

Enzymes That Hydrolyze Fungal Cell Wall Polysaccharides

I. PURIFICATION AND PROPERTIES OF AN ENDO- α -D-(1 \rightarrow 3)-GLUCANASE FROM *TRICHODERMA VIRIDE**

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SUMMARY

An endo- α -D-(1 \rightarrow 3)-glucanase capable of hydrolyzing various α -(1 \rightarrow 3)-glucans has been isolated and purified from the culture filtrate of the cellulolytic fungus *Trichoderma viride*, and its specificity has been examined. Of the compounds tested only those with α -(1 \rightarrow 3)-glucosidic linkages were attacked, and the enzymatic hydrolysis occurred with retention of configuration of the anomeric carbon atom involved in cleavage.

Some properties of the enzyme have been investigated. Optimum pH and temperature for activity are 4.5 and 50°, respectively. The values of K_m and V_{max} under standard assay conditions are 4.6×10^{-2} M glucose equivalents and 0.16 μ mole of glucose equivalent per min. The molecular weight of the enzyme estimated by column chromatography on Sephadex G-100 was found to be approximately 47,000. Zn^{++} and Fe^{++} were found to be reversible inhibitors of the enzyme while Ag^+ and Hg^+ abolished activity irreversibly.

The use of this enzyme in structural carbohydrate chemistry is discussed.

the structures of bacterial cell wall components (1, 2) and glycoproteins (3, 4).

There have been a few reports describing the presence of crude enzyme activities capable of hydrolyzing α -(1 \rightarrow 3)-glucosidic linkages in higher plants and seaweeds; however, no enzyme specific for this linkage has been reported. Peat and Reese (5) prepared a crude enzyme from *Porphyra umbilicalis* which hydrolyzed nigerose but was equally active on sucrose and maltose. A rather nonspecific nigerase activity has also been described in extracts of alfalfa, protozoa, and yeast (6). An activity in *Streptomyces* capable of hydrolysis of yeast α -(1 \rightarrow 3)-glucans has recently been reported (7).

Enzyme activity specific toward α -(1 \rightarrow 3)-glucosidic linkages was first observed by us in culture filtrates of *Trichoderma viride* (8). When grown in a medium containing the α -(1 \rightarrow 3)-glucan (pseudonigeran) isolated from *Aspergillus niger*, *T. viride* produces the enzyme in its culture fluid. Of a number of fungal strains tested this species showed the highest activity.

Polysaccharides which consist almost exclusively of α -(1 \rightarrow 3)-glucosidic linkages have only recently been reported but appear to be important in cell wall structure. Ralph and Bender (9) obtained a preparation from the walls of the basidiomycete *Polyporus tumulosus* which yielded an α -(1 \rightarrow 3)-glucan fraction. Similar polymers have now been isolated from *A. niger* (10), *Polyporus betulinus* (11), and, more recently, *Cryptococcus* and *Schizosaccharomyces* species of yeast (7). The present communication details the purification and properties of a highly specific α -(1 \rightarrow 3)-glucanase with activity toward a variety of glucose-containing oligo- and polysaccharides containing α -(1 \rightarrow 3)-linkages.

MATERIALS

A. niger NRRL 326 was obtained from the culture collection of the Northern Regional Research Laboratory, United States Department of Agriculture, Peoria, Illinois, and *T. viride* QM6A from the United States Army Natick Laboratories, Natick, Massachusetts. The exo- β -D-(1 \rightarrow 3)-glucanase was purified as previously described (12). *Polyporus tumulosus* glucan was a gift from Dr. B. J. Ralph. Other polysaccharides were available in our laboratory collection.

The biological functions of a great majority of polysaccharides, especially in the plant kingdom, are not understood. The key to much of the understanding of their roles lies in a knowledge of their chemical fine structure and the mechanisms of their synthesis and degradation. Studies of the structures of many polyglycans have been greatly facilitated by the use of enzymes with rather strict specificity that cleave glycosidic linkages. The use of enzymatic methods in structural analysis of complex polysaccharides has been of particular value in gaining an insight into

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Ion exchange resins IR120(H)⁺ and IR45(OH)⁻ were obtained from Malinckrodt. Sephadex gels and blue dextran were purchased from Pharmacia. Hexamethyldisilazane, trimethylchlorosilazine, crystalline pepsin, trypsin, and pancreatic ribonuclease were obtained from Nutritional Biochemicals. Bovine serum albumin was purchased from Sigma. Horseradish peroxidase and hemoglobin were obtained from Worthington and cytochrome *c* from Mann. Reagents for polyacrylamide gel electrophoresis were purchased from Distillation Products Industries Division of Eastman Kodak.

All other chemicals were reagent grade and carbohydrates used were of the D configuration.

Pseudonigeran was isolated from mycelia of *A. niger* NRRL 326, which was grown in submerged culture with a Micro-Ferm laboratory fermentor, model MF-107 (New Brunswick Scientific Company, New Brunswick, New Jersey). Growth medium consisted of the following in grams per 10 liters: glucose, 466; tartaric acid, 26.6; NH₄NO₃, 26.6; (NH₄)₂SO₄, 1.67; K₂CO₃, 4.66; MgCO₃, 2.66; (NH₄)₂HPO₄, 5.0; FeSO₄·7H₂O, 0.466; ZnSO₄·7H₂O, 0.466; and H₂O to 10 liters. The pH was adjusted to 5.0 with concentrated NH₄OH.

Two 150-ml portions of medium in 500-ml Erlenmeyer flasks were inoculated with *A. niger* spores and grown on a reciprocal shaker at 20–22° for 6 days. They were then used to inoculate sterile culture medium in the fermentor. This culture was grown with an aeration rate of 2 liters per min at 25° for 6 days. Agitation was regulated at 100 rpm. The amount of pseudonigeran in the cells was found to fluctuate with changes in culture conditions. One factor controlling α-(1 → 3)-glucan content in this particular strain of *A. niger* is the carbon source. Practically no pseudonigeran is present in cells grown in surface culture with either sucrose or glucose as carbon source. However, submerged culture favors the synthesis of pseudonigeran by the mycelia.

While the reasons for this are unclear, it may be related to the structural nature of the mycelium under two different environments. In addition to nutritional conditions, other factors seem to influence the formation of the polymer. Six-day-old mycelia generally contained the maximum amount of polysaccharide, and an initial culture medium pH of 5.0 and growth temperature of 25° resulted in the highest yield of the glucan.

Mycelia were harvested by filtering the culture through cheesecloth. The mycelia was then placed on a Buchner funnel, and excess water was removed by suction filtration. The cells (500 to 600 g) were suspended in 4 liters of water and filtered again. This was repeated until the washings became colorless. The washed cell mass was suspended in 4 liters of water and autoclaved at 15 pounds pressure for 20 min. The suspension was filtered through cheesecloth while hot, and the mycelia were again extracted with 4 liters of boiling water for 20 min and washed with 3 to 4 liters of boiling water until the washings became colorless. The washed cells were then cooled and suspended in 4 liters of 0.1 M sodium borohydride solution, and the mixture was allowed to stand for several hours with occasional stirring. Without adjustment of the pH of the suspension, it was made 1 N with respect to NaOH and heated on a steam bath for 3 hours. The alkaline suspension was filtered through cheesecloth, and the resulting brownish filtrate was cooled. Two volumes of methanol were added to the extract with vigorous stirring, and the mixture allowed to stand for 3 to 4 hours at room temperature. The precipitated polysaccharide was collected by centrifugation (1000 × *g*) and washed with a solution of meth-

anol and water (3:1, v/v) until the washings became free of color. The washed precipitate was dissolved in 1 liter of 1 N NaOH, and a small amount of insoluble material was removed by filtration through glass wool. The filtrate was treated with 2 volumes of methanol, and the resulting white precipitate was collected by centrifugation (1000 × *g*) and washed with methanol-water (3:1, v/v) three times. It was then suspended in a solution of methanol and 0.1 N acetic acid (3:1, v/v). The suspension was allowed to stand for several hours with occasional stirring. The neutralized precipitate was collected and washed with methanol-water (3:1, v/v) and then with water. It was again suspended in hot water, and the suspension was heated in a boiling water bath for 20 min and immediately centrifuged. The supernatant was discarded. This hot water extraction was repeated three times. The precipitate was finally washed with ethanol and ether three times each and dried under reduced pressure (yield, 5.5 to 6 g). Pseudonigeran of this purity (PN 1) was used for induction of α-1,3-glucanase by *T. viride*.

For further purification, 5 g of polysaccharide (PN 1) were re-extracted three times with 1-liter portions of boiling water. The suspension was finally centrifuged, the washed polysaccharide was dissolved in 400 ml of 1 N NaOH, and 450 ml of 0.3 M CuSO₄ solution were added (13, 14). The mixture was allowed to stand at 5° for 2 hours. The precipitate which formed was collected by centrifugation and washed with cold 1 N HCl until the washing became free of blue color. The polysaccharide was then washed with water until the washings became free of chloride ions. It was then redissolved in 400 ml of 1 N NaOH. Pseudonigeran (PN 2) was precipitated, washed, and dried as before (yield, 4.3 to 4.7 g, [α]_D +257°, 0.6 1 N NaOH).

ANALYTICAL METHODS

Deionization of sugar solutions was performed with either BaCO₃ or a mixed bed resin column consisting of Amberlite IR-120 (H)⁺ and Amberlite IR-45 (OH)⁻. All sugar solutions were concentrated under reduced pressure at 45–50°. Descending chromatography was carried out at room temperature on Whatman No 1 paper with, unless otherwise stated, the following solvent systems: Solvent I, butanol-acetic acid-H₂O, 2:1:1, v/v (14); Solvent II, butanol-pyridine-H₂O, 6:4:3, v/v (15); Solvent III, 2-propanol-acetic acid-H₂O, 54:8:18, v/v (16). Sugars were detected on paper by the silver nitrate method of Trevelyan, Proctor, and Harrison (17). Nigerodextrins were prepared from pseudonigeran essentially by the method described by Johnston (10), and the degree of polymerization was determined by a modification of the method of Whelan (18) of the phenol-sulfuric acid assay (19). Periodate oxidation of polysaccharides was performed by the method of Aspinall and Ferrier (20), but incubation was carried out with continuous stirring at 5° instead of 35°. Reduction of the products of periodate oxidation was performed by the method of Hamilton and Smith (21). Glycerol liberated from the Smith degradation of polysaccharide was determined by the method of Lambert and Neish (22). Total carbohydrate was estimated by the phenol-sulfuric acid method of Dubois *et al.* (19). Reducing sugars were determined by the method of Nelson-Somogyi (23).

Protein was determined by the method of Lowry *et al.* (24) with crystalline bovine serum albumin as a standard and by absorbance at 280 mμ. The molecular weight of the enzyme was determined by the method of Whitaker (25). The α-(1 → 3)-glucanase used in this particular experiment was an ammonium

sulphate fraction and was located by the standard assay described below. Peaks for cytochrome *c*, hemoglobin, and peroxidase were determined from absorbances of their solutions at 550, 405, and 405 $m\mu$, respectively. Other proteins were measured at 280 $m\mu$.

Analytical disc gel electrophoresis in polyacrylamide gel was carried out at pH 8.6 by the procedure of Ornstein and Davis (26) and at pH 4.5 according to the method of Reisfeld, Lewis, and Williams (27).

Location of enzyme activity in the analytical gels was achieved by cutting the gel into 0.5-cm sections and eluting each in 1 ml of 0.05 M citrate phosphate buffer, pH 4.5, containing 0.5 M sodium chloride in small test tubes. The gel section was smashed with a spatula, and the mixture was allowed to stand in the cold overnight. Twenty-five μ liters of each fraction were then assayed by the micro method described below for the ability to hydrolyze pseudonigeran.

Gas chromatography of products of the enzyme reaction was carried out according to the method of Sweeley *et al.* (28) and Parrish and Reese (29). The glass column (6 feet \times 1/4 inch) was packed with SE 30 (1%) on Anakrom 100 to 110 mesh. Column temperature was 140°.

Enzyme Assays—The standard assay consisted of an incubation of 0.5 ml of suitably diluted enzyme with 0.5 ml of 0.4% pseudonigeran suspension in 0.05 M citrate-phosphate buffer, pH 4.5, at 40° for 10 min. Aliquots were then withdrawn for determination of reducing sugar. When necessary, after arsenmolybdate reagent was added, insoluble pseudonigeran was centrifuged (200 $\times g$) directly in the tubes to prevent turbidimetric interferences with colorimeter readings.

One unit of enzyme is defined as that amount which will liberate 1.0 μ mole of reducing sugar equivalent, expressed as glucose, per min under the standard assay conditions. Enzyme concentration is expressed as units per mg of protein.

Determination of the ability of the enzyme to attack various polysaccharides or oligosaccharides was performed by the following micro scale procedure. Approximately 0.01 unit of enzyme was incubated with 100 μ g of carbohydrate in solution or suspension in 0.05 M acetate or 0.03 M citrate-phosphate buffer, pH 4.5, in a final volume of 50 μ liters. The incubation was carried out in a sealed melting point capillary tube at 40° overnight, and the entire contents were then chromatographed directly in Solvent II for identification of reaction products. Controls without enzyme were included for each incubation.

Enzyme Production—*T. viride* QM6A was grown in a medium containing glucose (0.3%), pseudonigeran (0.3%), and *Trichoderma*-salts solution (30).

The medium was disrupted by sonic oscillation until the pseudonigeran became a fine suspension. Five 500-ml Erlenmeyer flasks, each containing 100 ml of disrupted medium, were prepared. The autoclaved flasks were inoculated with *T. viride* QM6A spores and incubated on the reciprocal shaker at room temperature for 10 days. This time period allowed for maximum accumulation of enzyme in the culture filtrate.

Enzyme Purification—All steps were carried out a 0–4° unless specified otherwise. The culture filtrate (500 ml) was filtered through Whatman No. 1 paper, and the crude enzyme could be obtained by raising the cold solution to 75% of saturation with ammonium sulfate, followed by collection of the precipitate after 12 hours, or by the use of membrane filtration (UM-1 ultrafilters and concentrating device, Amicon Corporation, Lexington, Mas-

sachusetts). The pH was maintained between 4.5 and 6.5 during this process. The concentrated enzyme (9 ml) was dialyzed for 12 hours against several changes of distilled water. Any insoluble material in the dialyzed enzyme solution was removed by centrifugation at 12,000 $\times g$ for 15 min. Enzyme could be frozen as the concentrated extract for several months without significant loss of activity.

Three to 4 ml of the ammonium sulfate enzyme (15 mg of protein) were chromatographed on a column of Sephadex G-150 (2.5 \times 35 cm) equilibrated with 0.15 M citrate-0.03 M phosphate buffer, pH 7.0, and eluted with the same buffer. The flow rate was adjusted to 0.15 ml per min. Fractions were assayed for enzymatic activity and absorbance at 280 $m\mu$. Tubes containing enzyme were combined and concentrated under reduced pressure with semipermeable collodion bags (Schleicher and Schuell). The yields from two G-150 column preparations were usually pooled before proceeding to the next step.

A 3-ml sample of G-150 enzyme (8 to 10 mg of protein) was chromatographed on a DEAE-Sephadex column (1.8 \times 17 cm) which was equilibrated with 0.015 M citrate-0.03 M phosphate buffer, pH 7.0. A flow rate of 0.1 ml per min was usually maintained. Elution was carried out with 400 ml of buffer with a linear gradient of sodium chloride from 0 to 0.5 M. Fractions with enzymatic activity were combined and concentrated to 0.5 ml with a semipermeable membrane bag against water, and 0.5 ml of 0.3 M acetate buffer, pH 4.5, was added to the enzyme solution. The concentrated DEAE-Sephadex enzyme (2 to 3 mg of protein) was applied to a Sulfoethyl-(SE-) Sephadex C-50 column (1.8 \times 38 cm) previously equilibrated with 0.1 M acetate buffer, pH 4.5. A linear gradient of 0 to 0.5 M sodium chloride in the same buffer was again employed with a flow rate of 0.1 to 0.15 ml per min. Pooled fractions containing the enzyme were concentrated as above and stored at 3°. The enzyme was found to be most stable when maintained in the pH range of 4.5 to 6 and could be kept for several months before significant loss of activity occurred.

RESULTS

Structure of Polysaccharide¹—Pseudonigeran (PN 2, 5 mg) was suspended in 2 ml of 1 N H₂SO₄ and hydrolyzed for 12 hours at 100° in a sealed glass vial. The polymer completely dissolved during this time. The hydrolysate was neutralized with BaCO₃ and centrifuged. The concentrated supernatant was chromatographed in Solvents I and II. Glucose was the only product observed with the silver nitrate developer.

Pseudonigeran (PN 2, 85 mg) was suspended in 50 ml of 0.015 M sodium periodate. In addition, 104 mg of nigeran suspended in an identical volume of periodate and incubated under the same conditions served as a standard. The results shown in Fig. 1 indicate 3.8×10^{-2} mole of periodate consumed per mole of anhydrohexose residue in pseudonigeran. Nigeran consumed 0.5 mole per mole of anhydrohexose, which is consistent with its structure. The very small consumption of periodate by pseudonigeran supports the view that the material is (1 \rightarrow 3)-linked.

The average degree of polymerization of the polysaccharide was determined by a Smith degradation (32) of the molecule. When a linear glycan was subjected to this procedure, followed by mild acid hydrolysis, 1 molecule of glycerol released from the non-

¹ Detailed chemical studies of the polysaccharide have been published elsewhere (31).

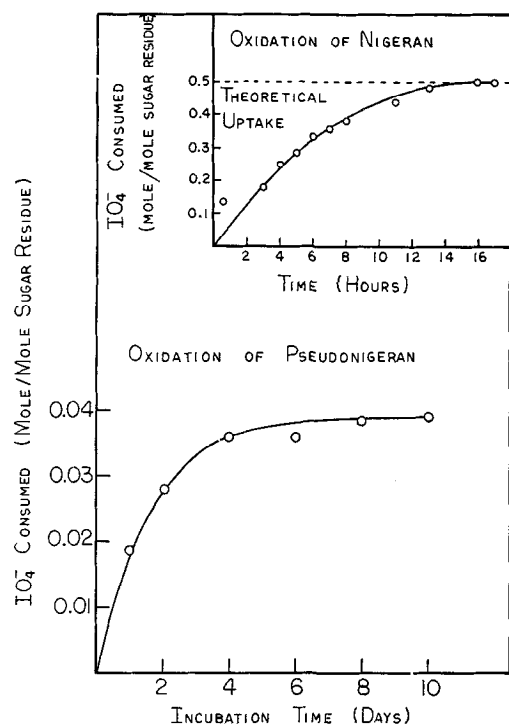


FIG. 1. Pseudonigeran (85 mg) and nigeran (104 mg) were each suspended in 50 ml of 0.015 M sodium periodate solution. The course of oxidation at 5° in the dark was followed spectrophotometrically according to the method of Aspinall and Ferrier (20). The theoretical uptake of periodate by nigeran is 0.50 mole per mole of sugar residue.

reducing end of each chain. It is therefore possible to calculate the degree of polymerization from the amount of liberated glycerol.

Pseudonigeran (PN 2, 39.8 mg) was suspended in 50 ml of 0.015 M sodium periodate in the dark at 5° with stirring. After 16 days, 0.2 ml of ethylene glycol was added to destroy excess periodate. The oxidized pseudonigeran was centrifuged, washed with water, and resuspended in 10 ml of water. Forty milligrams of sodium borohydride dissolved in 5 ml of water were added to the suspension, and the mixture was allowed to stand another 24 hours. The excess borohydride was destroyed by adding acetic acid to pH 6.0. The reduced polysaccharide was collected by centrifugation and washed exhaustively with water.

The polysaccharide alcohol was suspended in 10 ml of 0.1 N H_2SO_4 and stirred at room temperature for 24 hours. It was then centrifuged, and the supernatant was recovered. The precipitate was washed with water, and the washings and supernatant were combined. This solution was concentrated to dryness under reduced pressure at 50°, and 2 ml of water were added to the residue. It was then assayed for glycerol as described above in "Analytical Methods," and 1.34×10^{-3} mole of glycerol was liberated per mole of anhydrohexose. Assuming an unbranched molecule (31), the degree of polymerization of this pseudonigeran preparation appears to be about 700, based on these results.

Enzyme Production—The effects of growth period and concentration of inducer, pseudonigeran, on α -(1 \rightarrow 3)-glucanase production by *T. viride* were investigated. The organism was grown as described above with increasing amounts of pseudonigeran. The control medium contained 0.6% glucose but no

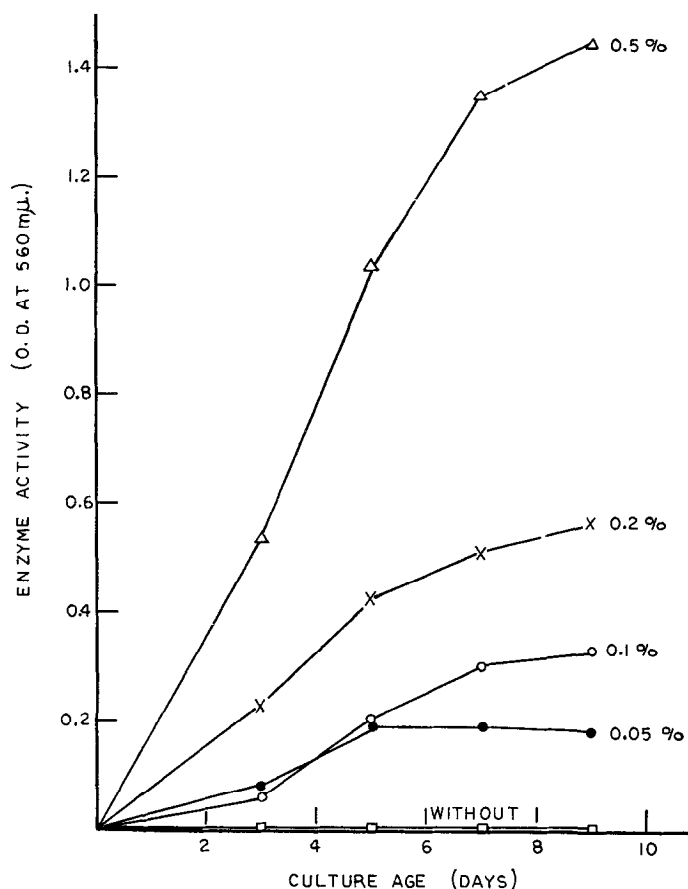


FIG. 2. Effect of growth period and inducer concentration on α -(1 \rightarrow 3)-glucanase production. *T. viride* QM6A was grown in *Trichoderma* salts medium (30) with increasing amounts of pseudonigeran. The control medium contained 0.6% glucose as carbon source. Aliquots of culture filtrate were assayed for activity at the indicated times. \square — \square , without pseudonigeran; \bullet — \bullet , 0.05%; \circ — \circ , 0.1%; \times — \times , 0.2%; and \triangle — \triangle , 0.5%, pseudonigeran, respectively. For details see text.

pseudonigeran. Periodically, 4-ml aliquots of growth culture were withdrawn, and the filtrate was assayed for enzyme activity by the standard enzyme assay. The results are shown in Fig. 2.

The amount of enzyme activity induced was proportional to the amount of inducer added. For producing the enzyme, 0.3% inducer was routinely used. Maximum yield of enzyme was usually obtained in cultures after 8 to 10 days of growth.

A much higher recovery of enzyme units was consistently obtained when the 75% ammonium sulfate fraction was allowed to stand overnight at 4° before the precipitated protein was collected by centrifugation. The bulk of the activity was found in the fraction obtained between 35 and 75% saturation. For large scale experiments, however, where 5 to 10 liters of culture filtrate were fractionated, ultrafiltration was a more efficient method of concentrating the enzyme.

The result of a typical Sephadex G-150 column is shown in Fig. 3. Three major peaks were generally observed. The first minor peak corresponds to the void volume, while the activity eluted as a second peak in tubes 40 to 56.

The elution pattern on DEAE-Sephadex is shown in Fig. 4. Enzyme activity was recovered in the small second peak eluting over the range of tubes 16 to 28. Fig. 5 shows the result of

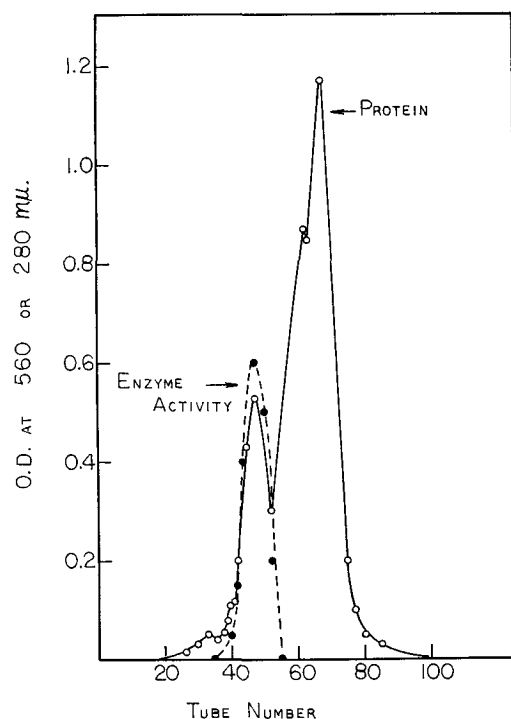


FIG. 3. Three milliliters of ammonium sulfate enzyme (15 mg of protein) were chromatographed on a column (2.5×35 cm) of Sephadex G-150. The eluting buffer was 0.015 M citrate-0.03 M phosphate, pH 7.0. Fractions of 2 ml each were usually collected. The void volume corresponds to tube 32. ●---●, enzyme activity; ○—○, protein. For details see text.

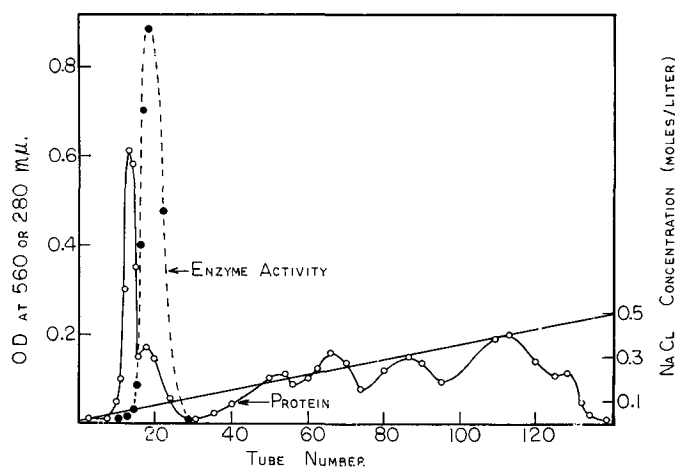


FIG. 4. Eight milligrams (47 units) of the Sephadex G-150 enzyme in 3 ml of 0.015 M citrate-0.03 M phosphate buffer, pH 7.0, were placed on a column (1.8×17 cm) of DEAE-Sephadex A-50. A linear gradient of 0 to 0.5 M NaCl was used to elute the enzyme; 1-ml fractions were collected. ○—○, protein; ●---●, enzyme activity. For details see text.

Sephadex C50 column chromatography of DEAE-cellulose enzyme. Three protein peaks were observed with the activity corresponding to the first peak.² A summary of the purification

² This step also served to remove a very weak α -(1 \rightarrow 3)-glucanase activity from the preparation. This enzyme was distinguished from the major activity by its ability to hydrolyze nigerose.

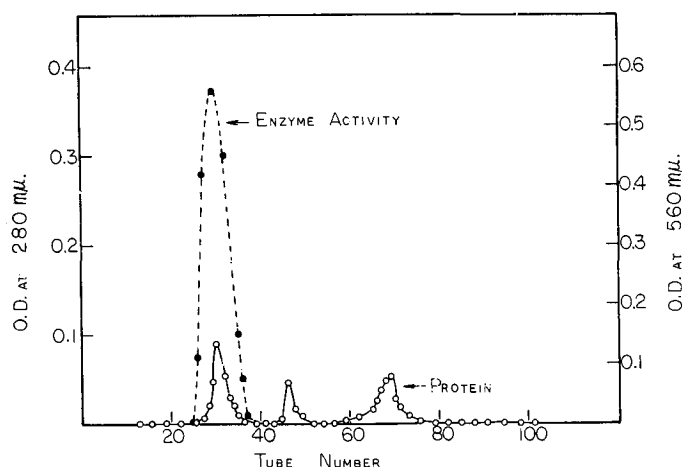


FIG. 5. One milliliter (2.15 mg of protein) of DEAE-Sephadex enzyme was chromatographed on a column (1.8×39 cm) of sulfoethyl Sephadex C-50 previously equilibrated with 0.1 M acetate buffer, pH 4.5. A linear gradient of 0 to 0.5 M NaCl was employed in eluting the enzyme; 1-ml fractions were collected. ●---●, enzyme activity; ○—○, protein. For details see text.

TABLE I
Enzyme purification

The data were obtained during the purification of 500 ml of culture filtrate. For details of experimental procedure see the text.

Purification step	Total volume	Enzyme concentration	Protein concentration	Specific activity	Yield	Purification
	ml	units/ml	mg/ml	units/mg protein		
Culture filtrate.....	500	0.18	0.25	0.73	100	
Ammonium sulfate.....	9.5	8.9	5.1	1.75	93	2.4
Sephadex G-150.....	90	0.85	0.143	5.95	84	8.2
DEAE-Sephadex A-50.....	19.5	2.5	0.157	16	54	22
SE-Sephadex C-50*.....	22.5	1.2	0.026	46	30	63

* SE-, Sulfoethyl.

procedure is outlined in Table I. The over-all purification of only 60-fold reflects the relatively pure state of the enzyme in the culture filtrate.

Properties of Enzyme—A photograph of an acrylamide gel of enzyme prepared by the above procedure is shown in Fig. 6. At pH 4.5 there appears to be a minor band moving slightly behind the enzyme. Only the major band contained any detectable enzymatic activity as evidenced by sectioning, eluting, and assaying the gel fractions as detailed in "Analytical Methods." At pH 8.3 the enzyme moved only slightly after several hours as a single band, but it could not be eluted in an active form. This was not unexpected since the enzyme does not retain its activity upon storage at pH values above 7.

The molecular weight of the enzyme was estimated by use of a Sephadex G100 column (2.3×30 cm) equilibrated with 0.15 M citrate-0.03 M phosphate buffer, pH 8.0. One milliliter of marker protein solution in the same buffer was applied to the column, and the flow rate was adjusted to 0.2 ml per min. Void volume was determined with a 1-ml sample of 0.2% blue dextran solution. A plot of relative elution volumes of markers and enzymatic activity versus the log of molecular weight for the



FIG. 6. Acrylamide gel electrophoresis of purified α -(1 \rightarrow 3)-glucanase. Enzyme, 100 μ g, was run at pH 4.5 according to the method of Reisfeld *et al.* (27). Migration took place from top (+) to bottom (-). The protein was stained using Coomassie brilliant blue in 10% trichloroacetic acid (33). Only the area which corresponds to the major band contained detectable enzymatic activity when assayed as described under "Analytical Methods."

markers is given in Fig. 7. The molecular weight determined from the experiment is approximately 47,000.

The proportionality of the reaction to enzyme concentration and time is illustrated in Fig. 8.

The influence of hydrogen ion concentration on enzymatic activity was investigated over the pH range 3 to 8. The assay was carried out under standard conditions, except for pH, and the results are shown in Fig. 9. The optimal pH, 4.5, typical of many carbohydrases (3, 12), was also found excellent for storage of the enzyme over long periods.

Experiments conducted to determine initial reaction velocity as a function of temperature indicated that at 50° the enzyme is rapidly and irreversibly inactivated under standard assay conditions. A calculation of the activation energy for the enzymatic hydrolysis with the integrated form of the Arrhenius equation yielded a value of 7000 calories per mole. The rather high temperature maximum may in part reflect the effect of partial solubilization of the substrate on reaction rate.

The influence of substrate concentration on the initial velocity of the reaction was determined, and the K_m and V_{max} values for the enzyme were calculated from the Lineweaver-Burk plot shown in Fig. 10.

The effect of various metal ions on enzyme activity was investigated. All metal solutions were prepared as chloride salts except Pb^{++} , Hg^{++} , and Ag^{+} , which were employed as their acetates.

Enzymes previously dialyzed against water (0.008 unit) was incubated in 0.03 M citrate-phosphate buffer, pH 4.5, containing 1 or 10 micromoles of metal ion in a final volume of 0.7 ml at 40° for 15 min. Then 0.3 ml of a 7% pseudonigeran suspension in the above buffer was added, and the incubation was continued

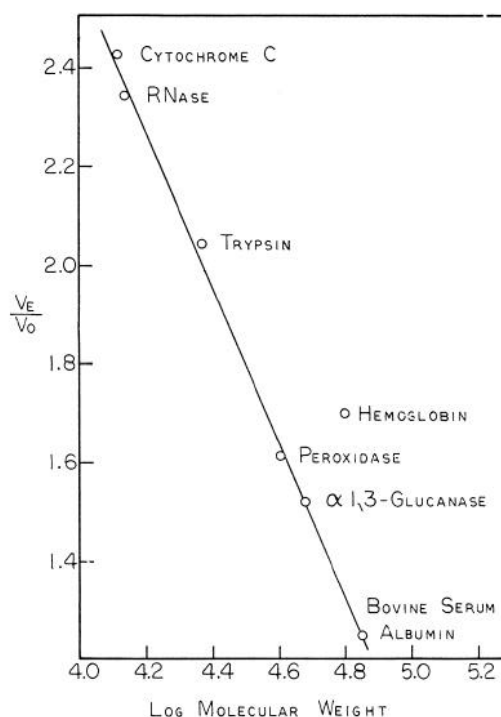


FIG. 7. Molecular weight determination of the enzyme by Sephadex G-100 column chromatography (25). The size of the column was 2.3 \times 30 cm; the flow rate was 0.4 ml per min, and fractions of 1 ml were collected. The temperature was 4°. All markers and the unknown were each dissolved in 1 ml of 0.015 M citrate-0.03 M phosphate buffer, pH 7.0. Concentrations employed for the proteins were in milligrams per ml; cytochrome c, 3.0; ribonuclease, 4.5; trypsin, 2.5; peroxidase, 3.0; hemoglobin, 5.0; serum albumin, 5.0; and α -(1 \rightarrow 3)-glucanase, 3.5. The elution volume of each protein was determined independently immediately after a void volume measurement for each run was made with blue dextran 2000.

for 10 min at 40°. Reducing power was measured in the various reaction mixtures, and all values obtained were corrected for any effect on the Nelson's test by using standard glucose solution assayed in the presence of each metal ion independently. The results are summarized in Table II. Activity is expressed relative to a control containing no added metal ion. Under the conditions of the assay Fe^{++} and Zn^{++} are strongly inhibitory toward the enzyme at 1.7×10^{-2} M, but the inhibition is freely reversible since practically full activity could be restored by dialyzing the inactivated enzyme against several changes of distilled water. The only other metals found to affect activity significantly were Hg^{+} and Ag^{+} , which completely and irreversibly abolish activity at 1.7×10^{-3} M. Other heavy metals have no effect on the enzyme while Co^{++} appears to stimulate the activity. The reason for this activation has not been investigated further.

Specificity and Mechanism of Action of Enzyme—Substrate specificity studies were carried out employing the micro procedure described in "Analytical Methods." The results are summarized in Table III. The preparation is free of glucosidase activity as well as β -(1 \rightarrow 3)- and β -(1 \rightarrow 4)-glucanase activities, all of which are common to fungal enzymes. No activity was noted with bacterial dextran, but in this molecule the glucose units bearing substitution at Position 3 of the hexose are also substituted on Position 6 (34), and thus activity was probably

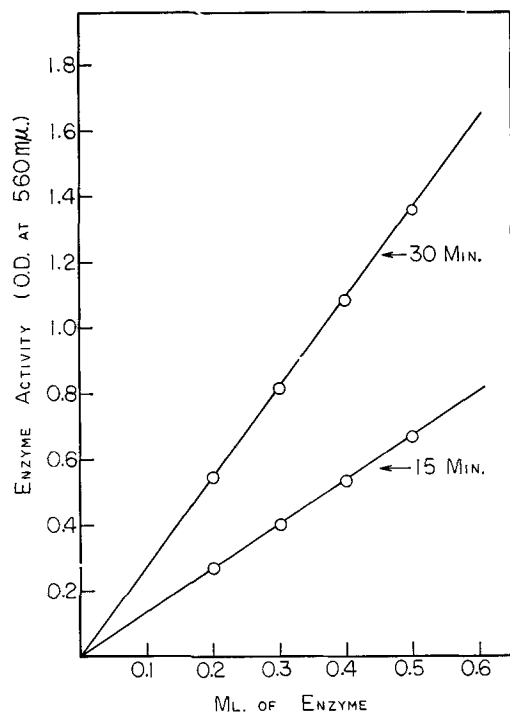


FIG. 8. Proportionality of the reaction to enzyme concentration and time. Various amounts of purified enzyme (0.04 unit per ml) were incubated for either 15 or 30 min at 40°. Other conditions were as described in the text for standard enzyme assay.

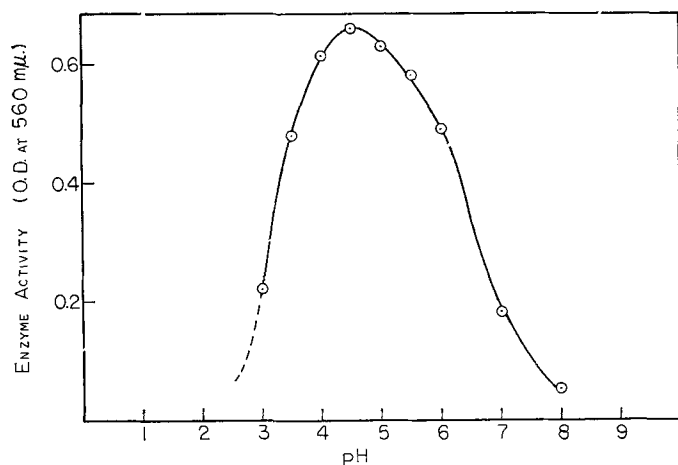


FIG. 9. Effect of pH on enzyme activity. Each tube contained 1 mg of substrate and 0.008 unit of purified enzyme in 0.14 M citrate phosphate buffer which had been adjusted to the various pH values, in a final volume of 1 ml. After 10 min at 40°, the reducing power in each tube was measured. For details see text.

abolished by this additional structural modification. The enzyme has rather strict structural requirements and provides a highly specific tool for application in certain structural carbohydrate problems.

If the α -(1 \rightarrow 3)-glucanase operates by an exo mechanism, it would be predicted that a modification of the nonreducing terminal glucose unit of pseudonigeran would affect the enzyme activity. If it is an endo enzyme, then little change in activity would be expected since the sites of initial attack would be located away from the area of modification. Pseudonigeran which has

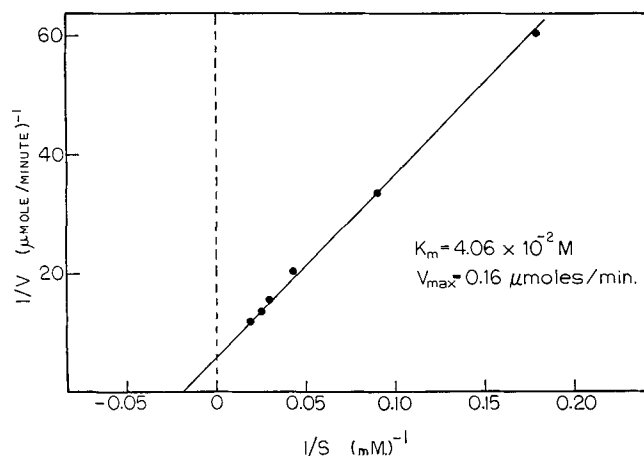


FIG. 10. Effect of substrate concentration on reaction velocity. Various amounts of pseudonigeran were incubated with 0.03 unit of purified enzyme in 0.05 M citrate phosphate buffer, pH 4.5, in a final volume of 1 ml. Incubation was carried out for 10 min at 40°. The reaction mixtures were then assayed for reducing power. Substrate is expressed as millimolar concentration of anhydrohexose units and the data is plotted by the method of Lineweaver and Burk.

TABLE II

Effects of metal ions on enzyme

The effect of incubation of the enzyme with selected metal ions on its ability to hydrolyze pseudonigeran. For details see text.

Metal ion	Concentration	Enzyme activity
	$M \times 10^2$	%
None	0	100
Co ⁺⁺	1.7	147
Mg ⁺⁺	1.7	114
Ca ⁺⁺	1.7	120
Pb ⁺⁺	1.7	123
Ba ⁺⁺	1.7	100
Fe ⁺⁺⁺	1.7	92
Fe ⁺⁺	1.7	21
Zn ⁺⁺	1.7	30
Ag ⁺	0.17	0
Hg ⁺	0.17	0

been treated with periodate and borohydride will serve as an excellent substrate for this type of study because periodate oxidation of (1 \rightarrow 3)-linked glucans modifies only the terminal units of the polysaccharide, leaving the remainder of the chain unaltered (35). For the preparation of modified substrate 300 mg of pseudonigeran (PN 1) were subjected to two successive Smith degradations. No acid hydrolysis was used following the second periodate oxidation and borohydride reduction. This leaves a residue of D-hydroxymethyldiethyleneglycol attached to the new terminal nonreducing glucose residue in each chain (32). Polysaccharide which was subjected to the first Smith degradation followed by mild acid hydrolysis was used as a control substrate. Assays were then carried out under standard conditions, and the results are shown in Fig. 11. No difference in rate of attack on the two substrates by the enzyme was observed. An independent method was used to confirm the endo mechanism (36). Steady state measurement of the products of the enzymatic reaction during the course of the hydrolysis revealed also that the glu-

TABLE III
Substrate specificity of enzyme

The ability of the enzyme to hydrolyze various carbohydrates was evaluated by incubation of the substrate and enzyme in sealed capillary tubes followed by paper chromatographic analysis of the reaction products. Plus sign indicates that the substance was hydrolyzed by the enzyme. Minus sign indicates no attack. For details see text.

Glucan	Major linkage(s)	Activity
Pseudonigeran.....	α -1,3	+
Nigeran.....	α -1,3; α -1,4	+
Soluble starch.....	α -1,4; α -1,6	-
Waxy starch.....	α -1,4; α -1,6	-
Isolichenin.....	α -1,3; α -1,4	+
<i>Polyporus tumulosus</i> polysaccharide.....	α -1,3	+
Dextran.....	α -1,6 (α -1,3)	-
<i>Sclerotium rolfsii</i> gum.....	β -1,3; β -1,6	-
Pachyman.....	β -1,3	-
Maltose.....	α -1,4	-
Sucrose.....	β -1,2	-
Gentiobiose.....	β -1,6	-
Isomaltose.....	α -1,6	-
Nigerose.....	α -1,3	-
Nigerotriose.....	α -1,3	+
Nigerotetraose.....	α -1,3	+
Nigeropentaose.....	α -1,3	+

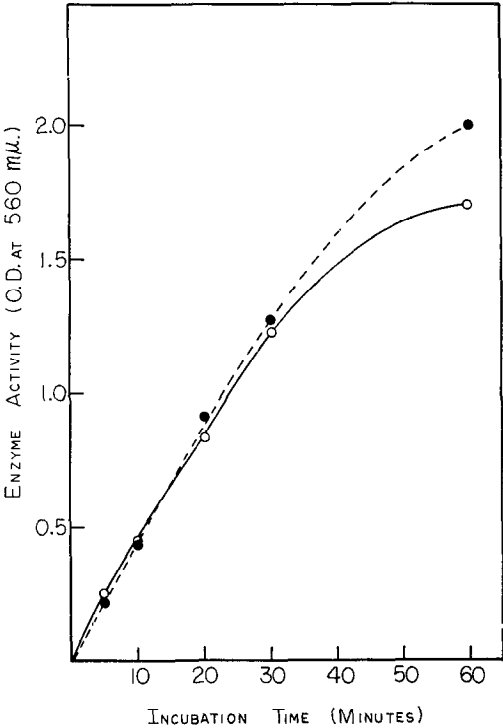


FIG. 11. Effect of modification of the terminal nonreducing unit of pseudonigeran on enzyme activity. Standard incubation mixtures contained polysaccharide modified as described under "Results." Each sample was analyzed over a period of time for the amount of reducing power liberated by the α -(1 \rightarrow 3)-glucanase. O—O, control substrate; ●---●, modified substrate. For details see text.

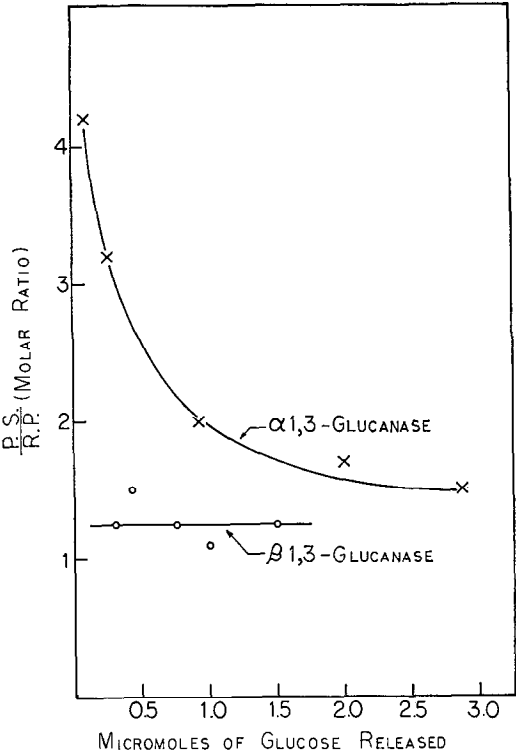


FIG. 12. Action pattern of the α -(1 \rightarrow 3)-glucanase. α -(1 \rightarrow 3)-Glucan, 5 mg, was incubated in 0.05 M citrate phosphate buffer (pH 4.5) with 0.01 to 0.48 unit of α -(1 \rightarrow 3)-glucanase at 40° for 5 min. Then, 0.2 to 2.0 units (36) of β -(1 \rightarrow 3)-glucanase was incubated with 5 mg of β -(1 \rightarrow 3)-glucan (laminarin) in 0.005 M acetate buffer (pH 4.8) at 37°. P.S., moles total carbohydrate solubilized; R.P., reducing power expressed as glucose equivalents. For details see Reference 36.

canase operates in an endo manner. Fig. 12 compares the enzyme with that of β -(1 \rightarrow 3)-glucanase, which operates in an exo fashion (35).

Fig. 13 shows the action of the enzyme on a series of nigerodextrins prepared from pseudonigeran. The mode of attack of the glucanase is such that under the conditions employed all homologous members of the series tested were degraded to nigerose and glucose. The enzyme is completely inactive on nigerose, however.

The stereochemistry of bond cleavage during enzymatic hydrolysis of pseudonigeran was also investigated. Purified enzyme, 0.15 unit, was incubated with 4 mg of substrate suspension, in 0.05 M citrate-phosphate, pH 4.5, in a final volume of 1 ml. Incubations were carried out at 40° for periods of 5, 10, 15, and 20 min. The reaction was stopped by freezing the reaction mixture in Dry Ice-acetone, and the frozen mixtures were then lyophilized. To each dry sample were added 0.5 ml of dry pyridine, 0.2 ml of hexamethyldisilazane, and 0.1 ml of trimethylchlorosilazane. Each solution was mixed thoroughly and allowed to stand for 20 min at room temperature and then evaporated under reduced pressure at 50°. Hexane, 2 ml, was then added to the dried material to extract the trimethylsilyl derivatives. Any insoluble material was removed by centrifugation. The hexane extract was again concentrated to dryness under reduced pressure, and it was redissolved in 0.1 ml of hexane. A suitable aliquot of the hexane extract was injected into the gas chromatograph. Samples of α - and β -D-glucose were trimethylsilylated

TABLE IV

Stereochemistry of products of enzyme reaction as revealed by gas chromatography

The anomeric configuration or configurations of the glucose released by enzymatic hydrolysis was analyzed as a function of time. For details see the text.

Sample	Retention time
	min
α -D-Glucose.....	20
β -D-Glucose.....	35
Product from 5-min incubation.....	20
Product from 10-min incubation.....	20
Product from 15-min incubation.....	20 (35) ^a
Product from 20-min incubation.....	20 (34) ^a

^a A small second peak corresponding to the derivative of the β anomer was detected only on increasing the sensitivity of the detector.

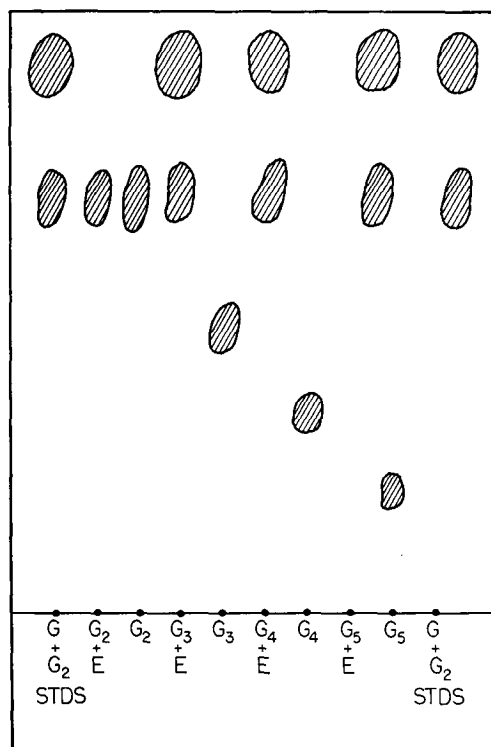


FIG. 13. Each oligosaccharide, 50 to 100 μ g, was incubated with 0.01 unit of enzyme in a final volume of 50 μ l of acetate buffer, pH 4.5. The incubation was carried out in a sealed melting point capillary tube at 40° overnight. The contents of the tube were applied directly on Whatman No. 1 chromatography paper and developed in Solvent III for 24 hours. Location of the various carbohydrates was accomplished with silver nitrate developer. The compounds were prepared from pseudonigeran by the procedure described by Johnston (10) and the degree of polymerization of each determined as described under "Analytical Methods." G, glucose; G₂, nigerose; G₃, nigerotriose; G₄, nigerotetraose; G₅, nigeropentose; E, enzyme.

in an identical way and chromatographed. The major compound to be detected in enzymatic incubation mixtures was almost exclusively the α -D-glucose derivative (Table IV). A small

β -D-glucose derivative peak was observed only in the samples incubated for 15 min or longer.

DISCUSSION

Structure of Polysaccharide—The combined results of periodate oxidation, acid hydrolysis, optical rotation, and determination of degree of polymerization suggest very strongly that pseudonigeran is an unbranched polymer composed almost entirely, if not exclusively, of α -(1 \rightarrow 3)-linked glycopyranosyl units. Results obtained in the present investigation and by Horisberger (31) suggest the possibility of a very small percentage of (1 \rightarrow 4) linkages near the reducing end of the molecule. This is indicated by a somewhat high amount of periodate consumed and formic acid produced compared to what is expected of a linear (1 \rightarrow 3)-glucan.

Johnston has also isolated from the cell wall of *A. niger* a highly dextrorotatory polysaccharide ($[\alpha]_D^{25} + 233^\circ$ N sodium hydroxide) and characterized it as an α -(1 \rightarrow 3)-glucan containing approximately 10% α -(1 \rightarrow 4) linkages (10). Since nigeran is hot water-soluble, while the α -(1 \rightarrow 3)-glucan is not, nigeran should be easily removed from hot water-insoluble materials. It is therefore not likely that the α -(1 \rightarrow 4) linkages found by Johnston are simply due to contaminating nigeran in his α -(1 \rightarrow 3)-glucan preparation. The evidence seems to indicate that perhaps the polymer may contain varying amount of (1 \rightarrow 4)-linked units.

Additional structural information about the polysaccharide was provided by an examination of the products of partial acid hydrolysis of the polysaccharide. This process yielded a series of oligosaccharides, which were separated and identified by paper chromatography, enzymic hydrolysis, and determination of degree of polymerization. The results are in good agreement with those reported by Johnston for nigerodextrins (10).

Properties of α -(1 \rightarrow 3)-Glucanase—Fungal α - and β -glucanases are generally extracellular enzymes with many properties in common. It is an easy task to remove the organism, leaving a cell-free culture filtrate, which can be used as a source of crude enzyme and which is partially purified by the fact that cell breakage is not needed to obtain the enzyme. Glucanases are also usually quite active enzymes even at relatively high temperatures, and the (1 \rightarrow 3)-glucanase fits this pattern also. The optimum pH for the many fungal glucanases in 4 to 5, with very low activity below pH 2 and above pH 7. The enzymes are usually inactive above pH 8. Maximum stability seems to be in the range of pH 5 to 7 (37). This is again the case with the α -(1 \rightarrow 3)-glucanase.

The K_m value obtained for the α -(1 \rightarrow 3)-glucanase (4.6×10^{-2} M) is quite large. It should be noted, however, that the initial velocity in the present system is measured by determining reducing sugars liberated during a 10-min incubation period. The evidence shows that the enzyme is of the endo type, and therefore the first reaction products are oligosaccharides rather than glucose. Since the molar reducing value of oligosaccharides is lower than that for glucose, the initial rate determined by measuring reducing power would result in a slightly lower apparent initial velocity at each substrate concentration tested, leading to a somewhat higher apparent K_m value. Another reason, and probably the most important, may be the insolubility of the substrate. K_m values determined with insoluble substrates do not characterize the enzyme in the same way as do values for

soluble substrates. The value of K_m is obtained in the form of the total concentration of linkages with insoluble substrate whereas only those linkages which are exposed and accessible to the enzyme can be expected to influence the rate of formation of the enzyme substrate complex. Indeed, the values obtained with various insoluble substrates may reflect solubilization processes rather than enzymatic affinity.

Substrate Specificity and Mechanism of Action—The enzyme freely attacks α -(1 \rightarrow 3) linkages, as evidenced by its ability to degrade certain compounds listed in Table III. Substitution near the glycosidic linkage being cleaved does not appear to be too critical since α -(1 \rightarrow 4) linkages on either side of the α -(1 \rightarrow 3)-nigeran being cleaved do not abolish activity. It definitely is inactive on β -(1 \rightarrow 3) linkages, however, and in this sense the anomeric configuration of the bond being cleaved is critical. Finally, it would appear that the inability of the enzyme to attack nigerose (Fig. 13) might indicate either that at least 3 glucose units are required for proper binding to the enzyme or that 2 glucose units must be located penultimate to the α -(1 \rightarrow 3) bond being hydrolyzed for there to be any demonstrable activity.

The two usual patterns of enzymatic attack on polysaccharides are the removal from the nonreducing end of successive monosaccharide or disaccharide units (exo enzymes) and the completely random attack on all glycosidic bonds (endo enzymes). In the first instance, only the terminal bond is susceptible to enzyme attack. In the second instance, all bonds are more or less equally susceptible. The modification of the terminal nonreducing unit did not alter the rate of attack by the enzyme, but this treatment has been shown to inhibit the rate of attack of an exo- β -(1 \rightarrow 3)-glucanase on laminarin by about 95% (35). The steady state determination of the degree of polymerization of the soluble products of the reaction also indicates quite clearly that the enzyme operates in an endo manner while the failure of the α -(1 \rightarrow 3)-glucanase to react with nigerose further supports the idea that the enzyme operates by an endo mechanism.

Analysis of the products of the enzymatic hydrolysis of pseudonigeran by gas chromatography clearly shows that the glucose liberated is of the α configuration. Parrish and Reese (29) have recently shown that exo enzymes attack with inversion of configuration while all endo carbohydrases analyzed hydrolyze with retention of configuration. The present results are in complete agreement with their observations. Koshland (38) has proposed a mechanism to explain inversion and retention of configuration with carbohydrases, as well as the exchange reactions noted with some of these enzymes.

A number of polysaccharides of microbial origin have been characterized as containing α -(1 \rightarrow 3)-glucosidic linkages. Their occurrence appears to be quite widespread in the cell walls of fungi, but their structural role in the organisms remains to be defined. The α -(1 \rightarrow 3)-glucan of *A. niger* discussed above contains a small percentage of α -(1 \rightarrow 4) linkages near the reducing end of the molecule, but whether these are structurally significant or arise from contamination by another polysaccharide has not been clearly established. It is interesting to note, however, that regions of both uniformity and nonuniformity of linkages have been established for certain mammalian and microbial polysaccharides involved in structural roles (39). Exhaustive digestion of these α -(1 \rightarrow 3)-glucan preparations with the α -(1 \rightarrow 3)-glucanase, followed by fractionation and characterization

of the digested material, might give some insight into the (1 \rightarrow 4)-linked segments.

Recent experiments in one of our laboratories has resulted in the isolation of a galactoglucan from the cell walls of *A. niger*. Preliminary work indicates that the polymer is composed of α -(1 \rightarrow 3)-linked glucopyranose units and (1 \rightarrow 5)-linked galactofuranosyl units.³ Because of the furanose form of galactose in the polymer, mild acid hydrolysis (pH 2) of the native material selectively cleaves the galactose units from the polymer and precludes the isolation of either galactofuranosyl oligosaccharides or those containing both glucose and galactose. However, the use of the endo- α -(1 \rightarrow 3)-glucanase has permitted the isolation of portions of the carbohydrate chains relatively intact and is aiding the study of this polymer's structure.

Since most fungal glucans characterized as α -(1 \rightarrow 3) polymers have been identified only on the basis of partial acid hydrolysis and infrared or x-ray diffraction analysis (7), the use of α -(1 \rightarrow 3)-glucanases in the determination of their fine structure will be useful, particularly if more than one type of glucosidic linkage is present or if branching occurs in these recently discovered molecules.

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**Enzymes That Hydrolyze Fungal Cell Wall Polysaccharides: I.
PURIFICATION AND PROPERTIES OF AN ENDO- α -D-(1 \rightarrow
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Shiro Hasegawa, John H. Nordin and S. Kirkwood

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