Purification and Mode of Action of a Chitosanase from *Penicillium islandicum*

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Penicillium islandicum produced an inducible extracellular chitosanase when grown on chitosan. Large-scale production of the enzyme was obtained using *Rhizopus rhizopodiformis* hyphae as substrate. Chitosanase was purified 38-fold to homogeneity by ammonium sulphate fractionation and sequential chromatography on DEAE-Biogel A, Biogel P₆₀ and hydroxylapatite. Crude enzyme was unstable at 37 °C, but was stabilized by 1.0 mM-Ca²⁺. The pH optimum for activity was broad and dependent on the solubility of the chitosan substrate. Various physical and chemical properties of the purified enzyme were determined.

Penicillium islandicum chitosanase cleaved chitosan in an endo-splitting manner with maximal activity on polymers of 30 to 60% acetylation. No activity was found on chitin (100% acetylated chitosan) or trimers and tetramers of *N*-acetylglucosamine. The latter two oligomers and all small oligomers of glucosamine inhibited the activity of chitosanase on 30% acetylated chitosan. The pentamer of *N*-acetylglucosamine and glucosamine oligomers were slowly cleaved by the enzyme. Analysis of the reaction products from 30% acetylated chitosan indicated that the major oligomeric product was a trimer; with 60% acetylated chitosan as substrate a dimer was also found. The new terminal reducing groups produced by chitosanase hydrolysis of 30% acetylated chitosan were reduced by sodium borohydride. The new end residues were found to be *N*-acetylglucosamine. The analyses strongly indicated that *P. islandicum* chitosanase cleaved chitosan between *N*-acetylglucosamine and glucosamine. Both residues were needed for cleavage, and polymers containing equal proportions of acetylated and non-acetylated sugars were optimal for chitosanase activity. The products of reaction depended on the degree of acetylation of the polymer.

INTRODUCTION

Chitin, a crystalline polymer of *N*-acetylated (1→4)- β -linked glucosamine residues, occurs widely in nature as a structural polymer in the integument of insects and crustacea and in the cell walls of many fungi. Chitosan, in contrast, is a non-crystalline polymer of glucosamine and *N*-acetylglucosamine residues. Prior definitions of chitosan have been based on its lack of crystallinity, its high solubility in dilute acid, and its degree of acetylation. As a practical approach, we define the chitosan polymer to contain glucosamine moieties partially *N*-acetylated, with the degree (percentage) of acetylation cited. In nature, the only reported occurrence of chitosan is in the walls of a limited but medically important group of fungi, the Zygomycetes (Bartnicki-Garcia, 1968; Bruck *et al.*, 1971; Fenton *et al.*, 1978; Kreger, 1954; Letourneau *et al.*, 1976; Ruiz-Herrera, 1978). These fungi are opportunistic invaders of man and can be major pathogens in burn wounds (Bruck *et al.*, 1971). Lysis of the cell walls of these fungi was first observed by Jones *et al.* (1968). Subsequently, chitosanases (EC 3.2.1.99), a new class of enzymes that hydrolyse chitosan, were independently discovered by

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two groups: Monaghan *et al.* (1972) in a study of the possible use of lytic enzymes to combat pathogenic fungi, and Ruiz-Herrera & Ramirez-Leon (1972) during a fundamental study of cell wall architecture.

Chitosanases are produced by many micro-organisms, including fungi, bacteria and actinomycetes (Monaghan *et al.*, 1972). Crude chitosanases exhibit a wide range of substrate specificities. Enzymes acting toward chitosan have been purified to homogeneity; those from *Streptomyces* no. 6 (Price & Storck, 1975) and *Bacillus* R-4 (Tominaga & Tsujisaka, 1975) have been found to degrade chitosan specifically, while a polysaccharase from *Myxobacter* Al-1 degrades both chitosan and carboxymethylcellulose but not chitin (Hedges & Wolfe, 1974). However, none of these reports has examined the effect of the degree of acetylation of the polymer on enzyme activity or the site of cleavage of the enzyme.

As crude extracellular preparations from *Penicillium islandicum* contained chitosanase, chitinase and carboxymethylcellulase activities (Monaghan *et al.*, 1972), we proposed to purify the enzyme that degrades chitosan and to determine its substrate specificity. In addition, since naturally occurring fungal chitosans can occur with a range of degrees of acetylation (Araki & Ito, 1974; Datema *et al.*, 1977), it was of interest to characterize the oligomeric products of enzymic degradation of chitosan of different degrees of acetylation and to determine the effect of polymer acetylation on enzyme activity and the site of enzymic cleavage.

METHODS

Cultures. *Penicillium islandicum* Sopp QM7571 was obtained from Emory Simmons, Culture Collection of Fungi (QM), Department of Botany, University of Massachusetts, Amherst, Mass. *Rhizopus rhizopodiformis* was isolated during an autopsy performed on a patient at the Fort Sam Houston Burn Unit, U.S. Army, Houston, Texas.

Media. *Penicillium islandicum* was maintained on chitosan agar, which contained (g): chitosan, 1.0; K_2HPO_4 , 1.0; KH_2PO_4 , 3.0; $MgSO_4 \cdot 7H_2O$, 0.7; NaCl, 0.5; $CaCl_2$, 0.5; agar, 20; and distilled water to 1.0 l. This is a modification of the medium of Tabata & Terui (1962). Chitosan serves as both the carbon and nitrogen source in this medium. The chitosan was about 30% acetylated (as received) and derived by alkaline treatment of King Crab chitin (Food Chemical and Research Laboratories, Seattle, Washington). The chitosan (10× concentration) was first dissolved in 0.2 M-HCl, brought to pH 5.5 with 1.0 M-NaOH, autoclaved separately, cooled to 48 °C, and combined with the sterile molten salts/agar solution, which was dispensed while stirring in order to keep the chitosan in suspension.

Rhizopus rhizopodiformis was maintained on yeast/peptone/glucose medium, containing (g): yeast extract, 3.0; Bactopeptone (Difco), 10.0; glucose, 20.0; agar, 20.0; and distilled water to 1.0 l.

Enzyme production. Large-scale preparation of the extracellular chitosanase was accomplished by growing *P. islandicum* in the presence of *Rhizopus* hyphae. The *Rhizopus* mycelium to be used subsequently as substrate was grown in a first-stage fermenter, and *P. islandicum* was grown on the autoclaved *Rhizopus* culture in a second stage. The *Rhizopus* mycelium was used as a convenient source of crude chitosan, because of the lack of large quantities of commercially available purified chitosan.

Tank I. *Rhizopus rhizopodiformis* was grown in 750 l medium consisting of (g l⁻¹): cerelese (technical-grade glucose; Clinton Corn Products, Englewood Cliffs, N.J.), 20.0; KH_2PO_4 , 2.4; $MgSO_4 \cdot 7H_2O$, 0.7; NaCl, 0.6; $(NH_4)_2SO_4$, 1.0; casein, 1.0; soya peptone, 5.0; yeast extract, 0.5. The initial pH was adjusted to 6.5. Incubation was at 37 °C with stirring (300 rev. min⁻¹) and an airflow of 500 l min⁻¹. After 24 h the pH was adjusted to 5.2. After 48 h the culture was autoclaved and two-thirds of the contents (500 l) were used as the substrate for enzyme production by *P. islandicum*. Only two-thirds of the culture was used to allow for addition of 30 l inoculum and to circumvent problems due to foaming.

Tank II. An inoculum of *P. islandicum* was prepared by growth in an F50 fermenter (New Brunswick Scientific Co.) containing 30 l medium consisting of (g l⁻¹): cerelese, 20.0; KH_2PO_4 , 2.0; $(NH_4)_2SO_4$, 1.0; NaCl, 0.66; $MgSO_4 \cdot 7H_2O$, 0.7; yeast extract, 0.5; peptone 0.5. The culture was grown at 28 °C with stirring (300 rev. min⁻¹) and an airflow of 15 l min⁻¹ for 3 d. This culture was transferred to tank I and *P. islandicum* was grown with *Rhizopus* hyphae as substrate for 7 d. The pH increased from 6.0 to 7.5. The mycelium was removed by centrifugation in a Sharples continuous centrifuge to yield 425 l broth. The clarified broth was concentrated in a flash evaporator (28 °C). The resulting 28 l was lyophilized and used as starting material for enzyme purification. Relatively poor yields of chitosanase (0.4 units ml⁻¹) were obtained in this particular large-scale production.

Enzyme assays. Amylase, cellulase, chitinase and chitosanase activities were assayed by measuring the release of reducing sugars by the Nelson–Somogyi method (Nelson, 1944). A standard assay mixture contained 0.2 M-sodium acetate buffer pH 5.0 (1.0 ml), 1.0% substrate (0.5 ml), enzyme and water to a final volume of 3.0 ml. This mixture was incubated at 37 °C for 15–60 min, depending on the enzyme activity, and assayed for the production of reducing sugar residues. One unit of enzyme was defined as the release of 1.0 μmol reducing sugar equivalent min^{-1} . Glucose, *N*-acetylglucosamine and glucosamine .HCl were used, as appropriate, for standard for amylase, cellulase, chitinase and chitosanase activities. Because the enzyme was subsequently to be evaluated as a prophylactic agent against mycotic infections, 37 °C was used as an incubation temperature. Sodium azide (final concentration 1 mM) was added as a microbial inhibitor in long-term (>60 min) incubations.

Viscometric comparisons were performed for chitosanase utilizing a Cannon 150 C18 8 viscometer, with efflux time for water of 24 s at 37 °C. Assay mixtures were as defined for reducing sugar assays. Incubation was at 37 °C.

Protein was determined by the method of Lowry, using crystalline bovine serum albumin as the standard.

Substrates for enzyme assays. Chitosan (30% acetylated) was prepared for assay by dissolving flakes (5.0 g) in 200 ml 0.2 M-HCl, adjusting to pH 5.0 with 1.0 M-NaOH, with blending, and diluting the mixture to a 1.0% (w/v) concentration. Glycol chitosan (Wako Industries, Osaka, Japan) was dissolved in distilled water to a concentration of 1.0% (w/v). To prepare 5% acetylated chitosan, 30% acetylated chitosan was boiled in 50% NaOH under nitrogen (Horton & Lineback, 1965).

Colloidal chitin was prepared by repeated alternate washing of chitin powder (Nutritional Biochemicals, Chagrin Falls, Ohio) with 0.1 M-NaOH and 0.1 M-HCl at 24 h intervals at 5 °C. The cleaned chitin was suspended in a small volume of acetone, added to cold concentrated HCl, stirred for 12 h at 5 °C, filtered through glass wool, dialysed against several changes of water, and adjusted to pH 5.5 with 0.1 M-NaOH; the concentration was adjusted to 1.0% (w/v).

Carboxymethylcellulose-1500 (degree of polymerization 1000; degree of substitution 15%; I.C.I., U.K.) was dissolved in deionized water to a concentration of 1.0% (w/v). Borohydride-reduced starch (1%, w/v) was used as substrate for amylase assays.

Polysaccharides and glycosides were obtained from Sigma. Gifts of laminaran (from *Laminaria hyperborea*) from the Norwegian Seaweed Institute and pullulan (from *Aureobasidium pullulans*) from E. T. Reese are gratefully acknowledged. Mucoran was prepared from *Rhizopus rhizopodiformis* by the method of Bartnicki-Garcia & Reyes (1968).

Oligomers of *N*-acetylglucosamine were prepared by the method of Rupley (1964). Glucosamine oligomers were prepared by hydrolysis of fully deacetylated chitosan with concentrated HCl (Horowitz *et al.*, 1957) and the products were fractionated by using Dowex cation exchange (50W-X1; H⁺ form) and Biogel P₆ chromatography. The elution profiles of the gel filtration columns were determined using standard sugar oligomers and application of 'French-Wild' plots (French & Wild, 1953).

Preparation of chitosans. A range of chitosans with different degrees of acetylation was prepared according to Hirano *et al.* (1976). Commercial chitosan derived from King Crab chitin was used as a starting material. It was about 30% acetylated. For chitosans with less than 30% acetylation, this material was first completely deacetylated (Horton & Lineback, 1965) and reacetylated to the desired value. For the preparation of chitosans of greater than 30% acetylation, the untreated commercial material was used. Portions of 25 ml of a 1% solution of chitosan at pH 3.5 were diluted with 50 ml methanol. Twice the stoichiometric amount of acetic anhydride required to yield a given percentage acetylation (i.e. 2.0 mol acetic anhydride per mol glucosamine) was added, with stirring. The solution was stirred overnight and the methanol and acetic acid were then removed by evaporation. The chitosans were precipitated with 1.0 M-NaOH, collected by centrifugation and redissolved in 0.2 M-HCl; the pH was adjusted to 5.0 with 0.1 M-NaOH.

Determination of the degree of acetylation of chitosan. The degree of acetylation of each chitosan was calculated by determination of glucosamine in each sample using fluorescamine (Hoffman LaRoche) (Udenfriend *et al.*, 1972). Samples of chitosan were degraded with excess chitosanase at 37 °C at pH 5.0 and the mixture was then adjusted to pH 8.0 with 0.1 M-NaOH. Samples (1 ml) containing 10, 20 or 30 μg chitosan were mixed with 0.5 ml fluorescamine (0.2 mg ml^{-1}) and the resulting fluorescence was immediately read at 475 nm, while exciting at 390 nm, using an Aminco Keirs Spectro-Fluorometer (American Instrument Co., Silver Springs, Maryland). The glucosamine content of the chitosan was determined by comparison with glucosamine as standard and the degree of acetylation calculated by difference. The percentage acetylation can only be determined to an approximate value ($\pm 2.0\%$), due to innate variability in fluorescence determinations. The percentage acetylation of chitosans of less than 50% acetylation was also determined by titration (Hayes & Davies, 1978), which gave values in agreement with the fluorescence determinations. Samples of greater than 50% acetylation behaved like chitins when titrated. They were considerably depolymerized by the concentrated HCl required in the titration procedure.

Enzyme purification. All purification steps were performed at 5 °C. The crude lyophilized culture broth was suspended in a minimal amount (0.9 g ml^{-1}) of 0.2 M-acetate buffer pH 5.0 containing 1.0 mM- Ca^{2+}

('concentrated culture broth' in Table 1). It formed a viscous and particulate slurry. This mixture was stirred for 1 h, centrifuged to remove insoluble materials (4300 g, 10 min) and the supernatant fluid was decanted and dialysed against several changes of 1.0 mM-Ca²⁺. The dialysed enzyme was subjected to 60–100% (NH₄)₂SO₄ fractionation. The precipitate was collected by centrifugation (3000 g, 15 min), and resuspended in a minimal amount of 0.005 M-Tris/HCl buffer pH 7.0.

The initial recovery of the enzyme was difficult to evaluate, since the original crude enzyme gave variable activity when assayed. In some instances, recoveries of greater than 100% were achieved, presumably due to removal of inhibitory materials. Thus, no yields are reported for the (NH₄)₂SO₄ fractionation step (Table 1). The (NH₄)₂SO₄ precipitate was difficult to redissolve in buffer. Therefore, relatively large volumes were used, resulting in a slight concentration of enzyme. Isoelectric focusing of the crude enzyme showed it to have an isoelectric point of 4.2. Thus a DEAE support was chosen as the ion exchanger in purification and the chitosanase was adsorbed on DEAE-Biogel A (Bio Rad Laboratories, Richmond, California), equilibrated with 0.005 M-Tris/HCl buffer pH 7.0. Enzyme elution was attained with a linear gradient of NaCl (0.05–0.3 M); 2 ml fractions were collected. This step afforded a partial separation of chitinase and chitosanase activities.

Chitosanase fractions 65–105 were combined, lyophilized and further purified by gel filtration using Biogel P₆₀. The Biogel P₆₀ column (35 × 3.0 cm) was equilibrated with 0.2 M-sodium acetate buffer pH 5.0, and was run at a flow rate of 20–30 ml h⁻¹; 2 ml fractions were collected. This step afforded a complete separation of chitinase and chitosanase activities. Fractions 25–31 were combined and lyophilized. Disc gel electrophoresis showed this chitosanase preparation to be composed of two proteins, a major chitosanase band and a minor contaminating amylase component. Chromatography on hydroxylapatite (Biogel HPT no. 13201, preswollen in 0.01 M-phosphate buffer pH 7.0 in a 1.0 × 15 cm column), with a linear gradient of pH 7.0 phosphate buffer (0.01–0.03 M) afforded a complete separation of chitosanase from the contaminating amylase. Fractions 21–29 were combined.

Carbohydrate and cystine content of the purified enzyme. The purified enzyme was analysed for carbohydrate according to the method of Krystal & Graham (1976). Chitosanase was hydrolysed in 2.0 M-HCl for 24 h at 100 °C. Interfering amino acids were removed by Dowex ion exchange chromatography, and sugars were analysed using ferricyanide with glucose as a standard. Cystine was determined as cysteic acid following performic acid oxidation (Hirs, 1967).

Analysis of products of chitosan degradation. Chitosanase reaction products were purified by gel filtration through Biogel P₂, P₆, or P₁₀ columns (90 × 1.5 cm). Elution was carried out at room temperature with H₂O adjusted to pH 8.0 with 0.1 M-NaOH; 2 ml fractions were collected, and products were located using fluorescamine.

Boro[³H]hydride (borotritide) reduction of reaction products. Enzyme products were prepared by reacting 100 mg chitosan with chitosanase overnight during dialysis against distilled water at 5 °C. The low molecular weight dialysable material was dried by lyophilization. Four of these incubations yielded 200 mg of product. These oligomeric products (175 mg) were dissolved in 1.0 ml water, pH 8.0, and reacted with 25 µCi sodium borotritide (Shiveley & Conrad, 1970). To trap the evolved tritium gas, the reaction was performed in vacuo, with excess tritium gas being drawn off into a separate flask.

Analysis of the borotritide-reduced oligomers. The reduced oligomers were deaminated with nitrous acid and were analysed by paper chromatography. Non-reduced products, nitrous acid-treated products and standards of glucosamininol, *N*-acetylglucosamininol, and 2-deoxyglucose were similarly compared. Solvent systems included ethyl acetate/acetic water/water (9:2:2, by vol.) and ethyl acetate/acetic acid/formic acid/water (9:3:3:1, by vol.). After the chromatogram was developed, it was dried and cut vertically to separate the radioactive and non-radioactive products. The non-radioactive products were located using *p*-anisidine (Hough *et al.*, 1950) or silver nitrate reagent (Trevelyan *et al.*, 1950). The chromatogram strip containing the separated radioactive products was cut into 2.5 cm horizontal sections, and the activity of products in each section was determined by use of liquid scintillation, using Aqua Sol as the fluor. Counting was performed on a Beckman LS 230 scintillation counter. All samples were monitored to a standard deviation of 5.0% and an efficiency of 91%.

Nitrous acid deamination. Nitrous acid deamination was performed basically according to the method of Shiveley & Conrad (1970). A sample of 1–2 mg of the amino sugar or polymer was deaminated by incubation with 0.5 ml 10% (w/v) sodium nitrite and 0.5 ml 33% (w/v) acetic acid for 1–2 h. The nitrous acid degradation procedure degrades glucosamine, both as a monomer and within a polymer, to 2,5-anhydromannose; it converts glucosamininol to 2-deoxyglucose, but does not attack *N*-acetylglucosamine (Datema *et al.*, 1977; Horton, 1969; Shiveley & Conrad, 1970).

RESULTS

Enzyme purification. The enzyme purification procedure yielded a 38-fold purification, giving a chitosanase having a specific activity of 10 units (mg protein)⁻¹ (Table 1). Analytical

Table 1. *Purification of P. islandicum chitosanase*

Treatment	Specific activity* [units (mg protein) ⁻¹]	Overall yield (%)	Purification factor at each stage
None (concentrated culture broth)†	0.26	100	—
(NH ₄) ₂ SO ₄ (60–100% saturation)	0.8	—‡	3.1
DEAE-Biogel A	3.3	72	4.1
Lyophilization	3.3	57	1.0
Biogel P ₆₀	10.0	43	3.3
Hydroxylapatite	10.1	42	1.0
Overall purification factor			38.8

* One unit represents the release of 1.0 μ mol reducing sugar equivalent min⁻¹.

† Crude lyophilized culture broth (0.9 g ml⁻¹) in 0.2 M-acetate buffer pH 5.0 containing 1.0 mM-Ca²⁺ (as chloride).

‡ Variable: see Methods.

Table 2. *Effect of divalent cations on chitosanase activity*

Portions (1 ml) of dialysed crude enzyme were incubated at 37 °C in a standard assay (see Methods) containing chitosan (30% acetylated) as substrate, and added cations (as chlorides) as shown. The activity of the control incubations was 0.5 units ml⁻¹.

Added cation		Activity (%)
None		100
Ba ²⁺	10 ⁻³ M	100
	10 ⁻⁴ M	98
	10 ⁻⁵ M	100
Ca ²⁺	10 ⁻³ M	225
	10 ⁻⁴ M	200
	10 ⁻⁵ M	200
Cu ²⁺	10 ⁻³ M	0
	10 ⁻⁴ M	0
	10 ⁻⁵ M	0
Hg ²⁺	10 ⁻³ M	0
	10 ⁻⁴ M	0
	10 ⁻⁵ M	0
Mg ²⁺	10 ⁻³ M	100
	10 ⁻⁴ M	101
	10 ⁻⁵ M	103
Zn ²⁺	10 ⁻³ M	90
	10 ⁻⁴ M	100
	10 ⁻⁵ M	100

disc gel electrophoresis (Davis, 1964) at pH 8.2 and 3.4 showed the purified chitosanase to be composed of a single protein (results not shown).

Effect of divalent cations on chitosanase activity. Since *P. islandicum* chitosanase was unstable in the temperature range 15–37 °C, the effect of several divalent cations (Ba²⁺, Ca²⁺, Cu²⁺, Hg²⁺, Mg²⁺ and Zn²⁺, added as chlorides) on enzyme stability was examined. Only Ca²⁺ showed any positive stabilizing effects at 37 °C (Table 2). Chitosanase with Ca²⁺ was stable for more than 48 h, while chitosanase without Ca²⁺ denatured in 1–2 h. All enzyme purification was therefore routinely performed in the presence of 1.0 mM-Ca²⁺. The enzyme remained labile in the absence of Ca²⁺ ions following purification. Among the other ions tested, Ba²⁺, Mg²⁺ and Zn²⁺ showed no effect on enzyme activity, while Hg²⁺ and Cu²⁺ were completely inhibitory (Table 2).

Table 3. *Stabilization of P. islandicum chitosanase by Ca²⁺*

Purified enzyme was used. Details of the procedures are given in Results. The activity of the undialysed control incubation was 0.5 units ml⁻¹.

Treatment	Ca ²⁺ in assay	Activity (%)
No dialysis	+	100
EDTA dialysis	+	95
EDTA dialysis	—	10
EDTA dialysis and preincubation for 30 min at 37 °C without Ca ²⁺	+	13

Fig. 1. pH optimum for activity and stability of *P. islandicum* chitosanase. For determination of the optimal pH for activity, purified enzyme diluted to 1.0 ml with distilled water was combined with 1.5 ml 0.5 M buffer (sodium acetate buffer for pH 3.6–5.6; sodium phosphate buffer for pH 5.8–7.6; sodium carbonate for pH 8.0–10.0), and 1.0 ml 30% acetylated chitosan. Activity was measured after 60 min incubation at 37 °C by the Nelson–Somogyi reducing sugar procedure. Maximum activity (0.1 units ml⁻¹) was equated to 100%. For determination of relative stability in relation to pH, unbuffered purified enzyme was incubated at 50 °C for 3 min at various pH values, in the presence of Ca²⁺. The pH of the reaction mixture was then adjusted to 5.0, and a standard enzyme assay was run. ●, Activity in relation to pH (glycol chitosan as substrate); □, activity in relation to pH (30% or 5% acetylated chitosan as substrate); ○, stability in relation to pH (30% acetylated chitosan as substrate).

To determine if Ca²⁺ was activating or stabilizing the chitosanase, the purified enzyme was dialysed overnight against 0.005 M-EDTA at 5 °C, and then assayed for activity in the presence and absence of Ca²⁺. In addition, buffered chitosanase without Ca²⁺ or substrate was preincubated under assay conditions (30 min at 37 °C) and then assayed (Table 3). EDTA-dialysed enzyme without Ca²⁺, assayed in the absence of Ca²⁺, showed greatly reduced activity (10% of that of the control, undialysed enzyme). With 1.0 mM-Ca²⁺ in the assay mixture optimal activity was maintained (95% of the control value). However, when the EDTA-dialysed enzyme was incubated at 37 °C for 30 min, and then assayed in the presence of 1.0 mM-Ca²⁺, only 13% of the initial control activity was measured. Thus Ca²⁺ acts to stabilize the labile enzyme at the relatively high temperature of the assay procedure. As shown in Table 2, this results in an activity of 200% of that of the unstabilized enzyme assayed at 37 °C.

Molecular weight determination. Gel filtration (Biogel P₆₀) of chitosanase, in comparison with known protein standards, allowed an estimation of its molecular weight (Andrews, 1964). This was found to be approximately 30000.

Carbohydrate content. Analysis of the carbohydrate content of the purified enzyme showed it to be less than 0.05 µg per 10 µg purified protein, i.e. <0.5%.

pH optimum for activity and stability. The pH optimum for activity of the chitosanase depended on the solubility of the chitosan. With 30% or 5% acetylated chitosan, a broad pH

Fig. 2. Lineweaver-Burk plot of *P. islandicum* activity. Purified enzyme was diluted with 0.02 M-sodium acetate buffer pH 5.0 and incubated for 10 min at 37 °C with a range of concentrations of 5% acetylated chitosan (a) or 30% acetylated chitosan (b). For 5% acetylated chitosan the K_m was 1.3 mg ml⁻¹ and the V_{max} was 1.2 $\mu\text{mol min}^{-1}$; for 30% acetylated chitosan the values were 1.4 mg ml⁻¹ and 2.7 $\mu\text{mol min}^{-1}$.

optimum was found between pH 4.5 and pH 6.0, with a rapid drop in activity below pH 4.0 and above pH 6.5 (Fig. 1). These chitosans precipitated when the pH was raised to 6.5 and thus presumably became more resistant to enzymatic cleavage on precipitation. With soluble glycol chitosan as substrate, a biphasic pH curve was found, with maxima between pH 4.5 and 6.8, and pH 7.1 and 8.0 (Fig. 1). *Penicillium islandicum* chitosanase was quite stable from pH 3.5 to pH 8.5, with optimum stability between pH 3.5 and pH 5.5 (Fig. 1). However, only 60% of initial activity was retained between pH 5.5 and pH 9.0, under the short-term conditions tested (50 °C for 3 min).

Effect of substrate concentration on chitosanase activity. The effect of substrate concentration on enzyme activity was determined and is displayed as a Lineweaver-Burk plot (Fig. 2). The apparent K_m for both 5% and 30% acetylated chitosan was approximately 1.4 mg ml⁻¹ at pH 5.2. However, *P. islandicum* chitosanase was twice as active toward 30% acetylated chitosan (V_{max} 2.7 $\mu\text{mol min}^{-1}$) as toward 5% acetylated chitosan (V_{max} 1.2 $\mu\text{mol min}^{-1}$).

Effect of temperature on enzyme activity. The effect of temperature on activity was determined by running assays at 20, 30, 40, 50 and 60 °C, using 30% acetylated chitosan as substrate. Optimal activity for chitosanase, assessed from an Arrhenius plot, was found to be at 45 °C; its activation energy was 20 kcal mol⁻¹ (84 kJ mol⁻¹).

Substrate specificity. The purified enzyme was specific in its degradation of chitosan. It did not degrade carboxymethylcellulose, colloidal chitin, dextran (from *Leuconostoc*), galactan, polygalacturonic acid, laminaran, mucoran, pectin, pullulan, salicin, xylan, *p*-nitrophenyl- β -D-*N*-acetylglucosamine or *p*-nitrophenyl- β -D-glucosamine.

Effect of the degree of acetylation of chitosan on enzyme activity. The degree of acetylation of the polymer greatly affected the activity of the chitosanase (Fig. 3). The chitosan polymers most amenable to hydrolysis were 30–67% acetylated, with polymers of lower and higher degrees of acetylation being more resistant. Thus, chitosan must contain both glucosamine and *N*-acetylglucosamine for optimal chitosanase activity. A bacterial chitosanase from *Bacillus* no. 8 prepared in our laboratory showed a similar preference (B. Davis, personal communication). However, crude *Serratia* chitinase (Fig. 3) and a crude chitinase from *Streptomyces griseus* (Monreal & Reese, 1969) showed markedly different action patterns, only being active toward highly acetylated polymers.

Fig. 3. Activity of *P. islandicum* chitosanase (●) and *Serratia marcescens* chitinase (○) in relation to the degree of acetylation of chitosan. Chitosans with differing degrees of acetylation were prepared and their susceptibility to hydrolysis was measured in a standard reaction mixture, containing either 1.0 unit of *P. islandicum* chitosanase or *S. marcescens* chitinase (see text). *Serratia marcescens* chitinase was obtained directly from culture broth and not purified. One unit of enzyme activity represents the release of 1.0 μmol reducing sugar equivalent min^{-1} .

Fig. 4. Reaction products from 30% acetylated chitosan (a) and 60% acetylated chitosan (b). A 500 ml sample of chitosan plus excess chitosanase was incubated for 48 h. The dialysable products were concentrated and chromatographed on a Biogel P₂ column and detected using fluorescamine. Dimer and trimer locations were defined by comparison with authentic standards of *N*-acetylglucosamine.

Viscometric comparisons of chitosanase action toward each type of chitosan tested showed a rapid decrease in viscosity in all cases, except with the 5% acetylated polymers. The chitosanase slightly decreased the viscosity of soluble chitin glycol in 24 h. Oligomeric products were detected from all chitosans tested, on analysis by paper chromatography and gel filtration.

Analysis of the products of enzymatic degradation of chitosan. (a) Gel filtration. Products of the enzymic degradation of polymers of different percentage acetylation were collected and analysed by gel filtration. Heteropolymeric standards composed of glucosamine and *N*-acetylglucosamine were not available and thus all comparisons were made with authentic oligomers of *N*-acetylglucosamine. A 48 h digest of 30% acetylated chitosan (Fig. 4a) contained a peak of higher molecular weight oligomers, a trimer peak and a smaller amount of *N*-acetylglucosamine. Analysis of products from 60% acetylated chitosan again showed a peak of high molecular weight oligomers, and a dimer peak and some trimer (Fig. 4b). Chitosan substrates of 80% acetylation yielded a mixture of only high molecular weight oligomers.

Table 4. Possible products of nitrous acid degradation of tritiated oligomeric chitosans

Tritiated oligomer reducing terminus	HNO ₂ degradation product
(A) GlcN · GlcNAc ^{ol*}	M + GlcNAc ^{ol*}
(B) GlcN · GlcN ^{ol*}	M + dGlc*
(C) GlcNAc · GlcN ^{ol*}	GlcNAc · dGlc*
(D) GlcNAc · GlcNAc ^{ol*}	GlcNAc · GlcN ^{ol*}

* Indicates the tritium label following reduction. GlcN, glucosamine; GlcN^{ol}, glucosaminitol; GlcNAc, *N*-acetylglucosamine; GlcNAc^{ol}, *N*-acetylglucosaminitol; M, 2,5-anhydromannose; dGlc, 2-deoxyglucose.

Fig. 5. Nitrous acid degradation of purified oligomer products from 30% acetylated chitosan. The enzyme products were reduced with borotritide and purified by gel chromatography, and half the samples were subjected to nitrous acid degradation. The products from the latter reaction were separated by paper chromatography and the relative migration rates of the radioactive nitrous acid products were measured by liquid scintillation. Dashed line, nitrous acid treated; continuous line, no nitrous acid treatment.

(b) *Determination of the new terminal reducing residue formed by chitosanase.* The new reducing residue formed by the enzyme was determined by tritium labelling of the reducing terminus of the enzyme reaction products, their subsequent degradation by nitrous acid, and chromatographic characterization. Enzymic degradation products from 30% acetylated chitosan were tritiated with borotritide and purified by repeated filtration on a Biogel P₂ column. The purified 'trimer' and oligomeric peaks were collected, samples were treated with nitrous acid, and the degradation products were chromatographed.

The purified reduced oligomers can have four different dimeric sequences at their reducing termini (Table 4). If *N*-acetylglucosaminitol (GlcNAc^{ol}) were the terminal reducing sugar (A, D in Table 4) nitrous acid degradation of the oligomeric products would yield either *N*-acetylglucosaminitol (A) or a dimer (D), as the only radioactive product. A trimer (GlcNAc · GlcNAc · GlcNAc^{ol}) containing *N*-acetylglucosaminitol would also be a possible product but would be a minor component in 30% acetylated chitosan. Analysis by paper chromatography showed no radioactive *N*-acetylglucosaminitol in the oligomers not treated with nitrous acid (Fig. 5). However, nitrous acid treatment released a radioactive component corresponding to *N*-acetylglucosaminitol from the oligomeric product (Fig. 5). No 2-deoxyglucose (dGlc), the nitrous acid degradation product from glucosaminitol (GlcN^{ol}), was found. As *N*-acetylglucosaminitol was released by nitrous acid degradation, *N*-acetylglucosamine (GlcNAc) was the reducing residue formed by the enzyme. Glucosamine linked to terminal glucosaminitol was not a possible major sequence, as 2-deoxyglucose was not detected.

Table 5. Possible products of nitrous acid degradation of tritiated trimers

Tritiated trimer	HNO ₂ degradation product
(A) GlcN · GlcN · GlcN ^{ol*}	M + M + dGlc*
(B) GlcN · GlcN · GlcNAc ^{ol*}	M + M + GlcNAc ^{ol*}
(C) GlcN · GlcNAc · GlcN ^{ol*}	M + GlcNAc · dGlc*
(D) GlcN · GlcNAc · GlcNAc ^{ol*}	M + GlcNAc · GlcNAc ^{ol*}
(E) GlcNAc · GlcN · GlcNAc ^{ol*}	GlcNAc · M + GlcNAc ^{ol*}
(F) GlcNAc · GlcN · GlcN ^{ol*}	GlcNAc · M + dGlc*
(G) GlcNAc · GlcNAc · GlcN ^{ol*}	GlcNAc · GlcNAc · dGlc*
(H) GlcNAc · GlcNAc · GlcNAc ^{ol*}	GlcNAc · GlcNAc · GlcNAc ^{ol*}

* Indicates the tritium label following reduction. Abbreviations as for Table 4.

Fig. 6. Nitrous acid degradation of purified 'trimer' product from 30% acetylated chitosan. The borotritide-reduced 'trimer' was purified by gel chromatography, and half the samples were subjected to nitrous acid degradation. The products of the reaction were separated by paper chromatography and their relative migration rates were measured by liquid scintillation. ●, Nitrous acid treated; ○, no nitrous acid treatment.

For further clarification of the enzyme's site of cleavage, the reducing termini of the purified trimers were also analysed. The eight possible trimer sequences and nitrous acid degradation products are listed in Table 5. Sequences *B*, *C*, and *F* are the most probable with a 30% acetylated polymer. If *N*-acetylglucosamine is the only reducing residue in the trimer peak (e.g. *B*), treatment with nitrous acid will show *N*-acetylglucosaminitol to be the only radioactive monomer formed. Paper chromatography showed that nitrous acid treatment of the trimer released *N*-acetylglucosaminitol and no 2-deoxyglucose (Fig. 6). Thus, sequence *B* (GlcN · GlcN · GlcNAc^{ol}) and sequence *E* (GlcNAc · GlcN · GlcNAc^{ol}) are the most probable sequences in the trimer. In either case, the enzyme cleaves the polymer at an *N*-acetylglucosamine unit.

Sequence of the purified trimer derived from 30% acetylated chitosan. A trimer composed of GlcN · GlcN · GlcNAc^{ol} (*B* in Table 5) is degraded to 2,5-anhydromannose (M) and *N*-acetylglucosamine by nitrous acid treatment. Other combinations that could be formed with *N*-acetylglucosamine as the terminal reducing residue would not be degraded (GlcNAc · GlcNAc · GlcNAc), or would be degraded to a monomer and a dimer (GlcNAc · M + GlcNAc or M + GlcNAc · GlcNAc). Paper chromatography of the products of nitrous acid treatment of the purified trimer product showed only *N*-acetylglucosamine and 2,5-anhydromannose to be formed, indicating that the sequence of the trimer is GlcN · GlcN · GlcNAc (results not shown). It should be noted that standard 2,5-anhydromannose is unstable and degrades rapidly. It was necessary to repeat this experiment several times in order to obtain satisfactory separation of the standards.

Table 6. *Oligomers as substrates and inhibitors of chitosanase activity*

For the substrate tests, the oligomers were used at 10 mg ml⁻¹. Substrate degradation is expressed as a percentage of that obtained in a standard assay with 30% acetylated chitosan as substrate. For the inhibitor tests, the oligomers were used at 1.0 mg ml⁻¹ in a standard 15 min assay with 30% acetylated chitosan as substrate and 0.5 unit of enzyme. The activity of the control incubation was 0.5 units ml⁻¹.

Oligomer	Degradation of substrate (%)	Inhibition (%)
(GlcNAc) ₃	0	22
(GlcNAc) ₄	0	15
(GlcNAc) ₅	1	20
(GlcN) ₃₋₅	61	30
GlcN · GlcN · GlcNAc	0	100
GlcN	NT	51
GlcNAc	NT	5

NT, Not tested.

Effect of small oligomers on enzyme activity. Purified oligomers of *N*-acetylglucosamine were tested for their ability to act as substrates (at 10 mg ml⁻¹) or inhibitors (at 1 mg ml⁻¹) for *P. islandicum* chitosanase (Table 6). The trimer and tetramer of *N*-acetylglucosamine were not substrates for the enzyme. These oligomers inhibited chitosanase cleavage of 30% acetylated chitosan by 15–25% when they were present in a standard assay mixture. The pentamer of *N*-acetylglucosamine inhibited chitosanase action by 20%, but was also a poor substrate for the enzyme, being 100-fold more resistant to hydrolysis than 30% acetylated chitosan. A mixture of oligomers of glucosamine (1.0 mg ml⁻¹) inhibited chitosanase activity by 30%. Upon long incubation (24 h) with the enzyme, these glucosamine oligomers were slowly degraded.

Thus, it would appear that both *N*-acetylglucosamine and glucosamine oligomers are capable of binding to chitosanase, inhibiting its action. It also appears that both *N*-acetylglucosamine and glucosamine are necessary within the polymer for optimal enzyme action (Fig. 3, Table 6). It is unfortunate that standard hetero-oligomers containing both glucosamine and *N*-acetylglucosamine were not available for testing. However, one such oligomer was prepared—the trimer product GlcN · GlcN · GlcNAc—from hydrolysis of 30% acetylated chitosan. This oligomer was not a substrate for chitosanase, even upon long incubation with highly concentrated enzyme solution. However, at 1.0 mg ml⁻¹ it completely inhibited the enzyme's action toward 30% acetylated chitosan (Table 6).

DISCUSSION

The *P. islandicum* chitosanase was purified 38-fold, with retention of 40% of initial activity, by (NH₄)₂SO₄ fractionation and sequential chromatography on DEAE-Biogel A, Biogel P₆₀ and hydroxylapatite. It was found to be a relatively small acidic protein, having a molecular weight of 30 000, a pI of 4.2, and, with 30% acetylated chitosan as substrate, a *K_m* of 1.4 mg ml⁻¹, a *V_{max}* of 2.7 μmol min⁻¹ and a *t_{max}* of 45 °C. It was unstable at room temperature, but was stabilized by 1.0 mM-Ca²⁺. The effect of pH on enzyme activity was dependent on the substrate used. With soluble chitosan of 5% and of 30% acetylation, a pH optimum of 4.5 to 6.0 was found. These soluble chitosans precipitate near pH 6.5, and thus presumably become more resistant to enzymatic cleavage. It should also be noted that Ca²⁺ in phosphate buffer would become insoluble at these higher pH values. Whether this loss of Ca²⁺ from solution would result in a decrease in the stabilizing action of this ion on the enzyme is unknown. The use of a buffer other than phosphate at alkaline pH may favour solution of Ca²⁺ ion and may increase the stability and activity of chitosanase at alkaline pH. With glycol chitosan, a

Table 7. *Microbial chitosanases*

	<i>Penicillium islandicum</i>	<i>Streptomyces</i> no. 6	<i>Myxobacter</i> Al-1	<i>Bacillus</i> R4	<i>Bacterium</i> K-1
Specificity					
Chitosan	+	+	+	+	+
Chitin	—	—	—	—	—
CM-cellulose	—	—	+	NT	+
Action: endo- or exo-splitting	Endo	Endo	Endo	NT	Endo
Constitutive (C) or inducible (I)	I	I	C	C	I
Mol. wt	30 000	30 000	28 900* 31 000†	NT	50 000
Stability at 37 °C	Stable (+1 mM-Ca ²⁺)	Unstable	±‡	Stable	Stable
Optimum pH					
Chitosan	4.5–6.0	7.5	5.0 and 6.8	7.6§	5.5–6.9
CM-cellulose	NA	NA	5.0	NT	5.0
Zygomycete protoplasts released by crude enzyme	+	+	+	+	+
Reference	This study	Price & Storck (1975)	Hedges & Wolfe (1974)	Tominaga & Tsujisaka (1975)	B. Davis (pers. comm.)

NT, Not tested; NA, not applicable.

* From amino acid composition.

† From gel filtration analysis.

‡ Stable at 70 °C in the presence of chitosan, but 75 % of activity lost if incubated at 38 °C in water.

§ Cell wall used as substrate.

substrate soluble over a wide pH range, a biphasic curve was seen. The biphasic response of *P. islandicum* chitosanase to pH using glycol chitosan as substrate is similar to that exhibited by *Myxobacter* Al-1 chitosanase (Hedges & Wolfe, 1974), although 5.0 % acetylated chitosan was used as a substrate in the latter case and would have been precipitated in colloidal state above pH 6.5.

A comparison of *P. islandicum* chitosanase with the other known chitosanases is presented in Table 7. They are all extracellular, although an intracellular chitosanase, presumably involved in cell wall modification, has been demonstrated in *Rhizopus rhizopodiformis* (Monaghan *et al.*, 1972). Both inducible and constitutive forms are known. All four purified chitosanases have molecular weights of approximately 30 000 or 50 000. The mode of action of all chitosanases is apparently endo-splitting (Fenton *et al.*, 1978). There are no apparent similarities in the amino acid composition of the two chitosanases analysed (Hedges & Wolfe, 1974; Price & Storck, 1975). It is noteworthy that both the *Streptomyces* and the *P. islandicum* chitosanase contain no cystine, and yet inhibition by Hg²⁺ was found with both enzymes. One possibility is that since chitosan has a well-documented chelation capacity (Muzzarrelli, 1973, 1977), Hg²⁺ could be associating with the polymer and sterically inhibiting chitosan cleavage (Storck & Price, 1977).

Purified *P. islandicum* chitosanase degrades chitosan specifically. It does not act on chitin or carboxymethylcellulose. It cleaves chitosans of 30–60 % acetylation with maximal activity (Fig. 3). Thus, both glucosamine and *N*-acetylglucosamine are necessary for optimal activity. The highly acetylated chitosans, chitin and chitosans of low acetyl content appear to be poor

substrates because they contain either insufficient *N*-acetylglucosamine or insufficient glucosamine residues for optimal activity. However, glycol chitin, the pentamer of *N*-acetylglucosamine, and oligomers of glucosamine are all slowly hydrolysed by chitosanase over a 24 h incubation period, indicating that cleavage of these homo-polymers can occur, but at a much reduced rate (Table 6).

The role of a chitosanase with greatest reactivity toward 30–60% acetylated chitosan is intriguing. Early studies implied that naturally occurring chitosan of Mucorales is a fully deacetylated polymer (Bartnicki-Garcia & Nickerson, 1962). Subsequent work has demonstrated chitosan to be partially acetylated in the cell wall of *Mucor mucedo* (Datema *et al.*, 1977) and *M. rouxii* (Davis & Bartnicki-Garcia, 1980). Highly acetylated chitosans could also occur in the walls of Mucorales following partial deacetylation of chitin (Araki & Ito, 1974), but no such chitosans have yet been reported.

Chitosan has been used as substrate for the characterization of crude chitinase preparations (Tracey, 1955; Vessey & Pegg, 1973). The use of chitosan in these instances is inappropriate since chitinase from *Serratia* (Fig. 3), *Streptomyces* sp. (results not shown) and *Streptomyces griseus* (Hirano & Yagi, 1980) show no activity toward chitosan. The activities observed using chitosan as a substrate for chitinase may reflect the presence of specific chitosanases in the crude preparations. There may be chitosanase–chitinase enzymes with broad specificity, but until this point is defined the use of chitosan as a substrate for chitinase is ill-advised.

The new terminal reducing groups of the reaction products from 30% acetylated chitosan were identified by tritiation of the reducing groups with sodium borotritide and subsequent analysis of the products of deamination with nitrous acid. *N*-Acetylglucosamine was shown to be the terminal reducing residue of the products (Figs 4, 5 and 6). The results of these analyses can be most simply explained if the enzyme cleaved the polymer optimally at those sequences where *N*-acetylglucosamine was linked by its reducing moiety to a glucosamine. That is, the enzyme cleaved the polymer to form *N*-acetylglucosamine as the new reducing terminus (Figs 5 and 6), but could not readily cleave the bond between two adjacent *N*-acetylglucosamine residues (Table 6, Fig. 7). A 30% acetylated polymer would contain, on the average, one acetylated sugar per three residues. A trimer composed of GlcN·GlcN·GlcNAc could be a major product of enzymic degradation, and this trimer appeared as a product (Fig. 4*a*). A 60% acetylated polymer should contain *N*-acetylglucosamine approximately every other residue, and this a dimer of glucosamine and *N*-acetylglucosamine (GlcN·GlcNAc) should be a major product; and a dimer product was found (Fig. 4*b*). Chitosan of 80% acetylation, a chitin-like polymer which was a poor substrate for chitosanase, could be cleaved at only two out of every ten residues, consequently releasing large oligomers. All our results indicate preferred cleavage to occur between *N*-acetylglucosamine and glucosamine residues. Cleavage at other sites would yield distinct products not found in this study (Fig. 7).

Inhibition of activity by trimers and tetramers of *N*-acetylglucosamine, and the slow cleavage of the pentamer, indicate that the minimum chain length for cleavage is probably at least five residues (Table 6). However, since these oligomers lack glucosamine, they were not ideal substrates for the enzyme. Unfortunately, no oligomers of greater than three residues and containing both glucosamine and *N*-acetylglucosamine residues were available to test their relative effects.

How does one define chitosanase and distinguish it from other glycosidases? Because chitinases have been defined as enzymes which act against chitin and yield products with *N*-acetylglucosamine terminal residues (Muzzarrelli, 1977), the initial simple concept of a 'chitosanase' was of a glycosidase that would hydrolyse chitosan to yield glucosamine terminal residues. However, *P. islandicum* chitosanase does not fit this concept since it preferentially releases *N*-acetylglucosamine terminal reducing moieties. It differs from chitinase in showing essentially no activity toward chitin, though it will react slowly with small chitin oligomers. Chitosanases that release terminal glucosamine residues may well exist, for

Fig. 7. Three idealized schemes for the enzymic degradation of 30% acetylated chitosan. A regular repeating unit is used for simplicity. Only scheme I yields *N*-acetylglucosamine as the reducing terminus. Schemes II and III yield oligomers with reducing terminal glucosamine, which were not found in this study. Only scheme I is consistent with the observation that no 2-deoxyglucose (the nitrous acid degradation product of glucosamine) was detected in this study. The small arrows indicate cleavage sites.

instance the specific chitosanases from *Streptomyces* (Price & Storck, 1975) and *Bacillus* (Tominga & Tsujisaka, 1975), but their site of cleavage has not yet been determined. Furthermore, as older cell walls of Mucorales contain chitosans with relatively low degrees of acetylation (5–30%; unpublished results), they could well be susceptible to intracellular chitosanases (Monaghan *et al.*, 1972), which have glucosamine residues as their sites of attack. The recent techniques that allow preparation of chitosans with a range of defined degrees of acetylation allow further study of this interesting grouping of enzymes.

Why did chitosanases of differing specificity evolve? Schindler *et al.* (1977) have hypothesized that 'evolutionary changes in substrate structure may have influenced the development of the active site of lysozyme so that it could function most efficiently with the particular natural substrate encountered by each species'. The chitosan of Mucorales exhibits a range of degrees of acetylation (Araki & Ito, 1974; Bartnicki-Garcia, 1980) and a range of chitosans is probably produced in the environment through deacetylation in the recycling of chitin (unpublished observation). Thus, similar evolutionary pressure may be postulated for 'chitosanases', leading to the development of enzymes having differing specificities.

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