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Extracellular Enzyme From Myxobacter AL-1 That Exhibits Both β-1,4-Glucanase and Chitosanase Activities

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An enzyme that has both β-1,4-glucanase and chitosanase activities is characterized. Evidence for homogeneity was obtained from electrophoresis and sedimentation velocity studies; only one N-terminal amino acid, valine, was found. Results of denaturation studies showed that β-1,4-glucanase and chitosanase activities decreased at equal rates. With carboxymethylcellulose as the substrate, a K_m of 1.68 g of carboxymethylcellulose per liter of solution and a V_{max} of 2.20×10^{-9} mol/min were found. With chitosan (the β-1,4-polymer of glucosamine) as the substrate, a K_m of 0.30 g of chitosan per liter of solution and a V_{max} of 0.75×10^{-9} mol/min were found. A pH optimum of 5.0 was found for β-1,4-glucanase activity, and pH optima of 5.0 and 6.8 were found for chitosanase activity. β-1,4-Glucanase activity had a temperature optimum of 38 C, and chitosanase activity had a temperature optimum of 70 C. Chitosan stabilized both enzyme activities at 70 C. Cellotriose was the smallest polymer capable of hydrolysis. Glucosamine was released by action of the enzyme upon cell wall preparations of several fungi.

In previous communications, two extracellular enzymes produced by myxobacter strain AL-1 have been described and characterized. One of these, protease I, has been shown to lyse cell walls of a number of bacteria by cleavage of the pentaglycine bridge, as well as by removal of the peptide moieties from the peptidoglycan (4, 11). Protease II was shown to exhibit specific peptide bond cleavage on the amino side of lysine (25). We present here the characterization of a third extracellular enzyme that has both β-1,4-glucanase and chitosanase activities. This is the first enzyme with both β-1,4-glucanase and chitosanase activities to be described; chitosan is the β-1,4-polymer of glucosamine and is prepared by the deacylation of chitin. By definition, a β-1,4-glucanase attacks amorphous forms of cellulose, such as carboxymethylcellulose (CMC), but not crystalline cellulose (12). Chitosanase activity has been reported in crude growth liquors; these growth liquors lysed certain fungi (16, 22). Purified chitosanases may provide a tool for the characterization of fungal cell walls.

MATERIALS AND METHODS

β-1,4-Glucanase and chitosanase activities were measured in a standard assay mixture which contained: 0.05 M sodium acetate buffer (pH 5.0); 1 to 3 enzyme units; either 7.5 mg of CMC (type 4M) or 0.75 mg of chitosan; and distilled water to make a final volume of 2.0 ml. The mixture was incubated at 38 C for 15 min after the addition of enzyme. A 0.1-ml sample was removed to assay for the reducing sugar that was released during incubation. The Park-Johnson reducing sugar assay (20) with the modification of Ghuysen and Tipper was used (5). One unit of enzyme is defined as the amount of enzyme that releases 10^{-6} mol of reducing sugar per 0.1 ml of assay mixture in 15 min.

Protein concentration was determined by the method of Lowry et al. (13), with bovine serum albumin (Sigma Chemical Co.) as the standard.

Viscosity determinations were made in a Cannon Ubeholde viscometer (size 25), which had a constant of 0.002403 centistokes per s. The viscometer and the material for which the viscosity was to be determined were equilibrated at 38 C in a constant-temperature water bath.

CMC was obtained from the Hercules Powder Co. Chitosan was prepared by the method of Horton and Lineback (9) for a 97% deacylated product, with chitosan from K & K Laboratories, Inc. used as the starting material. Cellobiose, xylan, laminarin, and dextran were obtained from the Nutritional Biochemical Company; chitin and pectin were from Calbiochem; cellulose powder was from W. R. Balston, Ltd.; and polygalacturonic acid was from Sigma Chemical Co. Phosphate-swollen cellulose powder was made by the method of Whitaker (24), and phosphate swollen chitin was made by the method of Monreal and Reese (17).

Molecular weight was estimated by gel filtration by method of Andrews (1). A 0.15-mg amount of each protein was used, and all peaks except cytochrome *c*

were detected by measuring optical density at 280 nm; cytochrome c was measured at 315 nm.

Cellodextrins were prepared by hydrolyzing cellulose powder with hydrochloric acid by the method of Miller (15). The unhydrolyzed cellulose was removed by filtration through Whatman no. 1 filter paper. The filtrate was concentrated and desalted through a Diaflo UM-05 membrane until no chloride ions were detectable upon addition of $AgNO_3$. Cellodextrins (degree of polymerization 2 to 6) were separated on Sephadex G-15 resin. Cellodextrins and products from enzymatic hydrolysis were separated by ascending chromatography in the solvent system water-ethanol-1-propanol (40:40:20) on Eastman Chromogram cellulose sheets that were held between Eastman Sandwich plates (10). Glucose and cellobiose were used as standards for identification of the products from the complete enzymatic hydrolysis of the cellodextrins.

Analytical electrophoresis of enzyme preparations was performed with a 7.5% acrylamide gel system. Gels were formed by mixing solutions A, B, and C (1:1:2). Solution A contained 0.46 ml of *N,N,N',N'*-tetramethylethylenediamine in 100 ml of the desired buffer at 0.04 M. Solution B contained 30.0 g of acrylamide and 0.8 g of *N,N'*-methylenebisacrylamide per 100 ml of aqueous solution. Solution C contained 0.12 g of ammoniumpersulfate per 100 ml of water. The reservoir buffer in each case was the same as the gel buffer except that a concentration of 0.01 M was used. Protein samples (0.1 to 0.3 mg) were dissolved in a 10% sucrose solution that had been made with reservoir buffer. Each sample was applied to the gel and subjected to electrophoresis at 6 mA with a Canalco model 200 power supply.

Sedimentation velocity determinations were performed in a Spinco model E ultracentrifuge with a double-sector cell and a standard AN-D rotor. Photographic plates were exposed for 30 s at 16-min intervals. A speed setting of 59,780 rpm, at 20 C, was used.

Dialysis bags were prepared by the method of McPhie (14). To prevent enzymatic hydrolysis of the cellulosic bonds of the dialysis bags, the enzyme was dialyzed against 1.25×10^{-4} M ammonium hydroxide.

For amino acid analysis, hydrolysis of the protein was carried out in duplicate for 24, 48, and 72 h at 110 C by the method of Moore and Stein (18). After hydrolysis, each hydrolysate was evaporated to dryness at 50 C under a stream of nitrogen. Each hydrolysate was dissolved in water and evaporated again, the procedure being repeated three times. The hydrolysate was then dissolved in 0.02 M citrate buffer (pH 2.2) and analyzed for amino acid content with a Beckman model 120 amino acid analyzer. Cystine was determined as cysteic acid by the performic acid oxidation method of Hirs (8). Tryptophan was determined by the method of Goodwin and Morton (6).

Dansylchloride derivatives were made by the method of Gross and Labouesse (7) and were separated by ascending chromatography for 90 min in an aqueous buffer system of 0.4% pyridine and 0.8% acetic acid on Brinkman cellulose MN300 sheets (2), followed by electrophoresis in the same buffer for 90 min in a Brinkman-Desage electrophoresis chamber at 400 V and 10 mA.

Rhizopus oligosporous NRRL 2710, *Geotrichium candidum*, *Trichoderma viride* QM9123, *Aspergillus niger*, *Penicillium digitatum* NRRL 1202, and *Alternaria* sp. were obtained from Z. J. Ordal. *Rhizopus rhizopodiformis* QM9395 was obtained from E. T. Reese. All of the fungi were grown at 30 C with shaking for 24 to 48 h in 1 liter of Pramer and Schmidt medium (21). The mycelia were harvested and washed three times with distilled water. Harvested mycelia were sonically disrupted and then passed through a French pressure cell three times at 15,000 psi to produce cell wall fragments. These fragments were harvested by centrifugation and washed three times with distilled water.

To assay for the amount of reducing sugar produced by the action of enzyme upon the cell walls, 3.0 ml of the cell wall suspension was placed in a 10-ml Erlenmeyer flask, and 0.2 ml of 1.0 M collidine-hydrochloride buffer (pH 6.8) was added. Enzyme (60 to 100 U) was added to the suspension. The total volume was made to 4.0 ml with distilled water, and the flask was sealed with a rubber stopper. After incubation with shaking at 38 C for 4 h, a sample was removed and appropriately diluted to assay for the amount of reducing sugar released. Incubation of the cell wall suspension was continued for an additional 20 h at room temperature without shaking and again was assayed for reducing sugar. After removal of the last sample, 1.0 ml of the suspension was removed and dried to a constant weight at 100 C. For assays with the purified *T. viride* cellulase (Miles Laboratories), 1.0 M sodium acetate buffer (pH 5.0) was used instead of the collidine-hydrochloride buffer.

Glucosamine that was released from the fungal cell walls by the myxobacter enzyme was identified by ascending, thin-layer chromatography on Brinkman MN 300 cellulose sheets with a pyridine-ethylacetate-acetic acid-water (36:36:7:21) solvent system (26). Reducing sugar spots were detected by the silver nitrate method (23).

RESULTS

A purification procedure for proteases in the growth liquor of the organism has been described previously (11, 25). For the present study, the growth liquor was poured over an excess of ice as it came out of a Sharples centrifuge, and the zinc chloride precipitation was performed immediately. This modification prevented loss of enzyme activity that would have occurred during slow cooling of the harvested liquor. Unwanted materials were removed from the zinc precipitate by extraction with 0.05 M mono-tris(hydroxymethyl)aminomethane (Tris) buffer (pH 9.0) that, contained 1 mM ethylenediaminetetraacetic acid. After centrifugation, the precipitate was retained and the enzyme was solubilized from

the precipitate by extraction with 0.15 M citrate buffer (pH 5.0). Recoveries of enzyme activities that were obtained at various steps of the purification are shown in Table 1. The enzyme was precipitated from the citrate buffer with 3.4 M ammonium sulfate at 0 C. The precipitate was recovered by centrifugation, dissolved in 0.025 M Tris buffer at pH 9.0, and dialyzed overnight against 15 liters of 1.25×10^{-4} M ammonium hydroxide. After dialysis, the protein solution was poured into 1 liter of SP-Sephadex C-50 resin that had been equilibrated with 0.01 M collidine-hydrochloride buffer (pH 6.5) and from which the excess buffer had been removed. After the resin was stirred for 30 min, the slurry was poured onto a scintered-glass filter, and the solution was removed by suction. Brown coloration was removed from the resin by elution with 0.01 M collidine-hydrochloride buffer (pH 6.5). The enzyme was eluted with 0.01 M collidine-hydrochloride buffer (pH 6.5) that contained 0.5 M NaCl. Modification of the purification procedure (25) to use SP-Sephadex C-50 resulted in better binding of the enzyme to the resin and greater recovery of the enzyme. The enzyme was then precipitated from the SP-Sephadex eluant with 4.5 M ammonium sulfate at 0 C. The solution was eluted from a Sephadex G-75 column (85 by 5 cm) with 0.05 M Tris buffer (pH 7.0).

Enzyme from the Sephadex G-75 column was dialyzed and lyophilized. Purification to homogeneity was achieved by column chromatography with an SP-Sephadex C-50 resin equilibrated with 0.01 M collidine-hydrochloride buffer (pH 6.5), a 0.0 to 0.1 M NaCl gradient being used. Figure 1 shows the coincidence of the β-1,4-glucanase, chitosanase, and protein peaks that were eluted from the column. Specific activity of 6.34×10^4 β-1,4-glucanase units per mg of protein and 2.66×10^4 chitosanase units per mg of protein were obtained.

Analytical gel electrophoresis of the enzyme at several different pH values revealed only one band migrating from the anode to the cathode (Fig. 2). No bands were found to migrate in the opposite direction. The isoelectric point occurs at pH 9.0 with barbital buffer.

Protein concentrations of 6.4 and 10.6 mg/ml were used in 0.05 M Tris buffer (pH 7.0) for sedimentation velocity analysis. Only one very broad peak, which moved slowly, was detected (Fig. 3). The enzyme had a total hexose content of 1.3% as determined by the method of Dubois et al. (3).

The enzyme was found to be inactivated by incubation in water, and an experiment was designed to follow each enzymic activity as the enzyme was allowed to incubate at 30 and at 38 C. At the times indicated in Fig. 4, samples were removed to assay for β-1,4-glucanase and chitosanase activity by the standard assay procedure; both β-1,4-glucanase and chitosanase activities decreased at equal rates.

The enzyme was bound to a cellulose powder column that had been equilibrated with 0.05 M sodium acetate buffer at pH 5.0. When the column was washed with 10 column volumes of the same buffer, enzyme activity did not elute from the column. Chitosan was added to the buffer to give a concentration of 0.02%. After addition of chitosan to the buffer, both β-1,4-glucanase and chitosanase activities eluted together (Fig. 5). All of the enzymatic activity loaded onto the column was eluted in one peak. In a control experiment, 1.0 M NaCl did not elute the enzyme from the column.

Several polymers were tested as possible substrates for the enzyme. Only the amorphous forms of cellulose, CMC and phosphate-swollen cellulose, and chitosan were attacked. Cellulose powder (a crystalline form of cellulose), starch,

TABLE 1. *Comparison of enzyme activities during purification procedures*[a]

Purification Step	Substrate (type)	Total units	Total protein (mg)	Sp act	% of original activity
Citrate extract	4M CMC	3.50×10^6	8.05×10^4	43.50	39.40
	Chitosan	1.27×10^6		15.80	39.40
Dialyzed ammonium sulfate precipitate	4M CMC	1.74×10^6	7.88×10^3	221.00	19.60
	Chitosan	6.30×10^5		80.00	19.60
SP-Sephadex elution	4M CMC	4.48×10^5	7.00×10^2	640.00	5.04
	Chitosan	1.63×10^5		233.00	5.00
G75 elution	4M CMC	1.64×10^5	78.75	2,100.00	2.00
	Chitosan	6.30×10^4		800.00	2.00

[a] Samples were withdrawn at the steps indicated for enzyme and protein assays. Appropriate dilutions were made, and boiled-enzyme blanks were used as controls in the enzyme assays; 8.88×10^6 β-1,4-glucanase units, and 3.22×10^6 chitosanase units were present in the starting 240 liters of growth liquor.

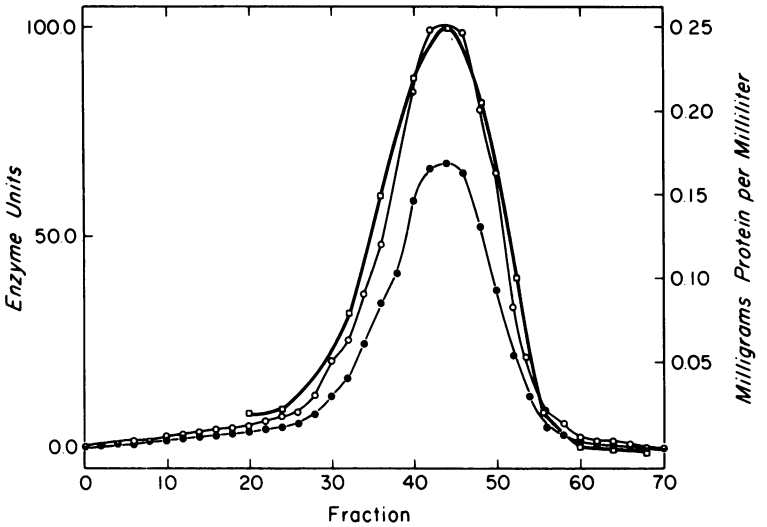

FIG. 1. *Elution of protein, β-1,4-glucanase, and chitosanase activities from an SP-Sephadex column. SP-Sephadex C-50 equilibrated with 0.02 M collidine-hydrochloride buffer (pH 6.5) was packed in a column to give bed dimensions of 3.0 cm in diameter and 35 cm in length. Enzyme (80 mg of protein) that had been eluted from a Sephadex G75 column, dialyzed, and lyophilized was dissolved in the column buffer and loaded on the column. A 0.0 to 0.1 M NaCl gradient with a flow rate of 40 ml/h was applied. Fractions indicated were assayed with appropriate dilutions for β-1,4-glucanase (○), chitosanase (●), and protein concentration (□). Each fraction had a volume of 12.0 ml.*

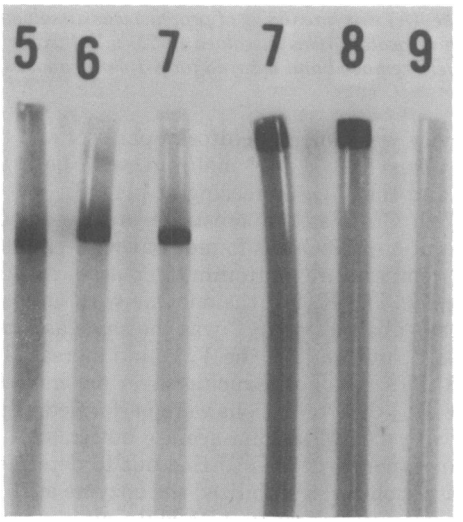

FIG. 2. *Electrophoresis of the enzyme at different pH values. A 0.3-mg amount of protein was used for each gel. The number above each gel indicates the pH at which electrophoresis was performed. The following buffer systems were used (left to right): collidine acetate (pH 5.0 and 6.0), collidine-hydrochloride (pH 7.0), phosphate (pH 7.0), and barbital (pH 8.0 and 9.0). All buffer concentrations were 0.01 M.*

dextran, laminarin, xylan, pectin, and poly-β-1,4-galacturonic acid were not attacked. Neither chitin, from which chitosan was made by deacylation, nor the amorphous, phosphate-swollen chitin was attacked. The enzyme showed a specificity for β-1,4-glucose and glucosamine polymers. Cellodextrins with a degree of polymerization from 2 to 6 were tested as substrates for the enzyme. Cellotriose was the smallest polymer to be attacked, cellobiose not being a substrate. When a sample of each reaction mixture was subjected to thin-layer chromatography, glucose and cellobiose were the only products found.

Viscometric studies of enzymatic hydrolysis of chitosan and CMC (type 7M) showed a logarithmic decrease of viscosity both with respect to time and reducing sugar produced. These results are indicative of an enzyme that attacks internal bonds of a polymer in a random fashion.

For K_m determinations, substrate concentration was measured in units of grams per liter of assay mixture. With CMC as the substrate, a K_m of 1.68 g of CMC (type 4M) per liter and a V_{max} of 2.20×10^{-9} mol of reducing sugar per min were found. With chitosan as the substrate, a K_m of 0.30 g of chitosan per liter and a V_{max} of 0.75×10^{-9} mol of reducing sugar per min were found. These determinations were performed at 38 C and pH 5.0.

With CMC (type 4M), as the substrate, a pH optimum of 5.0 was found (Fig. 6). With chitosan as the substrate, pH optima were found at 5.0 and 6.8. At pH 6.8 the K_m for chitosanase

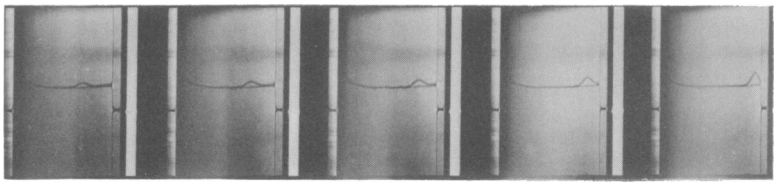

FIG. 3. *Sedimentation pattern of the enzyme. The enzyme was centrifuged at 59,780 rpm in 0.05 M Tris buffer (pH 7.0) at a concentration of 6.4 mg/ml, and at 20 C. The photographs were taken at 16, 32, 48, 64, and 80 min.*

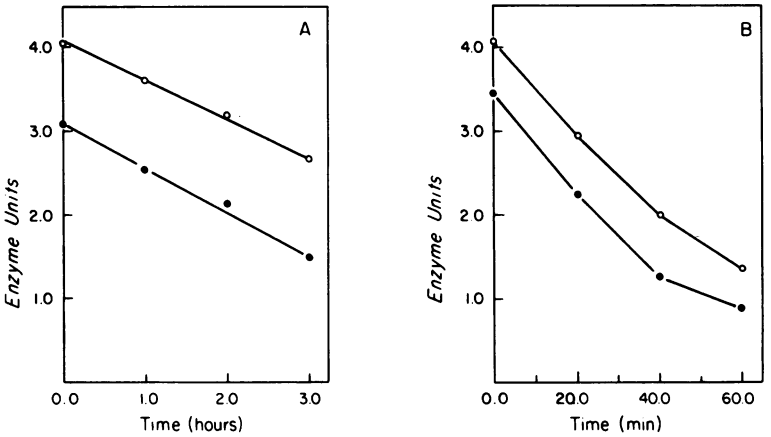

FIG. 4. *Thermal stability of β-1,4-glucanase and chitosanase. (A) Enzyme (68 μg of protein) was dissolved in 5.0 ml of water and incubated at 30 C. (B) Enzyme (108 μg of protein) was dissolved in 7.0 ml of water for incubation at 38 C. At the times indicated, 0.1-ml samples were removed and assayed for β-1,4-glucanase* (○) *and chitosanase* (●).

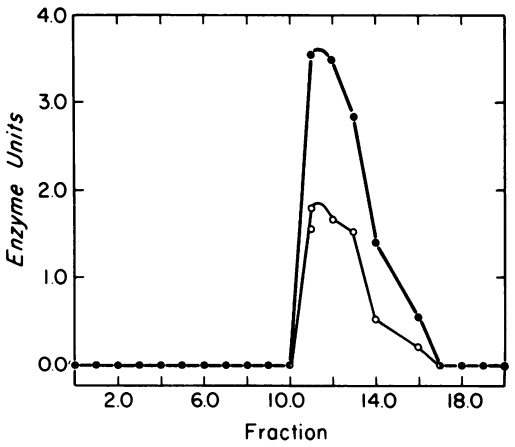

FIG. 5. *Elution profile of enzyme activities from a cellulose column. Cellulose powder that had been defined and equilibrated with 0.02 M acetate buffer (pH 5.0) was poured into a column to give bed dimensions of 1.5 by 10.0 cm. Enzyme (70 μg of protein) was dissolved in the buffer and loaded on the column, which was then washed with 1 liter of buffer. The elution profile of enzyme activities after addition of chitosan (0.02%) to the eluting buffer is shown:* ●, *β-1,4-glucanase activity;* ○, *chitosanase activity. Each fraction had a volume of 10.0 ml.*

activity was 0.52 g of chitosan per liter and the V_{max} was 1.42×10^{-9} mol/min, with both the K_m and the V_{max} increasing at pH 6.8.

With CMC as the substrate, a temperature optimum of 38 C was found, but with chitosan the temperature optimum increased to 70 C (Fig. 7). The K_m for the enzyme with chitosan as the substrate at 70 C was the same as found at 38 C, but at 70 C the V_{max} had increased to 2.43×10^{-9} mol of reducing sugar per min. K_m and V_{max} determinations were performed at pH 5.0 with 0.05 M sodium acetate buffer.

An experiment was carried out to determine whether chitosan stabilized the enzyme at 70 C. Enzyme was added to 0.5% chitosan in a standard reaction mixture that had been equilibrated at 70 C. At the times indicated in Fig. 8, samples were removed and assayed for chitosanase and β-1,4-glucanase activities at 38 C and for chitosanase activity at 70 C. The amount of reducing sugar released from the 0.5% chitosan incubation mixture was determined. As the enzyme became unsaturated, substrate protection decreased and both β-1,4-glucanase and chitosanase activities began to fall. The activities roughly paralleled each other; one activity was not preferentially lost. Incubation of the

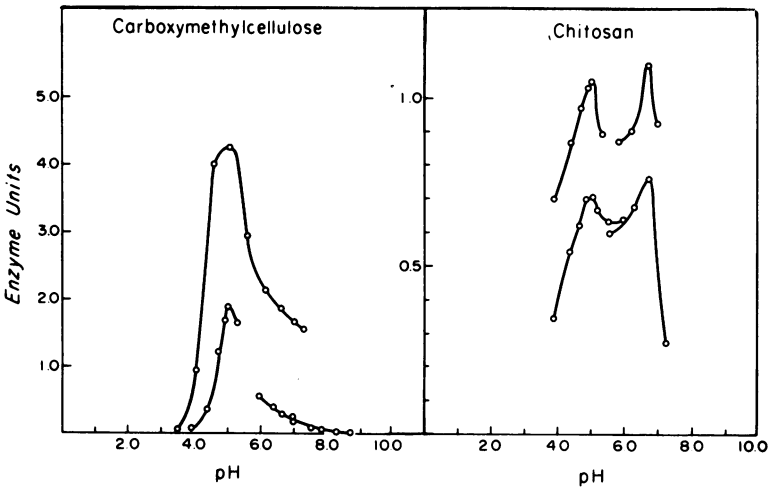

FIG. 6. *pH optima. The standard assay reaction mixture was used. (Upper curve) Citrate buffers from pH 3.5 to 7.3 were used with CMC type 4M; 11.3 μg of protein was used in each reaction mixture. (Lower curve) With CMC type 4M, 3.78 μg of protein was used in each reaction mixture; acetate buffer was used from pH 4.2 to 5.5; Tris-maleate buffer was pH 5.9 to 7.0 and Tris-hydrochloride buffer was pH 6.9 to 8.8. With chitosan as the substrate, 3.78 μg of protein was used in each assay for the upper curve. Acetate buffer was used at pH 3.9 to 5.3 and Tris-maleate was used at pH 5.8 to 6.9; 0.68 μg of protein was used for the lower chitosan curve. Pyridine-hydrochloride buffer was used at pH 3.9 to 6.0, and collidine-acetate buffer was used at pH 5.0 to 7.0. All buffer concentrations were 0.05 M. pH was determined directly on each reaction mixture.*

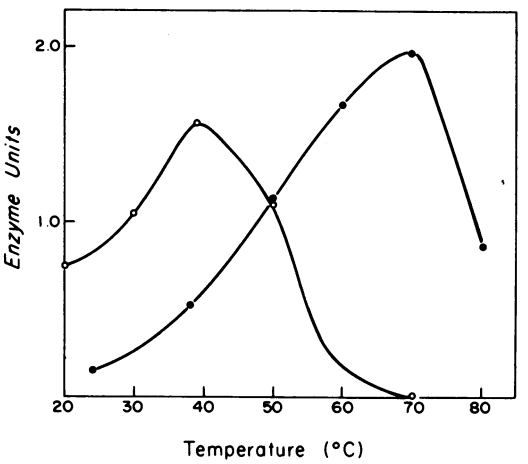

FIG. 7. *Temperature optima. Standard reaction mixtures were incubated at the temperatures indicated. A 1.8-μg amount of protein was used for CMC type 4M (○); 0.9 μg of protein was used for chitosan (●).*

enzyme with 1% CMC (type 4M) at 70 C revealed no enzyme activity even after a 1-min incubation period. Chitosan at saturating levels appears to stabilize the enzyme at 70 C, providing an explanation for the two temperature optima.

A number of salts were tested for their effect upon enzymatic activity. None was found to be stimulatory. $HgCl_2$, $AgNO_3$, $CdCl_2$, and $ZnCl_2$ were found to inhibit β-1,4-glucanase and chitosanase activity equally. They were effective only at concentrations of 10^{-3} and 10^{-4} M. All of the salts were tested at 38 and 70 C, but no salt was found to stabilize β-1,4-glucanase activity at 70 C.

Cellobiose, a product of enzymatic hydrolysis of the cellodextrin polymers, but not a substrate for the enzyme, was not found to be an inhibitor even at 10^{-2} and 10^{-3} M concentrations (the viscosity assay was used). CMC also was tested as a possible inhibitor of the enzyme. CMC (type 7M) that had been completely hydrolyzed by the enzyme was mixed in various ratios (2:0,2:1,2:3, and 2:4) with unhydrolyzed CMC (type 7M). The mixtures were brought to equal volumes and used as substrates in the standard assay procedure. Hydrolyzed CMC (type 7M) was not found to be an inhibitor of the enzyme.

The amino acid composition of the enzyme is shown in Table 2. Large amounts of the basic amino acids lysine and arginine are indicated. Results from electrophoretic mobility and binding to cation-exchange columns had previously indicated that the enzyme was a basic protein. The amide content was not determined. A molecular weight of 28,919 was calculated from the amino acid composition. A molecular weight estimation of 31,000 was determined with Sephadex G-75 gel filtration (Fig. 9).

Since cellulases have not been assayed for their ability to attack chitosan, several cellu-

lases were tested (Table 3); none attacked chitosan. Two bacterial isolates, A and B, were obtained from enrichments that selected for organisms capable of using chitosan as a carbon source. Growth liquor from isolate A attacked only chitosan, whereas growth liquor from isolate B attacked both CMC and chitosan. Although no further work was done with isolate B to determine the nature of the enzymatic activity, these results indicate that enrichment cultures of soil organisms with chitosan as substrate may yield organisms with interesting hydrolytic extracellular enzymes.

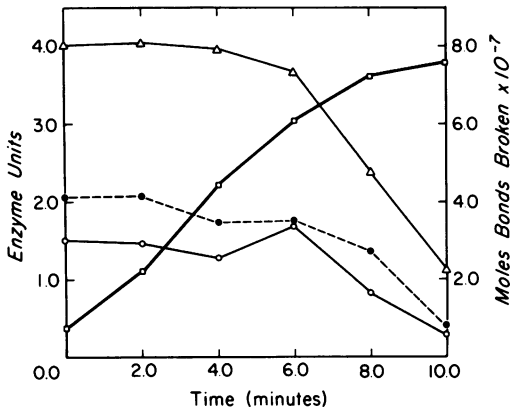

FIG. 8. *Stabilization of enzyme activity at 70 C by chitosan. A reaction mixture of 0.5% chitosan was made to a total volume of 4.0 ml in the same proportions as the standard reaction mixture. A 21-μg amount of protein was used in the reaction mixture. The reaction mixture was equilibrated at 70 C before the enzyme was added. At each 2-min interval, a 0.1-ml sample was removed to assay for enzyme activity; chitosanase was assayed at 38 C (●) and at 70 C (△). β-1,4-Glucanase activity was assayed at 38 C (○). A 0.1-ml sample also was withdrawn into 1.9 ml of water and placed in a boiling-water bath for a determination of reducing sugar released in the 4.0-ml reaction mixture (□) and to serve as a blank to correct for reducing sugar carried into the enzyme assays.*

Cell wall preparations from representatives of several genera of bacteria were tested as possible substrates for the enzyme. Neither the myxobacter homogeneous enzyme nor growth liquor would cause the release of reducing sugar from the bacterial cell wall preparations. Fungal cell wall preparations also were tested as possible substrates for the enzyme. Work by others has shown that growth liquors that contained chitosanase activity would lyse fungal cell walls (16, 22). With homogeneous enzyme, reducing sugar was released from all of the fungal cell wall preparations tested except *P. digitatum* and *Alternaria* sp. (Table 4). Because of the β-1,4-glucanase activity of the enzyme, purified *T. viride* cellulase (Miles Laboratory) was tested for its ability to release reducing sugar from the fungal cell wall preparations; no reducing sugar was released from any of the fungal cell wall preparations by the cellulase. After treatment of each cell wall preparation by homogeneous myxobacter enzyme, the soluble fraction was assayed by thin-layer chromatography. The reducing sugar detected had properties identical to a glucosamine standard.

TABLE 2. *Amino acid analysis of the enzyme*

Amino acid	Protein (g/100 g)	No. of residues	Assumed no. of residues	Amino acid residues per g
Lysine	5.06	10.32	10	1,462.0
Histidine	2.68	4.77	5	776.0
Arginine	8.43	13.92	14	2,438.8
Aspartic acid	13.35	29.09	29	3,859.9
Threonine	3.71	9.20	9	1,071.9
Serine	6.18	16.85	17	1,786.7
Glutamic acid	7.12	13.89	14	2,059.4
Proline	5.17	13.17	13	1,496.3
Glycine	5.97	23.15	23	1,727.3
Alanine	8.32	27.14	27	2,405.7
Valine	6.48	16.10	16	1,873.6
Methionine	1.55	3.00	3	477.6
Isoleucine	4.08	9.30	9	1,180.8
Leucine	7.71	17.10	17	2,230.4
Tyrosine	7.52	11.92	12	2,174.4
Phenylalanine	4.00	6.63	7	1,156.4
Cysteine	1.26	2.73	3	363.6
Tryptophan	1.41	2.19	2	408.8

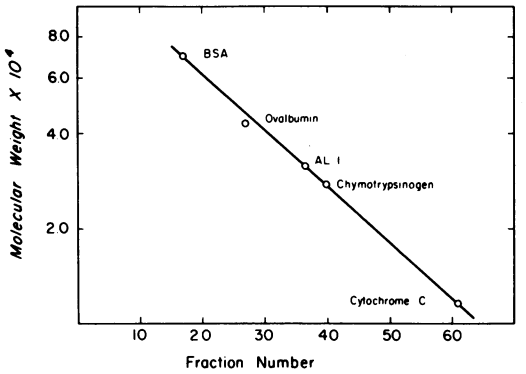

FIG. 9. *Estimation of enzyme molecular weight with Sephadex G75.*

DISCUSSION

The results presented here suggest that one protein species is responsible for both β-1,4-glucanase and chitosanase activities. No separation of the two activities was found during the various precipitation and solubilization steps of the purification procedure, or during gel filtration or cation-exchange chromatography. Only one N-terminal amino acid, valine, was found. Only one band was detected after analytical gel electrophoresis at several different pH values. Neither heat denaturation nor inhibition by salts preferentially affected one enzymatic activity. With the similarity between the chitosan and CMC, it is not unreasonable that one enzyme may attack both polymers.

An explanation for the higher temperature optima at 70 C resides in the evidence that chitosan was found to stabilize the enzyme at 70 C.

Cellulases and β-1,4-glucanases generally have a pH optimum of 5.0. The only pH optimum reported for a chitosanase is 7.6, which was obtained with growth liquor of a streptomycete with *Mucor rouxii* cell walls as a substrate (22). Two pH optima were found for the myxobacter enzyme, one at pH 5.0 with both CMC and chitosan as substrates and another at pH 6.8 with chitosan as the substrate. Tris-maleate buffer was used with both substrates at pH 6.8, indicating that the phenomenon at pH 6.8 was not an effect of the buffer. The two pH optima of the myxobacter enzyme may be due to the chitosan substrate. Above pH 6.8, the chitosan precipitates due to the loss of protons from the glucosamine monomers of the polymer. This change in the charge of the substrate may affect the formation of the enzyme-substrate complex and the release of the product after bond hydrolysis. The increase in K_m and V_{max} observed at the higher pH optimum is consistent with this hypothesis.

The effect of salts upon the myxobacter enzyme activity is similar to those found for other cellulases and β-1,4-glucanases. The streptomycete chitosanase activity reported by Ramirez-Leon and Ruiz-Herrera (22) was inhibited by divalent cations at pH 7.6 with cell walls as the substrate. Muzzarelli and Sipos (19) found that at pH 7.6 chitosan would bind some divalent cations.

Since gel filtration techniques yield a molecular weight estimation within a 10% error (1), the molecular weight estimation obtained by this method is in agreement with that calculated from the amino acid composition. Molecular weight studies performed with the analytical ultracentrifuge were unsatisfactory because of the rapid diffusion and slow migration of the enzyme during the sedimentation velocity studies. The enzyme contained 1.3% carbohydrate; homogeneous preparations of cellulases from bacteria and fungi contain carbohydrate. Cellulase B from *Pseudomonas flourescens* var. *cellulosa* contains more carbohydrate than cellulases A or C from the same organism and also has the broadest sedimentation velocity peak of

TABLE 3. *Comparison of extracellular β-1,4-glucanase and chitosanase activities from various organisms*[a]

Organism	Enzyme preparation[b]	Reducing sugar released (nmol/ml)	
		CMC type 4M	Chitosan
Myxobacter strain AL-1	H	1,060	650
Pseudomonas fluorescens var. *cellulosa* (cellulase A)	H	1,660	0
Ruminococcus albus strain 7	GL	648	0
Sorangium cellulosum	GL	680	2
Cellulomonas sp.	GL	480	0
Clostridium thermocellum	GL	630	0
Trichoderma viride (Miles Laboratory)	H	1,600	0
Aspergillus niger	CP	1,470	1
Rhizopus	CP	250	3
Trichoderma viride QM 9123	CP	560	2
Basidiomycete QM 806	CP	670	0
Chrysosporium pruinosum QM 826	CP	792	3
Streptomyces sp. B 814	CP	756	0
Myrothecium verrucaria QM 460	CP	1,750	0
Isolate A	GL	8	1,700
Isolate B	GL	226	112

[a] Enzymes were dissolved in water and used in the standard assay for β-1,4-glucanase and chitosanase activity.

[b] H, Homogeneous; GL, growth liquor; CP, crude preparation.

TABLE 4. *Lysis of fungal cell walls by myxobacter enzyme*

(Organism)	Reducing sugar (nmol) released per milligram of cell wall					
	Myxobacter enzyme		Myxobacter growth liquor		*T. viride* Cellulase	
	4 h	24 h	4 h	24 h	4 h	24 h
Rhizopus oligosporous NRRL 2710	234	344	161	165	0	0
Rhizopus rhizopodiformis QM 9395 ...	11	11	52	54	0	0
Geotrichium candidum	3	3	112	122	0	0
Trichoderma viride QM 9123	4	19	34	53	0	0
Aspergillus niger	24	63	25	71	0	0
Penicillium digitatum NRRL 1202	0	0	0	0	0	0
Alternaria sp	0	0	206	965	0	0

these three enzyme fractions (27). The myxobacter enzyme was found to be capable of attacking some fungal cell walls to release glucosamine. The enzyme may be useful as a tool for the classification of fungi and for the characterization of fungal cell walls.

No name has been given to the enzyme studied. It is the first enzyme with both β-1,4-glucanase and chitosanase activities to be purified to homogeneity. It is also the first enzyme with chitosanase activity to be purified to homogeneity. Growth liquors with both β-1,4-glucanase and chitosanase activities do exist. In addition to isolate B reported here, D. E. Eveleigh and R. L. Monaghan (personal communication) report that several of their chitosanase-producing organisms also produce β-1,4-glucanase activity. Until more enzymes are purified, it will not be known if the enzyme reported here is one of a kind or one of a class of enzymes having both β-1,4-glucanase and chitosanase activities.

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