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Communication

Practical Preparation of Lacto-*N*-biose I, a Candidate for the Bifidus Factor in Human Milk

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A one-pot enzymatic reaction to produce lacto-*N*-biose I (LNB), which is supposed to represent the bifidus factor in human milk oligosaccharides, was demonstrated. Approximately 500 mM of LNB was generated in 10-liter of reaction mixture initially containing 660 mM of sucrose and 600 mM of GlcNAc by the concurrent actions of four enzymes, sucrose phosphorylase, UDP-glucose—hexose-1-phospate uridylyltransferase, UDP-glucose 4-epimerase, and lacto-*N*-biose phosphorylase, in the presence of UDP-Glc and phosphate, indicating a reaction yield of 83%. LNB was isolated from the mixture by crystallization after yeast treatment. Finally, 1.4 kg of LNB of 99.6% purity was recovered after recrystallization.

Key words: lacto-*N*-biose I; human milk oligosaccharides; lacto-*N*-biose phosphorylase; bifidus factor; *Bifidobacterium*

Bifidobacteria, gram-positive intestinal anaerobes, have been recognized to be beneficial to human health. Rapid intestinal colonization of bifidobacteria in breast-fed infants has been known since early twentieth century.[1,2] The resulting intestinal flora is occupied by bifidobacteria and appears to prevent infection of some pathogenic bacteria and diarrhea.[3] Such rapid colonization does not occur in the intestine of bottle-fed infants.[3] It is widely accepted that oligosaccharides in human milk other than lactose (human milk oligosaccharides, HMOs) play a key role in the growth of bifidobacteria,[4,5] but the growth mechanism is a long-term enigma due to the difficulty of research into the complicated contents of HMOs, which include more than 100 kinds of molecules.[4] Now some prebiotic oligosaccharides such as lactulose[6] are added to formula milk, which is made from cow's milk, which contains no oligosaccharides other than lactose,[4,5,7] to grow bifidobacteria in the intestine, but the effect is considerably less than HMOs in forming intestinal flora, consisting 90% of bifidobacteria and 10% of enterobacteria.[8] It has often been reported that early bifidobacterial colonization may reduce allergy.[9] Thus, it is still desirable to improve the intestinal flora of bottle-fed infants to resemble the flora of breast-fed infants.

We found that bifidobacteria had a special galactose metabolism pathway specific to lacto-*N*-biose I (Galβ1→3GlcNAc, LNB) and galacto-*N*-biose (Galβ1→3GalNAc, GNB).[10] The metabolism starts with the phosphorolysis of disaccharides into α-galactose 1-phosphate (Gal1*P*) and corresponding *N*-acetylhexosamines by lacto-*N*-biose phosphorylase (LNBP, EC 2.4.1.211). Then the Gal1*P* is converted into glucose 1-phosphate (Glc1*P*) as in the Leloir pathway. Since bifidobacteria possess endo-α-*N*-acetylgalactosaminidase, which liberates O-α linked GNB,[11] the operon is considered to play a key role in intestinal colonization by utilizing GNB, which exists as a core I sugar chain in mucin.

Lacto-*N*-tetraose (Galβ1→3GlcNAcβ1→3Galβ1→4Glc) and lacto-*N*-fucopentaose I (Fucα1→2 Galβ1→3GlcNAcβ1→3Galβ1→4Glc), which show the LNB structure, are predominant components in HMOs as well as 2′-fucosyllactose.[4] We noticed another possibility in the operon, that the LNB structure in HMOs can be metabolized in the same pathway if bifidobacteria possess lacto-*N*-biosidase and α-fucosidase. Though the presence of lacto-*N*-biosidase has not been proved, α-fucosidase has been found in *Bifidobacterium bifidum*.[12] Hence we hypothesized that LNB is the real bifidus factor in human milk.[10] To prove this hypothesis, practical preparation of LNB is required. In this paper we explain kg-scale preparation of LNB, which is scale-up ready.

The scheme of the reaction is summarized in Fig. 1. Sucrose and GlcNAc were chosen as the starting materials. These compounds were industrially prepared and are available at reasonable cost. Four enzymes, sucrose phosphorylase (SP, EC 2.4.1.7), UDP-glucose—

The nucleotide and deduced amino acid sequences reported here are available in the GenBank sequence database under accession nos. AB303573 (GalT), AB303838 (SP), and AB303839 (GalE).

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Abbreviations: GalE, UDP-glucose 4-epimerase; GalT, UDP-glucose—hexose 1-phosphate uridylyltransferase; GNB, galacto-*N*-biose; HMO, human milk oligosaccharide; LNB, lacto-*N*-biose I; LNBP, lacto-*N*-biose phosphorylase; SP, sucrose phosphorylase

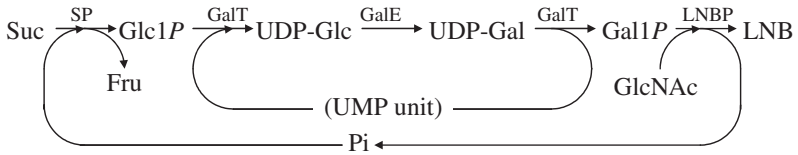

Fig. 1. Reaction Scheme for LNB Production.

Table 1. Nucleotide Sequences for Cloning Primers, Preparation of Expression Plasmids, and Productivity and Specific Activity of Recombinant Enzymes

Enzyme	Primer sequence (5′ → 3′)	Vector/Restriction endonuclease	Productivity (mg/l-culture)	Specific activity (U/mg)
SP	gtccctttcgaggtaatatcgaac cgggcgcacgtacggttccaacat gatatacatatgaaaaacaaagtgcaactcatc ggccaactcgaggtcgatatcggcaatcggcgggttggc	pET30/ *Nde*I and *Xho*I	34	66
GalT	gatttagtaaaggagctcgtgctggcc gaacagcttggtagcttgcgaaacgcc caaacatatggctgatttcgccaactac ttctgcggccgcgtcggcgatgtcgatgga	pET30/ *Nde*I and *Not*I	60	210
GalE	ggcaacacggccgtcttcaagcagaaagc ttcgaatcgcaccgagctcacccgctgggc gatatacatatgactactgttctggttacgggc ctgctcctcgagctccgcgtcgcggaaaccgttggggttc	pET30/ *Nde*I and *Xho*I	125	140
LNBP*			32	3.5

*derived from reference 12).
Primers are listed in the following order: cloning forward, cloning reverse, expression forward, and expression reverse. The restriction endonuclease sites are underlined.

hexose-1-phospate uridylyltransferase (GalT, EC 2.7.7.12), UDP-glucose 4-epimerase (GalE, EC 5.1.3.2), and LNBP, were involved in the reaction. Sucrose is phosphorolyzed into α-glucose 1-phosphate (Glc1*P*) and fructose by SP. Glc1*P* is converted into UDP-Glc concomitantly with the conversion of UDP-Gal into Gal1*P* by GalT. UDP-Glc is converted into UDP-Gal by GalE, and the resulting UDP-Gal is consumed in the reaction of GalT. Finally, LNB is synthesized from Gal1*P* and GlcNAc by LNBP. Because phosphate and UDP-Glc are recycled during the reaction system, the overall reaction is described as follows:

$$\text{Suc} + \text{GlcNAc} \rightarrow \text{LNB} + \text{Fructose}$$

LNBP was prepared with the expression plasmid of *lnpA2* from *Bifidobacterium bifidum* JCM1254 in *Escherichia coli* as described previously.[13] The SP, GalT, and GalE genes were isolated by PCR with KOD plus polymerase (Toyobo, Osaka, Japan) from *B. longum* JCM1217 genomic DNA prepared by InstaGene (Bio-Rad, Hercules, CA). Primers were designed based on the DNA sequence of the BL0536, BL1211, and BL1644 genes from *B. longum* NCC2705,[14] as shown in Table 1. After sequencing of the PCR products, each gene was amplified by expression primers and inserted into pET 30 vector (Novagen, Darmstadt, Germany) with the restriction endonuclease site using Ligation High (Toyobo), as shown in Table 1. Transformation of *E. coli* BL21 (DE3) and purification of recombinant enzymes from the transformant was performed according to the manufacturer's manual. Purification of recombinant enzymes from cell-free extract was performed using Ni-NTA agarose (Qiagen, Hilden, Germany) column chromatography. The homogeneity of the purified enzymes was confirmed by SDS–PAGE. Protein concentrations were determined using theoretical extinction coefficients calculated from the amino acid sequences of the protein.[15] The yield of the four enzymes per 1-liter culture was calculated from the concentration of the enzymes after the purification step (Table 1).

The activities of the four enzymes were measured in 100 mM MOPS buffer (pH 7.5) at 30 °C. SP and GalT activities were quantified by determining the increase in the concentration of Glc1*P*[16] from 100 mM sucrose and 100 mM phosphate (SP), and 4 mM UDP-Glc and 4 mM Gal1*P* (GalT), as substrates. GalE activity was determined by measuring the increase in UDP-Glc from 10 mM UDP-Gal as the substrate. LNBP activity was determined by measuring the increase in phosphate[17] from 10 mM Gal1*P* and 10 mM GlcNAc as substrates. One unit of enzyme was defined as the amount of enzyme that produced 1 µmol of product under above conditions. The specific activities of the four enzymes are shown in Table 1.

The production of LNB was performed in 10-liter of a reaction mixture consisting of 660 mM sucrose, 600 mM GlcNAc, 1 mM UDP-Glc, 30 mM phosphate buffer

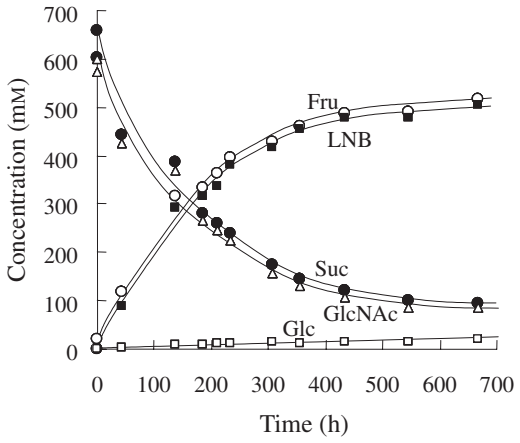

Fig. 2. The Time Course of LNB Production.
The concentrations of LNB, sucrose, fructose, GlcNAc, and glucose are shown by closed square, closed circle, open circle, open triangle, and open square respectively.

(pH 7.0), 10 mM $MgCl_2$, 26 mg/l (1716 U/l) SP, 22 mg/l (3080 U/l) GalE, 17 mg/l (3570 U/l) GalT, and 43 mg/l (150 U/l) LNBP at 30 °C. The concentrations of LNB, GlcNAc, sucrose, fructose, and glucose were monitored by high performance liquid chromatography (Shimadzu, Kyoto, Japan) with a Corona Charged Aerosol Detector (ESA Inc., Chelmsford, MA) using a Shodex Asahipak NH2P50-4E column (4.6 mm ID × 250 mm, Showa Denko, Tokyo, Japan) with acetonitrile-H_2O (75:25 by volume) as the solvent at a flow rate of 1 ml/min. The time course of the reaction is shown in Fig. 2. Production of LNB was observed with decreases in sucrose and GlcNAc at an early stage of the reaction. The LNB concentration was reached 500 mM after 600 h of reaction. The yield was 83% based on the GlcNAc used.

After the reaction, 50 g of DEAE-cellulose (Wako Pure Chemical Industries, Osaka, Japan) pre-equilibrated with 50 mM phosphate buffer (pH 7.0) were added to the reaction mixture to adsorb the enzymes. The mixture was stirred at room temperature for 90 min. After removal of DEAE-cellulose by filtration, 200 g of baker's yeast (Oriental Yeast, Tokyo, Japan) was added to the mixture, which was incubated at 30 °C for 12 h to eliminate fructose (byproduct, 516 mM) and sucrose (remaining substrate, 96 mM).[18] After centrifugation (18,000 × *g*, 30 min) to remove yeast cells, the supernatant was concentrated to 4.8-liter using a rotary evaporator. After crystallization at 4 °C for 12 h, 1.5 kg of crystalline LNB (96.9% purity) was recovered by filtration, followed by vacuum drying. In addition, 0.3 kg of crystalline LNB (95.0% purity) was recovered from the filtrate after concentration to 1.8-liter and crystallization under the same conditions.

The two LNB samples (1.5 kg and 0.3 kg) were mixed and dissolved in 3.6-liter water at 60 °C, followed by the addition of 3.6-liter of ethanol at 4 °C for crystallization with filtration and vacuum drying. Finally, 1.4 kg of crystalline LNB (99.6% purity) was obtained after filtration and vacuum drying. The ^{1}H-NMR spectum of the compound was taken in D_2O using an Avance 800 spectrometer (Bruker Biospin, Rheinstetten, Germany) at 298 K. It was identical with that of authentic LNB. Anomeric analysis using HPLC[19,20] revealed that the crystal was a α-anomer of LNB.

The above results indicate that LNB was produced by a one-pot enzymatic reaction. All the unit processes used in this method were scale-up ready. It should be noted that the reaction time can easily be shortened only by increasing the amounts of the four enzymes at the same ratio. Although this method requires expensive UDP-Glc, the amount of UDP-Glc used was only 1/600 of GlcNAc, indicating that UDP-Glc acted as a catalyst with a turn-over number of 500. Considering these facts, this method can be used in industrial production of LNB by constructing a bio-reactor system using immobilized enzymes. Since LNB has been hypothesized to be a bifidus factor in HMOs, LNB may be applicable as an additive to formula milk to achieve bifidus flora in bottle-fed infants as well as breast-fed infants.

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