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The Preparation and Identification of Hydrolysis Oligosaccharide from White Copra Meal by Yeast Fermentation and Sunflower Seed Enzymes

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Abstract

β-1,4-Mannotriose was prepared by the enzymatic hydrolysis of white copra meal (WCM) and the subsequent elimination of monosaccharides from the resultant hydrolysate with a yeast. The enzyme system from sunflower seed hydrolyzed WCM and produced monosaccharides and β-1,4-mannotriose without other oligomers at the final stage of the reaction. WCM (50 g) was hydrolyzed at 50°C and pH 4.5 for 24 hr with a crude enzyme solution (500 mL) from sunflower seed. By the elimination of monosaccharides from the hydrolysis products with a yeast (*Candida glaebosa*), 8.1 g of crystalline mannotriose was otained without the use of chromatographic techniques. After 48hr of yeast cultivation, the total sugar content decreased from 4.6% to 3.5%, whereas the average degree of polymerization increased from 2.3 to 3.1.

Key words: β-1,4-mannotriose, white copra meal, sunflower seed, α-galactosidase

INTRODUCTION

α-Galactosidase (EC 3.2.1.22) is of particular interest in view of its biotechnological applications. α-Galactosidase from coffee beans demonstrates a relatively broad substrate specificity, cleaving a variety of terminal α-galactosyl residues. *Cyamopsis tetragonoloba (guar)* α-*galactosidase effectively liberates* the α-galactosyl residue of galactomannan. Removal of a quantitative proportion of galactose moieties from guar gum by α-galactosidase improves the gelling properties of the polysaccharide and makes them comparable to those of locust bean gum (1).

In the sugar beet industry, α-galactosidase has been used to increase the sucrose yield by eliminating raffinose, which prevents normal crystallization of beet sugar (2).

Raffinose and stachyose in beans are known to cause flatulence. α-Galactosidase has the potential to alleviate these symptoms, for instance, in the treatment of soybean milk (3). α-Galactosidase is known to occur widely in microorganisms, plants, and animals, and some of its forms have been purified and characterized (4). Also, studies of the purification and substrate specificity of α-galactosidase from sunflower seeds (5) and earthworms (6) by affinity chromatography have been reported.

The objective of this paper is to apply the specific character of the enzyme and to carry out the preparation of mannotriose from WCM using a combined process. The combined process consists of namely hydrolyzing the WCM by the crude enzyme and eliminating monosaccharides from the resulting hydrolysate with a yeast.

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MATERIALS AND METHODS

White copra meal (WCM) and β-1,4-mannoligosaccharides

The preparation method and sugar compositions of WCM have been described by Park et al. (7). The substrate was also prepared by the method described in a previous paper (8).

Preparation of α-galactosidase solution

The galactosidase from sunflower seeds was prepared as described by Park et al. (5). The resulting crude enzyme was dialyzed at 4°C overnight against a 4-fold volume of distilled water, and then was used as the galactosidase solution in the hydrolysis of the WCM.

Determination of α-galactosidase activity

α-Galactosidase activity was assayed by measuring the amount of *p*-nitrophenol released from p-nitrophenyl-α-D-galactopyranoside (9). One unit of activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol from pNP Gal per min at pH 4.5 and 50°C.

Determination of reducing sugars

Reducing sugars were determined by the method of Somogyi (10). Total sugar content in the enzymatic hydrolysate was determined by the same method, after hydrolyzing oligosaccharides by 4% H_2SO_4 at 100°C for 2 hr.

Medium composition and conditions of yeast cultivation

Nutrient (consisting of 0.2 g peptone, 0.3 g yeast extact, 0.1 g potassium phosphate (monobasic) and magnesium sulfate), and 0.2 g calcium carbonate, were added to 100 mL of the surpenatant liquid of the enzymatic hydroysate of WCM.

The resulting medium (pH 5) was placed in a 500 mL shaking flask, and sterilized at 120°C for 5 min in an autoclave. The seed culture of the yeast was inoculated into the medium, and cultivated at 30°C on a reciprocal shaker. At certain time intervals, a small amount of culture broth was removed from the flask, followed by the removal of yeast cells from the broth by centrifugation. The supernatant solution thus obtained was subjected to the determination of sugar content and the TLC.

Preparation of β-1,4-mannotriose

WCM (50 g) was hydrolyzed with 500 mL of the enzyme solution at pH 4.5 and 50°C for 24 hr. After the removal of insoluble materials from the hydrolysate by centrifugation, a solution containing 15 g of total sugar was obtained. The final concentration of the nutrients added to the solution was 0.2% peptone, 0.3% yeast extract, 0.1% potassium phosphate (monobasic), 0.05% magnesium sulfate and 0.2% calcium carbonate respectively. About 100 mL of each of the solution supplement with the nutrients was placed into five 500 mL shaking flasks and sterilized under the same conditions mentioned above. After cooling, 5 mL of the seed culture of *Candida glaebosa* was inoculated into the medium in the flask. Cultivation was carried out at 30°C for about 48hr. After cultivation, the yeast cells were removed by centrifugation, and the supernatant liquid containing 12.5 g of total sugar was obtained. The solution was decolorized with active carbon, and was then desalted on columns of Cation (IR-200c) and anion (IRA-68) exchange resins. The resulting sugar solution was concentrated to a syrup by a vaccum rotary evaporator. Hot absolute ethanol was added to the syrup to reach a concentration of about 80% ethanol. After the seeding of crystalline mannotriose and the cooling, the mannotriose was crystallized. The crystals formed were isolated by centrifugal filtration, and crystalline mannotriose was obtained.

Thin-layer chromatography (TLC) and Bio-Liquid chromatography (Bio-LC)

TLC was carried out according to the method of McCleary (11). The sugar sample was dotted on a plate of Merck DC-Alufolien Kiesel gel 60 (0.2 mm), and developed with a solvent system of n-propanol : nitromethane : water (5:2:3, v/v) for about 4 hr at room temperature. The sugar on the plate was revealed by heating the plate at 120°C for about 10 min after spraying it with 30% H_2SO_4-ethanol. Then it was analyzed by a Bio-LC (Dionex Co., USA) equipped with a Carbo Pac TA 1 Grand column (Dionex Co.). The carrier gas was N_2 and the eluent was 150 mM NaOH. The flow rate was 1.0 mL per min.

Hydrogenation of saccharides

Saccharide was hydrogenated into their corresponding sugar alcohols by treating aqueous solutions of the sugar with sodium borohydride for 2 hr at room temperature. The resultant sugar solution was treated with Amberlite IR-200c (H+) to decompose the excess sodium borohydride, and to remove the base, and then evaporated with methanol to remove boric acid.

Methylation analysis

The sugar was methylated by the methods of Ciucanu and Kerek (12). The methylated sugar was hydrolyzed in 10% trifluoro acetic acid, hydrogenated with sodium borohydride and acetylated with an equal mixture of pyridine and acetic anhydride. The resultant alditol-acetate was analyzed by using a column of 3% ECNSS-M on GC (Gas Chrom Q) (Nippon Kuromato Kogyo, Japan) at 155°C, and a column of OV-210 on Spelcoport (Nippon Kuromato Kogyo, Japan) at 190°C.

Identification of component sugars

Oligosaccharide was hydrolyzed in 10% trifluoroacetic acid (in an ampoule), by heating at 100°C for 2 hr. The hydrolysate was evaporated to dryness on a rotary evaporator. The resultant sugar was converted into their alditol-acetate derivative and an analyzed by gas liquid chromatography (13) on a 3% ECNSS-M column.

RESULTS

Formation of β-1,4-mannotriose from white copra meal (WCM) with galactosidase solution

The WCM (3 g), which contained 1.51 g of total sugar (with 1.04 g of mannan), was hydrolyzed with 30 mL of the enzyme solution at pH 4.5 and 50°C for 24 hr. After the removal of insoluble materials from the resultant hydrolysates at certain time intervals (1,3,5,8 and 24 hr) by centrifugation, each 4 μL of the supernatant liquid was subjected to TLC for the characterization of the hydrolysis products.

Fig. 1 shows TLC of the time-course of hydrolysis of WCM with enzyme solution. At the reaction time between the first and the 24th hr, galactose and mannotriose were the products of the hydrolysis. From this result, the preparation of mannotriose by the enzymatic hydrolysate of WCM without using chromatographic separation techniques was tried. These results were possible with a combination of the hydrolysis of the WCM by crude enzyme and the elimination of galactose from the resultant hydrolysate by the selective fermentation with a yeast.

Elimination of galactose from enzymatic hydrolysate of WCM by yeast

Because *Candida glaebosa* is capable of metabolizing galactose in enzymatic hydrolysate, and of leaving mannotriose in the hydrolysate, which was selected. The time-course of the decrease of sugar in the yeast cultivation was followed by the method of Somogyi (10). Fig. 2 shows TLC of the time-course of yeast cultures. After 48 hours of cultivation, the yeast strain digested galactose, but left mannotriose in the medium.

Fig. 3 shows the course of yeast cultivation. As shown in Fig. 3, the sugar content decreased as the time passed till about the 30th hr, but no remarkable decrease was observed there-

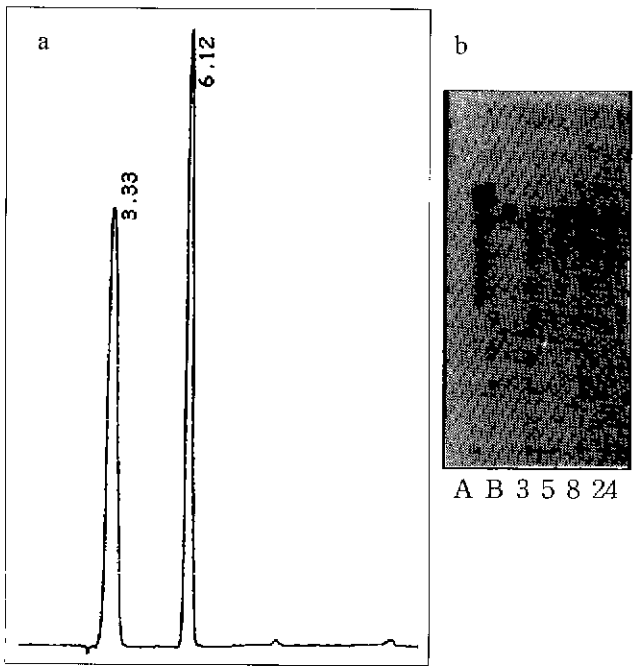


Fig. 1. Bio-liquid chromatogram (a) and thin-layer chromatogram (b) of hydrolysates of white copra meal with the enzyme. (a) RT time 3.33; galactose, RT time 6.12; mannotriose. (b) A: Authentic mannose, mannobiose, mannotriose, mannotetraose and mannopentaose from top to bottom, B: Authentic galactose

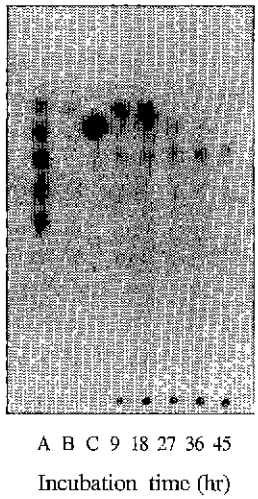


Fig. 2. Elimination of monosaccharides from enzymatic hydrolysate of white copra meal by yeast. A, Authentic mannose, mannobiose, mannotriose, mannotetraose and mannopentaose from top to bottom; B, Authentic glucose; C, Authentic galactose

after. After 48hr of cultivation, the total sugar content decreased from 4.6% to 3.5%, and the average degree of polymerization (DP), on the other hand, increased from 2.3 to 3.1.

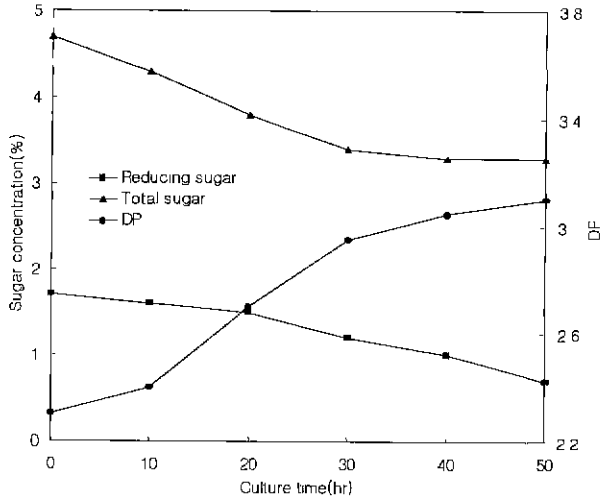


Fig. 3. Time course of cultivation of enzymatic hydrolysate of white copra meal with yeast.

Characterization of mannotriose

Fig. 4 shows the flow sheet on the preparation of crystalline mannotriose. Crude crystals of the mannotriose from above were recrystallized twice from about 80% aqueous ethanol. 8.1 g of crystalline mannotriose was obtained. The methylation analysis (Table 1) of crystalline mannotriose revealed the 2,3,4,6-Tetra-O-Me-D-Man (1 mol) and 2,3,6-Tri-O-Me-D-Man (2 mol). The methylation of the corresponding hydrogenated derivative revealed the appearence of 1,2,3,5,6-Penta-O-Me-D-Mannitol (1 mol), 2,3,4,6-Tetra-O-Me-D-Man (1 mol) and 2,3,6-Tri-O-Me-D-Man (1 mol) with the disappearence of 2,3,6-Me-Man (1mol) (Table 1). Based on the above results

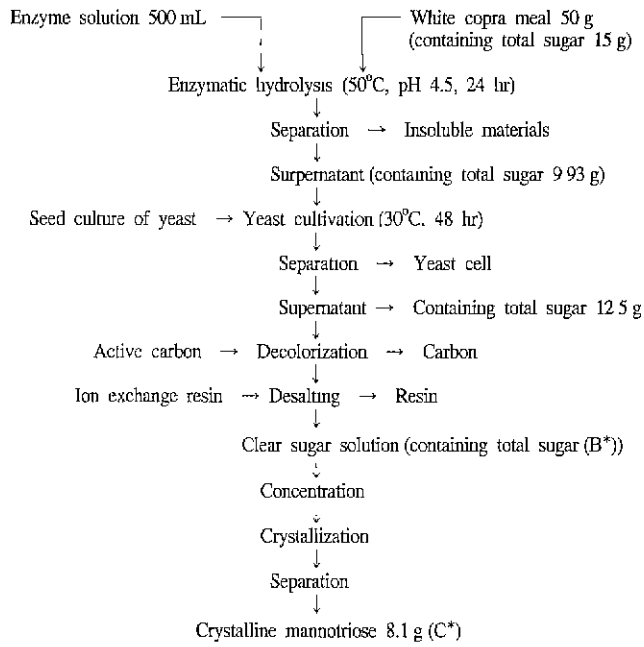


Fig. 4. Flow chart for the process of enzymatic preparation of crystalline mannotriose from white copra meal.

Table 1. Methylation analysis of the oligosaccharide and their hydrogenated derivative isolated from the enzymatic hydrolysate of white copra meal

Alditol acetate		1,2,3,5,6-Penta-O-Me-D-Mannitol	2,3,4,6-Tetra-O-Me-D-Man	2,3,6-Tri-O-Me-D-Man	2,3-Di-O-Me-D-Man
Retention time (min)		2.1	5.2	13.5	31.8
Reference mannotriose Sugar	A		+	++	
	B	+	+	+	
Sample	A		+	++	
	B	+	+	+	

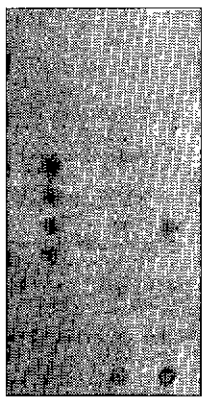
A, Original sugar; B, After hydrogenation with $NaBH_4$; +, 1 mol ; ++, 2 mol.

the proposed structure identified to β-1,4-mannotriose. Figure 5 also shows sugar samples at various stages of the preparation of β-1,4-mannotriose from the white copra meal.

DISCUSSION

The galactomannan consists of galactose and mannose in the ratio of 1:10~1:15. α-Galactosyl branches are irregular, and in some parts of the galactomannan chain, the distance between two branches is short. The degradation of the galactomannan by *Streptomyces* mannanase produced mannose and mannooligosaccharides, in addition to several kinds of heterooligomers consisting of galactose and mannose residues. On the other hand, the saccharides produced from the degradation of several galactomannans by mannanases originating from the various kinds of fungi, were almost the same with the saccharides described above (14). Accordingly, an enzyme system with the coexistence of β-mannanase and α-galactosidase is essential to avoid the formation of the galactomanno-oligosaccharides from copra galactomannan. Moreover, it is desirable to simplify the process of mannotriose production so that an enzyme system can hydrolyze directly the galactomannan in white copra meal and produce only monosaccharides and mannotriose from the copra meal.

In the connection with the mannooligosaccharides preparation, mannobiose preparation from brown copra meal by *Penicillium purpurogenum* have been reported (15). The strain produced β-mannanase extracellularly and the enzyme system directly attacked the galactomannan in the brown copra meal. Moreover, the optimum pH and temperature for the mannanase activity were 5 and 60°C, respectively. On the other hand, the optimum pH and temperature for the α-galactosidase activity, produced by the same strain, were 4.5 and 55°C, respectively. Therefore, the enzyme system seems to be most suitable for the hydrolysis of galactomannan in the brown copra meal, because the properties of the two enzymes are very similar to each other. The final products of the digestion of galactomannan by the enzyme system included monosaccharides (galactose, glucose and mannose) and mannobiose without a considerable amount of other oligomers. To prepare mannobiose without using any chromatographic techniques, *Candida parapsilosis var. komabaensis* k-75 having the selective fermentation is able to elininate only the monosaccharides. In addition, it was assumed that the enzyme system may also contain other kinds of enzymes, probably cellulase and β-glucosidase as glucose was detected in the enzymatic hydrolysate. In conclusion, the combination of the hydrolysis of white copra meal by the enzyme system of sunflower seeds and the elimination of galactose from the resultant hydrolysate with *Candida glaebosa* was suitable for the preparation of mannotriose from the copra meal.



A B C

Fig. 5. Thin-layer chromatograms of sugar samples at various stage of mannotriose preparation. A: Authentic mannose, mannobiose, mannotriose, mannotetraose and mannopentaose from top to bottom B*: sugar solution after yeast was grown in the enzymatic hydrolysate medium. C*. Crystalline mannotriose. Symbols B* and C* in this figure correspond to those in Fig. 4.

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