JOURNAL OF BACTERIOLOGY, Dec. 1975, p. 1574-1585

Vol. 124, No. 3
Printed in U.S.A.

# Production, Purification, and Characterization of an Extracellular Chitosanase from *Streptomyces*

J. S. PRICE[1] AND R. STORCK*
*Department of Biology, Rice University, Houston, Texas 77001*

Received for publication 18 September 1975

The synthesis by *Streptomyces* sp. no. 6 of an extracellular chitosanase was induced by glucosamine. The enzyme was purified to homogeneity by Sephadex G-100, carboxymethyl-cellulose, and diethylaminoethyl-cellulose chromatography. The purified enzyme hydrolyzed chitosan (the β-1,4-linked polymer of glucosamine) but not chitin nor carboxymethyl-cellulose. The only products of the hydrolysis detectable by paper chromatography were di- and triglucosamine. Sephadex G-100 chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that the molecular weight of the enzyme was between 29,000 and 26,000. Acid hydrolysates of the enzyme contained no cysteic acid or glucosamine or other carbohydrate. At 25 C, maximum activity was obtained between pH 4.5 and 6.5. The enzymatic hydrolysis of chitosan occurred over a wide range of temperatures and was maximal at 60 C. The rate of the reaction was inhibited by concentrations of soluble chitosan higher than 0.5 g/liter. The apparent $K_m$ calculated from a Lineweaver-Burke plot was 0.688 g/liter at pH 5.5. The enzyme prevented spore germination and caused a significant decrease in the turbidity of germinated spore suspensions of the *Mucor* strains tested. Such a decrease was the result of a partial lysis of the cell wall.

Chitosan is a β-1,4-linked glucosamine polymer which, unlike chitin, contains few, if any, *N*-acetyl residues. It was discovered in 1954 in the cell wall of *Phycomyces blakesleeanus* (17) and has since been found in other fungi, but so far only in those which, like *P. blakesleeanus*, belong to the order *Mucorales* (3). In *Mucor rouxii*, chitosan accounts for as much as 30% of the dry weight of the cell wall (4).

An unidentified *Streptomyces* species, known as no. 6, was shown, a few years ago, to be able to grow in a medium in which the sole carbon source was the cell wall of *Mucor rammanianus* or *Mucor plumbeus*. Cell-free filtrates of cultures several days old solubilized as much as 80% of the wall of these two organisms after about a week of incubation. The *Streptomyces* could also be grown at the expense of the cell wall of fungi unrelated to *Mucorales*. In that case, however, cell-free filtrates had no lytic activity toward the wall of the two mucors (15). A specific response to chitosan-containing walls was also observed with *Mortierella vinacea* (25), *M. rouxii* (26), and *Rhizopus rhizopodiformis* (22). Hexosamine, but not *N*-acetylglucosamine, was found among the products of the enzymatic hydrolysis of *M. rouxii* wall. Furthermore, chitosan, but not chitin, was attacked by the cell-free filtrates. These observations suggested that the *Streptomyces* responded to the presence of *Mucor* cell wall by producing chitosanase, a novel type of enzyme (26). The presence of chitosanase activity in cultures of a variety of fungi and bacteria has since been reported (13, 22, 31). In the case of a *Myxobacter* strain, an enzyme with both chitosanase and β-1,4-glucanase activities has been purified to homogeneity and characterized (13).

The work described in the present paper deals with the chitosanase of the *Streptomyces* species. We show that, if glucosamine rather than cell wall or chitosan was used as an inducer, maximum enzymatic activity was reached after a few hours instead of days of incubation. The chitosanase was purified to homogeneity. The enzyme was found to be stable, free of cysteine and carbohydrates, and specific for chitosan. The products of its activity detectable by paper chromatography were di- and trisaccharides, indicating that the chitosanase is an endoglycosidase. The enzyme caused considerable damage to the cell wall of the *Mucor* strains tested.

## MATERIALS AND METHODS

**Organisms.** The strain *Streptomyces*, originally isolated by Jones and Webley (16), was sent to us by

[1] Present address: Biology Department, Battelle-Pacific Northwest Laboratories, Richland, Wash. 99352.

J. Ruíz-Herrera (Instituto Politécnico Nacional, México City, México). Some *Mucor* strains originated from the collection of the Northern Regional Research Laboratory (NRRL), Peoria, Ill., through the courtesy of C. W. Hesseltine; the others were purchased from the American Type Culture Collection (ATCC), Rockville, Md.

**Mucor cultures.** The strains were maintained on tomato juice agar. Spores were collected aseptically as described elsewhere (12) and stored in sterile, distilled water at 4 C. They were counted with a standard hemacytometer. Germlings were produced at 30 C, under forced aeration, in a medium containing 0.3% yeast extract, 1.0% peptone, and 2.0% glucose (YPG).

**Streptomyces cultures.** The organism was maintained on 5-ml slants of the following medium: 1.0% glucose, 0.2% yeast extract, and 2% agar. Spores were collected in 5 ml of a sterile 0.1% aqueous solution of Tween 80 (E. H. Sargent and Co.) by scraping the surface of the medium with an inoculating loop. They were dispersed by vigorous shaking, and the suspension was used to inoculate liquid cultures, which were made in YPG medium buffered at pH 7.2 to 7.3 with $5 \times 10^{-3}$ M potassium phosphate (YPGS). Solutions of yeast extract plus peptone (adjusted to pH 7.0 with KOH) of glucose, and of phosphate buffer were autoclaved separately and then combined aseptically. The organism was grown under forced aeration at 28 C. Growth was followed by measuring turbidity with a no. 66 filter in the Klett-Summerson colorimeter and is expressed in Klett units. For large-scale enzyme production (see Results), a starter culture of 100 ml was inoculated with 5 ml of spore suspension. When its turbidity had reached 100 Klett units it was transferred to 4 liters of medium distributed into eight baffled 2-liter Erlenmeyer flasks. After about 6 h of growth corresponding to 100 Klett units, the contents of the eight flasks were transferred to 100 liters of medium contained in a New Brunswick Scientific Co. fermenter. When the turbidity had reached 150 Klett units (after about 7 h), the cells were harvested with a Carl-Padberg type 41G CEPA continuous-flow centrifuge.

**Preparation of cell-free solutions.** In some experiments, growth was also followed by dry-weight measurements. Samples of cell suspensions were removed periodically and filtered on Bac-T-Flex membrane filters, type B-6 (Schleicher and Schuell Co.). Dry weights were determined after incubation of the prepared filter disks at 100 C for 12 to 24 h. The cell-free filtrates were collected and used for measuring extracellular enzyme activity and protein. When necessary, they were dialyzed prior to assay.

**Purification and deacetylation of commercial chitosan.** Partially acetylated chitosan and deacetylated chitosan were used to assay for chitosanase activity. The first was a commercial product derived from chitin and had an estimated acetyl content of about 30% (Pfanstiehl, lot no. 5900). It was dissolved in 0.05 M maleic acid to a concentration of 2 mg/ml. Insoluble material was eliminated by centrifugation at $10,000 \times g$ for 10 min. The solution was dialyzed for 24 h at 25 C against 20 volumes of 0.05 M maleic acid to remove dialyzable reducing sugars. After dialysis, trace amounts of remaining insoluble materials were removed by centrifugation as above. The supernatant fraction was a colorless, viscous solution, which remained free of bacterial contamination during storage at 4 C for at least 6 months and storage at room temperature for at least 12 h. With Ludowieg and Dorfman's procedure (20), using ethylacetate as standard, this purified chitosan preparation was found to have an acetyl content of 22%. The final concentration of chitosan was determined by dry weight.

Purified, commercial, partially acetylated chitosan was deacetylated by the NaOH procedure described by Horton and Lineback (14) to yield a product with an acetyl content, determined by the same method as above, of less than 1%.

**Assays of chitosanase activity on partially acetylated chitosan.** Two types of assays were used. In the first type, the pH of the solution was adjusted to 7.2 by adding KOH. At such a pH a colloidal suspension of chitosan was obtained. A suitably diluted enzyme preparation was added to 0.5 mg of colloidal chitosan to a final volume of 1 ml. The decrease in turbidity was measured at 25 C and 520 nm in a recording spectrophotometer (Gilford model 2000). A unit of activity was defined as a decrease in absorbance of 1.000/min measured during the first 1-min period after addition of the enzyme. This assay was used (Fig. 1) to test cell-free filtrates during enzyme production. In the second type, the pH of the solution was adjusted to 6.0 by adding KOH. At such a pH, chitosan was soluble. The reaction mixture contained 0.5 mg of soluble chitosan per ml and a suitably diluted enzyme preparation in a final volume of 2.0 ml. The mixture was incubated at 25 C for 15 min. The reaction was stopped by the addition of 0.05 ml of 1 M KOH to precipitate chitosan. The mixture was incubated on ice for 30 min to complete the precipitation. The precipitate was eliminated by centrifugation at $5,000 \times g$ for 15 min. The supernatant fraction was assayed for hexosamine content by the indole method of Dische (6). (It should be noted that such a method does not distinguish between mono- and oligosaccharides.) A unit of chitosanase activity was defined as 1 $\mu$mol of hexosamine equivalent produced per min. This assay was used for testing the purification steps shown in Fig. 2, 3, and 4 and solutions 4, 5, 6, and 7 of Table 1. It was also used for testing solutions such as no. 1, 2, and 3 (Table 1), which had not undergone Sephadex G-100 chromatography. In that case, however, the assays were preceded by a 24-h dialysis of the enzyme preparations at 4 C against 0.01 M potassium maleate (pH 6.5) to eliminate contaminating hexosamines.

**Assay of chitosanase activity on deacetylated chitosan.** Deacetylated chitosan was dissolved in 0.01 M potassium maleate at pH 6.0. The reaction mixture contained 0.5 mg of chitosan per ml and a suitably diluted enzyme preparation, in a final volume of 2.0 ml. The mixture was incubated at 25 C for 5 min. The reaction was stopped by the addition of Nelson's alkaline copper reagent (23). The amount of reducing sugar resulting from the enzymatic hydrolysis was determined by the arsenomolybdate method of Nelson (23), using glucosamine-hydrochloride (Sigma) as

standard. In such an assay a unit of chitosanase activity was defined as 1 μmol of reducing sugar equivalent produced per min. This type of assay was used only for the characterization of the purified enzyme (Fig. 7-9).

**Protein determination.** In the cell-free filtrates protein was determined by the procedure of Lowry et al. (19), and in concentrated enzyme preparations it was determined by the biuret method (18), using in both cases crystalline α-chymotrypsinogen A (Worthington) as the standard.

**Materials used for concentration and purification of chitosanase.** The materials used for these processes (to be described in Results) were as follows: Amicon hollow-fiber cartridge no. H1DP10 (Amicon Corp.), polyethylene glycol (Carbowax) (molecular weight, 20,000; Fisher Scientific Co.), Spectrapor 1 dialysis tubing (Spectrum Medical Industries, Inc.), Sephadex G-100 (Pharmacia Fine Chemicals, Inc.), advanced microgranular ion exchange celluloses; carboxymethyl (CM)-cellulose and diethylaminoethyl-cellulose (Reeve Angel Co.).

**Analysis of the products of hydrolysis of chitosan by chitosanase.** To determine the nature and the degree of polymerization of the products of enzymatic hydrolysis of chitosan, a number of methods were used. A search for free glucosamine was made with the procedure described by Strominger et al. (28). In addition, hydrolysates were treated with trimethylsilylating reagents (Pierce Chemical Co.) and analyzed by gas-liquid chromatography according to Sweeley et al. (29), using D-glucosamine as standard. Hydrolysates supplemented with glucosamine were used as controls. Paper chromatography of concentrated hydrolysates was performed on either Whatman no. 1 or 3 MM paper according to Nelson et al. (24), using the butanol-pyridine-water (6:4:3) solvent system of Distler and Roseman (7). The carbohydrate spots were developed by the silver nitrate method (30). The standards used were D-glucosamine hydrochloride, chitobiose hydrochloride (acetyl free), and tri-*N*-acetylchitotriose. The latter two were a gift from Saul Roseman.

**Preparation of chitin for the determination of enzyme specificity.** A 100-mg amount of chitin (Calbiochem) was dissolved in cold concentrated HCl (5 mg/ml) and, after 3 h of incubation, sedimented by centrifugation at 5,000 × *g*. The clear supernatant fraction was neutralized with 10 M NaOH. The resulting white precipitate was washed five times with 20-ml volumes of distilled water by centrifugations at 10,000 × *g*. The pellet from each centrifugation was resuspended each time by a 10-s pulse of sonication with a Bronson sonifier, model W185. The salt-free precipitate was further washed with five 25-ml volumes of 0.05 M potassium maleate, pH 6.3, and then resuspended in 15 ml of that buffer by a brief pulse of sonication. The reducing sugar content of the chitin was negligible. The chitin solution was stored at 4 C. The final concentration was determined by dry weight.

**Physicochemical characterization of purified chitonase.** The purity of the enzyme was examined by electrophoresis in 7.5% acrylamide gels as described by Gabriel (10) and also by electrophoresis in sodium dodecyl sulfate (SDS)-12.0% acrylamide gels as described by Weber and Osborn (32), using in both cases a tris(hydroxymethyl)aminomethane (Tris)-glycine buffer at pH 8.9. The molecular weight of the enzyme was estimated by Sephadex G-100 chromatography (1) and SDS-acrylamide gel electrophoresis (32).

For determination of amino acid composition, hydrolysis of 1 mg of enzyme was carried out in duplicate for 20 h at 110 C in 2 ml of 6 M HCl. Hydrolysates were cooled and carefully evaporated to dryness under vacuum in a rotary evaporator. The dry residue was dissolved in 1 ml of 0.2 M citrate buffer (Beckman Co.) at pH 2.2. Aliquots of 0.2 ml were applied to each column of a Beckman model 120 amino acid analyzer. The results were highly reproducible. Therefore, to increase the resolution of amino acids present in low amount, 1 ml of the combined hydrolysates was applied to the "acidics" column. Tryptophan content was estimated by the method of Goodwin and Morton (11).

**Lysis of germinating spore suspensions.** Spores were germinated under the conditions described above. After various periods of incubation, the germlings were sedimented by centrifugation and resuspended in 0.01 M potassium phosphate buffer, pH 7.0. The suspensions, adjusted to given densities, were supplemented with a given volume of chitosanase solution. The decrease in turbidity was followed in a Klett-Summerson colorimeter using a no. 57 filter.

## RESULTS

**Induction of enzyme production.** In the absence of *Mucor* cell wall or chitosan, the *Streptomyces* grew rapidly in YPGS medium and remained viable during the stationary phase of growth, but did not produce chitosanase. If, however, that medium with or without glucose (YPS) was supplemented with glucosamine, extracellular chitosanase activity became detectable at the end of the exponential phase and accumulated rapidly during the stationary phase of growth. Intracellular chitosanase activity also became detectable at the end of the exponential growth phase, but reached a maximum that was only about 1% of that of the total of intracellular plus extracellular activity. Glucosamine was also effective as an inducer if added to glucose-grown cells that had been centrifuged, washed, and resuspended in 0.01 M potassium phosphate buffer at pH 7.0. Under such conditions, the amount of enzyme produced increased with increasing cell densities. Galactosamine had a slight inductive capacity, whereas mannosamine, *N*-acetylglucosamine, and glucose had none. If a mixture of glucosamine glucose was added, the amount of activity detectable in the cell-free filtrates was either small or nil, depending on the relative concen-

tration of the two sugars. Finally, addition of chloramphenicol at 100 μg/ml prevented enzyme induction by glucosamine. Various glucosamine concentrations were tried. A molarity of 0.05 was found to be optimal, whereas molarities greater than 0.1 were found to be inhibitory. In light of the results of these exploratory experiments, a procedure was developed for large-scale chitosanase production.

**Large-scale enzyme production.** The *Streptomyces* cells grown and harvested as described in Materials and Methods were suspended in a volume of glucosamine-containing medium such as to have a cell density 12.5-fold higher than in the culture at the time of harvest. The cell suspension was incubated for 6 h, at 25 C, and under forced aeration. The dry weight of the cell suspension and the protein content and enzyme activity of cell-free filtrates were determined hourly. The dry weight remained constant. The enzymatic activity increased during the first 3 h and then tended to stabilize (Fig. 1). Protein content continued to slowly accumulate during the entire 6-h incubation period. The protein concentration remained, however, relatively low, the maximum being 70 μg/ml, which indicated that autolysis in the concentrated cell suspension was minimal.

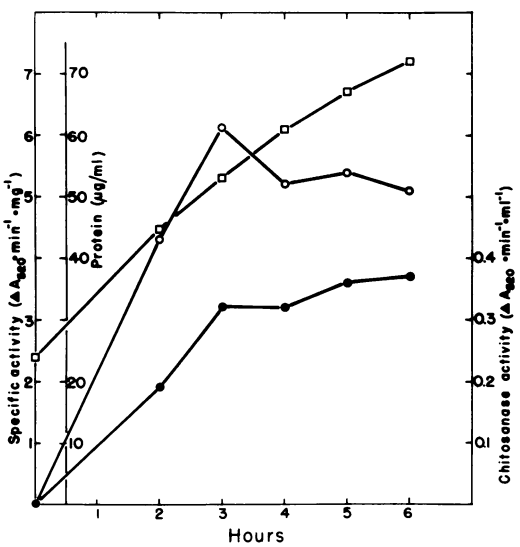


FIG. 1. *Extracellular accumulation at 25 C of protein* (□) *and chitosanase activity* (●) *in a concentrated suspension of resting cells of Streptomyces. Cells from a 100-liter culture in YPGS were suspended in 8 liters of a medium consisting of 0.05 M* D*-glucosamine-hydrochloride plus 0.1% yeast extract, pH 7.0, and incubated under forced aeration in a 14-liter fermenter. Protein was determined by the procedure of Lowry et al. (19). The enzyme activity was assayed turbidimetrically with colloidal, partially acetylated chitosan. Specific activity, units per milligram of protein* (○).

**Enzyme concentration.** After the 6-h incubation with glucosamine, the cell suspension was pelleted in the CEPA continuous-flow centrifuge. The supernatant fraction was collected, its pH was adjusted to 7.0, and its temperature was brought to 0 C. It was then clarified by one passage at 0 C through a hollow-fiber cartridge. The pale amber solution was concentrated 50-fold (Table 1) by a 72-h dialysis of 4 C against a total of 36 liters of 25% polyethylene glycol (PEG). Three dialysis tubes continuously fed at one end with filtrate were each immersed in 2 liters of PEG solution. Every 6 h the dialysis tubes were transferred to 2 liters of a fresh PEG solution.

**Enzyme purification.** The concentrate was dialyzed for 24 h against 2 liters of 0.01 M potassium maleate buffer, pH 6.5, and then lyophilized. The dry powder was dissolved in 17 ml of cold distilled water. The solution, chocolate brown in color, was applied to a G-100 Sephadex column. The peak of enzymatic activity coincided with a peak of absorbance at 280 nm (Fig. 2). The smallest absorbance peak eluting with the void volume of the column (fractions 32 to 40) contained no detectable enzyme activity. The largest absorbance peak eluting after fraction 80 was not due to protein but to "small" unidentified molecules, which were responsible for the chocolate brown color of the material applied to the column. (The high background equal to 0.5 units absorbance at 280 nm was due to the maleate buffer.)

Colorless fractions 58 to 73 (Fig. 2) were pooled and applied to a CM-cellulose column. Chitosanase activity eluted, between 0.08 and 0.1 M KCl, as a major sharp peak coincident with an equally sharp peak of absorbance at 280 nm (Fig. 3).

Fractions 65 to 69, eluted from the CM-cellulose column, were pooled and dialyzed overnight against 0.01 M Tris-hydrochloride buffer at pH 7.2. The enzyme solution was then applied to a diethylaminoethyl-cellulose column. The enzyme eluted again as a single peak of activity and of absorbance at 280 nm, between 0.08 and 0.1 M KCl (Fig. 4). Fractions 52 to 56 were pooled and used for further characterization of the chitosanase.

**Efficiency of the concentration and purification scheme.** Chitosanase activity, measured by the amount of soluble hexosamine released from soluble, partially acetylated chitosan, and protein concentration were determined after

TABLE 1. *Efficiency of the procedure for concentration and purification of chitosanase*

| Solution | Vol (ml) | Total enzyme (U) | Total protein (mg)[a] | Sp act (units/mg of protein) | Purification factor | Yield (%) |
|---|---|---|---|---|---|---|
| Culture supernatant[b] | 8,000 | 28,800 | 576 | 50 | 0 | 100 |
| Hollow-fiber filtrate | 7,830 | 28,188 | 564 | 50 | 0 | 98 |
| PEG concentrate | 145 | 14,355 | 288 | 50 | 0 | 50 |
| Lyophilization concentrate | 17 | 12,580 | 252 | 50 | 0 | 44 |
| Sephadex G-100 eluate | 80 | 12,960 | 171 | 76 | 1.5 | 45 |
| CM-cellulose eluate | 25 | 8,750 | 98 | 89 | 1.8 | 30 |
| Diethylaminoethyl-cellulose eluate | 25 | 8,025 | 76 | 105 | 2.1 | 28 |

[a] Protein in the first two solutions was assayed by the method of Lowry et al. (19) and by the biuret method (18) in the others.

[b] Protein and chitosanase were assayed for in cell-free filtrates.

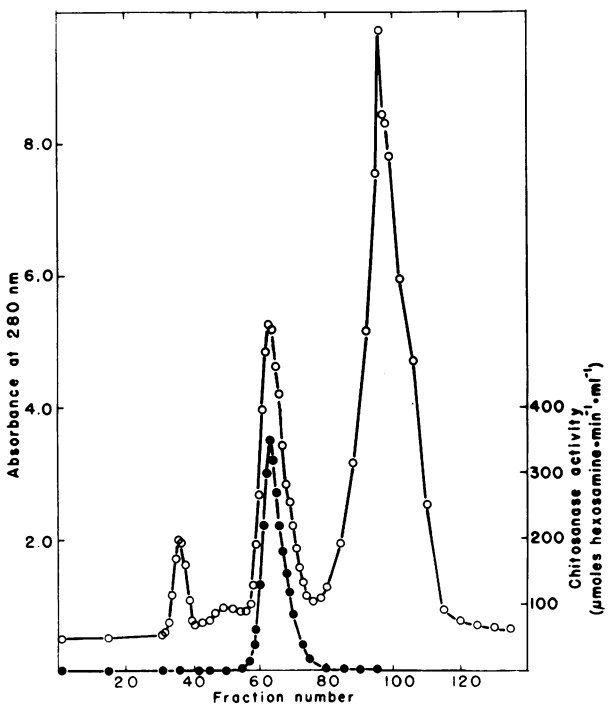


FIG. 2. *Chromatography of chitosanase on Sephadex G-100. The column (2.6 by 100 cm) was equilibrated with 0.01 M potassium maleate buffer, pH 6.5. The enzyme was eluted with the same buffer. Five-milliliter fractions were collected. Protein was estimated by absorbance at 280 nm* (○) *and enzyme activity was estimated by determination of hexosamine released from soluble, partially acetylated chitosan* (●).

each step. The values obtained are presented in Table 1. Two major features emerge from a comparison of these values. First, the greatest loss of enzyme occurred during the dialysis against 25% PEG, resulting in a final yield of recovery of 28%. Second, the final purification factor was 2.1. Specific activity of the purified enzyme, as determined by reducing sugar released from deacetylated chitosan in the standard assay at 25 C and pH 5.5, was 28 μmol/min per mg of enzyme.

**Molecular weight and chemical composition of the chitosanase.** The results of anionic and SDS-polyacrylamide disc gel electrophoresis of 150 μg of enzyme (applied to each gel) are shown in Fig. 5. In both the standard 7.5% acrylamide gel and in the 12.0% acrylamide-SDS gel, the enzyme formed a single band,

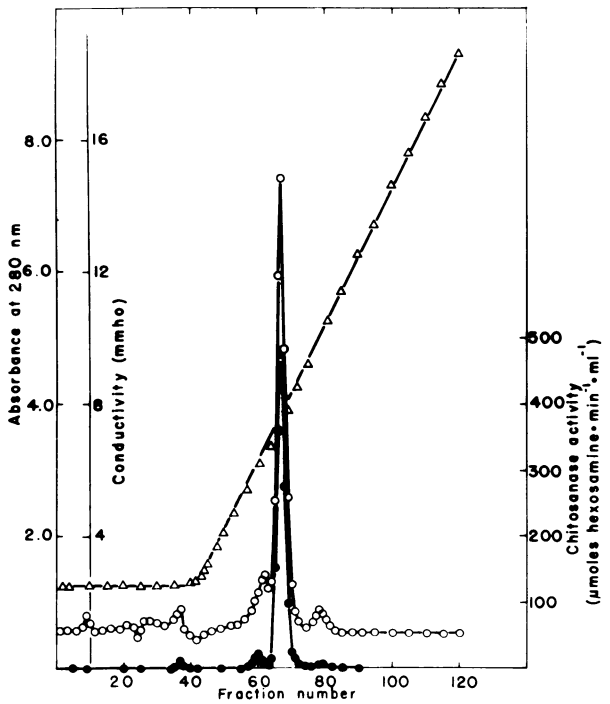


FIG. 3. *Chromatography of chitosanase on CM-cellulose. The column (2.5 by 10 cm) was equilibrated with 0.01 M potassium maleate buffer, pH 6.5. The enzyme was eluted in a linear KCl gradient; 5-ml fractions were collected. Protein was estimated by absorbance at 280 nm* (○), *enzyme activity by determination of hexosamine released from soluble, partially acetylated chitosan* (●), *and KCl concentration by conductivity* (△).

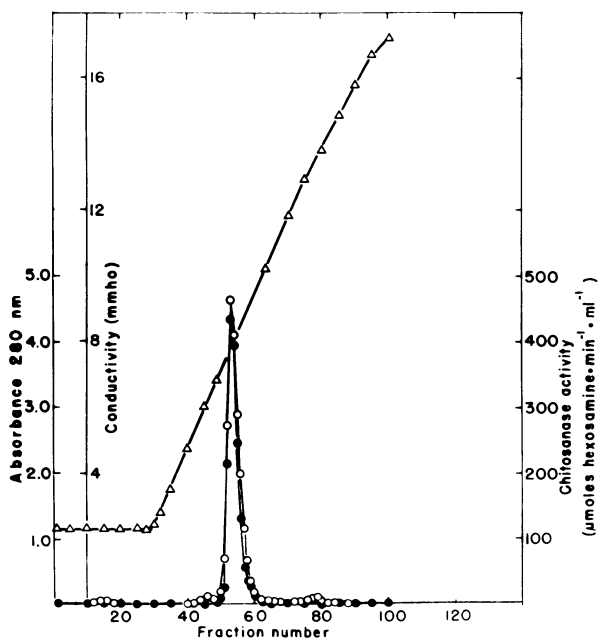


FIG. 4. *Chromatography of chitosanase on diethylaminoethyl-cellulose. The column (2.5 by 10 cm) was equilibrated with 0.01 M Tris-hydrochloride buffer, pH 7.2. The enzyme was eluted in a linear KCl gradient; 5-ml fractions were collected. Protein was estimated by absorbance at 280 nm* (○), *enzyme activity by determination of hexosamine released from soluble, partially acetylated chitosan* (●), *and KCl concentration by conductivity* (△).

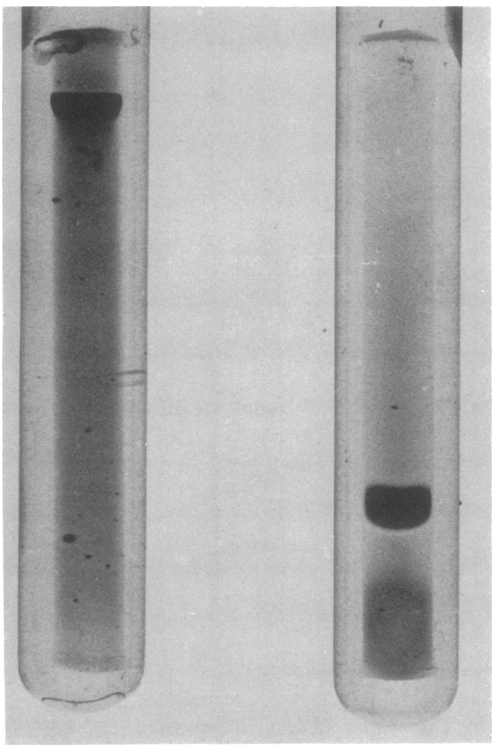

FIG. 5. *Polyacrylamide gel electrophoresis of purified chitosanase. On the left a 7.5% acrylamide gel was used at 2 mA and 4 C, and on the right a 12.0% acrylamide-SDS gel was used at 3 mA and 25 C. In both cases, 150 μg of enzyme was applied to the gel and electrophoresed for 2.5 h at pH 8.9 in a Tris-glycine buffer. The gels were fixed 1 h in 25% trichloroacetic acid at 25 C, stained 1 h at 37 C in a solution of 0.25% Coomassie blue R 250 in 45% methanol-10% acetic acid, and then destained in 5% methanol-7.5% acetic acid.*

indicating homogeneity. The high mobility of the protein in the SDS gel suggested a relatively low molecular weight. Estimation of the molecular weight by SDS-gel electrophoresis yielded a value of 26,000, and by Sephadex G-100 chromatography it yielded a value of 29,000 (Fig. 6). The mobility of chitosanase on the SDS gels was indistinguishable from that of α-chymotrypsinogen A, and the two proteins formed a single band (no broader than that obtained with either protein alone) when electrophoresed on the same gel.

The near-ultraviolet absorbance of the enzyme in 0.01 M Tris-hydrochloride buffer at pH 7.2 and containing 0.1 M KCl was recorded in a Cary 118B spectrophotometer. Maximum absorbance was at 278 nm. The extinction coefficient at 280 nm ($E^{280}_{1\%}$) was calculated to be 11.7. The spectrum was characteristic for protein and did not reveal the presence of contaminants.

Amino acid analysis of acid hydrolysates of the enzyme failed to indicate the presence of either half-cystine or cysteic acid in amounts of hydrolysates that would have permitted the detection of one residue per molecule. Glucosamine was also absent from the hydrolysates. In addition, no carbohydrate could be detected in polyacrylamide electrophoregrams of chitosanase stained with the periodic acid-Schiff reagent (33) or in 4 mg of chitosanase assayed by the anthrone procedure of Seifter et al. (27) and the phenol-sulfuric acid method of Dubois et al. (8). The amino acid composition of the enzyme is presented in Table 2. An inspection of that table reveals that, in addition to the absence of cysteine, the enzyme can also be characterized by a relatively high proportion of lysine and arginine residues. The amide content was not determined.

**Enzymatic properties of the chitosanase.** These properties were, as already indicated, determined by using the standard assay with deacetylated chitosan. The effect of pH on activity is shown in Fig. 7. The optimum extended from 4.5 to 6.5. As mentioned earlier, at pH values equal to or less than 6.5 the substrate was soluble. At high pH values it was colloidal. The possible primary or secondary effects that this change in the solubility of

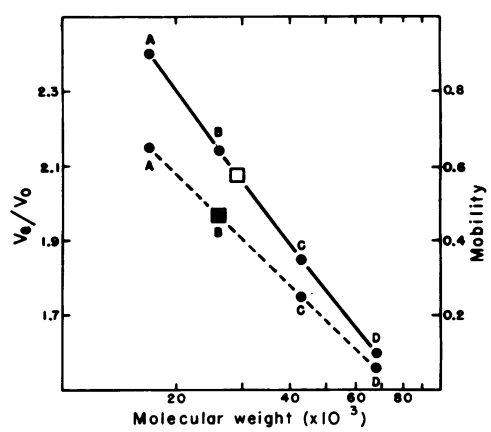


FIG. 6. *Estimation of the molecular weight of chitosanase. SDS-acrylamide (12.0%) gel electrophoresis was performed as described in Fig. 5 (●- - -●), and $V_e/V_o$, ratio of elution volume over void volume, was determined by Sephadex G-100 chromatography in a 2.5- by 45-cm column at 4 C in 0.01 M Tris-hydrochloride, pH 7.2, containing 0.02 M KCl (●——●). The position of chitosanase is indicated by a square. The markers and their respective molecular weights were: A, myoglobin (17,000); B, α-chymotrypsinogen A (26,000); C, ovalbumin (43,000); D, bovine serum albumin (68,000).*

chitosan might have on the reactivity of the chitosanase were not investigated. The effect of temperature was determined by incubating the enzyme with substrate for 5 min at each of the temperatures indicated in Fig. 8. As can be seen, the Arrhenius plot is characterized by an extended linear portion, which is evidence for thermal stability. The maximal rate was observed at about 60 C. The activation energy for the hydrolysis of chitosan, calculated from that plot, yielded a value of 10.4 kcal/mol.

TABLE 2. *Amino acid analysis of chitosanase*

| Amino acid | % Weight of protein | Calculated no. of residues per molecule[a] | Rounded no. of residues per molecule[a] |
|---|---|---|---|
| Lysine | 8.99 | 20.34 | 20 |
| Histidine | 2.34 | 4.95 | 5 |
| Arginine | 5.05 | 9.37 | 9 |
| Ammonia | 0.59 | | |
| Tryptophan | 1.93 | 3.01 | 3 |
| Aspartic acid | 12.56 | 31.65 | 32 |
| Threonine | 4.31 | 12.38 | 12 |
| Serine | 4.57 | 15.21 | 15 |
| Glutamic acid | 9.84 | 22.10 | 22 |
| Proline | 3.14 | 9.37 | 9 |
| Glycine | 4.52 | 22.99 | 23 |
| Alanine | 6.85 | 27.94 | 28 |
| Valine | 4.11 | 12.02 | 12 |
| Methionine | 2.24 | 4.95 | 5 |
| Isoleucine | 2.83 | 7.25 | 7 |
| Leucine | 7.38 | 18.92 | 19 |
| Tyrosine | 6.47 | 11.49 | 11 |
| Phenylalanine | 4.76 | 9.37 | 9 |
| Cysteine | 0.00 | 0.00 | 0 |

[a] Assuming a molecular weight of 29,000.

The rate of chitosanase activity was proportional to chitosan concentration up to about 0.3 mg/ml and reached a maximum between 0.4 and 0.5 mg/ml (Fig. 9). At higher concentrations there was a marked rate decrease which stopped at 2.0 mg/ml. It was found that the reducing capacity of glucosamine in the arsenomolybdate assay (23) was decreased in the presence of more than 0.5 mg of chitosan per ml and in the absence of enzyme. The results of enzymatic assays performed at such concentrations were therefore corrected for this effect. The rate inhibition shown in Fig. 9 may thus represent unspecific binding of enzyme to substrate or perhaps even product inhibition. From a Lineweaver-Burke plot of the values shown in Fig. 9, an apparent $K_m$ of 0.668 mg/ml at pH 5.5 was calculated.

Chitin (1.0 mg/ml) was readily hydrolyzed by commerical chitinase (Calbiochem) at 2 mg/ml after a few minutes of incubation at pH 7.0 and 25 C. In contradistinction, it was not hydrolyzed by purified chitosanase even after a 2-h incubation at 37 C. Like chitin, CM-cellulose, when tested under conditions identical to those described by Hedges and Wolfe (13), was also resistant to the *Streptomyces* sp. chitosanase.

No free glucosamine could be detected in enzymatic hydrolysates of chitosan, either colorimetrically or by the use of gas chromatography. This conclusion was reinforced by the

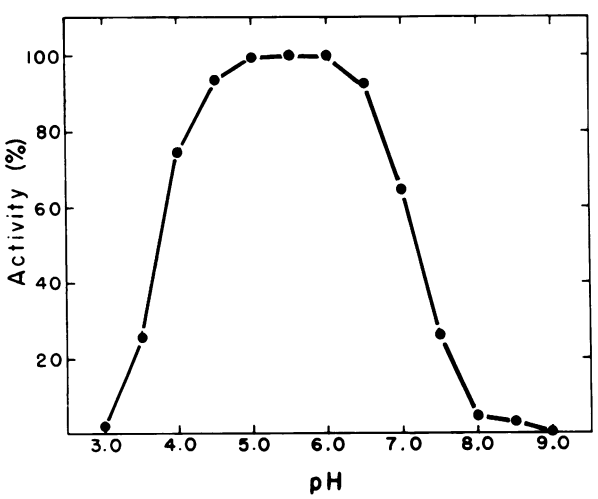


FIG. 7. *Chitosanase activity as a function of pH. The concentration of deacetylated chitosan was 0.5 mg/ml. The buffer used was composed of 0.02 M succinic acid, 0.02 M maleic acid, and 0.02 M Tris-hydrochloride. The pH values shown were obtained by adding the necessary amounts of KOH. The enzyme activity was measured by determination of reducing sugar released after 5 min of incubation of chitosanase with soluble deacetylated chitosan.*

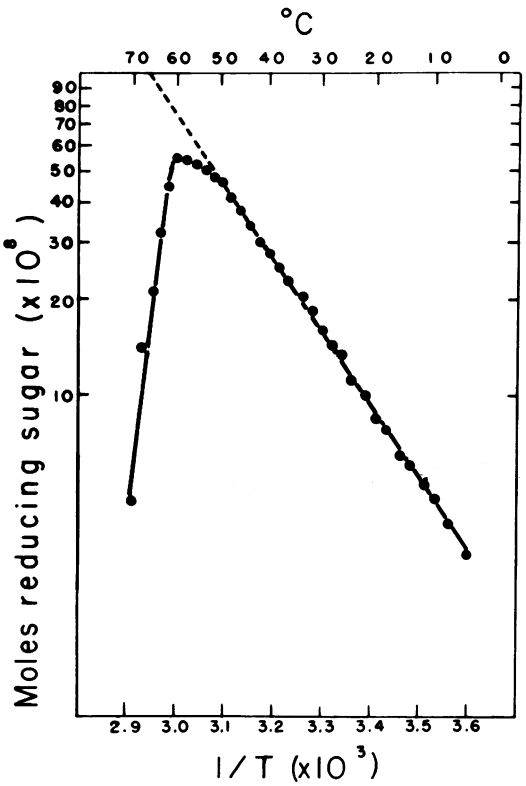


FIG. 8. *Chitosanase activity as a function of temperature plotted according to Arrhenius. The enzyme activity was measured by determination of reducing sugars released after 5 min of incubation of chitosanase with soluble deacetylated chitosan at each of the temperatures shown.*

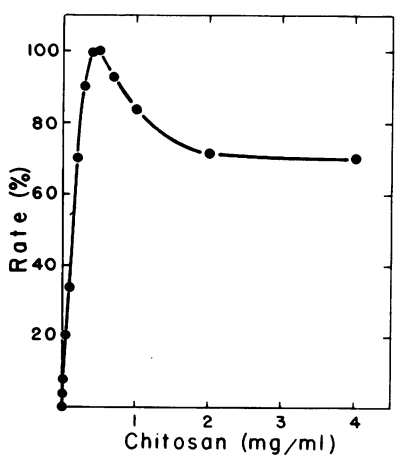


FIG. 9. *Effect of substrate concentration on the rate of reaction of chitosanase. The enzyme activity was measured by determination of reducing sugars released after 5 min of incubation of chitosanase with soluble deacetylated chitosan at each of the concentrations shown.*

direct visualization of the hydrolytic products by paper chromatography. The only migrating components were the di- and trisaccharides (Fig. 10). It follows that the *Streptomyces* sp. chitosanase is more than likely an endoglycosidase, which is inactive on the glucosamine trisaccharide.

Addition of pure chitosanase to suspensions of germinating spores of several *Mucor* strains resulted in a rapid decrease in turbidity (Fig. 11). As exemplified by the case of *Mucor mucedo* (ATCC 7941), such a decrease in turbidity was caused by microscopically visible damage to the cell wall (Fig. 12).

## DISCUSSION

The rate and extent of chitosanase accumulation in *Streptomyces* cultures with isolated

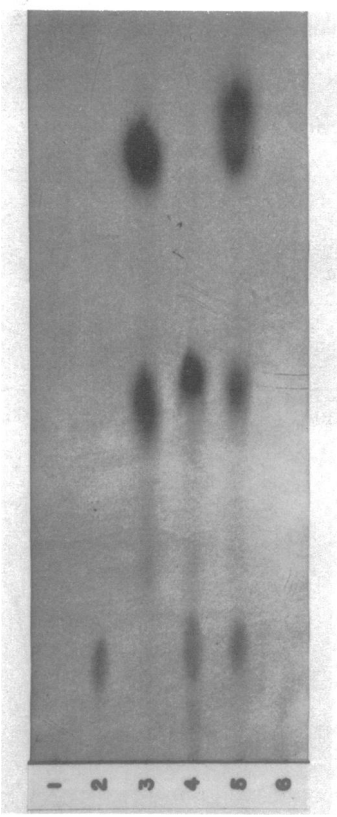

FIG. 10. *Paper chromatograms (50 h) of the products of hydrolysis after 14-h incubation at 25 C of chitosanase with soluble deacetylated chitosan. The substances spotted at the origin of each lane were as follows: 65 μg of chitosan (lanes 1 and 6), 10 μg of chitin trisaccharide (lane 2), 10 μg of glucosamine plus 20 μg of chitobiose (lane 3), 50 μg of enzymatically hydrolyzed chitosan (lane 4), 65 μg of chitosan, plus 10 μg of glucosamine, plus 20 μg of chitobiose, plus 10 μg of chitin trisaccharide (lane 5).*

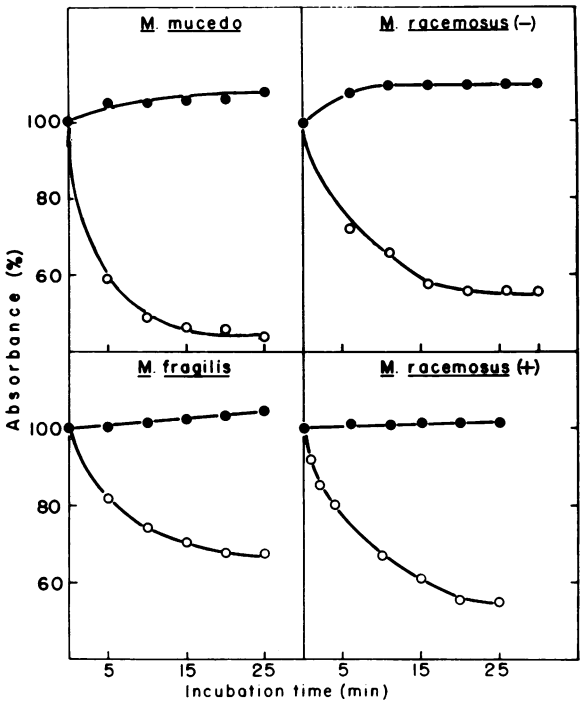


FIG. 11. *Lysis of germinated spores of Mucor strains in 0.01 M potassium phosphate buffer, pH 7.0, at 30 C. Chitosanase was added at 0 time. The decrease in turbidity* (○) *was measured in a Klett-Summerson colorimeter with a no. 57 filter. The respective concentrations of enzyme protein and densities of spores were as follows: M. mucedo (ATCC 7941), 6 μg/ml and 3.5 × 10⁶/ml; M. racemosus − (NRRL 1428), 24 μg/ml and 6.7 × 10⁵/ml; M. racemosus + (NRRL 1427), 30 μg/ml and 8 × 10⁶/ml; M. fragilis (ATCC 10777), 1.5 μg/ml and 5 × 10⁶/ml. The controls* (●) *were identical suspensions without chitosanase.*

chitosan or *Mucor* cell wall were too small for large-scale production. It was therefore desirable to find an inducer other than chitosan or cell wall. Ideally, an inducer should have been soluble and active even in the absence of growth, although not necessarily a substrate for chitosanase. As we have shown, glucosamine turned out to be such an inducer. The choice of glucosamine was based on the assumption that it was a product of the chitosanase activity and therefore endowed with inducing capacity, as are the products of other inducible polysaccharidases (9, 21). As we have demonstrated, glucosamine is not a product. Neither is it a substrate. These findings raise interesting questions. Thus, one may ask if other molecules, for example, glucosamine di- and trisaccharides, could substitute for glucosamine. Also, what mechanism is involved in the induction process itself? Several models come to mind, but in view of the absence of experimental results their presentation here is not warranted. What ought to be mentioned, however, is that during the period of time preceding the appearance of extracellular chitosanase activity, there was no detectable intracellular chitosanase activity. That, together with the observed inhibitory effects of glucose and chloramphenicol, suggests that there was no intracellular accumulation of active enzyme, followed by its extracellular secretion, but rather a de novo protein synthesis.

The specificity of the response to glucosamine was remarkable in the sense that during the active phase of its production chitosanase accounted for most of the protein that accumulated extracellularly. Such a minimal extracellular protein concentration also indicated that the extent of autolysis in the concentrated cell suspension was negligible.

Various procedures for concentration of the enzyme were tried. These included ultrafiltration and ammonium sulfate precipitation, which has been used by others in preliminary purification attempts (22, 26), and precipitation with organic solvents (acetone and ethanol). None of these procedures was as efficient as dialysis against PEG. The use of PEG was not ideal, however, since it resulted in a significant loss of protein and enzymatic activity (Table 1).

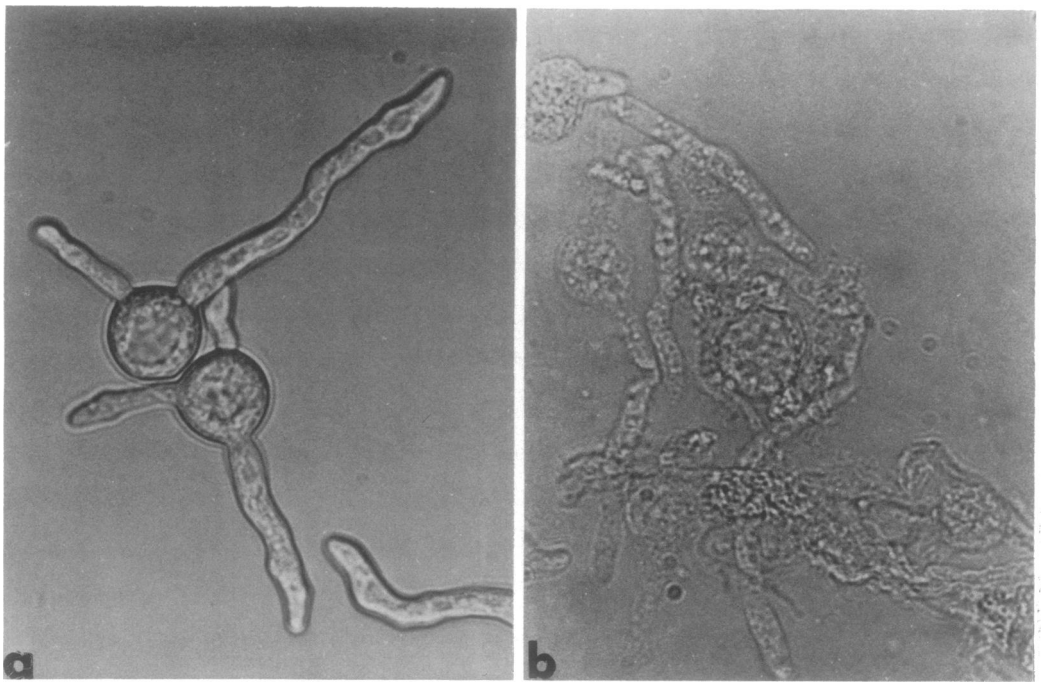


FIG. 12. *Germinated spores of M. mucedo (ATCC 7941) after incubation for 25 min in 0.01 M. potassium phosphate buffer (pH 7.0) in the absence (a) and presence (b) of 6 μg of chitosanase per ml.*

The purification scheme which was followed could probably be improved. It permitted, nevertheless, and for the first time, to purify to homogeneity an enzyme with activity restricted to the hydrolysis of chitosan.

The *Streptomyces* sp. enzyme, unlike that from *Myxobacter* AL 1 (13), had no β-1,4-glucanase activity. In addition, unlike for the *Myxobacter* enzyme, di- and trisaccharides rather than glucosamine were the products of chitosan hydrolysis. Other properties permit a further distinction between these two chitosanases. Thus, that from the *Myxobacter* had a temperature optimum of 70 C and two pH optima (5.0 and 6.8) versus 50 C and a single pH optimum (4.5 to 6.5), respectively, for that from the *Streptomyces*. Unlike the chitosanase described in the present paper, that from the *Myxobacter* contained 1.3% carbohydrate and an estimated three cysteine residues per molecule of enzyme. The proportions of several other amino acids, notably arginine plus lysine and aspartic plus glutamic acids, were also significantly different.

The *Streptomyces* chitosanase has several properties which could make its use practical. It is stable for hours at room temperature and for months in the frozen and dried states. Enzyme powder is readily soluble in water and a variety of buffers. The enzyme is active over wide ranges of pH and temperature. It could be used for taxonomic studies of fungi, and, in this respect, for diagnosis of some mycoses of plants and animals. It could also be used for the study of the structure and biosynthesis of the cell wall of *Mucorales*, not only by itself, but also in combination with other enzymes which degrade other important wall polymers, such as chitin (2). Worth mentioning in this respect is the observation that a commercial preparation of chitinase failed to cause a decrease in the turbidity of *Mucor* germling suspension. However, a mixture of chitinase and chitosanase caused a decrease in turbidity which was higher than that caused by chitosanase alone (Fig. 11). Finally, it can be used for the formation of protoplasts. It was found in this and another laboratory (5) that quantitative conversion of intact cells into protoplasts required, however, in apparent agreement with the observation described above, the presence of both enzymes.

The exact conditions required for optimum hydrolysis of *Mucor* cell wall components and for protoplasts formation are presently under investigation in this laboratory.

## ACKNOWLEDGMENTS

We thank A. D. Elbein (University of Texas Medical School in San Antonio) for his advice and for his hospitability. Amino acid analysis was performed in the laboratory of K. S. Matthews, near ultraviolet absorbance measurements in

that of F. B. Rudolph, and large-scale growth experiments in the laboratory of G. J. Schroepfer, Jr., of the Biochemistry Department. We also wish to express our gratitude to T. E. Nelson (Baylor College of Medicine) for advice, S. Roseman (Johns Hopkins University) for the gift of samples of chitobiose and chitin trisaccharide, and R. S. Wolfe (University of Illinois) for that of carboxymethyl-cellulose.

The work was supported in part by U. S. Public Health Service grants AI-07804 and AI-00106 from the National Institute of Allergy and Infectious Diseases.

## LITERATURE CITED

1. Andrews, P. 1965. The gel-filtration behaviour of proteins related to their molecular weight over a wide range. Biochem. J. **96**:595–606.
2. Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu. Rev. Microbiol. **22**:87–108.
3. Bartnicki-Garcia, S. 1970. Cell wall composition and other biochemical markers in fungal phylogeny, p. 81–103. *In* J. B. Harborne (ed.), Phytochemical phylogeny. Academic Press Inc., New York.
4. Bartnicki-Garcia, S., and W. J. Nickerson. 1962. Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii*. Biochim. Biophys. Acta **58**:102–119.
5. Binding, H., and H. J. Weber. 1974. Isolation, regeneration and fusion of *Phycomyces* protoplasts. Mol. Gen. Genet. **135**:273–276.
6. Dische, Z. 1955. New color reactions for determination of sugars in polysaccharides, p. 313–358. *In* D. Glick (ed.), Methods of biochemical analysis, vol. 2. Interscience Publishers, Inc., New York.
7. Distler, J., and S. Roseman. 1962. D-Glucosamine oligosaccharides, p. 303–305. *In* R. L. Whistler (ed.), Methods in carbohydrate chemistry, vol. 1. Academic Press Inc., New York.
8. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
9. Dubos, R. 1932. Factors affecting the yield of specific enzyme in cultures of the bacillus decomposing the capsular polysaccharide of type III *Pneumococcus*. J. Exp. Med. **55**:377–391.
10. Gabriel, O. 1971. Analytical disk gel electrophoresis, p. 565–578. *In* W. B. Jakoby (ed.), Methods in enzymology, vol. 22. Academic Press Inc., New York.
11. Goodwin, T. W., and R. A. Morton. 1946. The spectrophotometric determination of tyrosine and tryptophan in proteins. Biochem. J. **40**:628–632.
12. Haidle, C. W., and R. Storck. 1966. Control of dimorphism in *Mucor rouxii*. J. Bacteriol. **92**:1236–1244.
13. Hedges, A., and R. S. Wolfe. 1974. Extracellular enzyme from *Myxobacter* AL-1 that exhibits both $\beta$-1,4-glucanase and chitosanase activities. J. Bacteriol. **120**:844–853.
14. Horton, D., and D. R. Lineback. 1965. N-deacylation. Chitosan from chitin, p. 403–406. *In* R. L. Whistler (ed.), Methods in carbohydrate chemistry, vol. 5, General polysaccharides. Academic Press Inc., New York.
15. Jones, D., J. S. D. Bacon, V. C. Farmer, and D. M. Webley. 1968. Lysis of cell walls of *Mucor ramannianus* Möller by a *Streptomyces* sp. Antonie van Leeuwenhoek J. Microbiol. Serol. **34**:173–182.
16. Jones, D., and D. M. Webley. 1968. A new enrichment technique for studying lysis of fungal cell walls in soil. Plant Soil **28**:147–157.
17. Kreger, D. R. 1954. Observations on the wall of yeasts and some fungi by x-ray diffraction and solubility tests. Biochim. Biophys. Acta **13**:1–9.
18. Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins, p. 450–451. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press Inc., New York.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. Ludowieg, J., and A. Dorfman. 1960. A micromethod for the colorimetric determination of N-acetyl groups in acid mucopolysaccharides. Biochim. Biophys. Acta **38**:212–218.
21. Markovitz, A., and H. P. Klein. 1955. Some aspects of the induced biosynthesis of $\alpha$-amylase of *Pseudomonas saccharophila*. J. Bacteriol. **70**:641–648.
22. Monaghan, R. L., D. E. Eveleigh, R. P. Tewari, and E. T. Reese. 1973. Chitosanase, a novel enzyme. Nature (London) New Biol. **245**:78–80.
23. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. **153**:375–380.
24. Nelson, T. E., J. V. Scaletti, F. Smith, and S. Kirkwood. 1963. The use of enzymes in structural studies on polysaccharides. I. The mode of attack of a $\beta$-D-(1-3)-glucanase on laminarin. Can. J. Chem. **41**:1671–1678.
25. Peberdy, J. F. 1971. Protoplasts from *Mortierella vinacea*. Trans. Br. Mycol. Soc. **56**:67–72.
26. Ramirez-Leon, I. F., and J. Ruiz-Herrera. 1972. Hydrolysis of walls and formation of spheroplasts in *Mucor rouxii*. J. Gen. Microbiol. **72**:281–290.
27. Seifter, S., S. Dayton, B. Novic, and E. Muntwyler. 1950. The estimation of glycogen with the anthrone reagent. Arch. Biochem. **25**:191–200.
28. Strominger, J. L., J. T. Park, and R. E. Thompson. 1959. Composition of the cell wall of *Staphylococcus aureus*: its relation to the mechanism of action of penicillin. J. Biol. Chem. **234**:3263–3268.
29. Sweeley, C. C., R. Bentley, M. Makita, and W. W. Wells. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Am. Chem. Soc. **85**:2497–2507.
30. Trevelyan, W. E., D. P. Proctor, and J. S. Harrison. 1950. Detection of sugars on paper chromatograms. Nature (London) **166**:444–445.
31. Tsujisaka, Y., Y. Tominaga, and M. Iwai. 1975. Purification and some properties of the lytic enzyme from *Bacillus* R-4 which acts on *Rhizopus* cell wall. Agric. Biol. Chem. **39**:145–152.
32. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
33. Zacharius, R. M., T. E. Zell, J. H. Morrison, and J. J. Woodlock. 1969. Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. **30**:148–152.