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Expression of *manB* Gene from *Escherichia coli* in *Lactococcus lactis* and Characterization of Its Bifunctional Enzyme, Phosphomannomutase

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Phosphomannomutase (ManB) converts mannose-6-phosphate (M-6-P) to mannose-1-phosphate (M-1-P), which is a key metabolic precursor for the production of GDP-D-mannose used for production of glycoconjugates and post-translational modification of proteins. The aim of this study was to express the *manB* gene from *Escherichia coli* in *Lactococcus lactis* subsp. *cremoris* NZ9000 and to characterize the encoded enzyme. The *manB* gene from *E. coli* K12, of 1,371 bp and encoding 457 amino acids (52 kDa), was cloned and overexpressed in *L. lactis* NZ9000 using the nisin-controlled expression system. The enzyme was purified by Ni-NTA column chromatography and exhibited a specific activity of 5.34 units/mg, significantly higher than that of other previously reported ManB enzymes. The pH and temperature optima were 8.0 and 50°C, respectively. Interestingly, the ManB used in this study had two substrate specificity for both mannose-1-phosphate and glucose-1-phosphate, and the specific activity for glucose-1-phosphate was 3.76 units/mg showing 70% relative activity to that of mannose-1-phosphate. This is the first study on heterologous expression and characterization of ManB in lactic acid bacteria. The ManB expression system constructed in this study canbe used to synthesize rare sugars or glycoconjugates.

Keywords: *Lactococcus lactis*, phosphomannomutase, mannose-1-phosphate, mannose-6-phosphate

Introduction

Lactic acid bacteria (LAB) are used as starter in the various fermented foods such as kimchi, sauerkraut, yogurt [1]. LAB have numerous beneficial probiotic effects such as resistance to acid and bile, production of antimicrobial substances, and modulation of intestinal microflora [2]. Moreover, LAB have been given a 'generally recognized as safe (GRAS)' status according to the U.S. Food and Drug Administration (FDA). Consequently, LAB microbial systems offer alternatives to *Escherichia coli*, and are candidates for the production of bioresource materials [3]. Accordingly, several gene expression systems have been developedand among them, *Lactococcus lactis* is increasingly applied in food biotechnology, with a tightly controlled gene expression system using the bacteriocin nisin [4, 5]. Despite many advantages, *L. lactis* omits the metabolic pathway to production of nucleotide sugars such as GDP-D-mannose, GDP-L-fucose, GDP-D-rhamnose, and UDP-sialic acid. This results in the inability of the species to produce rare sugars, glycoconjugates, and lipo- orexo-polysaccharides.

Phosphomannomutase (ManB also PMM, E.C. 5.4.2.8), an enzyme belonging to a family of phosphohexomutase, catalyzes the interconversion of mannose-6-phosphate (M-6-P) to mannose-1-phosphate (M-1-P) and its gene (*manB*) is absent in *L. lactis*. M-1-P is essential for the production of GDP-D-mannose, whichis used for production of structural carbohydrates, alginates and ascorbic acid, lipo- and exo-polysaccharide of many pathogenic bacteria, and post-translational modification of proteins in prokaryotes and eukaryotes [6, 7]. In addition, GDP-D-mannose is a key

metabolic intermediate for the production of various glycoconjugates such as GDP-L-fucose, GDP-D-talose, and GDP-D-rhamnose [8, 9].

Therefore, the *manB* gene and encoded enzyme ManB which responsible for M-1-P synthesis was expressed in *L. lactis*. For this, the *manB* gene from *E. coli* was cloned and expressed in *L. lactis* subsp. *cremoris* NZ9000 (*L. lactis* NZ9000) using the nisin controlled expression system. Then, enzyme activity, optimal pH, optimal temperature, and substrate specificity of the purified enzyme were characterized.

Materials and Methods

Materials and Chemicals

L. lactis NZ9000 and the pNZ8008 plasmid (MoBiTec, Göttingen, Germany) were used as the host and gene expression vectors, respectively. *L. lactis* NZ9000 was grown in M17 medium (Difco, USA) with 0.5% glucose (GM17) at 30°C. *E. coli* MC1061 (MoBiTec) was used as the cloning host; it was grown in LB medium at 37°C under shaking conditions. Ten μg/ml chloramphenicol was used to select the transformants. The nisin which was used for induction of gene expression was prepared as follows: 2.5% nisin powder (Sigma, USA) dissolved in 0.05% acetic acid and the final concentration was fixed as 1 ng/ml. All chemicals and enzymes were purchased from Sigma Co. (USA).

Construction of Recombinant Plasmid

The *manB* gene from *E. coli* K12 (ATCC 10798) genomic DNA was amplified using the primers: *manB*-N (5′-TTCTGCAGATGA AAAAATTAACCTGCTTT-3′) and *manB*-C (5′-TTGAATTCTCTA GATTAGTGGTGATGATGGTGATGCTCGTTCAGCAACGTCA-3′). The *manB* open reading frame (1,371 bp) was inserted into pNZ8008 to construct pNZmanB (6,348 bp) recombinant plasmid. pNZmanB was transformed into *E. coli* MC1061 and the transformants were selected on LB agar containing chloramphenicol.

Expression of *manB*

For *manB* gene expression, the constructed recombinant plasmid pNZmanB was transformed into *L. lactis* [10]. Then, it was grown in GM17 broth containing chloramphenicol until $O.D_{600nm}$ 0.4. To induce the protein expression, 1 ng/ml nisin was added and it was further incubated for 3 h at 30°C. Cells were then collected by centrifugation at 10,000 ×*g* for 5 min, and the pellets were resuspended in 100 mM Tris-HCl buffer (pH 7.0). Cells were disrupted by sonication (on 50 sec, off 10 sec, for 10 min), and the supernatant fraction was used as the crude enzyme preparation. The enzyme fraction was purified using Ni-NTA chromatography with a His Trap-FF column (GE Healthcare, Uppsala, Sweden). Expression and purification of ManB were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Assay of ManB Enzyme Activity and Reaction Products

For assaying ManB activity, 50 μl crude or purified enzyme was reacted with 300 μl Tris-HCl buffer (100 mM, pH 7.0) containing 1.5 mM M-1-P, 0.25 mM glucose 1,6-bisphosphate, 2 mM $MgCl_2$, 2 mM $NADP^+$, 1 U phosphoglucose isomerase, 1 U phosphomannose isomerase, and 1 U glucose-6-phosphate dehydrogenase at 37°C. The reduction of 1 μmol $NADP^+$ per min, which was measured at 340 nm was defined as one unit of activity [11]. Protein concentration was measured by a Thermo BCA protein assay kit (Pierce Biotechnology Inc., USA). Product quantitation was performed using a high-performance anion exchange chromatography (HPAEC) system (Bio-LC ICS 3000; Dionex Co., USA) with a pulsed amperometric detector (ED50; Dionex Co.) and a CarboPac PA1 column (0.2 × 25 cm; Dionex Co.). The mobile phases were prepared as follows: 150 mM NaOH in water was used as solvent A and 150 mM NaOH and 600 mM sodium acetate in water was used as solvent B. Solvent B was increased from 0 to 35% from 0 to 35 min, and holding 100% solvent B from 35 to 45 min and 100% solvent A from 45 to 60 min. The flow rate of the mobile phase was 1.0 ml/min.

Characterization of ManB

The effects of pH on ManB were analyzed in 100 mM sodium phosphate buffer (pH 5.0–7.0), 100 mM Tris-HCl buffer (pH 8.0–10.0), and 100 mM glycine-sodium hydroxide (pH 11.0–12.0) at room temperature. The enzyme reaction solution was as mentioned above. To measure the effect of temperature on ManB activity, reactions were performed at a range of temperatures (10–60°C). In addition, glucose-1-phosphate was used as substrate for ManB to investigate substrate specificity.

Results and Discussion

Codon Usage Analysis

In order to determine whether the heterologous *manB* gene from *E. coli* could be expressed in *L. lactis* at high levels, codon usage was analyzed. For this, DNA sequences and codon information of both *manB* gene and *L. lactis* were obtained from GenBank (http://www.ncbi.nlm.nih.gov) and the SMS (http://www.bioinformatics.org/sms2/) site, respectively, and their respective codon usage was compared. The GC content of *manB* was 55%, which was higher than that of *L. lactis* NZ9000 (35.8%). However, the codon usage was found to be similar in the 2 cases, except 5 amino acid residues encoded by codons that the codon usage values wereless than 20% in *L. lactis*; they were CUG (Leu), CCG (Pro), UGC (Cys), CGC (Arg), GGC (Gly) (Table 1). In general, acodon usage value below 20% is considered as representing an "unfavorable codon" [12]. These results showed that the differences of codon usage between *manB* gene and *L. lactis* would not affect the

Table 1. Codon usage data of *manB* gene and *L. lactis* NZ9000 genome.

AA	Codon	*manB* gene[a]	*L. lactis*[b]	Codon usage (%)[c]	AA	Codon	manB gene[a]	*L. lactis*[b]	Codon usage (%)[c]
Phe	UUU	28.45	36	127	Ala	GCU	10.94	30.1	275
	UUC	17.51	11.6	66		GCC	32.82	12.3	37
Leu	UUA	13.13	30.9	235		GCA	10.94	23.1	211
	UUG	4.38	21.6	493		GCG	32.82	8.2	25
	CUU	0	25.4	-	Tyr	UAU	15.32	27.9	182
	CUC	17.51	8	46		UAC	10.94	7.7	70
	CUA	2.19	7.5	342	ter	UAA	2.19	2.4	110
	CUG	59.08	5.9	10		UAG	0	0.4	-
Ile	AUU	26.26	51.9	198		UGA	0	0.7	-
	AUC	30.63	16.2	53	Gln	CAA	4.38	30.8	703
	AUA	0	8.6	-		CAG	10.94	6.1	56
Met	AUG	26.26	24.8	94	Asn	AAU	17.51	40.4	231
Val	GUU	13.13	31.6	241		AAC	30.63	10.7	35
	GUC	15.32	12.3	80	Lys	AAA	43.76	61	139
	GUA	4.38	12.9	295		AAG	8.75	12.6	144
	GUG	37.2	9	24	Asp	GAU	43.76	38.1	87
Ser	UCU	0	16.5	-		GAC	30.63	14.7	48
	UCC	8.75	3.1	35	Glu	GAA	52.52	57.3	109
	UCA	0	21.3	-		GAG	15.32	12.6	82
	UCG	4.38	3.8	87	Cys	UAU	2.19	3.5	160
	AGU	0	14.4	-		UGC	8.75	1.1	13
	AGC	19.69	5.8	29	Trp	UGG	8.75	9.9	113
Pro	CCU	4.38	11.6	265	Arg	CGT	13.13	14.7	112
	CCC	10.94	2.9	27		CGC	45.95	4.5	10
	CCA	4.38	15	342		CGA	4.38	5.6	128
	CCG	26.26	2.9	11		CGG	2.19	2.3	105
Thr	ACU	4.38	20.8	475		AGA	0	8	-
	ACC	32.82	7.3	22		AGG	0	1.4	-
	ACA	0	22.1	-	Gly	GGU	26.26	24.1	92
	ACG	15.32	6.8	44		GGC	50.33	8.4	17
His	CAU	8.75	13.2	151		GGA	6.56	24.7	377
	CAC	13.13	4.5	34		GGG	8.75	8.3	95

[a]Frequency per thousand in manB gene; [b]Frequency per thousand in *L. lactis* NZ9000 genome; [c]Codon usage was defined as codon number in *L. lactis* divided by codon umber in *manB* gene.

expression levels. Therefore, the native *manB* gene was inserted into pNZ8008, resulting the recombinant plasmid pNZmanB, and it was transformed into *L. lactis* NZ9000. Codon usage analysis is very important when expressing heterologous genes. Due to the differences of codon usage between the expression gene and host, the rare codons would be accumulated during expression and low levels of heterologous gene expression could be caused [13, 14].

Gene Expression and ManB Enzyme Purification

L. lactis harboring pNZmanB was grown in GM17 broth containing chloramphenicol until $O.D_{600nm}$ 0.4. To induce the protein expression, 1 ng/ml nisin was added and it was further cultured for 3 h at 30°C. Non-induced cells of *L. lactis* harboring the same plasmid were also grown as a control. The cell growth of induced recombinant *L. lactis* ($O.D_{600nm}$ 0.9) was lower than that of non-induced cells

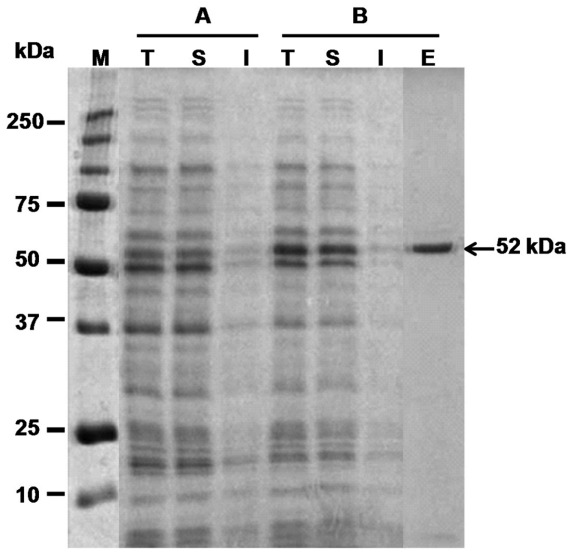

Fig. 1. SDS-PAGE analysis of ManB expressed in *L. lactis*.
A, *L. lactis* harboring pNZmanB without induction; B, *L. lactis* harboring pNZmanB with nisin induction; M, molecular weight markers; T, total fraction; S, soluble fraction, I, insoluble fraction of cell extracts; E, purified enzyme after Ni-NTA purification.

($O.D_{600nm}$ 1.1) (data not shown). The recombinant protein was expressed as the soluble fraction and it was purified by Ni-NTA affinity chromatography by using a histidine tag at the C-terminus. As shown in Fig. 1, 52 kDa recombinant protein of ManB which was similar with the theoretical molecular weight (50.5 kDa) was confirmed by SDS-PAGE analysis.

Analysis of Enzyme Function

Enzyme activities were measured before and after enzyme purification using M-1-P as substrate. As result, the crude and the purified enzymes had a specific activity of 0.59 U/mg and 5.34 U/mg, respectively, revealing that the enzyme activity was enriched 9.05 fold after the purification process (Table 2). Notably, ManB activity in this study was meaningfully higher than that of ManB found in previous studies, *i.e.*, 100 mU/mg for *Salmonella enterica*, 0.32 mU/mg for *Pseudomonas aeruginosa* PAO1, 2.0 mU/mg for *Pseudomonas aeruginosa* 8821, and 0.8–3.0 mU/mg for *Azotobactervinelandii* [15]. ManB enzymes from mammalian, fungal, and plant cells have been characterized as requiring bisphosphate sugars (glucose 1,6-bisphosphate used in this study) which used for the phosphoryl donor for the C1 or C6 phosphate in the reaction [16]. In the proposed reaction mechanism, it transfers the phosphoryl group of the active site of the enzyme to substrate to form a bisphosphorylated catabolic intermediate in the first step. Then, another phosphoryl group is transferred from the intermediate to the enzyme in the second step. Finally, the phosphoryl-transferred product is produced [17]. Therefore, the key feature of the activator, glucose-1,6-bisphosphate, is regarded as maintaining the enzyme in its phosphorylated state.

Table 3. Substrate specificity of purified ManB after expression in *L. lactis*.

Substrates	Specific activity (units/mg)	Relative activity (%)
Mannose-1-phosphate	5.34	100
Glucose-1-phosphate	3.76	70

Characterization of Bifunctional ManB

To further characterize the enzyme, the optimal pH and temperature of ManB were also measured. It exhibited relatively high activity between pH 7.0 and 10.0, with maximum activity at pH 8.0 (Fig. 2A). The effect of temperature on purified ManB activity was measured at temperatures range from 10°C to 60°C: the highest activity was obtained at 50°C and the relatively optimal temperature was between 40°C and 50°C. However, ManB did not show any activity at lower temperatures (≤20°C) or higher temperatures (≥60°C), revealing that the enzyme is very sensitive to the reaction temperature (Fig. 2B).

In a previous study, the ManB enzyme from *E. coli* was specific for M-6-P, but in contrast ManB enzymes from other bacteria and eukaryotes are bifunctional, showing activity for both M-6-P and glucose-6-phosphate [6, 7]. Therefore, we also measured ManB enzyme activity using

Table 2. Enzyme activities of ManB before and after purification.

Steps	Volume (ml)	Total activity (units)	Protein (mg)	Specific activity[a] (units/mg)	Purification fold	Yield (%)
Crude enzyme	100	28.6	48.81	0.59	1	100
Ni-NTA purification	100	25.7	4.81	5.34	9.05	90

[a]One unit of activity corresponds to the oxidation of 1 μmol NADPH per min.

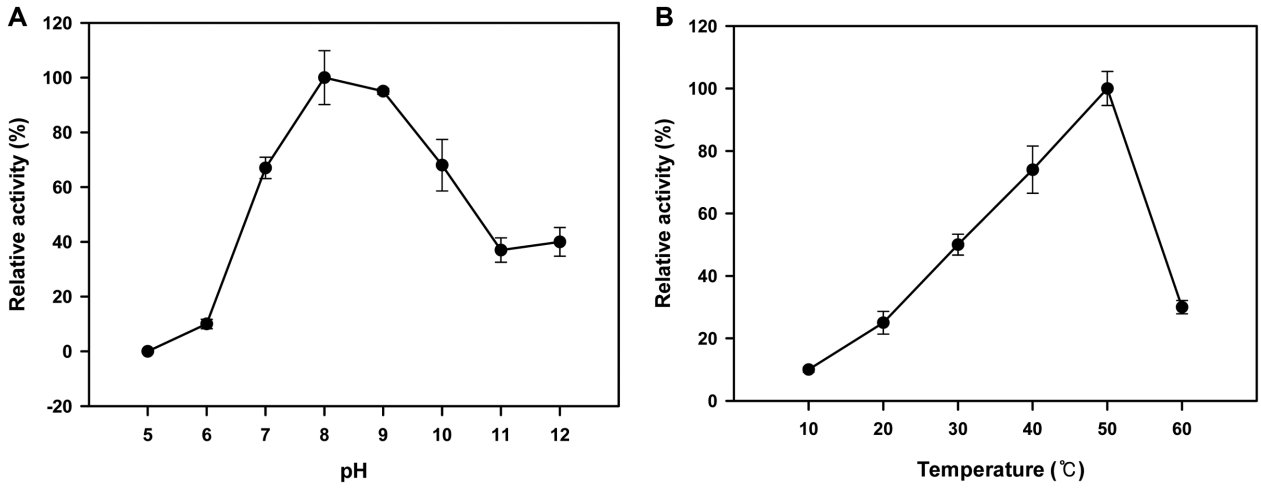

Fig. 2. Effects of pH (**A**) and temperature (**B**) on ManB activity. Maximum activity was defined as 100%.

glucose-1-phosphate as substrate. As shown in Table 3, the specific activity for glucose-1-phosphate was 3.76 units/mg, showing 70% activity relative to M-1-P. This result demonstrated that ManB used in this study has dual substrate specificity for both M-1-P and glucose-1-phosphate. ManB isolated from *Streptomyces coelicolor*, *Thermococcus kodakaraensis*, *Sphingomonas paucimobilis*, and rabbit muscle, had both phosphomannomutase and phosphoglucomutase activities [6, 7, 18–20]. Due to their similar preference for glucose and mannose-based phosphosugar substrates, ManB can be classified as a subgroup of the α-D-phosphohexomutase enzyme superfamily [21].

In this study, the *manB* gene from *E. coli* was cloned and expressed in *L. lactis*, and the characteristics of ManB enzyme were analyzed. The ManB used in this study has dual substrate specificity for both M-1-P and glucose-1-phosphate. To the best of our knowledge, this is the first study on heterologous expression and characterization of ManB in a LAB system. The expression system used in this study will allow production of GDP-D-mannose to link metabolic pathways to the synthesis of rare sugars or glycoconjugates.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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