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# Characterization of Three Chitosanase Isozymes Isolated from a Commercial Crude Porcine Pepsin Preparation

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Three chitosanases designated PSC-I, PSC-II, and PSC-III were purified from commercial pepsin preparation by sequentially applying pepstatin A-agarose affinity chromotography, DEAE-Sephacel ion-exchange chromatography, Mono Q column chromatography, and Mono P chromatofocusing. With respect to chitosan hydrolysis, the optimal pHs were 5.0, 5.0, and 4.0 for PSC-I, PSC-II, and PSC-III, respectively; optimal temperatures were 40, 40, and 30 °C; and the Km's were 5.2, 4.0, and 5.6 mg/mL. The molecular masses of the three isozymes were ∼40 kDa, as estimated by both gel filtration and SDS−PAGE, and the isoelectric points were 4.9, 4.6, and 4.4, respectively, as estimated by isoelectrofocusing electrophoresis. All three chitosanase isozymes showed activity toward chitosan polymer and *N*,*N*′,*N*″-triacetylchitotriose oligomer. Most effectively hydrolyzed were chitosan polymers that were 68−88% deacetylated.

**KEYWORDS: Chitosanase; purification; characterization; pepsin**

## INTRODUCTION

Chitosan is a linear polysaccharide composed of $\beta$-1, 4-linked D-glucosamine residues. In nature, this polymer is partially acetylated and, in fact, the name chitoasan describes a large family of polymers containing various proportions of D-glucosamine and *N*-acetyl-D-glucosamine residues. Chitosan occurs in the cell walls of some fungi (*1*−*5*) and in certain green algae, such as *Chlorella* (*6*), and is also produced commercially by alkaline deacetylation of shellfish (usually crab) chitin.

Chitosanases (EC 3.2.1.132) are hydrolases acting on chitosan and occur widely in soil microorganisms and in some plants. Plant chitosanase are distinguished from chitinases (EC 3.2.1.14) by their lower apparent molecular mass and their substrate specificity (*7*, *8*). Up to now, chitosanase activity has been studied primarily in bacteria (*9*−*11*) and fungi (*12*, *13*), most of which secrete chitosanases into their extracellular surroundings. These are generally endosplitting enzymes that hydrolyze chitosan into chitooligosaccharides and glucosamine. The ability of a given isozyme to hydrolyze $\beta$-glucosaminidic and *N*-acetyl-$\beta$-glucosaminidic chitosan linkages with differing degrees of deacetylation varies depending on the microorganism from which the chitosanase was obtained. Multiple forms of chitosanase have been detected in *Bacillus megaterium* P1 (*11*), *Bacillus licheniformis* UTK (*14*), *Mucor rouxii* (*15*), *Cucumis sativus* (*16*), and a few plant species (*17*, *18*). In addition, Osswald et al. (*19*) characterized 11 isoforms from *Citrus sinensis*, of which four exhibited both chitinase and chitosanase activities; the remainder exhibited only chitinase activity. Chitinases have also been identified in glandular tissues from the digestive systems of various animals, including many coelenterates, nematodes, mollusks, and arthropods (20). Chitosanase, by contrast, has generally not been detected in animals, with termites being the notable exception (21).

With that as background, Yalpani and Pantaleone (*22*) recently reported that a substantial number of commercially available crude enzyme preparations, including pepsin, lipase, and glucanases, among others, display lytic activity toward chitosan. These investigators, however, did not purify and characterize the chitosanolytic enzyme. On the other hand, Liao et al. (*23*) partially purified chitosanolytic enzyme from a commercial pepsin preparation and demonstrated the presence of chitosanase. Here, we describe our further purification and characterization of three chitosanolytic enzymes isolated from a commercial pepsin preparation. All three isozymes hydrolyzed chitosan polymer as well as *N*,*N*″,*N*‴-triacetylchitotriose oligomer.

## MATERIALS AND METHODS

**Chemicals.** Pepsin (P 7125, from porcine stomach mucosa), pepstatin A-agarose, *N*-acetyl-D-glucosamine (GlcNAc), *N*,*N*′-diacetylchitobiose ($(GlcNAc)_2$), *N*,*N*′,*N*″-triacetylchitotriose ($(GlcNAc)_3$), and 3-cyclohexylamino-1-propane sulfonic acid (CAPS) were purchased from Sigma (St. Louis, MO). The low molecular weight calibration kit (LMW), high molecular weight calibration kit (HMW), Mono Q HR 5/5, Mono P HR 5/20, Superose 12 HR 10/30, Ampholine PAGplate (pH 4.0−6.5), Polybuffer 94, and p*I* calibration protein (p*I* 2.8−6.55) were from Pharmacia (Uppsala, Sweden). Chitosan (82% deacetylation) was obtained from Ohka Enterprises Co. Ltd. (Kaohsiung, Taiwan).

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The silica gel plate 60 $F_{254}$ was from Merck (Darmstadt, Germany). The bicinchoninic acid protein assay reagent was from Pierce (Rockford, IL). Buffer salts and other chemicals used were of reagent grade.

**Purification of Chitosanase.** Twenty grams of pepsin preparation was dissolved in 160 mL of 0.1 M acetate buffer (pH 4.0), after which the precipitate was removed by centrifugation at 8000 × g for 10 min, and the resultant supernatant was dialyzed against 25 mM acetate buffer (pH 4.0). The dialyzate was lyophilized, dissolved in 0.1 M acetate buffer (pH 5.3, 0.35 M NaCl), and applied to a pepstatin A-agarose column (1.6 × 10 cm). The column was then washed with 50 mL of 0.1 M acetate buffer (pH 5.3, 0.35 M NaCl) and eluted with 40 mL of 0.1 M sodium bicarbonate buffer (pH 9.0, 1 M NaCl) at a flow rate of 25 mL/h. The fractions (2 mL) containing chitosanase activity were collected, dialyzed against 25 mM imidazole-HCl buffer (pH 7.0), and applied to a DEAE-Sephacel column (2.6 × 15 cm). The adsorbed chitosanase was eluted from the column with a linear NaCl gradient (0.2−0.8 M) in 25 mM imidazole-HCl buffer (pH 7.0) at a flow rate of 30 mL/h, and the fractions (3 mL) containing the enzyme activity were collected. After concentration by ultrafiltration, the enzyme solution was applied to a Mono Q HR 5/5 column and eluted with a linear NaCl gradient (0−0.4 M) in 25 mM imidazole-HCl buffer (pH 7.0) using an FPLC System (Pharmacia) at a flow rate of 30 mL/h. The fractions (0.5 mL) containing the enzyme activity were collected, dialyzed against 25 mM imidazole-HCl buffer (pH 7.4), concentrated by ultrafiltration, and applied to a Mono P HR 5/20 (Pharmacia) column. This column was eluted with 80 mL of diluted (1:8) Polybuffer 94 (Pharmacia) (pH 4.0) at a flow rate of 30 mL/h, and the fractions (0.5 mL) containing the enzyme activity were collected.

**Glycol Chitin Synthesis.** Glycol chitin was obtained by reacting chitin with ethylene chlorohydrin (2-chloroethanol) under alkaline conditions as follows according to the method (procedure B) of Hirano (*24*). Powdered chitin (4 g) was slurried with 60% (w/w) sodium hydroxide solution (16 mL) containing 0.2% sodium dodecyl sulfate at 4 °C for 1 h, after which the slurry was kept in a freezer overnight at −20 °C. After thawing and then refreezing it overnight at −20 °C, the slurry of frozen alkali chitin was added in portions over a period of 30 min to 70 mL of ice-cold 2-propanol containing ethylene chlorohydrin (16 mL). The mixture was mechanically stirred at room temperature for 1 h, after which the product was collected by filtration through a glass filter paper and washed well with ethanol to give a powdered material. The product was then N-acetylated by addition of acetic anhydride, neutralized with acetic acid, dialyzed against water, and lyophilized to give salt-free glycol chitin.

**Measurement of Chitosanase Activity.** Aliquots of 1% chitosan (0.5 mL) in 1% acetic acid, 1.4 mL of 0.1 M acetate buffer (pH 4.0), and 0.1 mL of properly diluted enzyme solution (1−3 units) in a total volume of 2 mL were incubated at 40 °C for 15 min. The reducing sugar produced was measured colorimetrically with a ferri-ferrocyanide reagent, as described by Imoto and Yagishita (*25*). One enzyme unit was defined as the amount of enzyme that produced an increase in sugar reduction equivalent to 1 μg of glucosamine per minute at 40 °C.

**Measurement of Chitinase Activity.** Chitinase activity was determined using glycol chitin as a substrate. One mL of 0.2% glycol chitin, 1.4 mL of 0.1 M acetate buffer (pH 4.0), and 0.1 mL of properly diluted enzyme solution in a total volume of 2 mL were incubated at 40 °C for 15 min. The reducing sugar produced was measured colorimetrically with a ferri-ferrocyanide reagent (*25*).

**Measurement of Protease Activity.** Protease activity was measured using hemoglobin hydrolysis as an index according to the method of Anson (*26*) with some modifications. The incubation mixture contained 5 mL of 2% hemoglobin (in 0.075 M NaOH) and 0.2 mL of properly diluted enzyme solution (30−60 units) in a total volume of 5.2 mL. The incubation was carried out at 37 °C for 10 min, after which the reaction was stopped by adding 5 mL of 0.11 M trichloroacetic acid containing 0.22 M sodium acetate and 0.33 M acetic acid, and the absorbance at 275 nm of the trichloroacetic acid (0.05 M) soluble product was measured. A protease unit was defined as the amount of enzyme that gave an increase in absorbance at 275 nm equivalent to 1 μg of tyrosine per minute at 37 °C.

**Determination of Optimal pH and pH Stability.** The optimal pH for the chitosanolytic activity of the purified chitosanase isozymes was assayed in a universal buffer (Britton and Robinson type) over a pH range of 3−10. To assess pH stability, enzyme solutions in a universal buffer (pH 3−10) were immersed for 30 min in a thermostatic water bath at 30 °C. The remaining activities were measured as described above.

**Determination of Optimal Temperature and Thermal Stability.** The optimal temperatures for chitosan hydrolysis by the purified chitosanase isozymes were assayed at their respective optimal pH over a temperature range of 20−90 °C. To assess thermostability, the enzyme solutions were immersed in a thermostatic water bath for 30 min at various temperatures (from 20 to 90 °C). The remaining activities were measured as described above.

**Estimation of Molecular Mass.** The molecular masses of the chitosanase isozymes were estimated using gel filtration on a Superose 12 HR using the FPLC System (Pharmacia) according to Andrews (*27*). Alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa) served as standards.

**Measurement of the Isoelectric Point (p*I*).** The p*I*'s of the purified chitosanase isozymes (PSC-I: 0.4 μg, PSC-II: 0.6 μg, PSC-III: 0.4 μg) were measured using isolelectric focusing (IEF) on a Pharmacia Ampholine PAGplate (pH 4.0∼6.5) and compared with standards from an IEF calibration kit (Pharmacia 17-0472-01, p*I* 2.8−6.55) according to the manufacturer's instructions.

**Michaelis Constant and Rate Constant Determination.** The Michaelis constant ($K_m$) and the maximal velocity ($V_{max}$) of chitosan hydrolysis by the purified chitosanase isozymes were determined at substrate concentrations ranging from 0.2 to 8 mg/mL. The $K_m$ and $V_{max}$ of each isozyme was calculated from a double reciprocal plot according to Lineweaver and Burk (*28*). Rate constant ($k_{cat}$) was calculated from the $V_{max}$ and the enzyme concentration used in the reaction mixture.

**SDS−PAGE.** SDS−PAGE was performed in 12.5% polyacrylamide gels using bromophenol blue as the tracking dye. The stacking gel was made of 4% (w/v) polyacrylamide (*29*). Samples were boiled for 5 min with 2% (w/v) SDS in 125 mM Tris-HCl buffer (pH 6.8). After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250.

**N-Terminal Amino Acid Sequence Analysis.** After dialysis against water and lyophilization, the purified chitosanase isozymes were sequenced by automated Edman degradation using an Applied Biosystems 477 A protein sequencer.

**Intrinsic Viscosity Determination.** Nine mL of each chitosanase isozyme [PSC-I (360 units), PSC-II (864 units), and PSC-III (477 units)] were incubated with 1 mL of 1% chitosan (in 1% acetic acid) at 40 °C for 2 h. After heating at 100 °C water bath for 10 min to stop the reaction, the intrinsic viscosity [$\eta$] of the chitosan hydrolysates at 25 °C was determined using a Cannon−Fenske capillary viscometer (Cannon 75). The [$\eta$] values were obtained by extrapolation from the $\eta_{sp}$/C versus C plot. The average molecular weights of the chitosan hydrolysates were calculated from the viscometric constant according to the equation $[\eta] = 3.04 \times 10^{-5}\ M^{1.25}$ (*30*).

**Thin-Layer Chromatography of Hydrolysis Products.** One hundred μL of each chitosanase isozyme [PSC-I (4 units), PSC-II (9.6 units), and PSC-III (5.4 units)] were incubated with 100 μL of 5 mM $(GlcNAc)_3$ at 40 °C for 24 h. The enzymatic products were subjected to thin-layer chromatography (TLC) on a silica gel plate 60 $F_{254}$ in a solvent system composed of *n*-propanol−water−ammonia water (70:30:1 v/v). The TLC plates were developed by first dipping them in acetone saturated with silver nitrate and then sprinkling them with ethanol containing 0.5 M NaOH.

**Chitosan Deacetylation Degree Determination.** A series of chitosans with varying degrees of deacetylation were prepared by alkaline deacetylation of crab chitin. To prepare 10−20% deacetylated chitosan, 20 g of chitin was mixed with 400 mL of 40% NaOH and heated at 100 °C for 1 and 1.5 h. To prepare 30−40% deacetylated chitosan, 20 g of chitin was mixed with 400 mL of 45% NaOH and heated at 100 °C for 1.5 and 2.5 h. To prepare 50−70% deacetylated chitosan, 20 g of chitin was mixed with 400 mL of 50% NaOH and heated at 100 °C for 0.5, 1, and 5 h. To prepare 90% deacetylated chitosan, 20 g of

**Table 1.** Purification of Chitosanase Isozymes from Commercial Pepsin[a]

| procedure | total activity[b] (units) | total protein (mg) | specific activity (units/mg) | purification (fold) | yield (%) |
|---|---|---|---|---|---|
| dialysis and lyophilization | 6737 | 667.4 | 10.1 | 1 | 100 |
| protease pepstatin A-agarose adsorption | 5434 | 82.9 | 65.5 | 6.5 | 80.6 |
| DEAE Sephacel ion-exchange chromatography | 1286 | 11.2 | 114.8 | 11.4 | 19.1 |
| Mono Q ion-exchange chromatography | 963 | 2.8 | 343.9 | 34.1 | 14.3 |
| Mono P chromatofocusing | | | | | |
| PSC-I | 40.1 | 0.054 | 742.6 | 73.5 | 0.6 |
| PSC-II | 192.0 | 0.392 | 489.8 | 48.5 | 2.9 |
| PSC-III | 159.9 | 0.218 | 733.5 | 72.6 | 2.4 |

[a] Data were obtained from 20 g of crude porcine pepsin. [b] One chitosanase unit is defined as the amount of enzyme needed to produce 1 $\mu$g of glucosamine per min at 40 °C, pH 4.0.

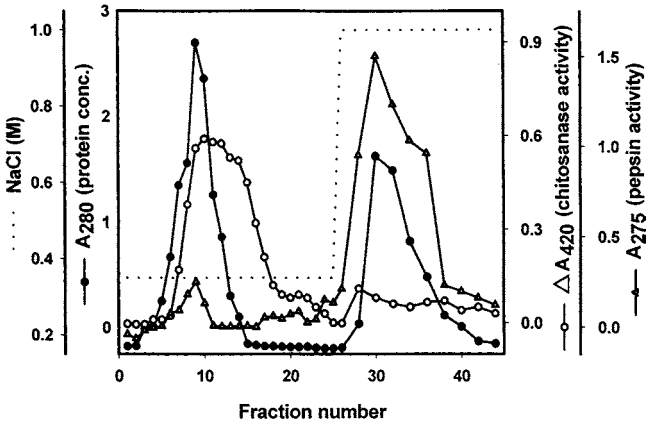


**Figure 1.** Column chromatography of chitosanase on pepstatin A-agarose. The column (1.6 × 10 cm) was equilibrated with 0.1 M acetate buffer (pH 5.3, 0.35 M NaCl), after which dialyzed commercial pepsin was applied. The column was then washed with equilibrium buffer and eluted with 0.1 M sodium bicarbonate (pH 9.0, 1 M NaCl) at a flow rate of 25 mL/h; 2-mL fractions were collected.

65% deacetylated chitosan was mixed with 400 mL of 60% NaOH and heated at 100 °C for 1 h. After cooling, the supernatant was decanted, and the remaining mixture was filtered by suction through a glass filter paper. The residue was then thoroughly washed with water and dried with hot air.

The degree of chitosan deacetylation was measured using the colloid titration method of Toei and Kohara (*31*). A 30-mL sample of 0.5% chitosan was 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 for the titration was marked by the color of the indicator changing from blue to pink.

**Protein Determination.** The protein concentration was determined using the bicinchoninic acid method (*32*) using bovine serum albumin as the standard.

## RESULTS

**Chitosanase Purification.** Purification of chitosanase using the protocols summarized in **Table 1** enabled us to isolate three chitosanase isozymes in the final step. These isozymes were purified 49- to 74-fold over the first partially purified chitosanase, and the total yield was 5.9%. Initially, affinity chromatography was performed on a pepstatin A-agarose column equilibrated with 0.1 M acetate buffer (pH 5.3, 0.35 M NaCl) (**Figure 1**). The chitosanase was not adsorbed by the affinity adsorbent and therefore emerged immediately from the column. Other proteins were adsorbed onto the gel and emerged only after being eluted. Protease, for example, was eluted with 0.1 M $NaHCO_3$ (pH 9.0, 1 M NaCl). Most proteins without chitosanase activity were removed by this procedure. The chitosanase obtained from the pepstatin A-agarose column was

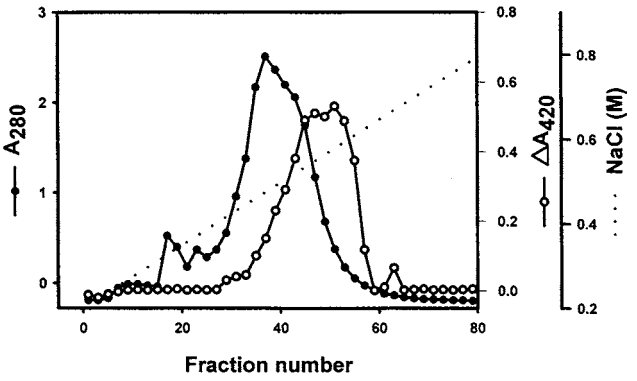


**Figure 2.** Ion-exchange chromatography of chitosanase on DEAE-Sephacel. The column (2.6 × 15 cm) was equilibrated with 25 mM imidazole-HCl buffer (pH 7.0), after which chitosanase from the pepstatin A-agarose column was applied. The column was eluted with a linear NaCl gradient (0.2–0.8 M) in equilibrium buffer at a flow rate of 30 mL/h; 3-mL fractions were collected.

further purified by ion-exchange chromatography on a DEAE-Sephacel column (**Figure 2**). One major protein peak containing chitosanase activity was eluted using a linear NaCl gradient (0.2−0.8 M), after which the fractions containing higher amounts of chitosanase-specific activity (fractions 45−60) were collected. The amount of enzyme activity recovered at this step was rather low, perhaps because some fractions containing lesser amounts chitosanase-specific activity (fractions 35−44) were removed. Further purification of the enzyme on a Mono Q column was performed using an FPLC System, with which a single protein peak with chitosanase activity and several peaks without enzyme activity were obtained (**Figure 3**). After collecting the fractions containing the chitosanase activity, final purification of the enzyme was carried out using chromatofocusing on a Mono P column (**Figure 4**). Chitosanase activity was found in five fractions (F1−F5), of which fractions 1 (fraction no. 19 and 20), 3 (fraction no. 31−34), and 4 (fraction no. 41−46) were homogeneous, as determined by isoelectrofocusing electrophoresis (**Figure 5**), and were designated as porcine stomach chitosanase isozymes PSC-I, PSC-II, and PSC-III.

**Effect of pH and Temperature on Enzyme Activity and Stability.** The optimal pHs for chitosan hydrolysis by PSC-I, PSC-II, and PSC-III were 5.0, 5.0, and 4.0, respectively (**Figure 6A**). Incubation for 30 min at 20 °C in a universal buffer at pHs ranging from 3 to 10 revealed PSC-I to be stable at pH 5−8, PSC-II at pH 3−7, and PSC-III at pH 6−9 (**Figure 6B**).

Optimal temperatures for chitosan hydrolysis by PSC-I, PSC-II, and PSC-III were 40, 40, and 30 °C, respectively (**Figure 7A**). Incubation for 30 min at temperatures ranging from 20 to 70 °C revealed all three isozymes to be stable over the entire range (**Figure 7B**).

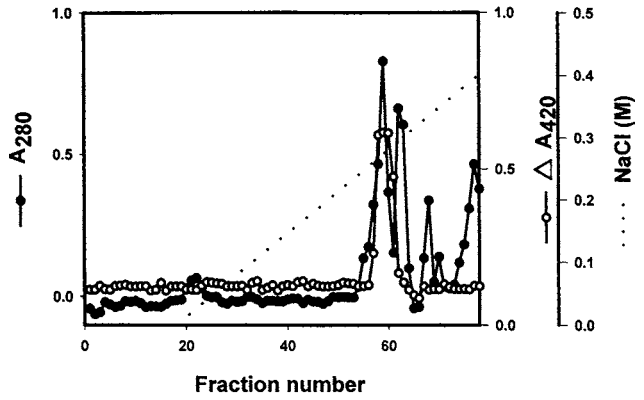


**Figure 3.** Ion-exchange chromatography of chitosanase on a Mono Q HR 5/5 column. The column (0.5 × 5 cm) was equilibrated with 25 mM imidazole-HCl buffer (pH 7.0), after which chitosanase from the DEAE-Sephacel column was applied. The column was washed with equilibrium buffer and eluted with a linear NaCl gradient (0–0.4 M) in equilibrium buffer at a flow rate of 30 mL/h using an FPLC System (Pharmacia); 0.5-mL fractions were collected.

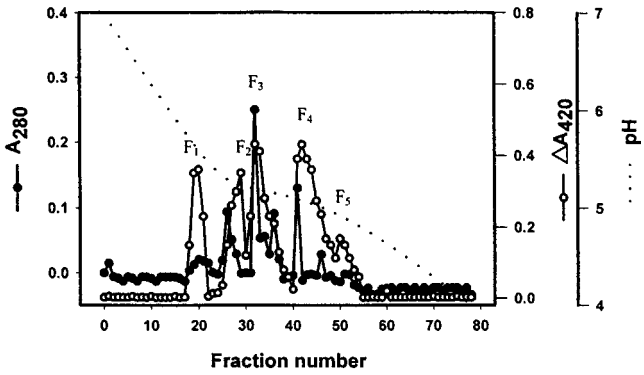


**Figure 4.** Chromatofocusing of chitosanase on a Mono P HR 5/20 column. The column (0.5 × 20 cm) was equilibrated with 25 mM imidazole-HCl (pH 7.0), after which chitosanase from the Mono Q HR column was applied. The column was eluted with 80 mL of diluted (1:8) Polybuffer (pH 4.0) at a flow rate of 30 mL/h; 0.5-mL fractions were collected.

**Table 2.** Kinetic Constants for Chitosan Hydrolysis by the Isolated Chitosanase Isozymes[a]

| chitosanases | $k_{cat}$ ($sec^{-1}$) | $K_m$ (mg $mL^{-1}$) | $k_{cat}/K_m$ (mL $mg^{-1}$ $sec^{-1}$) |
|---|---|---|---|
| PSC-I | 5.9 | 5.2 | 1.1 |
| PSC-II | 2.3 | 4.0 | 0.6 |
| PSC-III | 6.2 | 5.6 | 1.1 |

[a] Kinetic constants were determined using chitosan as the substrate at their respective optimal pH at 40 °C.

**Molecular Mass and p*I*.** The molecular masses of PSC-I, PSC-II, and PSC-III were 37.5, 40.6, and 43.7 kDa, respectively, as estimated by gel filtration on a Superose 12 HR in a FPLC System. These values were close to those estimated by SDS−PAGE (**Figure 8**), indicating all three isozymes to be monomers.

The p*I*'s of PSC-I, PSC-II, and PSC-III were 4.9, 4.6, and 4.4, respectively, as estimated by isoelectrofocusing electrophoresis (**Figure 5**).

**Michaelis Constant ($K_m$) and Rate Constant ($k_{cat}$).** The $K_m$, $k_{cat}$, and $k_{cat}/K_m$ for chitosan hydrolysis by the three isozymes are shown in **Table 2**. That PSC-II had a smaller $K_m$ value than PSC-I or PSC-III indicates it to have a lower apparent constant of dissociation of enzyme−substrate complex than the other isozymes. In addition, the $k_{cat}$ and $k_{cat}/K_m$ of PSC-II was much lower than that of PSC-I or PSC-III, indicating PCS-II had a much lower rate constant for chitosan hydrolysis at chitosan concentrations much higher or lower than $K_m$. PSC-I and PSC-III thus have higher apparent catalytic efficiencies for chitosan hydrolysis than PSC-II.

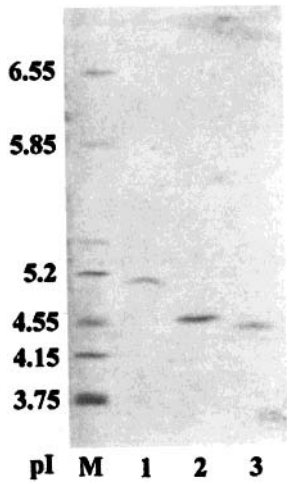


**Figure 5.** IEF electrophoresis of chitosanase isozymes PSC-I, PSC-II, and PSC-III: lanes 1–3, purified chitosanase isozymes PSC-I, PSC-II, and PSC–III, respectively; lane M, standard proteins [human carbonic anhydrase B (p*I* = 6.55); bovine carbonic anhydrase B (p*I* = 5.85); $\beta$-lactoglobulin A (p*I* = 5.2); soybean trypsin inhibitor (p*I* = 4.55); glucose oxidase (p*I* = 4.15); methyl red (p*I* = 3.75)]. IEF was performed on a Pharmacia Ampholine PAGplate (pH 4.0–6.5).

**Table 3.** Effect of the Degree of Chitosan Deacetylation on the Activities of Chitosanase Isozymes

| degree of chitosan deacetylation (%) | relative chitosanase activity (%)[a] | | |
|---|---|---|---|
| | PSC-I | PSC-II | PSC-III |
| 20.1 | 67 | 59 | 54 |
| 37.9 | 68 | 62 | 53 |
| 67.9 | 97 | 74 | 75 |
| 87.0 | 98 | 95 | 78 |
| 88.4 | 100 | 100 | 100 |

[a] Chitosanase activities were determined using 0.25% chitosans with differing degrees of deacetylation as substrates in 2 mL of 70 mM acetate buffer (pH 4.0) at 40 °C. The activity observed using 88.4% deacetylated chitosan as a substrate was assigned a value of 100.

**Effect of Chitosan Deacetylation on Enzyme Activity.** As shown in **Table 3**, chitosan polymers exhibiting varying degrees of deacetylation (20−88%) were all susceptible to the three chitosanase isozymes, with the most susceptible being 68−88% deacetylated and the least susceptible being less than 38% deacetylated. In addition, the three isozymes also acted on *N*,*N*′,*N*″-triacetylchitotriose, though an unknown oligomer with a chain length longer than three was detected (**Table 4**), suggesting the action of the isozymes on the triacetylchitotriose included transglycosylation or condensation in addition to hydrolysis.

**N-Terminal Amino Acid Sequence.** The sequence of the first 14 N-terminal amino acid residues of the three chitosanase isozymes are shown in **Figure 9**. The N-terminal of PSC-I and PSC-II show substantial sequence identity, as 9 of the first 14 residues are pairwise identical. The N-terminal of PSC-III, by contrast, had only one residue in common with PSC-I and none with PSC-II but showed a high degree of sequence identity with

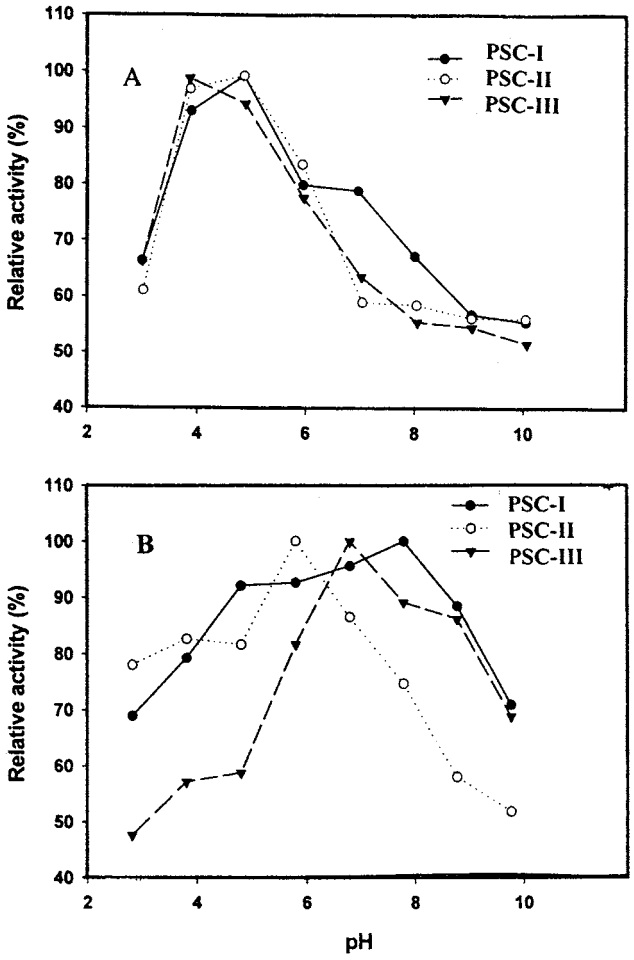


**Figure 6.** Effect of pH on porcine chitosanase activity and stability. Panel A: Chitosanase isozymes PSC-I, PSC-II, and PSC-III were assayed in a universal buffer (Britton and Robinson type); pH ranged from 3 to 10. Panel B: PSC-I, PSC-II, and PSC-III were immersed in a thermostatic water bath at 30 °C for 30 min in a universal buffer (pH 3–10), after which the remaining enzyme activities were assayed.

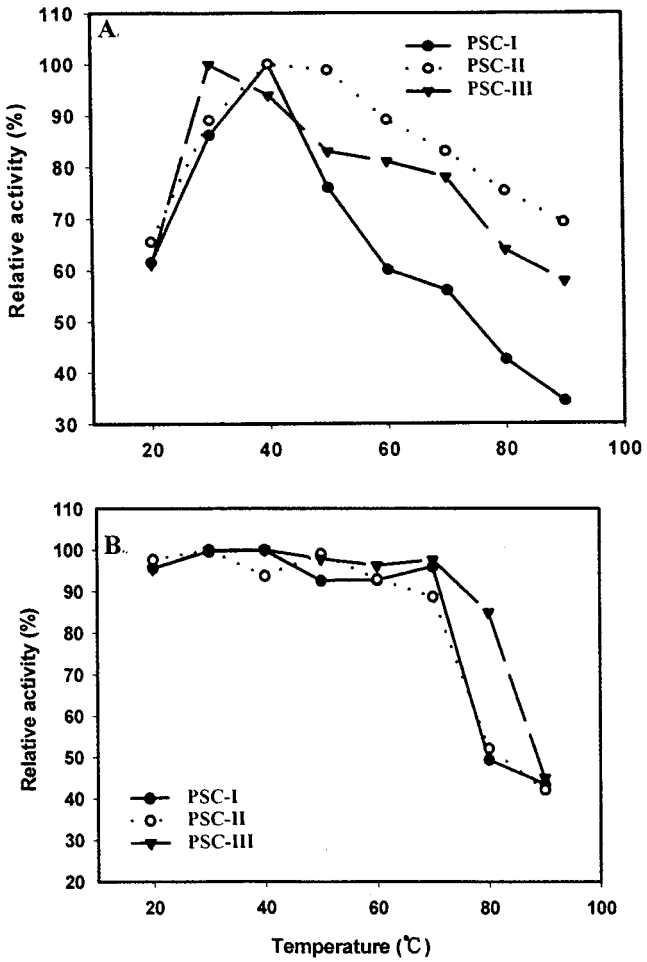


**Figure 7.** Effect of temperature on porcine chitosanase activity and stability. Panel A: Chitoasnase isozymes PSC-I, PSC-II, and PSC-III were assayed at their respective optimal pH; temperatures ranged from 20 to 90 °C. Panel B: PSC-I, PSC-II, and PSC-III were immersed in a thermostatic water bath for 30 min at various temperatures (from 20 to 90 °C), after which the remaining enzyme activities were assayed.

**Table 4.** Products of the Hydrolysis of *N*,*N*′,*N*″-Triacetylchitotriose Catalyzed by Chitosanase Isozymes

| chitosanases | substrates | products |
|---|---|---|
| PSC-I | $(GlcNAc)_3$[a] | GlcNAc, $(GlcNAc)_n$ $(GlcNAc)_3$ |
| PSC-II | $(GlcNAc)_3$ | GlcNAc, $(GlcNAc)_n$ $(GlcNAc)_2$ (trace) |
| PSC-III | $(GlcNAc)_3$ | GlcNAc, $(GlcNAc)_n$ $(GlcNAc)_2$ (trace) |

[a] GlcNAc indicates *N*-acetyl-D-glucosamine; the numbers outside the parenthesis indicate the degree of polymerization. $(GlcNAc)_n$ indicates an unknown chitin oligomer with a length greater than three.

**Table 5.** Average Molecular Masses of the Products of Chitosan Hydrolysis Catalyzed by the Three Chitosanase Isozymes[a]

| chitosanases | average molecular mass of products (kDa) |
|---|---|
| none | 1079 |
| PSC-I | 280 |
| PSC-II | 217 |
| PSC-III | 206 |

[a] Nine ml of 1% chitosan (in 1% acetic acid) was incubated with 1 mL of chitosanase isozyme at 40 °C for 2 h.

the N-terminal of pepsin B; 13 of the first 14 N-terminal amino acid residues of PSC-III and pepsin B were pairwise identical.

**Products of Chitosan Hydrolysis.** To assess the products of the chitosan hydrolysis catalyzed by the three isozymes, the intrinsic viscosities of the resultant hydrolysates were determined using a capillary viscometer. The average molecular masses of the products generated by PSC-I, PSC-II, and PSC-III were estimated to be 280, 217, and 206 kDa, respectively (**Table 5**), suggesting all three isozymes are endohydrolases. This was further confirmed by analyzing the degraded oligomers in the hydrolysates using thin-layer chromatography (TLC).

## DISCUSSION

Chitosanases are a class of hydrolases identified as being distinct from chitinases and lysozymes (EC 3.2.1.17) (*33*). The sources of the chitosanases characterized so far have been mainly bacteria (*11*, *34*) and fungi (*35*), though multiple forms have been identified in a few plant species (*16*, *17*, *19*), and some plant pathogenesis-related (PR) proteins have been shown to be extracellular chitosanases (*7*). For instance, four acidic hydrolases with both chitosanase and chitinase activities have been isolated from sweet orange callus (*19*), and chitosanase that degrades only chitosan has been identified in stressed barley, cucumbers, and tomatoes (*7*). In addition, chitosanase has been

**Table 6.** Comparison of Properties of Termite (*Macrotermes estherae*) and Porcine Chitosanase Isozymes

| | termite caste[a] | | | | porcine chitosanase | | |
|---|---|---|---|---|---|---|---|
| specificity | workers | soldiers | males | winged females | PSC-I | PSC-II | PSC-III |
| chitosan | + | + | + | + | + | + | + |
| chitin | – | – | – | – | + | + | + |
| action type | exo | exo | exo | endo | endo | endo | endo |
| optimal pH | 6.2 | 5.8 | 4.9 | 5.33 | 5.0 | 5.0 | 4.0 |
| optimal temperature (°C) | 24–26 | 23–25 | 24 | 25 | 40 | 40 | 30 |
| molecular mass (kDa) | 40 | 35 | 31 | 31 | 37.5 | 40.6 | 43.7 |
| isoelectric point (p*I*) | ND[b] | ND | ND | ND | 4.9 | 4.6 | 4.4 |

[a] Data obtained from Aruchami et al. (1982) (*21*). [b] ND: not determined.

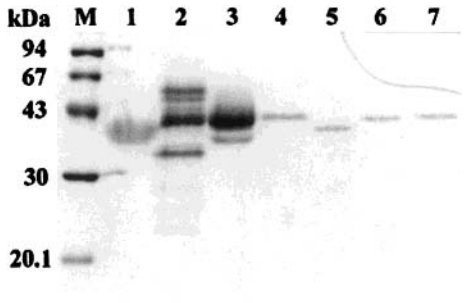


**Figure 8.** SDS–PAGE of chitosanase: lane 1, dialysis purified enzyme; lane 2, pepstatin A-agarose column purified enzyme; lane 3, DEAE-Sephacel column purified enzyme; lane 4, Mono Q column purified enzyme; lanes 5–7, Mono P column purified chitosanase isozymes PSC-I, PSC-II, and PSC-III, respectively; lane M, low molecular weight standard proteins.

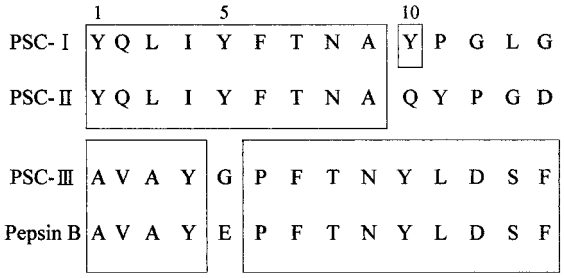


**Figure 9.** Comparison of the N-terminal amino acid sequences of PSC-I, PSC-II, PSC-III, and pepsin B.

identified in termites (*21*), which until now was the only report of an animal source of chitosanase. In the present study, however, we purified three chitosanase isozymes (PSC-I, PSC-II, and PSC-III) from a commercial porcine pepsin preparation that also displayed lytic activity toward the chitin oligomer *N*,*N*′,*N*′′-triacetylchitotriose. Hydrolases with dual chitinase and chitosanase activity have been isolated previously from microbial and plant sources (*11*, *15*, *19*, *36*−*38*), but this is the first report of chitosanase with chitinase activity from an animal source.

The characteristics of the three porcine chitosanase isozymes identified in the present study differed significantly from those isolated from microbes and plants, as well as from those isolated from the various termite castes (**Table 6**). For instance, PSC-I, PSC-II, and PSC-III all exhibited both chitosanolytic and chitotrioselytic activities, whereas the termite chitosanase degraded only chitosan. Moreover, with respect to chitosan hydrolysis, the porcine isozymes had a lower optimal pH and a higher optimal temperature than termite chitosanases. Still, the molecular masses of the porcine chitosanases (37−44 kDa) were similar to those from termites (31−40 kDa), which tended to be larger than many microbial (23−43 kDa) (*34*) and all plant (10−23 kDa) isoforms (*16*, *17*). One similarity between PSC-I, PSC-II, and PSC-III and most other chitosanases described in the literature (*34*) is that they are endohydrolases. Chitosanases from *Nocardia orientalis* (*39*) and termites (*21*) remain the only known exohydrolases. Another similarity is that the p*I*'s of the three porcine isozymes range from 4.4 to 4.9. These are the first p*I*'s determined for chitosanases from an animal source and are within the range of p*I* values determined for isoforms from various microbial and plant sources (4.0−10.1) (*34*).

The degree of deacetylation affected the efficiency with which the three porcine isozymes hydrolyzed chitosan. These enzymes were most active when the substrate chitosan was 68−88% deacetylated; chitosans that were less than 68% deacetylated were less susceptible to the enzymes' action. The effects of chitosan deacetylation on chitosanase activity have been described previously for enzymes from *Penicillium islandium* (*12*), *Bacillus circulans* MH-K1 (*40*) and *Bacillus* sp. 7I−7S (*41*). The fungal enzyme (*P. islandium*) was less active in hydrolyzing chitosan that was less than 40% or more than 70% deacetylated, whereas the *Bacillus* chitosanases were most active when hydrolyzing chitoasan with a high degree of deacetylation. In addition, the presence of *N*-acetylglucosamine residues was required for optimal activity by the enzyme from *B. circulans* MH-K1 (*40*) but was not required for optimal activity by the enzyme from *B.* sp. 7I−7S (*41*).

In summary, some commercial crude proteolytic enzyme preparations, including pepsin, papain, stem bromelain, ficin, and pancreatin (*22*, *42*−*44*), have recently been shown to contain chitosanolytic activity. In that regard, Liao et al. (*23*) reported that crude pepsin was strongly chitosanolytic because of the presence of chitosanase in the enzyme preparation. In the present study, we further purified and characterized three chitosanase isozymes from this commercial porcine pepsin preparation.

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