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Purification and Characterization of a Cell-Associated, Soluble Mannanase from *Bacteroides ovatus*

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***Bacteroides ovatus*, a human colonic anaerobe, utilizes the galactomannan guar gum as a sole source of carbohydrate. Previously, we found that none of the galactomannan-degrading enzymes were extracellular, and we characterized an outer membrane mannanase which hydrolyzes the backbone of guar gum to produce large fragments. We report here the purification and characterization of a second mannanase from *B. ovatus*. This enzyme is cell-associated and soluble. Using ion-exchange chromatography, gel filtration, and chromatofocusing steps, we have purified the soluble mannanase to apparent homogeneity. The enzyme has a native molecular weight of 190,000 and a monomeric molecular weight of 61,000. It is distinct from the membrane mannanase not only with respect to cellular location but also with respect to stability and isoelectric point (pI of 6.9 for the membrane mannanase and pI of 4.8 for the soluble mannanase). The soluble mannanase, like the membrane mannanase, hydrolyzed guar gum to produce large fragments rather than monosaccharides. However, if galactosyl side chains were removed from the galactomannan fragments by α-galactosidase, both the soluble mannanase and the membrane mannanase could degrade guar gum to monosaccharides. Thus either or both of these two enzymes, working together with α-galactosidase, appear to be sufficient for the breakdown of guar gum to the level of monosaccharides.**

Breakdown of the dietary galactomannan guar gum by *Bacteroides ovatus* is accomplished by cell-associated enzymes (5). Two types of enzyme are needed: mannanase, which hydrolyzes the mannosyl backbone of guar gum, and α-galactosidase, which removes galactosyl side chains. Previously, we found that approximately 30% of the cellular mannanase activity is tightly bound to cell membranes. The enzyme responsible for this activity is located in the outer membrane and hydrolyzes the backbone of guar gum to produce large fragments (5). No mannose was detectable even after prolonged incubation. This finding indicates that some other enzyme is required for complete breakdown of guar gum to monosaccharides. In preliminary experiments, the soluble mannanase activity, which accounted for about 70% of the total cellular activity, appeared to be distinct from the outer membrane mannanase because it was much more stable. It also appeared to be distinct from the α-galactosidase (α-galactosidase I), which is produced during growth of *B. ovatus* on guar gum, because it could attack intact guar gum. Although the α-galactosidase can remove galactose branches from large fragments of guar gum, it has no detectable activity when intact guar gum is the substrate (4). One possible function of a second mannanase could be to break guar gum or the large fragments produced by the membrane mannanase into mono- or disaccharides. To determine whether the soluble mannanase activity was, in fact, distinct from the membrane mannanase and, if so, whether the soluble enzyme might be able to produce monosaccharides from guar gum, we have purified the soluble mannanase and compared its characteristics with those of the membrane mannanase.

MATERIALS AND METHODS

Organisms and growth conditions. *B. ovatus* 0038-1 was obtained from the culture collection of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg. Bacteria were grown as previously described (5). Guar gum (lot G-20 B32 FG70-70) was obtained from Hercules Corp., Wilmington, Del. Guar gum was prepared for use in medium and enzyme assays as previously described (5).

Enzyme assays. Mannanase activity was measured as previously described (5). The concentration of reducing sugar was measured by the method of Dygert et al. (3). One unit of enzyme activity was defined as 1 μg of reducing sugar liberated per min in 0.1 M potassium phosphate (pH 6.5) at 37°C. When assaying column fractions during enzyme purification procedures or slices from isoelectric focusing gel, we took only two time points (0 and 30 min). α-Galactosidase acitivity was measured as previously described (4).

Purification of soluble mannanase. *B. ovatus* was grown in 3 liters of minimal medium with guar gum as the sole source of carbohydrate, harvested by centrifugation (10,000 × *g* for 15 min at 4°C), washed twice, and suspended in a final volume of 40 ml in 0.02 M potassium phosphate buffer (pH 6.5). The cells were disrupted by being passed through a French pressure cell (12,000 lb/in^2) twice, and the cell debris were removed by centrifugation (17,000 × *g* for 15 min at 4°C). The cell extract was used in the subsequent purification steps. All remaining steps were performed at 4°C unless stated otherwise.

(i) Ultracentrifugation. The cell extract was centrifuged at 200,000 × *g* for 2.5 h at 4°C. The supernatant solution was saved. The pellet was suspended in 40 ml of 0.02 M potassium phosphate buffer (pH 6.5) with a tissue homogenizer, and the resuspended membranes were centrifuged again at 200,000 × *g* for 2.5 h at 4°C. The supernatant was collected and combined with the supernatant from the previous centrifugation.

(ii) DEAE-Sephacel chromatography. The combined supernatant fractions were applied to a DEAE-Sephacel column (1.5 by 10 cm) which had been equilibrated with 0.02 M potassium phosphate buffer (pH 6.5). The column was

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washed extensively with buffer until no eluting protein was detected by A_{280}. The column was then eluted with a linear 0 to 0.2 M NaCl gradient, and 2-ml fractions were collected. The fractions were assayed for mannanase, α-galactosidase, NaCl concentration, and protein (A_{280}). The fractions which contained mannanase activity were pooled.

(iii) Chromatofocusing. The pooled fractions from the DEAE-Sephacel chromatography were dialyzed against 0.025 M imidazole hydrochloride buffer (pH 7.4) and applied to a PBE 94 chromatofocusing column (1 by 25 cm) equilibrated with the same buffer. The column was eluted with Polybuffer 74 (pH 4.0), and 2.5-ml fractions were collected as described in the *Pharmacia Technical Bulletin* (Pharmacia, Inc., Piscataway, N.J.). The fractions were assayed for mannanase and pH. Fractions containing mannanase activity were pooled and concentrated.

(iv) Bio-Gel A-1.5m chromatography. The concentrated enzyme from the previous purification step was applied to a Bio-Gel A-1.5 m column (1.5 by 75 cm) (Bio-Rad Laboratories, Richmond, Calif.) which had been equilibrated with 0.15 M NaCl in 0.02 M potassium phosphate buffer (pH 6.5). The column was eluted with the equilibrating buffer at a flow rate of 20 ml/h, and 1.75-ml fractions were collected. The fractions were assayed for mannanase and protein (A_{280}). The fractions which contained mannanase activity were pooled.

Bio-Gel A-1.5m chromatography, under the same conditions as used above, was used to estimate the native molecular weight of the purified soluble mannanase. The elution volume of the mannanase activity was compared with that of known standards, determined by monitoring A_{280}. Thyroglobulin (669,000), gelatin trimers (210,000), β-galactosidase subunit (116,000), and ovalbumin (45,000) were used as standards.

Analysis of products of enzymatic degradation of guar gum. Purified soluble mannanase (0.2 ml; 2 to 3 U) was added to 4.8 ml of soluble guar gum in 0.02 M potassium phosphate buffer (pH 6.5) and incubated at 37°C for 4 h. The final concentrations of protein and guar gum in the reaction mixture were 8 to 10 μg/ml and 10 mg/ml, respectively. Samples (1.75 ml) were removed at various times (0, 2, 4, and 18 h) and boiled for 5 min. Portions (0.75 ml) of these samples were analyzed by gel filtration chromatography on either a Bio-Gel A-5M or a Bio-Gel P-10 column (1.5 by 50 cm) eluted with 1 M NaCl, and 1.5-ml fractions were collected. Fractions were analyzed for carbohydrate by the method of Dubois et al. (2). Low-molecular-weight fragments were analyzed by descending paper chromatography on Whatman no. 1 paper (Whatman, Inc., Clifton, N.J.) as described previously (4, 5). Results for the 18-h sample were the same as those for the 4-h samples, so the 4-h time point was used for further experiments.

To determine whether further breakdown of large galactomannan fragments would occur if galactose residues were removed, samples of partially degraded guar gum from the previous reaction mixture (4-h time point) were first incubated with 5 U of purified α-galactosidase I from *B. ovatus* for 6 h at 37°C. The purification of α-galactosidase I has been described previously (4). Soluble mannanase (3 U) was added to this reaction mixture, which was then incubated for 4 h at 37°C. The 4-h incubation time was chosen because continued incubation produced no further breakdown. Products were characterized by chromatography on a Bio-Gel P-10 column and by paper chromatography, as described above. A similar experiment was done with outer membrane mannanase, which had been solubilized with 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) as described previously (5). The amount of enzyme, the concentration of guar gum, and the incubation times were the same as described above.

Electrophoresis. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (7) and visualized with Coomassie brilliant blue R-250. The protein standards had M_r 116,000, 97,000, 67,000, 45,000, 29,000, and 25,700.

Isoelectric focusing (IEF) was used to ascertain that the soluble and membrane mannanases were distinct enzymes. Modifications of the procedures of Righetti and Drysdale (11) and Kranz and Gennis (6) were used. Tube gels were 5 mm by 12 cm. The composition of the IEF gels was 5% acrylamide, 10% sucrose, 2% Triton X-100, and 2% ampholytes (Serva, Garden City Park, N.Y.). The ampholytes were a mixture of pH 5 to 8 and pH 7 to 9 and pH 3 to 10 at a ratio of 2:2:1 (vol/vol/vol). Special conditions had to be used to recover membrane mannanase activity because of its instability (5). IEF gels were prefocused for 1 h or until the amperage decreased to ≤ 2 mA and remained at that level for 15 min. Samples prepared as described below were then applied to the gels, and electrophoresis was carried out for 4 h at 400 V or until broad-range (pI from 4.6 to 9.6) prestained IEF standards (Bio-Rad) had focused. After electrophoresis, gels were cut into 0.5-cm slices, and each slice was placed in 0.5 ml of distilled water. Enzyme activity and pH were measured for each slice. Under these conditions, recovery of both the soluble and the membrane mannanase was approximately 10%. It was necessary to include Triton X-100 in the IEF gel mixture to focus the membrane mannanase. CHAPS-solubilized mannanase did not focus. Triton X-100 did not affect the focusing or activity of the soluble mannanase (data not shown).

Triton X-100 was added to the purified soluble mannanase (10 U) to a final concentration of 1%. For focusing of the membrane mannanase, 0.2 ml of 48% Triton X-100 in 0.15 M KCl–0.1 M potassium phosphate buffer (pH 7.0) was added to 4.8 ml of outer membranes (in 0.15 M KCl–0.1 M potassium phosphate buffer [pH 7.0]) and the mixture was gently stirred at 4°C for 4 h. Outer membranes were obtained as described previously (5). The reaction mixture was centrifuged for 1.5 h at 150,000 × *g* at 4°C. Extraction with Triton X-100 removed about 80 to 90% of the mannanase from the outer membranes. The supernatant was collected, passed through a DEAE-Sephacel column (0.5 by 7 cm) Pharmacia, Uppsala, Sweden), which had been equilibrated in 0.02 M potassium phosphate buffer (pH 7.4)–1% Triton X-100, and concentrated in an Amicon Centriflo-10 concentrator (Amicon Corp., Lexington, Mass.) at 4°C. In one experiment, approximately 10 U each of the soluble and the membrane mannanase preparations were mixed and applied to the same IEF gel.

Protein determinations. In most experiments, protein was measured by the method of Markwell et al. (8). In chromatofocusing experiments, protein was measured by the method of Bradford (1).

RESULTS

Purification of the soluble mannanase. The effect of various purification steps on the yield and specific activity of the soluble mannanase activity is shown in Table 1. Approximately 50% of the α-galactosidase activity passed through the DEAE column, whereas all of the mannanase activity was retained. The mannanase activity eluted from the DEAE

TABLE 1. Purification of mannanase from the soluble fraction of cell extracts of *B. ovatus*

Purification step	Total protein (mg)	Total enzyme activity (U)	Sp act (U/mg of protein)	Yield (%)	Degree of purification
Cell extract	693	384	0.55	100	
Ultracentrifugation	429	230	0.53	60	
DEAE chromatography	7.1	69	9.7	18	18
Chromatofocusing	0.64	22	34	6	63
Gel filtration	0.21	16	76	4	138

column as a single symmetric peak at a salt concentration of 0.09 M NaCl and was well separated from the α-galactosidase activity that remained on the column (Fig. 1). Most of the α-galactosidase activity was removed by this purification step. The mannanase activity eluted from the chromatofocusing column as a single symmetric peak at pH 5 (Fig. 2). Subsequent gel filtration step on the Bio-Gel A-1.5m column removed most of the ampholytes and several contaminating proteins. The final enzyme preparation was free of α-galactosidase activity and migrated as a single band on an SDS-gel (Fig. 3). Prior to the chromatofocusing step, the band corresponding to the soluble mannanase on an SDS-gel was sharp (data not shown). After the chromatofocusing step, it appeared as a broader, less-defined band. Thus the breadth of the polypeptide band seen in Fig. 3 is probably due to residual binding of polybuffer from the chromatofocusing step.

Characteristics of soluble mannanase. The pH optimum of the purified soluble mannanase was 6.2 to 6.5. Activity was not affected by Triton X-100 (1%). The enzyme was stable for 8 h at 37°C and for at least 96 h at 4°C. It could be thawed and refrozen without significant loss of activity. The native molecular weight of the enzyme was 190,000 ± 10,000, as determined by gel filtration chromatography on a Bio-Gel A 1.5m column. The mannanase was separable from the slightly larger α-galactosidase (molecular weight, 240,000) on this column (data not shown). The apparent molecular weight of the mannanase of SDS-polyacrylamide gel electrophoresis was 61,000 ± 2,000 (Fig. 3).

Mannanase products. When purified soluble mannanase was incubated with guar gum for 4 h at 37°C, fragments of various sizes were produced, as indicated by the gel filtration profile of the products on a Bio-Gel A-1.5m column (Fig. 4A). Some of these fragments eluted in the fully included volume of the Bio-Gel A-5M column; however, further chromatography of the reaction mixture on a Bio-Gel P-10 column showed that none of the fragments were small enough to migrate within the fractionation volume of this column. Thus the soluble mannanase did not release monosaccharides or short oligomers from guar gum.

The ability of the soluble mannanase to hydrolyze the mannose backbone into smaller segments could have been blocked by interference from the galactose branches. To test this, we first incubated a portion of the 4-h incubation mixture with purified α-galactosidase I from *B. ovatus* for 6 h at 37°C. Then, after removal of a portion for analysis, we added purified soluble enzyme (5 U) and incubated the mixture for another 4-h period. The results are shown in Fig.

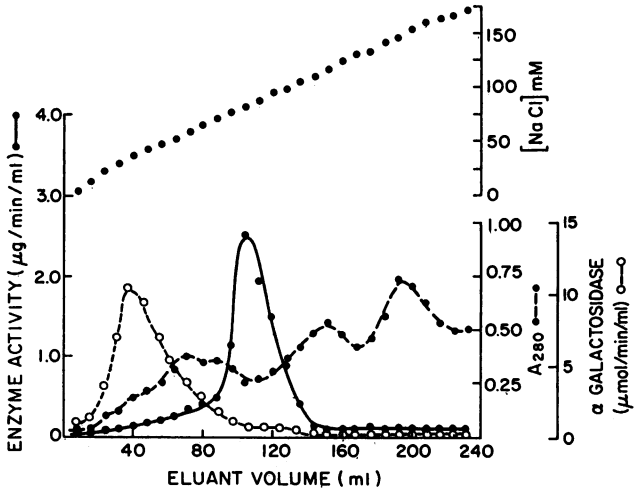

FIG. 1. Elution of mannanase activity (●—●) and α-galactosidase activity (○---○) from a DEAE-Sephacel column. Fractions corresponding to an eluant volume of 90 to 130 ml were pooled.

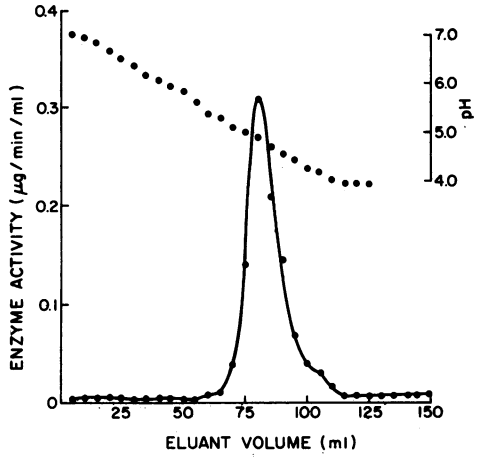

FIG. 2. Elution of mannanase activity (●—●) from a chromatofocusing column. Fractions corresponding to an eluant volume of 75 to 100 ml were pooled. ● ●, pH.

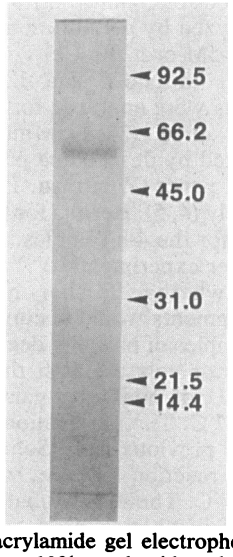

FIG. 3. SDS-polyacrylamide gel electrophoresis of the purified soluble mannanase on a 10% acrylamide gel. The gel was stained with Coomassie blue. Migration distances of molecular weight standards are indicated by arrows on the right side of the gel. The molecular weight of the enzyme was estimated to be 61,000.

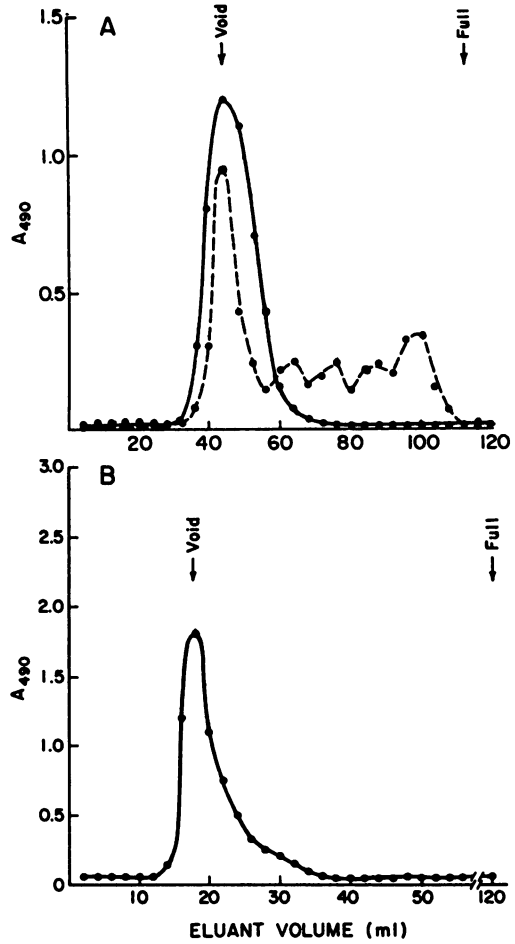

FIG. 4. Distribution of carbohydrate in a reaction mixture which contained guar gum and purified soluble mannanase. (A) Bio-Gel A5M column. ——, Elution profile of undigested guar gum; ------, distribution of carbohydrate after 4 h of incubation with the soluble mannanase. (B) Bio-Gel P-10 column. ——, Distribution of carbohydrate in the incubation mixture after 4 h. In both panels, the arrows indicate the void and fully included volumes of the columns.

5. The Bio-Gel P-10 profile of the products after incubation with α-galactosidase I showed a small peak which eluted in the fully included volume of the column. Further analysis of this sample by descending paper chromatography demonstrated that the low-molecular-weight material was free galactose. No free mannose or oligomers of galactose and mannose were detected. Most of the carbohydrate still migrated in the void volume of the Bio-Gel P-10 column. Thus the length of the mannose backbone after removal of galactose residues was still great enough to fall outside the fractionation range of this column. From the data shown in Fig. 5, we estimate that 75 to 80% of the galactose residues were removed by the α-galactosidase step. Once galactose residues had been removed, the soluble mannanase was able to degrade the mannan backbone fragments further to produce low-molecular-weight compounds which migrated in the fully included volume of the Bio-Gel P-10 column (Fig. 5). Analysis of this reaction mixture by paper chromatography showed that this low-molecular-weight carbohydrate consists mainly of mannose.

Previously, we found that the outer membrane mannanase, like the soluble mannanase, cleaved guar gum into

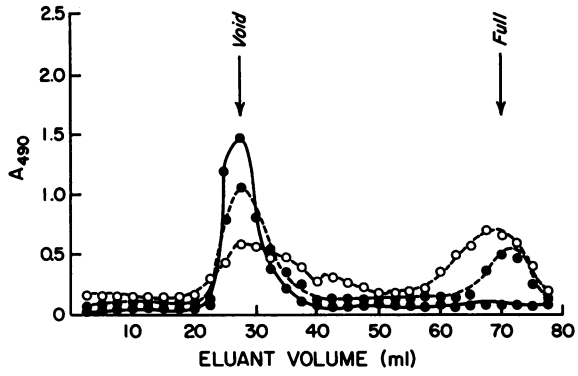

FIG. 5. Effect of removal of galactose residues on digestion products of the soluble mannanase. ●—●, Elution profile on a Bio-Gel P-10 column of guar gum which has been incubated with the soluble mannanase for 4 h; ●---●, profile of carbohydrate in the incubation mixture after incubation for 4 h with α-galactosidase I; ○---○, profile after a subsequent incubation with soluble mannanase.

fragments that were too large to elute within the fractionation range of a Bio-Gel P-10 column (5). To determine whether this enzyme was also able to degrade the mannan backbone fragments further if galactose residues were removed, we did a similar experiment with CHAPS- solubilized outer membrane mannanase instead of purified soluble mannanase. When α-galactosidase I was incubated with the products obtained from digestion of guar gum by the membrane enzyme, approximately 50% of the galactose residues were removed (Fig. 6). This step was probably less efficient than in the previous experiment because of the Triton X-100 in the incubation mixture. The mannan backbone fragments from which the galactose residues had been removed still eluted in the void volume of the Bio-Gel P-10 column (Fig. 6). Further incubation of the mannan backbone fragments with fresh membrane mannanase resulted in the breakdown of large mannan fragments to sugars that migrated in the fully included volume of the Bio-Gel P-10 column (Fig. 6). Analysis of the low-molecular-weight carbohydrate by descending paper chromatography confirmed that mannose was the main component. A reducing sugar which migrated more slowly than mannose, possibly a disaccharide, was also detectable.

Comparison of the soluble and membrane mannanases.

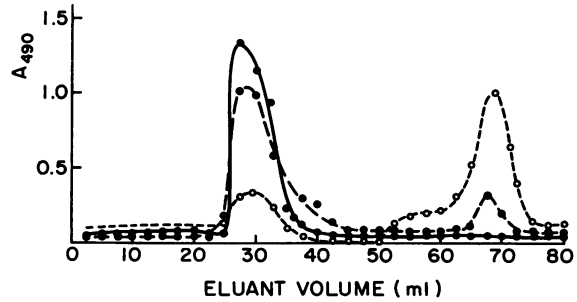

FIG. 6. Effect of removal of galactose residues on digestion products of CHAPS-solubilized outer membrane mannanase. ●—●, Elution profile on a Bio-Gel P-10 column of guar gum which had been incubated for 4 h with the membrane mannanase; ●---●, profile after incubation of this mixture with α-galactosidase I; ○---○, profile after a subsequent incubation with membrane mannanase.

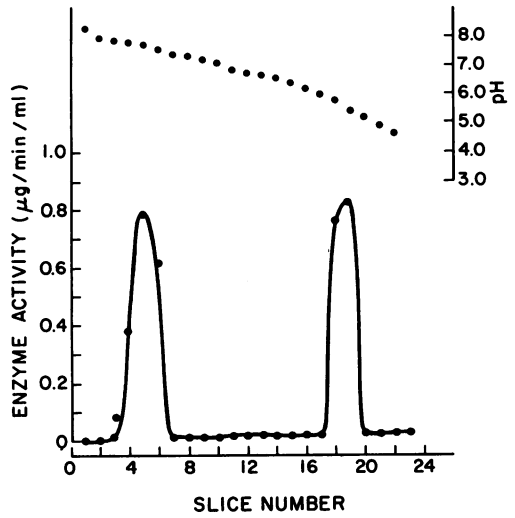

FIG. 7. Migration of mannanase activity in an IEF gel when a mixture of soluble mannanase (pI of 4.8) and Triton X-100-solubilized membrane mannanase (pI of 6.9) were focused on the same gel.

Although the soluble mannanase was much more stable than the membrane mannanase, both enzymes had the same pH optimum, both cleaved the guar gum backbone into large fragments, and both were similarly affected by galactose side chains. Comparison of molecular weights was not conclusive, because the membrane mannanase was not purified to homogeneity (5). To ascertain that the two enzymes were in fact distinct proteins, we compared their isoelectric points.

The membrane mannanase could be focused if it was solubilized with Triton X-100. Because of its instability, we had to find focusing conditions that made the focusing period as short as possible. Also, activity that was first solubilized with Triton X-100 had to be passed through a DEAE column before it was loaded on the IEF gel. This step eliminated any contaminating α-galactosidase activity as well as substances which gave a high background on the reducing-sugar assay. The use of gels fixed with 10% trichloroacetic acid and stained with Coomassie blue confirmed that all of the proteins from the sample had focused under these conditions. The pI of the solubilized outer membrane mannanase activity was 6.9, and that of the purified soluble mannanase was 4.8. When a mixture of solubilized outer membrane mannanase and purified soluble mannanase was focused on the same IEF gel, the two activities focused in two distinct regions of the gel (Fig. 7).

DISCUSSION

The results of this study indicate that the soluble mannanase activity that remains in the supernatant after membranes are removed from a disrupted cell suspension of *B. ovatus* by centrifugation is due to an enzyme that is distinct from the outer membrane mannanase which was described previously (5). The soluble enzyme is much more stable than the membrane enzyme and has a different pI. Moreover, the monomeric molecular weight of the soluble mannanase is 61,000, whereas the two major polypeptides in the most purified preparation of the membrane mannanase have molecular weights of 43,000 and 94,500 (5). Comparison of the native and SDS-polyacrylamide gel electrophoresis molecular weights of the soluble mannanase indicates that the enzyme is a trimer. However, we did not rule out the possibility that this enzyme is a glycoprotein or migrates anomalously on SDS-gels for some other reason.

Both the soluble and membrane mannanases acting alone hydrolyzed guar gum into large fragments. The smallest products of both enzymes were probably 10,000 daltons or larger because they eluted in the void volume of a Bio-Gel P-10 column even after most of the galactose side chains had been removed from the mannan backbone. However, precise sizing of polysaccharides, particularly branched ones, is difficult, because they are not globular molecules.

Both enzymes were capable of degrading the mannan backbone of guar gum to smaller fragments (including monosaccharides) if galactose residues were first removed by α-galactosidase. Recently, McCleary (10) has shown that the galactose residues of guar gum are not evenly distributed along the mannan backbone and can occur in clusters. Our results indicate that the *B. ovatus* mannanases are probably hydrolyzing the galactomannan backbone in regions that are free of galactose residues. Interference of galactose side chains with mannanase cleavage of guar gum has also been seen with mannanases from other organisms (9). Since free mannose is released only after galactose side chains are removed, and since the α-galactosidase can remove galactose residues only after the guar gum has been cleaved into large fragments by the mannanase, it seems likely that one or both of the mannanases are in close proximity to the α-galactosidase in the intact cell. Otherwise, an additional exo-acting mannanase would be needed for complete breakdown of the molecule.

Since the two mannanases have a similar mode of action and are also similarly inhibited by galactose branches, it is not clear why the cell needs both enzymes to degrade guar gum. One possibility is that the membrane mannanase breaks guar gum into large pieces, which are then transported into the cytoplasm, where they are further broken down by the soluble mannanase and the α-galactosidase acting in concert. Alternatively, all three enzymes could be closely associated in the periplasmic space. If they form a complex in the intact cell, it is easily dissociated, because no evidence of complexes was seen in our purification experiments. At present, no reliable method exists for ascertaining whether a soluble enzyme is in the periplasmic space of *B. ovatus*. No periplasmic marker enzymes have been identified. We have found that a procedure described previously (12) that appeared to release the soluble polysaccharidase chondroitin lyase selectively from *Bacteroides thetaiotaomicron* has not worked reproducibly. Moreover, spheroplasting of *B. thetaiotaomicron* does not release chondroitin lyase activity (L. Lipeski and Salyers, unpublished data). Future development of a method for selectively releasing periplasmic enzymes from *B. ovatus* may make it possible to determine whether the soluble mannanase and the α-galactosidase are in the periplasm or the cytoplasm.

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