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Chitosanolysis by a pectinase isozyme of *Aspergillus niger*—A non-specific activity

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Abstract

An isozyme of *Aspergillus niger* pectinase could depolymerize chitosan and its derivatives. The isozyme showed optimum depolymerization at pH 3.0 and 47 °C and exhibited classical Michaelis–Menten kinetics with a K_m and a V_{max} of 3.12 mg ml^{-1} and 153.85 nmol min^{-1} mg^{-1}, respectively, towards chitosan, which were higher than those for chitosan derivatives. The low molecular weight chitosans (LMWC) obtained were in the range of 6–20 kDa depending on the reaction conditions. Identification of glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) oligomers as depolymerized products indicated that the isozyme cleaved both GlcN-GlcN and GlcNAc-GlcN linkages. This study suggests the possible use of pectinase isozyme in place of chitosanase, which is expensive and unavailable in bulk quantity for the production of LMWC and chitooligomers.

Keywords: Chitosan; Pectinase; Low molecular weight chitosans; Chitooligomers

1. Introduction

Chitosanolytic enzymes are gaining increasing importance as low molecular weight chitosans (LMWC) and chitooligomers, the products of depolymerization, show innumerable applications in various fields such as medicine, agriculture and biotechnology (Muzzarelli, 1996, Muzzarelli, 1997; Skiak-Braek, Anthonsen, & Sandford, 1989). But the utility of chitosanase in such hydrolysis is limited because of its cost and unavailability in bulk quantity (Yalpani and Pantaleone, 1994). Pectinase (E.C.3.2.1.15) constitutes a complex enzymatic system responsible for the degradation of pectic substances. Recently, a non-specific hydrolytic action of pectinase (from fungi) on chitosan at acidic pH was reported (Sumise, 1990), although the purity of the enzyme preparation was in doubt (Pantaleone, Yalpani, & Scollar, 1992). Further, pectinase was subjected to modification, which also showed chitosan hydrolysing activity (Shin-Ya, Kajiuchi, Hinode, & Park, 1998; Shin-Ya, Lee, Hinode, & Kajiuchi, 2001). Such information is highly unusual and intriguing too.

Multiple substrate specificities of a number of glycanases and glycosidases are known in literature. β-1,4-Glucanases with hydrolyzing activity on mannan has been reported (Macarron, Acebal, Castillon, & Claeyssens, 1996). β-Glycosidases (designated as BgIA and BgIB) from *Bacillus* sp. *GLI* have been shown to cleave α- and β-linkages in *p*-nitrophenyl (*p*NP)-glycosides and positional isomers of β-1,4-glycopyranosyl linkages (Hashimoto et al., 1998). The product of BgIB gene was also identified as a gellan-degrading enzyme. Chitosanases with hydrolyzing activity on carboxymethylcellulose have been reported from *Myxobacter A-LI*, *Streptomyces griseus MUT 6037*, *Bacillus* sp. *7-M*, and *B. megaterium* (Hedges & Walfe, 1974; Ohtakara, 1988; Pelletier & Sygush, 1990). The β-1,3/1,4-glucanase from *B. circulans WL-12* was shown to possess chitosanase activity (Mitsutomi et al., 1998). Several explanations have been proposed for such enzyme actions on completely unrelated substrates. Gradually the concept of one enzyme-one activity is vanishing and several examples of multifunctional enzymes are being identified. The latter may result from gene sharing, gene fusion and

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exon shuffling. The present paper describes the non-specific hydrolysis of chitosan by a pectinase isozyme of *Aspergillus niger*, which is an alternative economical way of obtaining LMWC and chitooligomers from chitosan.

2. Materials and methods

Glycolchitosan, Celite, GlcN–HCl, GlcNAc, Sepharose CL-6B, pectinase were from Sigma Chemical Co., USA; pectin was from ICN Pharmaceutical Inc. (Cleveland), USA. Other reagents were of highest purity available.

2.1. Preparation of chitosan and its derivatives

Chitosan, prepared by heterogeneous N-deacetylation of shrimp chitin (Kittur, Kumar, & Tharanathan, 1998) was purified by dissolving in aqueous acetic acid (1%) to remove insolubles and precipitated with 2N NaOH. Carboxymethyl (CM) chitin was prepared as reported earlier (Hayes, 1986). *O*-Carboxymethylchitosan was prepared by N-deacetylating CM-chitin with NaOH (10%) containing $NaBH_4$ (0.1%) for 10 h at 80 °C (Hirano, Hayashi, & Hirochi, 1992). The average molecular weight of chitosan was calculated by (a) Viscometry (Wang, Shuqin, Shuqing, & Wen, 1991), (b) HPSEC (μ-Bondagel column), and (c) GPC (Sepharose CL-6B) (Tsaih & Chen, 1999) and the degree of acetylation determined by CP-MAS ^{13}C NMR (Ottoy, Varum, & Smidsord, 1996). The degree of substitution of *O*-carboxymethylchitin and *O*-carboxymethylchitosan were determined by IR spectroscopy (Varum, Egelandsdal, & Ellekjaer, 1995).

For viscometry, chitosan solutions of different concentrations were prepared in 0.2 M acetic acid − 0.1 M sodium acetate buffer and the intrinsic viscosity (η) was determined (Ostwald viscometer). Using Mark–Houwink equation, $(\eta) = K_m M^a$, where $K_m = 3.5 \times 10^{-4}$, molecular weight (M^a) was determined. HPSEC was done using Shimadzu HIC-6A system with E-linear and E-1000 column (30 cm × 3.9 mm i.d.) connected to RI detector (flow rate, 1 ml/min). Using a plot of $\log M^a$ of dextran standards versus retention time, M^a of chitosan was determined. GPC was done using 0.15 M acetic acid − 0.35 M sodium acetate as the eluent. The fractions were hydrolyzed using HCl and then the reducing sugar was analyzed (Imoto & Yagishita, 1971).

2.2. Polyacrylamide gel electrophoresis (PAGE, 7.5% *T*, where *T*-total percentage of acrylamide monomer)

(a) **Native and SDS-PAGE** were performed at pH 8.8 as described earlier (Laemmli, 1970). After electrophoresis, the protein bands were revealed by silver staining (Blum, Beir, & Gross, 1987).

(b) **Glycolchitosan** (0.05%, w/v) **incorporated PAGE** was performed at pH 8.8. The gel was stained for chitosanase activity by incubating at 37 °C for 1 h (crude enzyme) and 2 h (purified isozyme) in 0.1 M citrate buffer, pH 3.0, washed with distilled water and stained with Congo red (0.1%). The contrast was enhanced for the development of dark blue colour with the addition of 5% acetic acid where chitosanase activity band was observed as a clear area against a dark blue background.

(c) **Pectin** (0.05%, w/v) **incorporated PAGE** was performed as described above. After the run, the gel was incubated in 0.1 M citrate buffer, pH 3.0 at 37 °C for 1 h followed by staining with ruthenium red (0.05%, w/v). Pectinase activity appears as a clear zone against a pink background (Cruickshank & Wade, 1980).

2.3. Isolation of *A. niger* isozyme by electro-elution

A modified electro-elution setup was used for purification of the isozyme that showed both pectinase and chitosanase activities (Kittur et al., unpublished data). Following electro-elution, the contents were transferred to a centrifuge tube and spun at 2000 rpm for 10 min. The clear supernatant was then concentrated and used as the source of enzyme for subsequent analysis.

2.4. Reverse phase high performance liquid chromatography (RP-HPLC)

RP-HPLC of the commercial purified pectinase and electro-eluted isozyme was carried out using a Shimpak C18 reverse phase column (25 cm × 4.6 mm i.d.) (Shimadzu, Japan) at a flow rate of 1.0 ml min^{-1} using a linear gradient of (a) 0.1% trifluoroacetic acid (TFA) and (b) acetonitrile: water (70:30) and detected at 280 nm.

2.5. Enzyme assay

(a) **Pectinase assay**. This was done by the dinitrosalicylate (DNS) method (Miller, 1989) using sodium pectate (0.25% solution of pectic acid in 0.1 M sodium citrate buffer, pH 3.0) as substrate. The reaction mixture, consisting of 0.9 ml substrate and 0.1 ml (60 μg) enzyme solution was incubated at 25 °C for 30 min. Reaction was stopped by adding 1.0 ml DNS reagent and kept in a boiling water bath for 15 min. After cooling, double distilled water (3.0 ml) was added and the absorbance measured at 530 nm. One unit of pectinase activity is defined as the amount of enzyme required to liberate 1 μmole of reducing sugar per min at 25 °C.

(b) **Chitosanase assay**. To chitosan (1 ml, dissolved in 1% acetic acid and pH adjusted to 3.0), pectinase enzyme (0.1 ml, 60 μg) was added, incubated at 25 °C for 1 h, followed by addition of equal volume of 2N NaOH, as chitosan becomes insoluble above pH 6.5. The precipitate formed was removed by centrifugation

and the reducing sugar released into the supernatant was analyzed (Imoto & Yagishita, 1971). One unit of chitosanase activity is defined as the amount of enzyme required to liberate 1 μmole of reducing sugar per min at 25 °C.

2.6. Kinetic study

Chitosan solutions (1%) at various pH (2.0–6.0) were prepared by adjusting with either 0.1N HCl or 0.1N NaOH and used to study the effect of pH on isozyme activity. The temperature optimum and thermal stability of the isozyme were investigated over the temperature range 0–80 °C. The reaction mixture (Chitosan solution + isozyme) was pre-incubated at the desired temperature before adding the enzyme and the activity was monitored as described earlier. The thermal stability of the isozyme was measured by pre-incubating the enzyme in citrate buffer (0.1 M, pH 3.0) at the indicated temperature for 2 h before adding to the reaction mixture and the residual activity was measured. Kinetic studies were done taking chitosan and its derivatives as substrates under optimum conditions. Up to 20 mg ml^{-1} of chitosan solution was taken for the study of substrate concentration, as chitosan above this concentration is highly viscous. The apparent K_m and V_{max} values were calculated from double reciprocal plot.

2.7. Isolation and characterization of pectinase-depolymerized products

The degradation products of chitosan were isolated by alkali precipitation of high molecular weight products. The soluble fraction was loaded on a column of charcoal-Celite (3.5 × 16 cm). Salts and unadsorbed saccharides (GlcN) were removed by eluting with distilled water and the adsorbed oligomers (containing GlcNAc) mixture was eluted with 60% ethanol. The former fraction was re-N-acetylated with acetic anhydride and subjected to a second charcoal-Celite chromatography. The combined eluates were evaporated under reduced pressure and subjected to HPLC on Lichrosorb-NH_2 column (40 × 250 mm, E. Merck, Germany) using Shimadzu LC-3A system. The elution was performed with 70:30 acetonitrile: water at 27 °C at a flow rate of 0.8 ml min^{-1} and monitored using a RI detector.

MALDI-TOF-MS analysis of oligomers was carried out using Kompact analytical SEQ matrix assisted laser desorption ionization time-of-flight mass spectrometry (Kratos, UK) at an acceleration voltage of 30 kV using 2,5-dihydroxybenzoic acid as the matrix.

3. Results and discussion

The average molecular weight of chitosan, calculated by (a) Viscometry, (b) HPSEC, and (c) GPC was 98 ± 4 kDa and the degree of acetylation was 0.16. The degree of substitution of *O*-carboxymethylchitin and *O*-carboxymethylchitosan was 0.7.

Preliminary investigations indicated that a commercially available purified pectinase from *A. niger* could bring about the degradation of chitosan polymer, as revealed by a 61% reduction in the viscosity at pH 3.0. Chitosanolytic activity at pH 3.6 with a 99% reduction in viscosity by a pectinase G from *A. niger* has been reported (Pantaleone et al., 1992), wherein the presence of minor enzyme contaminants is not discounted (Pantaleone et al., 1992). To elaborate and identify the nature of enzyme responsible for the chitosanolysis, the commercial purified pectinase from *A. niger* was subjected to further purification by PAGE, followed by kinetic study for both pectinase and chitosanase.

Native PAGE and RP-HPLC of the commercial purified pectinase showed the presence of three proteins, indicating molecular heterogeneity (Fig. 1, lane 1 and Fig. 2). Specific staining for pectinase activity revealed the presence of three isozymes corresponding to the three protein bands visualized by protein staining (Fig. 1, lane 2). Zymogram analysis indicated the association of chitosanase activity with the pectinase isozyme of R_f 0.92 (Fig. 1, lane 3). The bifunctional isozyme was further electro-eluted and subjected to SDS-PAGE and RP-HPLC (Fig. 1, lane 2, Fig. 4) where it showed the presence of a single protein.

The specific activity of the commercial purified pectinase towards depolymerization of pectin was 9.85 units mg^{-1} whereas that of the electro-eluted isozyme, which also showed chitosanase activity, was 3.31 units mg^{-1}. The specific activity of the isozyme towards chitosanolysis was 0.65 units mg^{-1}. Optimum chitosanase activity for the electro-eluted isozyme was observed at pH 3.0 and at 47 °C. Pre-incubation of enzyme in the temperature range 0–80 °C for 2 h prior to enzyme assay showed that the enzyme was relatively more stable below 40 °C, lost 50% activity at 50 °C and was completely inactivated above 80 °C.

The effect of varying substrate concentration on chitosanolysis by pectinase isozyme displayed Michaelis–Menten

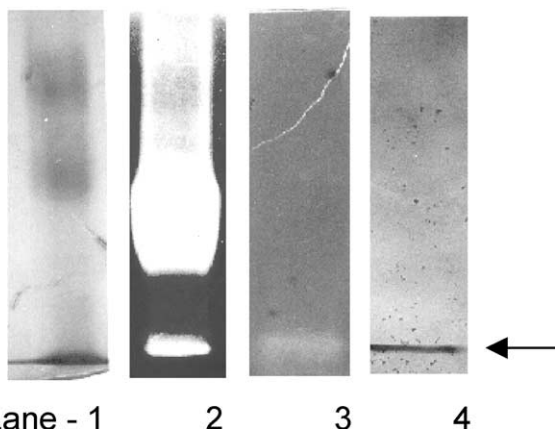

Fig. 1. Zymogram profile of *A. niger* pectinase: native PAGE (lane 1), pectinase activity (lane 2), chitosanolytic activity (lane 3) of commercial purified enzyme and SDS-PAGE of the electro-eluted isozyme (lane 4).

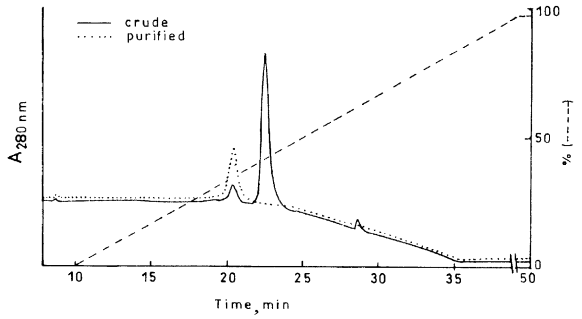

Fig. 2. Reverse phase high performance liquid chromatography (RP-HPLC) of *A. niger* pectinase: (a) crude (—) and (b) electro-eluted (···) enzyme.

kinetics (Fig. 3). Linear regression analysis of the double reciprocal plot showed a K_m of 3.12 mg ml^{-1} and V_{max} of 153.85 nmol min^{-1} mg^{-1}. The K_m for the carboxymethyl derivatives of chitin and chitosan was one fold lower than that for chitin (Table 1), suggesting that the derivatives bind to the enzyme with a higher affinity. On the basis of these data, the relative order of substrate-acceptor efficiency was *O*-carboxymethylchitin > *O*-carboxymethylchitosan > chitosan.

A lowering of chitosanolysis was observed at chitosan concentrations greater than 12 mg ml^{-1} suggesting

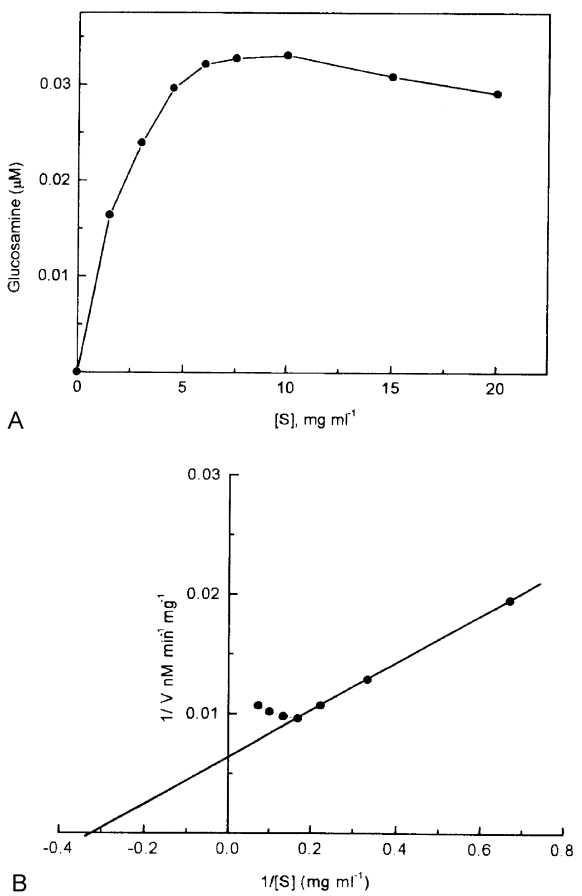

Fig. 3. (A) Effect of substrate (chitosan) concentration on pectinase activity, (B) Lineweaver–Burk plot for pectinase isozyme using chitosan as the substrate.

Table 1
Kinetic constants for aminopolysaccharide substrates

Substrate	V_{max} (nmol min^{-1} mg^{-1})	K_m (mg ml^{-1})
Chitosan	153.85	3.12
O-Carboxymethylchitosan	83.3	2.0
O-Carboxymethylchitin	76.9	2.0

the possibility of product or substrate inhibition (Fig. 3). The former was ruled out by adding chitooligomers (0.1 mM), the products of chitosan depolymerization, to the reaction mixture (containing 2.5–7.5 mg ml^{-1} chitosan), where no detectable effect was found, suggesting the susceptibility of the enzyme to substrate inhibition. Substrate inhibition has been described for a variety of enzymes and several mechanisms have been proposed to explain this effect (Cleland, 1979). This phenomenon can be rationalized by assuming that a second substrate molecule binds non-productively to the active site thereby inhibiting the productively bound one (Sanchez-Ferres, Franscisco, & Francisco, 1993).

The depolymerization of chitosan by pectinase isozyme was terminated by the addition of 2N NaOH. The precipitate obtained after centrifugation of the reaction mixture was LMWC and the supernatant contained chitooligosaccharides. The M^a of the LMWC varied between 6 and 20 kDa

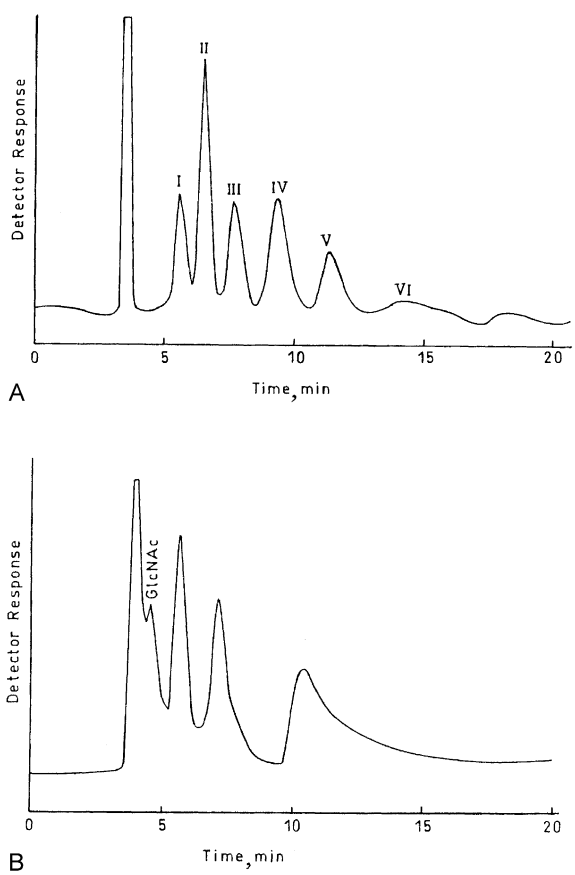

Fig. 4. HPLC of chitooligomers from hydrolysis of chitosan by *A. niger* pectinase (A) F1 and (B) F2 fraction.

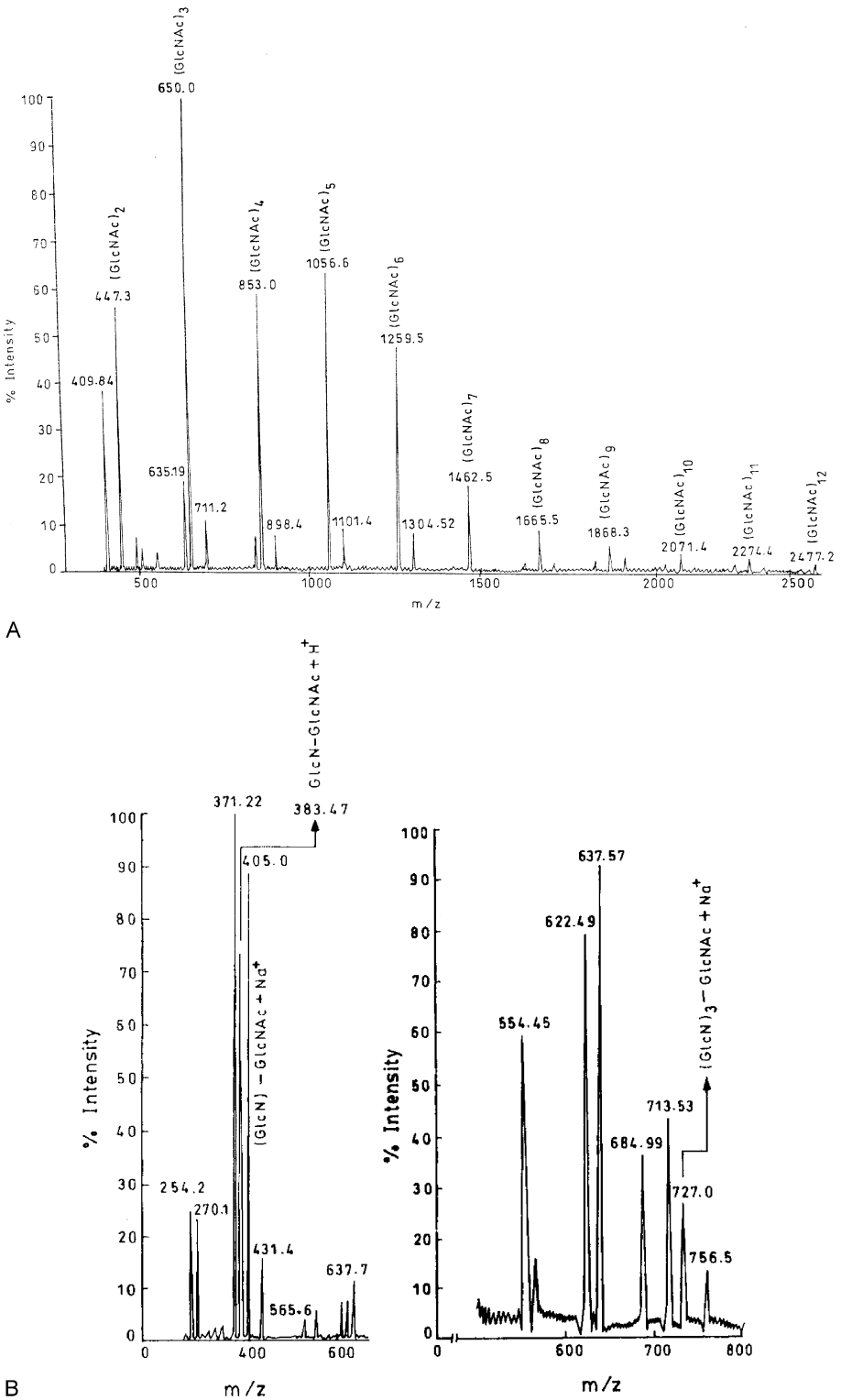

Fig. 5. MALDI-TOF-MS of chitooligomers from hydrolysis of chitosan by *A. niger* pectinase: (A) F1 and (B) F2 (I/II) fraction.

(by viscometry) depending on reaction time. Charcoal-Celite chromatography of the alkaline supernatant gave a GlcN-rich oligomeric fraction in the water eluent (F1), leaving behind the adsorbed GlcNAc oligomers (F2) in the column, which were subsequently eluted with 60% aqueous ethanol. Because of high salt content, the former was difficult to purify, whereas upon re-N-acetylation it could be easily refractionated. Both F1 and F2 fractions on HPLC (Fig. 4A and B) showed a mixture of several oligosaccharides. The MALDI-TOF-MS of F1 showed the presence of di- to hexamer in abundance (Fig. 5A), indicating that the pectinase isozyme can split the β-1,4-glycosidic linkages of

GlcN-GlcN in chitosan. However, detailed information on the specificity of chitosanolytic activity of pectinase could be obtained from the structures of minor products (F2) adsorbed on charcoal. MALDI-TOF-MS analysis of F2 afforded a number of $M + H^+$ and $M + Na^+$ molecular ions which could be categorized into two groups based on their abundance (i) *m/z* 350–410 (Fig. 5B, I); and (ii) *m/z* 500–800 (Fig. 5B, II). Abundant molecular ions at *m/z* 383 ($M + H^+$) and 405 ($M + Na^+$) were assigned to the protonated and sodium forms of GlcN-GlcNAc, whereas *m/z* 566 and 727 were assigned to sodium forms of $(GlcN)_2$-GlcNAc and $(GlcN)_3$-GlcNAc, respectively. These results are consistent with F2 being a mixture of heterooligomers, each of which carries a GlcNAc residue at the reducing end, suggesting that the pectinase isozyme can also cleave GlcNAc-GlcN linkages.

Chitinase and chitosanase are presently unavailable in bulk quantities for commercial exploitation. Therefore, the present result on the chitosanolytic activity of an inexpensive pectinase from *A. niger*, which is available in bulk opens up prospects for its commercial use in the modification of chitosan.

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