Biosci. Biotech. Biochem., **58** (2), 416–418, 1994

Note

Synthesis of Branched Oligosaccharides from Starch by Two Amylases Cloned from *Bacillus licheniformis*

In-Cheol KIM, Sang-Ho YOO, Sung-Joon LEE, Byung-Ha OH,* Jung-Wan KIM, and Kwan-Hwa PARK†

The Research Center for New Biomaterials in Agriculture and Department of Food Science and Technology, College of Agriculture and Life Sciences, Seoul National University, Suwon 441–774, Korea
**Department of Macromolecular Sciences, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania, U.S.A.*
Received June 14, 1993

An *E. coli* cell extract containing thermostable α-amylase and maltogenic amylase expressed from the clone pTMA322 was used to produce an anomalously linked oligosaccharides (Alo) mixture from starch. The liquefaction and saccharification of starch were done by one-step procedure using the above cell extract. The resulted Alo mixture contained over 40% oligosaccharides having DP 3 or more including branched forms.

Maltooligosaccharides mixture, maltotetraose syrup, and anomalously linked oligosaccharide (Alo) mixture are used as substitutes for sucrose and other saccharides in the food industry due to their lower viscosity, less sweet taste, and smaller freezing point depression. They also can be used to prevent crystallization of sucrose in foods and are useful in controlling microbial contamination as well as retrogradation of starchy foods because they have low water activity and high moisture-retaining capacity.[1] The low calorie content of branched oligosaccharides has a great appeal to low calorie dieters.

Our laboratory has reported the isolation of a novel maltogenic amylase gene from *Bacillus licheniformis*.[2] The protein (BLMA) purified from *E. coli* transformed with the clone produced maltose predominantly by hydrolyzing soluble starch. BLMA, a maltogenic amylase, hydrolyzed pullulan and cyclodextrin more efficiently than soluble starch. BLMA has both hydrolyzing and transferring activities. Thus, BLMA was capable of producing branched oligosaccharides *via* formation of α-1,6-linkages in the presence of an excessive amount of glucose. Another α-amylase gene of *B. licheniformis* (BLTA) was isolated in the laboratory[3] and found to be identical to the gene reported by Yuuki *et al.*[4] The gene encoded an amylase that is thermostable and hydrolyzes starch to glucose and oligosaccharides by cleaving α-1,4-glycosidic linkages.

Generally, branched oligosaccharides are manufactured by 2-step procedures that use α-amylase, β-amylase, and transglycosidase on a starch solution.[1] The starch slurry is liquefied to the degree of hydrolysis (DE) 6–10 by the action of a thermostable α-amylase, then saccharified by treatment with soybean β-amylase and transglycosidase of *Aspergillus niger* at 60°C for 72 h. In this study, we attempted to produce an Alo mixture containing branched oligosaccharides of short lengths from starch by a one-step procedure that uses a cell extract containing thermostable α-amylase and maltogenic amylase expressed from a clone harboring the two genes on an *E. coli* vector, pBR322. Liquefaction and saccharification of soluble starch were done simultaneously in the presence of the enzymes.

To construct a recombinant DNA containing both of the amylase genes, the 3.1 kb *Eco*RI fragment of pTA322 was ligated to the unique *Eco*RI site of pIJ322 (Fig. 1). The ligation mixture was used to transform the *E. coli* HB101 competent cells prepared by $CaCl_2$ treatment. The resulting transformants were tested for the amylase positive phenotype on 1% (w/v) starch agar plate by staining with iodine reagent (0.2% I_2 and 2.0% KI). The positive clone was designated as pTMA322 after analyzed by endonuclease digestion and agarose gel electrophoresis.

As an effort to prove that both of the genes were expressed in *E. coli*, the *E. coli* cell extract harboring pTMA322 was analyzed by SDS–PAGE. The *E. coli* cell extract containing pTMA322 had two bands with molecular weight of 64 kDa and 55 kDa corresponding to BLMA and BLTA, respectively, when it was analyzed by 10% SDS–PAGE (Fig. 2, lane D). Those two bands were not detected in the cell extract containing only the vector, pBR322 (Fig. 2, lane A). In the meanwhile, the *E. coli* cell extract containing either pIJ322 or pTA322 had only a 64 kDa or a 55 kDa protein band, respectively (Fig. 2, lanes B and C). The molecular

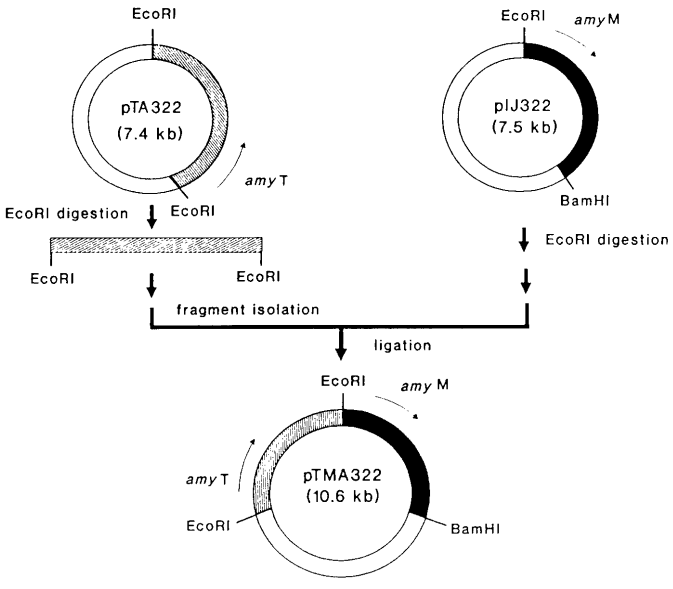

Fig. 1. Construction of pTMA322.

The 3.1 kb *Eco*RI fragment of pTA322 containing the gene for BLTA (*amy*T) was ligated to the *Eco*RI site of pIJ322 after eluted from an agarose gel by using a Gene Clean kit (Bio101, U.S.A.). The resulting clone, pTMA322, contained the *amy*T and *amy*M (for BLMA) genes in tandem. The arrows indicate the direction of each gene. The fragment with slashed lines contains the *amy*T gene, the solid fragment contains the *amy*M gene, and the open fragment represents the vector, pBR322.

† To whom correspondence should be addressed.

Abbreviations: Alo, anomalously linked oligosaccharide; DP, degree of polymerization; DE, degree of hydrolysis; BLTA, *B. licheniformis* thermostable amylase; BLMA, *B. licheniformis* maltogenic amylase; HPLC, high performance liquid chromatography; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; kDa, kilodaltons; kb, kilobases.

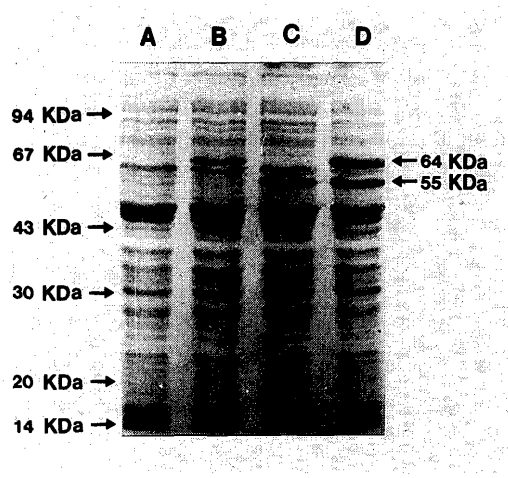

Fig. 2. SDS-PAGE Analysis of the *E. coli* Cell Extracts.

Samples were prepared from the *E. coli* overnight cultures in L-broth containing ampicillin, which had been transformed with one of the recombinant plasmids. The overnight cultures (100 ml) were centrifuged to collect the cells and the cells were resuspended in 10 ml of 50 mM Tris-HCl (pH 7.5). The suspension of the cells was sonicated for 5 min at 4°C. The debris of the cells were then precipitated by centrifugation (10,000 rpm, 50 min) and an approximately equal amount of the supernatant was subjected to 10% SDS-PAGE. Each lane contains the extract of the cells harboring; A) pBR322, the vector, B) pIJ322, C) pTA322, D) pTMA322, respectively. The positions of molecular weight markers are shown on the left side of the figure and those used are: 94 kDa, phosphorylase b; 67 kDa, bovine serum albumin; 43 kDa, chicken ovalbumin; 30 kDa, carbonic anhydrase; 20 kDa, myosin; and 14 kDa, lysozyme. The sizes of BLMA (64 kDa) and BLTA (55 kDa) are indicated on the right side of the figure.

weights of BLTA and BLMA deduced from the DNA sequencing data were 55,200 daltons and 66,883 daltons, respectively. From these it was concluded that the two enzymes were expressed at a similar level in *E. coli*.

To optimize the reaction conditions for the production of the Alo mixture, various combinations of reaction temperature and soluble starch concentrations were tested. The highest yield of branched oligosaccharides was obtained when 15% (w/v) starch solution was used. When starch was added at a low concentration (0.5%; w/v), the production of branched oligosaccharides was not detected.

The optimum temperature for the production of branched oligosaccharides was 40°C at all soluble starch concentrations tested (0.5%, 5%, 10%, 15%; w/v). The amount of branched oligosaccharides produced at 40°C, 50°C, and 60°C was 46%, 39%, and 10%, respectively. This can be explained by the instability of the maltogenic amylase at higher temperature. In the meanwhile, the optimum temperature of the purified BLTA enzyme was 70°C and its activity was stably maintained even at 80–90°C when 5 mM Ca^{2+} was added in the reaction mixture.[3] Therefore, mutagenesis study on the gene encoding BLMA is in progress in our laboratory to improve the stability of the protein at higher temperature. It would be beneficial in obtaining higher yields and preventing the reaction from contamination by microorganisms. From all these, it was determined that the highest yield of branched oligosaccharide could be obtained by doing the reaction at 40°C using 15% (w/v) starch solution.

The Alo mixture produced from starch solution by incubation with the cell extract containing BLTA and BLMA was analyzed by HPLC. The reaction was done at 40°C for 24 hours. The result of HPLC analysis is shown in Fig. 3. The Alo mixture contained glucose and maltose in fairly large amounts (peaks 1 and 2), while maltotriose (G3, peaks 3) was present as a small proportion. G4 and G5 were not detected as peaks on the chromatogram. The peak of maltose had a shoulder (peak 2) suggesting the presence of isomaltose. The shoulder was resolved as a peak of isomaltose

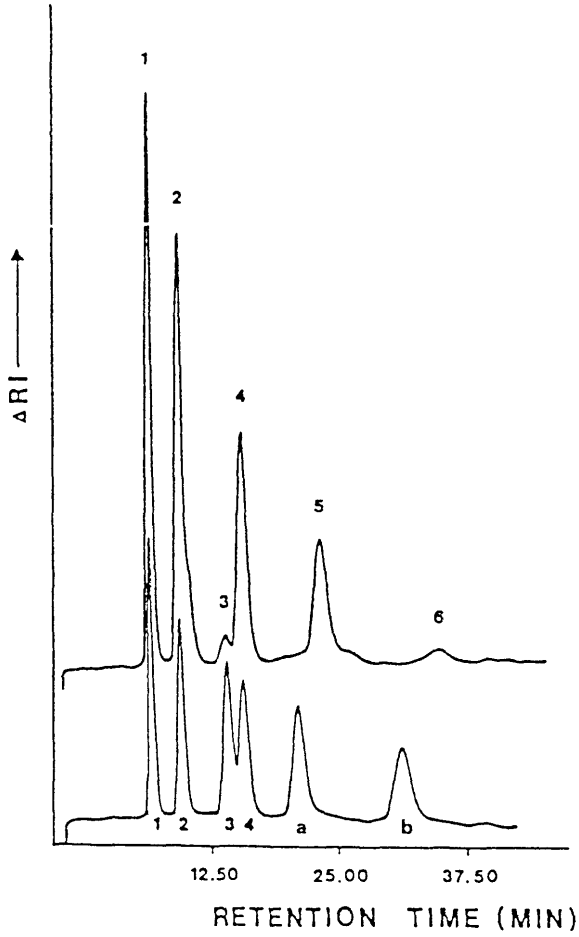

Fig. 3. The HPLC Analysis of the Starch Reaction Mixture Produced by the Action of the *E. coli* Extract Harboring pTMA322.

The samples for high performance liquid chromatography were prepared as follows; 0.5% or 15% (w/v) substrates was dissolved in 50 mM maleate-NaOH buffer (pH 6.8) containing 5 mM EDTA and then gelatinized by incubating in a boiling water bath for 5 min. The reaction was allowed to occur at 40°C for 24 hours by mixing the substrate solution and cell extract. The reaction mixture was then centrifuged at 10,000 rpm for 2 min and the supernatant was filtered through a 0.46 μm filter paper (Gelman Science, U.S.A.). Then the samples were analyzed by HPLC as described previously.[2] The samples were mixed with the same volume of cold acetonitrile before centrifugation. The standards are shown under the chromatogram of the reaction: 1, glucose; 2, maltose; 3, maltotriose; 4, panose; a, maltotetraose; b, maltopentaose.

Table Comparison of the Compositions of the Alo Mixture on Market and That Product in This Study

Sugars	Contents (%)	
	On market[a]	This work
Glucose	40.5	27.3
Maltose	6.7	21.1
Isomaltose	16.9	10.0
Others[b]	4.7	
Maltotriose	0.8	3.8
Panose	12.5	20.3[c]
Isomaltotriose	3.4	
Others[b]	2.3	
Maltotetraose		1.2
Anomalously linked tetraoligomers[b]	8.9	14.1
Anomalously linked pentaoligomers[b]	3.3	2.2

[a] See ref. 1.
[b] Structure not identified.
[c] May contain isopanose.

by ion-chromatography (Dionex, CarboPac PA1; chromatogram not shown). The retention time of the peak following the G3 peak corresponded to that of panose (peaks 4), indicating the production of the molecule in large amounts. The peak which was detected right after the elution of G4 seemed to represent a maltosylmaltose peak (DP4; peak 5). Another peak following the G5 peak was likely to contain branched G5 (DP5; peak 6). The content of the Alo mixture is compared to that of the Alo mixture on market in Table. The Alo mixture produced in our laboratory contained less glucose, but more maltotriose, panose, and anomalously linked DP4 molecules than that on the market. The Alo mixture produced in our laboratory would be beneficial in prevention of dental caries due to the low glucose content. The Alo mixture selectively promoted the growth of *Bifidobacteria*, which are beneficial intestinal bacteria.[5] Retrogradation of bread was delayed significantly when the Alo mixture was added in the concentration of 20% (Kweon and Park; unpublished data). The presence of either isomaltotriose or isopanose was not detected in the Alo mixture produced in our laboratory by the HPLC analysis. Recently, detection of a small amount of isopanose was made possible by ion-chromatography and the result will be discussed in detail elsewhere (manuscript in preparation). The DP4 fraction is likely to contain 6^2-*O*-α-maltosylmaltose since maltose was produced exclusively when the fraction was treated with pullulanase. From these, it is likely that anomalously linked oligosaccharides were formed in a complicated manner in which starch is hydrolyzed to glucose, maltose, and oligosaccharides by the action of BLTA; the resulting molecules were then transferred onto the cleavage site of another moiety of sugar by BLMA. The whole reaction was then followed by repeating hydrolysis and transfer reactions, thereby creating a new population of anomalously linked oligosaccharide molecules.

In this study, we showed that the procedure for Alo mixture manufacture can be simplified by applying the two amylases, BLTA and BLMA, to a starch solution simultaneously. The quality of the Alo mixture was improved as well in that the mixture contains less glucose by the procedure developed in this study. Further characterization of each HPLC fraction containing branched oligosaccharides and optimization of the conditions for branched oligosaccharides production have been undertaken in our laboratory and the manuscript is in preparation.

Acknowledgments. Dr. J. W. Kim is supported by the Korea Research Foundation. This work was supported by Research Center for New Bio-Materials in Agriculture.

References

1) H. Takaku, in "Handbook of Amylases and Related Enzymes: Their Sources, Isolation Methods, Properties and Applications," ed. by The Amylase Research Society of Japan, Osaka, Japan, Pergamon Press, New York, 1988, pp. 215–217.
2) I. C. Kim, J. H. Cha, J. R. Kim, S. Y. Jang, B. C. Seo, T. K. Cheong, T. S. Lee, Y. D. Choi, and K. H. Park, *J. Biol. Chem.*, **267**, 22108–22114 (1992).
3) I. C. Kim, PhD. ThD. Thesis, College of Agriculture and Life Sciences, Seoul National University, Suwon, Korea (1991).
4) T. Yuuki, T. Nomura, H. Tezuka, A. Tsuboi, H. Yamagata, N. Tsukagoshi, and S. Udaka, *J. Biochem.*, **98**, 1147–1156 (1985).
5) J. Park, J. Yoo, O. Shin, H. Shin, S. Lee, and K. Park, *Kor. J. Appl. Microbiol. Biotechnol.* (in Korean), **20**, 237–242 (1992).