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Extracellular Secretion of a Maltogenic Amylase from *Lactobacillus gasseri* ATCC33323 in *Lactococcus lactis* MG1363 and its Application on the Production of Branched Maltooligosaccharides

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Abstract A maltogenic amylase gene from *Lactobacillus gasseri* ATCC33323 (LGMA) was expressed in *Lactococcus lactis* MG1363 using the P170 expression system. The successful production of recombinant LGMA (rLGMA) was confirmed by the catalytic activity of the enzyme in liquid and solid media. The N-terminal amino acid sequencing analysis of the rLGMA showed that it was Met-Gln-Leu-Ala-Ala-Leu-, which was the same as that of genuine protein, meaning the signal peptide was efficiently cleaved during secretion to the extracellular milieu. The optimal reaction temperature and pH of rLGMA (55°C and pH 5, respectively) and enzymatic hydrolysis patterns on various substrates (β-cyclodextrin, starch, and pullulan) supported that rLGMA was not only efficiently secreted from the *Lactococcus lactis* MG1363 but was also functionally active. Finally, the branched maltooligosaccharides were effectively produced from liquefied corn starch, by using rLGMA secreted from *Lactococcus lactis*, with a yield of 53.1%.

Keywords: Branched oligosaccharides, lactic acid bacteria, *Lactobacillus gasseri*, *Lactococcus lactis*, maltogenic amylase

Lactic acid bacteria (LAB), a large group of Gram-positive cocci or bacilli belonging to a phylogenetically heterogeneous group, are best known for their widespread use in the manufacture of a variety of fermented food and feed products, such as fermented dairy products (cheeses, sour milks, yoghurts), meats, and sausages owing to their specific metabolic activities [12, 22]. Over the past decade, interest in the study of LAB has increased considerably. This reflects not only the growing industrial importance of these bacteria for a wide range of fermentation processes, but also the emergence of their application as probiotics to which human and animal health beneficial properties are attributed [16, 24, 26]. Recently, many researches have expanded their potential application as an important tool in the expression of foreign genes for industrial or medical uses [1].

Prebiotics are foods or nutrients defined as nondigestible or low-digestible food ingredients that benefit the host organisms by selectively stimulating the growth or activity of one or a limited number of probiotic bacteria in the intestine [6]. Branched oligosaccharides (BOS) are one of the major prebiotic carbohydrates, including isomaltose, panose, isopanose, branched maltotetraose, and branched isomaltopentaose, etc [10]. They are also called isomaltooligosaccharides, in which at least one α-1,6-glycosidic linkage is connected between glucooligosaccharides with degrees of polymerization (DP) ranging from 2 to 6 [3]. BOS are produced using a two-stage reactor system having two different enzymes [3]. Immobilized α-amylase (E.C.3.2.1.1) liquefies starch in the first stage, and then the liquefied starch is processed by both β-amylase (E.C.3.2.1.2) and α-glucosidase (E.C.3.2.1.20) in a second stage. The transglucosidase activity of α-glucosidase is mainly involved in the production of BOS.

Recently, it was shown that maltogenic amylases (MAase; E.C.3.2.1.133) could be employed for the production of BOS [10, 15]. MAase is an α-amylase-type enzyme in the glycoside hydrolase family 13, but it is distinguished from typical α-amylases by showing an extensive transglycosylation activity in addition to its hydrolysis activity. The coupled

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transglycosylation and hydrolysis activities of MAase were used for the production of BOS [15]. When *Bacillus stearothermophilus* maltogenic amylase (BSMA) and α-glucanotransferase from *Thermotoga maritima* acted simultaneously, the BOS content increased to 68%. Even BSMA alone produced various BOS up to 58% from liquefied corn syrup by its hydrolyzing and transglycosylation activities.

This report describes a successful extracellular secretion of maltogenic amylase of *Lactobacillus gasseri* ATCC33323 (LGMA) in *Lactococcus lactis* MG1363 using the P170 expression system [2] and structural characterization of the recombinant LGMA. Finally, the application of recombinant LGMA for the production of BOS was investigated.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Media

Lactobacillus gasseri ATCC33323 was obtained from the Korean Collection for Type Cultures (KCTC) and used for the amplification of the LGMA gene from the genomic DNA [20]. *Escherichia coli* DH5α [F^-Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1λ^-] was used as a host for the subcloning of plasmid DNA. *L. gasseri* and *E. coli* were grown in MRS and LB (Difco Laboratories, Detroit, MI, U.S.A.) media, respectively. *E. coli* transformants were grown in LB medium containing ampicillin (100 μg/ml) at 37°C. *L. lactis* strain MG1363 [5] was used as the host for recombinant production of LGMA. The expression vector, pAMJ2006, was kindly provided by Bioneer A/S (Hørsholm, Denmark). Plasmid pAMJ2006 is a medium copy number vector (10–20 copies per cell), where heterologous gene expression is controlled by the autoinducible promoter, P170 [18]. The P170 promoter is upregulated at low pH during the transition to stationary phase. Furthermore, an optimized signal peptide, SP310mut2, is included in the vector with the purpose of secreting heterologous protein products to the extracellular environment [23]. Recombinant *L. lactis* MG1363 were cultured at 30°C in M17 broth or on agar plate supplemented with 0.5% glucose [27]. For the selection of the recombinant *E. coli* and *L. lactis* harboring the recombinant LGMA gene, erythromycin was added into LB and M17 media at the concentrations of 100 μg/ml and 1 μg/ml, respectively.

Construction of an *lgma* Expression Vector and Transformation

A MAase gene (ORF 1468) in the *L. gasseri* ATCC33323 genome was isolated by the PCR method using the genomic DNA of *L. gasseri* ATCC33323 as a template [20]. Based on the *lgma* encoding sequence, two specific oligonucleotide primers (LGMA Sap, 5'-CTC GAT **GCT CTT C**CG CAA TGC AAC TAG CAG CTT TAA AAC AT-3', and LGMA Pst, 5'-CAG CTT CTT ATC **GCT GCA G**TT TCT TAT CAT AA-3') were designed. DNA fragments were amplified by *Pfu* DNA polymerase using the following protocol: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and an extension at 72°C for 1 min with an additional extension at 72°C for 5 min in the final cycle. The DNA sequence of the gene was confirmed by the chain termination method using an ABI377 Prism DNA sequencer (Perkin-Elmer, Wellesley, MA, U.S.A.). The amplified PCR product was purified with a QIAEXII gel extraction kit (Qiagen, Hilden, Germany) and inserted into the *L. lactis* expression vector, pAMJ2006, digested with SapI and PstI, finally creating pAMJ-LGMA, in which *lgma* was under the control of the autoinducible P170 promoter [18]. The gene encoding LGMA is translationally fused to the signal peptide SP310mut2 [23]. Transformation of *E. coli* DH5α was done by the Inoue method [25]. *L. lactis* was transformed by electroporation as described by Holo and Nes [7].

Assay of Hydrolytic Activity of LGMA

The recombinant LGMA was incubated with 0.5% (w/v) of β-cyclodextrin (β-CD) (Sigma Chemical Co., St. Louis, MO, U.S.A.) in a 50 mM sodium-acetate buffer (pH 5.0). The reaction was carried out at 55°C for 10 min. The reducing sugar generated by the hydrolytic activity of LGMA was determined by the 3,5-dinitrosalicylic acid method [19]. One unit of hydrolyzing activity for β-CD was defined as the amount of enzyme that forms 1 μmol of maltose per min. Protein concentration was measured by the Bradford method (Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin as the standard.

N-Terminal Amino Acid Sequencing of the Recombinant LGMA

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [13]. Samples were resolved on 10% polyacrylamide gels containing 0.1% SDS at room temperature. After electrophoresis, gels were stained with Coomassie brilliant blue R-250. The N-terminal amino acid sequence was determined by an automated Edman degradation apparatus (Model 477A, Applied Biosystems, Foster City, CA, U.S.A.) with online HPLC (Model ABI 120, Applied Biosystems). One μg of each sample was separated by SDS-PAGE and electroblotted onto a PVDF membrane. After staining the membrane with Coomassie brilliant blue R-250, the sample bands were excised and used for the N-terminal amino acid sequencing. The N-terminal amino acid sequencing was performed in the Korea Basic Science Institute (Seoul, Korea).

Thin-Layer Chromatography (TLC) and High Performance Anion-Exchange Chromatography (HPAEC) Analyses

TLC and HPAEC analyses were carried out to determine the reaction products of the recombinant LGMA generated from the various substrates. TLC analysis for the detection of hydrolytic activity of the recombinant LGMA was performed as described by Yang *et al.* [28]. HPAEC analysis was carried out with a CarboPac PA1 analytical column for carbohydrate detection (Dionex, Sunnyvale, CA, U.S.A.) and an ED40 electrochemical detector (Dionex). Filtered samples were eluted with a linear gradient from 100% buffer A (150 mM NaOH in water) to 30% buffer B (600 mM of sodium acetate in buffer A) over 30 min. The flow rate of the mobile phase was maintained at 1.0 ml/min.

RESULTS AND DISCUSSION

Extracellular Secretion of LGMA in *L. lactis* MG1363

A 1.7-kb SapI-PstI fragment, containing the *lgma* gene, was isolated from the genomic DNA of *L. gasseri* ATCC33323 by PCR. The final vector, pAMJ-LGMA, obtained by inserting the amplified PCR fragment into the expression vector pAMJ2006, was transformed into *L. lactis* MG1363. The secretion of the recombinant LGMA (rLGMA) was confirmed by the halo zone around the colony of *L. lactis* transformants grown in the M17 medium containing starch (Fig. 1). The major advantage of secreting heterologous proteins into culture media is that the heterologous protein is separated from the bulk of the intracellular components.

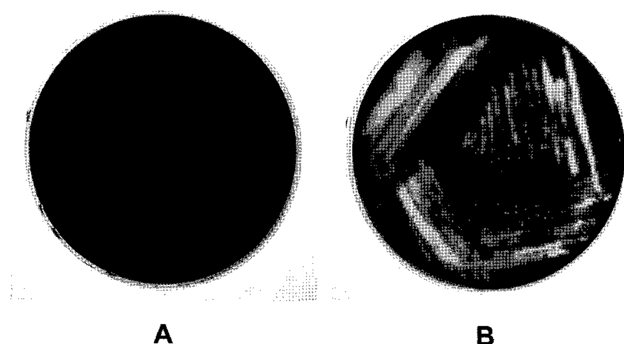

Fig. 1. Detection of the recombinant LGMA activity on M17-agar containing soluble starch (0.5%).

Both *Lactococcus lactis* MG1363 (**A**) and *Lactococcus lactis* MG1363 harboring pAMJ-LGMA (**B**) were grown on starch containing M17-agar plate containing 0.5% soluble starch with (**B**) or without (**A**) erythromycin at 30°C for 2 days. Plates were stained with iodine solution (2 g KI, 1 g I_2, and 100 ml H_2O) and the enzyme activity was detected as a clear zone around the colony.

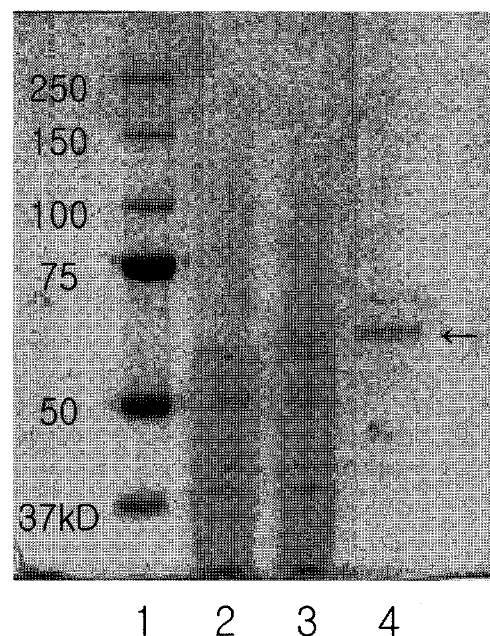

Fig. 2. SDS-PAGE analysis of proteins in *Lactococcus lactis* and *E. coli.*

Lane 1, Protein standard marker (Precision Plus Protein Standards, Bio-Rad, Hercules, CA, U.S.A.); lane 2, Concentrated culture medium of *Lactococcus lactis* MG1363; lane 3, Concentrated culture medium of *Lactococcus lactis* MG1363 harboring pAMJ-LGMA; and lane 4, affinity purified recombinant LGMA expressed in *E. coli.*

For some proteins it has been observed that the production yields of secreted forms in *L. lactis* are up to five-fold higher than those of cytoplasmic production [17]. It suggested that heterologous proteins produced in *L. lactis* are sometimes degraded intracellularly, whereas secretion allows the synthesized proteins to escape from proteolysis. The detection of MAase activity with which β-CD was hydrolyzed in the culture broth implied that the rLGMA was successfully secreted and escaped from the intracellular proteolysis.

The rLGMA in the culture supernatant was concentrated by filtration using a Vivaspin 6 membrane column (MW cutoff 50 kDa, Vivascience, Hannover, Germany) and the presence of the rLGMA in the culture supernatant was confirmed by SDS-PAGE (Fig. 2), which implies that the protein was efficiently secreted from the *L. lactis* cells. DNA sequence analysis showed that *lgma* encoded a protein (1,722 nucleotides) with 574 amino acids and had a calculated molecular mass of 66,634 Da. The concentrated rLGMA showed a molecular mass of around 65,000 Da (Fig. 2, lane 3), which is similar to the recombinant protein produced in *E. coli* (67,759 Da, Fig. 2, lane 4) [20]. The minor difference in molecular masses is likely due to the presence of the six-histidine tag and extra two amino acids

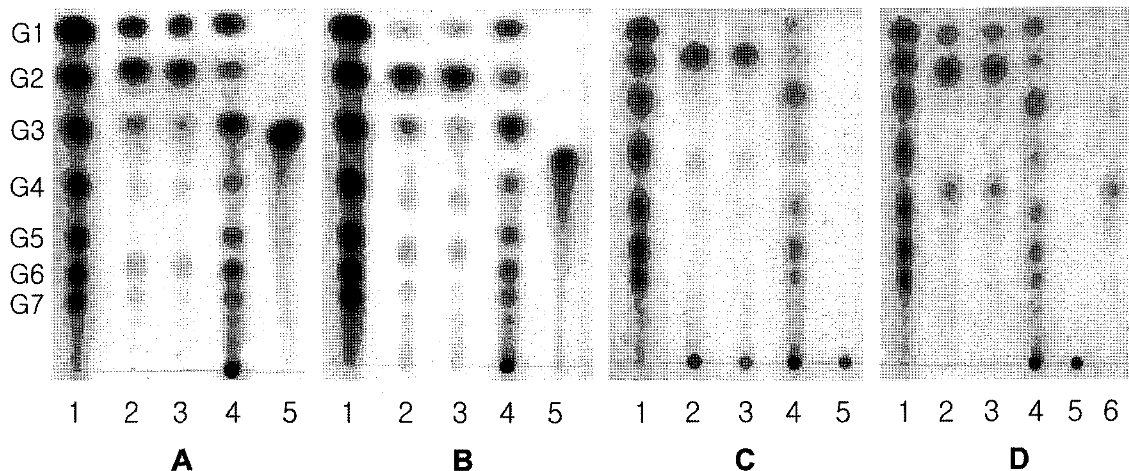

Fig. 3. Hydrolysis patterns of various substrates by two recombinant LGMAs produced in *Lactococcus lactis* MG1363 and *E. coli.*
Various substrates [1% maltotriose (**A**), β-cyclodextrin (**B**), starch (**C**), and pullulan (**D**)] were reacted with two recombinant LGMAs. Lanes 1 and 4, standards from glucose (G1) to maltoheptaose (G7); lane 2, reaction product with recombinant LGMA expressed in *E. coli*; lane 3, reaction product with recombinant LGMAs expressed in *Lactococcus lactis* MG1363; lane 5, substrate standards; and lane 6, panose standard.

(methionine and glutamic acid) attached to the N-terminus of the recombinant protein when synthesized in *E. coli* [20].

Authenticity of rLGMA

Amino-terminal (N-terminal) amino acid sequence analysis of rLGMA was carried out to determine the signal peptide cleavage site of rLGMA. The recombinant LGMA had Met-Gln-Leu-Ala-Ala-Leu as an N-terminal amino acid sequence, which is identical to the native sequence of LGMA. A signal peptide is known to be involved in targeting proteins for secretion via the Sec-dependent pathway [4, 23]. It is located at the N-terminus and absent from the mature protein. It usually comprises 25–35 amino acid residues and consists of three domains: the N-terminal, hydrophobic, and carboxy-terminal (C-terminal) regions. The N-region is rich in positively charged amino acids, whereas the hydrophobic region contains hydrophobic amino acid residues and tends to fold into an α-helix conformation upon spanning inside of the cell membrane. The C-terminal region is hydrophilic and contains the signal peptide cleavage site. Upon translocation, the secreted protein is released by cleavage of the signal peptide by a cell surface located signal peptidase. In the expression vector for LGMA, a mutated version of a signal peptide from a native lactococcal protein (SP310mut2) [23], which has proven to promote efficient secretion of foreign protein, was attached to the LGMA coding sequence in-frame. The authentic starting amino acid in the rLGMA implied that the signal peptide SP310mut2 was perfectly cleaved by signal peptidase in the right position and to direct the efficient secretion of authentic LGMA.

The enzymatic activity of rLGMA was examined and compared with the LGMA produced by recombinant *E. coli.* The optimal reaction temperature and pH of rLGMA on various substrates (β-CD, starch, and pullulan) was determined to be 55°C and pH 5, respectively, which were exactly the same as that of the LGMA synthesized by recombinant *E. coli.* The hydrolysis patterns of the recombinant LGMAs produced in *L. lactis* and *E. coli* on various substrates (maltotriose, β-CD, starch, and pullulan) were compared, as shown in Fig. 3. Both recombinant LGMAs hydrolyzed maltotriose, β-CD, and soluble starch mainly to maltose and glucose, and degraded pullulan to maltose, glucose, and panose, which is a typical action pattern of MAases [21], and no difference between the two recombinant enzymes was found, implying both enzymes were functionally equivalent to each other. Overall, the hydrolysis pattern on the various substrates combined with the correct N-terminal amino acid sequence of rLGMA confirmed that rLGMA had authentic structural and enzymatic properties.

Production of BOS by rLGMA

Production of BOS by rLGMA was investigated by a series of reactions using liquefied corn starch syrup. Prior to the experiments, the most appropriate amount of each enzyme for the highest yield of BOS was determined to be 100 U/g of substrate (data not shown). Recombinant LGMA was incubated with liquefied corn syrup (33 °Brix, DE 22, pH 6.5) at 50°C for 13.5 h (Fig. 4A). The proportion of BOS in the reactant produced by rLGMA reached about 50% in 3 h of incubation and remained almost constant during further incubation for 13.5 h (Fig. 4B). Lee *et al.* [15] employed two types of transferring activities of maltogenic amylase from *Bacillus stearothermophilus* (BSMA) and α-glucanotransferase (α-GTase) from *Thermotoga maritima* on liquefied corn

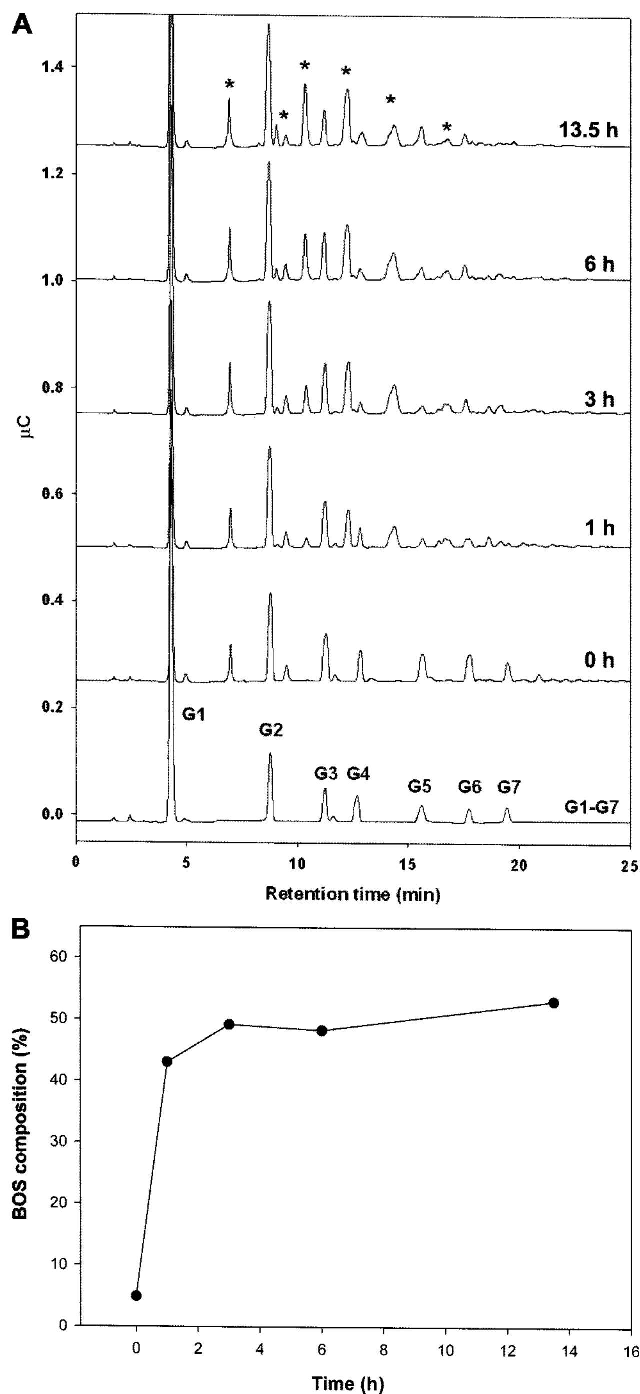

Fig. 4. Production of BOS from 30% liquefied corn syrup with recombinant LGMAs synthesized in *Lactococcus lactis* MG1363.

A. HPAEC analysis of the BOS produced by recombinant LGMAs expressed in *Lactococcus lactis* MG1363. BOS are marked by asterisks. **B.** The BOS content of the reaction mixture.

starch solution to stimulate the formation of IMOs in a cooperative mode. Using this method, up to 68% of BOS was obtained, which is 28% more efficient than the conventional method for BOS production. Therefore, there is a possibility of improving the yield of BOS production by the combined use of rLGMA and α-GTase from mesophilic bacteria.

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