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CHARACTERIZATION OF A BACTERIAL CHITINASE

A Thesis Presented

By

MARY PICKETT WEIR

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

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May 1978

Plant and Soil Sciences

CHARACTERIZATION OF A BACTERIAL CHITINASE

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ABSTRACT

Characterization of a Bacterial Chitinase

(May 1978)

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The chitinase system of an Erwinia species isolated from a healthy Lymantria dispar (L.) (gypsy moth) larva was investigated. The strain produced large amounts of chitinase when grown on chitin containing medium. However, chitinase activity was also found in culture supernatants when the cells were grown in a non chitinous medium. This suggests that the chitinase may in part be integral to cell autolysis rather than being exclusively induced by the presence of chitin.

The chitinase system contained two components: 1) chitin hydrolase which cleaves insoluble chitin into soluble oligomers; 2) chitobiase, which cleaves chitotriose and chitobiose and releases the monomer. The enzyme components were assayed as follows: total chitinase (chitin hydrolase and chitobiase) was determined by the release of N-acetylglucosamine from purified chitin, chitin hydrolase was measured by a decrease in turbidity of a purified chitin suspension, and chitobiase was measured by the release of a colored compound (ρ-nitrophenol) from an artificial substrate, ρ-nitrophenyl-N-acetylglucosaminide.

Several parameters were optimized for each assay. Optimum pH for the chitin hydrolase and total chitinase assays was determined to be 5.3-5.7, while the optimum pH for the chitobiase assay was established as 6.5-7.0. Greatest chitin hydrolase and total chitinase activity occurred when the assays were incubated at 50 C. Maximum chitobiase activity was found at an incubation temperature of 45 C. Examination of the relationship of substrate concentration to enzyme activity showed that 6-6.6 mg of chitin/ml assay was sufficient for the total chitinase assay. This indicated a low affinity for the substrate. Chitobiase was found to have a K_m of 0.25 mM which demonstrated a high affinity of the enzyme for the substrate.

It was found that a significant portion of the crude enzyme could be adsorbed onto chitin. Chitin hydrolase and chitinase were found to be relatively stable in the absence of substrate at 50 C while chitobiase rapidly lost activity.

From the results it could be concluded that chitin hydrolase is the rate limiting step in the enzymatic hydrolysis of chitin, while chitobiase is invariably present in sufficient quantities to rapidly complete the hydrolysis to the monomer.

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INTRODUCTION

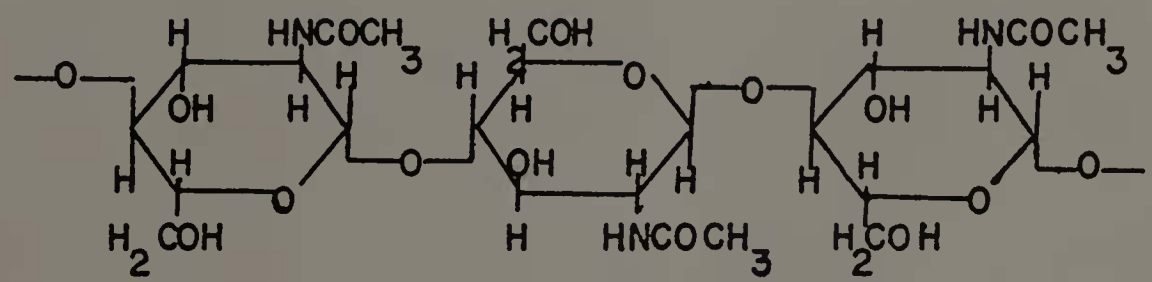
The growth of interest in agents of biological control of insects as well as of fungal pests has prompted growing attention to the enzymatic mechanisms underlying these agents. Most recently, impetus has been provided by the recognition of the deleterious effects imposed by chemical pesticides on the environment and on various biotic forms. Among the control mechanisms currently under investigation are those generated by microorganisms; in particular, those bacteria capable of producing enzymes lytic to specific tissues of insects or pathogenic fungi.

Of increasing importance appear to be those microorganisms capable of producing chitinases since this substrate is so universally found among pest insects and fungi of vast economic and health importance.

The description of bacterial chitinases is still at an early stage and it was therefore to further characterize these enzymes that this work was undertaken.

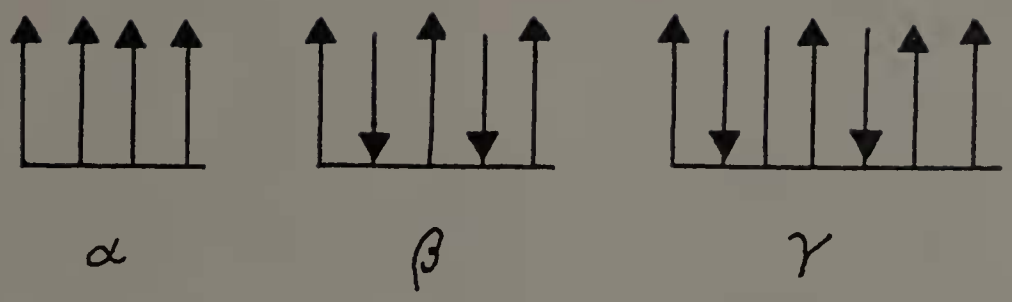
LITERATURE REVIEW

Chitin: structure and distribution. Chitin is a structural polysaccharide composed of N-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose) linked in a long straight chain polymer by a β(1→4) glycosidic bond (20). The structural repeating unit of the chitin polymer is the dimer of N-acetyl-D-glucosamine (NAG), chitobiose (33). The structure of chitin is shown below.



Braconnot, (9) in 1811, was the first to describe chitin. He extracted it from mushrooms and termed it fungine. He found that it contained more nitrogen than wood and was distinct from other substances found in plants. In 1823 this substance was named chitin by Odier (56). Chitobiose was not isolated until 1931 when it was proposed by Bergmann (7) as the repeating unit of chitin.

Three polymeric forms have been determined for chitin by x-ray crystallography (73). Schematic diagrams of these three forms are shown below (73). The arrows indicate the orientation of the polymer chain.



The α form is the most compact of the three forms. β chitin has a more open structure making it more penetrable by chemicals and enzymes (22). The γ form was proposed as an explanation for chitin which exhibited characteristics of both the α and β forms (73).

Chitin is the most abundant amino sugar containing polysaccharide (1). It is second only to cellulose as being the most common organic compound on earth.

The major source of chitin is fungal mycelium (74). It has been found in almost all fungi (16). Blumenthal and Roseman (8) in a survey of 25 strains found the chitin content to range from 2.6 to 26.2% of the dry weight of the fungal mycelia. Ruiz-Herrera found the chitin content of some fungi to be as high as 61%. Within the plant kingdom the only other group containing chitin is the Chlorophyceae (75). Chitin is found in many lower invertebrate in the animal kingdom, most notably in the arthropods (34).

Distribution of chitinolytic organisms. The yearly deposition of chitin in the environment is enormous yet little accumulation occurs. This suggests a wide distribution of active chitonolytic microorganisms capable of rapidly decomposing chitin.

The first chitin decomposing organism was isolated by Benecke (3) in 1905 from rotting plankton. In 1935, 250 different bacteria that could decompose chitin were isolated by Benton (4). These organisms were found in a great variety of environments which included: the intestines of insectivores, mud, soil, lake water, decaying crayfish and mayfly nymph shells. Later studies isolated other microorganisms from marine environments, both aerobic (90) and anaerobic, (71) and soils (76).

Among the bacterial genera with chitinase positive species are Achromobacter, Flavobacterium, Chromobacterium, Bacillus, Cytophaga and Pseudomonas (84), Bacillus (19), Beneckia (57), Klebsiella, Clostridium, Vibrio, Erwinia (10), Arthrobacter (23), and Serratia (45, 50).

Fungal genera containing chitinolytic species include: Aspergillus, Mortierella (84), Paecilomyces and Gleomastix, Verticillium and Tricoderma (19, 85), and Penicillium (58).

Many actinomycetes are also capable of hydrolyzing chitin. Some of the genera identified are Streptomyces, Micromonospora, and Nocardia (23, 84). Since many actinomycetes

possess chitinolytic ability, the clearing of chitin agar has been proposed by Hsu (23) as a criterion for identification of these organisms.

Synergistic interactions between organisms have been reported by Zobell and Rittenburg (90) and Okafor (57). Certain isolates which did not have chitinolytic ability alone, when used in conjunction with other isolates were able to clear chitin.

In addition to the many microbial chitinases mentioned briefly in this paper, chitinases have also been found throughout the plant and animal kingdoms. The diversity of chitinase containing organisms includes: snails (36), insects, (26, 37, 63, 86, 89), fish (13, 18, 60), birds (35, 81) and other vertebrates (30, 43) and in the plant world; beans and other seeds (64) and tomatoes (62). Several extensive reviews have been published on the distribution of chitinases (15, 31, 32).

Systems for chitin hydrolysis. Early investigations on chitin degradation dealt with physiological studies on the ability of microorganisms to clear chitin as an indication of chitin hydrolysis (70).

One of the earlier papers dealing with enzymological aspects of chitinase was Reynolds' (70) on the exocellular chitinase from a Streptomyces sp. He intimated that chitin decomposition followed the degradative pattern of other

polysaccharides where one or more enzymes would degrade chitin to chitobiose and another enzyme, chitobiase, would cleave the dimer. A comparison of chitin breakdown was made to that of amylose to maltose and of cellulose to cellobiose and of other systems which accumulate dimers. Reynolds also showed the chitinase of Streptomyces to be inducible. In a later paper, Reynolds in collaboration with Berger (5), described the chitinase system of Streptomyces griseus; these authors found 3 active protein fractions after zonal electrophoresis. Two of these were chitinases with similar activities which degraded chitin to NAG and chitobiose, while the third fraction was a chitobiase, having hydrolytic activity on the dimer and trimer of NAG but no activity on any of the higher oligomers.

Jeuniaux, in 1957 and 1959 (27, 29) described purification of a chitinase from another Streptomyces species. Three chitinases were found, all with similar activities. When these were recombined a synergistic effect on chitin hydrolysis was noted.

Winicur and Mitchell (89) in their investigation on Drosophila chitinase found 3 peaks of activity from fractions eluted from a DEAE-Sephadex column. It was suggested by these authors that when more than one chitinase is found, the additional "chitinase" fractions could actually be another type of enzyme, i.e., lysozyme or reverse chitin synthetase.

Powning and Irzykiewicz (64), in analyzing beans and other seeds for chitinase activity found, after DEAE cellulose fractionation, 2 chitinases and 1 acetylglucosamidase which had slight chitinase activity. They found the rate of hydrolysis of chitotetrose by both chitinase fractions was similar, but one fraction had a much greater affinity for the higher oligosaccharides. These authors also suggested that these chitinases in seeds could be fungicidal or fungistatic. This was also implied in the work of Mitchell and Alexander (49).

In another study, on the chitinase of Serratia marcescens, Monreal and Reese (51) proposed on the presence of a prehydrolytic factor (CH_1) similar to one in the cellulase system. They described chitin degradation as an induced system with the probable inducers being soluble chitinodextrins capable of entering the cell.

Jeuniaux (32) defined a chitinolytic system as; 1) a chitinase (poly-β(1→4)-(2-acetamido-2-deoxy)-D-glucoside glycanhydrolase) which breaks down chitin and oligomers of NAG to the trimer and dimer; 2) chitobiase (chitobiose acetamidodeoxy glucohydrolase) which hydrolyzes chitotriose and chitobiose. Most authors have agreed with this general scheme (although as previously mentioned, many have found more than one chitinase fraction upon purification).

It appears that many of the fungal chitinases are constitutive, producing chitinase when grown on non-chitinous

substrates (29, 40, 59, 61) while the bacterial and actinomycete chitinases are inducible (51, 54, 78) except for one described by Clarke and Tracey (10). The fungi may, however, be induced to produce chitinase by the chitin in their own cell walls.

Substrates and assays used for determination of chitinase activity. There are three basic types of assays for chitinase (32). These are: viscometric, where the decrease of viscosity is measured, turbidometric (nephelometric), where the reduction in turbidity of a colloidal chitin suspension is determined, and the colorimetric determination of end-products from chitin degradation including measurement of total reducing sugars and acetylhexosamines.

The viscometric method has been used by several authors (59, 62, 85, 89). One of the substrates used for viscometric assays is chitosan (62, 83, 85, 89). This is a chitin partially deacetylated by treatment with sodium hydroxide (55). Another substrate is glycol chitin (hydroxyethyl chitin) (59, 61) which is prepared by reacting alkali chitin with ethylene oxide (55). Carboxymethyl chitin (24) has also been used for this assay.

Glycol chitin was found by Otakara (61) and Okimasu (59) to be a convenient substrate for the analysis of chitinase. This chitin derivative is also similar to native chitin in both the degree of polymerization and in chemical

composition. Use of chitosan was also found to be a convenient method for certain applications (62, 83, 85, 89). A recent description (14, 66) of a chitosanase which is specific for chitosan raises some doubt to the validity of using this method for determination of chitinase activity. Jeuniaux, (31) stated that the carboxymethyl chitin of Hultin (24) was the best substrate for use in viscometry. He also questioned the use of chitosan as an enzyme substrate because of the variation in the degree of deacetylation (31, 32).

Another frequently used assay is the turbidometric method. This involves the use of colloidal chitin which is chitin dissolved in concentrated acid and then reprecipitated. This method has been used by several authors in conjunction with alternate assays (30, 43, 44, 64). It involves determination of reduction in turbidity caused by the hydrolysis of insoluble chitin to soluble endproducts. The turbidity reduction is measured by a nephelometer (32) or spectrophotometer (43). There is some controversy regarding the use of this technique, Jeuniaux (32) describes the method as fast and accurate but suitable only for samples of relatively high activity. Lundblad et al. (43) found the method "troublesome and difficult to reproduce." Hackman and Goldberg (22) in their proposal for new substrates for use with chitinases, commented that colloidal chitins precipitated from acids are considerably degraded and enzymes capable of

hydrolyzing the colloidal forms may not be able to act on chitin as it occurs in nature. In addition when native chitin precipitates from acid it tends to take the conformation of α chitin, the most compact and resistant to degradation of the three forms (73).

Hackman and Goldberg (22) proposed the use of a chitin dyed with Procion Red G dye. The dye is released upon degradation of the chitin. They suggested that the dyed chitin would be a more meaningful substrate for chitinase studies.

By far, the most commonly employed assay for chitinase is the colorimetric measurement of the endproduct of chitin degradation, specifically N-acetyl-D-glucosamine. This assay is more adaptable than the other two methods in that it allows the use of any form of chitin, including native chitin from any source or colloidal chitin prepared in a variety of ways. N-acetylhexosamines are detected by the method of Morgan and Elson (52) later modified by Reissig et al. (69). Some authors have used this as the only method of analysis for chitinase (37, 54, 77). Jeuniaux (32), however, has stated that if insufficient quantities of chitobiase is present, the results would not be an accurate measure of the chitinase activity. He suggested adding excess chitobiase to the enzyme assay mixture to insure complete hydrolysis to the monomer. Most authors have used the colorimetric assay in conjunction with other tests (5, 30, 43, 44, 60, 61, 62, 83, 85).

Some authors have used a more general method for quantifying the endproducts of chitin hydrolysis (5, 51, 70). This is the measurement of total reducing sugars instead of a specific test for N-acetylhexosamines. This test does not differentiate between glucose, D-glucosamine, N-acetylglucosamine or chitobiose. There is little evidence, however, for the breakdown of chitin to D-glucosamine or glucose and the measurement of NAG seems to be more specific and the preferred method.

Chitobiase assays. Several authors have reported analyses for chitobiase in addition to chitinase (5, 31, 44, 61, 63, 64). Other investigators have concentrated on the study of chitobiase (or β-N-acetylglucosaminidase) itself (2, 38, 65, 72). Many of these papers include methods with a substrate other than chitobiose (5, 31, 38, 44, 65, 72). Some of the substrates which have been used include; 4-methyl umbelliferyl N-acetyl-β-glucosaminide (65, 72), phenyl-N-acetylglucosaminide (5, 44, 64) β-methyl-N-acetylglucosaminide (61) and o-nitrophenyl-N-acetyl-β-glucosaminide (28, 41). Although these substrates are readily available and are easier to assay than chitobiose itself there is a question as to whether they are equivalent to chitobiose insofar as ease of hydrolysis is concerned. Kimura (41) studying an exo β-N-acetylglucosaminidase from the hemolymph in Bombyx mori and a chitobiase from moulting fluid, found that the exo β-N-acetylglucosaminidase had a greater affinity for the o-nitrophenyl-acetylglucosaminide

than for chitobiose, while the opposite was true for the moulting fluid chitobiase. He also found that the chitobiase appeared simultaneously with a chitinase and theorized from this circumstantial evidence that the chitobiase was, therefore, a true chitobiase. Bahl et al. (2) found the β-N-acetylglucosaminidase of Aspergillis niger to be an exo-glycosidase which could hydrolyze non reducing residues of NAG. The rate of hydrolysis was significantly greater with the dimer than with any of the higher oligomers.

Purification of chitobiase. Purification of chitobiase or β-N-acetylglucosaminidase has been accomplished by Robinson et al. (72) and Bahl and Agrawal (2). They have reported the K_m and V_{max} as 0.67mM and 676mumoles/mg/hr and 0.66mM and 71.4umole/min/mg, respectively while Jeuniaux (31) reported the K_m to range from 0.21 to 0.48mM. Although the K_ms reported by Bahl and Robinson and their colleagues are similar, they are from two different substrates, while Jeuniaux and Bahl both used chitobiose. Optimum pHs ranged from 3.2 to 4.8 (2, 41, 44, 72) with the exception of Berger and Reynolds (5) who used a pH of 6.3.

Chitinase: purification studies and characteristics. The greatest mass of data on the purification of chitinase has been published by Jeuniaux (27, 28, 29, 31, 32). His method of purification takes advantage of the insoluble nature of

chitin by absorbing the chitinase onto colloidal chitin. The chitin with the attached chitinase is centrifuged and then resuspended in buffer. The chitinase is then allowed to hydrolyze the chitin. When the hydrolysis is complete the chitinase is precipitated with ammonium sulfate. By this method, Jeuniaux obtained a 9.3 fold increase in specific activity. The chitinase studied (from Streptomyces antibioticus) was found to have 3 fractions when separated electrophoretically. These 3 fractions had a synergistic effect when recombined, as mentioned previously.

Reynolds (70) found in a preliminary study on the chitinase of a Streptomyces species that its chitinase could be concentrated by ultrafiltration and lyophilyzation with no significant loss of activity. He found that 3mg chitin/ml was an adequate concentration of substrate for assays.

Later in 1958, Berger and Reynolds (5) found that after ammonium sulfate fractionation they could separate chitinase from chitobiase by selective adsorption on bauxite, but they could not free chitobiase from chitinase activity. The chitinolytic system was separated by zonal electrophoresis on starch gels. Two chitinase and one chitobiase fractions were found. Both the chitinase and chitobiase assays were performed at pH 6.3 and 35 C.

Many other authors have reported purification schemes for chitinase (11, 39, 43, 45, 61, 62, 63, 64, 78, 82).

With the exception of Kosaki et al. (39) who used only ammonium sulfate fractionation for commercial preparation of chitinase all other authors mentioned used ammonium sulfate for fractionation and concentration in conjunction with various column chromatographic techniques.

Lysenko (45) used both DEAE-cellulose and Sephadex G-75 for purification. His yield was 4.7%. Pegg and Vessey (62) also used DEAE cellulose and chitinase was not absorbed. Results obtained from CM Sephadex dextran proved unsatisfactory. Powning and Irzykiewicz (63) found that with elution through Sephadex G-200 the acetylglucosaminidase appeared first and chitinase second but separation was not satisfactory. Two distinct fractions of chitinase were found after separation on DEAE cellulose.

Winicur et al. (89) used a UM-10 Diaflo membrane for concentration and encountered no loss of activity. Fractionation on DEAE Sephadex A-25 revealed three peaks of activity. They found no apparent cofactors which could be separated from the chitinase by running it through Biogel B-10 or CM 2.

Otakara, (61) using a column of hydroxyapatite, separated the chitinase system of A. niger into different fractions; one which caused a rapid decrease in viscosity of glycol chitin and another which released amino sugar from the oligosaccharides.

Skujins et al. (78) described purification of a Streptomyces chitinase on DEAE cellulose. They found that at pH 8.9 chitinase was not absorbed on the column while other proteins were retained. Elution from hydroxyapatite yielded 2 fractions which were both associated with proteinase activity. They found that most of the proteinase activity could be removed by elution through Biogel P 150. Elution through Sephadex 50 yielded only one fraction, in contrast to the 2 revealed by the hydroxyapatite column and the use of Sephadex was not pursued further. Skujins and his colleagues also needed to add Ca++ to maintain activity during purification procedures. They found only one band on polyacrylamide gels even though the preparation contained some proteinase.

Monreal and Reese (51) obtained poor recovery from DEAE Sephadex but they achieved a slight increase in specific activity from 3.0 to 3.6 in this purification step.

Morrissey et al. (54) used hydroxyapatite according to the method of Skujins and found little chitinase could be eluted from the column. They also recovered low activities from DEAE Sephadex. Their most effective column packing material was Sephadex G-75 where a 3 fold increase in specific activity was obtained above the crude ammonium sulfate cut. This amounted to a 12 fold increase in specific activity compared with the original culture supernatant.

Lundblad et al. (43) compared the efficiency of several column materials and obtained the best separation on Sephadex G-100 or G-150, after ammonium sulfate fractionation. The purification obtained was a 100 fold increase in specific activity surpassing that of other investigators with the exception of Takeda's (82) purification of a chitinase of Helix pomatia on Biogel P 30 and Amberlite CG50 which was also 100 fold purification. Huotari and co-investigators (25) pointed out, however, that frequently induced polysaccharases are found in culture media in such a condition that a 10 fold increase in specific activity will yield a pure enzyme. This may account for the discrepancy between the increases in specific activity of animal (43) chitinases and the chitinases of microorganisms.

Dandrifosse (11) purified chitinase from the pancreas and gastric mucosa of the frog by a modification of Jeuniaux's method involving adsorption onto chitin and chromatography on Biogel P 60. He obtained a 60 fold purification. He found two active fractions after polyacrylamide electrophoresis. These active fractions were tentatively identified as glycoproteins by selective staining.

The molecular weight of chitinase from microorganisms has been reported as about 30,000 Daltons by Jeuniaux, (31, 32) and Skujins et al. (78) and slightly higher (36,000) by Lysenko (45). The chitinase found by Lundblad and his colleagues

(43) in goat serum had a molecular weight of 60,000. Dandrifosse (11) in preliminary investigations found the chitinase from frogs to have a molecular weight of 90,000.

Several authors have reported values for the isoelectric points of different chitinases. These values are 3.8 (89), 4.85 (43), 6.8 (78) and 8.2 (32).

No cofactors are required for chitinase activity Jeuniaux (32, 89). However Skujins et al. (78) found that during purification, calcium had a stabilizing effect and 4 molecules of Ca were bound to each molecule of chitinase.

Vessey and Pegg (85) reported that $HgCl_2$, $CuSO_4$, and KCN were strongly inhibitory to chitinase and NaN_3 had no effect. $HgCl_2$ and $CuSO_4$ were also recognized as inhibitors by Jeuniaux. When Skujins and his co-workers (78) tested several cations at 0.2M concentration they found Mg to be the most inhibitory and Co and Zn slightly less inhibitory.

Most investigators have found the pH optimum for chitinases to be around 5.0 (32). Monreal and Reese reported a pH optimum for the chitinase of Serratia marcescens to be 6.4 while Lysenko (45) found the optimum for the chitinase of the same species to be 8.5 to 9.0. Berger and Reynolds (5) reported a pH optimum of 6.3 for Streptomyces griseus chitinase. The chitinase system described by Lundblad et al. (43) had a pH optimum on glycol chitin of 1.65 while the optimum on colloidal chitin was 5.5.

The optimum temperature for most chitinase assay systems was found to be 35-40 C (31, 32). Monreal and Reese (51), Morrissey (54) and Lundblad (43) with their co-workers reported optimum activity at 50 C.

Several papers have reported on Michaelis-Menten kinetics for chitinase. The chitinase from S. antibioticus (32) was reported to have a K_m of 0.010-0.011g/100ml. The K_m of chitinase found in tomatoes (62) was found to be 0.408 mg/ml while the V_{max} was 3.7ug NAG-N/hr/ml. McLaren (47, 48) and Skujins et al., (78) state that Michaelis Menten kinetics are not applicable to reactions at the surface because the concentrations of reactants at interfaces generally differ from those in adjacent solutions. McLaren (47) declared that "in micro gels and at interfaces the terms concentration, diffusion and pH cannot readily be applied.

MATERIALS AND METHODS

Isolation and selection of chitinolytic organism. Culture No. 134 is one of a group of microorganisms isolated from gypsy moth larvae (Lymantria dispar (L.)) in 1974. Larvae of three successive instars were collected from a light infestation of a mixed forest stand in Whately, Massachusetts. Upon collection, individual larvae were put into separate sterile screw cap tubes and brought back to the laboratory for processing.

In the laboratory 2 ml of a sterile 1.0% peptone solution was added to each tube. The larvae were then pulverized with a sterile glass rod and mixed thoroughly on a Vortex-Genie Mixer (Fisher Scientific Co.). Aliquots of 0.1 ml were smeared on to trypticase soy agar (TSA) and chitin agar plates with a sterile bent glass rod. TSA plates were incubated for 2 days at 28 C and chitin agar plates were incubated at room temperature for up to 15 days. Colonies which demonstrated ability to clear chitin were restreaked on TSA and chitin agar for positive isolation of single strains. All strains demonstrating chitinolysis were transferred to TSA slants and stored at 4 C or -60 C.

Strain 134 was chosen for this study since: 1) it was highly chitinolytic; 2) it had relatively consistent

growth characteristics; and 3) as a gram negative bacteria, it had less exocellular protein, which was found in early investigations with a gram positive bacillus, to interfere with chitinase production. Strain #134 was identified in this laboratory as an Erwinia species (12).

Preparation of crude and purified chitin.

Crude chitin. Commercial grade crude chitin was obtained from Pfaltz and Bauer, Inc. (375 Fairfield Ave., Stamford, Conn. 06902). Due to differences in quality of chitin available, one batch was used throughout this course of study.

Crude chitin was prepared for use by the following method: 500 g crude chitin was ground finely in an Osterizer for 3-5 min. The ground chitin was then boiled in 4 l of distilled deionized water for 10 min. The chitin was allowed to settle and the water was decanted off the top and more water added with mixing. This rinsing procedure was repeated a minimum of 10 times until the water decanted was clear and had no odor. The chitin was dryed at 80 C overnight.

Purified chitin. Purified colloidal chitin was prepared according to the following procedure modified from the method of Vessey and Pegg (85): 200 g ground commercial chitin was washed for 1 hour in 1 l of water on a shaker. The water was decanted off and the chitin was mixed with 505 ml of acidified ether and ethanol mix (250 ml diethyl ether, 250 ml ethanol and 5 ml conc. HCl). The mixture was shaken for

1 hr. and centrifuged in a Sorvall RC-2 refrigerated centrifuge at 10,000 rpm. The chitin pellets were then bleached with the addition of 500 ml of 0.2 M $NaClO_2$ and this mixture was held at 75 C for 1 hr. with constant stirring. After centrifugation 10 ml of acetone was added to the chitin with sufficient concentrated HCl at 0 C to dissolve as much of the residue as possible. The dissolved chitin-acid mixture was centrifuged to remove any undissolved particles and inorganic material. The supernatant was then carefully decanted into 8 l of ice water and allowed to precipitate for at least 2 hrs. The precipitate was centrifuged and rinsed 3 times with distilled water. The pH of the purified chitin was adjusted to 7.0 with KOH. The purified chitin was washed 3 more times and centrifuged after each wash to remove any salts resulting from the procedure. The preparation of chitin was divided up into 500 ml aliquots, put into bottles, autoclaved for 20 min. and stored at 4 C until needed.

Growth of organism and production of chitinase. A chitin containing modified Morris medium (53) (see appendix for composition) was used for the growth of cells and production of chitinase. This medium was tested for effect on chitinase yield of various nitrogen sources, omission of nitrogen source, and various pHs by adjusting the proportion of monobasic and dibasic potassium phosphate. The salts of modified Morris medium were made up in a double strength concentration

and chitin (either crude or purified) was added to a portion of distilled H_2O and autoclaved separately. Before use of the medium the salts were added aseptically to the sterile chitin. Studies were made to determine optimum amounts of purified and crude chitin to use for enzyme production. One percent purified and 3% crude chitin were routinely used for production of enzyme. All chitin broth cultures were grown on an oscillating shaker at 27 C in the dark.

Inoculum procedure. Several inoculum procedures were compared. For routine enzyme production and growth studies, the following procedures were used: 1) A loopfull of culture from a refrigerated TSA slant was put into 8 ml Trypticase Soy Broth (TSB) for 24 - 28 hr. incubation at 30 C. Five ml was then transferred into 50 ml TSB in a 125 ml erlenmeyer flask for 18 hrs. A 10% inoculum from this flask was introduced into chitin medium. 2) An alternate procedure was followed for faster enzyme production. Eight ml of TSB was inoculated with a loopfull of culture from a refrigerated TSA slant and incubated for 28 - 28 hrs. at 30 C. Five ml was transferred to 50 ml chitin broth and incubated on a shaker for 4 - 6 days. Ten ml was then transferred to fresh chitin broth (90 ml) and incubated until a high titer of chitinase was reached 6 - 8 days.

Bacterial enumeration. Bacterial counts were made using the plate drop count method of Reed (67). Cultures were

serially diluted and several aliquots of 0.01 ml each were spotted on a dry TSA plate with a 0.1 ml Mohr pipet. Plates were incubated overnight at 27 C and counted under a dissecting microscope.

Harvesting enzyme. Crude enzyme preparations were obtained from cultures with high chitinase titers. Cultures were centrifuged in a Sorvall RC-2 refrigerated centrifuge for 20 min. at 9,000 rpm. The supernatant was frozen at -60 C for long term storage and held at 4 C for short term shortage.

Assays.

Protein determination. Supernatant protein was measured using the method of Lowry et al., (42) modified to reduce interference from ammonium sulfate. Stock reagents were prepared (W/v) as follows:

2% $CuSO_4 \cdot 5H_2O$

4% Na · K tartrate

4% Na_2CO_3

Folin phenol reagent diluted 1:3 (frp/H_2O, v/v)

Before use the reagents were mixed:

100 ml 4% Na_2CO_3

1 ml 2% $CuSO_4 \cdot 5H_2O$

1 ml 4% Na · K tartrate

This solution was prepared fresh daily. Then to 0.5 ml of the protein sample, diluted if necessary, 5 ml of the mixed reagent was added. The protein + reagent solution was incubated at room temperature for 45 min. Diluted folin-phenol

(0.5 ml/tube) was added to each tube and mixed immediately. Color was allowed to develop for 15 min. and the samples read at 660 mμ in a Spectronic 20 (Bauch & Lomb). A reagent blank and a Bovine Serum Albumin (BSA) protein standard were included for each determination. Absorbence was converted into mg protein per ml by extrapolation on a standard curve prepared using known amounts of BSA.

Total chitinase and chitobiase assay. The standard chitinase reaction mixture contained 0.5 ml purified chitin solution (18 mg/ml), 0.5 ml of 0.1 M citrate buffer (pH 5.0), 0.5 ml enzyme. The assay mixture was incubated for 1 hr. at 50 C and immediately centrifuged for 15 min, at 15,000 rpm. The supernatant was decanted. An aliquot from it was diluted appropriately and tested for N-acetyl-D-glucosamine (NAG, obtained from Sigma Chemical Co. was used for the standard) colorimetrically according to the method of Reissig et al. (69). Product formation was shown to be linear with time. Controls for each determination consisted of a reagent blank, a known amount of NAG, and enzyme to determine the amount of NAG that was in the original supernatant. Results were translated from absorbence to μ moles NAG by plotting against a standard curve prepared with known amounts of NAG and then corrected for background interference from the original supernatant and the dilution factor. Units of chitinase activity were expressed as μ moles NAG released from purified chitin/ml

supernatant/hr. and specific activity as μ moles NAG/mg supernatant protein/hr.

Turbidometric (nephelometric) assay for chitin hydrolase. The standard assay mixture for chitin hydrolase contained 2 ml purified chitin solution (1 mg/ml), 0.5 ml of 0.2 M citrate buffer (pH 5.0) and 0.5 ml enzyme. These components were mixed thoroughly on a Vortex-Genie mixer and read immediately at 420 nm on a Spectronic 20. After incubation for 1 hr at 50 C, assay tubes were mixed and reread at 420 nm. Initial and final readings were plotted against a standard curve prepared with known amounts of chitin to give equivalents of mg purified chitin/ml assay. The subtraction of these two numbers reflected a decrease in absorbence due to the hydrolysis of chitin expressed as mg chitin hydrolyzed/ml assay/hr. Units of chitin hydrolase were corrected and activity expressed as mg chitin hydrolyzed/ml supernatant/hr. or mg chitin hydrolyzed/mg supernatant/hr. for specific activity.

Chitiobiase assay. The standard assay mixture for chitobiase contained the following: 0.2 ml of 0.2 M potassium phosphate buffer (pH 6.7), 0.4 ml of 4.0 mM ρ-Nitrophenyl-N-acetyl-β-D-glucosaminide (ρ-NPAG Sigma Chemical Co.) 0.1 ml H_2O, 0.1 ml enzyme (diluted). The assay mixture was incubated for 1 hr. at 45 C then 0.8 ml of 0.4 M glycine-NaOH buffer (pH 10.5) was added. The samples were read at 430 nm in a Beckman DU spectrophotometer. A reagent blank without

enzyme was run for each determination. Absorbence was plotted against a standard curve prepared using known amounts of p-Nitrophenol which was corrected by a factor of 1.6 to read µg NAG released from p-NPAG. Units were expressed as µg or µmoles NAG released from p-NPAG/ml supernatant/hr. and specific activity as µmoles NAG released/mg supernatant protein/hr.

Preparation of buffers. Buffers for pH studies were prepared by the method of Gomori (17). All other buffers were prepared by titrating the most acidic component to the proper pH with a solution of base and making up to the correct volume with distilled H_2O.

Determination of crude enzyme properties: optimization of the assays.

Optimization of pH for assays. Optimum pH was determined for all three enzyme assays. Crude supernatant and other assay ingredients were incubated with 0.1 M citrate buffer at 0.5 pH unit increments from pH 3.5 to 6.5 to optimize for total chitinase assay. Assay mixtures for chitin hydrolase were prepared using the same pH buffers but of 0.2 M concentration. The pH was measured in each assay mixture to compensate for the buffering effect of the supernatant itself.

Chitobiase assay mixtures were incubated with 0.2 M citrate buffer from pH 4.0 to 6.0 and potassium phosphate buffer from pH 5.7 to 8.0. It was assumed that the pH did not change

significantly because of the small amount of crude enzyme used for the assays.

Optimization of temperature for assays. The effect of temperature on release of product from the different assays was tested. A series of tubes, each containing identical enzyme and assay ingredients, were incubated at the following temperatures: 25, 30, 36, 45, 50 and 60 C respectively and analyzed for chitin hydrolase, chitinase and chitobiase activity. The total chitinase tubes were heated in a boiling waterbath for 5 min. to stop the reaction.

Determination of the effect of cations. Crude enzyme was prepared for the chitin hydrolase and chitobiase cation study by preincubation with EDTA (10 mM final concentration) in 0.05 M potassium phosphate buffer (pH 6.7) for 1 hr. at room temperature. Tne enzyme-EDTA mixture (a total of 10 ml) was then put into dialysis sacs prepared according to the method of Makula et al. (46) and dialyzed overnight against 2 l of 0.05 M potassium phosphate buffer (pH 6.7). The divalent cations were all chloride salts. In the chitobiase assay, the cation solutions were substituted for the 0.1 ml of H_2O and 0.2 M tris-maleate buffer (pH 6.7) was substituted for the potassium phosphate buffer, since the potassium phosphate buffer formed a precipitate with the cations and interfered with the assay. In the chitin hydrolase assay the cation solutions were compensated for by using 1.5 ml instead of 2.0 ml of chitin solution.

A study was also performed, to determine the effect of cations on the total chitinase assay. The crude enzyme was dialyzed against distilled water at 4 C for 24 hrs. Cations were made up in 0.1 M citrate buffer (pH 5.0).

Effects of reducing agents. Various concentrations of mercaptoethanol and dithiothreitol (DTT) were included in assay mixtures to test the effect of reducing agents on chitin hydrolase and chitobiase.

Enzyme stability. A preliminary test on the effect of freezing on chitinase (total) and chitobiase was performed. Crude enzyme supernatant was frozen at -60 C for 24 hr. and then thawed and used for chitinase and chitobiase assays. Enzyme preparations were frequently kept at 4 C for several months with no loss of activity.

Loss of enzyme activity in the absence of substrate at 50 C was determined. Crude enzyme supernatant was incubated in a waterbath at 50 C and aliquots were removed over a period of time and cooled immediately in an ice bath. All three assays were run on each aliquot.

Preliminary purification studies.

Adsorption of chitinase by purified and crude chitin. The degree of adsorption of chitinase by crude and purified chitin was determined. Five ml of crude enzyme was mixed with: 1 g crude chitin and, 2 ml purified chitin (20 mg/ml). Each mixture was stirred at room temperature on a magnetic

stirrer for 5 min. and then centrifuged at 10,000 rpm for 15 min. Total chitinase assays were run on the respective supernatants and percent loss of activity was determined.

Ammonium sulfate fractionation. Ninety ml of supernatant from a crude chitin culture was fractionated with ammonium sulfate (certified A.C.S. grade). Enough ammonium sulfate was added slowly to the crude enzyme while stirring in an ice bath to bring the concentration to 30% saturation. The sample was stirred for 30 min. and centrifuged at 5 C at 15,000 rpm. The concentration of ammonium sulfate was then increased in the supernatant to 60% saturation. The sample was then stirred for 30 min and centrifuged. A final addition of ammonium sulfate brought the concentration to 90% saturation and the sample was stirred, and centrifuged as before. All three pellets were dissolved in a minimum amount of 0.05 M potassium phosphate buffer (pH 7.0) and dialyzed overnight against 2.5 l of the same buffer. Percent saturation of ammonium sulfate was determined from a nomogram (P & L Biochemicals, Inc.). All fractions were assayed for protein concentration, chitin hydrolase, total chitinase and chitobiase activity.

Relationship of chitinase to cell lysis. An ancillary experiment was performed in an effort to determine if the appearance of chitinase is related to autolysis of the cell culture. One l of TSB was inoculated with culture 134 from

a slant and incubated for 48 hr. on an oscillating shaker at 27 C. The culture was then centrifuged for 15 min. at 9,000 rpm and the pellet was washed one time with modified Morris medium containing no carbon source and recentrifuged. The pellet was then suspended in 50 ml 0.05 M potassium phosphate buffer (pH 7.0) and a 10 ml aliquot was saved for ultrasonification. The remainder (40 ml) was shaken on an oscillating shaker overnight at 27 C and then centrifuged. This pellet was resuspended in 10 ml potassium phosphate buffer (0.05 M, pH 7.0). The 10 ml aliquot was sonicated for 10 sec. and allowed to cool for 15 sec. in ice. This procedure was repeated two more times. The sonicated portion was then centrifuged. The following portions of the experiment were saved, and tested for protein concentration, total chitinase, chitin hydrolase and chitobiase activity:

1) Supernatant from the TSB culture.

2) Supernatant from the modified Morris medium wash.

3) Pellet from cells incubated overnight in potassium phosphate buffer, suspended in 10 ml of buffer.

4) Supernatant from cells incubated overnight in buffer and centrifuged.

5) Pellet after sonication and centrifugation, suspended in 5 ml buffer.

6) Supernatant from sonication and centrifugation.

RESULTS

Enhancement of chitinase production: growth studies.

Effect of inoculum concentration on chitinase activity. The effect of different percent inocula from TSB and chitin medium on chitinase release is shown in Table 1. Similar chitinase activities were found in the supernatants of day 6 cultures inoculated from TSB, and day 7 cultures inoculated from chitin medium. In both experiments the 10% inoculum cultures had the greatest chitinase activity. Of the cultures inoculated with TSB, the culture inoculated with 10% also had the greatest specific activity, while of the chitin inoculum cultures the 1% had slightly higher specific activity.

Optimization of crude and purified chitin concentrations in chitin broth medium for enzyme yields. Protein concentration, chitobiase, chitinase and chitin hydrolase activities from culture 134 when grown on several concentrations of crude purified chitin are presented in Figure 1 (A, B, C, D). Exocellular protein concentration (Fig. 1A) was found to increase as chitin concentration in the medium was increased at both days 14 and 21. Chitobiase activity (Fig. 1B) also followed this pattern at days 14 and 21, with the greatest activity being found in cultures with greatest chitin concentration. However, chitin hydrolase activity (Fig. 1C)

TABLE 1

EFFECT OF INOCULUM CONCENTRATION ON TOTAL CHITINASE ACTIVITY

Percent inoculum from TSB medium*	Day 4 µmole NAG released /ml	Day 4 µmole NAG released /mg	Day 6 µmole NAG released /ml	Day 6 µmole NAG released /mg
1	38.9	47.5	29.9	31.5
5	29.9	32.4	38.9	43.4
10	28.5	25.8	43.6	54.9
from chitin medium**	Day 2		Day 7	
2	31.5	48.4	27.0	56.0
5	27.0	36.9	33.0	47.8
10	34.5	47.2	36.0	53.7

*A loopful of culture from a TSA slant was put into 8ml TSB for 24 hours at 30 C. Five ml of this was transferred into 50ml of TSB for 18 hr. This culture was then used to inoculate the chitin medium.

**Eight ml of TSB was inoculated from a slant culture and incubated for 24 hr. Five ml was then transferred to 50ml of chitin medium and incubated for 4 days. This culture was then used to inoculate fresh chitin medium.

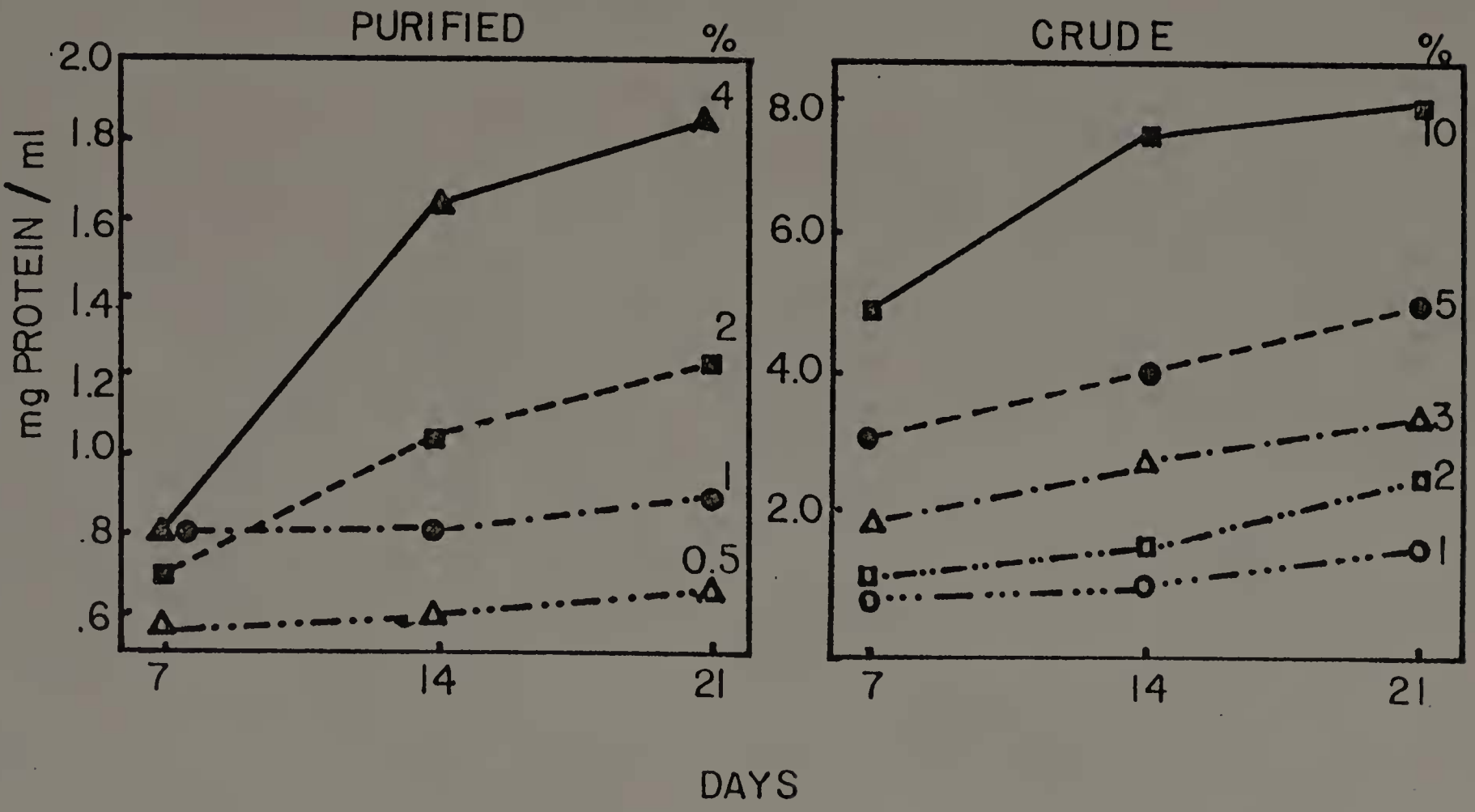


Fig. 1A. Supernatant protein concentrations from cultures grown in media containing different amounts of purified and crude chitin.

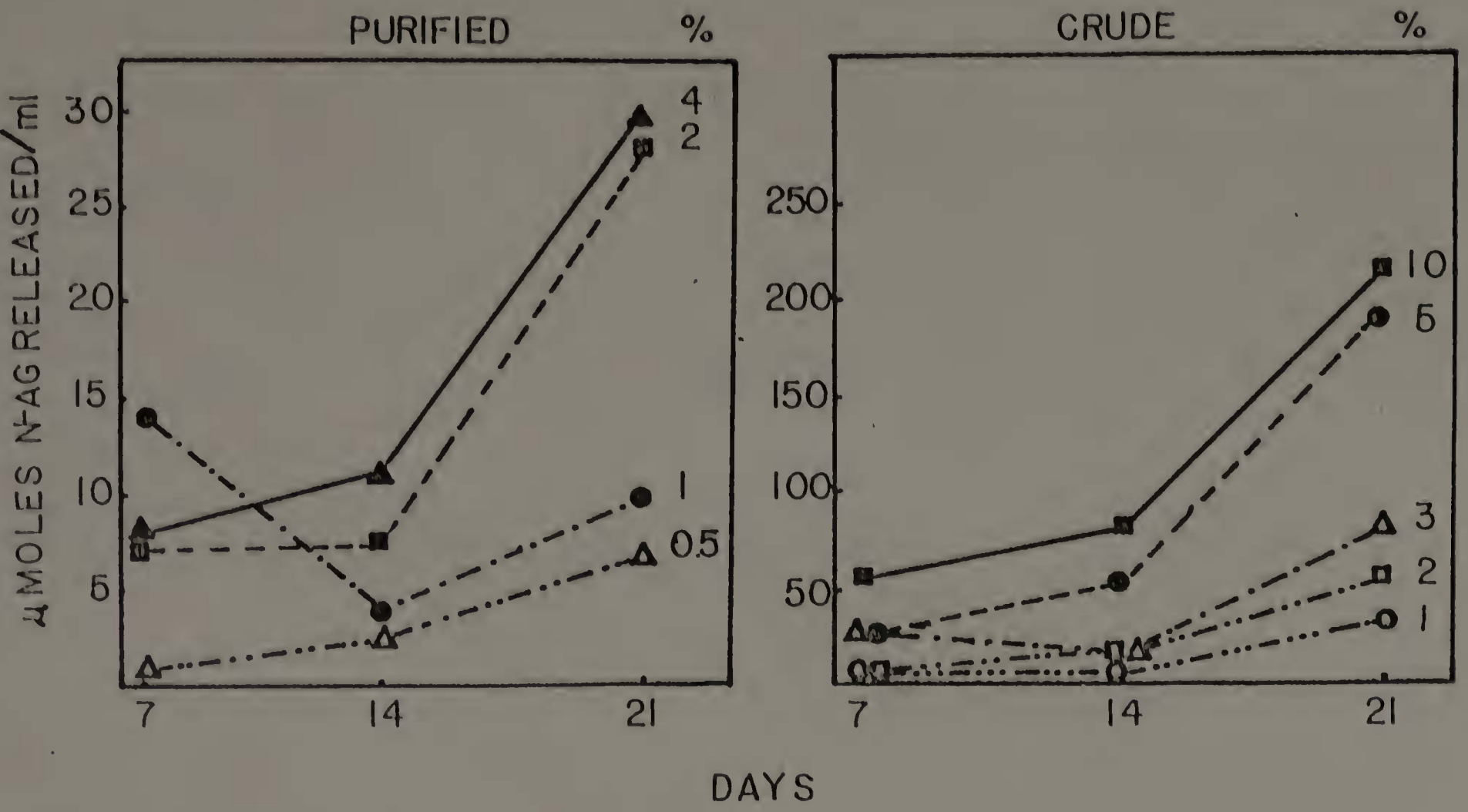


Fig. 1B. Chitobiase activities in supernatants from cultures grown in media containing different amounts of purified and crude chitin. Activities are expressed as μmoles NAG released from ρ-NPAG per ml culture supernatant per hr.

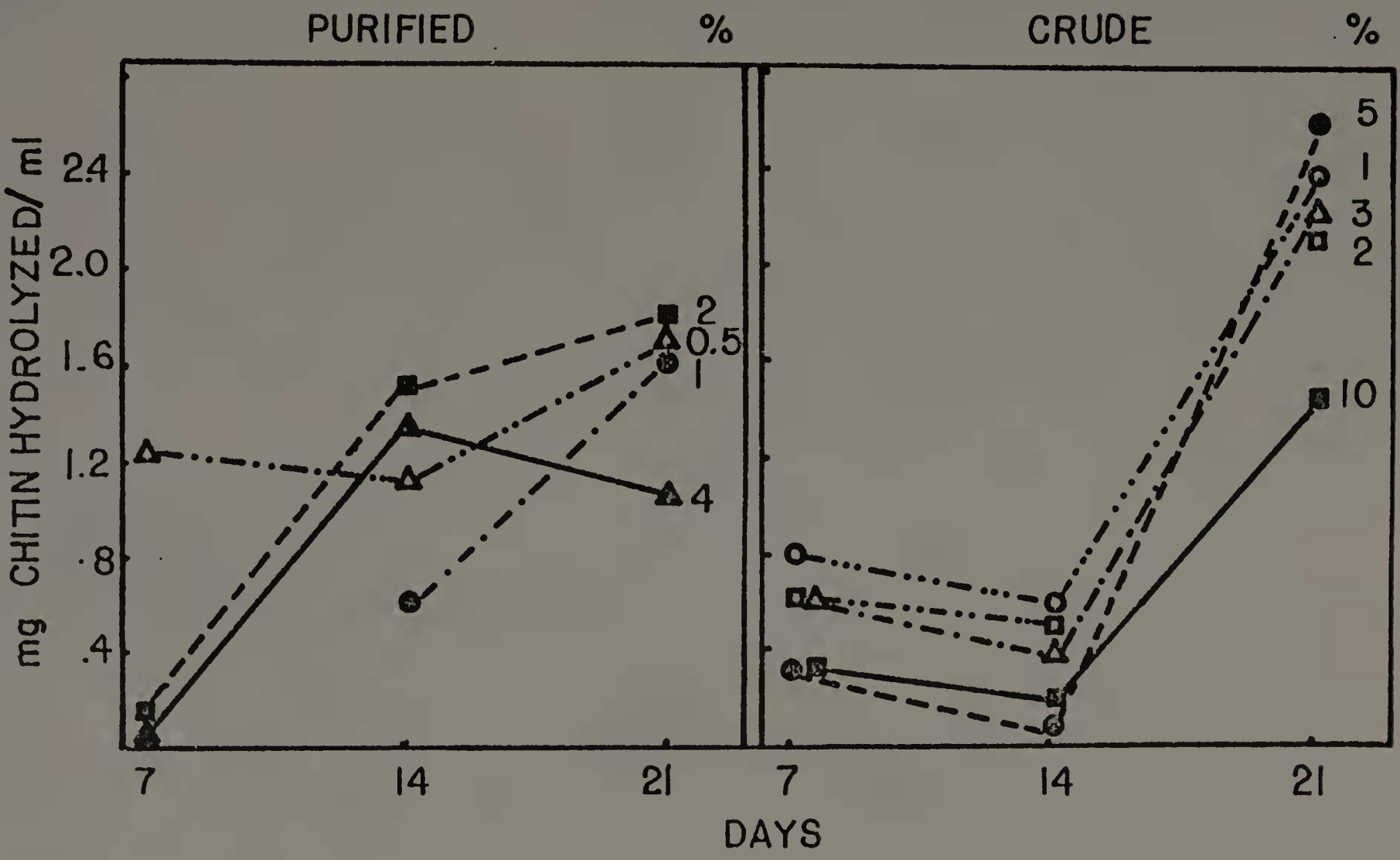


Fig. 1C. Chitin hydrolase activities in supernatants from cultures grown in media containing different amounts of purified and crude chitin. Chitin hydrolase activity is expressed as mg purified chitin hydrolyzed per ml supernatant per hr.

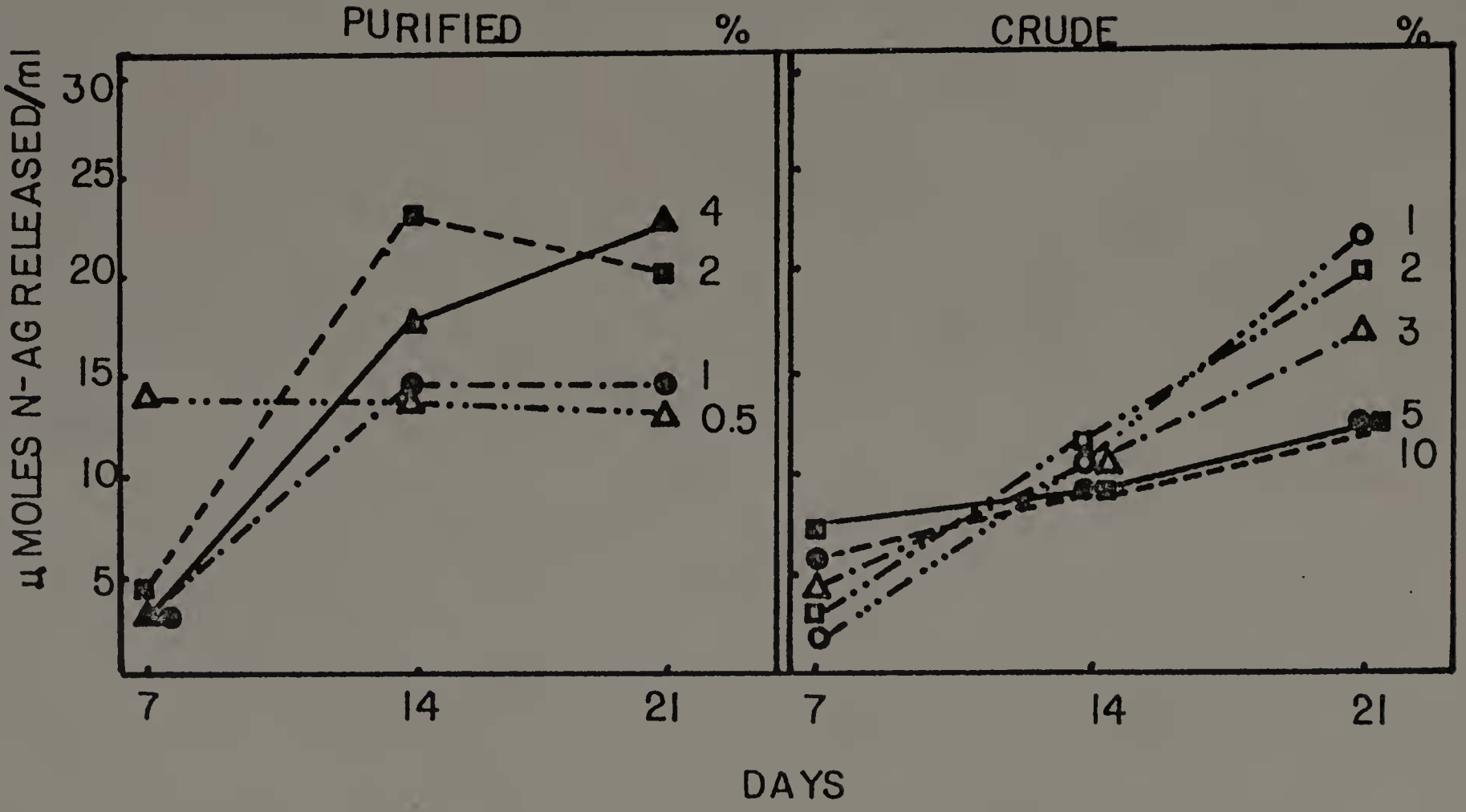


Fig. 1D. Total chitinase activities in supernatants from cultures grown in media containing different amounts of purified and crude chitin. Total chitinase activity is expressed as µmoles NAG released from purified chitin per ml supernatant per hr.

from purified chitin cultures at day 14 did not show the same pattern. At day 21 the enzyme preparations from the three lower concentrations of purified chitin had similar activities that were all higher than the enzyme from the culture with the highest chitin concentration. The crude chitin cultures exhibited similar activities at day 7 and 14, while at day 21 the enzymes from the lower concentration chitin cultures had greater activities than that of the 10% concentration. The total chitinase activities (Fig. 1D) from purified chitin cultures followed similar patterns to those of chitobiase and protein. The greatest chitinase activity at day 21 was found in the medium with the greatest concentration of purified chitin. Supernatants from cultures incubated with 1, 2, and 4% purified chitin increased in activity. The chitinase activity from the supernatant of the culture with the lowest concentration of chitin remained the same at all three sampling times. Chitinase activity from the supernatants of cultures incubated with crude chitin increased from day 7 to day 21 at all concentrations. In these cultures the greatest chitinase activity was associated with the 1% concentration while the lowest activity was derived from the cultures with a 5 and 10% concentration. This was a reversal of the trend found in the day 21 cultures with purified chitin.

Chitinase yields from culture 134 on purified and crude chitin. A comparison of chitinase yields per ml and per mg supernatant

protein from cells grown on 1% purified chitin and 3% crude chitin media, respectively, is presented in Table 2. The specific activity of chitinase from the cells grown in purified chitin was ten times that from the crude chitin culture at both day 8 and 11. However, the yields of chitinase per ml from the purified and crude chitin cultures were similar.

Release of chitinase by culture 134 in relation to cell numbers and extracellular protein concentration. Cell numbers reached a peak at day 3 and then remained at the same order of magnitude throughout the testing period as shown in Figure 2. Chitinase activity was greatest at day 5 and it then decreased gradually by one third until day 15, where it remained through day 23. Supernatant protein concentration increased rapidly until day 3, then continued to increase more slowly reaching a peak at day 8. This level of protein was maintained through day 23.

Total chitinase.

Standard assay for total chitinase. The release of NAG from purified chitin by chitinase at various intervals was measured and is presented in Fig. 3. The production of NAG maintained a linear course through 60 min. The rate of NAG release after 60 min decreased.

Effect of enzyme concentration on release of NAG. Decreasing crude chitinase concentration decreased relative enzyme activity proportionately as shown in Fig. 4.

TABLE 2

COMPARISON OF CHITINASE YIELDS* FROM CULTURE 134 GROWN ON 1% PURIFIED CHITIN AND 3% CRUDE CHITIN

Day	Type of chitin	Protein mg/ml culture supernatant	µmoles NAG released/ml supernatant	Specific activity µmoles NAG/ mg protein
6	purified	0.3	2.4	8.0
	crude	1.8	7.5	4.2
8	purified	0.41	10.8	26.0
	crude	3.4	10.5	3.0
11	purified	0.41	12.6	30.7
	crude	3.4	10.2	3.0

*Culture supernatants were assayed for total chitinase activity by determination of amount of NAG released from purified chitin in the assay.

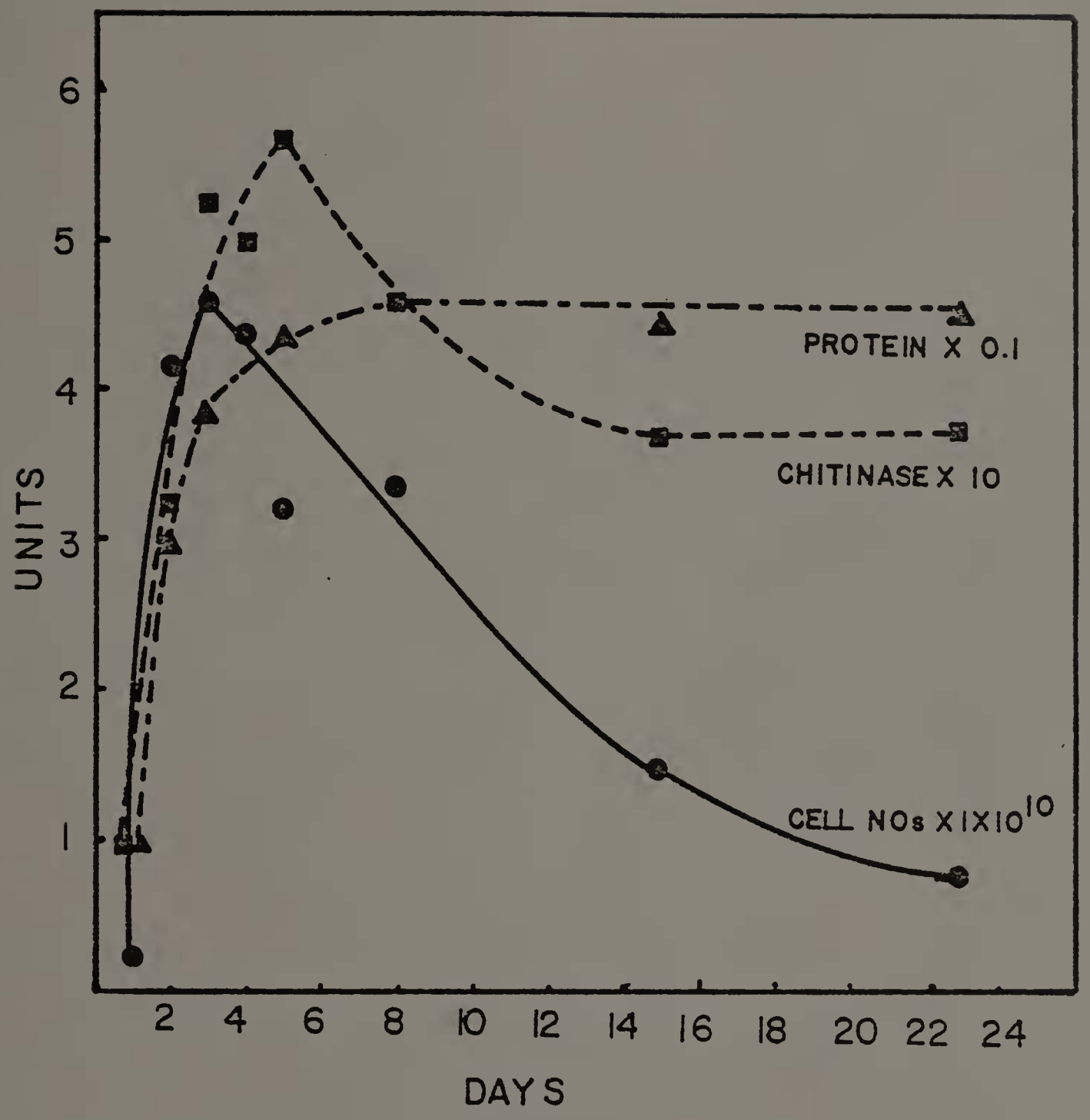


Fig. 2. Release of chitinase by culture 134 in relation to cell numbers and supernatant protein concentration. Protein units are mg supernatant protein per ml. Chitinase activity is expressed as μmoles NAG released from purified chitin per mg supernatant protein per hr.

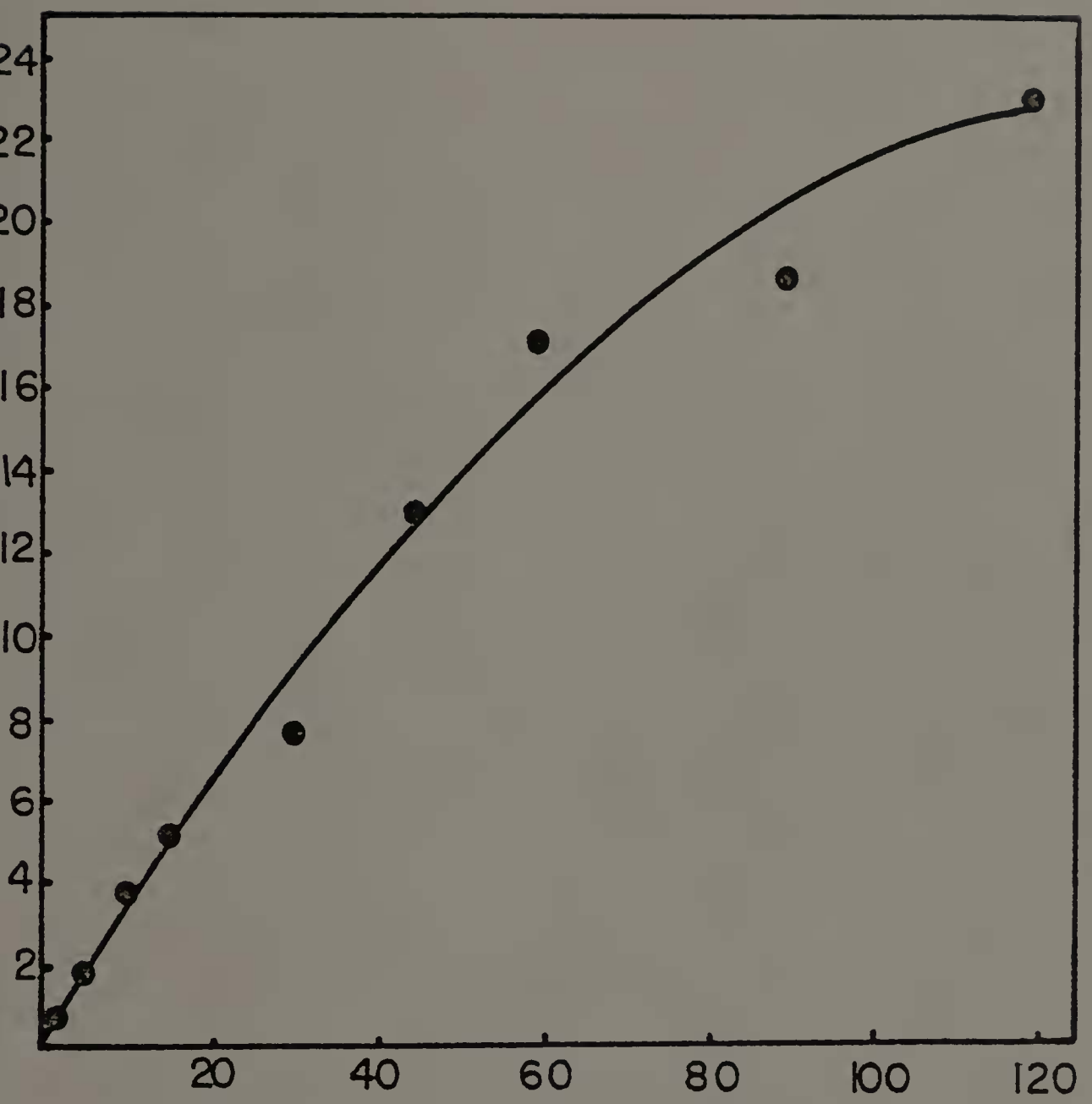


Fig. 3. Standard total chitinase assay. Total chitinase activity is expressed as μmoles NAG released from purified chitin per ml crude enzyme supernatant at various intervals.

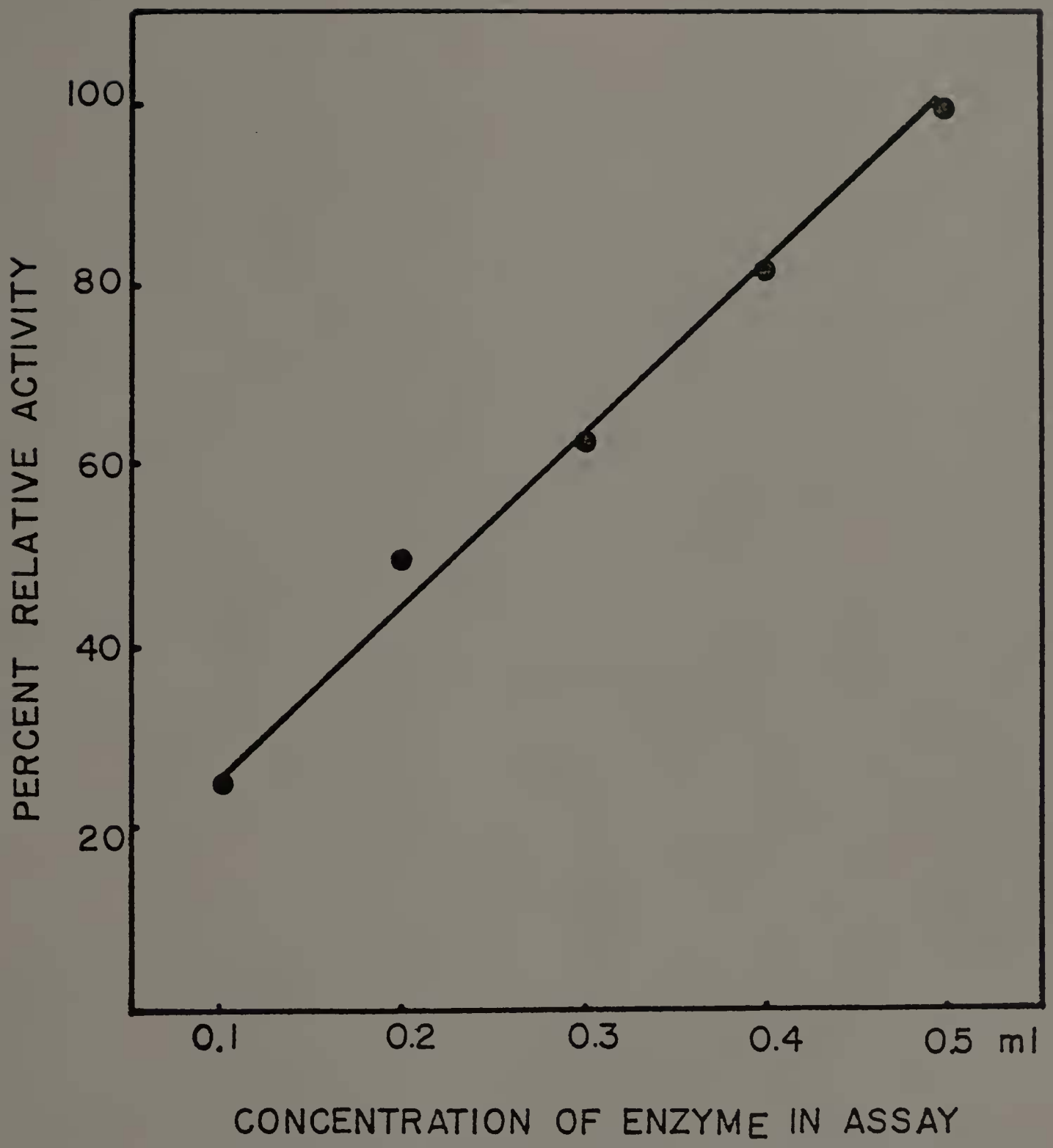


Fig. 4. Effect of enzyme concentration on the release of NAG from purified chitin. Standard total chitinase assays were run using 0.1, 0.2, 0.3, 0.4, and 0.5 ml of crude chitinase.

Influence of pH on release of NAG from purified chitin. Crude chitinase was incubated in citrate buffer at various pHs ranging from 3.5 to 6.9 as shown in Fig. 5. The optimum pH for release of NAG was found to be pH 5.3. Relative activity decreased to 56% at pH 3.6 and to 82% at pH 6.9.

Relationship of substrate concentration in assay to release of NAG. Crude chitinase was incubated in separate assay mixtures with increasing amounts of purified chitin. The results in Fig. 6 show that release of product proceeded in a linear fashion until a concentration of 6.0 mg purified chitin per ml assay was reached. Increasing the substrate concentration beyond 6.0 mg/ml did not increase the release of product.

Temperature: effect on release of NAG from purified chitin. The greatest release of NAG by crude chitinase from purified chitin was found at an incubation temperature of 50 C as presented in Fig. 7. Above 50 C the relative activity rapidly decreased to 37% when it reached 60 C, while at 30 C the relative activitiy decreased to 67% of that found at 50 C.

Divalent cations: effect on total chitinase activity. Crude enzyme supernatant was incubated with each of several divalent cations at 10 mM concentration (Table 3). All the cations tested were found to inhibit enzyme activity to some degree. Ca, Zn, and Mg had the least effect on the enzyme, reducing the relative activity to 91, 91 and 86% respectively.

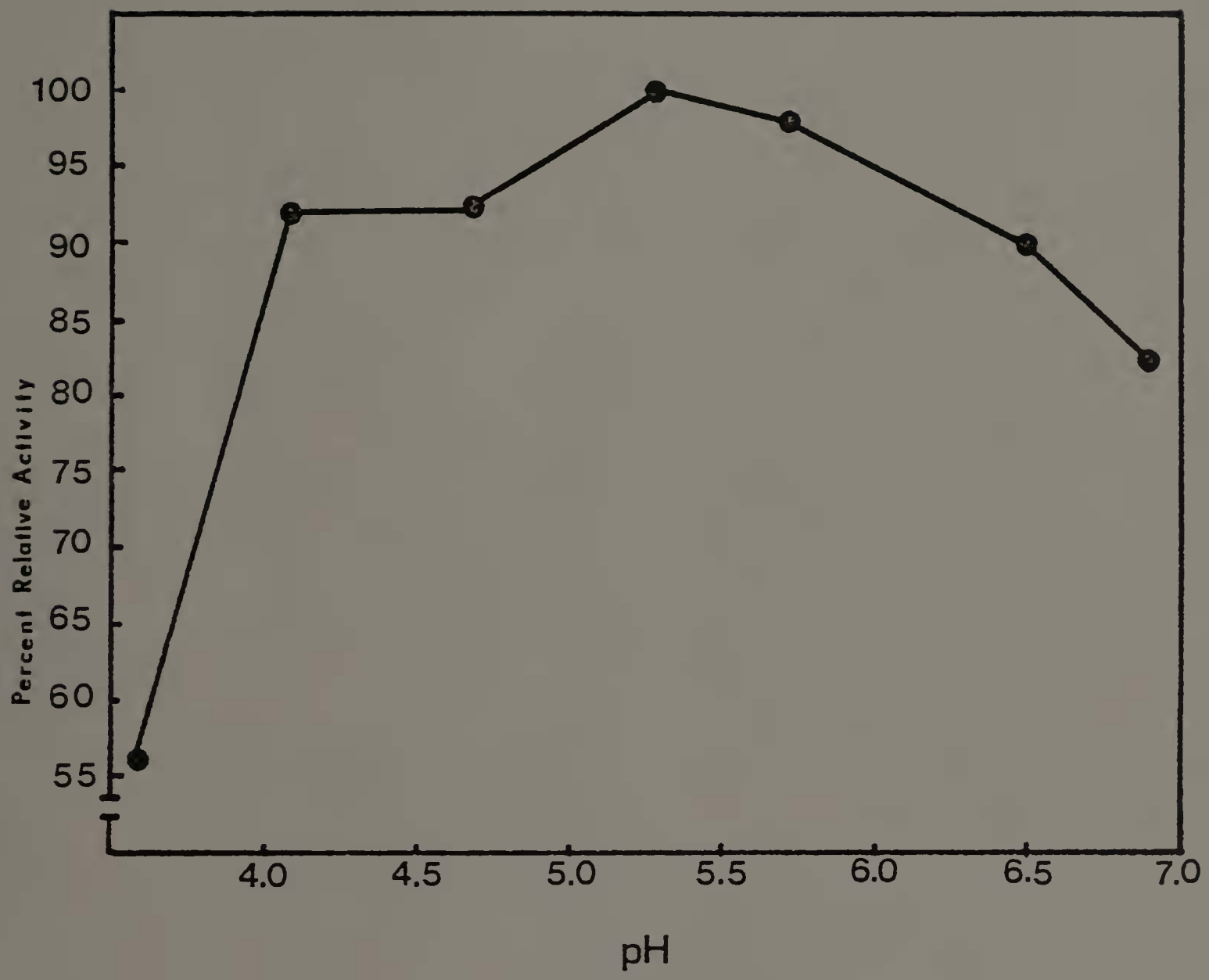


Fig. 5. Influence of pH on release of NAG from purified chitin.

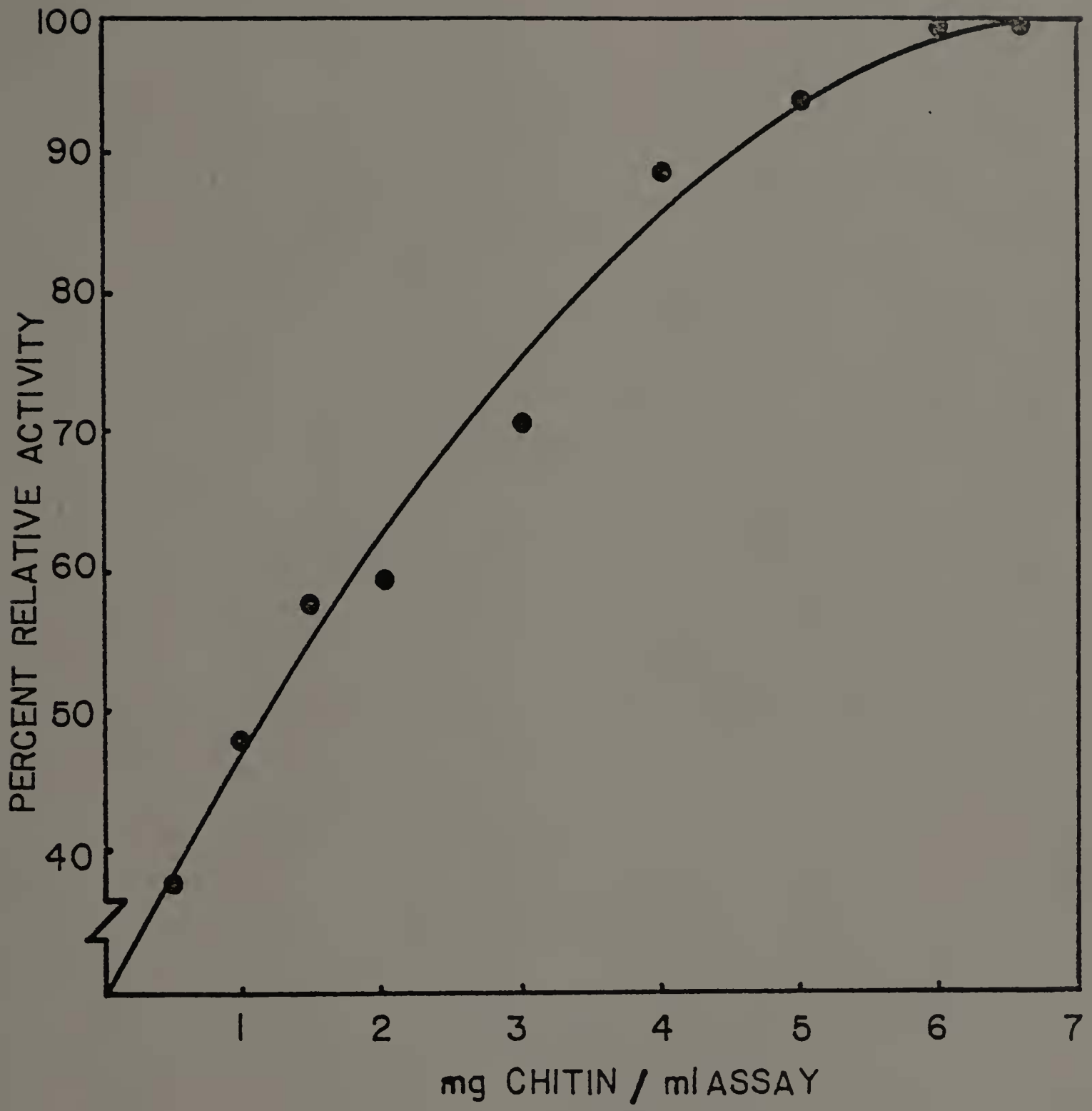


Fig. 6. Relationship of substrate concentration in total chitinase assay to release of NAG.

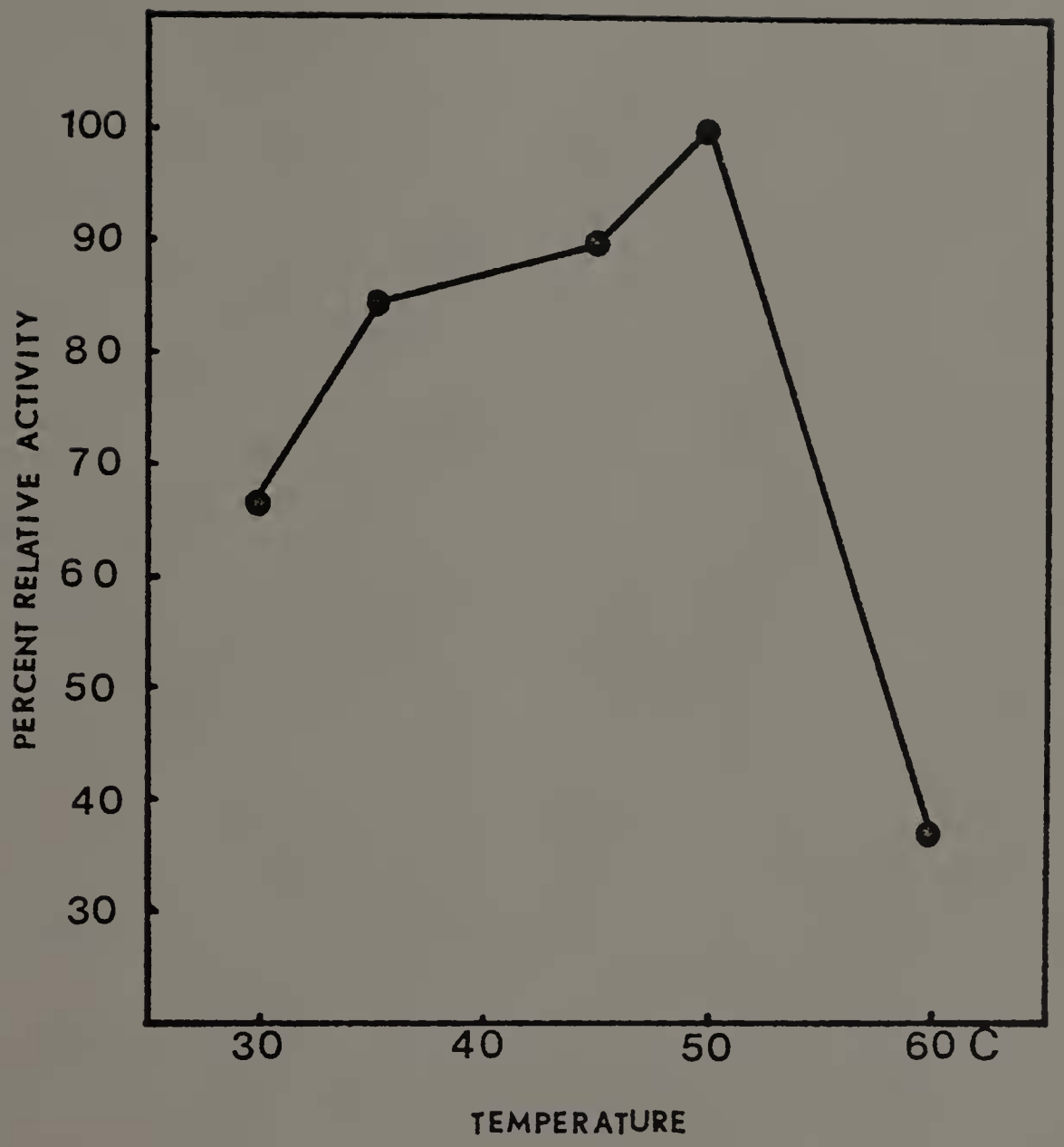


Fig. 7. Effect of temperature on release of NAG from purified chitin incubated with supernatant from culture 134.

TABLE 3

EFFECT OF DIVALENT CATIONS ON CHITINASE ACTIVITY

Divalent Cation 10 mM	µmoles NAG released/ ml supernatant	percent relative activity
Cu	9.7	59
Co	13.0	79
Ca	14.8	91
Zn	14.8	91
Mn	7.9	48
Mg	14.1	86
Fe	7.7	47
Control-	16.4	100

The assay incubated with Co had a relative activity of 79% and Cu, Mn and Fe had the greatest effect, decreasing the relative activity to 59, 48 and 47% respectively.

Chitobiase.

Standard chitobiase assay. The linearity of ρ-Nitrophenyl-N-acetyl-glucosaminide was measured under standard assay conditions and presented in Fig. 8. Chitobiase activity remained linear for 120 min and 60 min was subsequently chosen as the incubation time for standard assays.

Effect of pH on chitobiase activity. The activity of chitobiase was measured at various pH values. The pH optimum was found to range from 6.5 to 7.0 as shown in Fig. 9. The enzyme lost 30% of its relative activity at pH 5.7 and 22% at pH 8.0 using potassium phosphate buffer. Incubation of the same enzyme preparation with citrate buffer at pHs ranging from 3.5 to 6.0 demonstrated total loss of activity at pH 4.0 and below.

Effect of substrate concentration on chitobiase activity. Crude chitobiase was incubated with various concentrations of ρ-NPAG. Maximum enzyme activity was reached at a concentration of 0.9 mM ρ-NPAG as shown in Fig. 10. Subsequent assays were accordingly run with ρ-NPAG concentration of 2.0 mM to assure sufficient substrate.

Incubation of chitobiase assay mixtures at different temperatures. When crude chitobiase was incubated with

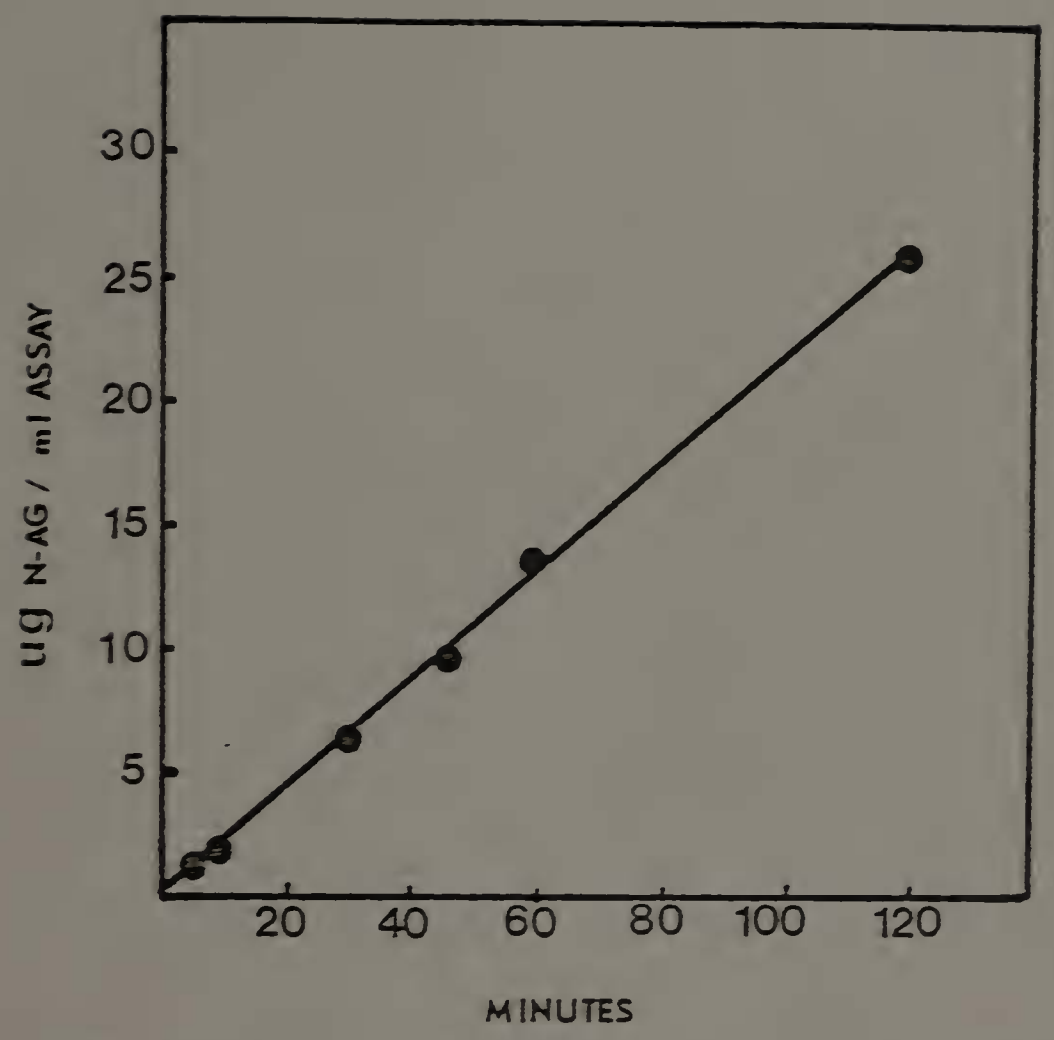


Fig. 8. Standard chitobiase assay. Chitobiase activity was determined by incubation of culture supernatant with o-NPAG.

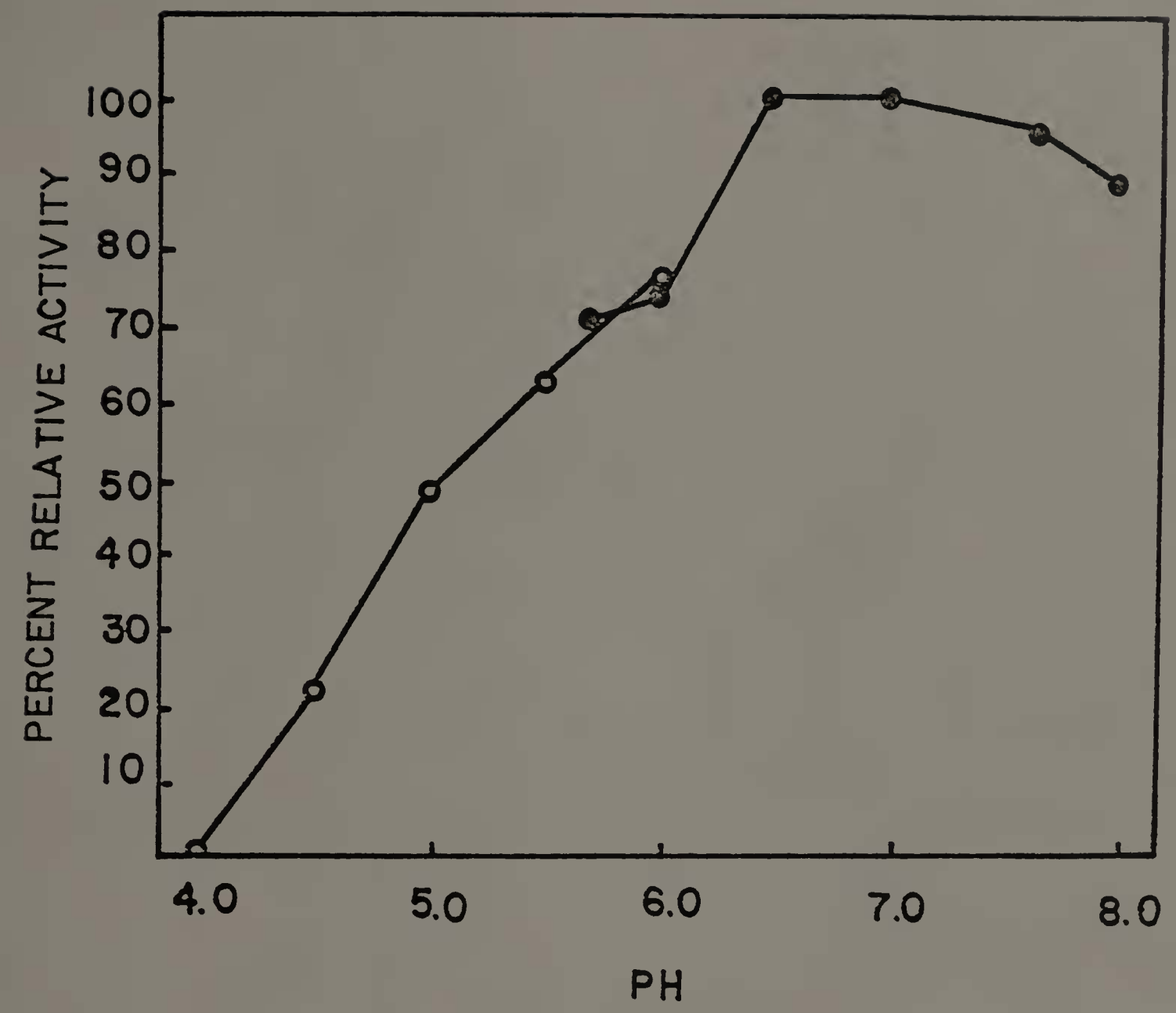


Fig. 9. Effect of pH on chitobiase activity with ρ-nitrophenyl-N-acetyl-β- D-glucosaminide as the substrate.

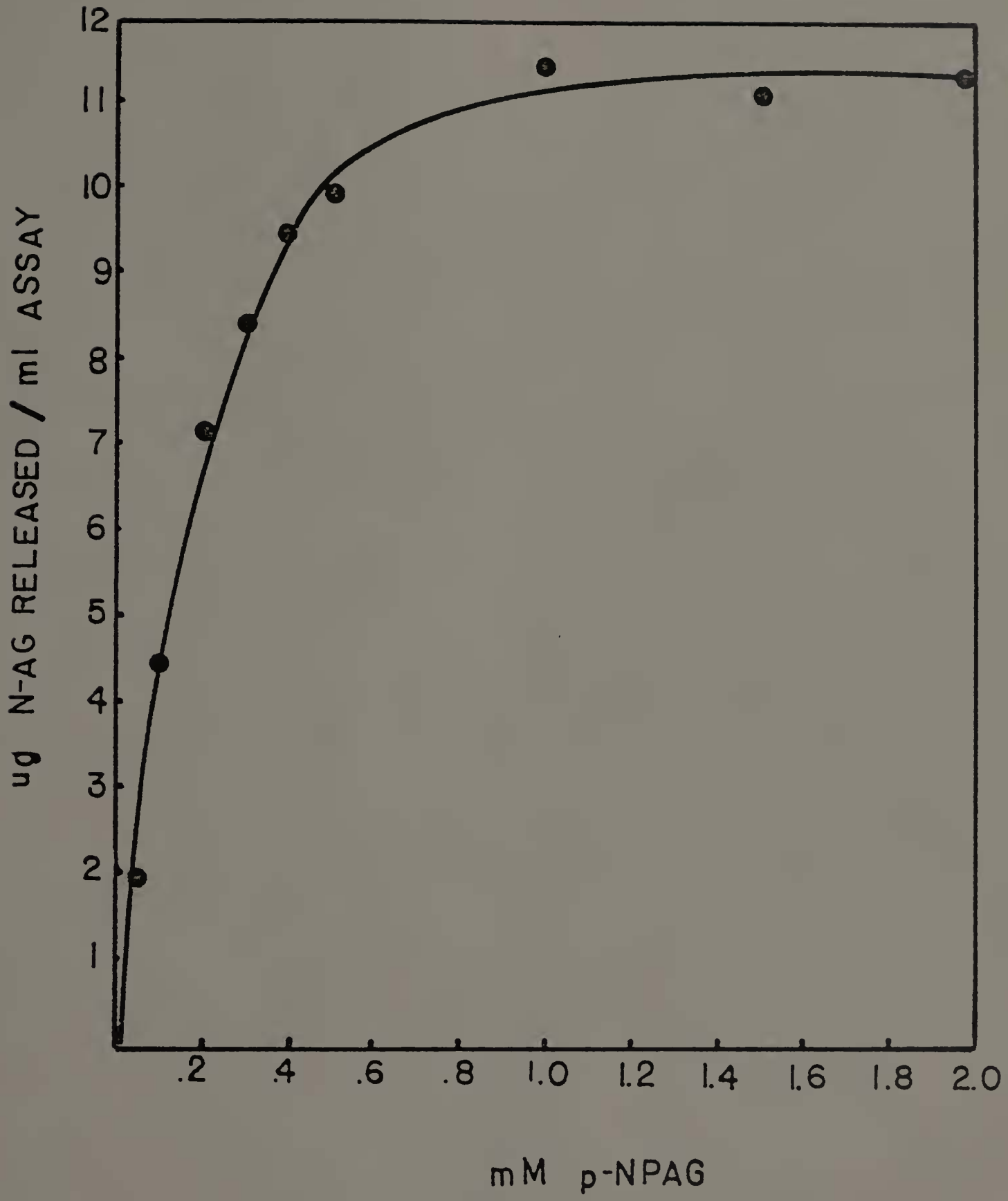


Fig. 10. Effect of substrate concentration on chitobiase activity.

standard assay mixtures at several temperatures, the greatest release of NAG from ρ-NPAG was found at 45 C (Fig. 11). The crude enzyme retained 25% of its activity at 25 C and 40% at 60 C.

Comparison of the effect of potassium phosphate and tris-maleate buffers on chitobiase activity. Potassium phosphate buffer was found to be slightly stimulatory when compared with tris-maleate buffer (Table 4). However, tris-maleate did not interfere with assays containing various cations as did potassium phosphate which formed a precipitate with the cations and tris-maleate buffer was chosen for use in subsequent tests with various cations.

Effect of divalent cations on chitobiase activity. Ca and Mg had little or no effect on enzyme activity as demonstrated in Fig. 12. Mn and Co appeared slightly deactivating; Cu and Zn were found to be strongly inhibitory with the enzyme losing 90% and 100% of its activity, respectively, at 10 mM concentration of these cations.

Effect of a monovalent cation on chitobiase activity. Incubation of several concentrations of Na^+(NaCl) with crude chitobiase had a slightly deactivating effect as demonstrated in Table 5. Of the total chitobiase activity 17.4% was lost by increasing the concentration of Na^+ from 0.01 M to 0.1 M.

Effect of EDTA on chitobiase activity. Preincubation of crude chitobiase with EDTA had a deactivating effect

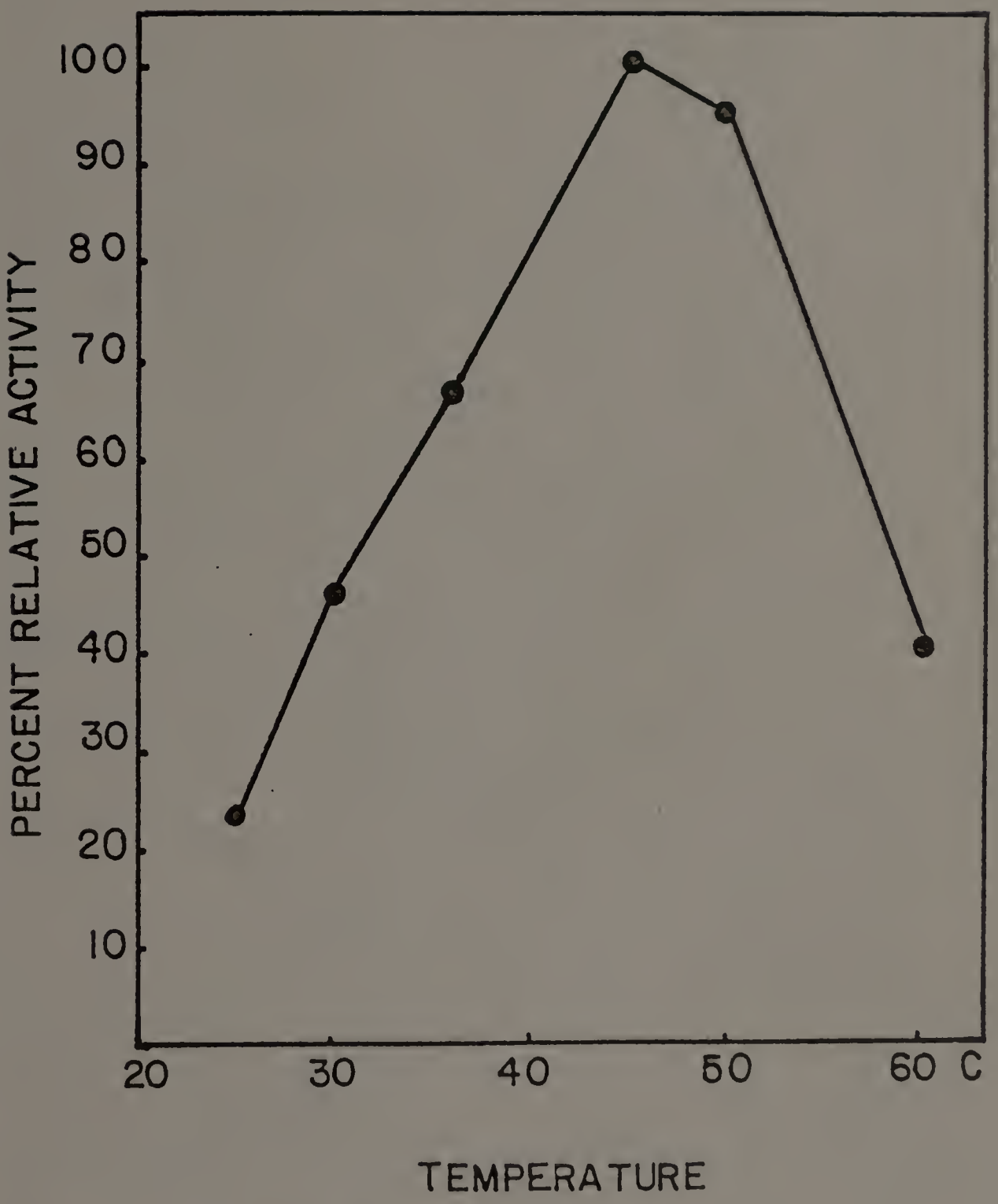


Fig. 11. Effect of temperature on chitobiase activity.

TABLE 4

CHITOBIASE ACTIVITY IN THE PRESENCE OF POTASSIUM PHOSPHATE AND TRIS-MALEATE BUFFERS

Buffer (pH 6.7)	Concentration	µg NAG/ ml assay	percent Relative Activity
Potassium phosphate	0.2M	11.0	100
Tris-Maleate	0.2M	10.1	91.8

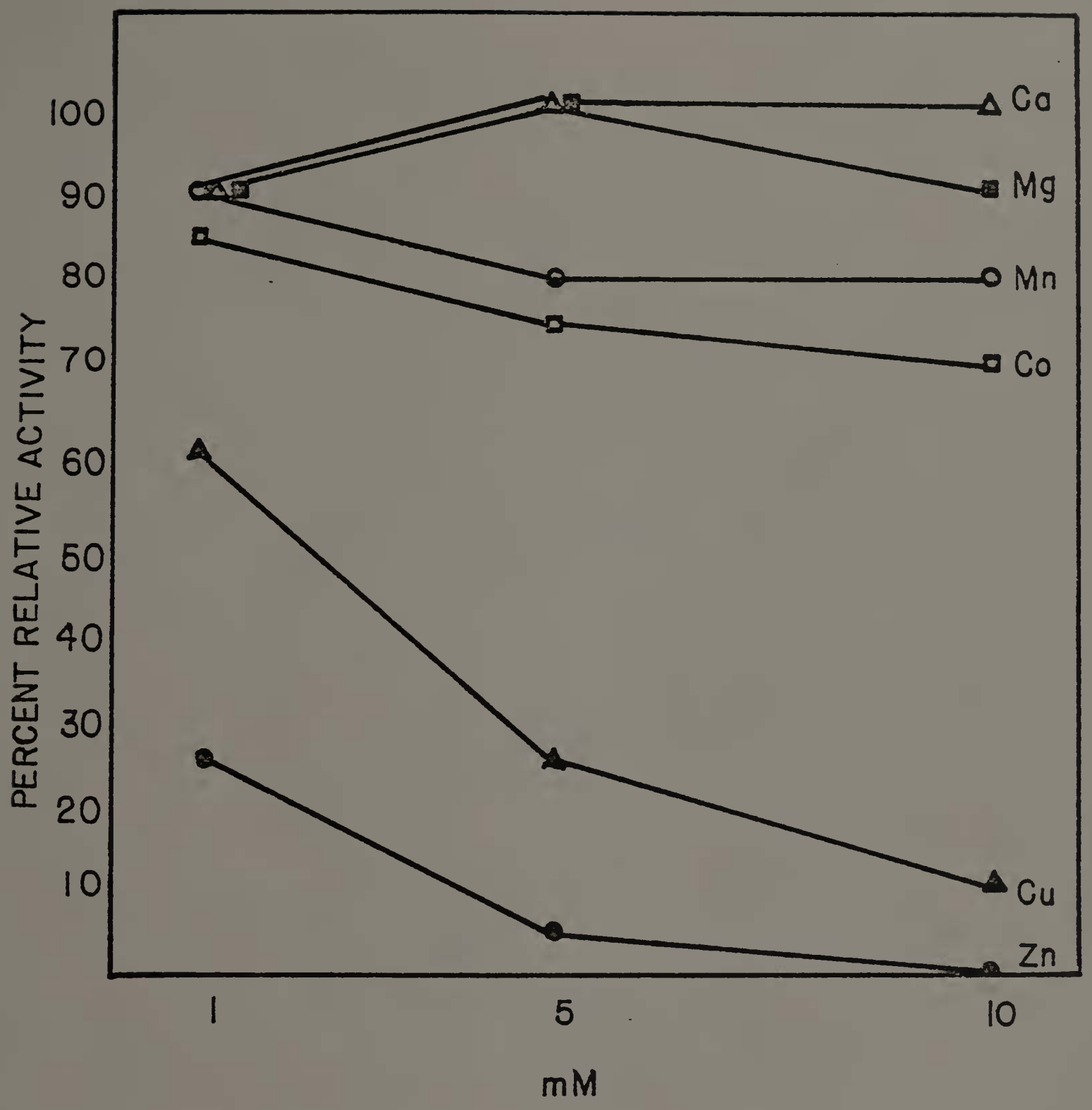


Fig. 12. Effect of divalent cations on chitobiase activity.

TABLE 5

THE EFFECT OF VARIOUS CONCENTRATIONS OF A MONOVALENT CATION Na+ (NaCl) ON CHITOBIASE ACTIVITY

Concentration of Na+ (M)	ug NAG released/ ml assay	Relative activity
0.1	8.1	82.6
0.05	8.5	86.7
0.01	9.8	100.0
0.0-control	9.8	100.0

(Table 6). Chitobiase incubated with 1.0 mM EDTA lost 5.5% of its relative activity; increasing the concentration to 10 mM, resulted in a loss of 27.8% of the relative activity.

Effect of two reducing agents on chitobiase activity. Incubation of crude chitobiase with mercaptoethanol and DTT at several concentrations had a slight activating effect as shown in Table 7.

Chitin hydrolase.

Standard chitin hydrolase assay. Results of the hydrolysis of purified chitin by chitin hydrolase at different intervals are presented in Fig. 13. The reaction rate remained linear for 60 min after which time no more chitin was hydrolyzed. Sixty minutes was therefore chosen as an appropriate incubation time in subsequent assays.

Effect of pH on chitin hydrolase activity. The activity of crude chitin hydrolase was determined at several pH values and the results are shown in Fig. 14. The pH optimum was found to be 5.8. The enzyme lost 34% of its activity at pH 4.2 and 31% at pH 6.8.

Effect of temperature on chitin hydrolase activity. Incubation of chitin hydrolase in a standard assay mixture at different temperatures showed that the greatest hydrolysis of purified chitin occurred at 50 C (Fig. 15). Raising the temperature to 60 C reduced the relative activity by 18.5% while at 25 C 59% of the relative activity was lost.

TABLE 6

EFFECT OF EDTA ON CHITOBIASE ACTIVITY

EDTA mM	µg NAG released/ml assay	Percent Relative Activity
1.0	8.5	94.5
5.0	7.5	83.4
10.0	6.5	72.2
0.0-Control	9.0	100.0

TABLE 7

EFFECT OF TWO REDUCING AGENTS MERCAPTOETHANOL AND DTT ON CHITOBIASE ACTIVITY

Reducing agent	Concentration mM	µg NAG released/ ml assay
Mercaptoethanol	1.0	11.0
	5.0	11.0
	10.0	12.0
DTT	1.0	11.0
	5.0	11.0
	10.0	11.5
Control	0	10.5

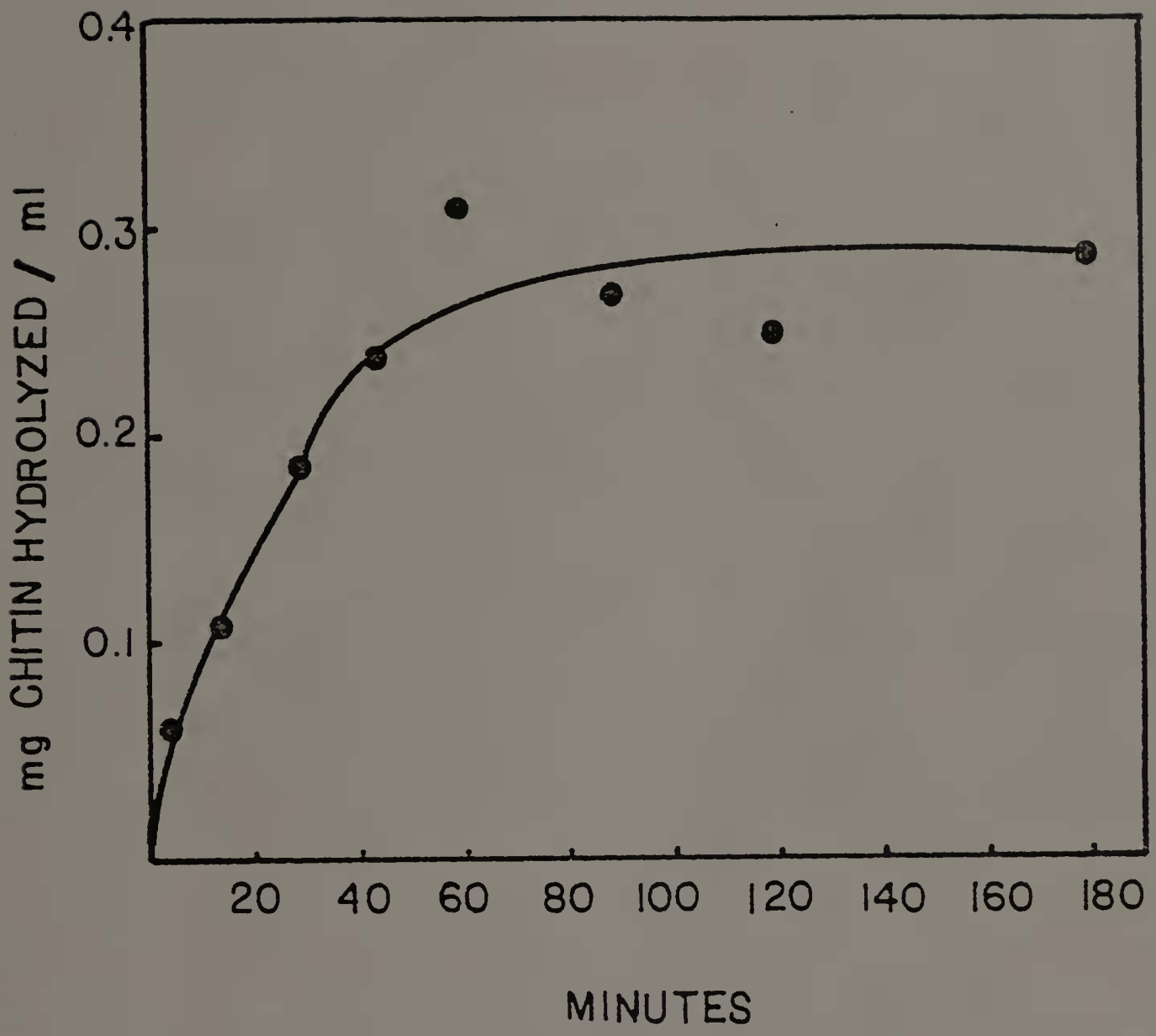


Fig. 13. Standard assay for chitin hydrolase. Crude chitin hydrolase was incubated with purified chitin and the decrease in turbidity due to the hydrolysis of chitin was measured over a period of time. Units of activity were expressed as mg chitin hydrolyzed per ml assay.

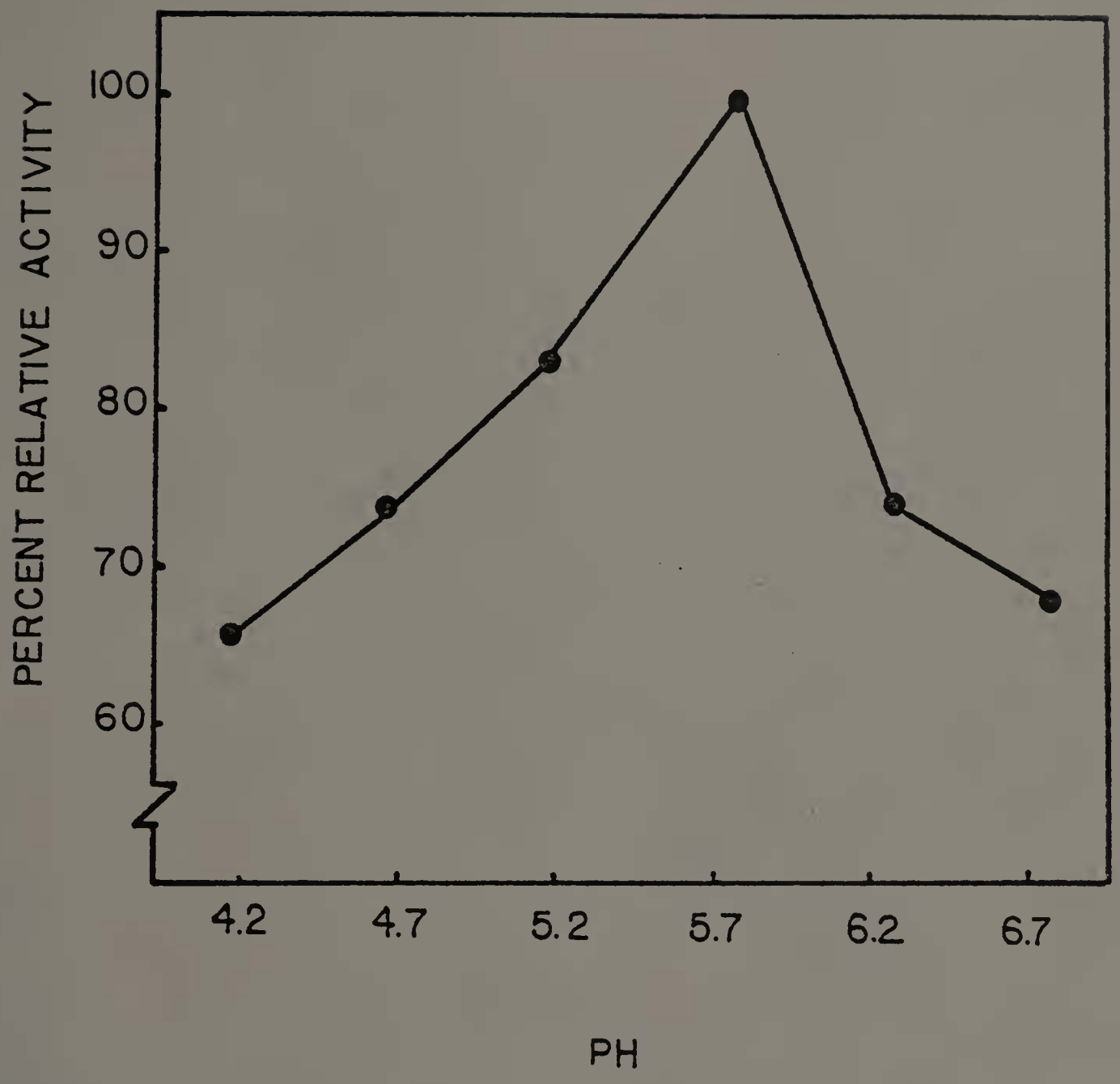


Fig. 14. Effect of pH on chitin hydrolase activity.

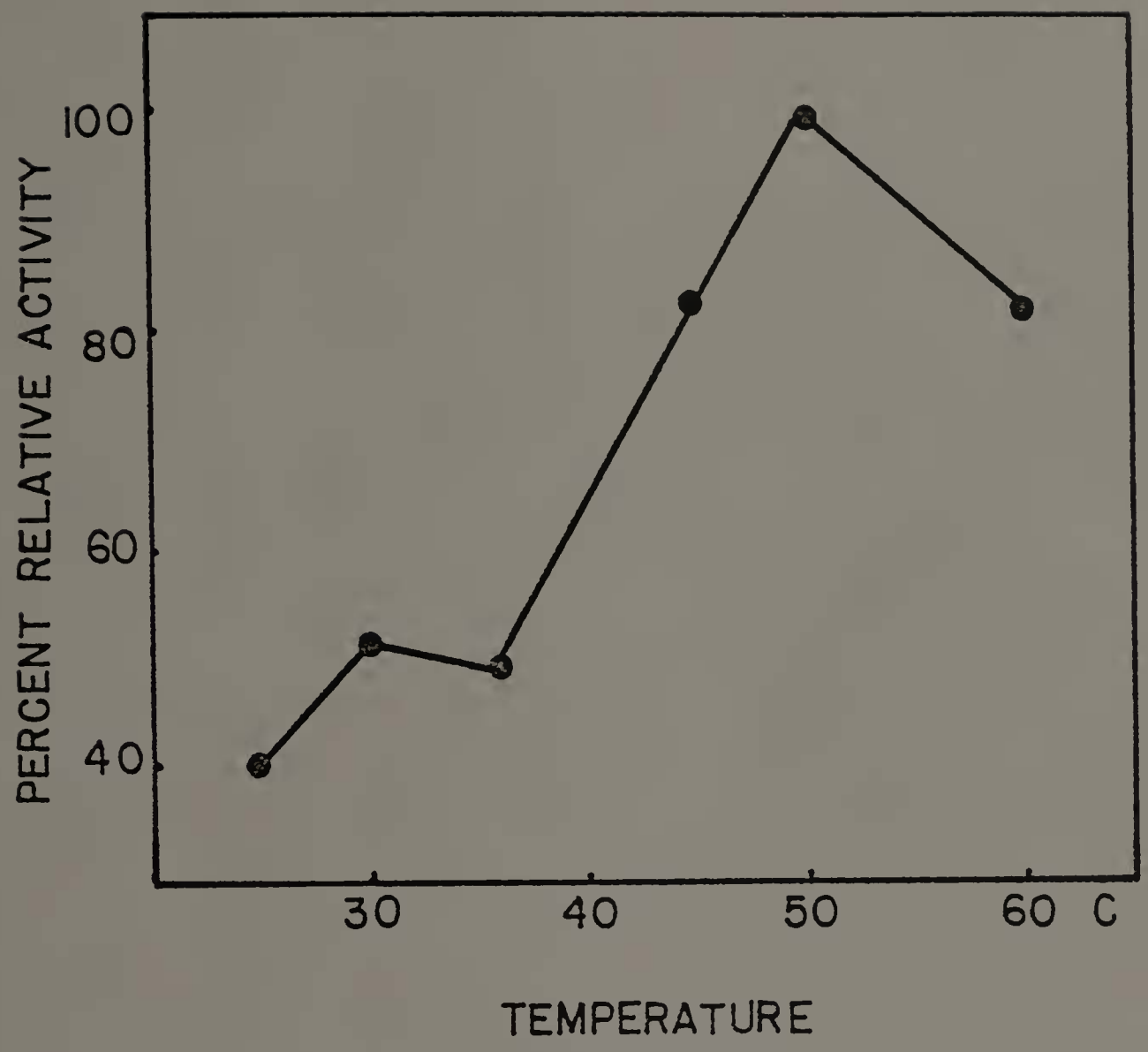


Fig. 15. Effect of temperature on chitin hydrolase activity.

Effect of divalent cations on chitin hydrolase activity. Chitin hydrolase was incubated with each of several divalent cations at 10 mM concentration (Table 8). All of the cations tested reduced enzyme activity. The greatest deactivation occurred in the presence of Ca where the relative activity was reduced by 58%. The least deactivation was found when the enzyme was incubated with Co and 21% of the activity was lost.

Effect of reducing agents on chitin hydrolase activity. The effects of incubating crude chitin hydrolase with different concentrations of mercaptoethanol and DTT are presented in Table 9. Both mercaptoethanol and DTT exhibited a slight activating effect.

Enzyme stability and preliminary purifications.

Heat stability of crude chitin hydrolase, chitinase and chitobiase at 50 C. Crude enzyme supernatant was incubated without substrate for various periods at 50 C and then tested by all three assays for activity (Fig. 16). Chitobiase lost 80% of its relative activity after 20 min and then continued to decline gradually over the 180 min test period to 5% of its original activity. Total chitinase activity declined more gradually over the 180 min period to 45% of its original activity, while chitin hydrolase activity dropped to 60% at 60 min and then increased to 82% at 180 min.

TABLE 8

EFFECT OF DIVALENT CATIONS ON CHITIN HYDROLASE ACTIVITY

Divalent cation (10 mM)	mg purified chitin hydrolyzed/ml assay	percent relative activity
Cu	.10	52
Co	.15	79
Ca	.08	42
Zn	.10	52
Mn	.12	63
Mg	.11	57
Fe	.12	63
Control	.19	100

TABLE 9

EFFECT OF REDUCING AGENTS, DTT AND MERCAPTOETHANOL, ON CHITIN HYDROLASE

Reducing agent	Concentration mM	mg chitin hydrolysed/ ml assay
DTT	1.0	.14
	5.0	.14
	10.0	.15
Mercaptoethanol	1.0	.16
	5.0	.12
	10.0	.12
Control	0	.12

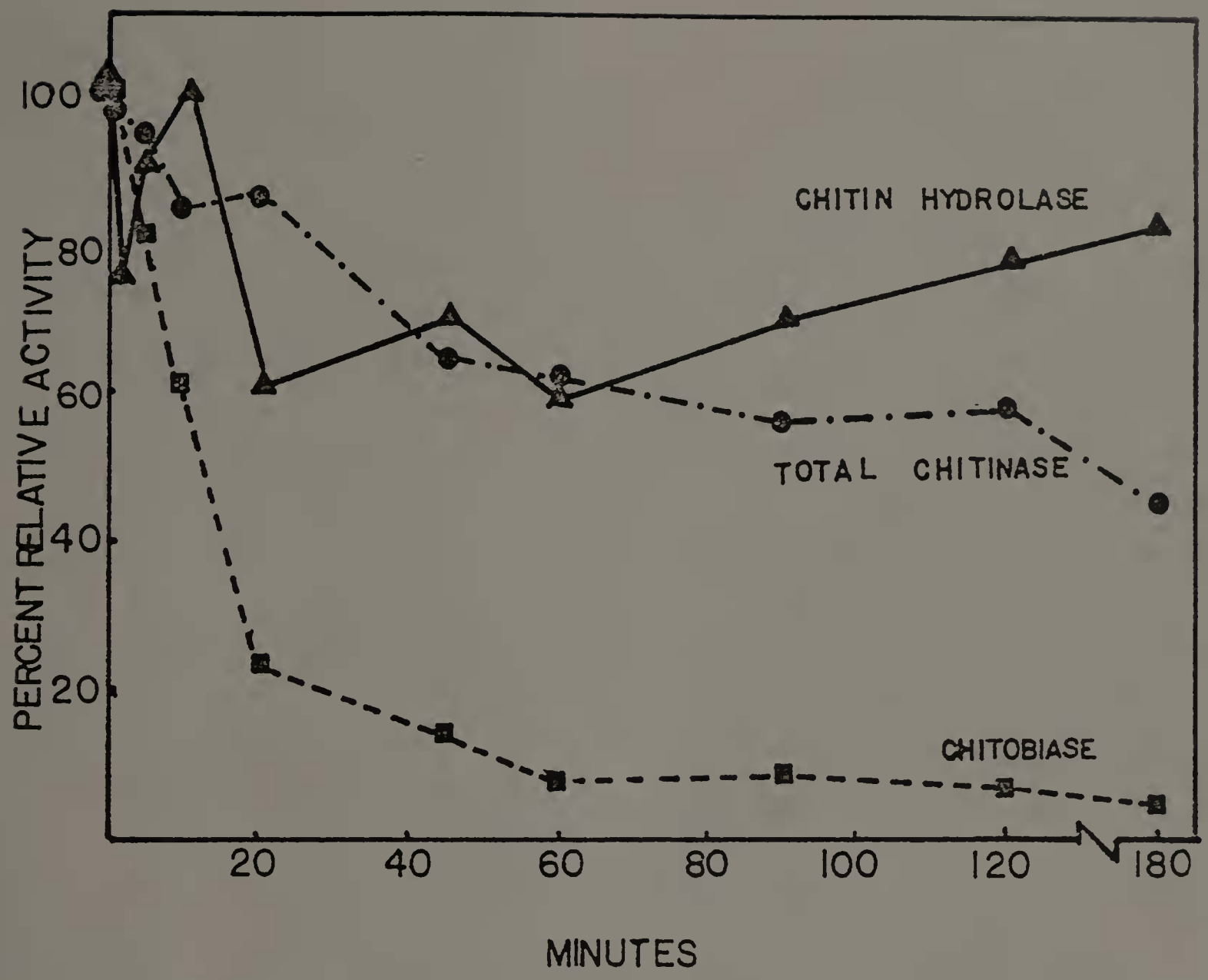


Fig. 16. Heat stability of crude chitin hydrolase, chitinase and chitobiase at 50 C. Crude enzyme was preincubated at 50 C and aliquots were taken at intervals and tested by the 3 assays for activity.

Effect of freezing on total chitinase and chitobiase activity. Crude enzyme supernatant was frozen for one day at -60 C with no discernible loss of total chitinase and only slight inactivation of chitobiase, as shown in Table 10. Other enzyme preparations, stored at -60 C for up to 6 months suffered no appreciable loss of activity.

Adsorption of chitinase by purified and crude chitins. Crude enzyme supernatant was tested for adsorbtion to crude and purified chitin. Results are presented in Table 11. When the crude enzyme was mixed with purified chitin and the supernatant subsequently assayed, it was found that 45% of its original activity was removed from solution and mixing with crude chitin adsorbed 36% of the chitinase activity.

Ammonium sulfate fractionation. Crude supernatant was fractionated by precipitation with ammonium sulfate (Table 12 A and B). There was a 97% recovery of chitinase units and the most activity was found in the 60 - 90% fraction. The greatest chitin hydrolase activity was also found in this fraction (44%) and recovery of chitin hydrolase units was 105%. Maximum chitobiase activity was found in the 60 - 90% fraction (58.5%) with a total recovery of 97.4%.

Ancillary studies.

Chitinase activity of commercial lysozyme. Commercial lysozyme (Sigma Chemical Co.) was assayed for total chitinase, chitin hydrolase and chitobiase activities and results

TABLE 10

EFFECT OF FREEZING ON TOTAL CHITINASE AND CHITOBIASE ACTIVITY

Sample	Total chitinase activity µmoles NAG released/ ml supernatant	Chitobiase activity µmoles NAG released/ ml supernatant
Crude enzyme control	18.2	65.0
Crude enzyme frozen at -60 C	18.2	54.0

TABLE 11

ADSORPTION OF CRUDE ENZYME SUPERNATANT ONTO COMMERCIAL AND PURIFIED CHITIN

Type of chitin used as absorbent	Activity µmoles NAG released/5ml enzyme	Percent adsorbed
Purified 40 mg total	46.2	45%
Commercial 1 g total	54.0	36%
None control	84.0	-

TABLE 12(A)

AMMONIUM SULFATE FRACTIONATION OF CRUDE SUPERNATANT FROM CULTURE 134

Fraction % saturation $(NH_4)_2SO_4$	Protein mg	% Recovery	Chitobiase units	Specific Activity	% Recovery
Original supernatant (90 ml)	193.5	100	1,540.0	7.9	100
0-30 (6.5 ml)	7.5	3.8	48.6	6.5	3.1
30-60 (6.0 ml)	8.4	4.3	567.5	67.5	36.8
60 - 90 (11.5 ml)	49.5	25.6	901.0	18.2	58.5
Total 0-90	65.4	33.7	1,517.0	-	97.4

TABLE 12(B)

AMMONIUM SULFATE FRACTIONATION OF CRUDE SUPERNATANT FROM CULTURE 134

% Fraction saturation $(NH_4)_2SO_4$	Chitinase units	Specific Activity	% Recovery	Chitin hydrolase units	Specific Activity	% Recovery
Original supernatant (90 ml)	2,308	11.9	100	132.3	.68	100
0-30 (6.5 ml)	487	64.8	21.1	48.8	6.5	37
30-60 (6.0 ml)	720	85.7	31.1	32.4	3.8	24
60 - 90 (11.5 ml)	1,035	20.9	44.8	58.6	1.2	44
Total 0-90	2,242	-	97.0	139.7	-	105

are presented in Table 13. Lysozyme was found to have appreciable chitin hydrolase activity and it could also release NAG from purified chitin. However, it had no demonstrable chitobiase activity as measured by degradation of p-NPAG.

Determination of chitin hydrolase, total chitinase and chitobiase activities in cells grown in TSB. The results of assays for total chitinase, chitin hydrolase and chitobiase activities from various treatments and fractions of cells grown in TSB are presented in Table 14. Chitinase activity was found in all supernatants and resuspended pellets. The chitinase activity from the supernatant of the cell culture may reflect interference from the color of TSB rather than the actual release of NAG from purified chitin. The highest activity per ml supernatants and cell suspensions were found in treatments 3, 5 and 6 and the highest activity per mg protein in 3, 4 and 6. Chitin hydrolase activity was found only in treatment 4 (supernatant from washed cells incubated overnight in buffer). Chitobiase activity was found also in all treatments. The activity per ml was greatest in treatments 3 and 5 while the specific activity was highest in treatment 3. Treatments 4, 5 and 6 had only 20% of the specific activity found in 3.

TABLE 13

CHITINASE ACTIVITY OF COMMERCIAL LYSOZYME DILUTED 1.0 MG/ML, IN STANDARD ASSAY MIXTURES

Replicate	Assay	Chitin hydrolase mg chitin hydrolysed/ml assay	Chitobiase µmoles NAG released from -NPAG /ml assay	Total chitinase µmoles NAG released from purified chitin/ml assay
1		.12	0	.23
2		.11	0	.23

KEY FOR TABLE 14

1. Supernatant from centrifugation of the culture grown in TSB for 2 days.

2. Supernatant from washing pellet with Modified Morris Medium (without carbon source).

3. Pellet from centrifugation of cells incubated overnight in buffer, resuspended in 10 ml potassium phosphate buffer (pH 7.0, 0.05 M).

4. Supernatant from centrifuged cells incubated overnight in buffer.

5. Pellet from centrifugation of sonicated cells, suspended in 5.0 ml 0.05 M potassium phosphate buffer (pH 7.0).

6. Supernatant from centrifugation of sonicated cells.

TABLE 14

CHITINASE, CHITIN HYDROLASE AND CHITOBIASE ACTIVITY DEMONSTRATED BY CELLS GROWN IN TRYPTICASE SOY BROTH

Treatment	Protein µg/ml	Chitinase activity µmole/ml	µmole/mg	Chitin hydrolase µg/ml	µg/mg	Chitobiase µg/ml	µg/mg
1	8.4	0.21	0.025	0	0	38	4.5
2	1.8	0.72	0.4	0	0	90	50.0
3	3.6	1.35	0.375	0	0	1,750	486.0
4	0.25	0.15	0.6	0.36	1.44	24	96.0
5	12.0	1.92	0.16	0	0	1,000	83.0
6	3.55	2.16	0.608	0	0	500	140.0

DISCUSSION

Clark and Tracy (10) in 1956 tested 6 species of *Erwinia* for chitinolytic ability and found only one to be chitinase positive. Other members of the Enterobacteriaciae which have been tested for chitin hydrolysis include *Vibrio*, *Serratia* and *Klebsiella* (10, 50). Only three studies describing the chitinase systems of gram negative bacteria are to be found in the literature two on Serratia (45, 51) and one on *Arthrobacter* (54).

This study was undertaken to characterize the chitinase system of an *Erwinia* strain pathogenic for gypsy moth larvae.

The history of this study is instructive in understanding certain of the characteristics of chitinase systems. This organism (strain 134) was initially isolated from a gypsy moth larva. It, among the many organisms isolated from this source, (fungi, actinomycetes, etc.) produced the most rapid clearing of chitin agar. In early growth studies, when a *Bacillus* sp. (also an isolate from gypsy moth) was compared with isolate 134 (see appendix no. 1) the *Erwinia* sp. produced a chitinase at day 24 that had a specific activity 65 times that of the *Bacillus* on purified chitin. On crude chitin, strain 134 had 55 times more chitinase activity/mg

protein than the other isolate. Thus choosing the Erwinia for this study immediately gave a much more active and possibly purer chitinase system to work with.

The purified colloidal chitin preparation which was being used at this time was stored as an acid paste at 4 C and as it was needed an aliquot of it was taken, adjusted to pH 7.0 with KOH, centrifuged and made into a slurry with distilled water. The slurry was dialyzed against 3 changes of water in the cold. This chitin was used as a growth substrate for approximately one year. In a period of about three months this preparation changed from producing actively growing cultures with high chitinase titers to cultures which did not increase in numbers from the original inoculum and released no chitinase at all. An effort was made to determine the cause of this anomaly but no satisfactory explanation was found. Finally a new batch of chitin was prepared and with a suspicion that the previous batch had somehow been degraded in storage as an acid paste, the new batch was adjusted to pH 7.0 immediately, washed several times and then sterilized by autoclaving. It was then stored in the refrigerator. When strain 134 was grown on the new chitin and the chitin supernatant tested in assays using both batches for the substrates, the assay using the new chitin had more than twice the amount of NAG released than the assay using the old chitin (see appendix no. 1).

The decreased release of NAG could reflect transformation of the chitin during storage to a less susceptible substrate, or release of D-glucosamine instead of NAG which would not react with the Reissig test for acetyl hexosamines.

In retrospect and in view of recent articles on chitosan and chitosanase (66) and reports on the lack of chitinase activity when cells were grown on chitosan (51, 54) it seems that the chitin must have been at least partially deacetylated and thus converted to chitosan or some other form of degraded chitin.

Although drastic changes in chitinase production were not seen after the new purified chitin was made, variability in results especially in growth studies frequently occurred with no other explanation than the intractibility of the substrate. The growth study reported in this thesis derives from previous findings on the various conditions which might maximize chitinase yields. Although the peak in cell numbers and chitinase activity was reached on incubation day 3 and day 5 respectively, the cell numbers remained stable between fairly narrow limits, 1×10^{10} and 4.5×10^{10} throughout the course of the experiment. The specific activity of the chitinase dropped from the peak of 57 µmoles NAG released/mg protein to 37.5 µmoles/mg protein. The peak of the chitinase activity was not reached until the cell numbers began to decline. This pattern was observed in other studies performed

in this laboratory (12). In other growth studies optimum chitinase activity was reached after 15 to 25 days incubation. This was also true when the cells were grown on non-chitinous substrates such as TSB, yeast extract, beef extract, and casein (12). The chitinase levels produced on these substrates were much lower than those found produced in chitin medium. This may represent an autolytic enzyme system rather than the presence of a true chitinase.

It is of interest that protein levels increased in the supernatant as the level of chitin in the medium increased. One possible source for such protein may be a result of enhanced cell autolysis in the presence of chitin and the liberation of cell protein. Such speculation is further reinforced by the fact that the protein concentration stays constant regardless of the change in cell numbers over the course of the experiment.

The levels of chitobiase as measured by the release of NAG from ρ-NPAG were found to be much greater when cells were grown on crude chitin in comparison with growth on various concentrations of purified chitin. The release of chitobiase, however, increased with increase in chitin concentration whether purified or crude. On the other hand chitin hydrolase and chitinase activity in the supernatants was unaffected by whether the chitin in the medium was crude or purified. A number of possible explanations may be advanced for the high

amount of chitobiase in the crude chitin cultures: 1) some stimulatory compound is present in the crude chitin that enhances chitobiase production; 2) the chitinase works more slowly on the crude chitin because of reduced surface area and smaller amounts of chitodextrins are released so the cells have compensated for this by increasing chitobiase production. Another interesting anomaly is suggested in the results recorded in Fig. 1. The highest concentration of chitinase was produced on the greatest amount of substrate with purified chitin while the trend was reversed with crude chitin. This could be a result of reduced surface area on the 1% crude chitin where more enzyme must be produced to obtain a similar amount of soluble polymer.

In another comparison, of 1% pure chitin and 3% crude chitin as substrates for enzyme production, the specific activity from the purified chitin culture was approximately 10 times that of the crude chitin culture. The total activity per ml however was almost the same in both cultures. Crude chitin was used in large batch production of crude enzyme preparations because of its more desirable characteristics in terms of cost, handling and consistency.

The use of three enzyme assays was adopted because of the probability that the measurement of release of NAG from chitin was an indirect method of measuring chitinase. The use of the assay was continued as a means of measuring the

total chitin hydrolytic system and as an indication of the efficiency of the overall system. If the production of one enzyme of the system was disproportionate it could more easily be perceived through the multi-assay analysis. In this regard, chitobiase was invariably found to be present in excess. Thus, the total chitinase assay was considered to be a valid measure of overall chitinase activity.

The chitin hydrolase assay was not found to be a very sensitive test. It tended to be more a gross indication rather than an index of specific activity. What is of significance, however, is that in this assay the reaction remained linear for about 60 minutes after which no additional hydrolysis occurred. This may indicate a feedback inhibition where the soluble oligomers above a certain concentration, interfere with the hydrolysis of the polymer. Conversely it may simply be a reflection of the limits of the assay indicating that substrate levels have fallen to the point where the activity of the enzyme slows or stops completely.

The total chitinase activity in the standard assay also remained linear through 60 min and then the reaction rate began decreasing slowly. In contrast the chitobiase assay remained linear throughout the entire testing periods.

A linear relationship was maintained throughout progressive dilution of the enzyme system as measured by the release of NAG from purified chitin indicating that inhibitors were not present and the enzyme(s) does not dissociate.

The optimum pH values of 5.3 to 5.8 for the chitin hydrolase and total chitinase were found to be consistent with those reported by several authors (13, 20, 32, 37, 43, 44, 53). The optimum pH for the chitobiase assay at pH 6.5 to 7.0 was significantly higher than has been previously reported. The chitobiase assay as described by Levvy and Conchie (41) was performed at a pH of 4.4. Bahl et al. (2), Lunt and Kent (44) and Jeuniaux (31) also assayed for chitobiase activity at pHs of 5.0 and less. Berger and Reynolds (5) used a pH of 6.3 in their assay using phenyl-β-D-2-deoxy-2-acetamidoglucopyranoside. The activity of chitobiase dropped to 0 at pH 4.0 while the relative activity of the chitin hydrolase retained 65% of its activity at pH 4.2. The total chitinase assay decreased from 92% relative activity to 56% when the pH was decreased from 4.2 to 3.6. The difference in pH optima may reflect the particularity of the bacterial strain from which it was derived. It may also suggest that chitobiases may have characteristics quite distinct from the chitin hydrolases with which they are associated.

The optimum pH of 5.3 for the total chitinase assay may be another indication that the chitin hydrolase is the rate limiting factor, with the chitobiase having high enough activity to compensate for the decrease in pH.

In attempting to optimize chitin and ρ-PNAG concentrations for the assay techniques used, it was found that the

activity of the chitinase assay appeared to reach maximum velocity at a substrate concentration of about 6 mg/ml assay. The concentration of the substrate could not be increased beyond this point because of its unwieldiness. Chitin at this concentration had a semi-solid consistency and was difficult to measure. Other authors used 1 and 2 mg/ml assay (63, 78) and Reynolds (70) suggested that 3 mg/ml assay was sufficient. Vessey and Pegg (85) used a concentration of 20 mg/ml and an incubation time of 24 to 48 hours. Chitinase appears to have a low affinity for the substrate. In contrast the substrate concentration-product relationship of chitobiase as measured by the degradation of ρ-NPAG,(initial results) indicated a high affinity of the enzyme for the substrate. The K_m was calculated by a Lineweaver Burk plot to be 0.25 mM. Bahl and his colleagues (2) found the K_m of a β-N-acetylglucosaminidase on ρ-nitrophenyl glycosides to be 0.66 mM and Robinson et al. (72) found the K_m of a rat kidney N-acetylglucosaminadase to be 0.67 mM when assayed on 4-methylumbelliferyl (N-acetyl)-β-glucosaminide.

When the activities of chitin hydrolase, total chitinase and chitobiase were measured at different temperatures, the results followed a similar pattern to that found in the pH studies. Both the chitin hydrolase and total chitinase had optimum activity when incubated at 50 C. In contrast Jeuniaux (32) reported that most chitinases had temperature

optimum of 40 C. Several authors have reported the optimum temperature for enzyme activity as 50 C (51, 54, 59, 61).

The temperature optimum for chitobiase was found to be 45 C with the activity decreasing slightly at 50 C. When the temperature was increased to 60 C the chitobiase lost 60% of its relative activity. No temperature studies were reported in the literature examined but other assays were conducted at 37 C (31, 72) and 38 C (41).

The effect of divalent cations at 10 mM on chitin hydrolase and total chitinase assays was significant. The chitin hydrolase assay was inhibited by all of the cations tested. The least inhibitory cation was Co which caused a decrease in activity of 21%. The most inhibitory cation was Ca, decreasing activity by 58%. The others, Mg, Fe, Cu, Mn and Zn produced inhibition ranging between that of Co and Ca. When a similar experiment was performed on the total chitinase assay Ca and Zn appeared the least inhibitory and Mn and Fe the most inhibitory. Chitobiase activity was tested against three concentrations of cations. Cu and Zn were found to be strongly and progressively inhibitory with the chitobiase retaining 10% and 0% of its relative activity at 10 mM concentration. Mn and Co were found slightly inhibitory while Mg and Ca exhibited little or no effect. Skujins et al. (78) stated "inhibition of chitinase by metal ions is characteristic" and several authors have reported

metal inhibition studies. Jeuniaux (32) describes chitinase as being inhibited by Cu and Hg and Reisert (68) found some non competitive inhibition of chitinase by Na, K, Li, and Co at 0.2M and complete inhibition by Cu and Cd. Wigert (88) also reported Cu to be strongly inhibitory, while Mg exerted some stimulation. Vessey and Pegg (85) describe inhibition by Cu, Hg and KCN at 0.5 M and he found that NaN_3 had no effect. Kimura (37) found Ca, Pb and Hg inhibit and Mg, Fe, Sn and Zn enhance activity.

Levvy and Conchie (41) suggest adding NaCl to the reaction mixtures for the assay of β-N-acetylglucasaminidase. The effect of NaCl on chitobiase was slightly inhibitory.

Slight stimulation of chitin hydrolase and chitobiase was observed when mercaptoethanol and DTT was added to the reaction mixtures. This may indicate the presence of sulfhydryl groups in the 2 enzymes.

Enzyme Stability and Preliminary Purification. Chitinase and chitobiase were found to be extremely stable. Enzyme activity in cultures remained high for at least 25 days when maintained on a shaking incubator at 27 C. Crude supernatants held at 4 C in the absence of insoluble chitin remain active for several months. It was noticed that even in the supernatants from crude chitin cultures which had high amounts of free sugars and protein no fungal contaminants were ever seen. Frozen preparations retained almost all of their activity also.

The stability of chitinase was similar to that described by Jeuniaux (32). He found frozen preparations to maintain their activity for at least 2 years, and chitinases in the absence of substrate had a half life of 40 days at 37 C. The chitinases were much more stable in the presence on chitin.

The effect of incubation at 50 C on a crude enzyme preparation showed that the relative stability as measured by the chitin hydrolase and total chitinase assays was much greater than that of chitobiase. This pattern was also found by Berger and Reynolds (5). They freed their chitinase preparations of chitobiase activity by heating them to 65 C for 10-20 min. One of their chitinase fractions was considerably more heat sensitive than the other. Skujins and his colleagues (79) found chitinase from a Streptomyces to decrease in activity to 12% after being held at 50 C for one hr and to 3% after 3 hr. Lundblad et al (43) found the goat serum chitinase lost most of its activity after storage at -20 C for three weeks. Monreal and Reese (51) found the chitinase of Serratia marcescens lost 50% of its activity after 1 hr at 50 C and Lunt and Kent (44) noticed when they incubated their chitinase at 37 C for a period of time that the release of NAG ceased while turbidity decrease continued to occur. They theorized that the enzyme which released NAG from the oligosaccharides was slowly deactivated at this temperature.

Preliminary results have indicated a relationship between autolysis of cells and production of chitinase. Although "chitinase" activities were found after lengthy incubations on non-chitinous substrates, cells grown in chitin medium had at least twice the amount of activity (12). These results indicate that at least part of the chitinase measured is due to the activity of an enzyme involved in the autolytic process. In an effort to determine if young cells grown on a non-chitin medium (TSB), cells were subjected to several treatments. After each treatment the preparations were assayed for chitin hydrolase total chitinase and chitobiase activity. It was determined that they did, in fact, have a capacity for chitinolysis. This gave some confirmation to the idea that the chitinase system is at least partly involved in autolysis.

Few authors have differentiated between chitinase and lysozyme. Lundblad and coworkers (43) found that purified chitinase from goat serum was free from muramidase (lysozyme) activity. Since two of the most common assays for chitinase, the nephelometric and colorimetric (release of NAG from purified chitin) will give similar results when lysozyme is used, it seems likely that some of the studies describing chitinase were actually performed on a lysozyme or other lytic enzyme. Lysozyme from hen egg white was found to possess activity as measured by the chitin hydrolase and total chitinase assays,

but it was not able to degrade ρ-NPAG. This agreed with the report of Berger and Weiser (6) who found that lysozyme could hydrolyze chitin but not β-phenyl-N-acetylglucosaminide. The ability of lysozyme to be adsorbed onto chitin has been used as a purification technique by Weaver et al. (87) and by Skujins et al. (79) who studied this phenomenon.

Berger and Reynolds (5) upon finding 2 chitinases proposed the following origins for the enzymes: "1) the production from the very beginning of 2 disparate proteins in cells of the Streptomycete or, 2) the hydrolysis of a single original chitinase molecule by intra or exocellular proteolytic enzymes which come into play in the later autolytic stages of the culture."

The adsorption of chitinase onto crude and purified chitin was found to be significant. A greater amount of chitinase was found to be removed from solution by purified chitin than by crude chitin. This is probably a reflection of the increased surface area for adsorption offered by the purified chitin. This demonstrated the potential of chitin-chitinase adsorption as an initial step in the purification as used by Jeuniaux (31), Dandrifosse (11) and Smirnoff (80).

Ammonium sulfate was also examined as a preliminary purification and concentration procedure. Excellent recoveries were obtained and the greatest amount of protein, chitinase and chitobiase was recovered from the 60-90% cut.

The specific activities of the protein in this cut were only increased 2 times over the original crude supernatant. The specific activity of the 30-60% cut was increased by 8, 5 times in chitobiase, 7 times in the total chitinase and 5.6 times in chitin hydrolase.

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APPENDIX 1

COMPARISON OF CHITINASE ACTIVITY PRODUCED BY BACILLUS ISOLATE 97B AND ERWINIA ISOLATE 134 GROWN ON 1% PURIFIED CHITIN AND 1.5% COMMERCIAL CHITIN

Culture growth substrate	Day 4 NAG released from purified chitin /ml	/mg protein	Day 11 NAG released from purified chitin /ml	/mg protein	Day 25 NAG released from purified chitin /ml	/mg protein
134 purified chitin	5.82	25.3	7.42	16.48	19.4	35.3
134 comm. chitin	1.59	1.4	1.53	1.09	19.4	11.08
97B purified chitin	.42	.72	.09	.08	.73	.56
97B comm. chitin	.12	.59	.63	.48	.4	.2

COMPARISON OF RELEASE OF N-ACETYLGLUCOSAMINE FROM ASSAYS USING 2 DIFFERENT BATCHES OF PURIFIED CHITIN

Preparation	mg chitin/ ml assay	µmoles NAG per ml crude enzyme	Percent relative activity
1	6.66	7.8	43
2	6.46	18.3	100

APPENDIX 2

Chitin - Modified Morris Medium

	Ingredients per liter
K_2HPO_4	7.0 g
KH_2PO4	3.0 g
$(NH_4)_2SO_4$	3.0 g
$MgSO_4 \cdot 7\ H_2O$	0.2 g
$FeCl_3 \cdot 6\ H_2O$	17.0 mg
$CaCl_2$	10.0 mg
H_3BO_3	5.6 mg
$MnCl_4 \cdot H_2O$	3.7 mg
$CuSO_4 \cdot 4\ H_2O$	0.4 mg
$NaMoO_4 \cdot 2\ H_2O$	1.5 mg
$CoCl_2 \cdot 6\ H_2O$	0.6 mg
$ZnSO_4 \cdot 2\ H_2)$	0.5 mg

These salts were made up at a double strength concentration and autoclaved. Chitin was autoclaved separately in a portion of water and added aseptically to the sterile salts solution.