Partial Purification of β -Mannanases from the Konjac Tubers and Their Substrate Specificity in Relation to the Structure of Konjac Glucomannan[†]

Hideo SHIMAHARA,* Hiroshi SUZUKI, Noboru SUGIYAMA,*
and Kazutosi NISIZAWA

*Department of Botany, Faculty of Science, Tokyo
Kyoiku University, Ohtsuka, Tokyo*

Received April 9, 1974

Two components of konjac β -mannanases, I and II, were purified by Amberlite CG-50 column chromatography and Sephadex G-100 and G-50 column chromatography. The enzyme preparations behaved as a homogeneous protein in ultracentrifugal analysis. Their molecular weights were estimated to be 32,000 and 29,000, respectively, by gel-filtration method.

Kinetic studies of these β -mannanases indicated that the hydrolysis of konjac glucomannan, mannoooligosaccharides and glucomannooligosaccharides proceeds in a random or an endwise mechanism, but the randomness of enzyme I is less than that of enzyme II.

Both β -mannanase I and II hydrolyzed β -1,4-mannoooligosaccharides and glucomannooligosaccharides, but the mannosidic bonds linked to the glucose residues were attacked slightly easier than those between mannose residues.

On the present and the previous results, a possible structure of the konjac glucomannan was proposed.

Tubers of *Amorphophallus konjac* spear on the ground in the middle of June, and mannan which was the main reserve polysaccharide has been consumed during the germination. This seemed to be due to the action of mannan hydrolyzing enzymes during this period.

In a previous work,¹⁾ the extraction procedure and the general properties of the crude mannanase preparation were reported. Later, we fractionated two components of β -mannanase from a crude extract of the konjac tubers, and purified them extensively, on which the substrate specificities were investigated in detail. This paper deals with these results. Furthermore, the possible unit structure of

konjac glucomannan was proposed on the basis of hydrolysis frequencies for given linkages of various oligosaccharides obtained from the glucomannan by the action of the β -mannanases.

MATERIALS AND METHODS

Enzyme source. Germinating konjac tubers were collected on July 10 in 1972 at Yukicho, Jinsekigun, Hiroshima. After being washed with water and removed from the stems, the tubers were dried in air, and used for the extraction of mannanases.

Substrates. (1) Konjac glucomannan was extracted from tubers of a domestic variety and purified by the procedure as reported in a previous work.²⁾ It was homogeneous in ultracentrifugal analysis and found to contain ash below 0.1%, and be free from nitrogen and phosphorus. Its average molecular weight, M_w , and the radius of gyration, R_g , were estimated to be 1,200,000 and 1,300 Å, respectively, from light scattering method.³⁾ (2) Soluble starch of the special grade was purchased from Wako Pure Chemical Ind., Ltd. (3) CMC and cellobiose of the special grade were purchased from Daiichi Pure Chemicals Co., Ltd.

[†] Studies on Mannan and Related Compounds. Part V.

This work was presented in part at the 29th Meeting of the Chemical Society of Japan, Hiroshima, October, 1973.

* Present address: Shimizu Chemical Laboratory, Kihara, Mihara, Hiroshima. The abbreviations used are: G, D-glucopyranosyl residue; M, D-mannopyranosyl residue; MH, D-mannitol; $\rightarrow$, β -1,4-glycosidic linkage.

(4) Following oligosaccharides were prepared from an enzymic hydrolysate of glucomannan as mentioned in a previous paper.⁴⁾ M→M; M→M→M; M→M→M→M; M→M→M→M→M; G→M; G→M→M; G→M→M→M; G→M→M→M→M; G→G→M; M→G→M. Following three oligosaccharides were kindly supplied by Dr. Koji Kato, the Faculty of Agriculture, Shizuoka University: M→G; M→M→G; M→M→M→G. Some of the above oligosaccharides were reduced with sodium borohydride to the corresponding sugar alcohols. (5) *p*-Nitrophenyl- β -glucoside was synthesized by the method of Glaser and Wulwek.⁵⁾

Enzyme assays

1) *Saccharifying activity.* The reaction mixture consisted of one volume of the substrate solution, one volume of the enzyme solution, and one volume of 0.1 M acetate buffer, pH 5.0. Unless otherwise indicated, 0.5% glucomannan, 1% CMC, 0.5% soluble starch, 0.5% *p*-nitrophenyl- β -glucoside, and 0.12% β -1,4-mannosyl-D-mannitol were used as the substrates. After incubation at 30°C, 1 ml aliquot of the reaction mixture was removed at intervals and heated for 2 min in a boiling water bath. Reducing power produced was determined colorimetrically by the method of Nelson-Somogyi⁶⁾ at 500 nm or 660 nm.

2) *Liquefying activity.* The reaction mixture consisted of 3 ml of 0.4% glucomannan solution, 1 ml of the enzyme solution, and 2 ml of 0.1 M acetate buffer, pH 5.0. After being incubated at 30°C for an appropriate period, the reaction mixture was heated in a boiling water bath for 2 min and decrease in the viscosity was measured at 30°C using an Ubbelohde type viscometer. The enzyme activity was expressed in terms of fluidity, the reciprocal of specific viscosity, η_{sp}^{-1} . The flow time for distilled water was 9.2 sec at 30°C.

Paper chromatography. Paper chromatography was carried out at room temperature for two days by the descending method using Toyo filter paper No. 51 for the quantitative determination of sugars,⁴⁾ and Whatman 3 MM filter paper for their preparation. The solvent system was a mixture of *n*-butanol:pyridine: water (6: 4: 3). The spots were detected with the silver nitrate-sodium hydroxide reagent.⁷⁾ D-Mannose was used as a reference sugar for calculation of *R_m* values.

Determination of protein. Protein in the effluents of column chromatography was estimated from the absorbancy at 280 nm. Protein content in mannanase solution was estimated from the absorbancy at 750 nm measured by the method of Lowry,⁸⁾ using bovine serum albumin as a standard.

RESULTS

Extraction of mannanase

A crude mannanase was extracted from 31.3 kg of the germinating konjac tubers in the same way as described in a previous paper.¹⁾ Yield, 13 g.

Fractionation of the crude mannanase by Amberlite CG-50 column chromatography

In 0.05 M acetate buffer, pH 5.5 (15 ml), containing 2 mM EDTA and 20 mM DFP (diisopropylfluorophosphate) was dissolved the crude mannanase (1.0 g). The mannanase solution was applied to an Amberlite CG-50 column (2.1×27 cm), which had been equilibrated with the same buffer containing 2 mM EDTA. After eluting the column with 660 ml of the same buffer, as shown in Fig. 1, further elution was carried out by a gradient of increasing concentrations of acetate buffer. The mannanase activities were resolved into the major and the minor peaks between the fractions 60~125. These activities were named mannanase I and mannanase II, respectively. The ratio of mannanase I to II in the capacity to produce reducing power was 3: 1, but the ratio in their liquefying activities was 2: 1. Mannanase II, therefore, was more random in the hydrolysis of glucomannan than mannanase I.

Then, the crude mannanase (10 g) was fractionated in the same way with a larger column (4.5×75 cm). Protein in the combined fractions 65~86 and 99~120 containing mannanase I and II, respectively, were salted out at 80% saturation with ammonium sulfate. The resulting precipitate was collected by centrifugation at 15000 rpm for 60 min, dissolved in each 50 ml of 0.02 M phosphate buffer, pH 7.5, and dialyzed against the same buffer with a cellulose membrane (Visking) for 48 hr. The dialyzed solution was concentrated to 10 ml in a collodion bag. The concentrates of mannanase I and II were then individually applied to DEAE-cellulose column (2.1×27 cm), which had been equilibrated with 0.02 M

FIG. 1. Amberlite CG-50 Column Chromatography of Crude Mannanase.

Elution was carried out by a gradient of increasing concentrations of acetate buffer (-----), pH 5.5, from 0.05 M to 1.0 M. Enzyme activities: Mannanase ($O.D._{660nm}/6$ hr), $\bullet$; amylase ($O.D._{660nm}/6$ hr), Δ ; β -mannosidase ($O.D._{660nm}/48$ hr), $\square$; β -glucosidase ($O.D._{660nm}/48$ hr), $\times$; protein ($O.D._{280nm}$), $\circ$.

FIG. 2. Gel-filtration Patterns of Mannanase I and II on Sephadex G-100 Column.

Enzyme activities ($O.D._{660nm}$) were measured by reducing method, after 30 min incubation for mannanase, $\bullet$, or 24 hr incubation for amylase, Δ ; protein ($O.D._{280nm}$), $\circ$.

phosphate buffer, pH 7.5, and eluted with the same buffer. Mannanase I and II passed through the column without adsorption, and were separated from a large amount of contaminating proteins and coloring substances.

Gel-filtration on Sephadex G-100 and G-50

Mannanase I and II were each applied to the two separate Sephadex G-100 (2.6×95 cm) columns which had been equilibrated with 0.1 M acetate buffer, pH 5.5, and eluted with the

same buffer (Fig. 2). Amylase activity was completely removed by this procedure. Active fractions 54~58 and 56~65 were respectively collected and each was concentrated to 5 ml in a collodion bag. For further purification, these mannanase concentrates were subjected to gel-filtration on Sephadex G-50 columns (2.6×95 cm), using 0.1 M acetate buffer, pH 5.5 as the eluent. As shown in Fig. 3, the peaks of protein and mannanase activities were all symmetrical and their posi-

FIG. 3. Gel-filtration Patterns of Mannanases on Sephadex G-50 Column.

Mannanase activities (O.D._{660nm}), ● were measured by reducing method, after 30 min incubation. Protein (O.D._{280nm}), ○.

tions entirely coincided with each other.

Purity and molecular weight of mannanases

Purified mannanases were analyzed for their homogeneity by sedimentation method. Both mannanases showed each single peak at various intervals of the centrifugation time (Fig. 4),

FIG. 4. Sedimentation Patterns of Purified Mannanases.

Sedimentation analysis was carried out under the following conditions: 4.0 mg protein/ml of 0.1 M acetate buffer, pH 5.0, at 6.0°C, 60,000 rpm and 70° of phase plate angle. These patterns were selected from 63 min run after reaching a maximum speed.

although several bands of proteins were found on the polyacrylamide disc-electrophoresis at pH 8.6 for these mannanases. These protein bands, however, do not seem to be enzymes which seem to relate with the enzymic hydroly-

sis of mannan, since those mannanase preparations were proved to be free from activities of β -mannanidase, β -glucosidase (Fig. 1) and cellulase.¹⁾

Approximate average molecular weights of the two kinds of mannanase were estimated by gel-filtration using Sephadex G-100 column (2.6 $\times$ 95 cm). The approximate average molecular weights of the enzyme I and II were estimated to be 32000 and 29000, respectively (Fig. 5).

FIG. 5. Determination of Molecular Weights of Mannanases by Gel-filtration on Sephadex G-100 Column.

FIG. 6. Fluidity *versus* Reducing Sugar in the Enzymic Hydrolysis of Glucomannan.

Mode of action of mannanase I and II

Relationship between the decrease in viscosity and the simultaneous increase in reducing power during the enzymic hydrolysis of glucomannan is shown in Fig. 6. The production of reducing sugars as D-mannose was very low as compared with decrease in the viscosity, suggesting the enzymic hydrolysis of mannan to proceed in a typical random mechanism. The ratios of specific fluidity (η_{sp}^{-1}) to reducing power (mμg D-mannose/ml) for mannanase I and II were found to be 1.88×10^{-4} , and 4.75×10^{-4} , respectively. The values indicated that mannanase II is more random than mannanase I; reduction in the viscosity of glucomannan attained 95% of the original, while production of the reducing sugar was less than 2% of the substrate. Such a high randomness in the hydrolysis of glucomannan indicates that the hydrolysis velocity of this polysaccharide to intermediate oligosaccharides is higher than that of the production of lower reducing saccharides from these oligosaccharides.

Time course of enzymic hydrolysis of mannan was determined (Fig. 7). Considerable difference in the production of reducing sugars was observed between two mannanases. The results also support that mannanase I is more active than mannanase II in saccharification of konjac glucomannan.

FIG. 7. Time Course of Enzymic Hydrolysis of Glucomannan.

A mixture of 1% glucomannan (2 ml), 0.1 M acetate buffer, pH 5.0 (1 ml), and enzyme solution (0.3 ml) was incubated at 30°C. The reducing power of 0.1 ml aliquot was determined at intervals by the Somogyi-Nelson method.

Quantitative determination of reaction products by mannanase I and II

Reaction products from glucomannan by the two mannanases were analyzed after 96 hr of incubation (Fig. 7). Although the glucomannan never forms precipitates from its solution, some insoluble materials were produced during the incubation. They were found to be composed of only glucose and mannose in almost the same ratios as that of the original glucomannan, so that they seemed to be glucomannan fragments (insoluble polymers in Table I). The cause of the production of these insoluble materials was not further investigated in the present work.

The supernatant fractions from the two digests were separately subjected to gel-filtration with Sephadex G-25 (Fig. 8).

These two gel-filtration patterns were remarkably different. From the hydrolysate by mannanase I, a soluble polymer fraction was obtained around the void volume of the column, while no such soluble polymer fraction was found from that by mannanase II. Moreover, the major oligosaccharides produced by mannanase I were dimer to tetramer, while those by mannanase II were trimer to pentamer.

The soluble polymer fractions and oligo-

FIG. 8. Gel-filtration Patterns of Glucomannan Hydrolysates by Two Mannanases.

Supernatant of the digest (Table I) was concentrated to *ca.* 0.5 ml *in vacuo* and applied to a Sephadex G-25 column (1.5 × 95 cm) which had been previously calibrated with cellooligosaccharides of DP=1-6. The column was eluted with water. Sugar contents in 0.1 ml aliquots were determined by the phenol-sulfuric acid method. Numbers in the figure represent the degree of polymerization of major oligosaccharides in each peak. Mannanase I, ○; mannanase II, ●.

TABLE I. YIELDS AND MOLAR RATIOS OF CONSTITUENT SUGARS OF THREE GROUPS OF THE PRODUCTS FROM ENZYMIC DIGESTS OF GLUCOMANNAN (Fig. 7)

Fraction ^{a)}	Mannanase I		Mannanase II	
	M/G	Yield, %	M/G	Yield, %
Original glucomannan	1.62	100	1.62	100
Insoluble polymers	1.57	2.6	1.58	17.4
Soluble polymers	1.60	1.0	—	not produced
Oligosaccharides	1.58	96.3	1.57	82.2

^{a)} Materials become insoluble during the incubation were collected by centrifugation at 4000 rpm for 30 min (Insoluble polymers). The supernatant was fractionated by gel-filtration with Sephadex G-25 (Fig. 8) into soluble polymers and oligosaccharides. These fractions were dried *in vacuo* and weighed. After complete acid hydrolysis of these fractions, the molar ratios of mannose and glucose were determined by quantitative paper chromatography.

saccharide fractions were individually combined. The yields of the total oligosaccharides by mannanase I and II were 96% and 82%, respectively (Table I). The molar ratios of D-mannose to D-glucose in insoluble polymer, soluble polymer and oligosaccharide fractions were roughly 1.6: 1.0, and they were practically the same as that of the original mannan.

Oligosaccharide fractions obtained from mannanase-digests were individually chromatographed on filter paper and the amount of sugars in each spot was determined. The results are shown in Table II. Yields of D-

TABLE II. YIELDS OF EACH OLIGOSACCHARIDE FROM ENZYMIC DIGEST OF GLUCOMANNAN

Oligosaccharides ^{a)}	By mannanase I		By mannanase II	
	Yield ^{b)} μg/spot	%	Yield ^{b)} μg/spot	%
Unknown substance	5.0	0.8	1.3	0.3
D-Mannose	62.4	9.6	8.6	2.0
D-Glucose	8.0	1.2	0.9	0.2
G→M	107.5	15.5	37.6	8.7
M→M	80.0	12.3	35.4	8.2
G→G (possibly)	5.9	0.9	3.0	0.7
Trisaccharides	178.0	27.4	139.0	32.1
Tetra- to heptasaccharides	210.0	32.3	207.0	47.8

^{a)} These oligosaccharides were identified by comparing their mobilities on the paper chromatogram with those of the authentic samples.

^{b)} The spot of each sugar on paper chromatogram was extracted with a small quantity of water, and the sugars were determined as D-mannose by the phenol-sulfuric acid method.

mannose and D-glucose were remarkably higher in the digest by mannanase I than in that by II, and it was supported by the result that man-

sential similarity in the bond-attack frequency of these mannanases, but the minor differences in particular linkages. From these results, the common features in the substrate specificities of the two kinds of mannanase can be summarized as follows:

A mixture of 0.2 μ mole of oligosaccharides, 4 μ g of mannanase as protein, and 2.5 μ moles of acetate buffer, pH 5.0, in a total volume of 25 μ l was incubated at 30°C with a drop of toluene. After 24 hr, the incubated mixture was subjected to paper chromatography. As indicated in Figs. 9 and 10 it can be first noticed that no remarkable differences are found between the mode of action on various manno- and glucomannooligosaccharides of two kinds of mannanase, except for a few oligosaccharides such as M→G→M, M→M→M→G, and G→M→M→M→M. This fact suggests an es-

Disaccharides	(M→M, M→G, G→M, G→G); not hydrolyzed
Trisaccharides	(M→M→M, M→G→M, M→M→G); slightly hydrolyzed
Tetrasaccharides	(M→M→M→M, M→M→M→G); readily hydrolyzed

Thus, the both kinds of mannanase seem to require a chain length of at least three consecutive hexopyranose residues for their activities. However, it is to be noticed that $M \rightarrow M \rightarrow M \rightarrow$

C, control; I, mannanase I; II, mannanase II.

FIG. 10. Relative Frequency of Bond-attack by Mannanases on Various Kinds of Oligosaccharide. $\Downarrow$, higher; $\downarrow$, moderate; $\downarrow\downarrow$, lower, and no arrow means no attack.

M is readily split into $M \rightarrow M \rightarrow M$ plus M and $M \rightarrow M \rightarrow M \rightarrow G$ is also into $M \rightarrow M \rightarrow M$ plus G, whereas $G \rightarrow M \rightarrow M \rightarrow M$ is split into $G \rightarrow M$ plus $M \rightarrow M$ or $G \rightarrow M \rightarrow M$ plus M. In addition, $M \rightarrow M \rightarrow G$ is split but slightly into $M \rightarrow M$ plus G, while $G \rightarrow M \rightarrow M$ is not split at any bond. The facts seem to indicate that M residue produced from $M \rightarrow M \rightarrow M \rightarrow M$ and $M \rightarrow M \rightarrow M$ may be originated in cleavage of the terminal bond toward the reducing end.

K_m and V_{max} values in the enzymic hydrolysis of glucomannan and some mannooligosaccharides

Effects of the substrate concentration on the rates of enzymic hydrolysis were measured. Glucomannan, mannotriose, mannotetraose, mannopentaose and mannotriosylmannitol were used as substrates. From Lineweaver-

Burk plots as shown in Figs. 11 and 12, the Michaelis constants (K_m) and maximum velocities (V_{max}) were calculated, and summarized

FIG. 11. Effect of Substrate Concentration on the Reaction Velocities of Mannanases.

Initial velocities were determined by reducing method, using $\Delta O.D._{660nm}$ per min per ml as a measure.

FIG. 12. Effect of Substrate Concentration on the Reaction Velocities of Mannanases.

A, mannotetraose; B, mannopentaose.

Initial velocities were determined by reducing method, using $\Delta O.D._{660nm}$ per min per ml as a measure.

in Table III. These results show that there are some differences between the affinities of mannanase I and II for the chain length of substrates. Mannopentaose had remarkably

TABLE III. RELATIONSHIP BETWEEN THE DP OF β -1,4-MANNO-OLIGOSACCHARIDES AND THE KINETIC CONSTANTS

The $V_{\max}$ values represent velocity in terms of Δ O.D. 660 nm per min per ml. No reliable values could be obtained for mannotriose and mannotriosyl-mannitol, because of their very low susceptibility to both mannanases.

Substrates	Mannanase I		Mannanase II	
	$Km(\text{mM})$	$V_{\max} \times 10^{-4}$	$Km(\text{mM})$	$V_{\max} \times 10^{-4}$
M→M→M→M	2.0	3.3	3.0	2.5
M→M→M→M→M	0.15	1.0	0.63	2.1
Glucomannan	0.035(%)	36.0	0.02(%)	8.7

higher affinity than mannotetraose. Moreover, the affinities of mannanase I for mannotetraose as well as -pentaose were much higher than those of II. The maximum velocity of mannanase I for mannan was far larger than that of mannanase II. This seems to support the fact that mannanase I showed a higher saccharification activity in the mannan hydrolysis. However, it is to be noticed that the Km values of the two mannanases increased as the

TABLE IV. INHIBITION CONSTANTS (K_i) OF SOME OLIGO-SUGAR ALCOHOLS FOR ENZYMIC HYDROLYSIS OF GLUCOMANNAN

Substrate	Inhibitor	Mannanase I	Mannanase II
		($\times 10^{-2}$)	($\times 10^{-2}$)
Glucoman- nan	none	3.50 ^{a1}	2.00 ^{a1}
	M→M→MH	6.25	33.3
	G→M→MH	1.72	1.61
	M→G→MH	1.10	1.00
	M→M→M→MH	0.88	0.82

^{a1} These values represent in terms of Km (%).

substrates decreased in their chain length; based on molar concentration, the affinity of mannanase I for mannan enhanced nearly 500 times that for mannopentaose, but it enhanced 3800 times with mannanase II.

Inhibition of reduced oligosaccharides for enzymic hydrolysis of glucomannan

The initial velocities of glucomannan hydrolysis were measured in the presence of 1 mM of the oligo-sugar alcohols such as M→G→MH, G→M→MH, M→M→MH, and M→M→M→MH. As seen in Lineweaver-Burk plots (Fig. 13), hydrolysis of mannan was competitively inhibited by these oligo-sugar alcohols, and their inhibition constants are summarized in Table IV.

FIG. 13. Inhibitory Effect of Some Oligo-sugar Alcohols on the Initial Velocities of Enzymic Hydrolysis of Glucomannan.

Initial velocities were measured by reducing method using Δ O.D. 660 nm per min per ml as a measure. Control, ●; M→M→MH, ○; G→M→MH, □; M→G→MH, △; M→M→M→MH, ×.

The inhibition by these sugar alcohols was larger with tetramer than with trimer. It is noticeable that the trimer containing G residue, particularly in the middle position, showed the most potent inhibition. Moreover, the inhibition toward mannanase I was higher in general than that toward mannanase II.

DISCUSSION

In the present work, the presence of two kinds of β -mannanase of slightly different molecular weight and substrate specificity was demonstrated in the germinating konjac tubers. This is the first finding in literature. The mannanases attack similarly not only konjac glucomannan but also manno- and glucomannooligosaccharides of the chain length of more than two. The relationship between fluidity and saccharification of glucomannan showed that the hydrolysis of the substrate by both mannanases is carried out in a typical random or endo mechanism, although $V_{\max}$ of mannanase I for glucomannan was higher than that of II and mannanase I showed less random hydrolysis of higher saccharifying activity. These mannanases did not hydrolyze reduced oligosaccharides of DP below 5 but they were inhibited competitively by these sugar-alcohols. Inhibition patterns were very similar for both mannanases, although the inhibition constants for mannanase I were higher than those for mannanase II. K_m values of the two mannanases for oligosaccharides were also very similar, but those of I were smaller than those of II for oligosaccharides while reverse was the case for glucomannan. All these findings suggest a similarity in the active centers of the two mannanases.

Recently, some microbial β -mannanases from *Aspergillus* sp.,⁹⁾ *A. niger*¹⁰⁾ and *Bacillus subtilis*¹¹⁾ have been reported. The M_w and isoelectric point of the *Bacillus* mannanase were estimated to be 22,000 and pH5.24, respectively. Thus, it appears that there are considerable differences between β -mannanases of konjac and the microbes in their physico-chemical properties. However, both mannanases of

konjac and *B. subtilis* exhibited somewhat similar mode of action on some β -1,4-manno-oligosaccharides, i.e. the *Bacillus* enzyme has also been found to attack mainly the linkages at the third or fourth position from the non-reducing end.

Substrate specificity of konjac mannanases for the oligosaccharides of known structure (Figs. 9 and 10) and the kinetic studies on the mannanases (Figs. 11 ~ 13; Tables III and IV) showed that the action of mannanases was of a typical random or endo type. However, since there was found some regularity in the hydrolyzable linkages and the sequence of M and G residues of oligosaccharides produced by enzymic hydrolysis, certain structural specificities may be assumed for the accessibility of binding sites of mannanase, and at least the following postulation may be possible for the explanation of the product pattern revealed by the mannanases:

- 1) The presence of at least three consecutive mannose residues (M) is necessary or preferable for the binding of substrates to the active center of mannanase, but the middle mannose residue of the three is replaceable for glucose residue (G) to accept a minimum catalytic activity.
- 2) The rates of hydrolysis of linkages in the glucomannan chain is in the order of $M \rightarrow G > M \rightarrow M \gg G \rightarrow M$. It is particularly noticed that the bond of $M \rightarrow G$ was more susceptible than that of $M \rightarrow M$.
- 3) The rate of hydrolysis becomes greater as the chain length of substrate increases, possibly due to achievement of a complete binding of the subsite as well as the active center of mannanase molecule to the substrate molecule.

Konjac mannan was hydrolyzed almost completely by mannanase I and II to produce a mixture of mono- to hepta-saccharides as the major products, and insoluble polymer and/or soluble polymer as the minor products (Table I). However, the ratios of M to G in all fractions were roughly equal to that of the original glucomannan; 1.6:1 or 8:5. In ad-

TABLE V. MOLAR RATIOS OF OLIGOSACCHARIDES OBTAINED FROM 13 GRAMS OF GLUCOMANNAN BY THE ACTION OF KONJAC MANNANASES

Data were cited from a previous paper.⁴⁾

It was strongly suggested in a previous paper⁴⁾ that oligosaccharides (l) and (m) were present in the enzymic digest from glucomannan, although their isolation was not succeeded.

Sugar		Yield, mg	mmole
M→M	(a)	178	0.52
M→M→M	(b)	161	0.30
M→M→M→M	(c)	73	0.11
M→M→M→M→M	(d)	34	0.04
G→M	(e)	119	0.35
G→M→M	(f)	126	0.23
G→M→M→M	(g)	96	0.14
G→M→M→M→M	(h)	91	0.11
M→G→M	(i)	84	0.16
G→G	(j)	85	0.25
G→G→M	(k)	69	0.13
G→G→M→M	(l)	—	—
G→M→M→G	(m)	—	—
Total		1116	2.341
		(ca. 9%)	

of konjac mannan chain.

- (1) Presence of di- to tetrasaccharides which occupied most part of the oligosaccharides in the enzymic digest (Table V),
- (2) a nearly equimolar yield of two groups of oligosaccharides, a~d and e~i (Table V), and
- (3) a nearly equimolar yield of two oligosaccharides, G→G→M and M→G→M (Table V), and the probable presence of G→G→M→M and G→M→M→G in the enzymic digest.

The structure of A-fragment in this figure does not seem to contradict with the partial fragments proposed by Kato *et al.*^{12,13)} and Satoh *et al.*¹⁴⁾ though their methods used for the structural investigations were quite different from ours.

On the other hand, as shown in Table V, 0.25 mmole of cellobiose (G→G) was isolated from the enzymic digest. According to the proposed structure, G→G should be originated in a high possibility from the B-fragment.

FIG. 14. Proposed Structure for Some of the Repeating Units of Konjac Glucomannan and Relative Frequency of Bond-split by Mannanases.

↓, higher; ↓, moderate; ···, lower. A and B denote the blocks of the hexose residues with each given sequence.

dition, G-residue in the glucomannooligosaccharides from the mannanase digest was mostly found at non-reducing end (Table V). The result led us to assume that some parts of the glucomannan chain may be constructed from a repeating or basic unit consisting of two kinds of hexose residue at a given ratio, and that the repeating or basic unit would be composed of at least 13 (8 plus 5) hexose residues.

From the results of following (1), (2) and (3), a structure would be proposed, as shown in Fig. 14, for one of the repeating or basic units

However, G→G→M was not attacked by mannanases I and II under the present incubation conditions, as indicated in Fig. 9.

Acknowledgement. The authors wish to thank Dr. Koji Kato, Department of Agriculture, Shizuoka University, for his kind supply of several valuable oligosaccharides.

REFERENCES

- 1) N. Sugiyama, H. Shimahara, T. Andoh and M. Takemoto, *Agr. Biol. Chem.*, **37**, 9 (1973).
- 2) N. Sugiyama, H. Shimahara, T. Andoh, M. Take-

- moto and T. Kamata, *Agr. Biol. Chem.*, **36**, 1381 (1972).
- 3) T. Kamata and H. Nakahara, *J. Coll. Inter. Sci.*, **43**, 90 (1973).
- 4) H. Shimahara, H. Suzuki, N. Sugiyama and K. Nisizawa, *Agr. Biol. Chem.*, **39**, 293 (1975).
- 5) E. Glaser and W. Wulwek, *Biochem. Z.*, **145**, 514 (1924).
- 6) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).
- 7) W. E. Trevelyan, D. P. Procter and J. S. Harrison *Nature*, **166**, 444 (1950).
- 8) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 9) E. Ahlgren, K. E. Erikson and O. Vesterberg, *Acta Chem. Scand.*, **21**, 937 (1967).
- 10) Y. Tsujisaka, K. Hiyama, S. Takenishi and J. Fukumoto, *Nippon Nôgeikagaku Kaishi*, **46**, 155 (1972).
- 11) S. Emi, J. Fukumoto and T. Yamamoto, *Agr. Biol. Chem.*, **36**, 991 (1972).
- 12) K. Kato, T. Watanabe and K. Matsuda, *ibid.*, **34**, 532 (1970).
- 13) K. Kato and K. Matsuda, *ibid.*, **36**, 639 (1972).
- 14) T. Satoh, A. Moriya, J. Mizuguchi and S. Suzuki, *Nippon Kagaku Kaishi*, **91**, 1070 (1970).
-