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Characterization of a Chitosanase Isolated from a Commercial Ficin Preparation

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A chitosanolytic enzyme was purified from a commercial ficin preparation by affinity chromatographic removal of cysteine protease on *p*HMB−Sepharose 4B and cystatin−Sepharose 4B and gel filtration on Superdex 75 HR. The purified enzyme exhibited both chitinase and chitosanase activities, as determined by SDS-PAGE and gel activity staining. The optimal pH for chitosan hydrolysis was 4.5, whereas the optimal temperature was 65 °C. The enzyme was thermostable, as it retained almost all of its activity after incubation at 70 °C for 30 min. A protein oxidizing agent, *N*-bromosuccinimide (0.25 mM), significantly inhibited the enzyme's activity. The molecular mass of the enzyme was 16.6 kDa, as estimated by gel filtration. The enzyme showed activity toward chitosan polymers exhibiting various degrees of deacetylation (22−94%), most effectively hydrolyzing chitosan polymers that were 52−70% deacetylated. The end products of the hydrolysis catalyzed by this enzyme were low molecular weight chitosan polymers and oligomers (11.2−0.7 kDa).

KEYWORDS: Fig chitosanase; ficin; purification; properties

INTRODUCTION

Chitosan is a linear polysaccharide composed of β-1,4-linked D-glucosamine residues. In nature, this polymer is partially acetylated, so that the name chitosan describes a large family of polymers containing various proportions of D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) residues. Chitosan occurs in the cell walls of a limited number of fungi (*1*−*4*) and in certain green algae, such as *Chlorella* (*5*), and is also produced commercially by alkaline deacetylation of shellfish (usually crab) chitin.

Chitosanases (EC 3.2.1.132) are hydrolases acting on chitosan. Most chitosanases are found in microorganisms (*6*−*9*), although a few are found in plants (*10*−*13*) and animals (*14*, *15*). Microbial chitosanases are classified into three subclasses based on their cleavage position specificity (*16*, *17*): subclass I (e.g., *Streptomyces* sp. N174 chitosanase) cleaves GlcN−GlcN and GlcNAc−GlcN linkages (*18*); subclass II (e.g., *Bacillus* sp. No.7-M) cleaves only GlcN−GlcN linkages (*19*); and subclass III (e.g., *Bacillus circulans* MH-K1 chitosanase) cleaves both GlcN−GlcN and GlcN−GlcNAc linkages (*20*). A few plant chitosanases also have been characterized and divided into two classes based on substrate specificity: class I, which includes chitosanases from stressed barley, cucumbers, and tomatoes (*21*), can degrade only chitosan, whereas class II, which includes chitosanases from tomato stems (*22*), *Citrus sinensis* (*10*, *11*), and pineapple stem (*23*), can degrade both chitin and chitosan. In addition, a few animal chitosanases have also been characterized from termite (*14*), Atlantic salmon (*24*), and a commercial crude porcine pepsin preparation (*15*). Chitosanases from termite and Atlantic salmon can degrade only chitosan, whereas chitosanase isozymes from a commercial pepsin preparation can degrade both chitosan polymer and *N*,*N*′,*N*″-triacetylchitotriose oligomer.

A number of commercially available crude protease preparations, including papain, pepsin, bromelain, and ficin, also have been found to possess chitosanolytic activity (*25*−*27*), although chitosanase, itself, was not initially identified. More recently, however, Liao et al. (*28*) partially purified a chitosanolytic enzyme from a commercial pepsin preparation and demonstrated the presence of chitosanase. Moreover, Fu et al. (*15*) further purified and characterized three chitosanase isozymes from crude pepsin, and Hung et al. (*23*) purified and characterized a hydrolase with dual chitinase and chitosanase activities from a commercial pineapple stem bromelain preparation. Ficin is a cystein protease produced commercially from fig latex. Commercial ficin also exhibits a substantial capacity to hydrolyze

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chitosan (*26*), strongly suggesting the enzyme preparation contains one or more chitosanolytic enzymes. Here we describe the purification and characterization of a 16.6-kDa (gel filtration) chitosanase from a commercial ficin preparation. The possible use of this enzyme for the preparation of low molecular weight chitosan is also discussed.

MATERIALS AND METHODS

Chemicals. *N*-Acetylimidazole (NAI), 1,2-cyclohexanedione (CHD), *p*-hydroxymercuribenzoate (sodium salt, *p*HMB), diethyl pyrocarbonate (DEPC), 2,4-dinitro-1-fluorobenzene (DNFB), ethyl acetimidate (EAM), phenylmethanesulfonyl fluoride (PMSF), *N*-ethyl-5-phenylisoxazoline-3′-sulfonate (Woodward's reagent K; WRK), dimethylformamide (DMF), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), ficin (F-4165), 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH), and glycol chitosan (G7753) were purchased from Sigma (St. Louis, MO). *N*-Bromosucinimide (NBS) was from Merck (Darmstadt, Germany). EAH−Sepharose 4B, ECH−Sepharose 4B, Superdex 75 HR column (16/60), and the low molecular weight calibration kit (LMW) were from Pharmacia (Uppsala, Sweden). Chitosan (84% deacetylation) was obtained from Ohka Enterprises Co. Ltd. (Kaohsiung, Taiwan). Bicinchoninic acid protein assay reagent was purchased from Pierce (Rockford, IL). All buffer salts and other chemicals used were of reagent grade.

Purification of Chitosanase. One hundred milligrams of ficin preparation was dissolved in 2 mL of 25 mM sodium phosphate buffer (0.15 M NaCl, pH 7.0). After centrifugation (3000*g*; 5 min), the supernatant was applied to a *p*HMB−Sepharose 4B column (1.6 × 12 cm), and the column was then washed with 60 mL of 25 mM sodium phosphate buffer (0.15 M NaCl, pH 7.0) at a flow rate of 20 mL/h to elute unbound proteins. Bound proteins were then eluted with 60 mL of 25 mM sodium phosphate buffer (0.15 M NaCl, 0.15 M β-mercaptoethanol, pH 7.0), and 1-mL fractions were collected. The fractions containing chitosanase activity were pooled, concentrated by ultrafiltration (MW cutoff, 10000), and run again on the *p*HMB column. After concentration by ultrafiltration, the resultant enzyme solution was applied to a cystatin−Sepharose 4B column (1.6 × 12 cm) preequilibrated with 25 mM sodium phosphate buffer (pH 7.0) for affinity adsorption of ficin protease. The unbound chitosanase was eluted with equilibrium buffer at a flow rate of 20 mL/h. Fractions (1 mL) containing chitosanase activity were pooled, concentrated by ultrafiltration, and applied to a Superdex 75 HR column. The enzyme was eluted with 25 mM sodium phosphate buffer (pH 7.0) at a flow rate of 21 mL/h, and 0.5-mL fractions containing chitosanase activity were pooled.

Purification of a Ficin−Papain Inhibitor (Cystatin) from Egg White. The whites of 20 fresh chicken eggs (≤2 days old) were separated from the yolks and blended with an equal volume of 0.25% NaCl using a mixer−homogenizer. The pH of the mixture was then adjusted to 6.0 with 1 N HCl, after which the resultant ovomucin precipitate was removed by centrifugation (2100*g*, 30 min) and discarded. Ammonium sulfate was then added to the supernatant to 50% saturation, and the resultant precipitate was dissolved in water (one-fifth the volume of the original egg white), dialyzed against water, and centrifuged. Ten-milliliter samples of the supernatant were then applied to a Sephadex G-75 column (1.6 × 100 cm) equilibrated with 0.1 M sodium acetate buffer (pH 6.0), after which the protein was eluted with the same buffer at a flow rate of 0.4 mL/min, and 2.5-mL fractions were collected. Fractions containing cystatin activity were pooled, dialyzed, and concentrated by ultrafiltration (*29*).

Preparation of Cystatin−Sepharose 4B Affinity Adsorbent. Twenty milliliters of packed ECH−Sepharose 4B (capacity = 12−16 μmol/mL) was suspended in 55 mL of 40% DMF, after which 600 mg of the purified cystatin were added, the pH was adjusted to 4.8, and 2.6 mmol of EDC was added. After the reaction had been allowed to run for 18 h while the mixture was swirled slowly at room temperature, the substituted Sepharose was washed with 1.5 L of 0.1 M $NaHCO_3$ (pH 8.8) over an 8-h period and then washed with water and 25 mM sodium phosphate buffer (pH 7.0).

Preparation of *p*HMB−Sepharose 4B Affinity Adsorbent. Twenty milliliters of packed EAH−Sepharose 4B (capacity = 7−12 μmol/mL) was suspended in 55 mL of 40% DMF, after which 2 mmol of sodium *p*-hydroxymercuribenzoate was added, the pH was adjusted to 4.8, and 2.6 mmol of EDC was added. The reaction and the subsequent washing steps were carried out as described above.

Preparation of Ethylene Glycol Chitin. Ethylene glycol chitin was obtained from the reaction of chitin with ethylene chlorohydrin (2-chloroethanol) under alkaline conditions according to the method (procedure B) of Hirano (*30*).

Measurement of Chitosanase Activity. *1. Viscosity Assay Method (23).* Viscosity was measured using a viscometer (Brookfield DV II$^+$) driven by a personal computer running DV loader software. The spindle rotor (SC4-18) was housed in a small sample adapter filled with 8 mL of 1% chitosan in 1% acetic acid (pH 4.0) and fitted into a flow jacket. The temperature was controlled at 30 °C. To this was added 0.05 mL of chitosanase solution with the aid of a syringe, after which the reaction was run for 5 min. A reaction mixture to which 0.05 mL of 25 mM sodium phosphate buffer (pH 7.0) was added instead of enzyme solution was used as a control. The difference between the viscosity observed in the absence and presence of chitosanase was taken as a measure of chitosanase activity.

2. Reducing Sugar Assay Method. Aliquots of 1% chitosan (0.15 mL) in 1% acetic acid (pH 4.0) and 0.05 mL of enzyme solution in a total volume of 0.2 mL were incubated at 37 °C for 5 min. The reducing sugar produced was measured colorimetrically using ferri-ferrocyanide reagent as described by Imoto and Yagishita (*31*).

Measurement of Protease Activity. Protease activity was measured using casein hydrolysis as an index according to the method of Anson (*32*). The reaction mixture contained 2.5 mL of 0.6% casein (in 0.05 M sodium phosphate buffer, pH 7.0) and 0.05 mL of enzyme solution. The reaction was run at 37 °C for 10 min and then stopped by adding 2.5 mL of 0.11 M trichloroacetic acid containing 0.22 M sodium acetate and 0.33 M acetic acid. Thereafter, the absorbance at 275 nm of the trichloroacetic acid (0.05 M)-soluble product was measured.

Measurement of Cystatin Activity. Cystatin activity was measured by preincubating 0.5 mL of protease (500 μg of ficin in 25 mM sodium phosphate buffer, pH 7.0) with 0.5 mL of cystatin at 37 °C for 10 min, after which 5 mL of 0.6% casein (in 0.05 M sodium phosphate buffer, pH 7.0) was added, and the remaining protease activity was measured as described above. The degree of enzyme inhibition was determined from the difference in the rate of reactions carried out in the presence and absence of the inhibitor. A unit of inhibitor was defined as the amount that inhibited the activity of ficin by 50% at pH 7.0 and 37 °C.

Determination of Optimal pH. The optimal pH for chitosan hydrolysis by the purified chitosanase was assayed in a sodium acetate buffer over a pH range of 2−6 using the reducing sugar assay method described above.

Determination of Optimal Temperature and Thermal Stability. The optimal temperature for chitosan hydrolysis by the purified chitosanase was assayed at optimal pH over a temperature range of 30−80 °C. To assess thermostability, the enzyme solutions were immersed in a thermostatic water bath for 2.5−30 min at various temperatures (from 40 to 90 °C). The retained activity was measured using the reducing sugar assay method described above.

Estimation of Molecular Mass. The molecular mass of the chitosanase was estimated by gel filtration on a Superdex 75 HR column (1.6 × 60 cm) on a FPLC system according to the method of Andrews (*33*). The column was equilibrated with 0.15 M sodium phosphate buffer (pH 7.0), after which the enzyme was eluted with equilibrium buffer at a flow rate of 21 mL/h, and 0.25-mL fractions were collected. Bovine serum albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A served as standards.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was carried out in 10% polyacrylamide gel for protein staining or in 12.5% polyacrylamide gels containing 0.1% (w/v) glycol chitosan or ethylene glycol chitin for activity staining using bromophenol blue as the tracking dye according to the method of Laemmli (*34*). For activity staining, samples were boiled for 5 min with 15% sucrose and 2% (w/v) SDS with or without 5% (v/v) 2-mercaptoethanol in 125 mM Tris-HCl buffer (pH 6.7). After

Table 1. Purification of Chitosanase from a Commercial Ficin Preparation[a]

procedure	total activity[b] (units)	total protein (mg)	specific activity (units/mg)	purification (fold)	yield (%)
ficin solution	4816	36.2	133	1	100
first *p*HMB–Sepharose 4 B affinity adsorption	4471	18.5	242	1.8	92
second *p*HMB–Sepharose 4B affinity adsorption	4420	10.5	421	3.2	92
cystatin–Sepharose 4B affinity adsorption	3864	7.0	552	4.2	80
Superdex 75 HR column gel filtration	2944	2.4	1227	9.2	61

[a] Data were obtained from 100 mg of commercial ficin preparation. [b] One chitosanase unit is defined as the amount of enzyme needed to decrease viscosity by 1 cP/min.

electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250. Chitosanase or chitinase activity was detected using Calcofluor White M2R staining; glycol chitosan or ethylene glycol chitin served as the substrate in the gel matrix as described by Ouakfaoui and Asselin (*10*, *35*). Renaturation of enzyme activity after SDS-PAGE was carried out in 100 mM acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 for 48 h at 30 °C with gentle shaking. Chitosanase or chitinase activity was detected as a dark (nonfluorescent) band against a UV fluorescent background of intact glycol chitosan or ethylene glycol chitin stained with Calcofluor White M2R.

Determination of the Degree of Chitosan Deacetylation. A series of chitosans with varying degrees of deacetylation were prepared by alkaline deacetylation of crab chitin as described previously (*15*), after which the degree of chitosan deacetylation was measured using a colloid titration method (*36*, *37*). A 30-mL sample of 0.5% chitosan was then dissolved in 0.5% (v/v) acetic acid and titrated against $^1/_{400}$ N potassium polyvinyl sulfate. A 0.1% solution of toluidine blue was used as an indicator. The end point of the titration was marked by a change in the color of the indicator from blue to pink.

Effect of Metal Ions. Purified chitosanase in 0.2 mL of 25 mM sodium phosphate buffer (pH 7.0) was incubated with 0.2 mL of buffer containing various metals (Hg^{2+}, Co^{2+}, Ba^{2+}, Pb^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, and Mg^{2+}) or EDTA (disodium salt) at concentrations of 0.1 or 1 mM for 30 min at 25 °C. The enzyme activity was then measured using the viscosity method described above. All of these metals were chloride salts. The relative activity was expressed as the percentage ratio of the specific activity (units per milligram) of the purified chitosanase in the presence of a metal to that in the absence of the metal.

Gel Filtration of Chitosan Hydrolysis Products. Two-tenths of a milliliter of purified chitosanase was incubated with 0.3 mL of 1% chitosan (in 1% acetate, pH 4.0) for 0.5−48 h at 37 °C. The reaction was then stopped by heating the mixture in a 100 °C water bath for 10 min, after which the hydrolysate was centrifuged (3000*g*, 5 min) to remove insoluble substances. The supernatant was applied to a Superdex 75 HR column (1.6 × 60 cm) and eluted with 0.1 M sodium acetate buffer (pH 4.5) at a flow rate of 30 mL/h, and 0.5-mL fractions were collected. Chitosan hydrolysis products eluted from the column were determined using a method for determination of hexosamines (*38*).

Protein Determination. Protein concentrations were determined using bicinchoninic acid (*39*); bovine serum albumin served as the standard.

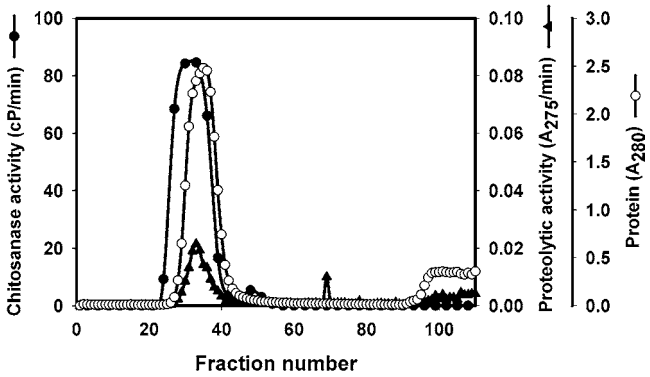

Figure 1. Affinity chromatography of protease and chitosanase on *p*HMB–Sepharose 4B. The column (1.6 × 12 cm) was equilibrated with 25 mM sodium phosphate buffer (0.15 M NaCl, pH 7.0), after which commercial ficin containing both protease and chitosanase activity was applied. The enzyme was eluted with equilibrium buffer at a flow rate of 20 mL/h; 1-mL fractions were collected.

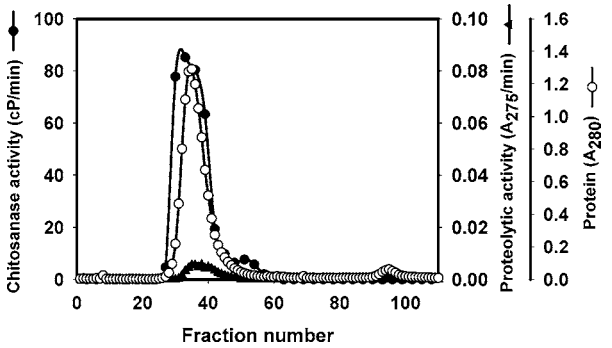

Figure 2. Affinity chromatography of protease and chitosanase on cystatin–Sepharose 4B. The column (1.6 × 12 cm) was equilibrated with 25 mM sodium phosphate buffer (pH 7.0), after which the chitosanase purified through two *p*HMB–Sepharose 4B column chromatography steps was applied. The enzyme was eluted with equilibrium buffer at a flow rate of 20 mL/h; 1-mL fractions were collected.

RESULTS

Chitosanase Purification. Purification of chitosanase using the protocols summarized in **Table 1** enabled us to remove proteolytic activity and isolate a chitosanolytic enzyme from a commercial ficin preparation. The chitosanase was purified 9.2-fold over the first commercial ficin enzyme solution, and the total yield was 61%. Initially, affinity chromatography was carried out twice on a *p*HMB−Sepharose 4B column for specific adsorption of thiol protease. Chitosanase was not adsorbed by the affinity adsorbent and emerged immediately from the column. Most of the protease was removed with this procedure (**Figure 1**). The chitosanase eluted from the *p*HMB−Sepharose 4B column was further purified by affinity adsorption of residual ficin protease on a cystatin−Sepharose column. Again, the chitosanase was not adsorbed onto the gel and was thus immediately eluted from the column (**Figure 2**). Although a trace of protease activity still contaminated the eluted chitosanase, almost all had been removed after this step. Finally, the chitosanase was purified by gel filtration on a Superdex 75 HR column.

The chitosanase preparations were analyzed by SDS-PAGE at each stage of the affinity chromatography and gel filtration (**Figure 3**). Following *p*HMB−Sepharose 4B affinity chromatography, the fractions contained four polypeptides with molecular masses of 30.2, 22.9, 17, and 14.5 kDa (**Figure 3**, lanes 1 and 2). After passage of the enzyme through cystatin−Sepharose 4B and Superdex 75 HR columns, the purified chitosanase contained only two polypeptides with molecular

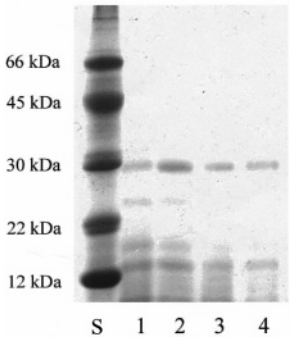

Figure 3. SDS-PAGE showing protein profiles of the chitosanase preparation at each stage of the purification. Electrophoresis was performed in 10% acrylamide gel: lane S, protein molecular weight markers; lane 1, eluate from the first *p*HMB–Sepharose column; lane 2, eluate from the second *p*HMB–Sepharose column; lane 3, eluate from the cystatin–Sepharose column; lane 4, eluate from the Superdex 75 HR column.

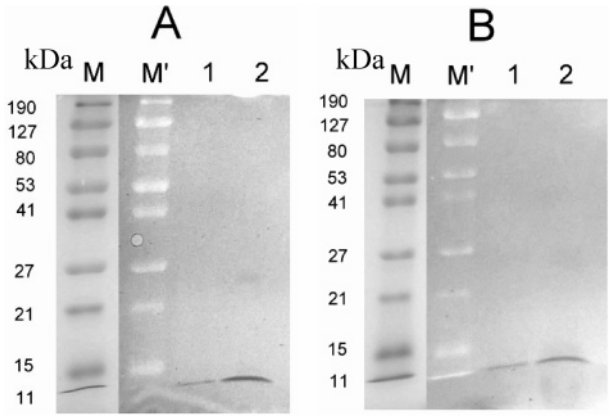

Figure 4. Activity staining after SDS-PAGE of the purified chitosanase. Electrophoresis was performed in 12.5% acrylamide gel containing 0.1% glycol chitosan (**A**) or 0.1% ethylene glycol chitin (**B**). Chitosanase or chitinase activity was detected by Calcofluor White M2R staining after lysis of glycol chitosan or ethylene glycol chitin in the gel: lanes M and M′, protein molecular weight markers before and after activity staining, respectively; lane 1, enzyme denatured with SDS plus 2-mercaptoethanol; lane 2, enzyme denatured with SDS alone.

masses of 30.2 and 14.5 kDa (**Figure 3**, lanes 3 and 4). An SDS-PAGE electropherogram of the commercial ficin preparation was not obtained because of interference from protein degradation catalyzed by the strong proteolytic activity. When the purified chitosanase was denatured with SDS without 2-mercaptoethanol and then subjected to SDS-PAGE, gel activity staining for chitosanase (**Figure 4A**) and chitinase (**Figure 4B**) revealed only one activity band with a molecular mass of ~13 kDa, indicating that although the purified enzyme contained at least two proteins (30.2 and 14.5 kDa), only one (14.5 kDa) was a chitosanolytic enzyme. Moreover, this enzyme showed dual chitinase and chitosanase activities, both of which could be rescued after removal of SDS. The apparent difference between the estimated molecular masses of the activity and protein bands (~13 kDa and 14.5 kDa, respectively) likely reflects the different protein molecular weight markers used in the two assays.

Effect of pH and Temperature on Enzyme Activity. The optimal pH and temperature for chitosan hydrolysis by this enzyme were 4.5 and 65 °C, respectively.

Thermostability. Incubation of the chitosanase for 2.5−30 min at temperatures ranging from 40 to 90 °C revealed that the enzyme was stable from 40 to 70 °C (**Figure 5**). At 75 °C,

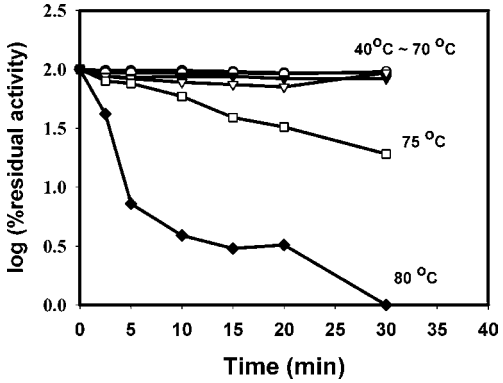

Figure 5. Thermostability of chitosanase. Plotted is the log of the percent activity remaining versus incubation time at various temperatures for the purified chitosanase: (●) 40 °C; (○) 50 °C; (▼) 60 °C; (▽) 70 °C; (□) 75 °C; (■) 80 °C.

Table 2. Effect of the Degree of Chitosan Deacetylation on the Activity of the Purified Chitosanase

degree of chitosan deacetylation (%)	relative chitosanase activity[a] (%)
94	15
80	62
70	88
60	94
52	100
40	80
31	41
22	7

[a] Chitosanase activity was determined using the reducing sugar assay method.

there was a comparatively modest and gradual loss of activity; there was a more rapid and substantial loss of activity at 80 °C. At 90 °C, the enzyme was dramatically inactivated. Note that the plot of the log of the percentage of residual enzyme activity versus incubation time is nonlinear; thus, the rate of thermal inactivation of this enzyme does not reflect denaturation proceeding according to first-order kinetics.

Molecular Mass. As estimated by gel filtration on a Superdex 75 HR column, the molecular mass of the chitosanase was 16.6 kDa. This value was close to that estimated from SDS-PAGE and gel activity staining for chitosanase and chitinase, which indicates chitosanase to be a monomeric enzyme.

Effect of Chitosan Deacetylation on Enzyme Activity. As shown in **Table 2**, chitosan polymers exhibiting varying degrees of deacetylation (22−94%) were all susceptible to hydrolysis by isolated chitosanase, with the most susceptible being 52−70% deacetylated and the least susceptible being <22% or >94% deacetylated.

Effect of Metal Ions and Chemical Modification Agents on Enzyme Activity. Metal ions or salts (0.1 and 1 mM) had no effect on this enzyme's activity (**Table 3**). On the other hand, chemical modification by 0.25 mM NBS, an oxidizing agent that usually acts specifically on tryptophan and cysteine (*40*), almost completely abolished enzyme activity (**Table 4**). That cysteine-specific modification by a mercaptide-forming agent (*p*HMB) and other modification agents had no effect on the enzyme's activity suggests that tryptophan is essential for this enzyme's catalytic activity.

Effect of Substrate Concentration. The substrate-saturation curve for this chitosanase was not a Michaelis−Menten type curve (**Figure 6**). The maximal velocity for chitosan hydrolysis was achieved at a substrate concentration of ~9 mg/mL

Table 3. Effect of Metal Ions on the Activity of the Purified Chitosanase

metal ion	relative chitosanase activity[a] (%) 1 mM	0.1 mM
none	100	100
Hg^{2+}	95	97
Co^{2+}	98	98
Ba^{2+}	100	99
Pb^{2+}	99	99
Ca^{2+}	100	100
Zn^{2+}	99	100
Cu^{2+}	100	101
Mg^{2+}	101	100
EDTA	102	100

[a] Chitosanase activity was determined using the reducing sugar assay method.

Table 4. Effect of Various Chemical Modification Reagents on the Activity of the Purified Chitosanase

reagent	concn (mM)	relative chitosanase activity[a] (%)
none		100
NBS	0.625	0
	0.25	4
	0.125	38
*p*HMB	1.25	99
	0.625	100
NAI	2.5	100
	1.25	100
CHD	5	99
	2.5	100
DNFB	5	76
	2.5	91
	1.25	97
DEPC	2.5	94
	1.25	98
PMSF	2.5	93
	1.25	97
EAM	2.5	98
	1.25	100
WRK	5	100
	2.5	100

[a] Chitosanase activity was determined using the reducing sugar assay method.

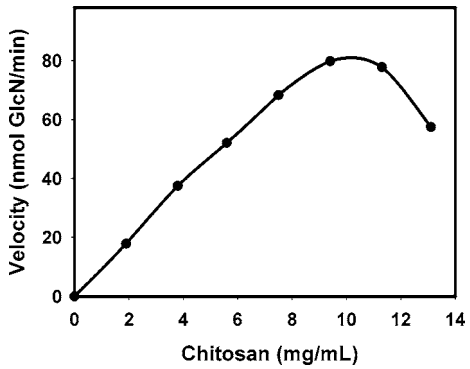

Figure 6. Effect of chitosan concentration on the activity of the purified chitosanase.

(reducing sugar assay method), but at concentrations >10 mg/mL substrate chitosan inhibited the enzyme's activity.

Substrate Specificity. As shown in **Table 5**, this enzyme also hydrolyzed water-soluble chitin (ethylene glycol chitin and CM-chitin), as measured by the reducing sugar assay method.

Table 5. Substrate Specificity of the Purified Chitosanase

substrate	relative activity[a] (%)
ethylene glycol chitin	100
crab chitosan (1100 kDa)	68
carboxymethyl chitin (CM-chitin)	55
crab chitosan (300 kDa)	34
shrimp chitosan (340 kDa)	24
glycol chitosan	7

[a] Enzyme activity was determined using the reducing sugar assay method.

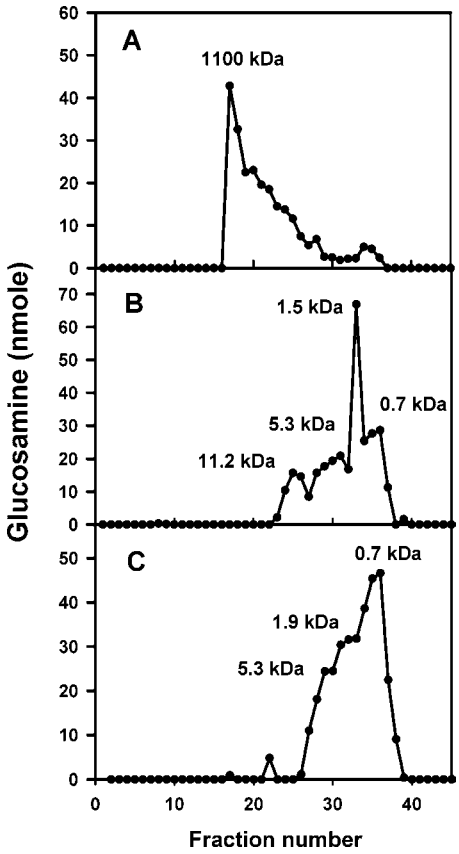

Figure 7. Gel filtration of chitosan hydrolysis products on a Superdex 75 HR column: (**A**) unhydrolyzed high molecular weight chitosan (1100 kDa); (**B**, **C**) products of 48 h of chitosan hydrolysis catalyzed by chitosanase, alone (**B**) or with subsequent degradation by H_2O_2 (0.5%) for 30 min (**C**). The column (1.6 × 60 cm) was equilibrated with 0.1 M sodium acetate buffer (pH 4.5). Chitosan and its hydrolysis products were eluted with equilibrium buffer at a flow rate of 30 mL/h; 0.5-mL fractions were collected.

These results are consistent with the gel activity staining for chitinase and chitosanase in SDS-PAGE. If the activity toward ethylene glycol chitin is arbitrarily assigned a value of 100, the activities toward crab chitosan (1100 kDa), CM-chitin, crab chitosan (300 kDa), shrimp chitosan (340 kDa), and glycol chitosan are 68, 55, 34, 24, and 7, respectively. In addition, the enzyme showed greater activity toward high molecular weight chitosan than toward low molecular weight chitosan.

Products of Chitosan Hydrolysis. The products of the chitosan hydrolysis catalyzed by this chitosanase were determined by gel filtration on a Superdex 75 HR column using dextran with various molecular masses as a calibration marker. As shown in **Figure 7**, unhydrolyzed, high molecular weight chitosan (1100 kDa) was eluted from the column with a major peak at fraction 17 (**Figure 7A**). The products found after 48 h of hydrolysis catalyzed by this enzyme were fractionated into several peaks with molecular masses ranging from 11.2 to 0.7

kDa (**Figure 7B**). If the resultant enzymatic hydrolysate was further degraded with 0.5% H_2O_2 for 30 min at 90 °C, the major products were chitosan oligomers with molecular masses ranging from 1.9 to 0.7 kDa and some oligomers with longer chain lengths (~5.3 kDa) (**Figure 7C**). On the basis of these results, we believe this chitosanase to be an endo-splitting enzyme.

DISCUSSION

Many plants contain latex that is exuded when leaves are damaged, and it has been suggested that latex secretion is a defense against wounding and/or predators such as insects and microorganisms. The defensive role of latex has been attributed in part to its sticky nature, which, for example, would enable the plant to capture and immobilize the mouth parts of insects (*41*, *42*). At the same time, however, latex contains proteases and a variety of other defense-related proteins, including chitinase, β-1,3-glucanase, hevein, and β-*N*-acetylglucosaminidase (*43*−*46*). Moreover, substantial amounts of cysteine protease (e.g., ficin) are found in the latex of several plants, including papaya and fig (*47*, *48*); thus, ficin is produced commercially from fig latex. In that regard, Yalpami and Pantaleone (*26*) recently reported that commercial ficin and several low-cost enzymes display chitosanolytic activity, although they did not demonstrate the presence of chitosanase in these commercial enzyme preparations. In the present study, we isolated and characterized a chitosanolytic enzyme from a commercial ficin preparation. This enzyme has a molecular mass of only ~13 kDa with dual chitinase and chitosanase activities, as determined by SDS-PAGE and gel activity staining, and is thus different from the chitinase (29 kDa) previously isolated from dried fig latex (*49*) and the basic class I chitinase (35.8 kDa) recently identified in fig tree latex (*50*). Like the other proteins present in fig latex, the enzyme isolated here is probably a defensive protein that was copurified with ficin during the protease's isolation from fig latex.

Chitosanases are a class of hydrolases identified as being distinct from chitinase and lysozyme (*51*). The sources of the chitosanases characterized so far have been mainly bacteria and fungi, although some have been identified in or characterized from a few plant species (*10*, *11*, *13*, *21*, *23*). For instance, Ouakfaoui and Asselin identified acidic and basic chitosanase isoforms with molecular masses between 10 and 23 kDa in the roots, leaves, fruits, and flowers of various dicots and monocots (*10*, *11*). Four acidic hydrolases with both chitosanase and chitinase activities have been isolated from sweet orange callus (*13*), whereas a hydrolase with only chitosanase activity was identified in stressed barley, cucumbers, and tomatoes (*10*); one with dual chitinase and chitosanase activity was purified from a commercial pineapple stem bromelain preparation (*23*); and three chitosanolytic enzymes with chitotriosidase activity were characterized in a commercial porcine pepsin preparation (*15*). As mentioned above, the molecular mass of the chitosanase identified in the present study is ~13 kDa (SDS-PAGE; or 16.6 kDa by gel filtration), which is much less than those of pineapple chitosanase (31.4 kDa), porcine chitosanase (~40 kDa), or most microbial chitosanases (23−43 kDa).

The degree of deacetylation affects the efficiency with which chitosanase hydrolyzes chitosan. The chitosanase identified in the present study degraded 52−70% deacetylated chitosan most effectively. The effects of chitosan deacetylation on chitosanase activity have been described previously for enzymes from the pineapple stem bromelain preparation (*23*), the porcine pepsin preparation (*15*), *Penicillium islandium* (*52*), *Bacillus circulans* MH-K1 (*37*), *Bacillus* sp. 7I-7S (*53*), and *Bacillus subtilis* IMR-NK1 (*54*). Chitosan polymers exhibiting 20−90% deacetylation were all susceptible to the pineapple chitosanase, with the most susceptible being 60−80% deacetylated, whereas 68−88% deacetylated chitosan was most susceptible to porcine chitosanase. The fungal enzyme (*P. islandium*) degraded 40−70% deacetylated chitosan most effectively, whereas the *Bacillus* chitosanases were most active toward chitosan with a high degree of deacetylation. The major products of chitosan hydrolysis catalyzed by the purified fig chitosanase are low molecular weight polymers and oligomers with molecular masses of 11.2−0.7 kDa. These products could then be further degraded by 0.5% H_2O_2 for preparation of chitosan oligomers with varying chain lengths and molecular masses ranging from 5.3 to 0.7 kDa. The activity pattern of this enzyme is distinct from most known microbial chitosanases, which normally produce chitobiose as one of their major products. In addition, this enzyme is more thermostable than the microbial ones. We suggest that this chitosanase from fig would be useful for production of low molecular weight chitosan preparations, although further characterization of the enzyme remains to be done.

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