PRODUCTION AND PROPERTIES OF CHITINASE FROM AN ISOLATE OF A STREPTOMYCES SP.

Thesis for degree of M. S.

Michigan State University

Robert L. Noveroske

1959

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AN ABSTRACT

Submitted to the College of Science and Arts Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

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Approved by _____

ABSTRACT

Natural soil and 18 Streptomyces isolates, most of which lysed living and killed mycelium of Glomerella cingulata (Ston.) Spauld. and Schrenk, dissolved chitin in agar. Degradation of chitin was always correlated with growth of bacteria or actinomycetes from the soil onto the agar. When Chloromycetin was added to natural soil and agar, no lysis of chitin occurred, although fungi from the soil grew abundantly throughout the agar and soil.

An extracellular chitinase was produced when Streptomyces isolate 8 was grown on a mineral medium with colloidal chitin. The enzyme was adaptive. The addition of soluble carbon or nitrogen sources to the medium decreased chitinase production. Maximum production of chitinase by Streptomyces isolate 8 occurred using a mineral medium plus 0.25% colloidal chitin which was incubated at 27° C for 4 days.

Chitinase was concentrated by pervaporation to 1/5 original volume, then chilled to 1° and precipitated with chilled (-18°) ethanol (ETOH). The enzyme was resuspended in 0.005 M phosphate buffer at pH 7.0. Activity was measured by recording changes in turbidity of a mixture of chitinase and chitin. Dilutions of such preparations plotted against optical density gave a straight line on log-log paper. Such preparations when stored in the cold (-18°) remained stable with no change in position or slope of the dosage-response curve during the study. Dosage-response curves made of preparations of chitinase in culture filtrate

and pervaporated culture filtrate had the same slope as the ETOH-precipitated preparations. Chitinase concentrated by pervaporation then precipitated with ETOH was purified 3.5 times on a dry weight basis, had a specific activity of 988, and contained 95.6% of the original activity from the culture filtrate. These preparations had a broad optimum pH range of 5.5-7.5 and showed maximum activity when assayed at 37°. Five minutes of boiling inactivated the enzyme preparation.

Each mg of chitin degraded by ETOH-precipitated chitinase formed 0.45 mg of N-acetylglucosamine (NAGA). This enzyme preparation had no effect on NAGA. Paper chromatograms of the products of chitinase reacted with chitin substrate showed the formation of 2 sugars, having R_f values of 0.40 and 0.30. NAGA standard had an R_f of 0.40. The enzyme preparation was moved 11.5 cm toward the cathode at pH 7.8, and remained as one detectable component.

Inhibition of germ tubes of G. cingulata following germination was produced by culture filtrates, $(NH_4)_2SO_4$-precipitated chitinase, and supernatant (free of ETOH) from ETOH-precipitated chitinase preparations. No such effect occurred with chitinase precipitated by ETOH.

Quantitative determinations of the effect of ETOH-precipitated chitinase on mycelium of G. cingulata were made at 37°. Chitinase on killed mycelium produced NAGA and a 16% loss in dry weight. Percent chitin removed (based on mg NAGA converted in terms of mg chitin) was 4.8. Chitinase

on living mycelium produced high quantities of NAGA, but no detectable weight loss. The reaction mixtures when chromatographed with n-butanol-acetic acid-water showed 2 sugars having the same R_f values (0.40 and 0.30) as those produced when chitinase was reacted with chitin. The NAGA standard again had the same R_f of 0.40.

Chitinase lysed killed agar cultures of G. cingulata at 24°, 28°, and 37°, but lysed living mycelium only at 37°.

Detectable quantities of chitinase were recovered from soil 51 hours after soil had been supplemented with the enzyme. Soil containing chitinase lysed chitin in agar after standing at room temperature one week.

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ACKNOWLEDGMENTS

The writer wishes to express his sincere thanks to Dr. J. L. Lockwood for his constructive criticism and encouragement throughout the course of the research and during the preparation of the thesis and to Dr. L. G. Wilson for his valuable suggestions.

Thanks are also due to Dr. R. L. Kiesling for the use of his equipment, and to Drs. R. S. Bandurski, E. S. Beneke, and R. P. Scheffer for reviewing the manuscript.

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INTRODUCTION

If chitinase-producing organisms can degrade chitinous residue in soil, such an enzyme might have a role in natural soil fungitoxicity. Evidence that living and dead mycelium of Glomerella cingulata (Ston.) Spauld. & Schrenk and other fungi can be lysed by natural soil, sterile soil inoculated with Streptomyces isolates, (13) and Streptomyces isolates themselves (5) would suggest that enzyme systems may be playing an active part in soil fungitoxicity. Since mycelium of G. cingulata contains chitin in its cell wall, an active chitinase enzyme system must have accounted for dissolution of the chitin portion of killed and possibly living mycelium (4). Therefore, a study of the chitinase system of one of the Streptomyces isolates known to lyse living and dead mycelium was undertaken to determine what role, if any, chitinase may play in natural soil fungitoxicity.

HISTORICAL REVIEW

Early works on chitin degradation by soil microbes dealt mainly with classification of chitin-decomposing organisms for taxonomic purposes. Benton (2) cites Benecke as the first to describe a bacterium which used chitin as food. Benecke prepared chitin from crab shell and added it to water containing a weak mineral solution. He inoculated this medium with rotting plankton from the bottom of Kiel Harbor and isolated an organism named Bacillus chitinovorous Benecke. Benton reviewed earlier attempts to classify chitinovorous bacteria, then described 250 isolates herself. She also found that actinomycetes produced chitinase but made no attempt to classify them. Johnson (11) did much the same work in describing bacterial growth on and decomposition of crab shell chitin. First attempts at a quantitative estimate of numbers of chitin-lysing organisms in soil were made by Skinner et al (18). They inoculated a mineral medium containing a strip of bleached crab shell chitin with 21 soil types and found that soil has a larger population of true bacteria than of molds or actinomycetes which are capable of destroying chitin.

Early works by Karrer and Hoffman (12) and others helped to establish the chemical nature of chitin. They worked with chitinase secured from snails and used HCl-precipitated chitin. They detected 50% N-acetylglucosamine production from chitin breakdown, but found only traces of glucosamine. Zobell and Rittenberg (22) isolated

chitinoclastic bacteria from sea water and studied 31 pure cultures. They detected the liberation of ammonia or acid from chitin and detected some reducing sugars. Veldkamp (21) isolated 2 strains of chitinovorous bacteria and grew them in a mineral medium containing chitin. The concentrated culture filtrate was chromatographed, and the presence of acetylglucosamine and glucosamine coupled with the formation of acetic acid and ammonia was shown. He concluded that both strains had the ability to form ammonia by deamination, but was not able to detect any glucose. Recently, Reynolds (17) found that when a concentrated cell-free chitinase preparation from a Streptomyces sp. (later identified as S. griseus (Krainsky) Waksman and Henrici) was reacted with chitin, 2 sugars were found, N-acetylglucosamine and the corresponding disaccharide, N-N-diacetylchitobiose. Jeuniaux (8) found that when chitin was used as the sole carbon source in a medium, 98 of 100 Streptomyces cultures liberated chitinase into the medium. The addition of glucose was found to decrease chitinase production. Recently, Jeuniaux (10) working with a Streptomyces chitinase has shown that chitinase activity can be concentrated 70 times by mass adsorption of chitinase onto chitin under acid conditions, followed by washing and restoring the pH. This method removed proteolytic and cellulolytic activity as well as pigments and reducing sugars. Ultracentrifugation indicated that the solution was homogeneous and had a molecular weight of 30,000. Using electrophoresis at pH 8.2, the proteins

were found to migrate to the cathode as 3 components. When these different fractions were quantitatively analyzed, 3 chitinases having the same specific activity per mg protein were resolved. Berger and Reynolds (3) showed 3 separate enzyme components, two chitinases and a chitobiase by starch electrophoresis of a crude chitinase produced by S. griseus.

Recent work by Horikoshi and Iida (6) showed that a crude chitinase preparation from a strain of S. griseus lysed living mycelium as well as dead washed cells of Aspergillus oryzae (Ahlburg) Cohn especially when accompanied by a lytic bacterial enzyme.

Jenson (7) strongly implicated bacteria and actinomycetes in the biological utilization of chitin in soil. He added several grams of powdered cell wall from a Lepotia sp. into 2 different soil types and showed a tremendous increase in numbers of bacteria and actinomycetes in soil after 20 days.

MATERIALS AND METHODS

Cultures of G. cingulata and Streptomyces isolates.-- Seventeen Streptomyces spp. lytic to living and killed cultures of G. cingulata isolated from soil by Lockwood (14) were grown in pure culture on yeast extract-glucose agar (per liter: yeast extract, 2 g; glucose, 20 g; agar, 20 g) in tubes. Streptomyces scabies (Thaxter) Waksman and Henrici was also included. For studies on the lysis of mycelium and of chitin, conidial suspensions from tubes were streaked in duplicate onto living and killed cultures of G. cingulata on peptone agar (per liter: agar 20 g; peptone, 5 g), and on chitin plates supplemented with Czapek's salts (per liter: $NaNO_3$, 2 g; K_2HPO_4, 1 g; KCl, 0.5 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; $FeSO_4$, 0.01 g).

Cultures of G. cingulata were maintained on potato-dextrose agar (PDA) (per liter: extract from 200 g potatoes; glucose, 20 g; agar, 20 g) in tubes. For the preparation of cultures of G. cingulata for assay purposes, approximately 1×10^7 conidia in 50 ml water were mixed with 200 ml of cooled (42°C) peptone agar. Fifteen ml aliquots were pipetted into sterile petri plates. For studies on inhibition of spore germination the plates were used immediately by placing sterile stainless steel cylinders on the surface of the agar. Material to be assayed was placed inside the cylinders.

Living cultures of G. cingulata were prepared by incubating freshly prepared plates at 26° for 4 days. Killed cultures were prepared by placing 3 day-old cultures with

the lids ajar in a desiccator containing 20 ml of propylene oxide in a beaker. Plates were removed 8 hours later, closed, and allowed to stand 18 hours before use.

Mycelial mats of G. cingulata were prepared by inoculating 50 ml of 0.5% peptone broth in 250 ml Erlenmeyer flasks with 2 x 10^4 conidia of G. cingulata. The flasks were incubated 1 week, unshaken, at 25° until a mycelial mat developed on the surface. Killed mats were prepared by autoclaving week-old cultures for 30 minutes at 121°.

Preparation of chitin--Crude chitin (Nutritional Biochemical Co. Cleveland, Ohio) was bleached by soaking 20-30 g in a 250 ml Erlenmeyer flask for 8-12 hours alternately with 50 ml of N HCL and N NaOH for a period of 5 days. Flasks were placed on a rotary shaker at 27°. The product was extracted with hot 95% ethanol (ETOH) in a Soxhlet apparatus for 2 hours (18 extractions) at which time the solvent was colorless. The extracted chitin was dried at 100°.

Milled chitin was prepared by grinding the bleached chitin to a fine powder using a 60-mesh screen in a Wiley mill.

Colloidal chitin was prepared in the following manner: Fifty g of bleached chitin was dissolved with stirring for 20-30 minutes in one liter of a mixture of H_2SO_4 and H_2O (50/50 by volume) which was prechilled to 10° to reduce hydrolysis. The dissolved chitin was filtered through glass wool in a Büchner funnel and the filtrate was stirred into 15 volumes of distilled water, precipitating the chitin

in a colloidal state. This material was dialyzed against running tap water for 3 days at which time the pH was near neutral, then against distilled water for 2 weeks. The colloidal chitin was concentrated by hanging up the dialysis bags, allowing the chitin to settle and pipetting off the excess water. The volume of the chitin suspension was determined and aliquots were oven-dried to determine the weight per ml. Such a procedure gave an 80-90% recovery of chitin in a colloidal state. The chitin suspension was then chromatographed on paper to determine if sugars were present. A colorimetric determination for N-Acetylglucosamine was also made.

Using a 2-fold dilution series of colloidal chitin, a standard curve was made. Optical density (O.D.) readings were taken for each dilution in a spectrophotometer (Bausch and Lomb Spectronic 20) at 525 mμ. The O.D. readings were also plotted on log-log paper against concentration of chitin in mg.

Chitin-agar plates were prepared by aseptically pipetting 10 ml of 2% water agar (Difco) into petri plates. After cooling, 5 ml of a solution containing 1.5% water agar (Difco) and 0.25% colloidal chitin was pipetted on top of the first layer with swirling.

Preparation of soils.-- "Natural soil" was prepared by mixing equal portions of muck and Conover loam soil. The mixture was sifted to remove any debris and water was added with mixing until the soil was fairly mosit, but still

retained its structure. "Sterile soil" was prepared by adding moistened natural soil to 250 ml Erlenmeyer flasks to a depth of 3/4 inch (60-70 g). The flasks were plugged with cotton and covered with aluminum foil to prevent drying and were autoclaved one hour each day for 5 days. "Inoculated soil" was prepared by inoculating cool, sterile soil with a heavy conidial suspension of Streptomyces isolate 3, 8, or 14. The inoculated soil was incubated 7 days before using, then was sprinkled on agar media containing living or killed G. cingulata or chitin so that the surface was covered with soil to a depth of 1/4 to 1/2 inch. At 1 and 2 weeks, 1/2 cm-square pieces of agar containing living or killed mycelia of G. cingulata from beneath the soil were squashed in duplicate between 2 microscope slides and examined with a microscope. Degree of lysis was rated according to the method of Lockwood (15).

Production of chitinase.--Streptomyces isolate 8 was grown on Reynold's mineral medium (per liter: K_2HPO_4, 0.7 g; KH_2PO_4, 0.3 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; $FeSO_4 \cdot 7\ H_2O$, 0.01 g; $ZnSO_4$, 0.001 g) using 0.5% milled or colloidal chitin to determine which medium gave maximum production of chitinase. A soluble carbon source was sometimes added. The pH of all media was adjusted to 7.0, placed in Erlenmeyer flasks to 1/5 their volume and autoclaved. The cooled media was inoculated with 1 ml of a suspension of conidia from Streptomyces isolate 8 and flasks were placed on a rotary shaker at 27°. After an appropriate length of time, the culture

was filtered through a Büchner funnel containing a cotton pad over a medium filter paper (Whatman No. 1), then through a fine porosity filter paper (Whatman No. 50).

Measurement of chitinase activity.--Chitinase activity was measured by a modification of the Morgan-Elson colorimetric determination for amino sugars (16) or by measuring a decrease in turbidity of a mixture of chitinase and colloidal chitin. The colorimetric method was as follows: P-dimethylaminobenzaldyhyde (PDAB) was recrystallized by dissolving in a minimum amount of concentrated HCl and diluted with 3 volumes of distilled water (20). Saturated sodium acetate was added with stirring until precipitation occurred. Additional sodium acetate was added to the supernatant until no more precipitation occurred. The precipitated PDAB was washed with cool distilled water and air-dried.

Ten grams of recrystallized PDAB were dissolved in 100 ml of glacial acetic acid which contained 12.5% (v/v) 10 N HCl. This color reagent was stored at 10° and diluted with 9 volumes of glacial acetic acid when used. An 0.8 M borate buffer (H_3BO_3) was prepared by adjusting to pH 8.9 with KOH. The color reagent was used in the following manner: One-half ml of a sample being tested for NAGA was placed in a test tube and 0.1 ml of borate buffer was added. The mixture was boiled 3 minutes, cooled, and 3 ml of diluted color reagent was added (2.7 ml glacial acetic acid and 0.3 ml of color reagent). The tube was incubated for 20 minutes at 37° and read in a spectrophotometer at 585 mμ.

A standard curve for NAGA was made from a 2-fold dilution series from a stock solution of NAGA containing 1 mg per ml. Colorimetric determinations were made, read in a spectrophotometer at 585 mμ and values were plotted on log-log paper against concentration expressed in mg.

The turbidity assay for chitinase was used in the following manner; 3 ml of a chitinase preparation and a suspension of chitin containing 1.6 mg per ml in 0.005 M phosphate buffer (pH 7.0), were warmed separately for 10 minutes in a 37° water bath. One ml chitin suspension was then added to the 3 ml of enzyme, gently mixed, and an initial reading quickly taken using a spectrophotometer at 525 mμ. The stock solution of colloidal chitin was prepared so that when diluted with the enzyme preparation, the initial reading was 0.40 O.D. units. The tube was then returned to the 37° water bath and readings were taken at appropriate times, generally every 10 minutes for 30 minutes.

Using a 2-fold dilution series of the enzyme preparation, dosage-response curves were determined for culture filtrates, pervaporated culture filtrates, and ammonium sulfate-and ETOH-precipitated enzyme preparations.

Protein determinations were made according to the method of Stadtman et al (19). Three ml-aliquots from a 2-fold dilution series of a standard bovine serum preparation were placed in test tubes and 3 ml of 5% trichloroacetic acid (TCA) were added, shaken, and a determination made with a spectrophotometer at 540 mμ 30 seconds later. Controls

included a water blank, a 0.005 M phosphate blank, a 3 ml protein plus 3 ml water blank and a TCA blank. The readings were plotted on log-log paper against mg of protein, giving a standard curve for TCA-precipitated protein. Protein determination was made for culture filtrate, pervaporated culture filtrate, and ETOH-precipitated chitinase. Units of chitinase were also determined for each preparation so that specific activities (units of chitinase per mg protein) could be determined.

Paper chromatography.--One dimensional paper chromatograms using n-butanol-acetic acid-H_2O (40:10:22) were run in an ascending column on Whatman No. 1 chromatography paper. Samples from reaction mixtures of chitinase plus chitin or chitinase plus mycelium of G. cingulata were spotted along with controls. After the solvent had dried, the spots were developed by dipping the paper in saturated aqueous $AgNO_3$ diluted in 200 volumes of acetone. After drying, the paper was dipped in a N NaOH solution diluted 5 times in methanol, until the spots developed sufficiently. The paper was then rinsed several times in tap water and dipped finally in a 5% solution of $Na_2S_2O_3$. After drying, spots were circled and R_f values computed.

Electrophoresis.--Attempts to migrate the enzyme by electrophoresis (RECO Model E-800-2 Migration Chamber) were made on beds of 1 and 1.5% agar. PH levels of 6, 7, and 7.8 were tried, using 0.005 M phosphate buffer. One-tenth ml of an ETOH-precipitated chitinase preparation containing

512 units per ml was placed at the origin and allowed to migrate for 8-18 hours at 250 volts. Activity was detected by spraying the agar bed with colloidal chitin and looking for clear zones formed by the dissolving of chitin 6 hours later.

Preparation of soils with chitinase.--One-half ml aliquots of an ETOH-precipitated chitinase preparation containing 140 units per ml were placed in test tubes containing 1 g air-dried natural or sterile soil. One -half ml was also placed in tubes containing no soil to serve as controls. At appropriate times, duplicate tubes were diluted with 3 ml of 0.005 M phosphate buffer, pH 7.0. The soils were mixed and centrifuged. Equal volumes of clear supernatant from the soils and the control were assayed for chitinase activity using the turbidimetric method. The residue was drained of excess supernatant and placed directly on chitin plates to qualitatively assay for chitinase activity. Within 18 hours the soil was removed and the plates were observed for lysis of chitin. To further test the stability of chitinase in soil, 1/2 ml of a chitinase preparation containing 512 units per ml was placed in 1 g natural soil and left at room temperature. Samples were placed on chitin plates daily and assayed within 18 hours to determine how long chitinase remained active in soil. Natural soil was used as a control.

RESULTS

Effect of Streptomyces isolates on living and killed cultures of G. cingulata and on chitin.--Eighteen isolates most of which were known to have lytic action on living or killed mycelium of G. cingulata were streaked on living and killed cultures of G. cingulata and on chitin plates to determine if these isolates were chitinase producers. After 10 days, all isolates lysed chitin and almost all lysed living and killed cultures of G. cingulata (Table 1). Results suggest that chitinase may be involved in lysis of living or killed mycelium, but there was no quantitative relationship between diameters of zones on living and killed mycelium and chitin plates. However, chitinase production appears to be a common phenomenon among lytic streptomyces. Streptomyces isolates 3, 8, and 14 were selected for further studies because of their superior ability to lyse killed mycelium of G. cingulata.

Lysis of living and killed cultures of G. cingulata and chitin by soil.--Natural soil and sterilized soil inoculated with Streptomyces isolates 3, 8, or 14 were placed on agar plates containing chitin or living or killed mycelium of G. cingulata. Sterilized soil and untreated cultures were used as controls. After 1 week both living and killed mycelium were lysed by natural and inoculated soil, whereas sterile soil had no effect. Chitin was lysed underneath and at a distance from the soil by inoculated and natural soils (Table 2). Both natural and inoculated

Table 1.--Lysis of living or killed cultures of G. cingulata or chitin in agar media by Streptomyces isolates streaked on the surface for 10 days.

Isolate	Diameter of lytic zone, mm		
	Living Mycelium	Killed Mycelium	Chitin
1	13	2	6
2	13	3	6
3	8	5	5
4	*	4	4
5	*	1	5
6	10	2	5
7	12	0	3
8	11	4	6
9	2	3	4
10	12	2	5
11	15	3	3
12	*	3	4
13	10	2	5
14	4	5	4
15	*	2	6
16	*	2	7
17	*	1	3
S. scabies	13	3	5

* No growth of isolate

Table 2.--Lysis of living and killed cultures of G. cingulata and of chitin in agar media by natural soils and soils inoculated with Streptomyces isolates.

Substrate	Soil	Lysis index[a] 1 week	2 weeks
Living G. cingulata	Natural	1	0
	Streptomyces # 3	2	2
	Streptomyces # 8	2	2
	Streptomyces # 14	2	1
	Sterile	4	4
	None	4	4
Killed G. cingulata	Natural	0	0
	Streptomyces # 3	2	1
	Streptomyces # 8	2	0
	Streptomyces # 14	2	0
	Sterile	4	4
	None	4	4
Chitin	Natural	✓	✓
	Streptomyces # 3	✓	✓
	Streptomyces # 8	✓	✓
	Streptomyces # 14	✓	✓
	Sterile	-	-
	None	-	-

[a]Lysis index from 0-4 with 0 indicating all mycelium lysed; 1, 1-5% of original mycelium remaining; 2, 5-50%; 3, 50-90%; 4, 90-100%; ✓ indicates lysis; - indicates no lysis.

soils and the Streptomyces isolates themselves all have the ability to lyse living and killed mycelium of G. cingulata and chitin in agar. Such results would tend to implicate actinomycetes as a factor in the lysis of fungi by natural soil. The fact that lysis occurred whether the lytic Streptomyces isolates were tested as streaks directly on the agar surface or in soil suggests that the chemical agents responsible for lysis of mycelium of G. cingulata and chitin are not inactivated by soil colloids.

To determine the time required for natural soil and soil inoculated with Streptomyces isolate 8 to lyse chitin in agar, samples of these soils were placed on chitin plates and were assayed daily by removing a portion of soil and observing for disappearance of chitin. Half the plates were covered with 5 ml of a 1% clear agar layer. Other treatments included natural and inoculated soils to which was added 20 ml of 100 ppm Chloromycetin per 60 g of moist soil. These soils were placed on chitin plates containing 100 ppm Chloromycetin per ml. This concentration of Chloromycetin did not interfere with the activity of the enzyme (Table 3). To determine the time required for lysis of chitin by isolate 8, conidia of this organism were streaked directly on chitin plates supplemented with Reynold's mineral medium. Half of these plates contained 100 ppm Chloromycetin. Lysis of chitin occurred underneath and adjacent to natural and inoculated soils and under the streaks of isolate 8 within 2 to 3 days except when Chloromycetin

Table 3.--Effect of Chloromycetin on chitinase activity.

Treatment	Units of chitinase per ml[a]
Enzyme + Substrate	36
Enzyme + Substrate + 10% Acetone[b]	20
Enzyme + Substrate + 1% Acetone	36
Enzyme + Substrate + 0.1% Acetone	36
Enzyme + Substrate + 10% Acetone + 1000 ppm Chloromycetin	16
Enzyme + Substrate + 1% Acetone + 100 ppm Chloromycetin	32
Enzyme + Substrate + 0.1% Acetone + 10 ppm Chloromycetin	32

[a] Average of 2 readings when assayed in 20 minutes.

[b] Acetone was used as the solvent for Chloromycetin.

was present (Table 4). The 5 ml layer covering the chitin did not interfere with the lysis of chitin. Even though fungal mycelium grew abundantly throughout the agar and natural soil containing Chloromycetin, no lysis of chitin was apparent. Whenever lysis of chitin occurred, colonies of bacteria or actinomycetes were visible. The results did not change after one week. It appears that bacteria and actinomycetes are active producers of chitinase and that lysis of chitin is associated with the biological portion of soil. Streptomyces isolate 8 was selected for further enzymatic studies.

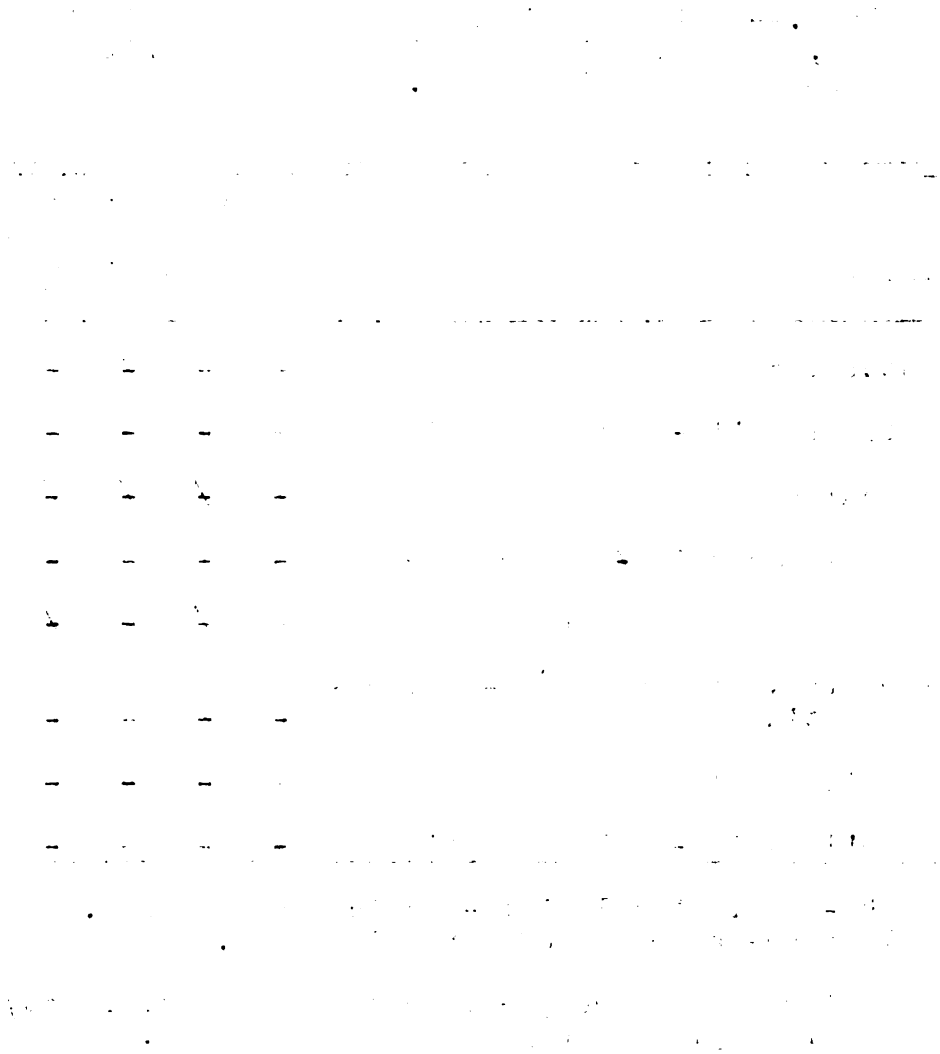
Production of chitinase.--To determine the biological effect of chitinase on G. cingulata, its stability in soil, and other properties, cell-free preparations of an active chitinase were prepared using Streptomyces isolate 8. All cultures were incubated at 27°. Preliminary studies with a medium rich in a soluble carbon source (Fries modified (1)) with and without the addition of 0.5% chitin showed that chitinase is an adaptive enzyme (Fig. 1). A much more active enzyme preparation was produced when Reynold's mineral medium plus chitin with no other carbon or nitrogen source was used. A comparison of Reynold's mineral medium plus 0.5% milled or colloidal chitin showed that higher levels of chitinase were produced and that peak production occurred earlier with colloidal chitin. (Fig. 2). Later, it was found that 0.25% colloidal chitin instead of 0.5% in culture media produced as active an enzyme preparation and sooner

Table 4.--Lysis of chitin by Streptomyces isolate 8, natural soil and soil inoculated with Streptomyces isolate 8.

Treatment	Lysis at indicated days after inoculation[a]			
	1	2	3	7
Natural soil	-	-	≠	≠
Natural soil ≠ Chloromycetin[b]	-	-	-	-
Inoculated soil	-	≠	≠	≠
Inoculated soil ≠ Chloromycetin	-	-	-	-
Streptomyces isolate 8	-	≠	≠	≠
Streptomyces isolate 8 ≠ Chloromycetin	-	-	-	-
Sterile soil	-	-	-	-
Sterile soil ≠ Chloromycetin	-	-	-	-

[a] ≠ indicates lysis; - indicates no lysis. All values are averages of 4 replications.

[b] 100 ppm Chloromycetin per ml in agar. Twenty ml of 100 ppm Chloromycetin per 60 g soil.



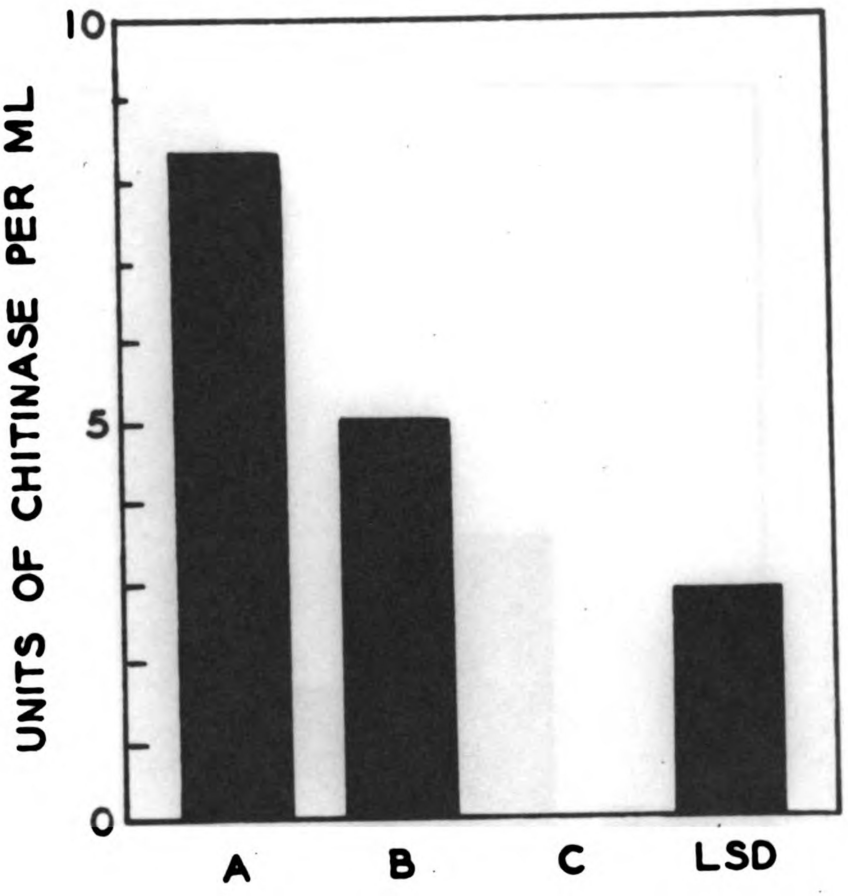


Fig. 1. Effect of varying carbon source in culture media on production of chitinase by Streptomyces isolate 8. A) Mineral medium plus 0.5% colloidal chitin, incubation period 3 days. B) Mineral medium plus 0.5% colloidal chitin plus 2% glucose, incubation period 3 days. C) Mineral medium plus 2% glucose, incubation period 3 days. LSD) Least significant difference at 5% level.

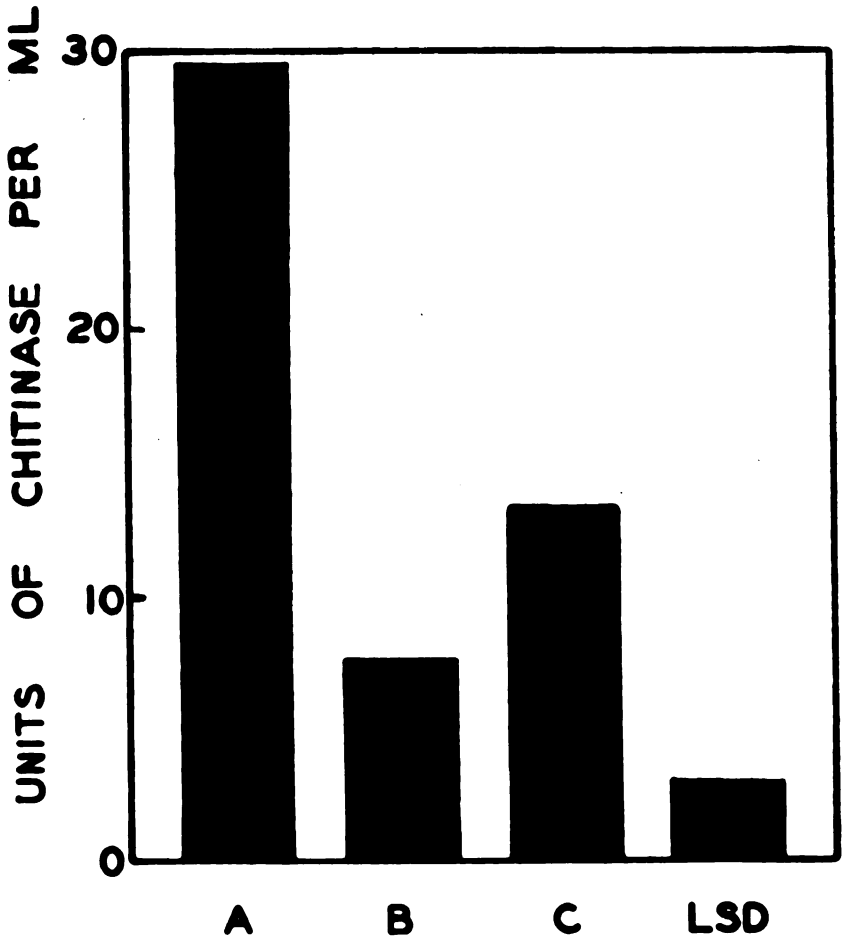


Fig. 2. Effect of different chitin preparations in culture media on production of chitinase by Streptomyces isolate 8. A) 0.5% colloidal chitin, incubation period 3 days. B) 0.5% Wiley-milled chitin, incubation period 3 days. C) 0.5% Wiley-milled chitin, incubation period 6 days. LSD) Least significant difference at 5% level.

than 0.5, 0.75, or 1.0% concentrations of colloidal chitin (Fig. 3). Therefore, for routine production of chitinase, Reynold's mineral medium plus 0.25% colloidal chitin was used, and cultures were harvested on the fourth day after incubating at 27° with Streptomyces isolate 8.

Characteristics of the turbidimetric assay.--A standard curve was prepared using a 2-fold dilution series of chitinase concentrated by pervaporation to approximately 1/5 the original volume, then precipitated with 95% ETOH and resuspended in 0.005 M phosphate buffer at pH 7.0. The O.D. readings for the 2-fold dilutions gave a straight line when plotted on log-log paper (Fig. 4). Activity of this preparation was assigned at 512 units per ml. A 1/8 dilution of this standard containing 64 units per ml produced a change in O.D. of 0.14 after 20 minutes at 37°. The standard chitinase preparation was stored at -18° and a dilution series was made whenever any experiment on chitinase activity was made. The slope and position of the dosage-response curve remained unchanged during the course of the chitinase studies, indicating chitinase is very stable when stored under these conditions. All other preparations of chitinase gave dosage-response curves with the same slope as the standard curve.

Increasing the concentration of chitin in the assay tubes from a concentration of 0.07 mg per ml to 0.45 mg per ml gave a straight line when plotted against units of activity on log-log paper (Fig. 5). At concentrations higher than 0.45 mg chitin per ml, the curve flattened,

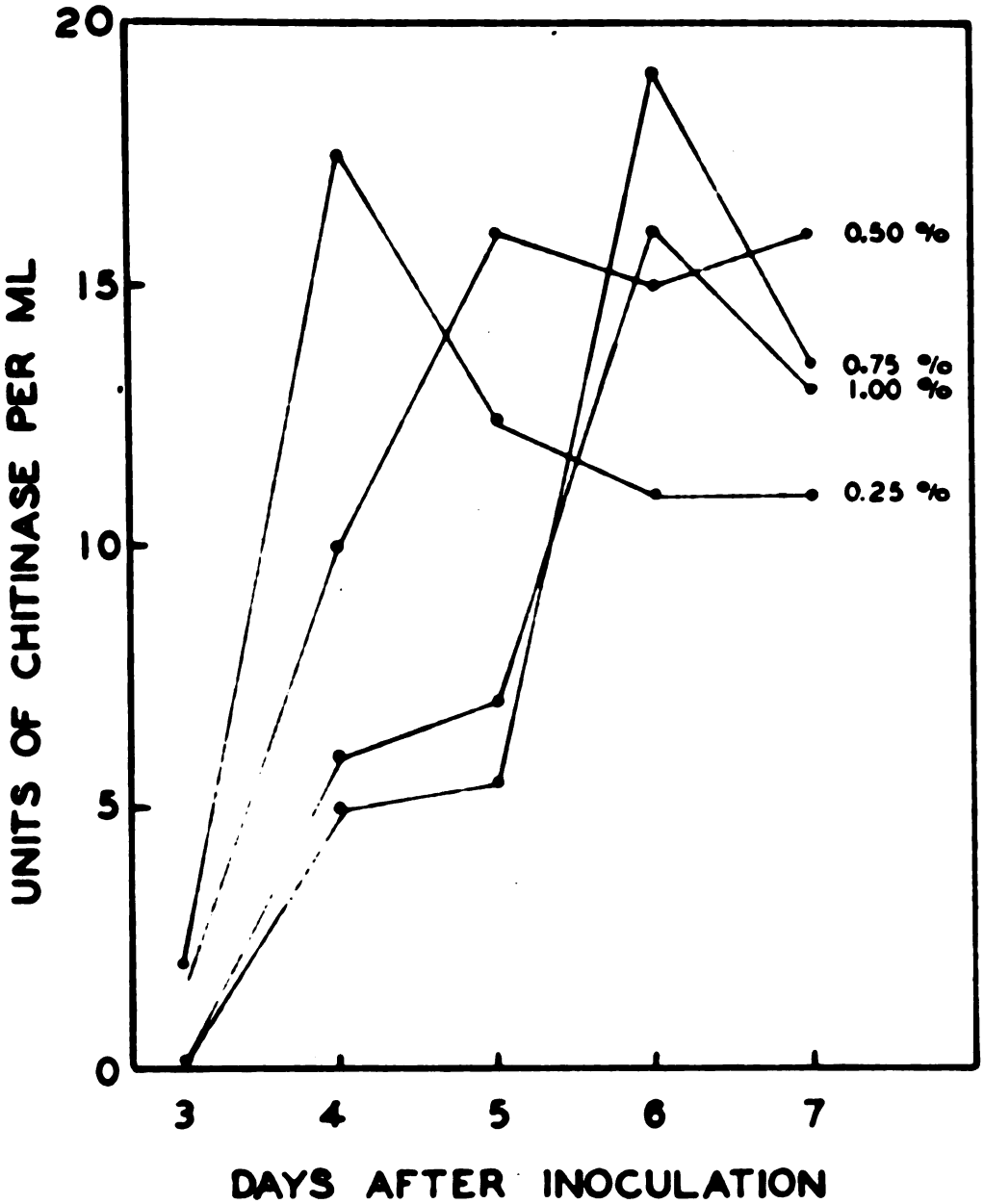


Fig. 3. Effect of varying concentrations of colloidal chitin in culture medium in chitinase production by Streptomyces isolate 8.

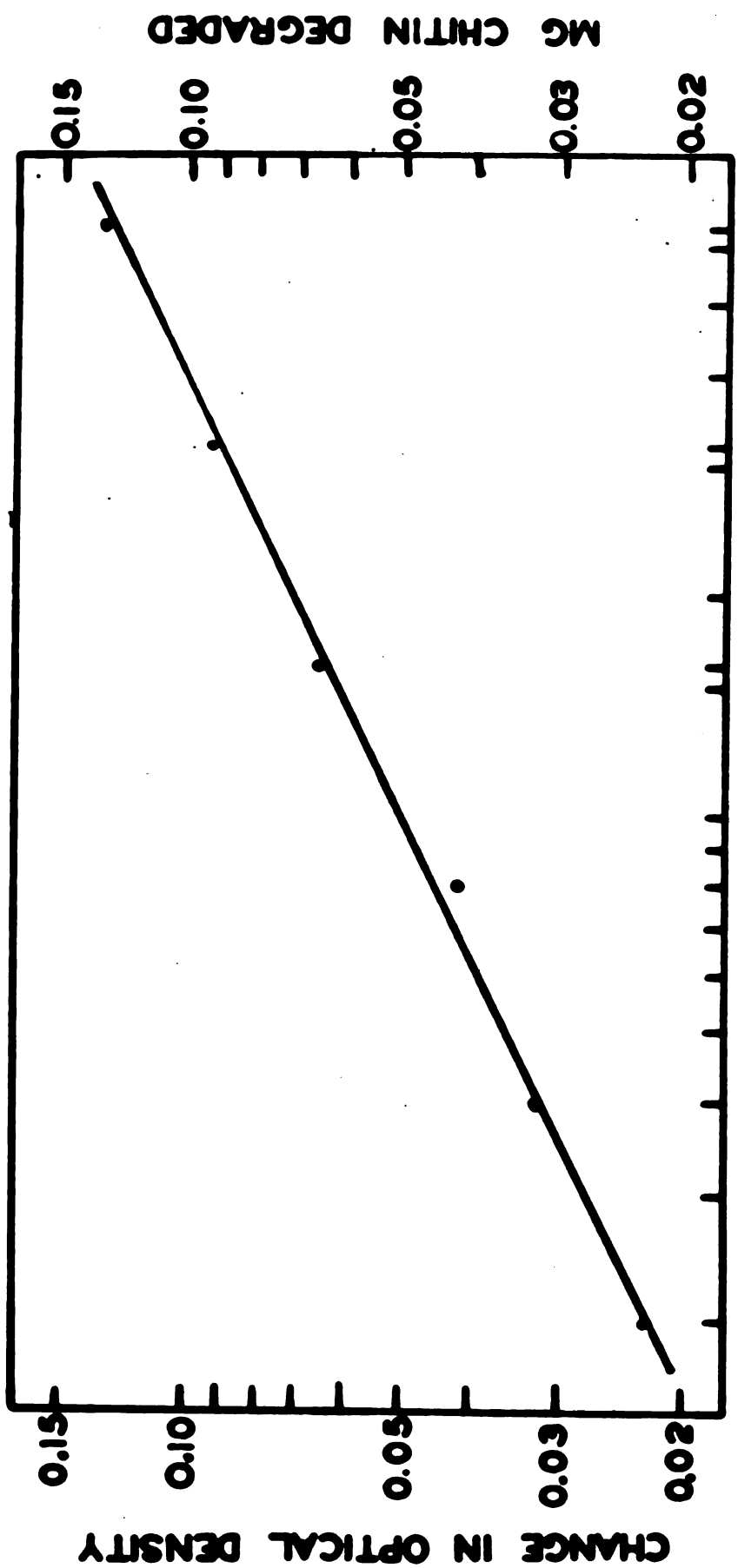


Fig. 4. Dosage-response curve for chitinase activity expressed as change in O. D. and mg chitin degraded.

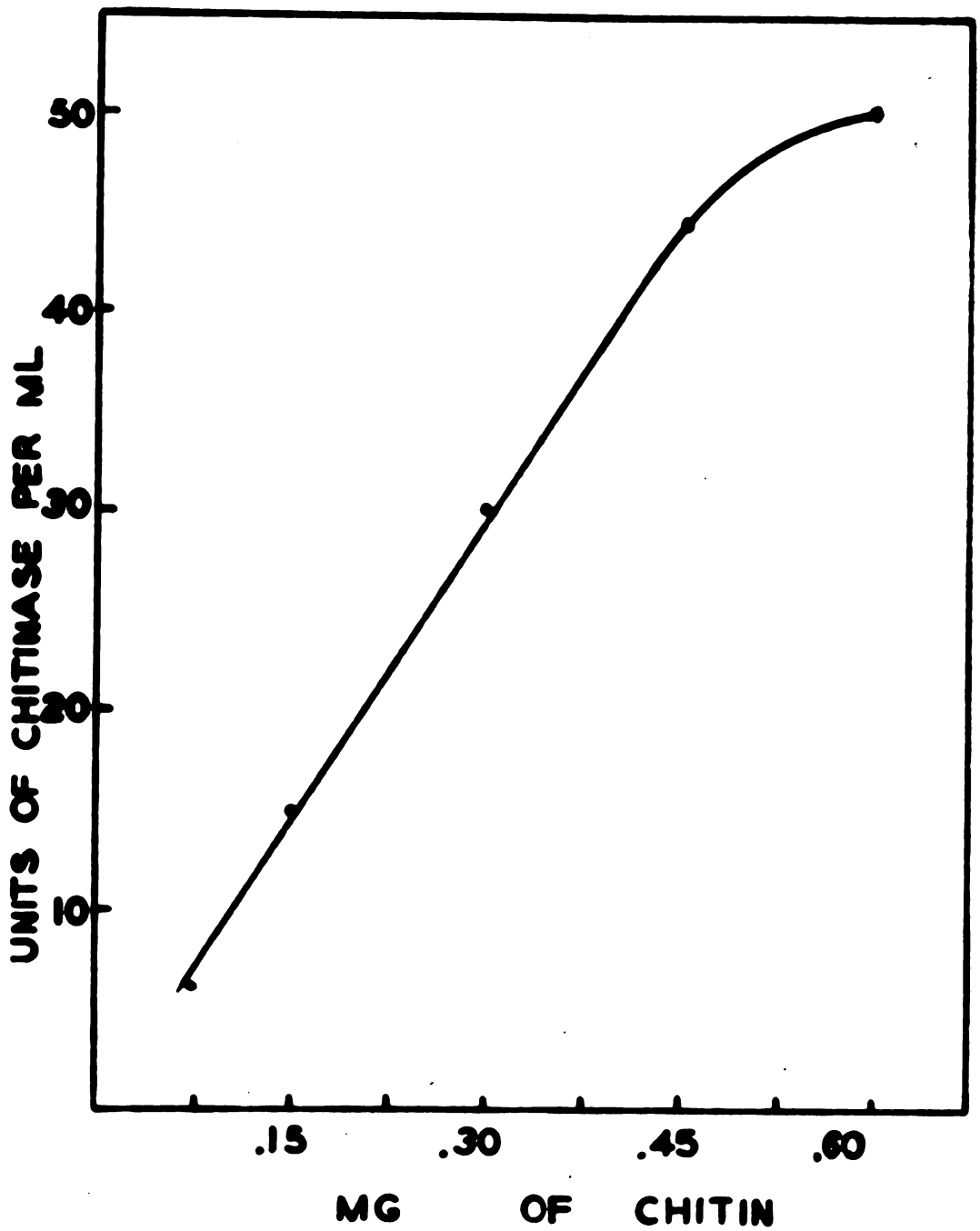


Fig. 5. Effect of substrate concentration on chitinase activity. Various concentrations of chitin were reacted for 20 minutes with the same amount of chitinase.

indicating that further concentrations of substrate did not increase the reaction rate appreciably.

When units of chitinase were plotted on log-log paper against mg of chitin degraded, a straight line resulted having the same slope and position as the standard curve for chitinase (Fig. 4). By coincidence, the O.D. reading itself was a measure of the weight in mg of colloidal chitin present in the assay tube. A concentration of 0.40 mg of chitin per ml in the assay tube was selected as the concentration of substrate for all enzymatic assays.

With the 2-fold dilution series used in the preparation of the standard curve, mg of chitin degraded per unit of chitinase was plotted on log-log paper. A straight line curve resulted with a descending slope (Fig. 6). The amount of chitin degraded per unit of enzyme decreased with increasing enzyme concentration. However, more concentrated preparations were used for assays to minimize the experimental error resulting from trying to detect small changes with the spectrophotometer. Generally, preparations containing at least 32 units of chitinase per ml were used for enzymatic studies.

Effect of concentrating on chitinase activity.--Freshly harvested cell-free filtrate was placed in dialysis tubing and pervaporated to about 1/5 its original volume. The enzyme was precipitated from the pervaporated culture filtrate in 2 ways: 1) Chilled pervaporated culture filtrate (1^{o}) was brought to 0.75 saturation with $(NH_4)_2SO_4$ and left

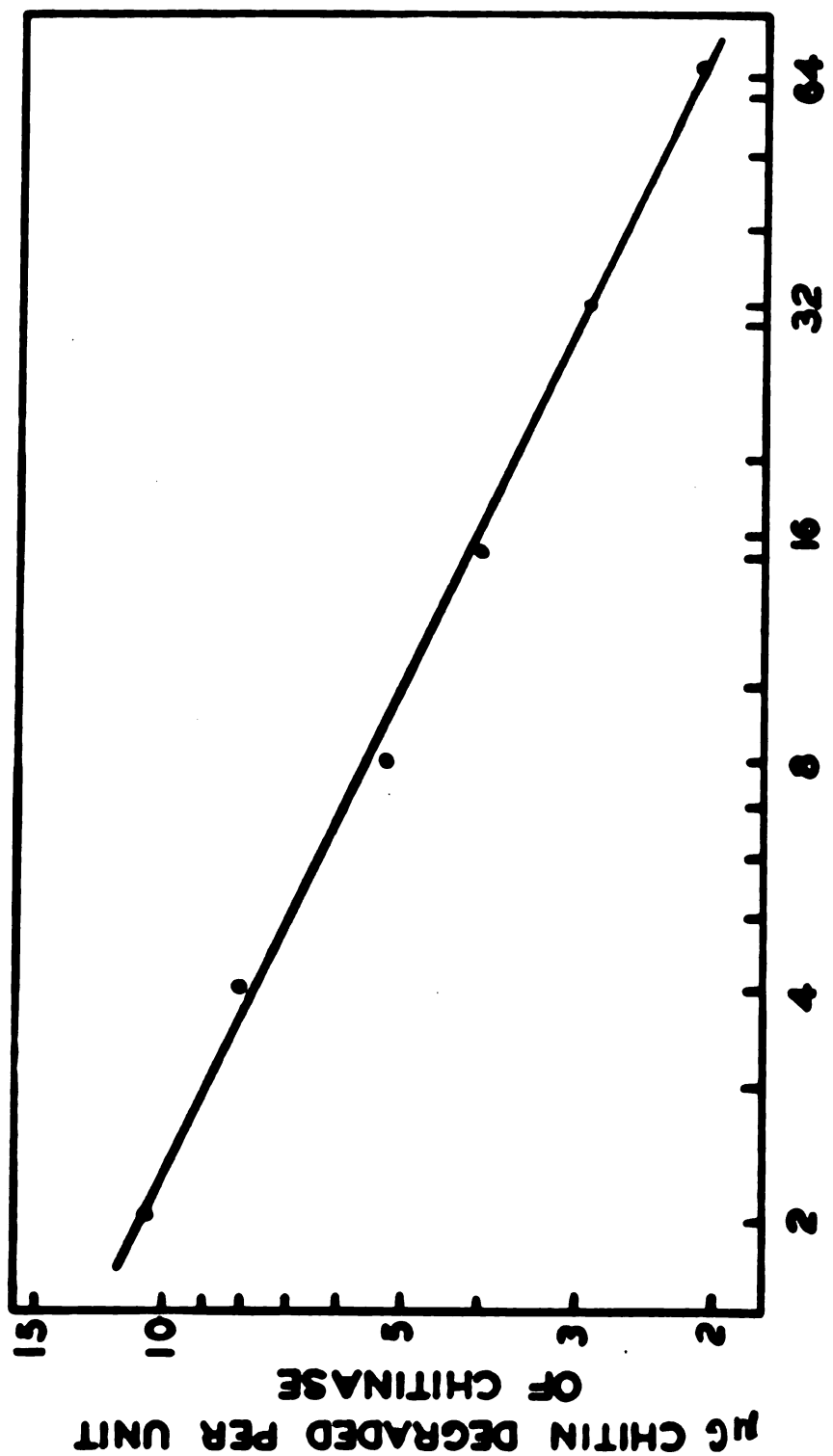


Fig. 6. Effect of chitinase concentration on efficiency of chitin degradation.

standing overnight at pH 6.5-7.0 at 1°. The culture fluid was centrifuged at 1° for 5 minutes at 6000 rpm and taken up in 0.005 M phosphate buffer at pH 7.0. 2) Chilled ETOH (-18°) was added to chilled, (1°) pervaporated culture filtrate so that the final concentration of ETOH was 70%. The precipitated protein fraction was immediately centrifuged at 1° for 5 minutes at 6000 rpm. The precipitate was resuspended in a minimum volume of 0.005 M phosphate buffer at pH 7.0. The denatured protein was centrifuged down for 5 minutes at 5000 rpm and the supernatant used for enzymatic assays. This final preparation was concentrated 45 times as compared with original culture filtrate. Dry weight determinations, total protein, and assays of chitinase activity were made with the culture filtrate, pervaporated culture filtrate, and ETOH-precipitated chitinase preparations (Table 5). Concentrating chitinase by the ETOH method gave a very efficient recovery. Little or no loss in activity resulted by pervaporation or precipitation with ETOH. The final concentrated preparation contained 95.6% of the total activity calculated in the culture filtrate. Specific activity (units of activity per mg of protein) was found to increase slightly, probably due to the removal of the denatured protein. No attempt was made to separate the enzyme from the protein fraction although preliminary experiments indicated this was possible, following the purification technique of Jeuniaux (10). Based on dry weight, the final ETOH-precipitated preparation was found to contain

Table 5.--Chitinase activity and specific activity of various chitinase preparations.

Treatment	Units/ ml	Total ml	Total units	Units/ mg dry wt.	Specific activity
Culture filtrate	16	1000	16,000	7.0	762
Pervaporated culture filtrate	70	216	15,120	7.5	875
Ethanol-precipitated preparation	680	22.5	15,300	24.6	988

3.5 times more activity than the culture filtrate.

PH optimum for activity of chitinase.--Colloidal chitin and ETOH-precipitated chitinase were placed in separate tubes and adjusted to appropriate pH values with HCl or NaOH. Controls included boiled enzyme plus substrate, enzyme only, and substrate only. Duplicates were made of all assay tubes. After warming separately for 10 minutes at 37°, the aliquots were mixed and read against a buffer blank every 10 minutes for 1 hour. Using concentrated enzyme preparations from 2 different batches of culture filtrate, a broad optimum pH range from 5.5-7.5 was found (Fig. 7). The two preparations, however, gave different values at pH 6.5 (test 1 and test 2). A third concentrated enzyme preparation was tested (test 3) at pH 6.0, 6.5, and 7.0. The results were quite similar to test 1. All other enzymatic studies were made at pH 7.0.

Effect of temperature on chitinase activity.--Preliminary studies showed little difference in enzymatic activity between 37° and 24° when the assay time was one hour or more. Preparations assayed under still or shaken conditions at 24° did not differ in activity. Since very active preparations were prepared when ETOH was used to precipitate the enzyme, a shorter assay time became possible. Aliquots from an ETOH- precipitated preparation were equilibrated in duplicate for 10 minutes at 24°, 37°, and 42°. Colloidal chitin in 0.005 M phosphate buffer at pH 7.0 was warmed separately. After mixing, turbidity measurements were made

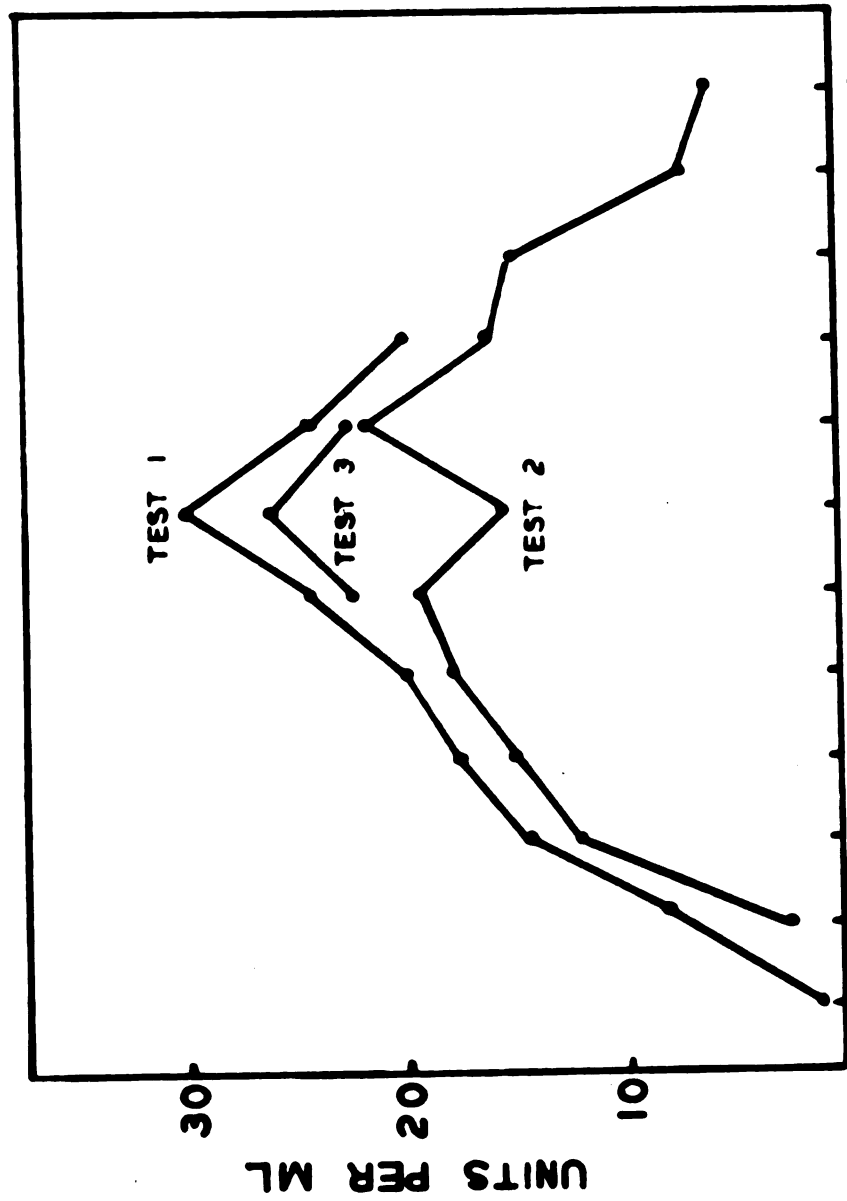


Fig. 7. Effect of pH on chitinase activity produced by Streptomyces isolate 8.

every 5 minutes for 30 minutes. Results showed that 37° was the optimum temperature for chitinase activity (Fig. 8). This temperature was selected for enzymatic assays. Five minutes of boiling completely inactivated the enzyme when a boiled enzyme preparation was desired as a control.

Relationship between chitin degradation and NAGA formation.--NAGA colorimetric determinations were used in biological assays to measure chitinase activity on mycelium of G. cingulata. Since quantitative assays of chitinase were based on turbidimetric measurements, it was desirable to know the relationship between chitin degradation and NAGA formation. A 2-fold dilution series of a concentrated enzyme preparation containing 360 units per ml was reacted with colloidal chitin for 30 minutes. Checks included boiled enzyme and substrate, enzyme alone, and substrate alone. The turbidimetric O.D. readings were referred to the standard curve for colloidal chitin to determine mg of chitin degraded. At the end of 30 minutes all tubes were boiled and NAGA determinations made. The values were referred to the standard curve for NAGA to determine mg of NAGA formed. It was found that 0.45 mg of NAGA was formed per mg of chitin degraded. This value remained constant for any dilution for which NAGA was determined when compared to the corresponding dilution of chitin.

To determine if any enzyme might be present which could further degrade NAGA formed from action of chitinase on chitin, duplicates of a preparation of chitinase containing

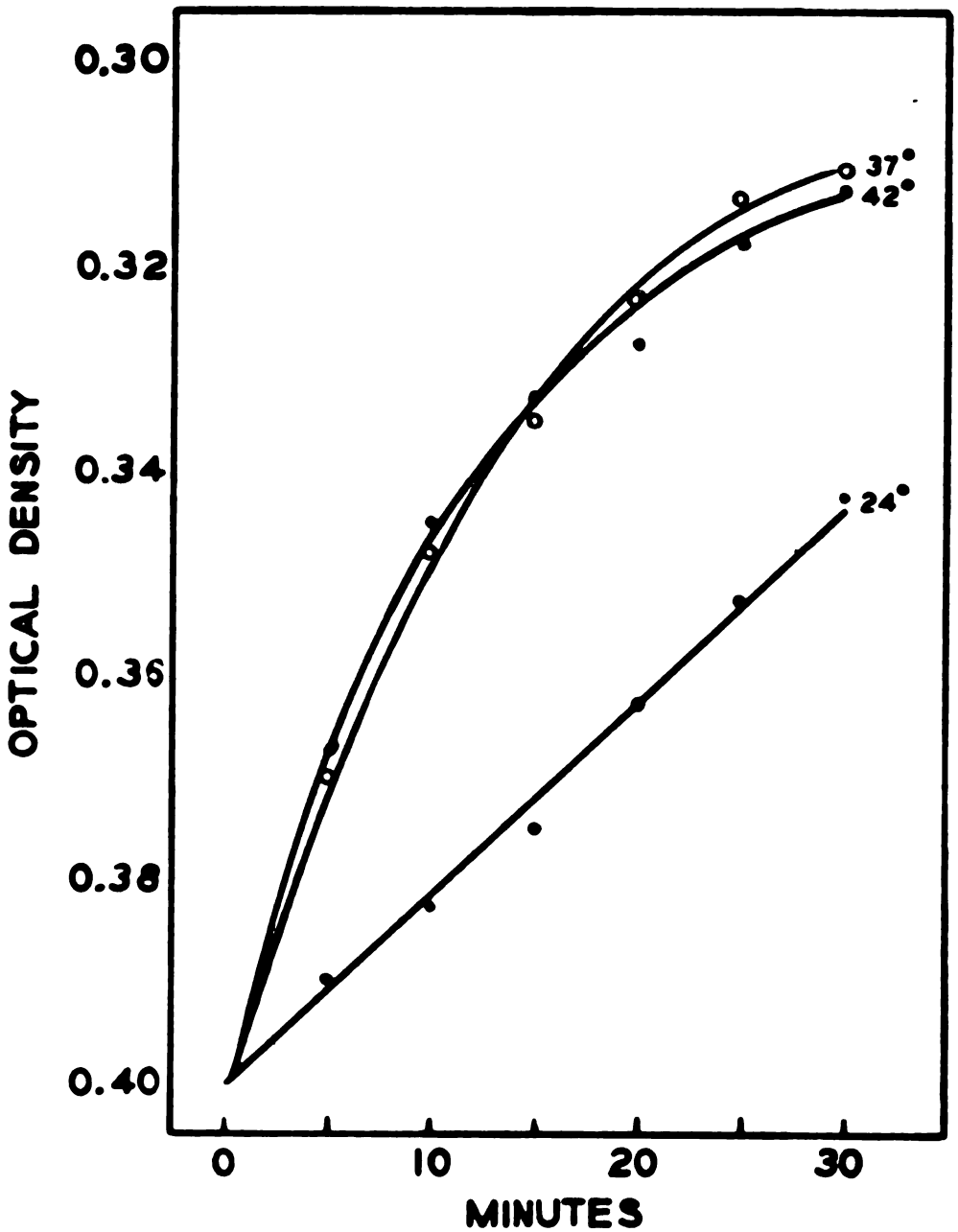


Fig. 8. Effect of temperature on chitinase activity produced by Streptomyces isolate 8.

360 units per ml were mixed with a 2-fold dilution series of NAGA and allowed to react for 30 minutes. Controls included boiled chitinase plus NAGA, chitinase alone, and NAGA alone. The chitinase preparation had no effect upon NAGA. It was concluded that the value of 0.45 mg of NAGA formed for every mg of chitin degraded by this chitinase preparation represented the actual amount of NAGA formed from this reaction.

Paper chromatography was undertaken to determine the sugars formed by the action of chitinase on colloidal chitin. Three ml of a preparation of chitinase containing 512 units per ml were reacted with 1 ml of a suspension containing 2.43 mg of chitin for 6 hours at 37°. The excess chitin was centrifuged down and the supernatant chromatographed. The standard solution of NAGA, chitinase only, and chitin only were also chromatographed. The NAGA standard had an R_f of 0.40 (Fig. 9). No sugars were apparent in the enzyme only and substrate only. Two spots from the reaction mixture of enzyme and substrate were found. One had an R_f of 0.40 and was identified as NAGA, the other had an R_f of 0.30 and was not identified, although it may be a polysaccharide such as was found by Reynolds (17). It was concluded that more than one enzyme component which degrades chitin was present in the chitinase preparation.

Electrophoresis of chitinase preparations.--Attempts to separate a chitinase preparation into more than one

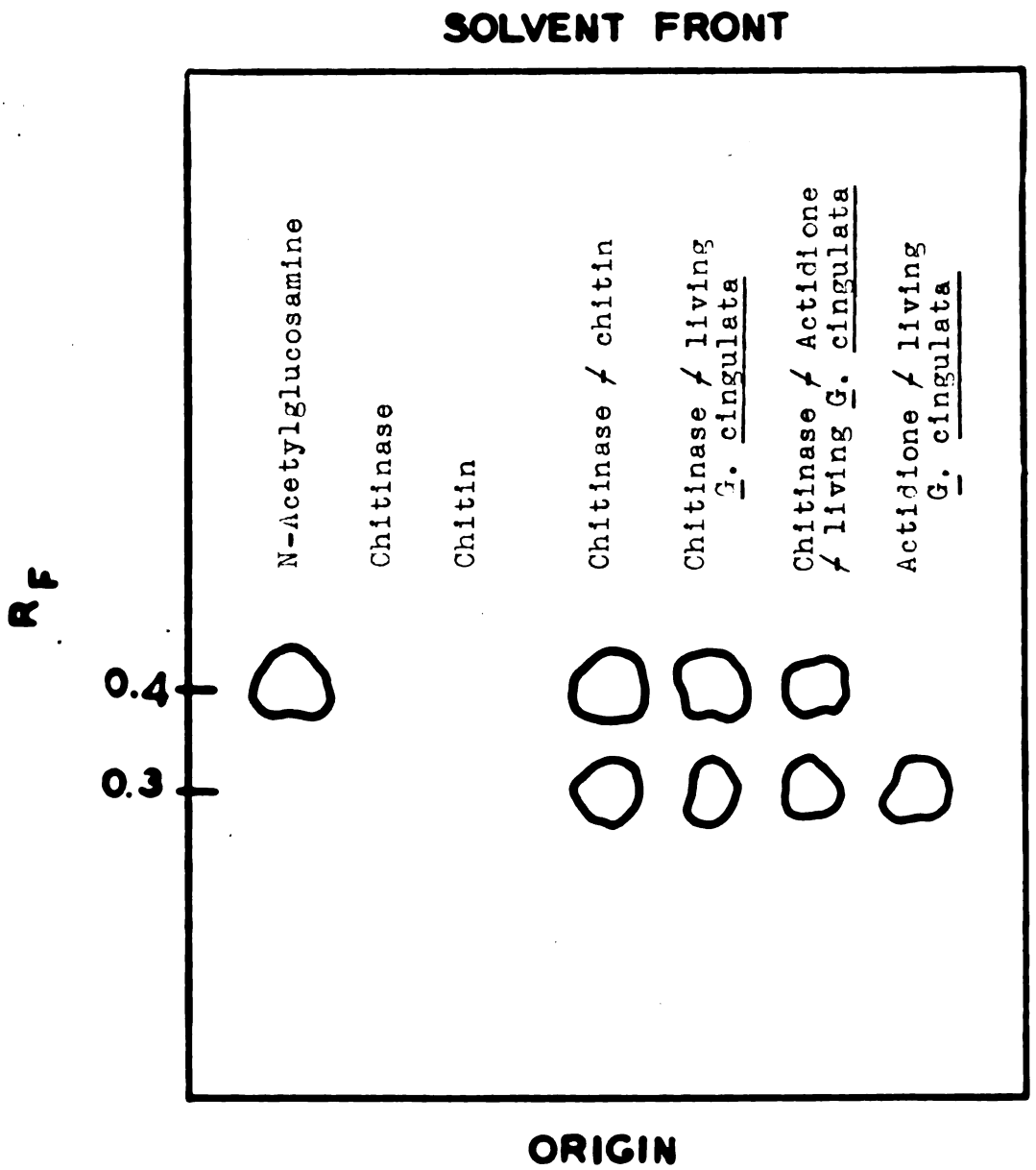


Fig. 9. Paper chromatogram of reaction mixtures. Chromatograms were developed by dipping in $AgNO_3$, then in NaOH, followed by washing in tap water, then dipping in $Na_2S_2O_3$.

component were unsuccessful. Activity was shown as far as 11.5 cm toward the cathode from the origin when 0.1 ml of a preparation containing 512 units per ml was allowed to migrate for 20 hours at pH 7.8, then sprayed with chitin and assayed after 6 hours.

Biological activity of chitinase.--The effect of chitinase on conidia and mycelia of G. cingulata was studied by placing material to be assayed on agar media containing living or killed mycelium and on chitin plates. Controls included distilled water and 0.005 M phosphate buffer at pH 7.0 (higher concentrations of buffer were found to be toxic to G. cingulata). Culture filtrate, ammonium sulfate and ETOH-precipitated chitinase preparations all dissolved chitin in agar. Culture filtrate and ammonium sulfate-precipitated chitinase both inhibited development of germ tubes following spore germination. Faint lysis zones were produced on living mycelium. ETOH-precipitated chitinase did not manifest these toxic effects. Figure 10 shows the inhibitory effect of ammonium sulfate-precipitated chitinase on germinating conidia of G. cingulata after 18 hours as compared to a control (Fig. 11). The supernatant from the ETOH-precipitated enzyme was tested on conidia of G. cingulata after removing the ETOH by concentrating to original volume in a flash evaporater under vacuum at 32° for 4 hours. Inhibition of germ tube growth was also caused by this fraction. Therefore, all enzymatic studies and biological assays were made with the ETOH-precipitated enzyme.

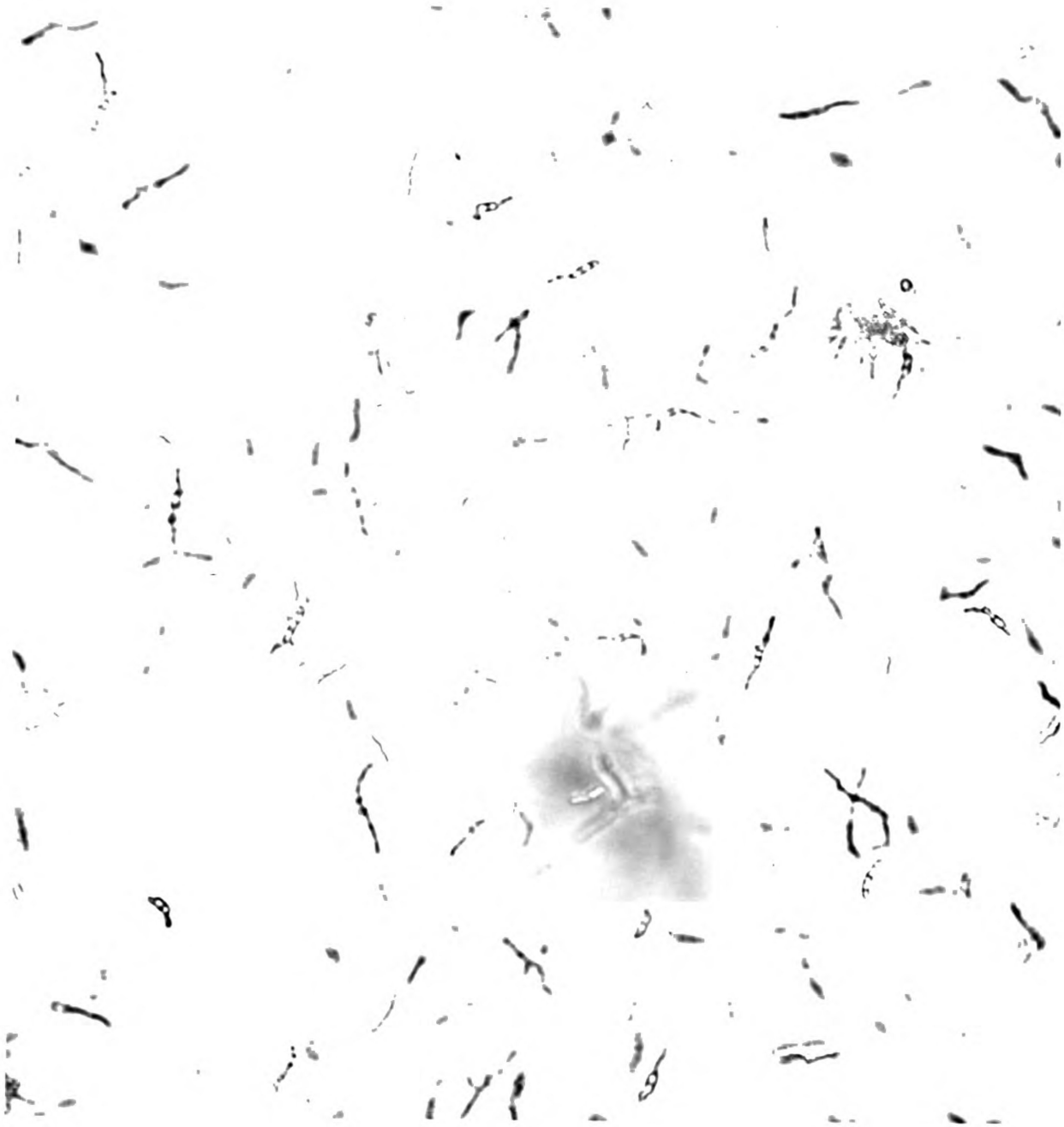


Fig. 10. Inhibition of germ tubes of G. cingulata in 0.5% peptone-agar 18 hours after being in contact with an $(NH_4)_2SO_4$-precipitated chitinase preparation.

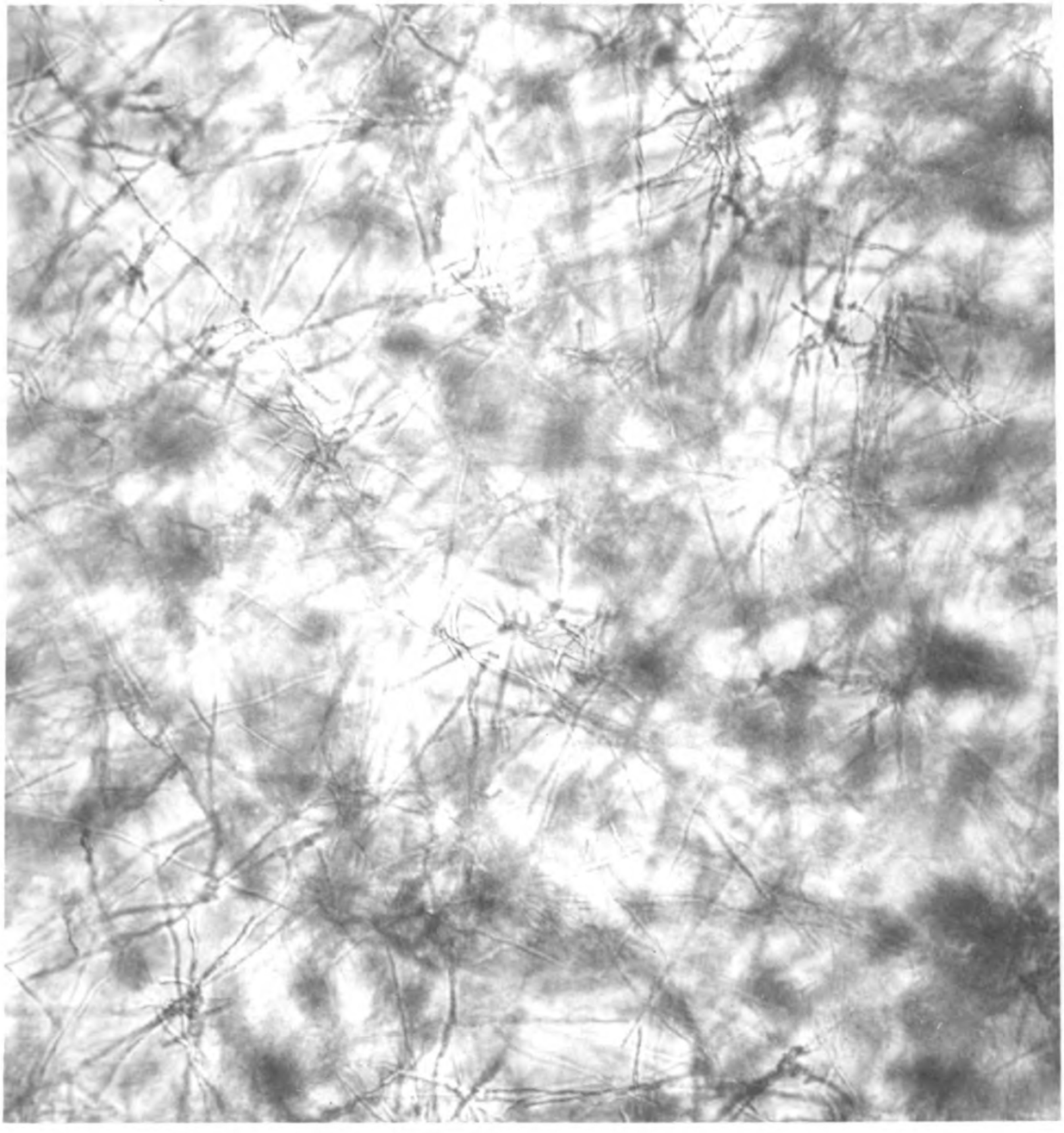


Fig. 11. Untreated mycelium of G. cingulata in 0.5% peptone-agar 18 hours old.

Since the toxic factor was apparently not present in the ETOH-precipitated chitinase preparation, an attempt was made to determine if chitinase itself had any effects on living and killed mycelium of G. cingulata. Disks of washed mycelium from living and killed mats produced in broth were punched out with a 12 mm diameter cork borer. Disks from the same mat were distributed uniformly among the different treatments. Each assay tube received 7 disks of living or killed mycelium. The following reaction mixtures were made: ETOH-chitinase plus living mycelium, ETOH-chitinase plus killed mycelium, ETOH-chitinase plus living mycelium plus 1000 ppm Actidione, and living mycelium plus 1000 ppm Actidione. This concentration of Actidione only slightly inhibited chitinase activity, apparently due to the ETOH it was dissolved in (Table 6).

Controls included living or killed mycelium without chitinase, chitinase only and buffer only. Four tubes were used for each of the treatments. All tubes were incubated at 37° for 7 hours, then colorimetric determinations were made. Living or killed mycelium or Actidione plus living mycelium produced no detectable NAGA. Large amounts of NAGA were formed when the enzyme acted upon living mycelium with or without the presence of Actidione (Table 7). Smaller amounts of NAGA were formed when chitinase acted on killed mycelium. This was correlated with a 16% decrease in weight, a fact not evident when the enzyme acted on living mycelium. Since enzyme systems in the living mycelium

Table 6.--Effect of Actidione on Chitinase Activity.

Treatment	Units of chitinase per ml[a]
Enzyme ≠ substrate	32
Enzyme ≠ Substrate ≠ 10% ETOH	25
Enzyme ≠ substrate ≠ 1% ETOH	28
Enzyme ≠ substrate ≠ 10% ETOH ≠ 1000ppm Actidione	25
Enzyme ≠ substrate ≠ 1% ETOH ≠ 100 ppm Actidione	28

[a] Average of 2 readings when assayed at 20 minutes.

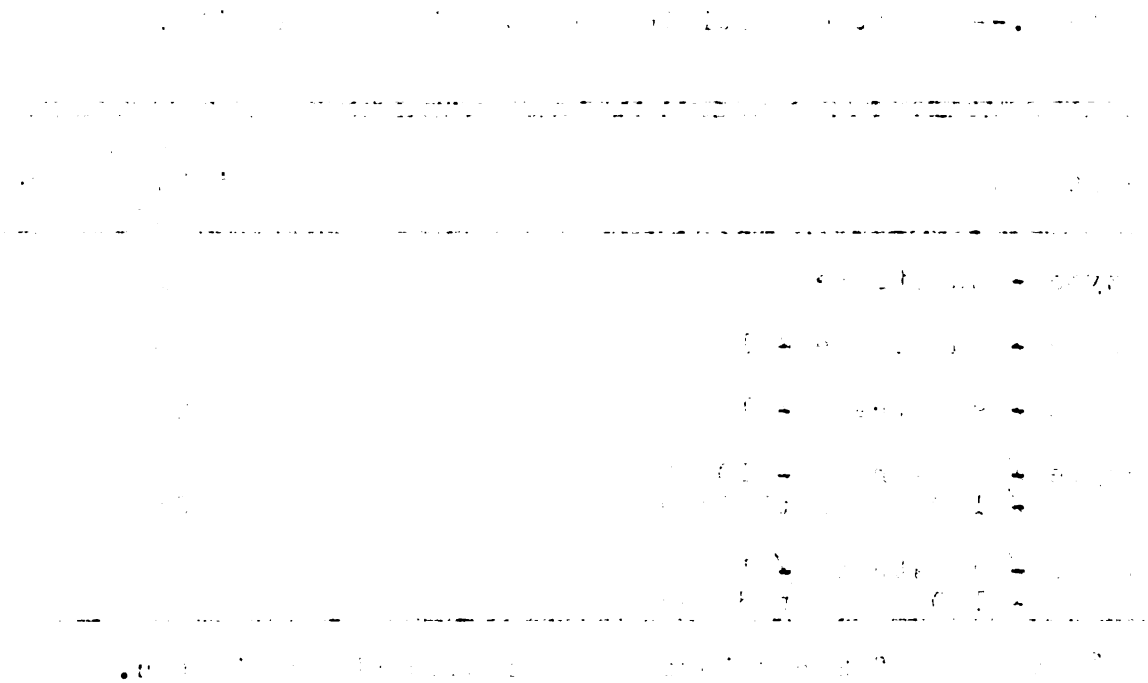


Table 7.--Effect of chitinase on mycelium of G. cingulata after 7 hours at 37° [a].

Treatment	mg NAGA formed	mg dry wt.	% loss in dry wt.
Enzyme + living mycelium	0.485	5.1	0
Enzyme + killed mycelium	0.095	3.7	16
Enzyme + 100 ppm Actidione + living mycelium	0.275	5.0	
1000 ppm Actidione + living mycelium	0.00	4.9	4
Living mycelium control	0.00	5.1	
Killed mycelium control	0.00	4.4	

[a] Figures given are averages of 4 replicates.

itself apparently induced production of NAGA in the presence of the chitinase preparation, the value for killed mycelium was accepted as a more valid estimation of actual NAGA formed from chitin degradation of mycelium of G. cingulata. A small loss in dry weight of mycelium was found when Actidione acted on living mycelium, with or without chitinase.

Paper chromatograms of the reaction mixtures in the above experiment were made in duplicate and showed 2 sugars having R_f values of 0.40 and 0.30 when enzyme and living mycelium were reacted together (Fig. 9). Two spots having the same R_f values were found when the enzyme was reacted with colloidal chitin. Another sugar having an R_f value of 0.30 was formed by the action of Actidione on mycelium. It is not known if this sugar and that formed by the action of chitinase on mycelium are the same. Standard NAGA again had an R_f value of 0.40.

From the above experiment the amount of NAGA formed when chitinase was reacted with killed mycelium was determined. Using the relationship between NAGA formation and chitin disappearance which had previously been established (0.45 mg NAGA formed per mg chitin degraded) the amount of chitin removed from the mycelium during the course of the reaction was computed. Four and eight-tenths percent of the total dry weight of killed mycelium of G. cingulata had chitin which was dissolved. This estimate agrees more closely with the amount of chitin in cell wall of killed G. cingulata as found by Blumenthal and Roseman (4) than the 16% calculated on a dry weight basis. Since high quantities

of NAGA without any measurable weight loss were found when the enzyme was reacted with living mycelium, the possible toxic effect of chitinase on living G. cingulata could not be determined. Qualitative tests were attempted to determine this. One-tenth ml aliquots of chitinase containing 512 units per ml were applied to living and killed cultures of G. cingulata at 24°, 28°, and 37° for 12 hours. Killed cultures showed a lysis zone at every temperature, but living cultures were lysed only at 37°. Killed cultures of G. cingulata, but not living mycelium, appear to be readily attacked by chitinase. The lysis of living mycelium at 37° may have occurred either because the temperature first killed G. cingulata or because the factor resisting chitinase invasion in living mycelium broke down at the high temperature.

Stability of chitinase in soil.--To determine if chitinase was stable in soil, both quantitative and qualitative assays were made. One-half ml aliquots of a chitinase preparation containing 140 units per ml were added to 1 g of air-dried samples of natural and sterile soil in centrifuge tubes, and to tubes containing no soil. Controls included natural and sterile soils with no enzyme and 0.005 M phosphate buffer (pH 7.0) which was placed in natural and sterile soil, recovered and enzyme added to it. All treatments were done in duplicate at 26°. Chitinase activity was expressed as units per gram of oven-dried soil. Aliquots of the residue

were placed on chitin plates and examined 18 hours later. Lysis of chitin within this time is assumed to occur by virtue of the chitinase added, since 48-72 hours are required to lyse chitin by natural soil, sterile soil inoculated with Streptomyces isolate 8, and by Streptomyces isolate 8 itself. (Table 4). Figure 12 shows the rate of decrease in activity of the enzyme recovered from soil. A rapid drop in activity occurred for both natural and sterile soil containing enzyme, and also for the control. Therefore, this loss in activity cannot be attributed to inactivation by soil. When the last determination was made 51 hours after addition of chitinase to soil, activity was still detectable in both soils. In addition, all residues lysed chitin in agar after 18 hours, indicating that all the chitinase enzyme was not recovered in the supernatant. Even if the soil had bound some of the enzyme, preventing its recovery in the supernatant, the residue was still able to exert a localized chitinase effect. Natural or sterile soil controls containing no chitinase did not lyse chitin in less than 48 hours.

One ml of a chitinase preparation containing 280 units per ml was mixed with 2 g of natural soil and incubated at 26°. Natural soil without enzyme was included. Assays made each day for a week lysed chitin underneath the enzyme-supplemented soil within 18 hours. If chitinase can be recovered in supernatant and show activity a week after being stored in soil, at room temperature, it may be important as one of many factors accounting for the decomposition of biological material in soil.

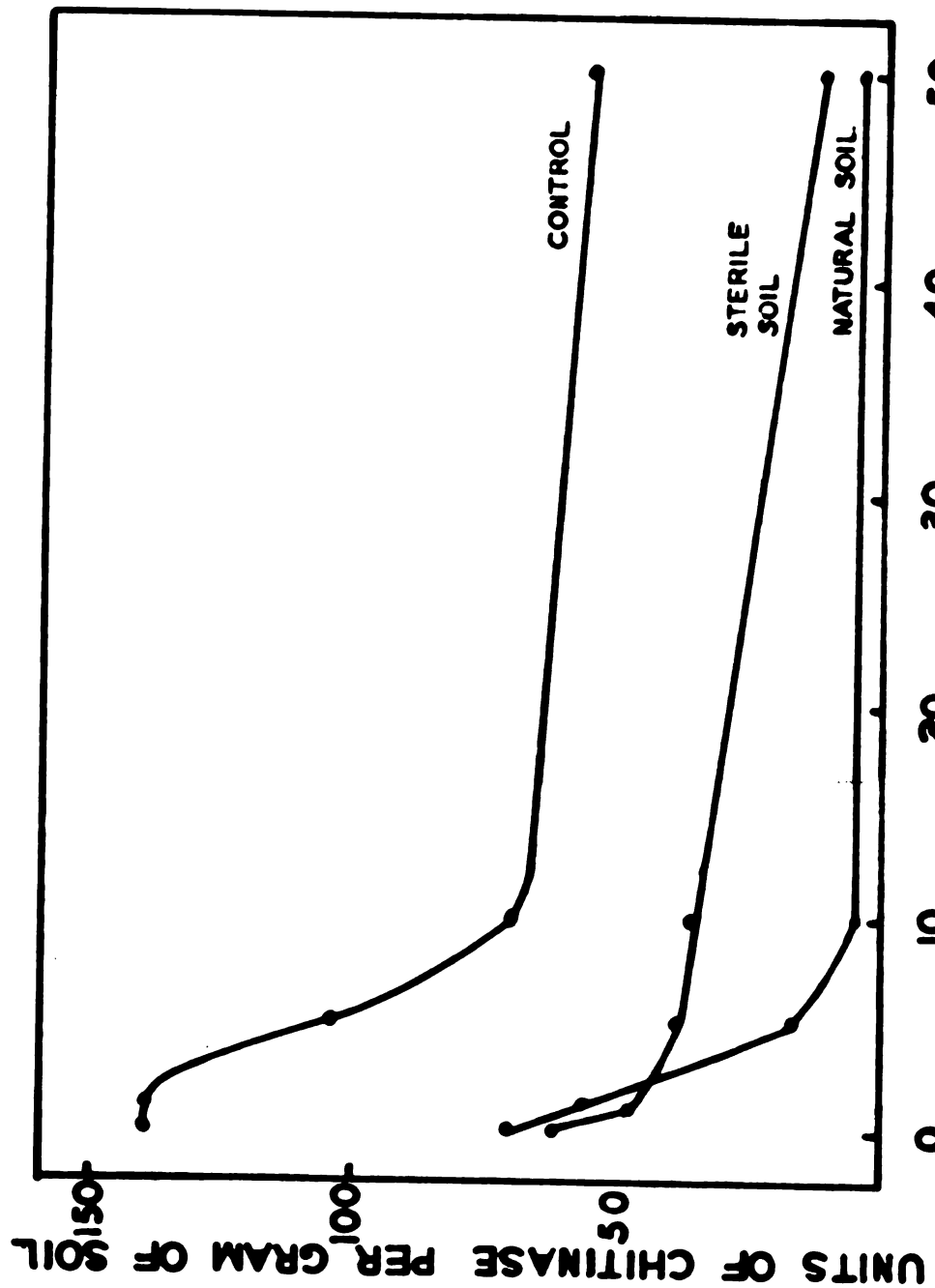


Fig. 12. Recovery of chitinase activity from soil. The soils were mixed with 0.005 M phosphate buffer (pH 7.0), centrifuged, and the supernatant was assayed for chitinase activity.

DISCUSSION

Concentrating chitinase by ETOH-precipitation gave excellent (95.6%) recovery of enzyme from culture filtrates of Streptomyces isolate 8. This technique increased chitinase activity per mg of dry weight 3.5 times. Jeuniaux (10) obtained a preparation of Streptomyces chitinase by adsorption of the enzyme on colloidal chitin. This preparation undoubtedly was of a higher purity. Apparently no one has previously reported the efficiency of concentrating with ETOH or of any other method used to collect and concentrate chitinase from culture filtrate, therefore, a comparison of techniques is not possible. In addition to efficient recovery, materials toxic to living mycelium of G. cingulata were left behind in the supernatant when the enzyme was precipitated. This preparation had a distinct advantage for studying biological effects of chitinase over culture filtrate, pervaporated culture filtrate, or $(NH_4)_2SO_4$-precipitated enzyme preparations, all of which retained this toxic principle.

ETOH-precipitated chitinase lysed killed mycelium of G. cingulata at 24°, 28°, and 37°, but had no effect on living mycelium except at 37°. Horikoshi and Iida (6) working with a crude chitinase preparation from S. griseus reported lysis of living cells of A. oryzae. It may be that A. oryzae is more sensitive to chitinase activity than is G. cingulata, or that the crude chitinase contained toxic agents which autolysed A. oryzae allowing chitinase activity

to ensue.

An unusually broad pH range for enzyme activity was found for the ETOH-precipitated chitinase preparation. Attempts to resolve this preparation into more than one enzyme component by electrophoresis as others have shown (3,10) were not successful, however, chromatography of enzymatic products revealed that 2 sugars were formed. One was identified as NAGA and although the other was not identified it had an R_f value similar to that of a disaccharide, N-N-diacetylchitobiose, found by Reynolds (16). It would appear that there is more than one chitin-degrading enzyme present in the ETOH-precipitated chitinase preparation, possibly accounting for the broad optimum pH range. Such a range encompasses the pH range usually existing in soils, supporting the possibility that chitinase may exert a biological effect in soil.

Natural soil, various Streptomyces isolates and sterilized soils inoculated with any of several of the Streptomyces isolates all destroyed mycelium of G. cingulata. The same soils and isolates also demonstrated chitinase activity. Enzyme activity on chitin in agar was apparent only when a colony of bacteria or actinomycetes was present. Since Chloromycetin prevented lysis of chitin by natural soil, even though fungi from natural soil grew abundantly through the chitin-agar plates, bacteria and actinomycetes are thought to be the principle organisms which produce chitinase in soil.

Chitinase added to natural and sterile soil at room temperature was recovered from such soils after 1 week. Moreover, since it does not appear to be inactivated by soil colloids and is probably diffusible, more than a localized effect might be produced by this enzyme in soil. Consequently, a chitinase-producing organism which is stimulated to produce this enzyme by coming in contact with a chitinous cell wall from an adjacent microorganism could conceivably account for the dissolution of chitin in this or adjacent cell walls. These results would suggest that chitinase may be of ecological significance as a factor acting on mycelial residues in soil.

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