

Production of Galacto-manno-oligosaccharides from Guar Gum by β -Mannanase from *Penicillium oxalicum* SO

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β -Mannanase from *Penicillium oxalicum* SO efficiently hydrolyzed guar galactomannan to galacto-manno-oligosaccharides. Gel filtration estimated the molecular weight of the β -mannanase as 35 000 and SDS-PAGE as 29 000. The optimum pH was around 5 while a stable pH was reached in the range of 3–6. Optimum temperature was around 60 °C at pH 5, while under 60 °C activity was stable. HPLC analysis detected oligosaccharides with degrees of polymerization (DP) of 2 to 7 and 2 to 6 released on hydrolysis of guar and locust bean gums, respectively; about 92% of the released sugars were oligosaccharides. In analysis of the sugar distribution on MALDI-TOF-MS, major products of DP 6 and 7 and DP 5 and 6 were confirmed in hydrolysates of guar gum and locust bean gum, respectively. One of the main oligosaccharides released from guar gum, with DP 7, had a high galactose content (Gal/Man = 0.76) and corresponded to a blockwise galactose-substituted mannan type in galactomannan.

KEYWORDS: β -Mannanase; guar gum; galactomannan; locust bean gum; oligosaccharides; *Penicillium oxalicum*

INTRODUCTION

Guar gum is produced from the endosperm of guar seeds (*Cyamopsis tetragonolobus*) that are cultivated in northwest India and Pakistan. Guar gum can hydrate rapidly in cold water and form a highly viscous state (1, 2); therefore, it is used as a natural food additive. Guar galactomannan consists of a β -1,4 linked D-mannose backbone and α -1,6 linked D-galactose side chains, in which the D-galactose and D-mannose contents are 38% and 62%, respectively. Partially hydrolyzed guar gum (molecular weight: 1000–100 000 Da) prepared by endo- β -mannanase (3, 4) not only has low viscosity but also has various health benefits such as improving conditions of constipation, a hypocholesterolemic and hypolipidemic effect, and improved intestinal microflora balance (5–10). Many β -mannanases can hydrolyze pure mannan of ivory nut (*Phyttelephas macrocarpa*) into mannooligosaccharides such as mannobiose and mannotriose (11–13), which also function in the selective growth of human-beneficial intestinal microflora (14). However, in the hydrolysis of guar galactomannan it is difficult to obtain oligosaccharides because the high content of galactose, which is distributed on the mannan backbone as a side chain, causes steric hindrance to β -mannanase. β -Mannanase (endo type enzyme) only attacks galactose-unsubstituted mannose blocks on galactomannan, resulting in the production of partially hydrolyzed guar gum. Exo type enzymes (β -mannosidase and α -galactosidase) are also inhibited by the galactosyl residue. In

fact, whether β -mannanase can hydrolyze guar gum into galacto-manno-oligosaccharides of low molecular size remains unknown. Oligosaccharides from guar gum are expected to improve the functional properties of partially hydrolyzed guar gum because of their small molecular size.

In the present study, a guar gum hydrolyzing strain, *Penicillium oxalicum* SO, was isolated from soil using a medium plate with 0.5% guar gum and 1.5% agar. We investigated and discuss here the culture conditions for production of β -mannanase and the enzymatic properties.

MATERIALS AND METHODS

Materials. Guar gum (*C. tetragonolobus*) and locust bean gum (*Ceratonia siliqua*) were purchased from Sigma Ltd; sugar compositions were 38% galactose–62% mannose and 23% galactose–77% mannose, respectively. 3,5-Dihydroxybenzoic acid (DHB) was purchased from Aldrich Chemical Co. All other chemicals were reagent grade.

Culture Conditions. *P. oxalicum* SO was separated from soil using plate culture with a medium consisting of 0.5% guar gum and 1.5% agar at 30 °C. The SO strain was cultivated in liquid medium containing 1% carbon source, 0.5% yeast extract, and 0.2% Na₂HPO₄·12H₂O (5 mL) in a 180 × 18 mm i.d. test tube at 30 °C and at 220 rpm for 3–5 days. Mannose, galactose, glucose, sucrose, maltose, glucomannan, locust bean gum, and guar gum were used as the carbon sources. The SO strain was liquid cultured at 30 °C and 140 rpm for 4 days in a 500 mL flask using medium consisting of 1% guar gum, 0.5% yeast extract, and 0.2% Na₂HPO₄·12H₂O (100 mL). The supernatant of the culture broth after centrifugation at 3000 rpm (1500g) for 10 min was used to determine enzyme activities.

Purification of β -Mannanase. The SO strain was cultivated in liquid medium containing 1% guar gum, 0.5% yeast extract, and 0.2% Na₂

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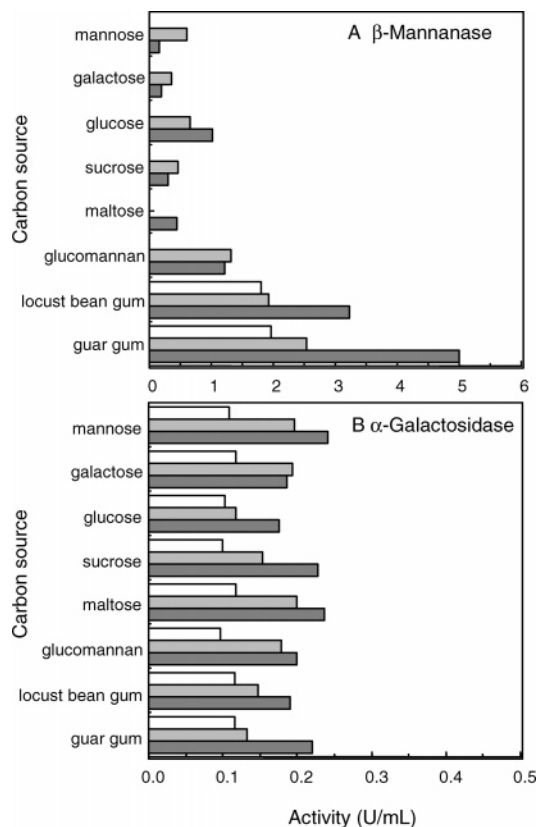


Figure 1. Effects of the carbon source on enzyme production in a liquid culture of the SO strain. SO strain was cultivated with culture medium consisting of 1% carbon source, 0.5% yeast extract, and 0.2% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (5 mL) in a 180×18 mm i.d. test tube at 30°C and 220 rpm for 3–5 days. Symbols: (white bars) 3, (light gray bars) 4, and (dark gray bars) 5 days in β -mannanase (A) and α -galactosidase (B).

$\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (100 mL) in a 500 mL flask (three duplications) at 30°C and 140 rpm for 4 days. The culture filtrate (290 mL) was desalted by being saturated in 90% ammonium sulfate. After filtration, the precipitate was dissolved in 100 mM sodium acetate buffer (pH 5). The crude β -mannanase (guar gum hydrolase) was purified by the following chromatography steps performed at 4°C . The crude enzyme solution (1 g/10 mL of water) was subjected to gel filtration on a Sephadex G-25 column (50×2.5 cm i.d.) pre-equilibrated with 10 mM sodium acetate buffer (pH 5). Proteins were eluted with the same buffer at a flow rate of 1.3 mL/min, and fractions were collected every 3 min. Next, fractions with β -mannanase activity were pooled and subjected to anion-exchange chromatography on a Super Q Toyopearl column (50×1.0 cm i.d.; Toso Ltd, Japan) pre-equilibrated with 10 mM sodium acetate buffer (pH 5). Proteins were eluted with a linear gradient of 0 to 0.6 M NaCl at a flow rate of 0.16 mL/min. Fractions with β -mannanase activity were pooled, and the aliquot was subjected to gel filtration on 60×2.15 cm i.d. TSKgel G3000SW column (Toso Ltd.) pre-equilibrated with 50 mM phosphate buffer (pH 6.8) containing 0.3 M NaCl. The proteins were eluted at a flow rate of 2.0 mL/min, and fractions were collected every 1 min.

Determination of Enzyme Activity. β -Mannanase (Guar Gum Hydrolase). Soluble guar gum (1%; 0.5 mL) was incubated with an enzyme sample (0.5 mL) at pH 5 and 40°C for 10 min. The reaction was stopped by addition of 0.5 mL of 1 M Na_2CO_3 , and then the mixture was centrifuged at 3000 rpm (1500g) for 10 min. The concentration of reducing sugar in the supernatant was determined using the 3,5-dinitrosalicylic acid method (15). One unit was defined as the amount of enzyme that could produce 1 μmol of reducing sugar (glucose base) for 1 min.

α -Galactosidase. *p*-Nitrophenyl α -D-galactopyranoside (1 mM) was incubated with an enzyme sample in 0.1 M acetate buffer (pH 5) at 40°C for 10 min (working volume: 1 mL). The reaction was stopped by addition of 0.5 mL of 1 M Na_2CO_3 and the absorbance of the released

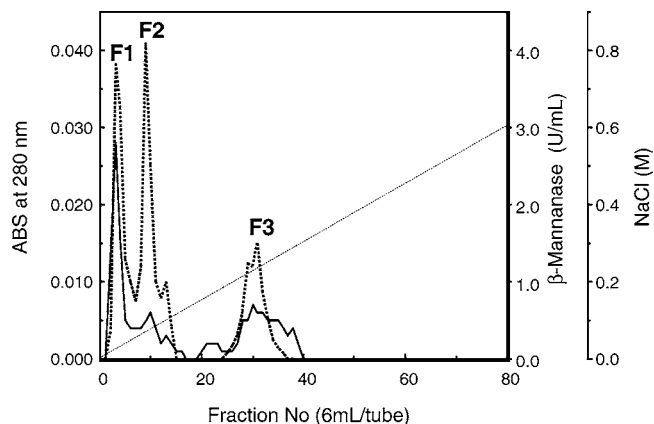


Figure 2. Separation of β -mannanase from crude enzyme on a Super Q Toyopearl column: (—) ABS at 280 nm, (---) β -mannanase activity, and (···) NaCl.

Table 1. Enzymatic Activities in Three Fractions

fraction	β -mannanase (units/mL)	α -galactosidase (units/mL)
F1	3.83	1.79
F2	4.13	0.00
F3	1.51	0.00

p-nitrophenol at 400 nm then determined. One unit was defined as the amount of enzyme that could produce 1 μmol of *p*-nitrophenol for 1 min.

Enzymatic Hydrolysis of Guar Gum and Locust Bean Gum. Fifty milligrams of guar or locust bean gum was rapidly mixed with an enzyme solution (1 mL) and incubated at pH 5 and 40°C for 24 h. After deactivation by heating in boiling water for 5 min, the reaction mixture was centrifuged at 3000 rpm (1500g) for 10 min, and then the supernatant was applied to high performance liquid chromatography (HPLC) and MALDI-TOF-MS analysis of sugars after filtration with a 0.22 μm membrane filter.

Determination of Component Monosaccharides by Hydrolysis with Sulfuric Acid. Supernatants of the reaction mixture from guar and locust bean gums, or the separated oligosaccharide fractions, were diluted with distilled water and filtrated. The aliquot (0.05 mL) was then mixed with 0.2 mL of 2 N sulfuric acid in a 10 mL test tube. The capped tube was put in a block heater and incubated at 100°C for 2 h. After cooling to room temperature, 0.2 mL of distilled water and about 50 mg of CaCO_3 were added to neutralize the reaction solution. The sample was filtered through a 0.22 μm membrane filter and then subjected to HPLC (column NH2P-50) analysis to measure the released monosaccharides (mannose and galactose).

HPLC Analysis of Sugars. Sugars released from guar gum in the enzymatic hydrolysis were analyzed by HPLC under the following conditions: column, 250×7 mm i.d. GL-C610 (Hitachikasei Ltd.); mobile phase, water; column temperature, 60°C ; flow rate, 1.0 mL/min; and detector, Hitachi model L-3300 differential refractive index monitor. For analysis of monosaccharides of mannose and galactose, a 250×4.6 mm i.d. NH2P-50 column (Asahikasei Ltd.) was used in the mobile phase, water:acetonitrile = 25:75, and the column temperature was 25°C .

MALDI-TOF-MS Analysis of Sugars. A supernatant (1 μL) of the reaction mixture from enzymatic hydrolysis was mixed with 10 mg/mL DHB (9 μL), and then an aliquot (1 μL) was spotted on a sample plate. After drying in a desiccator, this was subjected to MALDI-TOF-MS (Voyager DE STR, Applied Biosystem Ltd., MA). Detection was performed in the reflector mode with an accelerating voltage of 20 kV.

RESULTS AND DISCUSSION

Penicillium oxalicum SO was isolated from soil by growth on 0.5% guar gum and 1.5% agar medium. **Figure 1** shows the

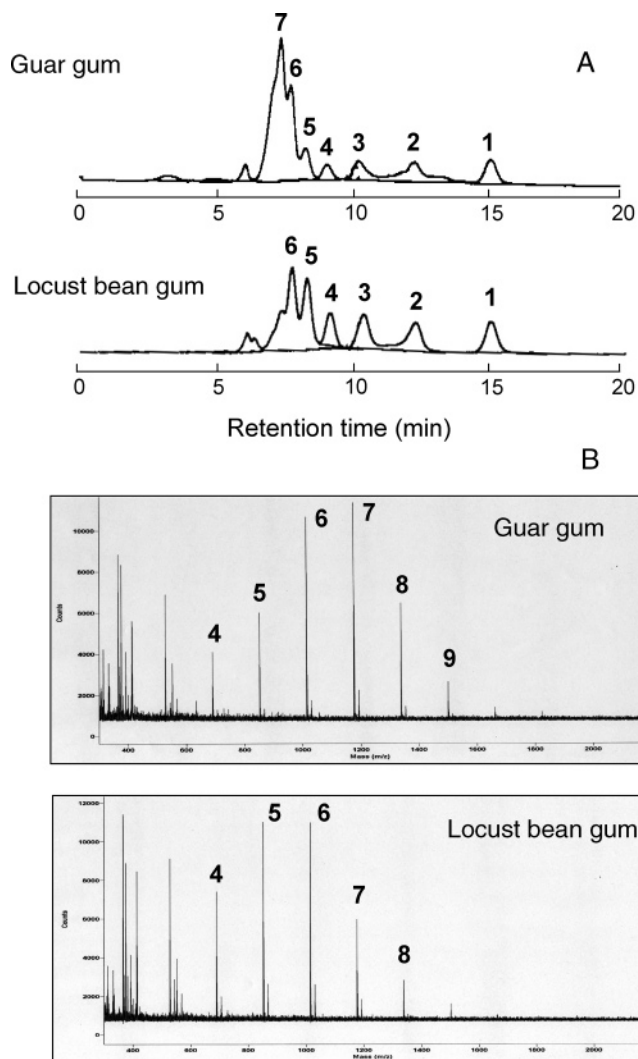


Figure 3. HPLC chart (A) and MALDI-TOF-MS spectrum (B) of the reaction mixture in the hydrolysis of guar and locust bean gums by F2 fractions. The substrate (50 mg) was incubated with 1 mL of enzyme solution at pH 5 and 40 °C for 24 h. The reaction mixture was applied to each analysis after dilution and filtration with a 0.22 μ m membrane filter. The peak number shows the degree of polymerization of the sugar detected.

effect of each carbon source (mannose, galactose, glucose, sucrose, maltose, glucomannan, locust bean gum, and guar gum) on enzyme production from the SO strain in a liquid culture with a 1% carbon source, 0.5% yeast extract, and 0.2% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$. β -Mannanase (guar gum hydrolase) activity in the culture broth was increased by galactomannan, reaching a maximum with guar gum as a carbon source. Guar gum has a higher galactose content (38% galactose) than does locust bean gum (23% galactose). α -Galactosidase (exo type enzyme) activity was the same low level with all carbon sources. Little β -mannosidase activity was detected. These results show that a carbon source of guar gum most efficiently induced β -mannanase.

SO β -mannanase was purified from culture grown in a medium containing 1% guar gum as a carbon source. A crude fraction from the SO strain, isolated by Sephadex G-25 chromatography, was separated by anion-exchange chromatography on a Toyopearl Super Q column (Figure 2). Three fractions with β -mannanase activity, peaks F1, F2, and F3, were eluted at around 0.008, 0.060, and 0.23 M NaCl, respectively. F2 and F3 had no α -galactosidase nor β -mannosidase activity

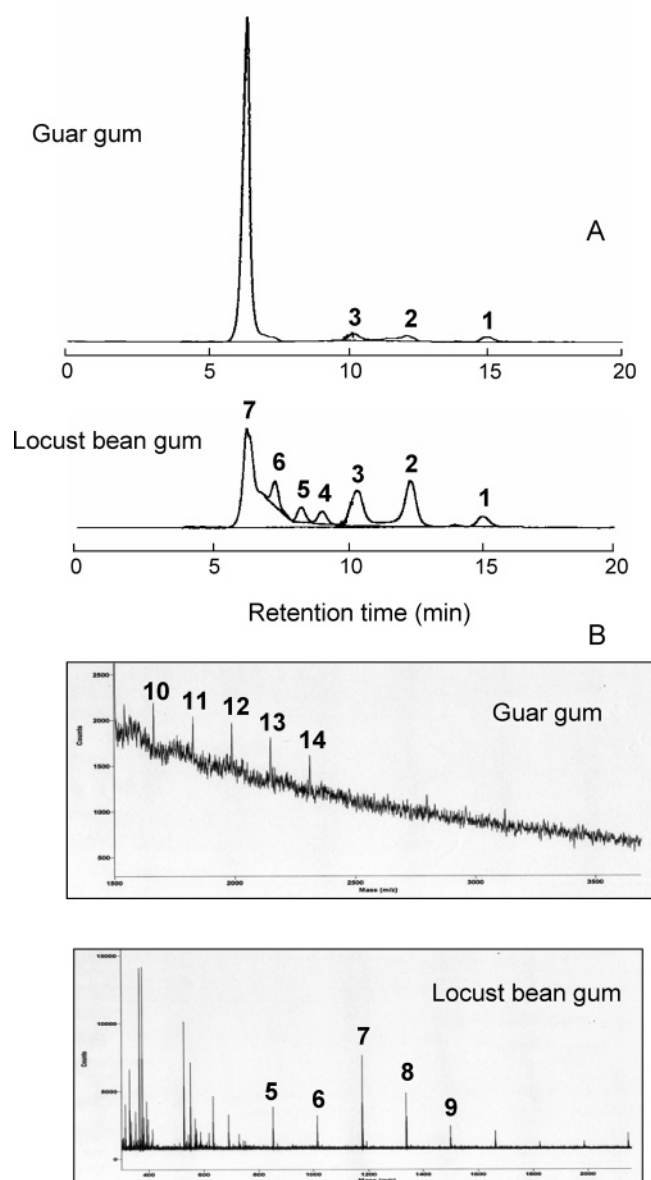


Figure 4. HPLC chart (A) and MALDI-TOF-MS spectrum (B) of the reaction mixture in the hydrolysis of guar and locust bean gums by F3 fractions. The substrate (50 mg) was incubated with 1 mL of enzyme solution at pH 5 and 40 °C for 24 h. The reaction mixture was applied to each analysis after dilution and filtration with a 0.22 μ m membrane filter. The peak number shows the degree of polymerization of the sugar detected.

(Table 1). This means that the F2 and F3 fractions contained only endo- β -mannanase activity.

The ability of the three fractions to hydrolyze guar and locust bean gums was also investigated. A reaction mixture containing 5% substrate had an initial high viscosity but was gradually liquefied during enzymatic hydrolysis at 40 °C, to a completely liquid state after about 5 h. The sugars released from the guar and locust bean gums were analyzed and are shown in Figure 3. In the HPLC charts of the F2 enzyme (Figure 3A), oligosaccharides of DP 2–7 and DP 2–6 were detected in the hydrolysis of both gums, respectively. The amount of monosaccharide (DP 1) was relatively small, and the peak was a mixture of mannose and galactose. The distribution of oligosaccharides was analyzed in detail by MALDI-TOF-MS. The central major products of DP 6 ($990 + \text{Na}^+$), 7 ($1152 + \text{Na}^+$), and DP 5 ($828 + \text{Na}^+$), 6 ($990 + \text{Na}^+$) sugars were confirmed in the

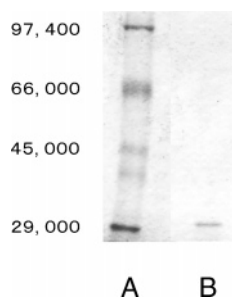


Figure 5. Analysis of β -mannanase from SO strain by SDS-PAGE. Lane A: marker, Lane B: β -mannanase.

Table 2. Purification of SO β -Mannanase from Crude Enzyme

purification steps	activity (units)	yield (%)	specific activity (units/mg)
crude enzyme	337	100	17
Sephadex G-25	110	33	22
Super Q Toyopearl	37	11	148
TSKgel G3000SW	18	5.3	160

hydrolysis products from guar and locust bean gums, respectively (**Figure 3B**). In addition, the same hydrolysis products were detected after treatment with F1, the fraction containing α -galactosidase activity (data not shown). On the other hand, with F3 (**Figure 4**), oligosaccharides of DP 2–7 from guar gum were almost never detected; instead there was a major hydrolysis product eluting at 6 min. By TOF-MS, signals ($1638 + \text{Na}^+ - 2286 + \text{Na}^+$) relating to oligosaccharides of DP 10–14 were detected (**Figure 4B**). Upon hydrolysis of locust bean gum by F3, a higher size distribution than that with F2 was detected and the major sugars shifted to DP 7 from DP 6.

Fraction F2, which produced oligosaccharides, was subjected to a further purification step. Two protein peaks were separated on a TSKgel G3000SW column, with the first, of estimated molecular size of 35 000, shown to have enzyme activity. On SDS-PAGE one band was detected with an estimated molecular weight of 29 000 (**Figure 5**). The purification steps are summarized in **Table 2**. This molecular size was almost the same as those of *Thielavia terrestris* NRRL 8126, *Sporotrichum cellulophilum* ATCC 20493, and *Trichoderma harzianum* T4 β -mannanases (16–18).

The effects of pH and temperature on purified β -mannanase were also investigated. As shown in **Figure 6**, at 40 °C the optimum pH was around 5 and a stable pH was reached at a relatively acidic area in the range of 3–6. The optimum temperature was around 60 °C at pH 5, while under 60 °C the activity was stable. Although an acidic optimum pH (5) has been seen in β -mannanases from many fungi, a higher optimum and stable temperature (60 °C) characterized the enzymatic properties of SO β -mannanase from those of *Sclerotium rolfisii*, *Thermotoga neapolitana* 5068, *Caldocellum saccharolyticum*, and *Trichoderma harzianum* T4 (12, 18–20). Stability at 60 °C is important in industrial applications because the risk of contamination is thus avoided.

Products from the hydrolytic treatment of galactomannan by the purified enzyme were the same as the results shown in **Figure 3**. On hydrolysis of konjac glucomannan, which has no side chain, DP 1–4 oligosaccharides were released and the central major product in the distribution was DP 2 (data not shown). **Table 3** gives the sugar composition of liquefied guar and locust bean gum samples after enzymatic hydrolysis. About 92% of the released sugars were oligosaccharides. This shows that β -mannanase efficiently produced oligosaccharides from galactomannan with a higher galactose content. Previously, guar

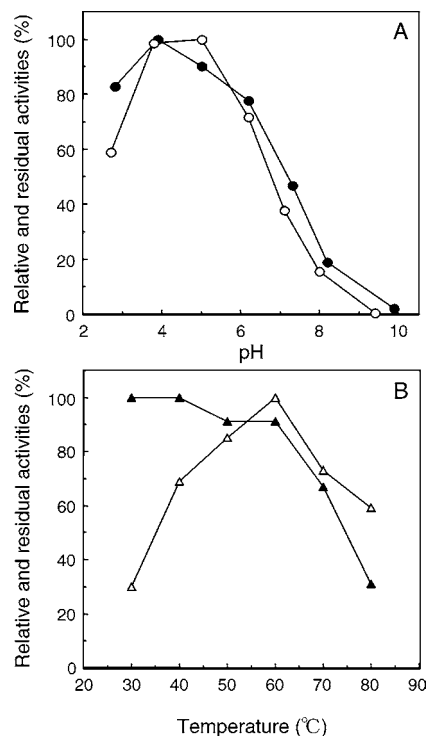


Figure 6. Effects of pH and temperature on SO β -mannanase activity. Relative activities were measured at 40 °C and pH 5, respectively, and residual activities then were measured after incubating at 40 °C for 60 min and at pH 5 for 30 min, respectively. Symbols: pH, (○) relative and (●) residual activities (A); temperature, (△) relative and (▲) residual activities (B).

Table 3. Composition of Oligosaccharides in the Reaction Mixture^a

substrate	monosaccharides (%)	oligosaccharides (%)
guar gum	7.8	92.2
locust bean gum	7.3	92.7

^a The amount of oligosaccharides was determined by analyzing the component sugars released in hydrolysis with 1.6 N sulfuric acid at 100 °C for 2 h.

gum (38% galactose) has not generally been hydrolyzed by β -mannanases into smaller molecular weight oligosaccharides; for example, the degree of hydrolysis effected by *Aspergillus niger* and *Bacillus circulans* K-1 enzymes was about 5% (4, 21). Fraction F3, separated from the SO enzyme in this work, corresponded to these types of β -mannanase. This lack of hydrolytic activity against gums with high galactose content is attributed to the steric hindrance of the galactose side chain. In fact, the release of galactose residues by α -galactosidase enhances subsequent hydrolysis by β -mannanase (22).

The galactosyl distribution in galactomannan has previously been investigated by analyzing products that could be released by β -mannanase (23, 24). The distribution of galactose substituted mannose is classified into three types, ordered, random, and blockwise. Commercial guar gum reportedly shows a blockwise pattern (24). The hydrolysis products generated by the SO β -mannanase, which has a higher hydrolyzing activity against guar gum, were analyzed. The main oligosaccharides (DP 5–8) from guar gum were separated by HPLC with a 250 × 10 mm i.d. NH2P-50 column and the component monosaccharides then analyzed. As shown in **Table 4**, the oligosaccharides consisted of only mannose and galactose, with the ratio of galactose to mannose highest in the DP 7 fraction (Gal/Man = 0.76). This high proportion of galactose to mannose suggests

Table 4. Oligosaccharides of DP 5–8 Fractions Released from Guar Gum^a

DP	galactose/mannose
5	0.49
6	0.45
7	0.76
8	0.54

^a The amount and sugar ratio of each oligosaccharide were determined by analyzing the component sugars released in hydrolysis with 1.6 N sulfuric acid at 100 °C for 2 h.

that this oligosaccharide was derived from a blockwise type. The ratios of released oligosaccharides were near that given by total guar gum polysaccharide and likely corresponded to a random type area. This result suggests that guar galactomannan has both blockwise and random types of galactose substituted mannan.

SO β -mannanase hydrolyzed blockwise and random types of guar gum to smaller molecular oligosaccharides (DP 5–8). In a subsite model of β -mannanase (25), SO β -mannanase is thought to have more subsites occupied by both non- and galactose-substituted mannose residues than enzymes of other origins. Furthermore, it would be able to recognize galactosyl residues more clearly. For example, it is known that xyloglucan endoglucanase also recognizes xylosyl residues of the side chain on the glucan backbone in xyloglucan (26).

In conclusion, β -mannanase from *P. oxalicum* SO efficiently hydrolyzed guar galactomannan to galacto-manno-oligosaccharides (mostly with DP 6 and 7). Released oligosaccharides of DP 7 sugar had a high galactose content (Gal/Man = 0.76). These oligosaccharides are likely to have physiological properties as a food additive, similar to those exhibited by partially hydrolyzed guar gum or other oligosaccharides.

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