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The Preparation of Crystalline β-1,4-Mannotriose from Poonac Using the Enzyme System and Yeast Fermentation

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Abstract Beta-1,4-mannotriose was prepared by the enzymatic hydrolysis of poonac and the subsequent elimination with yeast of monosaccharides and disaccharide from the resultant hydrolysate. The enzyme system hydrolyzed poonac and produced monosaccharides, disaccharide and β-1,4-mannotriose without other oligomers at the final reaction stage. Poonac (50 g) was hydrolyzed at 50°C and pH 6 for 48 hr with the crude enzyme solution (500 mL) from *Trichoderma harzianum*. The elimination of monosaccharides and disaccharide from the hydrolysis products with a yeast (*Candida guilliermondii*) produced 10.5 g of crystalline β-1,4-mannotriose without the use of chromatographic techniques. After 48 hr of yeast cultivation, the total sugar content fell from 4.8% to 3.4%, and the average degree of polymerization (D.P) rose from 2.5 to 3.2. The preparation method presented was confirmed to be suitable for the preparation of mannotriose from poonac.

Keywords: β-1,4-mannotriose, *Trichoderma harzianum, Candida guilliermondii,* methylation method

Introduction

The main objective of the coconut industry is to extract coconut oil for use as a raw material. In the process of extracting oil from copra, a large amount of residual cake (poonac) is discharged as a by-product. The poonac contains 40~50% of galactomannan (Gal : Man=1 : 10~15). By using the *Streptomyces* β-mannanase system, β-1, 4-mannooligosaccharides (1) were prepared for the application of functional mannooligosaccharides. It was further reported in this study that direct hydrolysis of the poonac by mannanase is easier and more economical for the preparation of mannooligosaccharides.

Mannotriose has been prepared from partial acid and enzymatic hydrolysates of plain mannans. The traditional preparation methods used, however, are not suitable for the preparation of a substantial quantity of mannotriose because of the low yields and complexity in operation.

The characteristic features of α-galactosidase from *Penicillium purpurogenum* (2) and the properties of the purified mannanase were reported, previously (3). There are many reports dealing with β-mannanase from various microorganisms (4) but only three kinds of the enzyme from *Streptomyces* sp. No. 17 (5), *Leucaena glauca* (6) and *Bacillus subtilis* (6) have been studied for the specificity of the enzyme to galactomannan.

The objective of the present paper was to apply the specific characteristic of the enzyme and carry out the preparation process. The combined process consists of mainly hydrolyzing the poonac by the crude enzyme and using a yeast to eliminate the monosaccharides and disaccharide from the resulting hydrolysate.

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Materials and Methods

Poonac and β-1,4-mannooligosaccharides Poonac, which is a by-product of oil extraction from copra, was kindly supplied by Blue Bar Inc. of the Philippines. The meal contained 49.9% as total sugar, which was composed of 63.4% mannose, 24.9% glucose, 6.6% galactose and 4.4% arabinose. Poonac was hydrolyzed with 72% sulfuric acid at 30 for 30 min, followed by 4% sulfuric avid at 100 for 2 hr. The preparation method and sugar compositions of poonac have been reported in a previous paper (2). The substrate was also prepared by the method described in another previous paper (1).

Preparation of β-mannanase solution The mannanase from *Trichoderma harzianum* was prepared by a submerged-culturing, as described in a previous paper (7). Briefly, the resulting culture filtrate was dialyzed at 4 overnight against a 4-fold volume of distilled water, and was then used as the mannanase solution in the hydrolysis of the poonac.

Determination of β-mannanase activity The assay mixture, containing 129.4 mg of copra mannan (3) (equivalent to 100 mg of polymannose), 4.0 mL of McIlvaine buffer solution (pH 6) and 5.0 mL of water, was deposited into an L-shaped tube. The tube was then preincubated at 50°C for 10 min on a Monod shaker with agitation of 60 oscillations per min. Enzyme solution (1.0 mL) was added to the mixture, after which the mixture was incubated for 30 min at the same temperature. The reducing power produced by the enzyme reaction was determined as mannose by the method of Somogyi (8). Briefly, one unit of the enzyme activity was defined as the amount which liberated reducing sugar equivalent to 1 μm of mannose per min under the above conditions.

Determination of reducing sugar Reducing sugar was determined by the method of Somogyi (8). Briefly, 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. The average degree of polymerization (D.P) of the sugar was calculated from the ratio of the total sugar to the reducing sugar.

Nutrients compositions and conditions of yeast cultivation Nutrients (consisting of 0.2 g peptone, 0.3 g yeast extract, 0.1 g potassium phosphate (monobasic) and magnesium sulfate) and 0.2 g of calcium carbonate were added to 100 mL of the supernatant liquid of the enzymatic hydrolysate of poonac. The resulting medium was placed a 500 mL shake 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, after which yeast cells were removed from the broth by centrifugation. The supernatant solution thus obtained underwent sugar content determination and thin layer chromatography (TLC).

Preparation of β-1,4-mannotriose The poonac (50 g), which contained 28.8 g of total sugar (with 16.5 g of mannan), was hydrolyzed with 500 mL of the enzyme solution at pH 6 and 50°C for 48 hr. After removal of insoluble materials from the hydrolysate by centrifugation, a solution containing 20.3 g of total sugar was obtained. The final concentration of the nutrients added to the solution was 0.2% of peptone, 0.3% of yeast extract, 0.1% of potassium phosphate (monobasic), 0.05% of magnesium sulfate and 0.2% of calcium carbonate. About 100 mL of each of the five solutions supplemented with the nutrients were placed into one 500 mL shake flask each and sterilized under the above conditions. After cooling, 5 mL of the seed culture of *Candida guilliermondii* (9) was inoculated into the medium in the flask. Cultivation was carried out at 30°C for about 48 hr. After cultivation, the yeast cells were removed by centrifugation, and the supernatant liquid containing a total of 15.7 g of 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 consistency by a vacuum rotary evaporator. Hot absolute ethanol was added to the syrup to achieve an ethanol concentration of about 80%. After the seeding of crystalline β-1,4-mannotriose and cooling, the mannotriose was crystallized. The mannotriose crystals formed were isolated by centrifugal filtration.

Thin layer chromatography (TLC) TLC was carried out according to the method of McCleary (10). Briefly, 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 1-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.

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

Methylation analysis The sugar was methylated by the methods of Ciucanuet *et al.* (11). Briefly, the methylated sugar was hydrolyzed in 10% trifluoroacetic acid, and 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 Gas Chrom Q (Nippon Kuromato Kogyo, Japan) at 155°C, and a column of OV-210 on Supelcoport (Nippon Kuromato Kogyo, Japan) at 190°C.

Identification of sugar component 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 sugars were converted into their alditol-acid derivatives and analyzed by gas liquid chromatography (12) on a 3% ECNSS-M column.

Results

Production of β-mannanase system Fig. 1 shows the course of cultivation. There was a rapid increase in the production of the enzyme at about 40 hr after the beginning of cultivation, and the mannanase activity in the culture filtrate peaked at 45 hr. After 45 hr, mycelium was filtered off through a Buchner funnel with Toyo-roshi No. 2 filter paper. The resulting filtrate was then dialyzed against a 4-fold volume of distilled water, and the dialyzed solution was used as the mannanase solution for the following experiments.

Formation of β-1,4-mannotriose from poonac with mannanase solution The poonac (5 g), containing 2.88

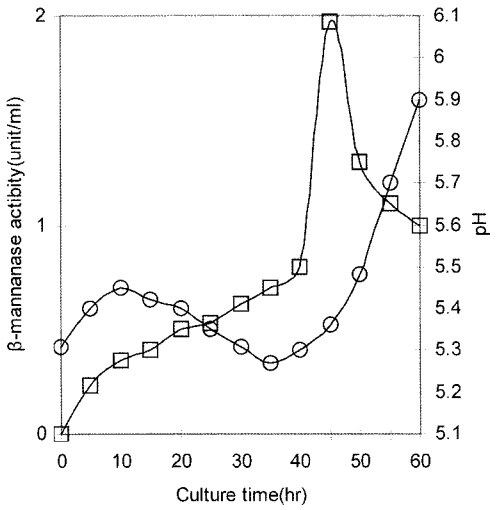

Fig. 1. Time course of the production of â-mannanase from *Trichoderma harzianum*. –□–, β-mannanase activity; –○–, pH.

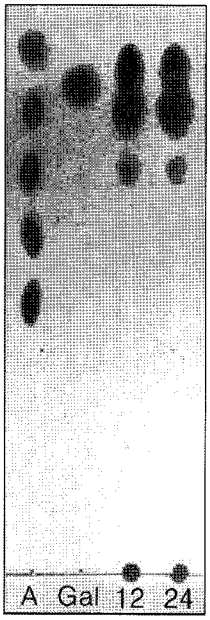

Fig. 2. Time course of hydrolysis of poonac with enzyme solution. A: authentic mannose, mannobiose, mannotriose, manno-tetraose and mannopentose from top to bottom; Gal: galactose.

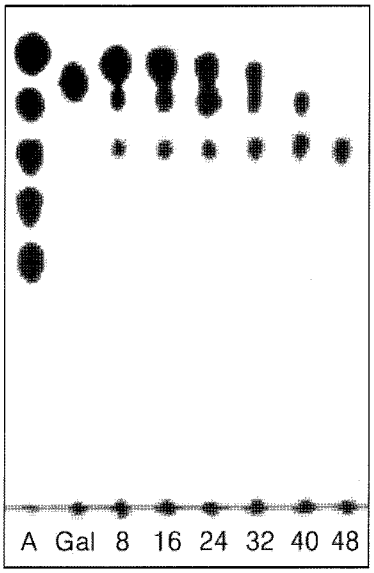

Fig. 3. Elimination of monosaccharides and disaccharide from enzymatic hydrolysate of poonac by yeast. A: authentic mannose, mannobiose, mannotriose, mannotetraose and manno-pentose from top to bottom; Gal: galactose.

g of total sugar (with 1.65 g of mannan), was hydrolyzed with 50 mL of the enzyme solution at pH 6 and 50°C for 48 hr. After the removal by centrifugation of insoluble materials from the resultant hydrolysates at 12 and 24 hr, each 4 μL sample of the supernatant liquid was subjected to TLC for the characterization of the hydrolysis products.

Fig. 2 shows TLC of the hydrolysis time-course of poonac with enzyme solution. Between 12 hr and 24 hr, monosaccharides, mannobiose and mannotriose were the hydrolysis products. From this result, the preparation of mannotriose from the enzymatic hydrolysate of poonac without using chromatographic separation techniques was studied. Also investigated was the combination of the hydrolysis of the poonac by crude enzyme and the elimination of monosaccharides and disaccharide from the resultant hydrolysate by the selective fermentation with *Candida guilliermondii*.

Elimination of monosaccharides and disaccharide from enzymatic hydrolysate of poonac by yeast Twenty-eight yeast strains were screened to find one capable of metabolizing monosaccharides and disaccharide in enzymatic hydrolysate and of leaving mannotriose in the hydrolysate, from which *Candida guilliermondii* was selected.

The time-course of the sugar decrease in the yeast cultivation was followed by the method of Somogyi (8). Fig. 3 shows TLC of the time-course of yeast cultures. After 48 hr of cultivation, the yeast strain had digested monosaccharides and disaccharide, but left mannotriose in the medium.

Fig. 4 shows the course of yeast cultivation. The sugar content decreased with passing time until about 24 hr, after which no remarkable decrease was observed. After 48 hr of cultivation, the total sugar content decreased from 4.8% to 3.4%, while D.P, on the other hand, rose from 2.5 to 3.2.

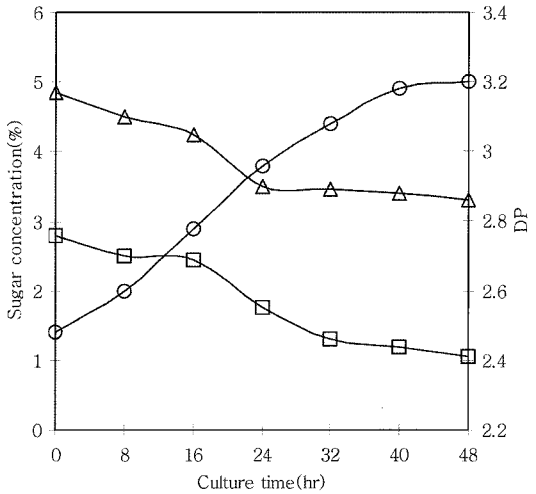

Fig. 4. Time course of cultivation of enzymatic hydrolysate of poonac with yeast. –△–, Total sugar; –□–, Reducing sugar; –○–, DP.

Characterization of mannotriose Table 1 shows the results of methylation analysis of β-1,4-mannotriose. This sugar was composed only of mannose and the position of M_3 on TLC was the same as that of β-1,4-mannotriose (Fig. 2). The results of methylation analysis further supported this structural interpretation (Table 1). The methylation analysis of M_3 revealed the presence of 2,3,4,6-Me-Man (1 mol) and 2,3,6-Me-Man (2 mol). The methylation of the corresponding hydrogenated derivative revealed the appearance of 1,2,3,5,6-Me-Mannitol (1 mol) with the disappearance of 2,3,6-Me-Man (1 mol). This result indicated that M_3 has a 1,4-linkage.

Discussion

In terms of its partial chemical structure (5), poonac

Table 1. Methylation analysis of the oligosaccharide and its hydrogenated derivative from hydrolysate of poonac with *Candida guilliermondii*

Alditol acetate		1,2,3,4,5.6 - Penta-O-Me-D-Mannitol	2,3,4,6 - Tetra-O-Me-D-Man	2,3,4,6 - Tetra-O-Me-D-Gal	2,3.6 - Tri-O-Me-D-Man	2.3 - Di-O-Me-D-Man
Retention time (min)		1.9	5.7	7.2	12.9	30.7
Reference sample						
Mannotriose	A		+		++	
	B	+	+		+	
M_3	A		+		++	
	B	+	+		+	

A: original sugar, B: after hydrogenation with $NaBH_4$, +: 1 mol, ++: 2 mol.

consists of galactose and mannose in the ratio of 1 : 10~1 : 15. The α-galactosyl branches irregularly, and in some parts of the poonac chain, the distance between a pair of branches is short. Therefore, in this experiment, the final products (5, 13) from the poonac degradation by *Streptomyces* mannanase were mannose and mannooligosaccharides, in addition to several kinds of hetero-oligomers consisting of galactose and mannose residues. On the other hand, the saccharides produced from the degradation of several poonac samples by mannanase originating from various kinds of fungi, were almost the same as those described above (14-16). Accordingly, an enzyme system with the coexistence of β-mannanase and α-galactosidase is essential to avoid the formation of galactomanno-oligosaccharides from poonac. Moreover, it is desirable to simplify the process of mannotriose production so that an enzyme system can hydrolyze directly the galactomannan in poonac to produce only monosaccharides, mannobiose and mannotriose. This requirement necessitates the screening of a microorganism which secretes an enzyme system suitable for the above purpose.

The yeast strain, *Candida guilliermondii*, produced β-mannanase extracellularly and the enzyme system attacked directly the galactomannan in the poonac. Moreover, the optimum pH and temperature for the mannanase activity were 6 and 50°C, respectively, whereas those for the α-galactosidase activity, produced by the same strain, were 4.5 and 55°C, respectively. Therefore, this enzyme system seems to be the most suitable for the hydrolysis of galactomannan in the poonac, 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), mannobiose and mannotriose without a considerable presence of other oligomers. Elimination of the monosaccharides and disaccharide with selective fermentation by yeast achieved the preparation of mannotriose without using any chromatographic technique. 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. Fig. 5 shows the flow sheet for the preparation of crystalline mannotriose. Crude crystals of the mannotriose from above were recrystallized twice from about 80% aqueous ethanol to produce 10.5 g of crystalline mannotriose.

In conclusion, the preparation method presented here, which combined the hydrolysis of poonac by the enzyme system of *Trichoderma harzianum* and the elimination of monosaccharides and disaccharide from the resultant hydrolysate with the yeast strain *Candida guilliermondii*, was suitable for the preparation of mannotriose from poonac. The oligosaccharide structure was elucidated by the methylation method.

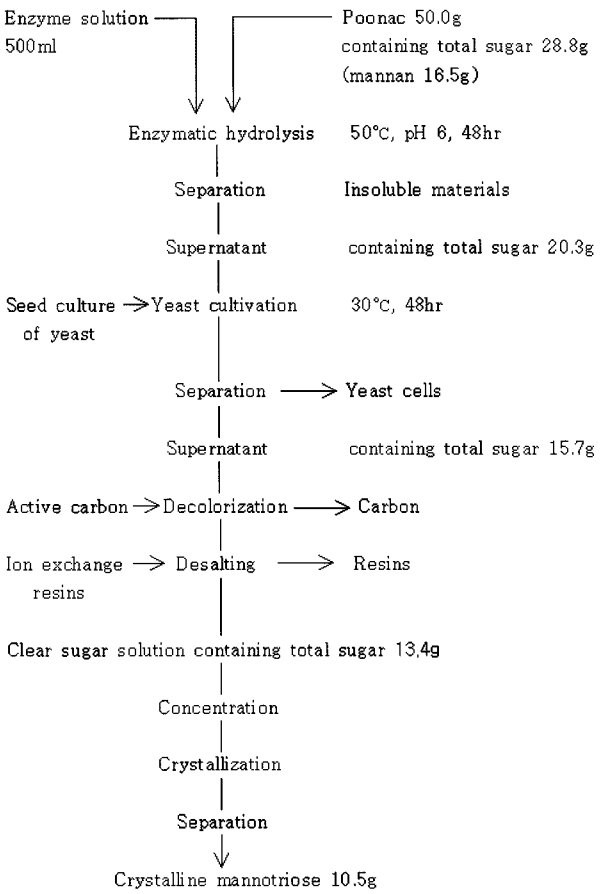

Fig. 5. Flow chart for the process of enzymatic preparation of crystalline mannotriose from poonac.

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