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Production of Lacto-*N*-biose I Using Crude Extracts of Bifidobacterial Cells

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Shuntaro Machida,[1,*] Katsuichi Saito,[1,**] Mamoru Nishimoto,[1,†] and Motomitsu Kitaoka[1,2,†]

[1] *Institute of Food Research, National Agriculture and Food Research Organization*
(*2−1−12 Kannondai, Tsukuba, Ibaraki 305−8642, Japan*)
[2] *Faculty of Agriculture, Niigata University*
(*8050 Ikarashi 2-no-cho, Nishi-ku, Niigata 950−2181, Japan*)

Abstract: Lacto-*N*-biose I (LNB) is supposed to represent the bifidus factor in human milk oligosaccharides, and can be practically produced from sucrose and GlcNAc using four bifidobacterial enzymes, 1,3-β-galactosyl-*N*-acetylhexosamine phosphorylase, sucrose phosphorylase, UDP-glucose-hexose 1-phosphate uridylyltransferase, and UDP-glucose 4-epimerase, recombinantly produced by *Escherichia coli*. Here the production of LNB by the same enzymatic method without using genetically modified enzymes to consider the use of LNB for a food ingredient was reported. All four enzymes were produced as the intracellular enzymes of *Bifidobacterium* strains. The mixture of the crude extracts contained all four enzymes, with other enzymes interfering with the LNB production, namely, phosphoglucomutase, fructose 6-phosphate phosphoketolase, and glycogen phosphorylase. The first two interfering enzymes were selectively inactivated by heat treatment at 47 ˚C for 1 h in the presence of pancreatin, and glycogen phosphorylase was disabled by hydrolyzing its possible acceptor molecules using glucoamylase. Finally, 91 % of GlcNAc was converted into LNB in the 100-mL reaction mixture containing 300 mM GlcNAc.

Key words: lacto-*N*-biose I, human milk oligosaccharides, bifidus factor, *Bifidobacterium*, enzymatic production

INTRODUCTION

A breast-fed infant rapidly obtains bifidobacteria-predominant intestinal flora after birth.[1)2)] The colonization of bifidobacteria in the gut is beneficial for the health of the infant. Human milk oligosaccharides (HMO), sugars in breast milk other than lactose with DP 3 or larger, have been considered to be the factor growing bifidobacteria since the middle of the 20th century.[3)] However, the complex composition of HMO consisting of more than 200 oligosaccharides had made it challenging to understand how bifidobacteria used HMO to obtain their predominant growth. In the component of HMO, type I sugars possessing lacto-*N*-biose I (LNB, Galβ1,3GlcNAc) structure at the non-reducing end are much more abundant than type II sugars possessing *N*-acetyllactosamine (Galβ1,4GlcNAc) structure.[4)5)6)] The type I predominant composition in the milk oligosaccharides is the specific feature of human milk within other mammalians' milk, including primates and anthropoids.[7)8)] Because the LNB linkage is resistant to most β-galactosidases,[9)10)] the metabolism of type I sugars by *Bifidobacterium* species is essential to understand how HMO plays the role as the bifidus factor.

It has been revealed that infant-resident species of *Bifidobacterium*, such as *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. breve,* and *B. bifidum*, generally possess the specific intracellular pathway to catabolize LNB and galacto-*N*-biose (GNB, Galβ1,3GalNAc), named GNB/LNB pathway, coded in a gene cluster.[9)11)] The cluster contains the genes encoding components of an ATP-binding-cassette sugar transporter specific to GNB and LNB.[12)] The intracellular enzymes convert GNB and LNB into the sugar phosphates that enter the glycolytic pathway. In the pathway, GNB and LNB are phosphorolyzed into α-galactose 1-phosphate (Gal1*P*) and the corresponding *N*-acetylhexosamines by 1,3-β-galactosyl-*N*-acetylhexosamine phosphorylase (GNB/LNB phosphorylase, GLNBP, EC 2.4.1.211).[9)13)] Most intestinal bacteria except for a part of *Bifidobacterium* did not use LNB.[14)] Additionally, these *Bifidobacterium* species grew in an LNB medium, but adult-resident species, such as *B. adolescentis* and non-human-resident species, such as *B. animalis* subsp. *lactis* did not.[15)] Cultivation of infant feces in an LNB medium caused an increase in *Bifido-*

†Corresponding author (MN, Tel. +81−29−838−7321, Fax. +81−29−838−7996, E-mail: manishi@affrc.go.jp; MK, Tel./Fax. +81−25−262−6665, E-mail: mkitaoka@agr.niigata-u.ac.jp).
*Present address: National Institute of Technology, Ube College (Tokiwadai, Ube, Yamaguchi 755−8555, Japan).
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Abbreviations: CE, Crude extract; F6PPK, fructose 6-phosphate phosphoketolase; Fru6*P*, fructose 6-phosphate; G6PDH, glucose 6-phosphate dehydrogenase; Gal1*P*, α-galactose 1-phosphate; GalE, UDP-glucose 4-epimerase; GalT, UDP-galactose–hexose 1-phosphate uridylyltransferase; Glc1*P*, α-glucose 1-phosphate; Glc6*P*, glucose 6-phosphate; GLNBP, 1,3-β-galactosyl-*N*-acetylhexosamine phosphorylase; GNB, galacto-*N*-biose; GP, glycogen phosphorylase; HMO, human milk oligosaccharides; LNB, lacto-*N*-biose I; MOPS, 3-(*N*-morpholino)propanesulfonate; PGM, phosphoglucomutase; SP, sucrose phosphorylase.

bacterium, especially *B. bifidum*. It resulted in a significantly higher acetic acid/lactic acid ratio compared to media with other prebiotic oligosaccharides.[16] Thus, it has been suggested that LNB is the potential bifidus factor.

A practical enzymatic production of LNB from sucrose and GlcNAc, using four recombinant enzymes derived from *Bifidobacterium* produced in *Escherichia coli*, sucrose phosphorylase (SP), UDP-glucose-hexose-1-phosphate uridylyltransferase (GalT), UDP-glucose 4-epimerase (GalE), and GLNBP was reported. The scheme of the reaction is summarized in Fig. 1 inside the solid line. About 1.5 kg of pure LNB was successfully produced from a 10-L-scale reaction.[17][18]

Unfortunately, the LNB production has not been commercialized yet. The reason may be related to the fact that the general uses of recombinant enzymes in food production have not received public acceptance in Japan. Thus, an attempt was made to produce LNB using the non-recombinant enzymes produced by *Bifidobacterium* species. Furthermore, considering the production of LNB as the food ingredient, purification of the enzymes was avoided due to the cost. Here, the practical production of LNB using crude extracts of *Bifidobacterium* species containing the four enzymes with several treatments to inactivate the possible interfering enzymes is reported.

MATERIALS AND METHODS

Preparation of the crude extract of Bifidobacterium cells. The frozen cells of *Bifidobacterium breve* MCC1320 possessing GLNBP and *B. longum* MCC135 possessing SP[19] (each single lot) were kindly donated by Morinaga Milk Industry Co., Ltd. (Tokyo, Japan) were used as enzyme source. These samples were lyophilized before use.

The lyophilized cells (2:1 mixture of MCC1320 and MCC135, 50 mg/mL) were resuspended in 50 mM sodium phosphate buffer (pH 7.0) and disrupted using an ultrasonic homogenizer (Sonifier 250 or Sonifier 450, Branson Ultrasonics Co., Brookfield, CT, USA). Sonifier 250 was used for cell suspension of 5 mL or less with conditions, duty cycle 50 %, output control 2, standing on ice, 10 min. Sonifier 450 was used for cell suspension of 5 mL or more with conditions, duty cycle 50 %, output control 5, stirring on ice, 10 min, four cycles. The sonicated solution was centrifuged, and the supernatant was harvested as the crude extract (CE).

Removal of the low-molecular-weight compounds from CE. The low-molecular-weight compounds, including ATP were removed from CE. When sample volume was small, CE was dialyzed against 50 mM sodium phosphate buffer (pH 7.0) for 16 h or more at 4 ˚C in a dialysis tube (Spectra/Pore 2, Molecular weight cutoff = 12,000−14,000, Repligen Corporation, Waltham, MA, USA). If the sample volume was 100 mL or more, the low-molecular-weight compounds were removed by cross-flow filtration using hollow fiber modules, PMP-003 (pore size 0.25 μm) and SLP-0053 (Molecular weight cutoff = 10,000, Asahi Kasei Corporation, Tokyo, Japan) as described below. The sample was filtrated using PMP-003 to remove the cell debris that remained in CE after centrifuge. When the remaining sample became approximately 50 mL, 50 mL of 50 mM sodium phosphate buffer (pH 7.0) was added to the sample, and the filtration was continued. After nine times repetition of filtration and the addition of buffer, the debris-free filtrate was obtained. This process prevents the clogging of the ultrafiltration hollow fiber in the next step. SLP-0053 concentrated the filtrate until the sample volume became half the initial volume (before the filtration PMP-003). Then, an equivolume of 50 mM sodium phosphate buffer (pH 7.0) was added to the concentrate. The filtration was continued until the volume became half. After four repetitions of filtration, the concentrate was obtained and made the initial volume using 50 mM sodium phosphate buffer (pH 7.0). Hereafter, CE after removing the low-molecular-weight compounds is called CE-L.

Pancreatin treatment of CE-L in the presence and absence of glucoamylase. GlcNAc, UDP-glucose, and pancreatin were added to CE-L to be the concentration of 10 mM, 0.5 mM, and 1 mg/mL, respectively, in the presence or absence of 2 U/mL glucoamylase. The mixture was heated at 47 ˚C for 1 h. After centrifugation, pH of the supernatant was adjusted to 7.5 with 4 M NaOH. Hereafter, CE-L treated with pancreatin is called CE-LP and additionally treated with glucoamylase is called CE-LPG.

Production of LNB using CEs (CE, CE-L, CE-LP, CE-LPG). For the LNB production, sucrose and GlcNAc as substrate were added to CEs. The reaction mixture was sterilized by passing a sterile 0.22 μm filter (Millex-GV or Stericup-GV, Merck Millipore, Burlington, MA, USA) and then incubated at 30 ˚C. For example, in the case of 100 mL reaction mixture preparation, including 300 mM sucrose and 300 mM GlcNAc, 30 mmol sucrose, and 30 mmol GlcNAc were added to about 70 mL CE-LPG, and volume was adjusted to 100 mL with CE-LPG.

Purification of LNB. LNB was isolated by crystallization basically as described previously.[17] Baker's yeast (Groupe Lesaffre, Marcq-en-Baroeul, France) was added to the reaction mixture (10 mg/mL) and incubated for 24 h at 30 ˚C by constant stirring using a magnetic stirrer to remove residual substrate and reaction by-products such as sucrose, glucose, and fructose. After removing the yeast cells through centrifugation (21,500 × G, 10 min) and filtration (Glass microfiber filter GF/B pore size 1.0 μm, GE Healthcare Life Science, Marlborough, MA, USA), the solution was concentrated thrice with a rotary evaporator. An equal volume of ethanol with the concentrated solution was added, and precipitated impurities were quickly removed by centrifugation. The supernatant was kept at 4 ˚C for a few days to crystalize the produced LNB.

HPLC analysis. Sucrose concentrations in the reaction mixture were measured by high-performance liquid chromatography using LC20A system (Shimadzu Corporation, Kyoto, Japan) equipped with a corona-charged aerosol detector (ESA Biosciences, Inc., Chelmsford, MA, USA), and a column SCR101C (7.9 mm ID, 300 mm L, Shimadzu Corporation) with H_2O as the solvent, at a flow rate of 1 mL/min at 80 ˚C. LNB and GlcNAc were also quantified using a UV-VIS detector SPD-10A (Shimadzu Corporation) at the wavelength of 215 nm. Each calibration curve was prepared with 200, 400, 800, 1,600, and 2,000 pmol of each component (sucrose, GlcNAc, and LNB).

Enzymes used in this study. The following proteases were

examined for the selective inactivation of the disrupting enzymes; bromelain, thermolysin, protease from *Streptomyces griseus*, papain, pepsin (from the porcine stomach), pancreatin (from the porcine pancreas), trypsin (from the porcine pancreas) (these 7 proteases, FUJIFILM Wako Pure Chemical Co., Osaka, Japan), and α-chymotrypsin (from bovine pancreas, Tokyo Chemical Industries Co., Tokyo, Japan). Glucoamylase (from *Rhizopus* sp., FUJIFILM Wako Pure Chemical Co.) was used to cleave the glycogen/dextrins existing in CE of bifidobacterial cells.

The following enzymes were bought for quantifying the enzymatic activities: glucose 6-phosphate dehydrogenase (G6PDH, from *Leuconostoc* sp., recombinant, FUJIFILM Wako Pure Chemical Co.), phosphoglucomutase (PGM, Sigma-Aldrich Japan K.K., Tokyo, Japan), peroxidase (PEO-131, Toyobo Co., Ltd., Osaka, Japan), glycerol kinase (GYK-313, Toyobo Co., Ltd.), and L-α-glycerophosphate oxidase (G3O-321, Toyobo Co., Ltd.). Recombinant GalT from *Bifidobacterium longum* subsp. *longum* JCM 1217 (BLLJ_0398 protein) was prepared as previously described.[17] Recombinant acetate kinase from *B. longum* subsp. *longum* JCM 1217 (BLLJ_0643 protein) was prepared as briefly described below. The *bllj_0643* gene was amplified from *B. longum* subsp. *longum* JCM 1217 genomic DNA by PCR with the forward and reverse primers, 5′-ATGCCCCATATGGCGAAAACCGTCCTTGTC-3′ and 5′-CGGTCTCGAGCTTGGCGAAGGTGTTGCCGT-3′, possessing NdeI and XhoI sites (underlined), respectively, and inserted into NdeI-XhoI site of pET30 vector. BLLJ_0643 protein was produced in *E. coli* BL21 (DE3) harboring the expression plasmid and purified using Ni-NTA agarose column chromatography through standard procedures.[17]

Assay of enzymatic activities. Each enzymatic reaction was conducted at 100-μL scale in a well of standard 384-well microplate at 30 °C, and each designated absorbance (400 nm to quantify thio-$NADH^+$ with 8.91 mM^{-1} for GLNBP, SP, GalT, GalE, PGM, and GP; 550 nm to quantify the product of peroxidase with 10.91 mM^{-1} for fructose 6-phosphate phosphoketolase (F6PPK)) was measured continuously to determine the activity using a microplate reader (Multiskan-go, Thermo Scientific Inc., Waltham, MA, USA).

GLNBP activity was determined by measuring the increased rate of Gal1*P* produced from LNB and phosphate (Fig. S1A; see J. Appl. Glycosci. Web site).[20] The enzymatic reaction was conducted in a solution containing 10 mM LNB and 10 mM sodium phosphate buffer (pH 7.0) as the substrates with 5 U/mL G6PDH, 5 U/mL PGM, 2 μM glucose 1,6-bisphosphate, 5 mM $MgCl_2$, 0.25 mM thio-NAD^+, 0.25 mM UDP-glucose, and 2 U/mL GalT in 50 mM 3-(*N*-morpholino) propanesulfonate (MOPS) buffer (pH 7.0) to quantify Gal1*P*. One unit of GLNBP was defined as the amount of enzyme that produces 1 μmol Gal1*P* per min.

SP, GalT, and GP activities were determined by measuring the increased rate of glucose 1-phosphate (Glc1*P*)[21] produced from sucrose and phosphate, UDP-glucose and Gal1*P*, and dextrin and phosphate, respectively (Figs. S1B, S1C, and S1G; see J. Appl. Glycosci. Web site). Each enzymatic reaction were performed in a solution containing 10 mM sucrose and 10 mM sodium phosphate buffer (pH 7.0) (for SP), 1 mM UDP-glucose and 1 mM Gal1*P* (for GalT), or 10 mg/mL dextrin hydrate (FUJIFILM Wako Pure Chemical Co.) and 10 mM sodium phosphate buffer (pH 7.0) (for GP), respectively, as the substrates with 5 U/mL G6PDH, 5 U/mL PGM, 2 μM glucose 1,6-bisphosphate, 5 mM $MgCl_2$, 0.25 mM thio-NAD^+, in 50 mM MOPS buffer (pH 7.0). One unit of the enzyme was defined as the amount of enzyme that produces 1 μmol Glc1*P* per min.

GalE activity was determined by measuring the increased rate of UDP-glucose from UDP-galactose (Fig. S1D; see J. Appl. Glycosci. Web site). The enzymatic reaction was performed in a solution containing 1 mM UDP-galactose as the substrates with 5 U/mL G6PDH, 5 U/mL PGM, 2 μM glucose 1,6-bisphosphate, 5 mM $MgCl_2$, 0.25 mM thio-NAD^+, 0.5 mM Gal1*P*, and 2 U/mL GalT from *B. longum* subsp. *longum* JCM 1217 in 50 mM MOPS buffer (pH 7.0). One unit of the enzyme was defined as the amount of enzyme that produces 1 μmol UDP-glucose per min.

PGM activity was determined by measuring the amount of glucose 6-phosphate (Glc6*P*) produced from Glc1*P* (Fig. S1E; see J. Appl. Glycosci. Web site) in a solution containing 1 mM Glc1*P* as the substrate with 5 U/mL G6PDH, 5 mM $MgCl_2$, and 0.25 mM thio-NAD^+ in 50 mM MOPS buffer (pH 7.0). One unit of the enzyme was defined as the amount of enzyme that produces 1 μmol Glc6*P* per min.

F6PPK activity was determined by measuring the amount of acetyl phosphate produced from fructose 6-phosphate (Fru6*P*) and phosphate (Fig. S1F; see J. Appl. Glycosci. Web site). The enzymatic reaction was performed in a solution containing 1 mM Fru6*P* and 10 mM sodium phosphate buffer (pH 7.0) as the substrates with 0.15 mg/mL acetate kinase, 10 U/mL glycerol kinase, 5 U/mL glycerol 3-phosphate oxidase, 5 U/mL peroxidase, 5 mM $MgCl_2$, 0.1 mM ADP, 1 mM glycerol, 0.5 mM *N*-ethyl-*N*-(3-sulfopropyl)-3-methylaniline (Dojindo Laboratories Co., Ltd., Mashiki, Japan), and 0.5 mM 4-aminoantipyrine in 50 mM MOPS buffer (pH 7.0). was used. One unit of the enzyme was defined as the amount of enzyme that produces 1 μmol of acetyl phosphate per min.

Trypsin and chymotrypsin activities were determined using the method reported by Erlanger *et al*.[22] and Shibata *et al*.,[23] respectively. Trypsin and chymotrypsin activities in 1 mg/mL pancreatin were 8.7 and 1.8 mU/mL, respectively.

RESULTS AND DISCUSSION

LNB production with CE.

The practical production of LNB from sucrose and GlcNAc using the four recombinant enzymes, GLNBP, SP, GalT, and GalE from *Bifidobacterium*, (Fig. 1 inside of solid line) was reported.[17] Considering LNB production without any recombinant enzyme, the bifidobacterial strains, *B. breve* MCC1320 and *B. longum* MCC135,[19] showing high GLNBP and SP activities, respectively, were employed. The enzymatic activities in crude extract from each strain (50 g/L) are indicated in Table 1. To prepare an enzyme solution showing the GLNBP and SP activities more than 0.3 U/mL, lyophilized cells of MCC1320 and MCC135 were mixed with the ratio of 2:1. The activities of GLNBP, SP, GalT, and GalE of CE are indicated in Table 1.

An attempt was first made to produce LNB by preparing 600 mM sucrose, 300 mM GlcNAc, and 0.5 mM UDP-glucose in the CE, followed by incubation at 30 °C.

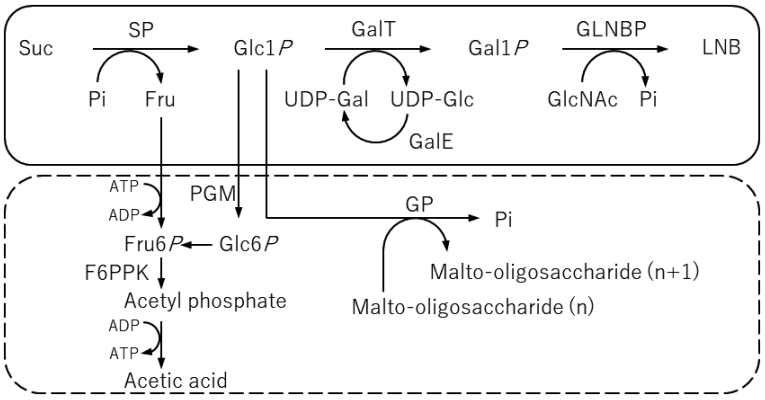

Fig. 1. Reaction scheme for LNB production and interference.
The schemes in the solid line and the dotted line indicate the LNB production and the interfering reaction, respectively.

Table 1. Used dried cells and each enzyme activity in cell-free extract.

Used dried cell	Activity (U/mL)						
	GLNBP	SP	GalT	GalE	PGM	F6PPK	GP
MCC1320 (50 g/L)	0.82	0.07	25	18	11	4.5	-
MCC135 (50 g/L)	0.22	1.5	7.5	22	9.4	4.7	-
MCC1320 (33.3 g/L) + MCC135 (16.6 g/L)*	0.59 ± 0.04	0.37 ± 0.04	21 ± 1.0	23 ± 3.0	35 ± 2.3	4.8 ± 0.4	0.95 ± 0.06

-, not measured. *This mixture is referred as CE in the text. The data of MCC1320 (33.3 g/L) + MCC135 (16.6 g/L) are means ± SD of three independent experiments. The data of MCC1320 (50 g/L) and MCC135 (50 g/L) are result of single experiment.

Quick termination of the reaction within a day was observed, resulting in a low concentration of LNB (19 mM at maximum, yielding 6 % from GlcNAc) (Fig. 2). Furthermore, the pH in the reaction mixture reduced to 4.8 within 1 day (Fig. S2; see J. Appl. Glycosci. Web site), suggesting that the substrate flows into acid production rather than LNB production.

The presence of PGM was suspected, which catalyzes the isomerization of Glc1*P* to Glc6*P* to compete with the GalT reaction (Fig. 1) in CE. Significant reduction in pH with the consumption of glucose was observed when 100 mM glucose or fructose was prepared in CE (data not shown), suggesting that the bifid-shunt pathway[24] remained in CE to phosphorylate the monosaccharide allowing it to enter into the bifid-shunt pathway. The PGM and F6PPK (the key enzyme in the bifid-shunt)[25] activities were measured in CE (Table 1) to be 35 and 4.8 U/mL, respectively. Thus, an attempt was made to remove PGM and F6PPK from CE.

Effect of removing low-molecular-weight compounds.

PGM requires glucose 1,6-bisphosphate for its activity, and kinases in the bifid-shunt (glucokinase, fructokinase, and acetate kinase) need ATP/ADP for the phosphorylation. First, there was an attempt to remove low-molecular-weight compounds from CE to inactivate PGM and the kinases (CE-L). It should be noted that PGM activity in CE-L was detectable without adding glucose 1,6-bisphosphate in the assay conditions and was insignificantly reduced from that of CE (data not shown). We prepared 600 mM sucrose, 300 mM GlcNAc, and 0.5 mM UDP-glucose in the CE-L, followed by incubation at 30 °C. Removing the low-molecular-weight compounds resulted a slight increase in the LNB production, but the effect was insufficient. The pH still decreased rapidly (Fig. S2; see J. Appl. Glycosci. Web site), and the reaction was terminated within a day (Fig. 2).

The effect of heating and protease treatment.

To remove the PGM and bifid-shunt activities, the selective removal of PGM and F6PPK, the key enzyme of

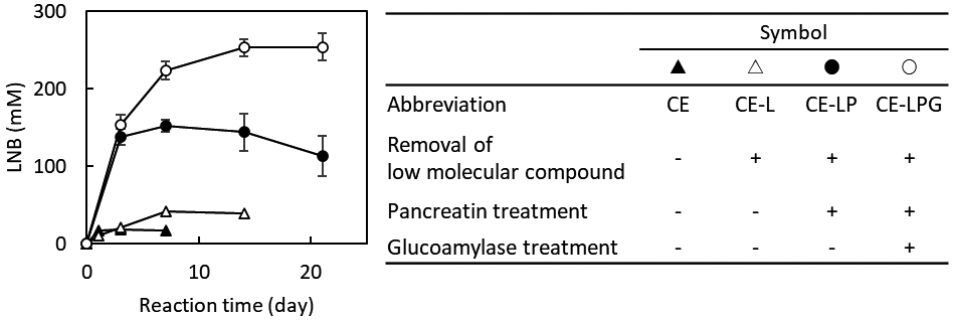

Fig. 2. The time course of LNB production using the crude extracts with/without the further treatments.
The time course of LNB production is shown. The legend for each symbol is indicated in the Table. The data of closed and open circles are means ± SD of five independent experiments. The data of closed and open triangles are result of single experiment.

bifid-shunt located at its entrance, was considered. Though GLNBP, SP, GalT, and GalE may be purified from CE to remove PGM and F6PPK by chromatography, the process is not practical due to the cost of purification. Therefore, the selective inactivation of PGM and F6PPK while maintaining the activities of GLNBP, SP, GalT, and GalE was considered.

To aim for the selective inactivation of PGM and F6PPK, the effect of protease during the heat treatment for 1 h at 47 °C in the presence of GlcNAc and UDP-glucose was examined, which is a condition that the activities of GLNBP, SP, GalT, and GalE are maintained. PGM and F6PPK were more stable than GalE under the simple heating conditions even though 10 mM GlcNAc and 0.5 mM UDP-glucose were supplemented to increase the stabilities of GLNBP and GalE (Table 1, control), suggesting that PGM and F6PPK cannot be selectively inactivated by simple heat treatment. Therefore, six proteases, bromelain, thermolysin, protease (from *S. griseus*), papain, pepsin, and pancreatin, were examined. CE-L added each protease (1 mg/mL) was heated at 47 °C in the presence of 10 mM GlcNAc, and 0.5 mM UDP-glucose for 1 h. The results are summarized in Table 2. Though no unacceptable reduction in the enzymatic activities to produce LNB was observed in the absence of a protease under the conditions, PGM and F6PPK activities did not decrease. It was found that the treatment with pancreatin caused the selective inactivation of the interfering enzymes. The residual activities of GLNBP, SP, GalT, and GalE were sufficient (69, 105, 74, and 47 %, respectively), and those of PGM and F6PPK were negligible (both < 1 %). Pancreatin contains two proteases, trypsin and chymotrypsin. Effects of trypsin, and chymotrypsin were examined, and it was found that trypsin in pancreatin is the major factor for the degradation of PGM and F6PPK in CE (Table 2).

The LNB production using the pancreatin-treated CE-L (CE-LP) was examined. Sucrose and GlcNAc were added to CE-LP to be 600 and 300 mM, respectively, and the mixture was incubated at 30 °C. As a result of the reaction, the concentration of LNB reached 138 mM after 3 days (Fig. 2), indicating that the pancreatin treatment significantly increased the productivity of LNB by the selective inactivation of PGM and F6PPK. It should be noted that the increase in the LNB concentration continued more than 7 days without inactivating pancreatin.

The effect of glucoamylase.

Though the pancreatin treatment improved the LNB productivity, the concentration of LNB did not increase after 3 days (Fig. 2). Maltooligosaccharides were detected in the reaction mixture made from CE-LP (Fig. S3; see J. Appl. Glycosci. Web site), evidenced by the digestion with glucoamylase (data not shown). It was supposed that the maltooligosaccharides were produced by the concerted action of glycogen phosphorylase (GP) of the bifidobacteria and α-amylase contained in pancreatin. At first, the GP elongated the nonreducing-ends of glycogen-like polysaccharides contained in CE-LP and the α-amylase hydrolyzed product to increase the number of molecules that acted as the primer substrate for GP. The activity of GP should be suppressed because it consumes Glc1*P* (Fig. 1). However, the GP was confirmed stable during the heat and protease treatment (Table 2). Thus, the removal of possible acceptor molecules, such as dextrins/glycogen/maltooligosaccharides contained in CE-LP by glucoamylase was conducted. Glucoamylase was added during the pancreatin treatment (CE-LPG). In CE-LPG containing 600 mM sucrose and 300 mM GlcNAc, LNB concentration continuously increased after 3 days. The final concentration reached 254 mM LNB (Fig. 2), clearly indicating that glucoamylase effectively digested the acceptors of GP to avoid the Glc1*P* consumption.

Substrate concentration.

Next, the concentration of sucrose was optimized against

Table 2. Activity and residual activity ratio of each enzyme in the treated extract.

Treatment	Activity (U/mL)						
	GLNBP	SP	GalT	GalE	PGM	F6PPK	GP
Before treatment 1	0.62 ± 0.00	0.34 ± 0.00	20.7 ± 1.18	21.3 ± 1.57	34.7 ± 2.41	5.02 ± 0.03	0.91 ± 0.04
Control	0.64 ± 0.05 (105 ± 10 %)	0.36 ± 0.00 (105 ± 1.5 %)	20.5 ± 1.36 (99 ± 12 %)	14.5 ± 1.52 (68 ± 2.1 %)	34.8 ± 2.45 (101 ± 0.1 %)	4.47 ± 0.12 (89 ± 1.8 %)	1.02 ± 0.02 (112 ± 7.1 %)
Bromelain	0.07 ± 0.00 (11 ± 0.0 %)	0.36 ± 0.04 (105 ± 13 %)	9.09 ± 0.58 (44 ± 0.3 %)	14.3 ± 1.04 (67 ± 0.1 %)	0.58 ± 0.30 (1.7 ± 0.7 %)	0.38 ± 0.02 (7.5 ± 0.5 %)	1.01 ± 0.09 (111 ± 5.2 %)
Thermolysin	0.04 ± 0.00 (6.7 ± 0.3 %)	0.35 ± 0.01 (103 ± 3.8 %)	0.00 ± 0.00 (0.00 ± 0.00 %)	0.25 ± 0.04 (1.2 ± 0.3 %)	0.01 ± 0.00 (0.04 ± 0.00 %)	0.14 ± 0.02 (2.8 ± 0.4 %)	0.91 ± 0.01 (100 ± 3.3 %)
Protease (Str)	0.02 ± 0.00 (3.2 ± 0.2 %)	0.20 ± 0.01 (60 ± 3.5 %)	0.00 ± 0.00 (0.00 ± 0.00 %)	0.03 ± 0.02 (0.13 ± 0.08 %)	0.00 ± 0.00 (0.01 ± 0.00 %)	0.08 ± 0.00 (1.6 ± 0.1 %)	0.02 ± 0.00 (2.7 ± 0.2 %)
Papain	0.43 ± 0.05 (70 ± 9.5 %)	0.37 ± 0.02 (108 ± 5.7 %)	17.6 ± 1.5 (85 ± 2.2 %)	10.3 ± 3.9 (48 ± 22 %)	11.4 ± 6.4 (33 ± 16 %)	0.69 ± 0.12 (14 ± 2.4 %)	0.92 ± 0.01 (101 ± 2.5 %)
Pepsin	0.53 ± 0.05 (86 ± 6.8 %)	0.38 ± 0.03 (110 ± 8.0 %)	21.9 ± 1.4 (106 ± 0.9 %)	8.73 ± 0.21 (41 ± 2.1 %)	33.7 ± 0.56 (97 ± 5.2 %)	1.94 ± 0.39 (39 ± 7.4 %)	0.90 ± 0.00 (98 ± 4.5 %)
Pancreatin	0.43 ± 0.00 (69 ± 0.7 %)	0.36 ± 0.03 (105 ± 9.3 %)	15.4 ± 0.2 (74 ± 5.4 %)	10.0 ± 2.0 (47 ± 6.0 %)	0.03 ± 0.01 (0.07 ± 0.03 %)	0.04 ± 0.01 (0.71 ± 0.18 %)	0.89 ± 0.06 (98 ± 2.5 %)
Before treatment 2	0.69	0.43	22.5	19.1	39.0	4.61	1.01
Trypsin	0.53 (77 %)	0.39 (92 %)	17.9 (80 %)	6.36 (33 %)	0.02 (0.06 %)	0.04 (0.93 %)	0.93 (92 %)
Chymotrypsin	0.52 (75 %)	0.40 (93 %)	17.8 (79 %)	5.5 (29 %)	4.04 (10 %)	0.72 (16 %)	0.92 (91 %)

The numbers in the parentheses indicate the residual ratio of the activities after the heat treatment. "Control" means the treatment without any protease. The data except trypsin and chymotrypsin are means of two independent experiments. The data of trypsin and chymotrypsin are result of single experiment.

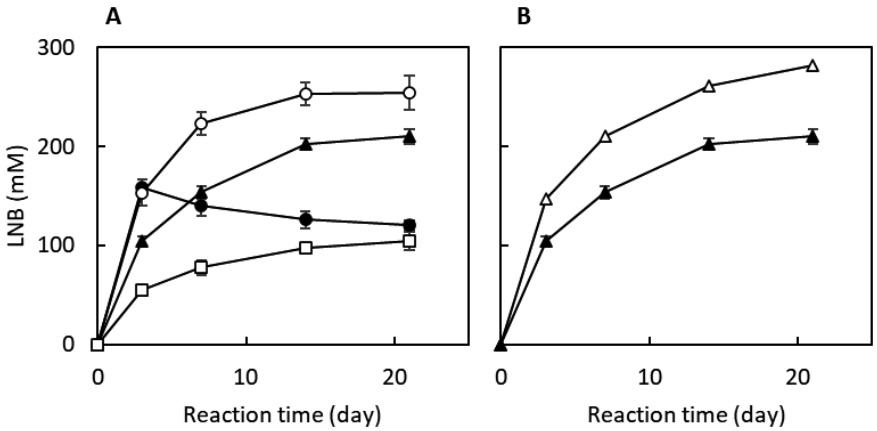

Fig. 3. The time course of LNB production in the presence of various concentrations of sucrose.

Panel A and B show the time course of LNB production. (A) The LNB concentrations in the reaction mixture added 300, 600, 900, or 1,200 μmol sucrose per 1 mL CE-LPG is shown using the closed circle, open circle, closed triangle, and open square, respectively. The data are means ± SD of three independent experiments. (B) Closed triangle indicates the LNB concentration in the reaction mixture added 900 μmol sucrose per 1 mL on day 0. The open triangle shows the LNB concentration in the reaction mixture added 300 μmol sucrose per 1 mL on days 0, 3, and 7, respectively. The data of closed triangles are means ± SD of three independent experiments. The data of open triangles are result of single experiment.

300 mM GlcNAc. LNB concentration in the reaction mixture made from CE-LPG starting with 300 or 600 mM sucrose was the highest after the reaction for 3 days. The LNB productivity was reduced with higher sucrose concentration (Fig. 3A), suggesting that LNB production was inhibited by high sucrose concentration. Therefore, there was an attempt to keep the sucrose concentration low in the reaction mixture. The reaction was started with 300 mM sucrose, and the 300 mmol sucrose per liter reaction mixture was added twice on days 3 and 7. As a result, the maximum concentration of LNB increased 30 % compared with the mixture starting from 900 mM sucrose (Fig. 3B).

LNB production in 100 mL reaction.

Based on the conditions examined above, the LNB production was conducted in a 100 mL reaction mixture. Sucrose and GlcNAc were dissolved in the 100 mL CE-LPG to be the final concentrations of both 300 mM. The mixture was incubated at 30 ˚C with constant stirring. Sucrose (30 mmol) was added on days 3 and 7. The LNB concentration reached 288 mM after 21 days, and the yield was 91 % based on the remaining 27 mM GlcNAc (Fig. 4). The yield is acceptable for the practical production of LNB, even though the total productivity of LNB by the method presented here was less than that using the recombinant enzymes (producing 500 mM LNB from 600 mM GlcNAc and 660 mM sucrose).[17)]

The produced LNB was isolated by crystallization after treating the reaction mixture with baker's yeast as previously described.[17)] After crystallization of the solution of the mixture at 4 ˚C, 9.35 g crystalline LNB (95 % purity) was recovered, which was 77 % yield based on the GlcNAc used.

The above results indicate that LNB can be produced using a crude extract of bifidobacterial cells as a non-genetically modified enzyme source with pancreatin and glucoamylase, both of which are allowed to be used to manufacture food. The procedure does not include any chromatography, which often costs too much for the food manufacturing process. It is hoped that the new method established in this study would contribute to the development of industrial LNB production and human health improvement.

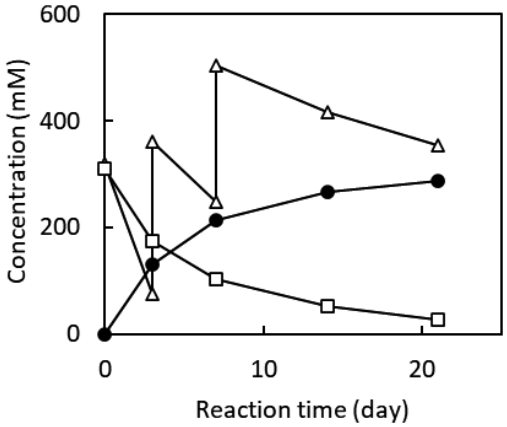

Fig. 4. The time course of LNB production in the 100 mL reaction mixture.

The concentrations of LNB, sucrose, and GlcNAc are indicated by a closed circle, open triangle, and open square, respectively. Sucrose was added on days 3 and 7 respectively. The data are result of single experiment.

CONFLICTS OF INTEREST

Part of this study was funded by Moringa Milk Industry Co., Ltd. National Agriculture and Food Research Organization and Moringa Milk Industry Co., Ltd. have jointly applied for a Japanese patent with the data given in the report, and all the authors are the inventors of this patent application. The authors have no other COI to disclose.

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species containing the enzymes.

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