A crude enzyme preparation hydrolyzing konjac mannan was extracted from germinating konjac tubers, and purified by chromatography with DEAE-cellulose and alkali-swollen cellulose, and by gel-filtration on Sephadex G-100. The purified enzyme preparation showed optimal activity at pH 4.7, optimum temperature at 40°C. It was considerably stable at pH's between 4.0 and 8.0, but inactivated rapidly by temperatures above 50°C. Hydrolysis of the mannan by this enzyme proceeded by typical random mechanism, and the rate was in agreement with an empirical equation, \( p = 0.43 E^{0.77} t^{0.5} \). As the \( K_m \) and \( V_{max} \) values for mannan, 7.14 \( \times 10^{-2} \) (%) and 23.8 \( \times 10^{-3} \) (\( \Delta \O_{500nm} \)) were obtained, respectively.

Mayeda\(^1\) and Ohtsuki\(^2\) have reported earlier that an enzyme hydrolyzing konjac mannan (called simply mannan hereafter) in Mollusca such as snail, earthworm and slug. Hydrolysis of mannan by bacteria has been also reported by Sawamura,\(^3\) Uyeda,\(^4\) Mayeda, Ohtsuki,\(^5\) and Inoue.\(^6\) For examples, mannan was found to receive an extensive hydrolysis by bacteria such as Bacillus mesentericus, B. aroideae and Aerobacter cloacae and to produce a quantity of mannose.

In 1922, the enzymatic hydrolysis of the mannan was studied by Mayeda\(^7\) for the first time. He obtained an enzyme preparation, which is capable of hydrolyzing mannan, from a bacterium isolated from potato tuber. The optimum pH of the enzyme was found in the neutral region, and the optimum temperature was at 50°C. A trisaccharide called laevidulin was obtained by this author from the digest of mannan by B. mesentericus valgatus. By treatment of mannan solution with a Taka-amylose preparation, Miyake\(^8\) obtained a liquefied reaction mixture containing no reducing sugars. Afterward, however, a liquefied mannan mixture containing reducing sugars was obtained by Ohtsuki\(^9\) after similar treatment. He fractionated two kinds of enzymes which hydrolyzed mannan, and studied some of their properties, but he carried out no detailed examination on the reducing products. Most recently, however, Kato et al.,\(^10\) and Satoh et al.,\(^11\) have determined the chemical structure of oligosaccharides, which were produced from the mannan by the action of Meicelase, a commercial product of cellulases.

In Japan, the parent tubers of Amorphophallus konjac C. Koch are generally planted in the ground in the middle of May. The tubers spear in the middle of June, and the stems and leaves continue to grow until the middle of July. Since then the plants show their full activity until their blighting by frost during the last half of October. Mannan, which is the main reserve polysaccharide in parent tubers, is having been consumed during germination and maturation of the plant as the nourishment for new stems, leaves, and young tubers, and consequently the parent tubers have been withering gradually. The young tubers grow thereafter without supply of the nourishment from the parent tubers. These facts suggest that the konjac mannan in parent tubers transported to young tubers after conversion to soluble forms,
possibly to mono- or oligosaccharides, and that there must be existent some hydrolases of the mannan in parent tubers and some synthetases of the mannan in young tubers.

However, there has been found no report so far in the literature for enzymes hydrolyzing the mannan in parent tubers of konjac plant. Therefore, the present authors attempted to isolate the konjac-mannanase (called simply mannanase hereafter) from the germinating tubers of this plant. In the present paper, extraction and purification of the mannanase and some of its properties will be described.

MATERIALS AND METHODS

1. Enzyme source. Tubers, which had been planted on May 15, 1971, were collected on July 3, 1971. An average weight of tubers was 200 g. At the time of the collection no leaf has developed surrounding the shoot which had begun to appear above the ground about a fortnight ago.

2. Substrate. (1) Konjac mannan was extracted from Zairai-shu and purified by the procedure reported previously. It was homogeneous on ultracentrifuge analysis and was found to contain ash below 0.1%, and free from nitrogen and phosphorus. Its weight average molecular weight, \( M_w \), and the radius of gyration, \( R_g \), were estimated to be 1,120,000 and 1,300 Å, respectively, from light scattering measurement. (2) Soluble starch was of the special grade purchased from Wako Pure Chemicals Ind., Ltd. (3) CMC and cellulose were of the special grade purchased from Daiichi Pure Chemicals Co., Ltd.

3. Enzyme assay

   1) Saccharifying activity. The reaction mixture consists of one volume of substrate solution, one volume of enzyme solution, and one volume of 0.1 M acetate buffer, pH 5.0. Unless otherwise indicated, 1% mannan, 1% CMC, 1% soluble starch, and 0.5% cellulose were used as the substrate solution. After incubation for appropriate period at 30°C, 1 ml aliquot of the reaction mixture was taken out at given intervals. After heating the solution for 2 min at 100°C, the reducing power of the reaction mixture was determined by the colorimetry of Nelson-Somogyi, in which the absorption at 300 nm was measured by a Hitachi Model 101 spectrometer. One mannanase unit was defined as an enzyme amount which produces the reducing power equivalent to 1 μg of glucose in 1 ml of the reaction mixture for 1 min.

   2) Liquefying activity. The reaction mixture consists of 3 ml of 0.4% mannan solution, 1 ml of enzyme solution, and 2 ml of 0.1 M acetate buffer, pH 5.0. After incubation for appropriate period at 30°C, the reaction mixture was heated for 2 min at 100°C and decrease in the viscosity of the reaction mixture was measured at room temperature (between 20.5 and 22.5°C) using an Ubbelohde type viscometer. The enzyme activity was expressed in terms of the reciprocal of specific viscosity, \( \eta^1_\infty \). The flow time for distilled water was found to be 9.2 sec at 22.5°C.

4. Thin-layer chromatography. Thin-layer chromatography was carried out by a double ascending method on a commercial cellulose thin-layer (Avicel SF, Funakoshi Chemicals Co.). A mixture of ethyl acetate, pyridine, acetic acid, and water (5:5:1:3 v/v) was used as the developing solvent. The reducing sugars were detected by spraying a silver nitrate-NaOH solution.

5. Determination of protein. Amount of protein in the eluents obtained by column chromatography was estimated from the absorbancy at 280 nm by a Hitachi Model 101 spectrometer. Protein content in enzyme solution was estimated from the absorbancy at 750 nm measured by the method of Lowry using a bovine serum albumin as a standard.

6. Preparation of alkali-swollen cellulose. It was prepared by the method of Hash and King as follows. After treatment of 40 g of a Whatman cellulose powder for column chromatography with 35% NaOH under the argon atmosphere, the resulting cellulose powder was washed thoroughly with distilled water, and equilibrated with a buffer solution of pH 4.0.

7. Thermostability of mannanase. (a) Each 1 ml of enzyme solution containing 30 μg of protein and 0.01 M acetate buffer (pH 5.0) was heated for each period of 1, 3, 6, 10, and 20 min at 80°C.

   (b) Each 1 ml of the same enzyme solution as above was heated for 5 min at each temperature of 10, 30, 40, 50, 60, 70 and 80°C.

   After cooling the reaction mixture with ice-water and keeping it for 2 hr under the conditions described above (3. Enzyme assay, (1) Saccharifying activity), the residual enzyme activity was measured.

8. pH-stability. To each 0.1 ml of enzyme solution containing 100 μg/ml of protein, was added 0.9 ml of 0.01 M acetate buffer of pH 3.50, 4.19, 4.92 and 0.01 M phosphate buffer of pH 6.32, 7.20 and 8.00. The mixture was incubated for 20 hr at 25°C. The enzyme solutions were adjusted to pH 5.0 by adding 1 ml of 0.1 M acetate buffer of pH 5.0. After incubating for 20 hr under the conditions described above (3. Enzyme assay, (1) Saccharifying activity), the residual
enzyme activity was measured.

RESULTS

1. Extraction and purification
   Extraction from konjac tubers

   A crude extract of ca. 3 liters was obtained from 4.5 kg of raw tubers by crushing with a centrifuge juicer, and placed in a vessel containing ice. Since the viscosity of this extract due to the solubilizing mannan was too high to be subjected to further treatment, the extract was kept in a refrigerator overnight for the enzymatic degradation of the contaminating mannan. After the insoluble substance being removed by centrifugation at 15,000 rpm for 30 min, a deep browned supernatant was obtained. It was dialyzed against distilled water for 30 hr through a cellulose membrane (Visking). The black insoluble substance produced during the dialysis was removed by centrifugation, the supernatant was salted out by 70% saturation with ammonium sulfate. The resulting precipitate was collected, dissolved in distilled water, and dialyzed against distilled water for 60 hr. All these treatments were carried out at 5°C. After removing the insoluble substance produced during dialysis by centrifugation, supernatant was lyophilyzed. A light brown crude enzyme preparation was obtained, whose yield was 1.6 g.

   This enzyme preparation exhibited the activities for mannan and starch, but not for CMC and cellobiose. It was stored in a refrigerator below -20°C and used for the following experiments.

   Fractionation of the crude enzyme preparation by DEAE-cellulose column chromatography

   The crude enzyme preparation (406 mg) was dissolved in 10 ml of the 0.033 M phosphate buffer and was applied to a DEAE-cellulose (2.7×40 cm) activated with 0.3 N HCl and 0.3 N NaOH and equilibrated with the phosphate buffer pH 7.0. The elution was run, as indicated in Fig. 1, with step-wise increasing of the sodium chloride concentration in

   ![Figure 1](image)

   **Fig. 1.** DEAE-Cellulose Column Chromatography of a Crude Enzyme Preparation Obtained from Konjac Tubers.

   Fractions indicated by I, II, III and IV were eluted with 33 mM phosphate buffer (pH 7.0) containing zero M, 0.02 M, 0.2 M, and 0.4 M NaCl, respectively. Enzyme activities were measured under the conditions of the enzyme assay (1), after 6-hr incubation. Enzyme activities: △, mannanase (O.D. 500 nm); ◊, amylase (O.D. 500 nm). Protein (O.D. 250 nm), O.
0.033 M phosphate buffer pH 7.0. The mannanase activity was detected in fractions of 4~10 which was accompanied with amylase activity. The fractions (frac. No. 4~10) were combined and dialyzed against distilled water for 2 days through the cellulose membrane (Visking) and lyophilized. An yield of 58 mg of protein was recovered.

Alkali-swollen cellulose column chromatography of the mannanase fraction

As the mannanase fraction obtained above exhibited the amylase activity, it was fractionated by an alkali-swollen cellulose column chromatography as follows.

A solution of the mannanase preparation (30.5 mg) in 3 ml of the McIlvaine buffer pH 4.0 (0.1 M phosphate-0.05 M citric acid) was applied to the alkali-swollen cellulose column. The elution was run with the same buffer until almost mannanase activity was eluted. As indicated in Fig. 2, the major amylase activity was found in the fractions of No. 30~39. All mannanase fractions, which have no amylase activity, were combined and lyophilized to about 1/10 volume, and dialyzed against distilled water for 24 hr to remove inorganic ions. The mannanase solution thus obtained was further purified through Sephadex G-100 column.

Gel-filtration on Sephadex G-100

A mannanase solution obtained above (35 ml) was applied to the Sephadex G-100 column previously washed with distilled water. The column was eluted with distilled water. As shown in Fig. 3, the mannanase activities of the elutes formed almost a symmetrical peak.

Yields of the mannanase and its specific activities during purification are summarized in Table I. This mannanase preparation after incubation for 22 hr under the condition of the assay (1) exhibited neither amylase activity nor activity for CMC or cellobiose.

**TABLE I. PURIFICATION OF MANNANASE**

Mannanase activity was measured under the condition of the enzyme assay (1).

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Specific activity&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Purification</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme powder</td>
<td>5.4</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Cellulose chromatography</td>
<td>38.4</td>
<td>7.1</td>
<td>14.3</td>
</tr>
<tr>
<td>Alkali-swollen cellulose column chromatography</td>
<td>81.6</td>
<td>15.1</td>
<td>1.87</td>
</tr>
<tr>
<td>Sephadex G-100 gel filtration</td>
<td>84.9</td>
<td>15.7</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> Specific activity was expressed in terms of unit per mg protein.

dnanase were determined at pH values from 3.5 to 7.2, and the result is shown in Fig. 4. The optimum activity of the mannanase was found at pH 4.7.

**Temperature effect on mannanase activity**

Rates of the hydrolysis of mannan in 0.05 M acetate buffer by mannanase were determined at temperatures from 7 to 51°C, and the result is shown in Fig. 5. The temperature for the maximum activity was found to near 40°C.

**pH-stability**

Residual activities of the enzyme solutions were measured after incubation for 20 hr at 25°C at various pH values, and the results are shown in Fig. 6. The enzyme was inactivated by exposure to pH values below 4.0, but fairly stable at pH values above 4.0 to 8.0.

**Thermal inactivation**

Heating only for 1 min at 80°C brought about almost complete inactivation of mannanase, although it was not shown here.

The residual enzyme activities after heating for 5 min at various temperatures are shown in Fig. 7. Temperatures above 50°C gave evidently a rapid damage to the mannanase activity.

**Relationship between the viscosity decrease of mannan and the simultaneous formation of reducing sugars**
Fig. 2. Alkali-swollen Cellulose Column Chromatography of the Fractions of 4–10 Recovered from Fig. 1.

Enzyme activities were measured under the conditions of the assay (1): △, mannanase (O.D.500 nm/6 hr); ×, amylase (O.D.280 nm/6 hr). Protein (O.D.280 nm), ---
FIG. 3. Gel Filtration on Sephadex G-100 Column of Mannanase Preparation Recovered from
the Alkali-swollen Column Chromatography.

Mannanase activities (%, O.D.500 nm) were measured under the conditions of assay (1), after 1-hr incubation. Protein (O.D.280 nm), ○.

Fig. 4. Effect of pH on the Saccharification Activity of Mannanase.
Mannan was dissolved at a 0.5% final concentration in 0.01M sodium acetate buffer. Reaction mixture was incubated at 30°C for 5 hr or 24 hr.

Relationship between decrease in viscosity and the simultaneous increase in reducing power during the hydrolysis of mannann by mannanase is shown in Fig. 8. As clearly seen from Fig. 8, production of reducing sugar as glucose was very slow as compared with decrease in the viscosity. This fact suggests that the enzymic hydrolysis of mannan proceeds by a typical random mechanism.

Ultraviolet absorption spectrum of mannanase preparation.

Ultraviolet absorption spectrum of a mannanase solution (73 μg/ml) was measured. Besides an absorption maximum at 277~283 nm characteristic of protein a small shoulder is observed at 290 nm, possibly due to coloring substance contaminating the enzyme solution.

Determination of kinetic constant

Effect of the substrate concentration on the rates of enzymic hydrolysis of mannan was measured. The mannan whose Mw and Rg are 1,120,000 and 1,300 Å, respectively, was used as the substrate. After addition of mannanase to mannan solutions of various

Fig. 5. Effect of Temperature on the Saccharification Activity of Mannanase.
Mannan was dissolved at an 1% concentration in 0.05M sodium acetate buffer, pH 5.00. After 0.5 ml of enzyme solution was added to 0.5 ml of the mannan solution, the reaction mixture was incubated at each temperature for 10 hr.
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FIG. 6. pH-stability Profile of Mannanase.
The procedure and the reaction conditions were described in Materials and Methods.

FIG. 7. Thermostability Profile of Mannanase.

FIG. 8. Fluidity Versus Reducing Sugar in the Enzymic Hydrolysis of Mannan.
Mannan was dissolved at a 0.2% final concentration in 0.02 M sodium acetate buffer, pH 5.00. After incubation for each appropriate period, the viscosity of the reaction mixture was measured at 20°C using an Ubbelohde type viscometer, and the reciprocals of specific viscosities were plotted against simultaneous production of reducing sugars.

FIG. 9. Effect of Substrate Concentration on the Reaction Velocities of Mannanase.
Initial velocities were measured under the conditions of the assay (1), and expressed as Δ O.D. per min per ml.

Effect of enzyme concentration on the hydrolysis of mannann

Initial velocities of enzymic mannan hydrolysis were estimated from the reducing sugars liberated in the reaction mixture. The results are summarized in Figs. 10 and 11. Figure 11 shows clearly a relationship, \( \log_{10} \frac{p}{t} = k \log_{10} E \), where \( p \) is the amount of the reducing sugar as glucose, \( t \) is the reaction time (min), and...

FIG. 10. Formation of Reducing Sugars during the Hydrolysis of Mannan by Different Concentration of Mannanase.
Numbers in the figure represent µg protein/ml of the mannanase solution. Enzyme activity was measured under the conditions of the assay (1).
at 30°C, \( E \) is the relative concentration of the enzyme, and \( k \) is a proportional constant. Thus the results will be expressed by the following equation.

\[
p = 0.42E^{0.77}t^{0.5}
\]

**Enzymic hydrolysis products from mannan**

A mixture of 2 ml of 1% mannan solution, 1 ml of mannanase solution (26 \( \mu \)g of protein/ml) and 0.5 ml of 0.1 M acetate buffer pH 5.0 was incubated at 30°C for 24 hr. After heating in a boiling water bath for about 2 min, the mixture was dialyzed against 6 ml distilled water through the cellulose membrane (Visking). The dialyzates were lyophilized, and the resulting solid substances were separated by cellulose thin-layer chromatography, as shown in Fig. 12.

Lyophilization of the solution remained inside the cellulose membrane gave light, fine powder of lower molecular weight mannan fragments. From their intrinsic viscosity an average molecular weight was estimated as 20,000 - 30,000.

In Fig. 12, furthermore, there are existent various kinds of unknown oligosaccharides and unknown substance (X5), probably monosaccharide, in addition to mannose. Judging from the values in the literature,\(^{10}\) X2 seems to be disaccharide, X3, X4 and X6 trisaccharides, and X6 tetrasaccharide. The identification of these oligosaccharides is under way.

**DISCUSSION**

A crude mannanase preparation was obtained in a 0.036 w/w\% yield from the spearing konjac tubers, and it was purified by chromatography with DEAE-cellulose and alkali-swollen cellulose, and by gel-filtration on Sephadex G-100. The alkali-swollen cellulose column chromatography achieved very effective removal of amylase. Similar result was observed by Toda et al.\(^{19}\) in removing the amylase from a fungal cellulase preparation, although the elution patterns differ from each other. The mannanase preparation thus obtained showed neither cellulase activity nor amylase activity. By further purification by Sephadex G-100 column chromatography, the mannanase activity
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showed only a single symmetrical peak.

The purified mannanase was most active at pH 4.7, and considerably stable at pH values above 4.0 to 8.0. Its Michaelis constant, $K_m$ was $7.14 \times 10^{-3} (%)$ and $V_{max}$ was $23.8 \times 10^{-3} (\text{OD}_{500\text{nm}})$ for the mannan of Mw, 1,120,000 and $R_G$, 1,300 Å.

The purified mannanase showed an almost similar kinetics to the cellulases of microbes. The reactions by this mannanase proceeded according to the following equation, which is originally presented by Shütz for protease:

$$p = k E^m t^n$$

Where $k$ is a proportional constant, $p$ is a hydrolysis extent effected by an enzyme amount $E$ after the reaction time $t$. The value of $n$ for the mannanase obtained in this experiment is 0.5. The value of the similar order has been reported for the hydrolysis of the colloidal hydrocellulose by *Pseudomonas*-cellulase. The value of $m$ of the mannanase was found to be 0.77 for mannan when $k$ was taken as 0.43. The values of $m$ for *Pseudomonas*-cellulase, however, have been reported to be 1.0 for CMC and 0.43 for hydrocellulose when $k$ is 11.7.

It was found in the present work that the mannan is hydrolysed by mannanase in the typical random mechanism. Even when viscosity of the substrate was reduced by 95% of the original one, reducing sugar as glucose less than only 2% of the substrate was produced (Fig. 8). The fact suggests that $k_1 > k_2$, where $k_1$ and $k_2$ are velocity constants in the reaction from substrate to intermediates and in that from the intermediates to end products, respectively.

In the digestive products of the mannan by the mannanase, three monosaccharides and at least six oligosaccharides were detected. Of the three monosaccharides, one was glucose and the other was mannose but the third one ($X_i$) was not yet identified. The identification of the oligosaccharides are of special interest for the investigation of substrate specificity of the mannanase, although it was not tried in this work.

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