APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1995, p. 4454–4458 Vol. 61, No. 12
0099-2240/95/$04.00+0

Purification and Characterization of an Extracellular β-1,4-Mannanase from a Marine Bacterium, *Vibrio* sp. Strain MA-138

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Received 5 May 1995/Accepted 26 September 1995

A β-mannanase (EC 3.2.1.78) from *Vibrio* sp. strain MA-138 was purified by ammonium sulfate precipitation and several chromatographic procedures including gel filtration, adsorption, and ion-exchange chromatographies. The final ion-exchange chromatography Mono Q yielded one major active fraction and three minor active fractions. The major active fraction was purified to homogeneity on the basis of native polyacrylamide gel electrophoresis (PAGE). This purified enzyme was identified as a glycoprotein by periodic acid-Schiff staining and a monomeric protein with a molecular mass of 49 kDa by sodium dodecyl sulfate-PAGE. The pI of the enzyme was 3.8. The purified enzyme exhibited maximal activity at pH 6.5 and 40°C and hydrolyzed at random the internal β-1,4-mannosidic linkages in β-mannan to give various sizes of oligosaccharides. The first 20 N-terminal amino acid sequence of the purified enzyme showed high homology with the N-terminal region of β-mannanase from *Streptomyces lividans* 66.

β-Mannan is an important structural component of some marine algae (15, 17) and terrestrial plants such as ivory nuts (7) and coffee beans (26). β-Mannanase (1,4-β-D-mannan mannanohydrolase, EC 3.2.1.78), which catalyzes the random hydrolysis of β-1,4-mannosidic linkages in β-1,4-mannan, glucomannan, and galactomannan, is a useful tool for the structural analysis of these polysaccharides and for protoplast isolation from marine algae and terrestrial plants (3). There have been several papers on the purification and characterization of β-mannanases from bacteria (1, 2, 14, 19, 25, 27), fungi (20, 24), and higher plants (21), but no information is so far available on an enzymatical study of β-mannanase from *Vibrio* spp.

We previously isolated a mannanase-producing bacterium, *Vibrio* sp. strain MA-138, from a marine environment (5). The organism secretes multiple β-mannanases into the growth medium in the presence of an inducer such as β-mannan, konjac powder, or mannose. This diversity of β-mannanases was supported by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the culture supernatant followed by activity staining (unpublished results). This paper describes the purification and characterization of a major extracellular β-mannanase from *Vibrio* sp. strain MA-138. The first 20 N-terminal amino acids of this enzyme were determined, and the sequence was compared with those of β-mannanases from terrestrial microorganisms.

MATERIALS AND METHODS

Materials. β-Mannan (17) and glucomannan (23) were prepared from *Codium fragile* (a green alga) and *Amorphophallus konjac* (konjac powder), respectively. Bovine serum albumin and *p*-nitrophenyl-β-D-mannoside were obtained from Sigma Chemical Company. The other chemicals were commercial special grade products from Wako Pure Chemical Industries.

Organism. *Vibrio* sp. strain MA-138 was isolated from seaweed collected at Ise Bay, Japan, in 1990. The characteristics of the strain were as follows: gram negative, polar flagella, aerobic, rod shaped, 49.3 mol% GC content, and sensitive to *Vibrio* static agent (5). The organism remained viable in storage at −80°C for at least 20 months in a peptone medium (0.5% Polypepton, 0.1% yeast extract, 3% NaCl, 0.05% $MgSO_4$, 0.2% K_2HPO_4, and 0.04% KH_2PO_4, pH 7.0) after addition of glycerol to a final concentration of 30%.

Preparation of crude enzyme from culture supernatant of *Vibrio* sp. strain MA-138. Cells of *Vibrio* sp. strain MA-138 were grown at 25°C for 24 h with shaking in 20 ml of a peptone medium containing 0.5% konjac powder in a 100-ml Erlenmeyer flask and then were transferred to 800 ml of the same medium in a 2,000-ml Erlenmeyer flask. After 24 h of incubation, the culture was centrifuged at 9,000 × *g* at 4°C for 40 min. The supernatant was collected and used in the following steps.

Purification of β-mannanase. Unless otherwise stated, all purification steps were carried out at 0 to 4°C. The last two steps of chromatography were run by a fast-protein liquid chromatography system (Pharmacia). **Step 1.** The active fraction from the culture supernatant (1,600 ml) at 75% saturation with solid ammonium sulfate was collected by centrifugation (9,000 × *g*, 30 min, 4°C), dissolved in a small volume of 50 mM Tris-HCl buffer (pH 7.3), and dialyzed against the same buffer.

Step 2. The dialyzed enzyme solution was applied to a column of Q Sepharose Fast Flow (20 by 250 mm; Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 7.3). After being washed with five bed volumes of the same buffer, the column was eluted with a continuous linear gradient of NaCl (0 to 1.5 M) at a flow rate of 30 ml/h. The active fractions eluted around 0.6 M NaCl were collected and concentrated by reverse salting out against saturated ammonium sulfate. The concentrated enzyme solution was dissolved in a small volume of 50 mM Tris-HCl buffer (pH 7.3) and dialyzed against the same buffer.

Step 3. The dialyzed enzyme solution (5 ml) was applied onto a Toyopearl HW-55S column (26 by 900 mm, Tosoh Co., Tokyo, Japan) equilibrated with 50 mM Tris-HCl buffer (pH 7.3). The active fractions were concentrated by ultrafiltration (Toyo Ultrafilter UK-10) and dialyzed against 1 mM potassium phosphate buffer (pH 6.8).

Step 4. The dialyzed enzyme solution (5 ml) was applied onto a Gigapite column (10 by 100 mm; Seikagaku Kogyo Co., Tokyo, Japan) equilibrated with 1 mM potassium phosphate buffer (pH 6.8) and eluted with a linear gradient between 1 and 200 mM of potassium phosphate buffer (pH 6.8). Holding operation was carried out at 50 mM of the buffer concentration in the course of the gradient. The active fractions eluted at 50 mM potassium phosphate buffer were pooled and dialyzed against 50 mM Tris-HCl buffer (pH 7.3).

Step 5. For further purification, the dialyzed enzyme solution (5 ml) was applied onto a Mono Q column (5 by 50 mm, Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 7.3). After being washed with 15 bed volumes of the same buffer, the column was eluted with a gradient of NaCl (0 to 1.0 M) combined with stepwise gradients of 0.15, 0.2, 0.35, and 0.4 M NaCl. The flow rate was 30 ml/h. One major active peak appeared at 0.4 M and three minor peaks appeared at 0.15, 0.2, and 0.35 M of NaCl.

Enzyme and protein assays. The reaction mixture consisted of 100 μl of enzyme solution, 500 μl of 0.5% (wt/vol) glucomannan, and 400 μl of 100 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES)-NaOH buffer (pH 7.0). The mixture was incubated at 37°C for 10 min, and then the reducing end group gener-

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ated was measured colorimetrically by the Somogyi-Nelson method (22). One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of D-mannose per min under the above conditions. The protein content of the enzyme preparations was determined by the method of Lowry et al. (18) with bovine serum albumin as a standard. The A_{280} was used for monitoring protein in the column effluents.

PAGE. Native PAGE and SDS-PAGE were done by the method of Davis (11) on a 15% polyacrylamide slab gel and by the method of Laemmli (16) on a 12.5% polyacrylamide slab gel, respectively. Pharmacia low-molecular-weight standards (M_rs, 20,100, 30,000, 43,000, 67,000, and 94,000) were used as standards. The gel was stained with Coomassie brilliant blue R-250. Glycoproteins were detected by PAS reagent (13).

Zymography. After the electrophoresis was completed, renaturation of the enzymes in the gel was done by the method of Blank et al. (10) with minor modifications as follows. SDS was removed from the gel by washing twice for 1 h in 0.1 M phosphate buffer (pH 6.8) at room temperature on a slowly rotating platform shaker. The gel was then transferred onto a glass plate, and excess liquid on the gel surface was removed. Detection of mannanase activity in the polyacrylamide gel was done by the method of Béguin, which was originally designed for the detection of cellulase activity (9). The gel was placed on a 1.5% agarose gel sheet (10 mm thick) containing 0.15% glucomannan and 0.1 M phosphate buffer (pH 6.8) prepared on a glass plate. The polyacrylamide gel and the agarose gel sheet on the glass plate were gently wrapped and incubated for 15 min at 37°C. The polyacrylamide gel was torn off from the agarose gel sheet, and proteins remaining in the gel were stained with Coomassie brilliant blue R-250. The agarose gel sheet was dipped into 1% Congo red solution and stained for 15 min. The Congo red was poured off, and the gel sheet was washed with 1 M sodium chloride until excess stain was removed.

Enzymatic digestion. Enzymatic digestion with collagenase was performed by SDS-PAGE; 5 μg of collagenase was incubated with 2 μg of purified enzyme in 10 μl of Tris-HCl (0.1 M, pH 7.3) for 20 h at 37°C. Digests were subjected to electrophoresis on a 12.5% acrylamide gel. After electrophoresis, zymography was done as previously described.

Isoelectric point. Activity profiles of the purified enzyme were obtained with Ampholine Electrofocusing Equipment (LKB8100-10; LKB-Producer AB) by the method described in the LKB instruction manual. The concentration of the Ampholine carrier (pH range: 3.5 to 10) was 1%. The sample was layered in the middle part of the column during the formation of the sucrose gradient, and isoelectric focusing was done at 600 V for 48 h.

Effect of pH, temperature, and various reagents on enzyme activity. Mannanase activity was measured at different pH values under standard assay conditions using 100 mM of sodium acetate (pH 3.2 to 6.0), MES-NaOH (pH 4.0 to 8.0), 2-*N*-cyclohexylamino ethanesulfonic acid (CHES)-NaOH (pH 8.0 to 10.0), and glycine-NaOH (pH 9.0 to 12.0) buffers. The initial activity of the enzyme was determined under standard assay conditions. After the enzyme solution was allowed to stand at 4°C for 20 h at various pH values (pH 3.2 to 12.0) without the substrate, the remaining activity was measured. The effect of temperature was also examined under the standard assay conditions by varying the temperature (20 to 100°C). For inhibitor sensitivity studies, the enzyme was preincubated with a final concentration of 1 mM of various reagents dissolved in 50 mM MES-NaOH buffer (pH 6.5) at 37°C for 30 min. The residual activity was then measured by using the standard assay conditions.

Purification of mannooligosaccharides. Mannooligosaccharides (mannobiose-mannohexaose; M_2-M_6) were prepared from hydrolysis products of β-mannan with β-mannanase from *Vibrio* sp. strain MA-138 by chromatographies on charcoal (20 by 400 mm; Wako Pure Chemical Industries, Osaka, Japan) and Sephadex G-15 (20 × 850 mm, Pharmacia, Uppsala, Sweden) columns. These oligosaccharides were confirmed to contain a single mannose residue as characterized by thin-layer chromatography of their acid hydrolysis products. Thin-layer chromatography of the hydrolysis products was performed on a silica gel 60-plastic sheet (Merck & Co. Inc.) developed with a solvent of *n*-butanol–acetic acid–water (2:1:1), and the oligosaccharides were visualized by spraying the plate with a diphenylamine-aniline-phosphate reagent (8).

N-terminal amino acid sequence. The N-terminal amino acid sequence of the purified enzyme was determined by automated Edman degradation using a protein sequencer (model 476A; Applied Biosystems).

RESULTS

Purification of β-mannanase. A β-mannanase from *Vibrio* sp. strain MA-138 was purified by ammonium sulfate and chromatographic procedures as described in Materials and Methods. The Gigapite-fractionated enzyme was separated by Mono Q chromatography into one major active fraction and three minor active fractions (Fig. 1). The major fraction gave a single protein band (Fig. 2A) and a single active band (Fig. 2B) following native PAGE. The enzyme was also proved to be a glycoprotein by PAS staining following native PAGE (Fig. 2C). The purified enzyme gave a single band following SDS-PAGE

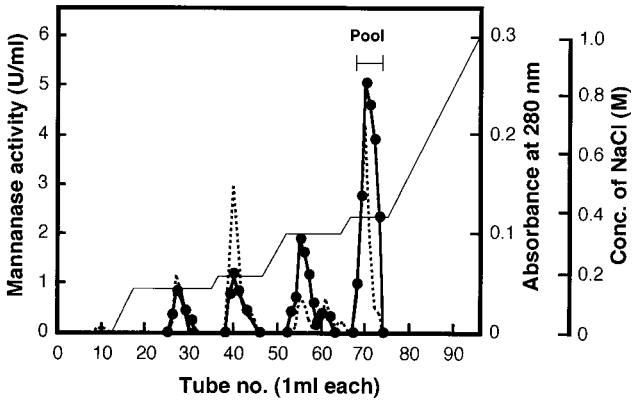

FIG. 1. Chromatography of β-mannanases from *Vibrio* sp. strain MA-138 by Mono Q column. Symbols: ●, mannanase activity; – – –, A_{280}; ——, concentration of NaCl (M).

(Fig. 3A), but two active bands were detected following activity staining (Fig. 3B). This is because the purified enzyme initially revealed one faint protein band below the major one following SDS-PAGE, yet it disappeared during the destaining procedure.

The purification steps are summarized in Table 1. The final enzyme preparation was purified 633-fold with a yield of 23%.

Molecular mass analysis and isoelectric point. The molecular mass of the purified enzyme was determined to be 49 kDa by SDS-PAGE and was slightly higher than that estimated by gel filtration (data not shown) (Fig. 3A). The purified enzyme had an isoelectric point of 3.8.

Enzymatic digestion of β-mannanase. Enzymatic digestion patterns are shown in Fig. 4. Limited proteolysis digestion with collagenase led a part of two active bands of the purified enzyme to a single active band of low molecular mass on zymogram.

Effects of pH and temperature on enzyme activity. The effects of pH and temperature on mannanase activity are shown in Fig. 5. The optimum pH of the purified enzyme was 6.5 (Fig. 5A). When incubated in various buffers at 4°C for 20 h, the

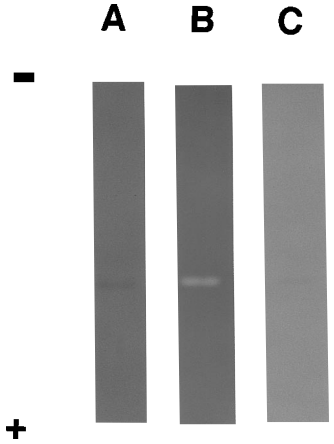

FIG. 2. Native PAGE of the purified β-mannanase from *Vibrio* sp. strain MA-138. (A) Native PAGE on a slab gel. The protein was stained with Coomassie brilliant blue R-250. (B) Zymography. One microgram of the purified enzyme was applied to a Native-PAGE followed by activity staining with glucomannan as the substrate. (C) PAS staining. A glycoprotein was stained with PAS reagent.

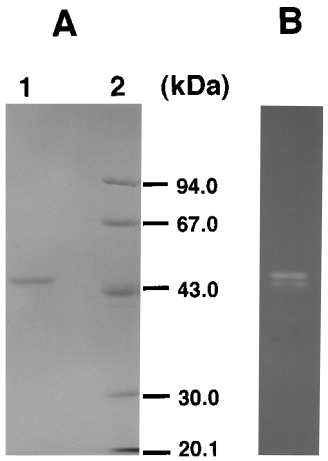

FIG. 3. Analysis of the purified enzyme by SDS-PAGE and zymography. (A) SDS-PAGE. Lane 1, purified enzyme (1 μg); lane 2, molecular mass markers. Numbers on the right are molecular masses (in kilodaltons) of the markers. (B) Zymography. One microgram of the purified enzyme was applied to an SDS-PAGE gel, followed by activity staining with glucomannan as the substrate.

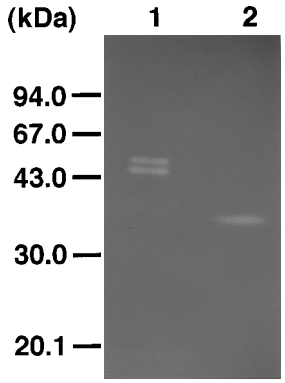

FIG. 4. Zymography analysis of digestion product of the purified enzyme with collagenase following SDS-PAGE. Numbers on the left are molecular masses (in kilodaltons) of the markers. Lane 1, purified enzyme (2 μg); lane 2, digestion product with collagenase.

enzyme was stable in the range of pH 5.5 to 10.0. The optimum temperature for the purified enzyme was 40°C (Fig. 5B) with stability up to 45°C.

Effect of various reagents. The effects of various metal ions and chemical reagents on mannanase activity is shown in Table 2. The metal ions Ag^{+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+}, Al^{3+}, and Fe^{3+} strongly inhibited mannanase activity, and Mn^{2+} inhibited the activity by about 50%. The chemical reagent *N*-bromosuccinimide completely inhibited mannanase activity, and other chemical reagents inhibited the activity by about 20 to 30%.

Kinetics of the mannanase reaction. The effect of various concentrations of substrates on mannanase activity was examined. Lineweaver-Burk double reciprocal plots were used to calculate the K_m values for β-mannan and glucomannan. The K_m values of the purified enzyme were 10 mg ml^{-1} for β-mannan and 4.7 mg ml^{-1} for glucomannan. Maximum velocities (V_{max}) were 450 U · mg of $protein^{-1}$ for β-mannan and 200 U · mg of $protein^{-1}$ for glucomannan.

Action pattern of β-mannanase. Hydrolysis patterns of the purified enzyme from *Vibrio* sp. strain MA-138 against β-mannan, glucomannan, and mannooligosaccharides are shown in Fig. 6 and 7. The purified enzyme hydrolyzed β-mannan to produce mannooligosaccharides such as mannose, mannobiose, mannotriose, and mannotetraose, and it also hydrolyzed glucomannan and produced several oligosaccharides. The purified enzyme cleaved mannotetraose to form mannotriose, mannobiose, and mannose. Mannopentaose was hydrolyzed in the same way to produce either mannotriose and mannobiose or mannotetraose and mannose. The main products from mannohexaose were mannotriose rather than mannobiose and mannotetraose, but the enzyme did not act on mannobiose, mannotriose, or *p*-nitrophenyl-β-D-mannoside. The major extracellular mannanase from *Vibrio* sp. strain MA-138 seems to hydrolyze preferentially the β-1,4-mannosidic linkages situated at the third position and followed by the fourth and second positions from the nonreducing end.

N-terminal amino acid sequence. The purified enzyme was subjected to N-terminal amino acid sequence analysis. The first 20 and 23 N-terminal amino acid sequences of β-mannanases from *Vibrio* sp. strain MA-138 and *Streptomyces lividans* 66, respectively, are shown in Fig. 8. The N-terminal sequence displayed 60% homology between them (6).

TABLE 1. Purification of β-mannanase from the culture supernatant of *Vibrio* sp. strain MA-138

Purification step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	473	5,738	0.082	1	100
Ammonium sulfate precipitate	364	119	3.06	37	77
Q Sepharose FF	238	14.9	16.0	195	50
Toyopearl HW-55S	173	10.0	17.3	211	37
Gigapite	166	3.7	29.6	361	35
Mono Q	109	2.1	51.9	633	23

DISCUSSION

The species of the genus *Vibrio* are widely distributed in the marine environment, and some of these would be expected to contribute to degradation of seaweed which contains β-1,4-mannan (4). However, to our knowledge there have been no reports on an enzymatic study of β-mannanase from *Vibrio* spp.

The procedures described here resulted in the purification of the major extracellular mannanase fraction. The purified β-mannanase from *Vibrio* sp. strain MA-138 gave a single band

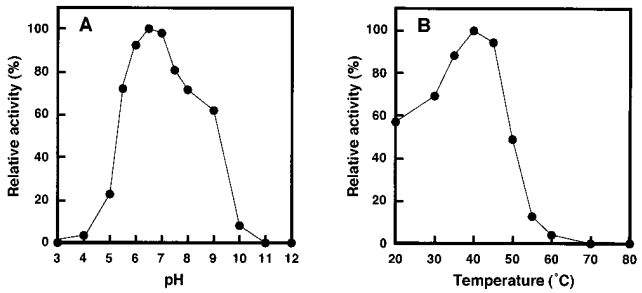

FIG. 5. Effects of pH and temperature on β-mannanase activity of the purified enzyme. (A) The buffers used were 100 mM sodium acetate (pH 3.2 to 6.0), 100 mM MES-NaOH (pH 4.0 to 8.0), 100 mM CHES-NaOH (pH 8.0 to 10.0), and 100 mM glycine-NaOH (pH 9.0 to 12.0). (B) The enzyme activities were assayed at various temperatures in 100 mM MES-NaOH buffer (pH 6.5).

TABLE 2. Effects of various metal ions and chemical reagents on the activity of mannanase

Reagents (1 mM)	Residual activity (%)
None	100
NaCl	107
KCl	113
$AgNO_3$	9
$MgCl_2$	107
$CaCl_2$	117
$Ca(CH_3COO)_2$	79
$MnCl_2$	48
$CuCl_2$	3
$CuSO_4$	0
$ZnCl_2$	11
$ZnSO_4$	0
$BaCl_2$	100
$HgCl_2$	0
$Pb(CH_3COO)_2$	0
$AlCl_3$	13
$FeCl_3$	15
Dithiothreitol	107
EDTA	78
Iodoacetic acid	82
N-Bromosuccinimide	0
N-Ethylmalaimide	73
p-Chloromercuribenzoic acid	68

of protein and activity following nondenaturing conditions. However, the same sample exhibited two active bands following denaturing conditions. Since the purified enzyme is a glycoprotein and since it exits a monomer (by SDS-PAGE), we consider that the two forms may be derived by the SDS-PAGE procedures, possibly resulting from a release of carbohydrate linked to the purified enzyme. This is because treatment of the purified enzyme with collagenase revealed a single active band following activity staining. Furthermore, the N-terminal amino acid sequences of these two bands were identical (data not shown).

The physicochemical and enzymatic properties of β-mannanase from *Vibrio* sp. strain MA-138 fell within the range of the values reported for other microbial mannanases. The enzyme activity was strongly inhibited by Ag^+, Cu^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+}, Al^{3+}, and Fe^{3+} at 1 mM concentration. Inhibition by these heavy metal ions was also observed with *Streptomyces* sp. strain no. 17 (24), *Aeromonas* sp. strain F-25 (2), and

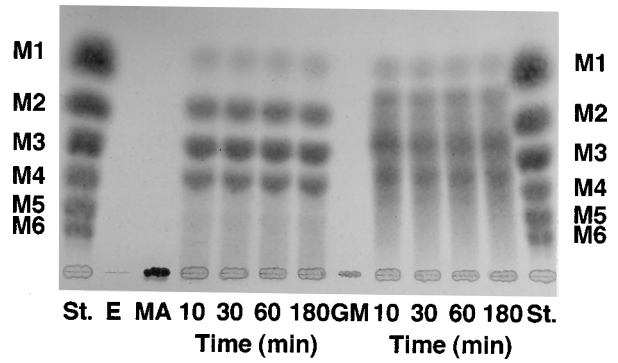

FIG. 6. Thin-layer chromatogram of hydrolysis products of β-mannan and glucomannan with purified β-mannanase. The reaction mixture contained 1 U of β-mannanase and 1% mannan or 0.5% glucomannan in 1 mM sodium acetate buffer (pH 6.0) at 37°C. St., standard sugars: M1 to M6, mannose to mannohexaose; MA, β-mannan; GM, glucomannan; E, purified β-mannanase.

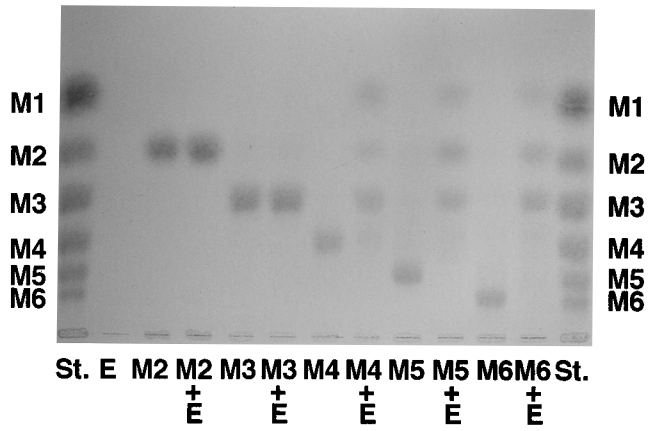

FIG. 7. Thin-layer chromatogram of hydrolysis products of mannooligosaccharides with purified β-mannanase. The reaction mixture contained 1 U of β-mannanase and each mannooligosaccharide in 1 mM sodium acetate buffer (pH 6.0) at 37°C. St., standard sugars: M1 to M6, mannose to mannohexaose; E, purified β-mannanase.

Pseudomonas sp. strain PT-5 (27) enzymes. However, β-mannanases from *Enterococcus casseliflavus* FL2121 were strongly inhibited by Ag^+ and Hg^{2+} and not inhibited by Cu^{2+} and Pb^{2+} (19). β-Mannanases from *Bacillus* sp. strain AM-001 (1) were also strongly inhibited by Ag^+ and slightly inhibited by Hg^{2+}. Although the number of reports on microbial mannanases is increasing, the catalytic mechanisms of these enzymes are not clear. It has been reported that tryptophan and carboxy groups are involved in the catalytic activities of carbohydrases such as lysozyme, amylase, and cellulase (12). Among the chemical modifiers tested, β-mannanases from *Streptomyces* sp. strain no. 17 (24), *Bacillus* sp. strain AM-001 (1), and *E. casseliflavus* FL2121 (19) were completely inhibited by *N*-bromosuccinimide. β-Mannanase from *Vibrio* sp. strain MA-138 was also completely inhibited by *N*-bromosuccinimide. This result may suggest that the tryptophan residue(s) play an important part at the active site or the substrate binding site of β-mannanase.

The action pattern of many mannanases is determined on mannooligosaccharides. β-Mannanase from *Bacillus* sp. strain AM-001 and *Streptomyces* sp. strain no. 17 hydrolyzed mannooligosaccharides larger than mannobiose (1, 24) and those from *Aeromonas* sp. strain F-25, *Bacillus subtilis*, *E. casseliflavus* FL2121, and *Pseudomonas* sp. strain PT-5 (2, 14, 19, 27) hydrolyzed mannooligosaccharides larger than mannotriose to produce several oligosaccharides. The purified mannanase from *Vibrio* sp. strain MA-138 was endo-β-mannanase, which hydrolyzed β-mannan, glucomannan, and mannooligosaccharides larger than mannotriose to produce several oligosaccharides but could not cleave mannotriose, mannobiose, and *p*-nitrophenyl-β-D-mannoside. Thus, β-mannanase from *Vibrio* sp.

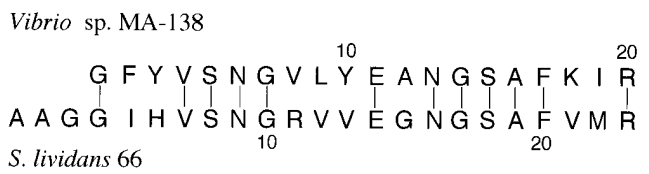

FIG. 8. Comparison of N-terminal amino acid sequence of β-mannanase from *Vibrio* sp. strain MA-138 with that from *Streptomyces lividans* 66. Vertical lines represent identical amino acids.

strain MA-138 was ascertained to be similar to the endo-β-mannanase of the latter group.

Further research is planned to clone the gene for β-mannanase and deduce the complete primary structure of the protein from the nucleotide sequence of the gene. Manipulation of the gene will provide detailed information on the multiplicity and activity domain of β-mannanase.

ACKNOWLEDGMENTS

We thank T. Aoki (Mie University) for the assay of glycoprotein. We also thank S. Karita (Center for Molecular Biology and Genetics, Mie University) for the use of an Applied Biosystems model 476A gas phase sequencer.

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