specificities to produce functional monosaccharides [18-21]. From the substrate specificities of RhaAs, it has been reported that the RhaAs, especially, have enzyme activity for L-form monosaccharides such as L-fructose, L-lyxose, L-talose. The L-form monosaccharides have been recently attracted much attention as potential functional materials for the various industries including food and pharmaceutical industries [1].

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Generally, *B. subtilis* has been known as GRAS (Generally Recognized As Safe) microorganisms and isolated from fermented food such as Natto, Chungkook-Jang, and Doenjang, *etc* [22-24]. Therefore, the enzymes from *B. subtilis* can be used safely in the fields of various biological industries. From these reasons, it is suggested that the L-rhamnose isomerase from *B. subtilis* can be used as a useful producer for functional monosaccharides.

In this study, we report that the gene encoding an L-rhamnose isomerase from the *B. subtilis* was cloned and expressed in *E. coli*. To produce functional monosaccharides, the characters of enzyme for pH, temperature, metal ions, and molecular mass were investigated and substrate specificity of the enzyme for monosaccharides was also evaluated. Moreover, the L-rhamnose isomerase from *B. subtilis* exhibited enzyme activity for L-form monosaccharides such as L-lyxose, L-xylulose, L-mannose, and L-fructose. Thus, the production of L-lyxose and L-mannose by RhaA from *B. subtilis* was attempted.

2. Materials and Methods

2.1. Bacterial strains, plasmid, and culture conditions

Genomic DNA from *B. subtilis* ATCC 23857, *E. coli* ER2566, and pET-24a (+) were used as the source of RhaA gene, host cells, and expression vector, respectively. The recombinant *E. coli* for protein expression were cultivated with shaking at 200 rpm in a 2,000 mL-flask containing 500 mL Luria-Bertani (LB) medium at 37°C with 20 μg kanamycin/mL until the OD_{600} reached 0.6. Isopropyl-β-D-thiogalctopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce enzyme expression and then the culture was grown at 16°C for 16 h.

2.2. Gene cloning

The gene encoding a putative protein was amplified by PCR using *B. subtilis* genomic DNA as a template. The sequence of the oligonucleotide primers used for gene cloning was based on the DNA sequence of the protein from *B. subtilis* Bsn5 (GenBank accession number NC_014976). Forward (5'-AAGCTAGCATGACCATAAAAGCCAATTAT-3') and reverse primers (5'-TTCTCGAGTTAGACAATCGGAGGAGGATG-3') were designed to introduce the *Nhe*I and *Xho*I restriction sites (underlined) and were synthesized by Bioneer (Daejon, Korea). The amplified DNA fragment obtained by PCR was purified and inserted into the pGEM-T easy vector (Promega, Madison, WI). *E. coli* Top10 strain was transformed with the ligation mixture and plated on LB agar containing 50 μg ampicillin/mL, 0.1 mM IPTG and 80 μg X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)/mL. Ampicillin-resistant white colonies were selected, and plasmid DNA from these transformants was isolated using a plasmid purification kit (Solgent, Daejon, Korea). The *Nde*I-*Xho*I fragment from the T-vector containing the gene encoding RhaA was subcloned into the same sites of pET-24a (+) plasmid and the resulting plasmid was obtained. The plasmid was transformed into *E. coli* ER2566 strain and grown on LB medium containing 20 μg kanamycin/mL. The expression of the gene encoding RhaA was analyzed by both SDS-PAGE and assay of enzyme activity.

2.3. Enzyme purification

The cells were harvested from culture broth by centrifugation at 6,000 ×*g* for 20 min at 4°C, washed twice with 0.85% (w/v) NaCl, and resuspended in lysis buffer (pH 8.0) containing 50 mM NaH_2PO_4 and 300 mM NaCl with 1 mg lysozyme/mL. The resuspended cells were disrupted on ice using a sonicator. The supernatant of disrupted cell was applied onto a His-Trap HP chromatography column (Amersham Biosciences, Uppsala, Sweden) and eluted with a linear gradient from 10 to 250 mM imidazole at 1 mL/min. The active fraction was collected and dialyzed against 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid) (EPPS) buffer (pH 8.0). After dialysis, the resulting solution was used as the purified enzyme.

2.4. Molecular mass determination

The subunit molecular mass of RhaA from *B. subtilis* was examined by SDS-PAGE under denaturing conditions, using the proteins of a pre-stained ladder (MBI Fermentas, Hanover, MD) as reference proteins. All protein bands were stained with Coomassie blue for visualisation. The molecular mass of native enzyme was determined by gel filtration chromatography using a Sephacryl S-300 HR 16/60 preparative-grade column. The enzyme solution was applied to the column and eluted with 50 mM citric acid buffer (pH 5.5) containing 150 mM NaCl at a flow rate of 1 mL/min. The molecular mass of the native enzyme was calculated by comparing with the migration length of reference proteins.

2.5. Effects of metal ions, pH, and temperature

Unless otherwise stated, the reaction was performed in 50 mM EPPS buffer (pH 8.0) containing 10 mM L-rhamnose and 0.5 U enzyme/mL in the presence of 1 mM Mn^{2+} at 60°C for 10 min. To investigate the effect of metal ions on the activity of RhaA, the enzyme activity was measured after treatment with EDTA at 4°C for 1 h or after adding 1 mM of each metal ion, such as $BaCl_2$, $CaCl_2$, $CoSO_4$, $CuSO_4$, $FeSO_4$, $MgCl_2$, $MnSO_4$, $NiSO_4$, or $ZnSO_4$ to EDTA-treated enzyme. To find the maximum activity of the enzyme, pH was varied from 6.5 to 8.5 using 50 mM

piperazine-*N*, *N*'-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.5 ~ 7.5) and 50 mM EPPS buffer (pH 7.5 ~ 8.5) at 85ºC, and the temperature range was from 45 to 70°C at pH 8.0. Thermal inactivation of RhaA from *B. subtilis* was also carried out from 55 to 75°C at pH 8.0. For investigating thermostability, the enzymes were incubated at temperatures ranging from 55 to 75°C for varying periods of time. A sample was withdrawn at each time interval and was assayed for the remaining enzyme activity in optimum conditions. The experimental data for thermal deactivation of enzyme were fitted to a first order curve and the half-lives of the enzyme calculated using Sigma plot 9.0 software (Systat Software, San Jose, CA, USA). The relative activity of 100% was 0.5 U enzyme/mL.

One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of L-rhamnulose from L-rhamnose per min at 60°C and pH 8.0.

2.6. Substrate specificity

To measure the specific activity for aldoses, ketoses, and aldose phosphates, enzyme reactions were performed at 60ºC in 50 mM EPPS buffer (pH 8.0) with 10 mM monosaccharide and 1 mM Mn^{2+} by adjusting the enzyme concentration (0.5 ~ 10 U/mL) and reaction time (10 ~ 20 min). The specific activity was defined as the produced amount of aldose, ketose, or ketose phosphate as a product per enzyme amount per reaction time. The enzyme kinetic parameters, K_m (mM), and k_{cat} (/sec) for substrates were determined by fitting the data to the Michaelis-Menten equation.

2.7. Equilibrium ratio

The equilibrium ratio between aldose and ketose was determined from the average value using three initial ratios of 0:100, 50:50, and 100:0 in 50 mM EPPS buffer (pH 8.0) at 60ºC for 6 h. Each reaction mixture contained 1 mM monosaccharides and 20 U/mL of enzyme.

2.8. Production of L-lyxose and L-mannose

To determine the optimal enzyme concentration, the enzyme concentration was varied from 2 to 10 U/mL for L-lyxose production and from 5 to 25 U/mL for L-mannose production. The reaction was performed in 50 mM EPPS buffer (pH 8.0) containing L-xylulose 100 g/L or L-fructose 100 g/L at 60ºC for 2 h.

The time-course of L-lyxose production by *B. subtilis* RhaA was investigated in 50 mM EPPS buffer (pH 8.0) containing 6 U/mL of enzyme and L-xylulose 100 g/L at 60ºC for 2 h, while that of L-mannose production was investigated in the same buffer containing 15 U/mL of enzyme and L-fructose 100 g/L for 2 h.

2.9. Analytical methods

The concentration of monosaccharides was determined using a Bio-LC system (Dionex ICS-3000, Sunnyvale, CA) with an electrochemical detector and a CarboPac PAI column. The column was eluted at 30°C with 200 mM sodium hydroxide at 1 mL/min.

3. Results and Discussion

3.1. Gene cloning, purification, and molecular mass determination of the RhaA from *B. subtilis*

The gene (1,275 bp) encoding the putative protein from *B. subtilis* which has 99.5% of identity with sequence as a L-rhamnose isomerase gene reported in GenBank (accession number NC_014976), was cloned and expressed in *E. coli*. In the comparison of amino acid sequence, the RhaA from *B. subtilis* showed 16, 18, 57, and 58% identity with those of RhaAs from *Pseudomonas stutzeri, Thermotoga maritime, E. coli*, and *A. pallidus*, respectively. Although the amino acid composition of RhaA form *B. sutilis* has low identity with other RhaAs, the active site residues of Trp 185, Glu226, Lys228, Asp258, His261, His286, Asp294, Asp296, and Asp326 in *E. coli* RhaA were absolutely conserved in those of RhaA (Fig. 1) [20].

The expressed enzyme was purified as a soluble protein from crude extract by His-Trap HP chromatography with a final purification of 25-fold, a yield of 47%, and a specific activity of 3.58 U/mg. The molecular mass of the purified enzyme from *B. subtilis*, determined by SDS-PAGE, was approximately 48.6 kDa (Fig. 2), which is consistent with the calculated value of 49,468 Da with Compute pI/Mw software based on the 424 amino acid residues including 6 His residues. The native enzyme was estimated as a tetramer with a molecular mass of 194 kDa as determined by gel filtration chromatography using a Sephacryl S-300 HR 16/60 column.

3.2. Substrate specificity of the RhaA from *B. subtilis* for monosaccharides

The specific activity of the RhaA from *B. subtilis* was investigated with the D- and L-forms of all pentoses and hexoses (Table 1). Among aldose substrates, the highest specific activity was observed with L-rhamnose, followed by L-lyxose, L-mannose, D-allose, D-gulose, D-ribose, L-talose. From the result of substrate specificity of RhaA from *B. subtilis* for aldoses, we knew that the enzyme displayed activity only with aldose substrate that possess hydroxyl groups oriented in the right-handed configuration (Fischer projections) at the C-2 and C-3 positions, such as L-rhamnose, L-lyxose, L-talose, D-gulose, D-ribose, L-mannose,

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BsRhaA·····MT--IKANYDSAKQAYEKWGIDVEEALRQLEQVPISIHCWQGDDIEGFEVNKGELSGGID
EcRhaA·····MTTQLEQAWELAKQRFAAVGIDVEEALRQLDRLPVSMHCWQGDDVSGFENPEGSLTGGIQ
ApRhaA·····MV—IKESFEIARQVYEKWGINIEEVLENLQQASISHPLLAGDDVKRFEADASELSGGID
           *·········*·*·····**··**·*··*····*·····***···**·····*·***·

BsRhaA·····VTGNYPGKAQTPEELRRDLEKALSLIPGKHRVNLHAIYAETNREAVERDELKPQHFENWV
EcRhaA·····ATGNYPGKARNASELRADLEQAMRLIPGPKRLNLHAIYLES-DTPVSRDQIKPEHFKNWV
ApRhaA·····VTGNYPGKQEMLKNKAGFRESAVINS--RKTSTYTPIMQKTNGEKVERRSVRTETFGKLG
···········*******·····*·*·····*·····*·*·····*···

BsRhaA·····KWAKNLGLGLDFNPTLFSHEKAADGLTLSHPDPDIREFWIRHCIACRRIGEYFGKELGTP
EcRhaA·····EWAKANQLGLDFNPSCFSHPLSADGFTLSHADDSIRQFWIDHCKASRRVSAYFGEQLGTP
ApRhaA·····NWGKKNGYRAGFKSYLFSHEKQQMDGRGLFRIRELENLGSTHCIRSRRIGEYLARAG---
············*·*·····*···***·····**···**···*·····

BsRhaA·····CLTNIWIPDGYKDIPSDRLTPRKRLKESLDRIFSEEISEQHNLDSIFSKLFGLGSESYVV
EcRhaA·····SVMNIWIPDGMKDITVDRLAPRQRLLAALDEVISEKLNPAHHIDAVFSKLFGIGAESYTV
ApRhaA·····HMLTIYDPDAIRRSKRP-IDSKEAIKRIMDKIFSVEINEKYNLDAVFSKLFGIGSESFVV
···········*··**·····*···*·····*··******·*·**··*

BsRhaA·····GSHEFYLAYALTNHKLCLLDTGHFHPTETVSNKISSMLLYTDKLALHVSRPVRWDSDHVV
EcRhaA·····GSNEFYMGYATSRQTALCLDAGHFHPTEVISDKISAAMLYVPQLLLHVSRPVRWDSDHVV
ApRhaA·····GSHEFYLGYALQNNKIYLLDTGHFHPTETVSNKISSILLYSDRLALHVSRPVRWDSDHVV
···········**·***··**·····**·*******·*·***···**···*·**********

BsRhaA·····VLDDELREIALEIVRNHALEKVAIGLDFFDASINRVAAWTIGTRNMIKALLYALLLPNGY
EcRhaA·····LLDDETQAIASEIVRHDLFDRVHIGLDFFDASINRIAAWVIGTRNMKKALLRALLEPTAE
ApRhaA·····ILDDELREIALEIVRNDALHKVLIGLDFFDASINRLAAWVIGTRNMIKALLYAMLMPHEY
···········****···**·****·····*·**********·***·******·****·*·*·*···

BsRhaA·····ASSPIV
EcRhaA·····------
ApRhaA·····------
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Fig. 1. Alignment of amino acid sequences of RhaA. The alignment of amino acid sequences of BsRhaA, L-rhamnose isomerase from *B. subtilis* (YP_004204944); EcRhaA, L-rhamnose isomerase from *E. coli* (CAA43002), and ApRhaA, L-rhamnose isomerase from *A. Pallidus* (BAF80456) was performed using ClustalW program. The Amino acids that are conserved in all the amino acid sequences are marked by asterisks (*). The residues related with active site of RhaA from *E. coli* are highlighted with black.

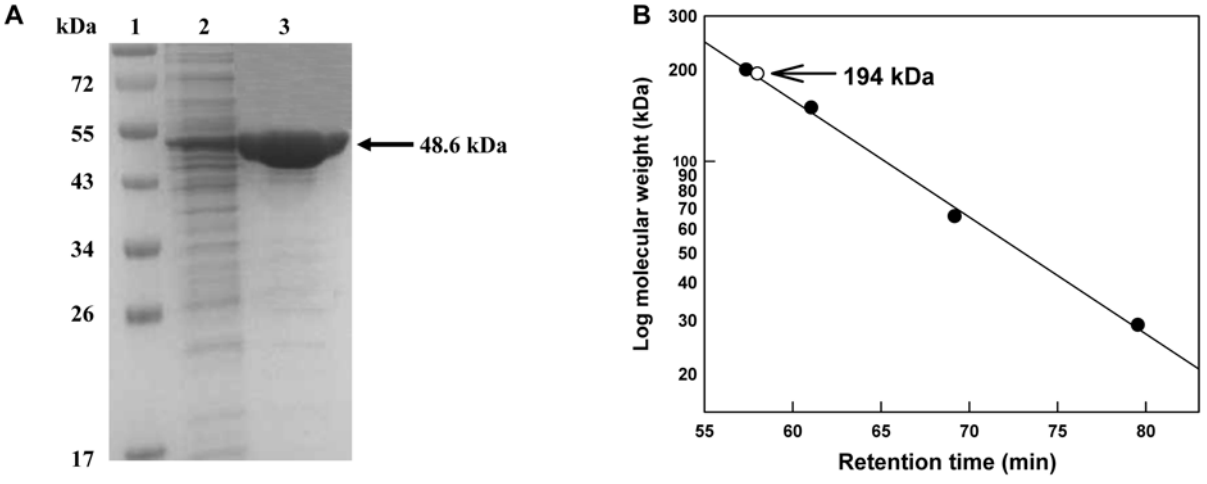

Fig. 2. Molecular mass of RhaA from *B. subtilis*. (A) SDS-PAGE analysis of purified enzymeat each purification step, lane 1, prestained marker proteins; lane 2, crude extract; lane 3, His-Trap HP column product (purified enzyme). (B) Determination of molecular mass of the native RhaA from *B. Subtilis* by gel-filtration chromatography, β-Amylase (200 kDa), aldolase (158 kDa), albumin (67 kDa), and ovalbumin (43 kDa) as reference proteins (●); RhaA from *B. subtilis* (○).

Table 1. Specific activity for monosaccharides of RhaA from *B. subtilis*

	Substrate	Product	Specific activity (μmol/min/mg)
Aldose	L-Rhamnose	L-Rhamnulose	3.58 ± 0.03
	L-Lyxose	L-Xylulose	2.58 ± 0.05
	L-Mannose	L-Fructose	0.92 ± 0.01
	D-Allose	D-Psicose	0.467 ± 0.01
	D-Gulose	D-Sorbose	0.136 ± 0.04
	D-Ribose	D-Ribulose	0.094 ± 0.001
	L-Talose	L-Tagatose	0.029 ± 0.001

Data represent the means of three experiments with standard deviation.

Table 2. Kinetic parametersof RhaA from *B. subtilis*

Substrate	K_m(mM)	k_{cat}(/sec)	k_{cat}/K_m(/M/sec)
L-Rhamnose	53 ± 0.3	153 ± 1.8	2,887 ± 18
L-Lyxose	86 ± 0.9	53 ± 0.9	616 ± 5.7
L-Mannose	97 ± 1.7	37 ± 0.2	381 ± 3.9

Data represent the means of three experiments with standard deviation.

and D-allose (Table 2). The open-chain structures of the monosaccharides involved in the aldose-ketose isomerization reactions catalyzed by the enzyme are illustrated in Fig. 3. Although the substrate specificity of RhaA from *B. subtilis* showed very similar character with those of Rhas from *E. coli*, *P. stutzeri*, *A. pallidus*, and *T. maritime* because these enzymes had enzyme activity for L-rhamnose, L-lyxose, L-mannose, D-allose, and D-ribose [12,20,21], this enzyme can be also applied to production of L-form monosaccharides due to its enzyme activities for L-form monosaccharides such as L-rhamnose, L-lyxose, L-mannose, and L-tagatose.

The kinetic parameters of the enzyme for L-rhamnose, L-lyxose, and L-mannose are shown in Table 2. The K_m for L-rhamnose was 1.6-fold lower than for L-lyxose. The k_{cat} for L-rhamnose was 2.9-fold higher than that for L-lyxose. As a result, the k_{cat}/K_m for L-rhamnose was 4.7-fold higher than that for L-lyxose. The specific activity and k_{cat}/K_m were the highest for L-rhamnose among substrates. From these results, we confirm experimentally that the sugar isomerase from *B. subtilis* is a RhaA.

3.3. Effects of metalions, pH, and temperature on the activity of RhaA from *B. subtilis*

In the metal ions experiment, Mn^{2+} was the most effective for the isomerization of L-rhamnose by RhaA from *B. subtilis*, resulting in 25-fold increase activity relative to no treatment (data not shown). The optimal Mn^{2+} concentration was 1 mM (data not shown). It have been reported that RhaAs from *E. coli*, *P. stutzeri*, *B. pallidus*, and *T. maritima* are metal-dependent enzymes and their activities were the highest with Mn^{2+} [12,18,20,21]. Consequently, all subsequent experiments were performed in the presence of 1 mM Mn^{2+}. The activity of RhaA from *B. subtilis* was examined over a pH range of 6.5 ~ 8.5 at 60ºC and a temperature range of 45 ~ 70°C at pH 8.0. The maximum enzyme activity was observed at pH 8.0 and 60°C (data not shown). The thermostability was examined by measuring the activity at temperatures ranging from 55 to 75°C. The RhaA from B. subtilis exhibited authentic enzyme activity after heating 6 h at the 60°C. Moreover,

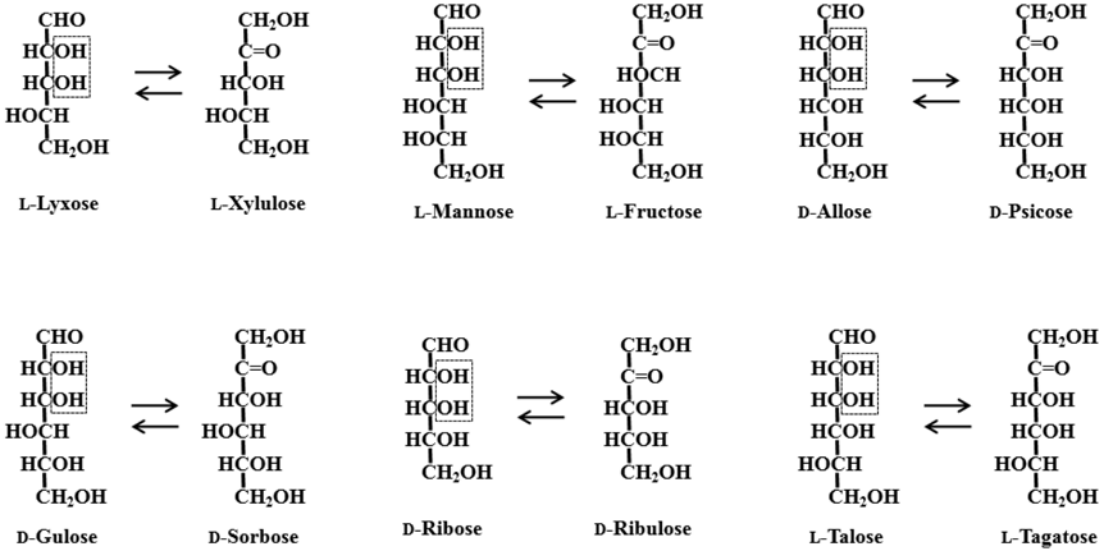

Fig. 3. Schematic representation of aldose-ketose isomerization reactions catalyzed by RhaA from *B. subtilis*. The boxed structure indicates the hydroxyl configurations at C-2 and C-3 positions of the sugars.

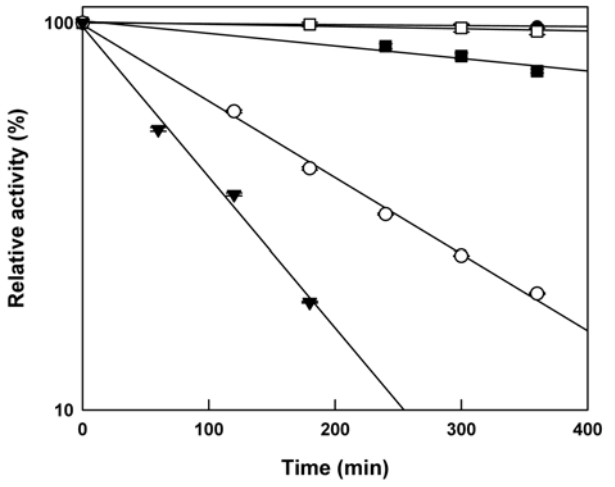

Fig. 4. Thermal inactivation of RhaA from *B. subtilis.* The enzymes were incubated at 55 (●), 60 (□), 65 (■), 70 (○), and 75°C (▼). A sample was withdrawn at each time interval and was assayed for the remaining enzyme activity. Data represent the means of three experiments and error bars represent standard deviation.

the enzyme remained approximate 53% enzyme activity in the incubation at 70°C for 2 h (Fig. 4). From these results, it is suggested that the enzyme has enough thermostability to produce functional monosaccharides at the optimum temperature. Except for RhaA from *T. maritima*, the optimum temperatures of RhaAs from the mesophiles *E. coli*, *P. stutzeri*, and *A. pallidus* have been reported as 60, 60, and 65. And Optimum pH of RhaA has been reported range of pH 7.0 ~ 8.5. From these results, the RahA from *B. subtilis* has similar enzyme characters with reported RhaAs. Although RhaA from *B. subtilis* has similar enzyme characters with reported RhaAs, the enzyme has significant merit to produce functional monosaccharides because the enzyme produces only one product from one substrate. The RhaA from *P. stutzeri* produces two products, the counterpart ketose and aldose for substrate, from one substrate because of its broad substrate specificity for monosaccharides. This acts serious problem on the production of the specific monosaccharides by RhaA because one product in the both products is regarded as by-product. Although the RhaA from *T. maritime* produce one product from one substrate, the enzyme has maximum activity at the 85°C which is too high temperature to treat monosaccharides because the high reaction temperature is causative of the maillard reaction of monosaccharides. The RhaAs from *A. pallidus* has the most similar characters with the RhaA from *B. subtilis* in the substrate specificity for monosaccharides and maximum temperature. However, the optimum temperature of RhaA from *B. subtilis* is lower than that of RhaA from *A. pallidus*. From these results, it is suggested that the RhaA from *B. subtilis* has proper characters for substrate specificity and temperature to produce functional monosaccharides. Moreover, the RhaA from *B. subtilis* is the first reported RhaA from GRAS microorganisms because the reported RhaAs have been isolated from *Pseudomonas* sp. *Thermotoga* sp. and *Aeribacillus* sp. This means that RhaA from *B. subtilis* can be applied also usefully in the various food related industries. In the previous report, the RhaA from *A. pallidus* was reported as RhaA from *B. pallidus* in 2007 [20], however, the strain is renamed as *A. pallidus* from 16S rRNA gene sequence divergence and the presence of unique phenotypic characteristics in 2010 [25]. Therefore, the RhaA from *B. subtilis* is characterized as the first RhaA from *Bacillus* strain in this study.

To determine the equilibrium ratio between aldose and ketose, the enzyme reactions were performed with a low substrate concentration (1 mM monosaccharides), and a high enzyme concentration (20 U/mL) in stable extended reactions (60ºC and 6 h). The equilibrium ratio between L-lyxose and L-xylulose was 40:60, while that of L-mannose and L-fructose was 25:75 (data not shown)

3.4. Production of L-lyxose and L-mannose by *B. subtilis* RhaA

The enzyme concentration for L-lyxose production was varied from 2 to 10 U/mL with L-xylulose 100 g/L as the substrate. L-Lyxose conversion yield was increased with increasing the amount of enzyme, whereas it reached a plateau at concentrations above 6 U/mL of enzyme. The optimal enzyme concentration for effective L-lyxose production was determined to be 6 U/mL (Fig. 5A). L-Mannose production was tested at the enzyme concentrations ranging from 5 to 25 U/mL with L-fructose 100 g/L and its optimal enzyme concentration was determined to be 20 U/mL (Fig. 5B).

The production of L-lyxose by *B. subtilis* RhaA was performed with 6 U/mL of enzyme and L-xylulose 100 g/L for 2 h (Fig. 6A). When the reaction time was increased, the concentration of L-lyxose increased and reached a maximum (40 g/L) at 50 min, corresponding to a conversion yield of 40%. The production of L-mannose was performed with 20 U/mL of enzyme and L-mannose 100 g/L for 2 h (Fig. 6B). The enzyme produced L-mannose 25 g/L for 80 min with a conversion yield of 25%.

L-Lyxose is a useful L-form monosaccharide because of its potential usage such as functional sweetener and in chemotherapy [26]. L-Mannose has much potential applications in food and pharmaceutical industry as an unnatural sugar [27]. Thus, the discovery of active RhaA from *B. subtilis* in the present study has important meaning for the production of functional L-form monosaccharides.

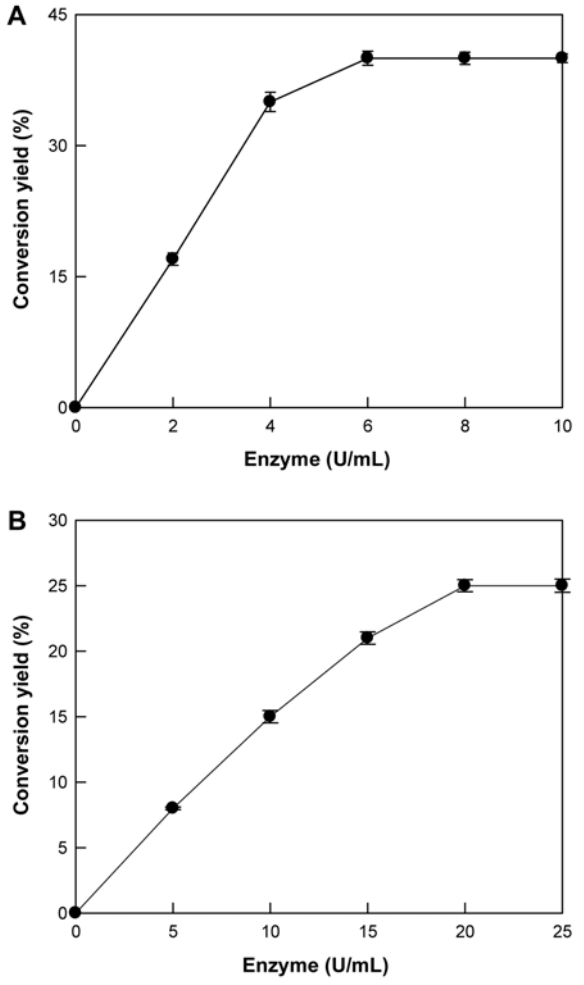

Fig. 5. Effect of enzyme activity on the production of L-lyxose (A) and L-tagatose (B) by RhaA from *B. subtilis*. Data represent the means of three separate experiments.

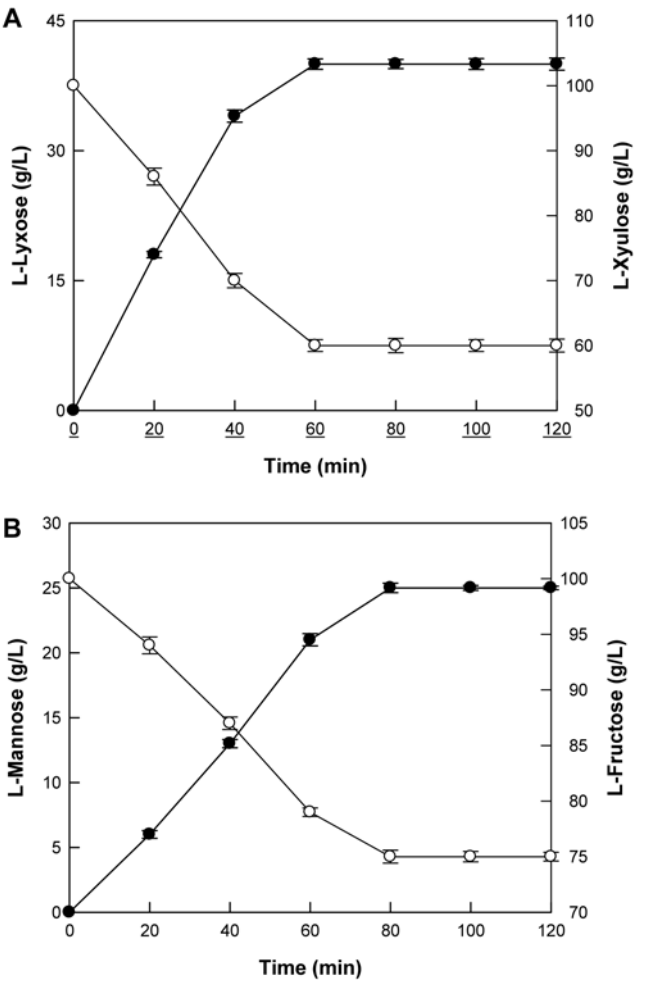

Fig. 6. Production of L-form monosaccharides by RhaA from *B. subtilis*: (A) L-Lyxose production (●) from L-xylulose (○). (B) L-Tagatose (●) production from L-talose (○). Data represent the means of three separate experiments.

4. Conclusion

A gene encoding L-rhamnose isomerase (RhaA) from *B. subtilis* was cloned, expressed, purified, and characterized. In the comparison of amino acid sequence, the amino acid sequence of RhaA from *B. subtilis* showed low identity with those of RhaAs from *Pseudomonas stutzeri, Thermotoga maritime, E. coli*, and *A. pallidus*, respectively. And the RhaA from *B. subtilis* shows enzyme activity for various substrates such as L-lyxose, L-mannose, D-allose, D-gulose, D-ribose, L-talose as well as L-rhamnose. From the result of substrate specificity of RhaA from *B. subtilis* for aldoses, we knew that the enzyme displayed activity only with aldose substrate that possess hydroxyl groups oriented in the right-handed configuration (Fischer projections) at the C-2 and C-3 positions. In the conditions of pH 8.0 and 60ºC, the RhaA from *B. subtilis* produced L-lyxose and L-mannose from L-xyulose and L-lyxose with 40 and 25% of conversion rate, respectively. From these results, we concluded that the RhaA from *B. subtilis* can be applied to produce L-form sugars as a useful producer.

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