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Biochemical Properties of a Chitin-Binding Class III Chitinase in Pumpkin Leaves

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When we compared the chitinase activity of various plant sources using colorimetric or active gel-staining assay methods, the specific activity of pumpkin leaves was the highest among the samples we analyzed. The highly active chitinase from pumpkin leaves (designated PL-ChtIII) was purified to homogeneity using affinity chitin gel and HPLC Mono-Q anion-exchange column chromatographies. In contrast to other members of the class III chitinase family, PL-ChtIII showed a strong binding affinity to the regenerated chitin gel column. The apparent molecular weight of PL-ChtIII was estimated to be 29 kDa on SDS-PAGE gel, while its optimum pH and temperature were shown to be pH 6.0 and 60°C, respectively. Analyzing the reaction products of PL-ChtIII with swollen chitin as substrate, the dimer and tetramer of *N*-acetylglucosamine were produced as major products in the first hour of the enzymatic reaction along with a small amount of monomers and trimers. As the reaction time increased, dimeric *N*-acetylglucosamine became the predominant form of reaction product.

Keywords: Chitin binding activity, Class III chitinase, Enzymatic properties, Pumpkin leaves.

Introduction

Like animals, plants are continuously challenged with various types of pathogens in their environment including viruses, bacteria, and fungi. Defense mechanisms against pathogenic attack are essential to the persistence of the plant species. Plants respond to pathogenic invasion with an altered pattern of protein synthesis to inhibit the growth of parasitic invaders (Linthorst, 1991). These responses include enzymes involved in the process of lignification and the synthesis of phytoalexins, protease inhibitors, as well as enzymes capable of hydrolyzing structural components of the pathogen (Boller *et al.*, 1983; Lamb *et al.*, 1989). Among these, chitinases catalyze the hydrolysis of chitin, a major structural component of the cell wall of many phytopathogenic fungi (Raikel *et al.*, 1993). Chitinase activity in plant cells is induced by pathogenic attack (Rasmussen *et al.*, 1992), wounding (Ming-Mei *et al.*, 1995), and plant hormones (Mauch and Staehelin, 1989) as well as a number of other environmental stimuli (Fink *et al.*, 1990). Induction of chitinases is often co-ordinated with the induction of β-1,3-glucanases and other pathogenesis-related proteins (Joosten and de Wit, 1989). Most plants have a number of chitinase isozymes that differ in their primary structure, molecular weight, isoelectric point, and cellular localization (Collinge *et al.*, 1993). Molecular cloning of chitinases from various plants has facilitated the classification of these proteins. At least four classes of plant chitinases have been proposed based on their primary structures as follows (Collinge *et al.*, 1993). Class I chitinases are enzymes containing an *N*-terminal cysteine-rich domain of approximately 40 amino acids and a highly-conserved main structure, separated by a variable hinge region. Class II chitinases lack the *N*-terminal cysteine-rich domain, but have a high amino acid sequence identity to the main structure of class I chitinases. Class IV chitinases contain a cysteine-rich chitin-binding domain and a conserved main structure which resemble those of class I chitinases, but are significantly smaller due to deletions. Lastly, class III chitinases show no sequence similarity to classes I, II, or IV enzymes and lack the *N*-terminal cysteine-rich domain.

In search of a more active plant chitinase, we screened many plant sources using the methods of colorimetric

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assay and active-staining for chitinase activity on SDS-PAGE gel. As a result, we found a highly-active class III chitinase from pumpkin leaves which shows strong chitin-binding activity, contrary to other classes of chitinase (Lucas *et al.*, 1985; Shinshi *et al.*, 1990). In this paper, we describe the biochemical properties of this novel class III chitinase after purification of the protein from pumpkin leaves.

Materials and Methods

Materials Reagents for SDS-PAGE including low molecular weight protein standards were obtained from Bio-Rad Co. (Richmond, USA). Nitrocellulose membrane (BA-85) was purchased from Schleiher and Schull (Keene, USA), and peroxidase-conjugated goat anti-rabbit IgGs and substrates were purchased from Hyclone (Logan, USA). Concentrating apparatus (cut-off size, 10 kDa) was from Amicon Co. Practical-grade crab shell chitin, chitosan powder, glycol chitin, calcoflour white M2R, and Triton X-100 were from Sigma (St. Louis, USA). All other reagents were of the highest purity available. Pumpkin (*Cucubita maxima*) leaves for enzyme purification were harvested in September from a fully-grown plants field grown in Namhae, Korea, and stored at −70°C until use.

Active-staining of chitinase on SDS-PAGE gel SDS-PAGE was performed with 12% (w/v) polyacrylamide gel containing 0.01% (w/v) glycol chitin. After electrophoresis, gels were incubated at 37°C for 24 h with slow shaking in 0.1 M Tris·HCl buffer, pH 7.0, containing 1% (v/v) Triton X-100. The gels were stained with 0.01% (w/v) Calcofluor white M2R in 0.5 M Tris·HCl buffer (pH 8.9) for 30 min in the dark and destained for 1 h at room temperature in sterilized water. The lytic zones were analyzed under an UV transilluminator. Glycol chitin was prepared by acetylating glycol chitosan by the method described by Molano *et al.* (1979).

Purification of a pumpkin chitinase and *N*-terminal peptide sequencing Pumpkin leaves were finely chopped and homogenized using a polytron (Kinematica, UK) with 0.1 M Tris·HCl buffer, pH 7.0, buffer containing 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF. After the precipitate was discarded by centrifugation, solid ammonium sulfate was slowly added to the supernatant to a find concentration of 80% saturation. After stirring overnight, the precipitate obtained from ammonium sulfate fractionation was collected and dialyzed in the same buffer. The dialyzed protein was applied to a regenerated chitin gel affinity column (3 × 30 cm), which was pre-equilibrated with 0.1 M Tris·HCl (pH 7.0) buffer. After completely washing the unbound proteins from the column with the equilibrium buffer, proteins bound to the chitin gel were eluted using a 20 mM NaOH solution at a flow rate of 1 ml/min. After promptly adjusting the pH of the eluted fractions to pH 7.0, eluted fractions were pooled and concentrated using a YM-10 Amicon concentrator. The concentrated protein was dialyzed against 20 mM sodium bicarbonate buffer, pH 8.3, and applied to an HPLC Mono-Q column. Proteins were eluted by increasing the concentration of NaCl from 0 to 0.4 M with a flow rate of 1 ml/min. Using these purification steps, a single fraction containing chitinase activity and one protein band on SDS-PAGE gel was obtained. We designated the protein as PL-ChtIII, a class III pumpkin leaf chitinase. Regenerated chitin was obtained by the method of Hirano and Ohe (1976). The *N*-terminal sequence of the purified chitinase was determined by the Edman degradation method using an Applied Biosystem model 473A protein sequencer.

Preparation of polyclonal antibody and Western blot analysis To prepare a polyclonal antibody specifically binding to PL-ChtIII, 200 μg of purified PL-ChtIII in complete Freund's adjuvant was subcutaneously injected into rabbits four times every two weeks. Three days before bleeding, the rabbit was finally boosted with 500 μg of the purified PL-ChtIII in Tris·HCl buffered saline lacking adjuvant (50 mM Tris·HCl, pH 8.2, 0.1% Tween 20, 150 mM NaCl, TTBS). The collected serum was stored at room temperature for 1 h and centrifuged for 10 min at 3000 rpm. The supernatant was collected and Western blot analysis was carried out using the procedures described by Towbin *et al.* (1979).

Chitinase activity assay Reaction mixture containing chitinase enzyme and 1 to 2 mg of swollen chitin in 300 μl of 100 mM sodium acetate (pH 5.2) was incubated at 37°C for 1 h. The reaction was terminated by centrifugation in a microfuge for 5 min at 14,000 × g. A 150 μl aliquot of the supernatant was transferred to a new tube containing 15 μl of 1.0 M potassium phosphate (pH 7.1) and 10 μl proptoplast-forming enzyme (30 mg/ml solution, Boehringer Mannheim Biochemicals, Indianopolis, USA) that was dissolved in 10 mM KCl and 1 mM EDTA (pH 6.8). The solution was incubated at room temperature for 30 min to hydrolyze the chitooligosaccharides. The resulting monomeric *N*-acetylglucosamine was determined spectrophotometrically after reaction with *p*-dimethyl aminobenzaldehyde as described by Reissig *et al.* (1955). Swollen chitin was prepared by stirring 50 g of chitin with 500 ml of 85% phosphoric acid (Monreal and Reese, 1969).

Measurement of optimum pH and temperature To determine the optimum pH of the enzyme, 100 μl of the enzyme solution was added to 200 μl of 5% swollen chitin in various buffers ranging from pH 2 to 10. Solutions of 20 mM acetic acid, sodium acetate, and sodium bicarbonate buffer were used for the adjustment of pH 2 to 4, pH 5 to 7, and pH 8 to 10, respectively. Mixtures were incubated for 1 h at 37°C and then chitinase activity was determined. Optimum temperature was determined by varying the reaction temperature from 10°C to 90°C.

Analysis of the chitinolytic product HPLC was performed to analyze the end-products of the reaction of PL-ChtIII with swollen chitin as a substrate. The HPLC system (Waters model 590) was equipped with a carbohydrate analysis column (3.9 × 300 mm) and refractive index detector. Approximately 10 μg of enzyme was reacted with substrate. The reaction was stopped at various time intervals (30 min, 1, 2, 4 h) and then 40 μl of supernatant was injected into the HPLC column. The reaction products were eluted with 80% acetonitrile in water (v/v) with a flow rate of 1 ml per min. The elution profiles were then compared to those of standard *N*-acetylglucosamine monomer, dimer, trimer, tetramer, and hexamer profiles.

Results and Discussion

Screening of a highly-active chitinase from various plant sources To screen highly-active chitinases, soluble proteins were extracted from various plant leaves and their chitinase activities were measured by a colorimetric assay. As a result, we found that the cytosolic proteins extracted from cucumber and pumpkin leaves contained relatively higher chitinase activity compared to those of other enzyme sources (Fig. 1A). By comparing the chitinase activity on active-staining SDS-PAGE gel, we selected pumpkin leaves as the preferable chitinase source from the various enzyme sources examined. Since the active-staining SDS-PAGE gel contained 0.01% glycol chitin as the chitinase substrate, the separated chitinases on SDS-PAGE gel locally hydrolyzed the substrate. Lytic zones were loaded under UV irradiation. Even though the protein profile of Coomassie-staining did not show much difference between the two samples (Fig. 1B), chitin-hydrolyzing activity in pumpkin leaves was significantly higher than that of cucumber leaves, as shown in Fig. 1C.

Purification of the chitinase from pumpkin leaves and its *N*-terminal amino acid sequence determination The chitinase that stained strongly in active-staining SDS-PAGE gel was purified to homogeneity using ammonium sulfate fractionation, regenerated-chitin affinity gel, and anion-exchange column chromatographies (Fig 2). Since the chitin-binding affinity of PL-ChtIII was so strong, most of the protein impurities could be removed during the washing procedure, and the chitinase fraction was eluted from the chitin gel by a highly alkaline solution of

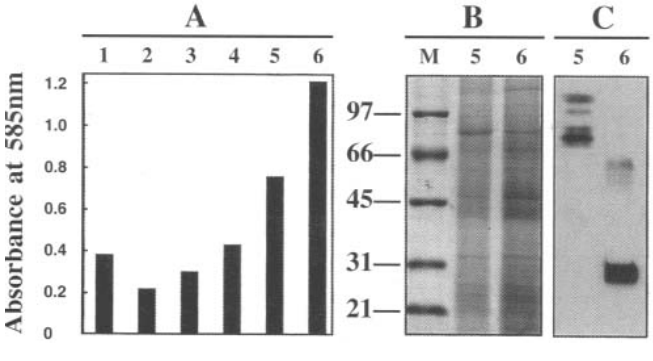

Fig. 1. Comparison of chitinase activity in the cytosolic fraction of various plant leaves. **A.** Chitinase activity in 10 μg soluble proteins extracted from various plant leaves was measured as described in Materials and Methods. Approximately 20 μg of soluble proteins from the two samples having relatively higher chitin hydrolyzing activities (lanes 5 and 6) were separated on a 12% SDS-PAGE gel containing 0.01% glycol chitin and either stained with Coomassie Blue (**B**) or subjected to chitinase active staining of the gel (**C**). Lanes 1 to 6 represent the proteins extracted from the leaves of red pepper (lane 1), tomato (lane 2), soybean (lane 3), arrow root (lane 4), cucumber (lane 5), and pumpkin (lane 6).

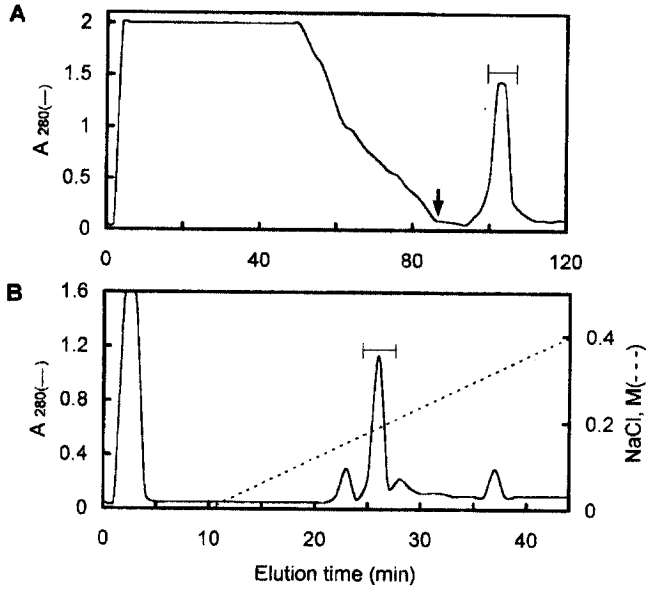

Fig. 2. Purification of the chitinase (PL-ChtIII) from pumpkin leaves. Proteins obtained from ammonium sulfate fractionation were applied to a regenerated chitin affinity gel and eluted with 20 mM NaOH solution. The arrow (↓) indicates the starting point of enzyme elution (**A**). After promptly adjusting the eluted fractions to pH 7.0, they were combined and concentrated in a YM 10 Amicon concentrator. The protein was further purified by HPLC Mono-Q column chromatography (**B**). Protein concentration was measured by the absorbance change at 280 nm (A_{280}). Fractions containing chitinase activity were pooled, as indicated by bars in the chromatogram.

20 mM NaOH. The active fractions pooled from the chitin column were further purified by HPLC Mono-Q column chromatography. From these chromatographic procedures, an essentially pure protein whose molecular mass was estimated to be 29 kDa on SDS-PAGE gel was obtained as shown in Fig. 3A (lane 3). Using the purified protein, we prepared a specific polyclonal antibody for the PL-ChtIII protein (Fig. 3B). In addition, we confirmed that the protein band obtained from HPLC Mono-Q chromatography was the chitinase enzyme by active-staining on SDS-PAGE gel. After electrophoresis, the chitinase was renatured by removing SDS from the gel using purified Triton X-100. The gel was stained with Calcofluor white M2R, which interacted with glycol chitin and completely destained the unreacted reagent. The hydrolytic zone was visualized by placing the gel on a UV-transilluminator and photographed (Fig. 3C). Even though pretreatment of β-mercaptoethanol prior to SDS-PAGE would have breakage disulfide bonds, the protein purified from HPLC Mono-Q column contained chitin hydrolytic activity and was renatured in polyacrylamide gel. From the renaturation of PL-ChtIII in SDS-PAGE gel, it is postulated that the enzyme may exist as a monomeric protein.

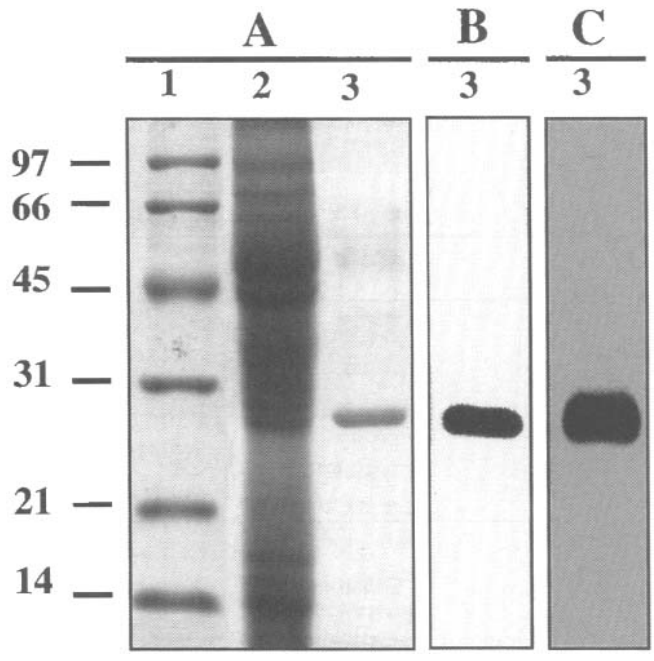

Fig. 3. SDS-PAGE, Western blot, and active-staining analysis of PL-ChtIII. Proteins obtained from ammonium sulfate fractionation (lane 2) and HPLC Mono-Q column chromatography (lane 3) were separated on a 12% SDS-polyacrylamide gel and either stained with Coomassie Blue (**A**) or immuno-probed with anti-PL-ChtIII antibody (**B**). Panel C indicates active-staining of the purified PL-ChtIII. Lane 1 contains low molecular weight standard proteins.

In order to prepare the oligonucleotide probe for cloning the gene encoding PL-ChtIII, we determined the *N*-terminal amino acid sequence of the protein by the Edmann degradation method and obtained the following result: *N*-Ala-Gly-Ile-Ala-Ile-Tyr-Trp-Gly-Gln-Asn-Gly-Asn-Glu-Gly-Ser-*C*. Comparing the *N*-terminal amino acid sequence of PL-ChtIII with the protein database using the NCBI-BLAST program, the enzyme had a considerable sequence similarity to class III chitinases of cucumber, pokeweed, *Arabidopsis*, and tobacco (Table 1). Furthermore, assignment of the purified protein as a class III group chitinase was confirmed by nucleotide sequence comparison of the cDNA clone encoding the protein, whose sequence was determined and deposited in the GenBank database under accession number AF082284. It was quite surprising that PL-ChtIII strongly bound to the regenerated chitin affinity gel and fell into the class III chitinase category because there had been no previous report on class III chitinases containing chitin-binding activity. Our results suggest that the PL-ChtIII is a novel type of class III chitinase.

Optimum pH and temperature of the purified PL-ChtIII Purified PL-ChtIII was quite stable at a wide range of pHs and temperatures as shown in Fig. 4. The

Table 1. Comparison of the *N*-terminal amino acid sequence of PL-ChtIII with those of other class III chitinases in plants.

Enzyme sources[1]	*N*-terminal amino acid seqeunces[2]
PL-ChtIII	A G **I** A **I** **Y** **W** **G** **Q** **N** **G** N **E** **G** S
Cucumber	A G **I** A **I** **Y** **W** **G** **Q** **N** **G** N **E** **G** S
Tobacco	G **D** **I** **V** **I** **Y** **W** **G** **Q** **N** **G** N **E** **G** S
Arabidopsis	G **G** **I** A **I** **Y** **W** **G** **Q** **N** **G** N **E** **G** N
Pokeweed	A G **I** A **I** **Y** **W** **G** **Q** **N** **G** **G** **E** **G** T

[1] *N*-terminal amino acid sequence of cucumber (Metraux *et al.*, 1989), tobacco (Lawton *et al.*, 1992), Arabidopsis (Verburg *et al.*, 1993), and pokeweed (Ohta *et al.*, 1995) were compared with that of PL-ChtIII.
[2] The conserved amino acids are marked as bold characters.

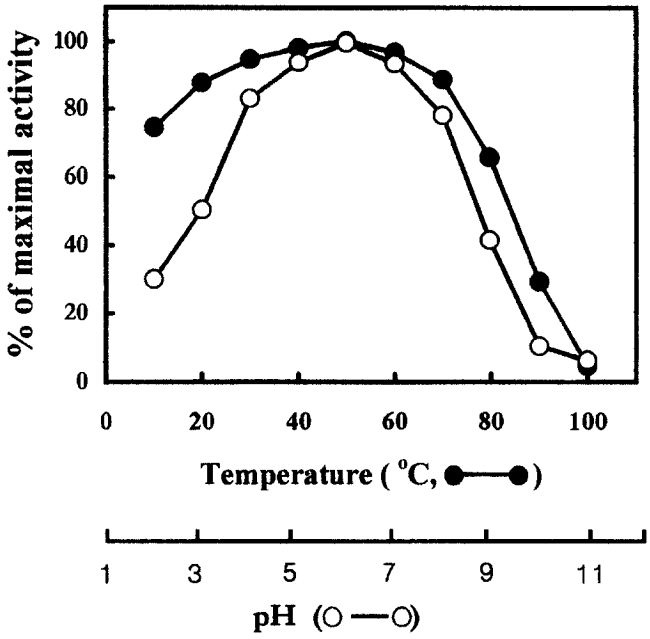

Fig. 4. Effects of temperature and pH on the PL-ChtIII activity. Chitinase activity was determined as described in Materials and Methods at various pH and temperatures.

optimum pH of the purified PL-ChtIII was pH 6.0, and it retained over 80% of maximal activity between pH 4.0 and 7.0. However, the chitinase activity of PL-ChtIII rapidly decreased beyond pH 2.0 or pH 9.0. The optimum temperature of the purified PL-ChtIII was about 50°C, although it contained more than 75% of its original activity between 10°C and 70°C.

Product analysis of PL-ChtIII The reaction products of PL-ChtIII with swollen chitin were analyzed using an HPLC carbohydrate column. The major products in the first hour were the dimers and tetramers of *N*-acetylglucosamine, together with a small amount of

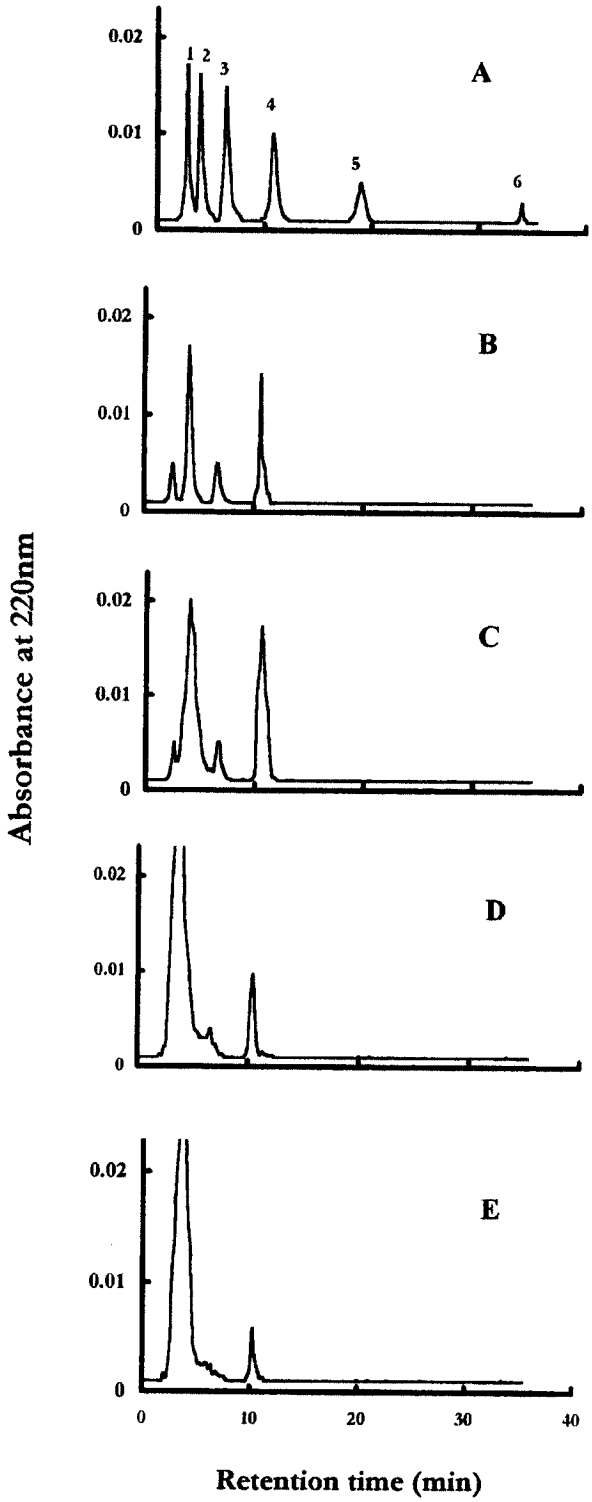

Fig. 5. Analysis of reaction products of the purified PL-ChtIII using swollen chitin as a substrate. Purified PL-ChtIII was incubated with swollen chitin for 30 min (B), 1 h (C), 2 h (D), and 4 h (E). After the reaction, 40 μl of reaction supernatant was collected at the indicated time-intervals and injected into a carbohydrate analysis HPLC column. Panel A indicates the mixture of chitooligosaccharide standards, *N*-acetylglucosamine (1), chitobiose (2), chitotriose (3), chitotetrose (4), chitopentose (5), and chitohexose (6).

monomers and trimers. As the reaction time increased, most oligomeric *N*-acetylglucosamine reaction products were dimeric, leading us to believe that dimeric *N*-acetylglucosamine is the final end-product of the PL-ChtIII enzyme action on swollen chitin (Fig. 5). In order to prove that the reaction products were generated by the PL-ChtIII, we analyzed the reaction product of swollen chitin incubated for 4 h without the addition of PL-ChtIII enzyme. Then, no signal was obtained from the supernatant of the reaction performed as a negative control experiment (data not shown).

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