Crystallization and Some Properties of Mannanase[†]

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A mannan-hydrolyzing enzyme produced by a certain strain of *Bacillus subtilis* was purified from the culture broth and isolated in a crystalline state by being treated with several ion-exchangers. The optimal pH of the enzyme was 6.0. The enzyme was stable in a pH region of 5.0 to 9.5 and at temperatures less than 55°C. The enzyme attacked only β -1,4-mannosidic linkages in the main chain of galactomannan of soybean seed coat, guar gum and coffee bean, and of glucomannan of konjak (*Amorphophalus konjac*). Investigation of the hydrolysis mode revealed that the enzyme attacked coffee bean galactomannan endwise to form mannobiose, mannotriose and mannotetraose. The action patterns on several mannohomooligomers prepared from a partial hydrolysate of coffee bean galactomannan were also investigated, indicating that the enzyme preferentially attacked the β -1,4-mannosidic linkages that were present apart three to four mannose residues from the non-reducing end of the mannose chain.

Our previous paper¹⁾ has reported purification, crystallization and some properties of an arabinogalactanase that is inducibly formed by a certain strain of *Bacillus subtilis*.

Recently, the above bacterium was found also to secrete an enzyme which hydrolyzed certain kinds of mannan. There have been published many papers on microbial mannanase.^{2,3)} However, most of them have been made about the enzymes derived from various fungi. Thus, studies on purification and some properties of our bacterial mannanase were undertaken. The present paper deals with crystallization of the mannanase, its some properties and specificity, especially its action patterns on manno oligomers prepared from coffee bean galactomannan.

MATERIALS AND METHODS

1. *The bacterium used, its culture method and preliminary treatment of the culture broth.* A strain of *Bacillus*

subtilis K-50 which was selected as an arabinogalactanase-producing bacterium was used throughout the present work. Culture of the bacterium was carried out under the same condition as that which was employed for cultural production of arabinogalactanase. Also, the cultural broth was treated in the same way as in the case of arabinogalactanase to obtain a crude preparation of mannanase. Namely, the culture filtrate of the bacterium was precipitated by adding ammonium sulfate; the precipitate was dialyzed and decolorized by passing through a column of an anion exchanger, Duolite A-2. The decolorized enzyme solution was again precipitated with ammonium sulfate and the precipitate was used as the crude enzyme preparation.

2. *Enzyme assay.* To 4 ml of a 1.0% solution of galactomannan (prepared from coffee bean) in M/50 acetate buffer, pH 6.0, was added 1 ml of enzyme and after 5 min incubation at 40°C, the reducing sugar formed was determined as mannose by the Shaffer-Somogyi method.⁴⁾ The enzyme quantity that produced 1.0 μ mole of mannose per minute under the above condition was defined as 1 unit enzyme activity. The specific activity of the enzyme to compare the purity during purification was tentatively expressed by the enzyme activity per unit of absorbance at 280 m μ of the enzyme solution, as reported previously.

[†] Studies of Hemicellulolytic Enzymes of *Bacillus subtilis*. Part II.

3. *Substrates employed.* In the present paper, galactomannans prepared from coffee bean,⁵⁾ soybean seed coat⁶⁾ and guar gum⁷⁾ and glucomannan from konjak⁸⁾ were used. The constituent sugars of the galacto- and gluco-mannan prepared are presented in Table I.

TABLE I. CONSTITUENT SUGARS OF SEVERAL MANNANS USED AS SUBSTRATE

Mannan	Total reducing sugar as mannose after acid hydrolysis, %	Constituent sugars
Coffee bean	90.0	Man ≫ Gal
Soybean	91.5	Man : Gal = 67.7 : 32.3
Konjak	94.0	Man : Glu = 61.3 : 38.7
Guar gum	96.5	Man : Gal = 65.0 : 35.0

Man, mannose; Gal, galactose; Glu, glucose.

4. *β-Mannosidase.* In order to investigate the constituent sugars of the oligosaccharides formed in the mannan digests with bacterial mannanase, β-mannosidase was used which was prepared from *Rhizopus niveus* according to the method of Hashimoto.⁹⁾ The purified enzyme was free from activities of mannanase and galactanase.

5. *Determination and identification of hydrolysis products of mannans.* Reducing sugar of hydrolysis products was determined by the Shaffer-Somogyi and Somogyi-Nelson method.¹⁰⁾ Total sugar content was estimated by the phenol-sulfuric acid method.¹¹⁾

Paper chromatography was carried out by the descending method using Toyo Roshi No. 50 as the paper and butanol:pyridine:water (6:4:3) as the solvent. Location of sugars on paper was detected by spraying an alkaline alcoholic solution of silver nitrate. Aniline hydrogen phthalate was also used as a locator of sugar for quantitative paper chromatography and the color density was measured at 440 mμ on a densitometer (Toyo Densitol) with a self-recording instrument. Also, thin layer chromatography (TLC) was applied using Silica Gel as the adsorbent and anisaldehyde-sulfuric acid^{12,13)} and tetrazolium blue,¹⁴⁾ as the locator of sugars and reducing sugars, respectively.

EXPERIMENTAL RESULTS

1. Purification of the bacterial mannanase

The crude enzyme preparation obtained as

described in Materials and Methods was dissolved in a proper amount of water and dialyzed against a large quantity of M/500 calcium acetate, pH 7.0, for two days in the cold. The dialyzed enzyme was treated with a column of SE-Sephadex C-25 equilibrated with M/50 acetate buffer, pH 5.8, containing M/500 calcium acetate, where mannanase was passed while arabinogalactanase and protease were adsorbed on the resin. In the breakthrough solution, however, α-amylase of starch liquefying type was found to exist and thus the amylase was removed by filtering through a layer of raw corn starch after adding ammonium sulfate to the enzyme solution in 0.3 saturation.¹⁵⁾ To the filtrate obtained here was added ammonium sulfate to become 0.6 saturation and the resulting precipitate after standing overnight was collected by centrifugation. The precipitate was then dissolved in a least quantity of M/500 calcium acetate solution and dialyzed against the same salt solution.

The dialyzed enzyme was then subjected to chromatography on a column of DEAE-Sephadex A-50 equilibrated with M/50 acetate buffer, pH 5.8, containing M/500 calcium acetate. The result is presented in Fig. 1 showing that mannanase was eluted from the

FIG. 1. Chromatography of Mannanase of *Bacillus subtilis* K-50 on a DEAE-Sephadex A-50 Column.

Column, 3 × 45 cm, M/50 acetate buffer containing M/500 Ca-acetate, pH 5.8; enzyme applied, 200 ml (activity, 65 U/ml; $E_{1\text{cm}}^{280\text{m}\mu}$, 4.0); flow rate, 30 ml/hr.

○—○, $E_{1\text{cm}}^{280\text{m}\mu}$; ●—●, enzyme activity; —, conc. of NaCl.

resin with sodium chloride solution of concentrations between 0.1 and 0.2M.

The eluted enzyme was precipitated by adding ammonium sulfate to 0.6 saturation and, after dissolving in the minimum quantity of M/500 calcium acetate, the solution was passed through a column of Sephadex G-75 conditioned with the salt solution as above. In Fig. 2 is illustrated the elution pattern of the enzyme, showing that specific activity of the enzyme was also greatly increased by this gel filtration procedure. The enzyme fractions whose specific activities were equal were combined and concentrated with a collodion bag. After the addition of calcium sulfate of a small quantity and acetone of the amount that caused a slight turbidity, the enzyme solution was allowed to stand in the cold when crystals of the enzyme appeared in a few days (Fig. 3). Activity of the crystalline preparation was 2,700 units per mg proteinous nitrogen by the standard assay. Recovery yield of activity as the crystalline enzyme was about 7.0%. The specific activity of the enzyme determined by

FIG. 2. Gel Filtration of Mannanase of *Bacillus subtilis* K-50 on a Sephadex G-75 Column.

Column, 2.6×140 cm, buffered with M/500 Ca-acetate; enzyme applied, 10 ml (activity, 1,000 U/ml; $E_{1\text{cm}}^{280\text{m}\mu}$, 18.0); flow rate, 20 ml/hr.

○—○, $E_{1\text{cm}}^{280\text{m}\mu}$; ●—●, enzyme activity.

FIG. 3. Crystalline Mannanase of *Bacillus subtilis* K-50.

the method employed in the present paper was 238 and 2,400 times as much higher as that of the bacterial culture broth. The crystalline mannanase was used in the experiments described below.

2. Some physicochemical properties of the mannanase

Isoelectric point of the bacterial mannanase was determined by the isoelectric focusing test using Ampholine and sucrose as the pH- and

FIG. 4. Isoelectric Focusing of Crystalline Mannanase of *Bacillus subtilis* K-50.

Column, 110 ml; enzyme applied, 2 ml (activity, 1,200 U/ml; $E_{1\text{cm}}^{280\text{m}\mu}$, 5.0); pH gradient, pH 3.0~10.0 ampholyte; 300 V, 4 mA for the first 3 hr and then 700 V, 0.6 mA for the later 40 hr at 5°C.

○—○, $E_{1\text{cm}}^{280\text{m}\mu}$; ●—●, enzyme activity; ----, pH.

FIG. 5. Elution Volume of Mannanase by Gel Filtration on a Sephadex G-75.

Column, 2.4×90 cm, equilibrated with M/20 acetate buffer, pH 6.0, containing M/500 Ca-acetate; enzyme applied, 2 ml ($E_{1\text{cm}}^{280\text{m}\mu}$, 2.0 each); molecular weight of the referring enzymes; 1, egg white lysozyme (14,307);¹⁶⁾ 2, mannanase; 3, alkaline protease of *Bacillus subtilis* var. *amylosacchariticus* FUKUMOTO (22,700);¹⁷⁾ 4, neutral protease of the above bacterium (33,800);¹⁸⁾ 5, *Bacillus subtilis* liquefying α -amylase (48,900).¹⁹⁾

FIG. 6. Ultraviolet Absorption Spectra of Mannanase of *Bacillus subtilis* K-50.

Enzyme, 95.2 U/ml; protein N, 35.2 γ /ml; —, in M/100 phosphate buffer, pH 7.0; ----, in N/10 NaOH.

density-gradient, respectively. As shown in Fig. 4, the isoelectric point of the enzyme was estimated to be 5.24. Figure 4 also indicates that the enzyme preparation was electrophoretically homogeneous.

In order to presume the molecular weight, behavior of the enzyme in gel filtration by a column of Sephadex G-75 was investigated. The result is presented in Fig. 5 and the molecular weight of the enzyme was estimated supposedly to be 2.2×10^4 .

In Fig. 6 is shown the ultraviolet absorption pattern of the enzyme. From absorbance at 280 m μ and 294.4 m μ in solutions of pH 7.0 and 0.1 N sodium hydroxide, tryptophan and tyrosine were estimated to be five and nine moles per mole protein, respectively, on the assumption that the molecular weight of the enzyme was 2.2×10^4 .

3. Stability and activity of the mannanase

The purified mannanase was stable at pH values between 5.0 and 9.5. In the presence of EDTA, however, the enzyme became unstable, especially at alkaline side as shown in Fig. 7.

The enzyme was stable at temperatures less

FIG. 7. pH-Stability of Mannanase.

30°C, 24 hr with a M/20 buffer solution: pH 4.0~6.0, acetate; pH 7.2~9.0, Tris-HCl; pH 9.0~11.0, glycine-NaOH.

O—O, containing M/500 Ca-acetate; ●—●, containing M/500 EDTA.

than 55°C in the presence of calcium ions. However, the addition of EDTA made the enzyme unstable at 55°C, resulting in the complete inactivation of the enzyme in a 15 min incubation (Fig. 8).

The optimal pH of the enzyme was found to lie around 6.0. The pH-activity profile of the enzyme was not affected by the presence

FIG. 8. Thermal-Stability of Mannanase.

Heated for 15 min at the temperature indicated, M/20 Tris-HCl buffer, pH 7.5.

○—○, containing M/500 Ca-acetate; ●—●, containing M/500 EDTA.

FIG. 9. pH-Activity of Mannanase.

1.0% coffee bean galactomannan, 40°C, 15 min reaction; buffer (M/20) used: pH 4.0~6.0, acetate; pH 7.2~9.0, Tris-HCl.

○—○, containing M/500 Ca-acetate; ●—●, containing M/500 EDTA.

FIG. 10. Temperature-Activity of Mannanase.

M/500 acetate buffer, pH 6.0, 15 min reaction.

○—○, containing M/500 Ca-acetate; ●—●, containing M/500 EDTA.

of EDTA at least at 40°C, as illustrated in Fig. 9.

Temperature-activity curve of the enzyme in the presence of calcium ions is presented in Fig. 10, showing that the highest activity occurred at 60°C. However, the addition of EDTA at temperatures higher than 40°C greatly decreased the enzyme activity and no enzyme action was observed at 60°C.

4. Kinetics of the mannanase reaction

Hydrolysis curves of several mannans by the mannanase are illustrated in Fig. 11. The enzyme hydrolyzed soybean galactomannan, konjak glucomannan and coffee bean galactomannan in the decreasing order of the initial reaction velocity. The hydrolysis limits of the substrates, however, were rather opposite to the order of the initial hydrolysis velocity and the highest hydrolysis degree was observed with coffee bean galactomannan. Guar gum galactomannan was hydrolyzed with an extremely slow velocity to only a slight hydrolysis limit.

In Fig. 12 are shown the Lineweaver-Burk plots of the enzyme for several mannans. The K_m values for coffee bean- and soybean-galactomannan and konjak glucomannan were

FIG. 11. Time Course of Hydrolysis of Several Mannans by Bacterial Mannanase.

6 ml of 0.5% mannan solution containing M/50 acetate buffer, pH 6.0, reacted with 1 ml of enzyme (activity, 1 U/ml).

○—○, soybean; ●—●, coffee bean; △—△, konjak; ×—×, guar gum.

FIG. 12. V_{max} - and K_m -Values of Bacterial Mannanase on Several Mannans.

○—○, coffee bean; ●—●, soybean; △—△ konjak.

calculated to be 0.28%, 0.59% and 0.34%, respectively.

5. Action patterns of the mannanase

The thin-layer chromatograms which were obtained with the mannan hydrolysates after

a twenty-four hour incubation with the enzyme (Fig. 11) are presented in Fig. 13. Of the hydrolysis products from soybean galactomannan, the oligomers symbolized as 2 and 3 were mannobiose and mannotriose, respectively, from the facts that their R_f values on the thin-layer chromatogram were consistent with those of the authentic samples and that they yielded only mannose in the theoretical quantity on hydrolysis by either β -mannosidase or acid. The spots numbered 2' of the hydrolysate of konjak glucomannan was an oligomer consisting of glucose and mannose. Number 3 of the hydrolysate was mannotriose.

The main products of coffee bean galactomannan were manno-triose, -tetraose and -biose in the decreasing order (Fig. 14). They were identified by hydrolysis with β -mannosidase and acid as described above. The spots marked A and B in Fig. 14 were hetero-

FIG. 13. Thin Layer Chromatogram of Hydrolysis Products of Several Mannans by the Mannanase.

M, markers: M 1~M 8, mannose~mannooctaose; Glu, glucose; Gal, galactose; C, coffee bean galactomannan; S, soybean galactomannan; K, konjak glucomannan; G, guar gum galactomannan; locator, anis-aldehyde-sulfuric acid.

oligomers consisting of galactose and mannose, the ratio of galactose to mannose being 1:3 for A and 1:4 for B (Fig. 15).

The action mode of the enzyme on several β -1,4-mannosidic oligomers was investigated.

FIG. 14. Quantitative Thin Layer Chromatograms of Hydrolysis Products of Coffee Bean Mannan by the Mannanase.

Hydrolysis degree, 36%; M, markers; M 1~M 8, mannose~mannooctaose; locator, anis-aldehyde-sulfuric acid.

The substrates were prepared from a partial hydrolysate of coffee bean galactomannan with the bacterial mannanase. A 2 g of galac-

FIG. 15. Constituent Sugars of Heterooligosaccharide A and B Formed in Digestion of Coffee Bean Galactomannan with Bacterial Mannanase.

M, markers; M 1~M 8, mannose~mannooctaose; Gal, galactose; A, oligosaccharide A in Fig. 14; B, oligosaccharide B in Fig. 14; H, hydrolysate of oligosaccharide A and B with 1 N HCl, 2 hr; locator, anis-aldehyde-sulfuric acid.

FIG. 16. Isolation of Mannooligosaccharides by a Charcoal Column.

Column, 1.8×45 cm; charcoal, activated charcoal for chromatography (Wako Pure Chemical Industries, Ltd.); oligosaccharides applied, 20 ml (reducing sugar as mannose, 144 mg; total sugar after acid hydrolysis, 650 mg), mannose (2.5 mg) appeared in the breakthrough solution, flow rate, 50 ml/hr; 20°C .

tomannan was suspended in 200 ml of M/50 acetate buffer, pH 6.0, containing 50 units of the mannanase and the suspension was incubated at 40°C. Four hours later, the suspension

(hydrolysis degree, 15%) was mixed with two volumes of ethanol and the supernatant was concentrated under reduced pressure. An adequate quantity of the concentrate was subjected

FIG. 17. Thin Layer Chromatograms of Hydrolysis Products of Several Mannooligosaccharides with β -Mannosidase of *Rhizopus niveus*.

The oligosaccharide, 2.0 μ moles, was reacted with 2 ml of enzyme solution (activity, 1.0 U/ml) containing M/50 acetate buffer, pH 5.0, at 40°C; M, markers; M1~M8, mannose~mannooctaose; locator, anis-aldehyde-sulfuric acid.

FIG. 18. R_f Values of Mannooligosaccharides Isolated from Coffee Bean Mannan. M1, mannose; locator, anis-aldehyde-sulfuric acid.

to a charcoal column chromatography (Fig. 16) and each fraction was chromatographed by the thin layer method. As shown in Figs. 17 and 18, each fraction was found to contain a sole sugar component consisting only of mannose. Polymerization degree of the manno-oligomers was determined by the graphical method reported by French²⁰⁾ (Fig. 18) and from the ratio of reducing sugar to total sugar content (Table II).

To 0.5 ml of 1.0% solution of each manno-oligomers was added 0.5 ml of the mannanase solution (total activity, 0.5 units) in M/50 acetate buffer, pH 6.0, and at certain intervals of time

TABLE II. RATIOS OF REDUCING SUGAR^{a)} TO TOTAL SUGAR^{b)} CONTENT OF THE ISOLATED MANNOOLIGOMERS

Symbols in Fig. 18	Ratio
A	2.02 (M 2)
B	3.00 (M 3)
C	3.90 (M 4)
D	5.05 (M 5)
E	5.85 (M 6)
F	6.75 (M 7)
G	7.85 (M 8)

^{a), b)}; determined by the Somogyi-Nelson method and by the phenol-sulfuric acid method, respectively.

FIG. 19. Action of the Mannanase on Several β -1,4-Mannosidic Oligomers. M 1 ~ M 8, mannose ~ manno-octaose as the markers; locator, anis-aldehyde-sulfuric acid.

FIG. 20. Action of Bacterial Mannanase on Manno-triosyl-, -tetraosyl-, -pentaosyl-, -hexaosyl- and -heptaosyl-mannitol.

M, markers: M 1~M 8, mannose~mannooctaose.

at 40°C an aliquot of the reaction mixture was subjected to thin-layer chromatography. In Fig. 19 are presented the results, showing that the bacterial mannanase does not attack β -1,4-mannobiose and mannotriose. However, mannotetraose was hydrolyzed to produce mannose, mannobiose and mannotriose. Mannopentaose was hydrolyzed in the ways to produce either mannose and mannotetraose or mannobiose and mannotriose. In the case of mannohexaose, mannobiose and mannotetraose rather prevailed over the formation of either mannotriose or mannose and mannopentaose. Mannoheptaose and mannooctaose were also hydrolyzed to mannotetraose and mannopentaose, the former being produced in larger quantities.

The action pattern of the enzyme was also investigated on several mannooligosylmannitols which were prepared by reduction of the mannooligomers with sodium borohydride. The hydrolysis products were detected by etrazolium blue whose reaction was positive only for reducing sugars. As shown in Fig. 20, the enzyme produced mannotriose and mannobiose from mannotriosylmannitol, indicating that the enzyme hydrolyzed the substrate to form either mannobiose and mannosylmannitol or mannotriose and mannitol. The main digestion products by the enzyme were as follows: Mannotriose from mannotetraosylmannitol,

manno-triose and -tetraose from mannopentaosyl-mannitol, manno-tetraose and -pentaose from mannohexaosyl- and mannoheptaosyl-mannitol, respectively.

The above results clearly indicate that the bacterial mannanase preferentially attacks the β -1,4-mannosidic linkages situating at the third or fourth from the non-reducing end of the mannose chain.

DISCUSSION

It appears quite reasonable from the ecological character that *Bacillus subtilis* secretes several hemicellulolytic enzymes. Our previous paper¹¹ has reported that arabinogalactanase of the bacterium is secreted inducibly. However, the mannanase in the present paper was found to be formed in the culture medium without the enzyme substrates or their related compounds.

The mannanase purified in the present paper was stabilized by the addition of calcium ions, especially at high temperatures. As the enzyme was active even in the presence of metal chelators at low temperatures, calcium ion may act only to protect the enzyme from heat inactivation.

From the action patterns, it is quite clear that the enzyme attacks mannan endowise. However, when reacted with β -1,4-mannosidic

FIG. 21. Action of Bacterial Mannanase on Several Mannooligosaccharides.

*, reducing end.

oligomers prepared from a partial hydrolysate of coffee bean galactomannan, the enzyme was found to preferentially attack the β -1,4-mannosidic linkages situating at the third or fourth position from the non-reducing end, as shown in Fig. 21 which schematically summarizes the results in Figs. 19 and 20.

It was of particular interest that when guar gum galactomannan was incubated with the bacterial enzyme, the viscosity of the mannan solution was rapidly decreased even on hydrolysis of less than 1.0%, indicating that the β -1,4-mannosidic linkages available for the enzyme attack strongly contribute to maintain viscosity of the galactomannan.

In the present study, several β -1,4-mannosidic oligomers with no galactose residues were prepared from a partial hydrolysate of coffee bean galactomannan with the bacterial enzyme. This fact may be the evidence that galactose residues are quite few in coffee bean galactomannan. The quantitative analyses of the galactomannooligosaccharides, A and B in Fig. 14 revealed that the ratio of mannose to galactose of coffee bean galactomannan was about 44~45:1.

A kinetical study of hydrolysis of mannan by our enzyme is now under investigation and the results will be published elsewhere. Studies

of the effect of the enzyme on various plant cell walls will be left in the future.

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