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Characteristics of Chitosanases from *Aspergillus fumigatus* KB-1

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Two chitosanases produced by *Aspergillus fumigatus* KB-1 were purified by ion exchange and size exclusion chromatographies. Molecular weights of chitosanases were 111.23 kDa (chitosanase I) and 23.38 kDa (chitosanase II). The *N*-terminal amino acid sequence of chitosanase II was determined as follows: YNLPNNLKQIYDKHKGKXSXVLAKGFTN. The optimum pH of the chitosanase I and II was 6.5 and 5.5, respectively. The optimum temperatures were 60°C for chitosanase Iand 70°C for chitosanase II. Hydrolysis products of two chitosanases were analyzed by HPLC and GPC. Chitosanase I hydrolyzed substrate to glucosamine. Chitosanase II produced chitooligosaccharides.

Key words: Chitosan, Chitosanase, *Aspergillus fumigatus* KB-1, *N*-terminal amino acid sequence

INTRODUCTION

Chitosan is a poly-β(1→4)-D-glucosamine, which is produced by deacetylation of chitin (Bartnicki-Garcia, 1968). Chitosan has many diverse applications such as antifungal (Hadwiger *et al.*, 1984), flocculating (Muzzarelli *et al.*, 1980) and hydrating agent (Hirano *et al.*, 1991). In addition, low molecular weight chitosan oligomers have been found to be useful for lowering blood cholesterol (Jennings *et al.*, 1988), healing wound (Muzzarelli *et al.*, 1980), stimulating immune system and treating tumor (Suzuki *et al.*, 1986).

Chitosan oligomers have been prepared by acidic hydrolysis in which concentrated hydrochloric acid is employed (Muzzarelli *et al.*, 1980). An alternative mild method is the use of chitosanolytic enzymes (Izumo *et al.*, 1992). The chitosanolytic activities have been found in several microorganisms such as *Bacillus* sp., *Streptomyces* sp., *Pseudomonas* sp. (Somashekar and Joseph, 1996), and *Penicillium* sp. (Cheng and Li, 2000). However, most chitosanases from the isolated microorganism hydrolyzed the chitosan to oligomers containing glucosamine monomer. For the production of higher molecular weight oligomers, novel endo-type chitosanase is required.

In this report, we described the characteristics of chitosanases from *Aspergillus fumigatus* KB-1.

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MATERIALS AND METHODS

Materials

Chitosan (75~85% deacetylated) was obtained from Showa Chemicals Inc. and chitooligosaccharide mixture was purchased from Seikagaku Co., Japan. All other chemicals were extra-pure or first grade. The freeze-dried culture broth of *Aspergillus fumigatus* KB-1 was kindly supplied by Korea Chitosan Co. DE52 (DEAE cellulose) was obtained from Whatman International Ltd. *S*-Sepharose fast flow was purchased from Sigma Chemical Co. Superdex 75 HR 10/30 column was from Amersham Bioscience Co. PVDF membrane (Hybond™-PVDF) was a product of Amersham Life Science.

Purification of chitosanase protein

Freeze-dried culture broth (2 g) was dissolved in 40 mL of 10 mM sodium acetate buffer, pH 5.0 (buffer A) and used as concentrated culture broth. Protein purification steps were carried out at 4°C. The concentrated culture broth was dialyzed with 10 kDa pore size dialysis tube against buffer A. Resulting solution was used as crude enzyme. Crude enzyme was loaded onto DE52 anionic exchange column (3 cm×15 cm) which was preequilibrated with buffer A. Elution was performed with a linear gradient of NaCl upto 1 M. Active fractions were pooled and loaded onto *S*-Sepharose fast flow column (3 cm×12 cm).

Eluting the column with a linear gradient of NaCl upto 1 M resulted in two active peaks A1 and A2. Among these two active peaks, peak A1 was loaded onto Superdex 75 HR 10/30 and eluted with buffer A at the flow rate of 1.0 mL/min in FPLC system (AKTA FPLC). To estimate degree of purification and protein molecular weight, 12% SDS-PAGE was carried out (Laemmli, 1970).

Protein concentration was determined by Bradford method (Bradford, 1976) by using Bio-Rad Protein Assay Dye reagent concentrate. Bovine serum albumin (Sigma Chemical Co.) was used as a protein standard.

Molecular mass determination of a chitosanase by MALDI-TOF-MS

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was carried out by using a HP G2025A system (Hewlett Packard, U.S.A.). Samples were prepared by a 1:1 dilution with the UV-absorbing matrix sinapic acid (50 mM) followed by rapid vaccum evaporation in the HP 2024A sample preparation accessory. 28 laser shots were summed for the spectrum (Lee *et al.*, 1999).

N-Terminal amino acid sequence determination

The purified chitosanase protein was electrophoresed and transfered to the PVDF membrane. After coomassie brilliant staining, the *N*-terminal amino acid sequence of the purified chitosanase was determined by using Procise Protein Sequencing System (Applied Biosystems).

Chitosanase activity assay

Chitosanase activity was assayed using chitosan (deacetylation degree 75~85%) dissolved in buffer A. 0.2 mL of 0.5% chitosan solution was mixed with 0.8 mL of chitosanase solution in buffer A and incubated at 30°C for 30 min in shaking water bath. The pH of reaction mixture brought to alkaline by addition of 1 M NaOH and phenolphthalein indicator. The amount of D-glucosamine released was determined by using the method of Rondle-Morgan and Morgan (Rondle, 1955). One unit of enzyme activity was defined as the chitosanolytic activity that released 1 μmol of D-glucosamine per min at 30°C.

Effects of temperature and pH on the chitosanase activity

Chitosanase activities were assayed at various temperatures (30~90°C) at pH 5.0 under chitosanase activity assay condition. The activities were also measured at different pHs (pH 3.5~8.5) using 10 mM citric acid-NaOH buffer (pH 3.5~4.5), 10mM sodium acetate buffer (pH 5.5), 10 mM sodium phosphate buffer (pH 6.5) and 10 mM Tris-HCl buffer (pH 7.5~8.5) at 30°C.

Effects of temperature and pH on the chitosanase stability

Chitosanases were preincubated for 1 h at various temperature (0~70°C) or pHs (pH 3.0~11.0) without additon of substrate. The remaining activity was determined by using chitosanase activity assay.

Effect of deacetylation degree of chitosan on enzyme activity

The effect of deacetylation degree of chitosan on enzyme activity was tested by using 38.3%, 51.2%, 64.6%, 80%, 92% and 100% deacetylated chitosan. These various chitosans were kindly supplied by Dr D.W. Chun, Ewha Womans University.

Effects of metal ions and chemical agents

Chitosanases were preincubated with various metal ions or chemical reagents at 1 mM concentration for 1 h and assayed the remaining chitosanase activity (Kim *et al.*, 1998).

Hydrolysis products of purified chitosanases

For the identification of chitooligosaccharides produced by chitosanase I and II, HPLC (Agilent 1100 series) was carried out with a carbohydrate analysis column (3.9 mm× 300 mm, Waters Co., Milford, U.S.A). Elution was perfomed at 1.0 mL/min flow rate with water:acetonitrile (28:72, v/v) solvent system (Lee *et al.*, 1999). The elution pattern was monitored by measuring the absorbance at 210 nm. *N*-acetylation of chitooligosaccharides was achieved by the method of Amano *et al.* (Amano *et al.*, 1977). The molecular weights of the enzyme hydrolysis products were determined by gel permeation chromatography (Agilent 1100 series). 4.5 mg of chitosan (Mw = 79,390) was incubated with 6.28 mU of chitosanase at 30°C for 1, 2, 4, 8, 16 and 32 min. After incubation, the reaction mixtures were boiled for 10 min to terminate the enzyme reaction. The lyophilized reaction mixtures were dissolved in elution solution (0.3 mol acetic acid and 0.2 mol $NaNO_3$ in 1 L H_2O) and injected to TSK-gel G2000SW column (TosoHass, 7.5 mm×30 cm). PL polysaccharide molecular weight standards (Polymer Laboratories Co.) were used for molecular weight calibration. The flow rate was 0.7 mL/min (Yoon *et al.*, 1998).

RESULTS AND DISCUSSION

Chitosanase protein purification

The freeze-dried powder of culture broth was dissolved in buffer A. After dialysis against same buffer, the enzyme protein was separated in DE-52 column (Fig. 1). The active fractions of DE-52 were loaded on S-Sepharose column. Two active fractions were appeared as shown in

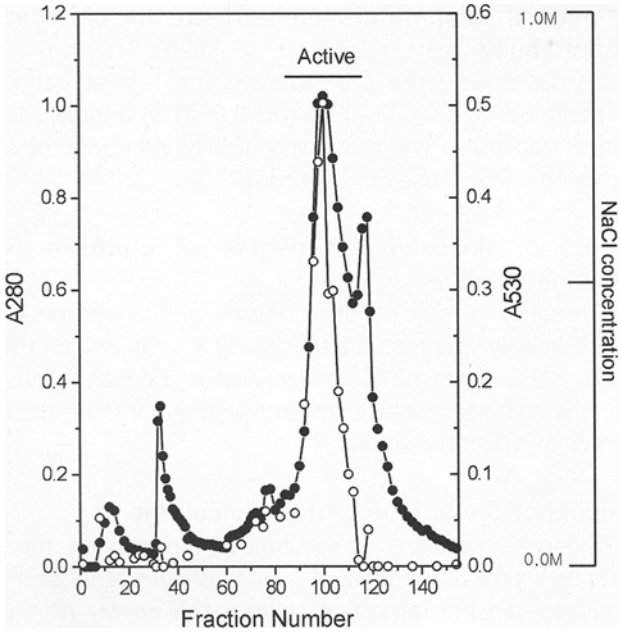

Fig. 1. DE-52 column chromatography of concentrated culture broth from *A. fumigatus* KB-1. Fractions 91~111 were collected for S-Sepharose column purification step. –●– Protein elution profile, –○– Activity elution profile.

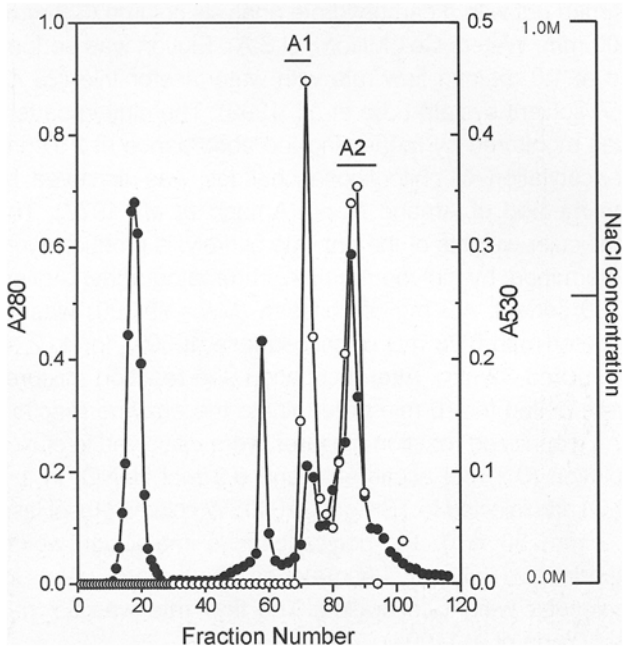

Fig. 2. S-Sepharose column chromatography of the active peak of DE-52 column. A1 (fractions 70~74) was collected for Superdex75 column chromatography. A2 (fractions 82~91) was collected as chitosanase II. –●– protein elution profile, –○– activity elution profile.

Fig. 2. A1 fraction was further separated by Superdex 75 HR 10/30, and the purified chitosanase was named as chitosanase I (Fig. 3). A2 fraction from S-Sepharose seemed to be pure, so we named it as chitosanase II (Fig.

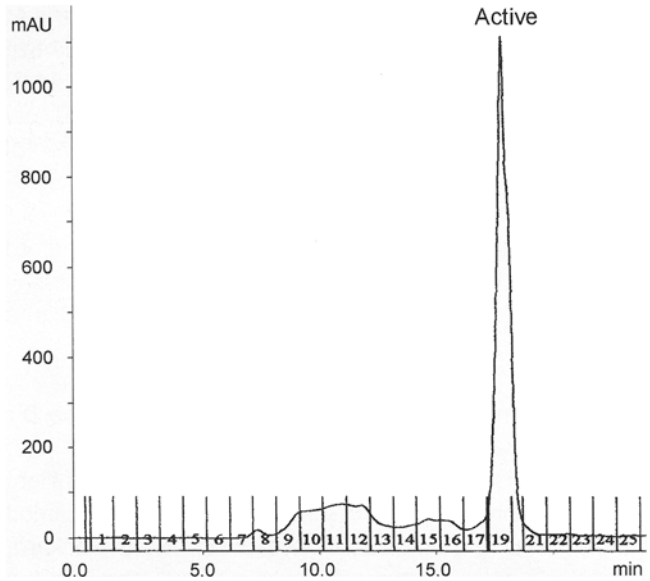

Fig. 3. Superdex 75 HR 10/30 fast protein liquid chromatography of A1 from S-Sepharose column chromatography. Fractions 18~20 were collected as chitosanase I.

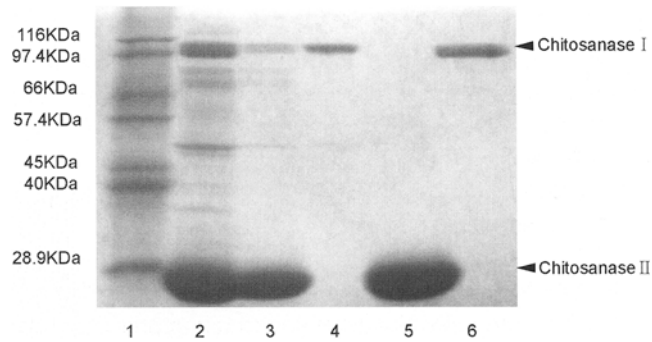

Fig. 4. 12% SDS-PAGE analysis of chitosanases during protein purification steps. Lane 1: size marker, Lane 2: crude enzyme, Lane 3: DEAE cellulose active fraction pool, Lane 4: S-Sepharose active fraction pool A1, Lane 5: S-Sepharose active fraction pool A2 (used as chitosanase II), Lane 6: Superdex 75 HR 10/30 active fractions (used as chitosanase I).

4). Molecular weight of chitosanase I and chitosanase II was 111.23 kDa and 23.38 kDa, respectively, based on SDS-PAGE and MALDI-TOF-MS analysis. Molecular weights of chitosanase I and II were similar to those of chitosanases (108 and 25.5 kDa) from *Aspergillus fumigatus* KH-94 (Kim *et al.*, 1998). 22 kDa Chitosanase from *A. fumigatus* was reported previously(Cheng and Li, 2000). It seems that *A. fumigatus* produce closely related chitosanase regardless of the strains.

N-Terminal amino aicd sequence analysis

N-terminal amino acid sequence of chitosanase II was YNLPNNLKQIYDKHKGKXSXVLAKGFTN (X is not determined). The determined N-terminal sequence of *Aspergillus*

chitosanase II	1																										28
from *A. fumigatus*	Y	N	L	P	N	N	L	K	Q	I	Y	D	K	H	K	G	K	X	S	X	V	L	A	K	G	F	T	N
endo-chitosanase precursor	18																										45
from *A. fumigatus*	Y	N	L	P	N	N	L	K	Q	I	Y	D	K	H	K	G	K	C	S	K	V	L	A	K	G	F	T	N
chitosanase B	18																										45
from *A. oryzae*	Y	D	L	P	D	N	L	K	Q	I	Y	E	K	H	K	G	K	C	S	K	V	Y	Q	K	G	F	T	N
chitosanase	18																										46
from *A. oryzae*	Y	D	L	P	E	N	L	K	Q	I	Y	E	K	H	K	S	G	K	C	S	K	E	L	Q	G	G	Y	D	N

Fig. 5. Comparison of the amino acid sequence of *A. fumigatus* KB-1 chitosanase II with other fungal chitosanases.

fumigatus KB-1 chitosanase II was similar to the endo-chitosanase precursor of *Aspergillus fumigatus* (92% identical) (Cheng, and Li, 2000), chitosanase B of *Aspergillus oryzae* (75% identical) (Shimosaka, 2002) and chitosanase of *Aspergillus oryzae* (62% identical) (Zhang *et al.*, 2001). The sequence homology was shown in Fig. 5.

Factors affecting on chitosanase activities

The optimum pHs of the chitosanase I and II were 6.5 and 5.5. The optimum temperatures were 60°C and 70°C. Chitosanase I stability to pH was declined as the pH value increased. But chitosanase II was stable in the pH range of 3.5~8.5. Chitosanase I was most stable at 10°C. The stability was dropped at higher temperature and most activities were lost above 60°C. In contrast to chitosanase I, chitosanase II was stable in the range of temperature 0 to 60 °C.

At 1 mM concentration, Bi^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}. Mo^{6+} and Pb^{2+} inhibited the chitosanase I by 48%, 59%, 50%, 49%, 81%, 42%, 94%, and 69%, respectively. At the same concentration, Cu^{2+}, Hg^{2+} and Mo^{6+} also inhibited the chitosanase II by 48%, 80% and 88%.

Deacetylation degree of chitosan affected hydrolysis activities of chitosanase I and II. As shown in Fig. 6, the higher the degree of deacetylation, the higher were the activities of chitosanase I and II. This is common property in chitosanase rather than chitinase (Somashekar and Joseph, 1996). These results indicate that the purified proteins were chitosanolytic enzymes.

Analysis of enzyme hydrolysis products

The hydrolysis products of chitosanase I and II were analyzed by high performance chromatography. The results are shown in Fig. 7. Chitosan hydrolysate by chitosanase I was composed of D-glucosamine mainly. However chitosanase II produced chitobiose, chitotriose, chitotetraose and higher degree of oligomers. Chitosanase II is different from the endo-chitosanase precursor that produced trimer, tetramer, pentamer as major products (Cheng and Li, 2000). These results indicate that

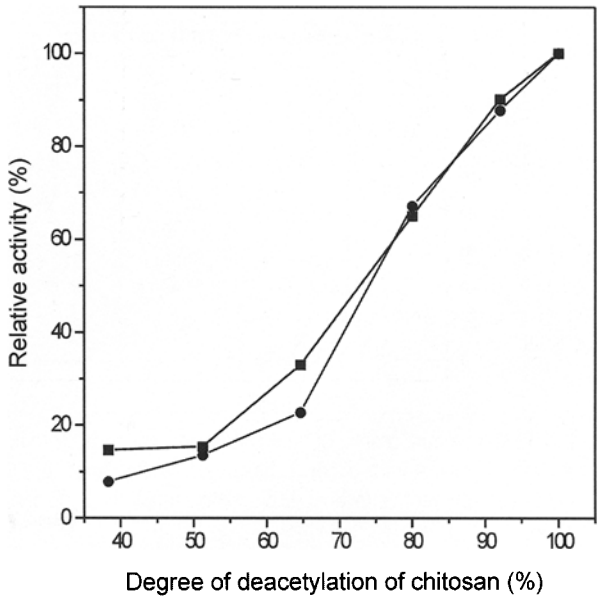

Fig. 6. Substrate specificity of chitosanases from *A. fumigatus* KB-1 –■– chitosanase I, –●– chitosanase II.

chitosanase I is exo-type enzyme for producing glucosamine and chitosanase II is endo-type to produce oligosaccharides.

There were several reports about chitosanases from *Aspergillus* spp., but gel permeation chromatography (GPC) analysis results were not reported. In this studies the higher molecular weight distribution of reaction products was analyzed by GPC. The average molecular weight of chitosan substrate was 79,390. The mixture of chitosanase I and II resulted in lower average molecular weight hydrolysis products than the employment of only one of chitosanase I or chitosanase II (Fig. 8). These suggest that two enzymes cooperate to hydrolyze chitosan substrate efficiently for carbon and energy source production in *Aspergillus fumigatus* KB-1. Based on HPLC and GPC analysis results, we might choose chitosanase I for glucosamine production and chitosanase II for chitooligomers.

In conclusion, the use of purified chitosanase or selec-

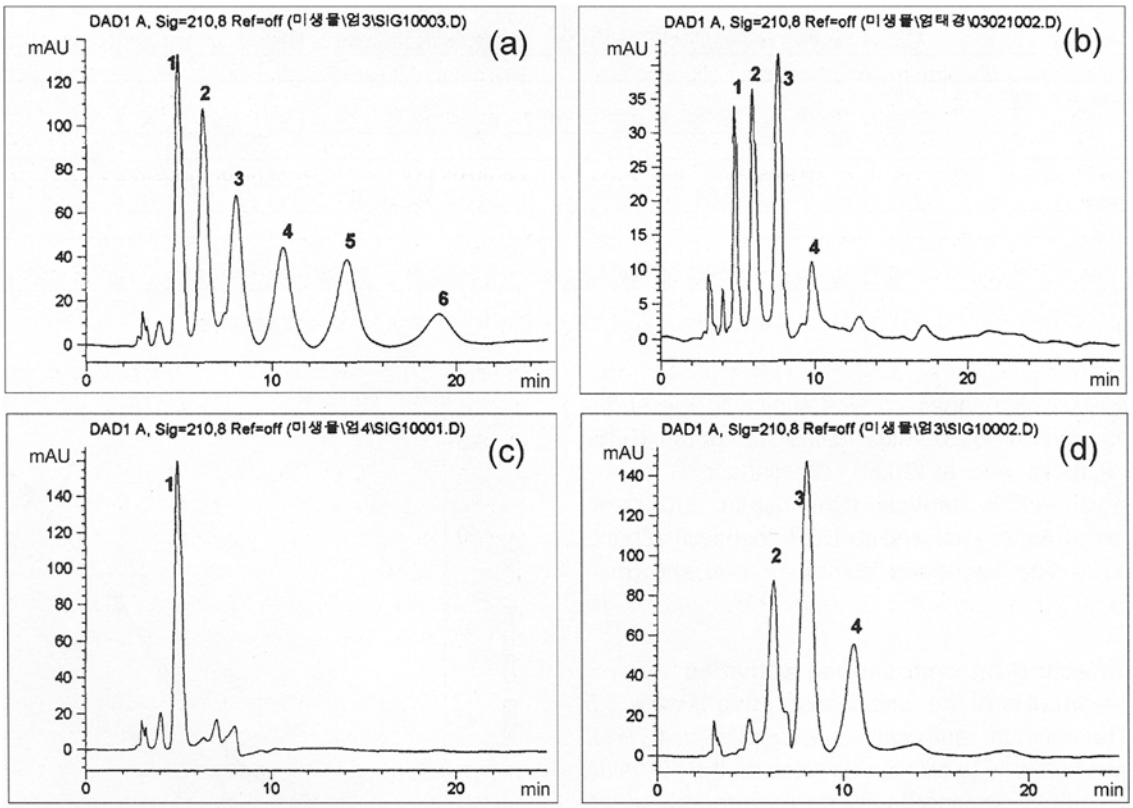

Fig. 7. HPLC chromatograms of the chitooligosaccharides produced by enzyme reaction. (a) chitooligosaccharide mixture(glucosamine and glucosamine oligomers from dimer to hexamer) as standard. (b) DEAE cellulose column active fraction. (c) chitosanaseI. (d) ChitosanaseII. The peak numbers represent as follows. **1**: glucosamine(GlcN), **2**: $(GlcN)_2$, **3**: $(GlcN)_3$, **4**:$(GlcN)_4$, **5**: $(GlcN)_5$, **6**: $(GlcN)_6$.

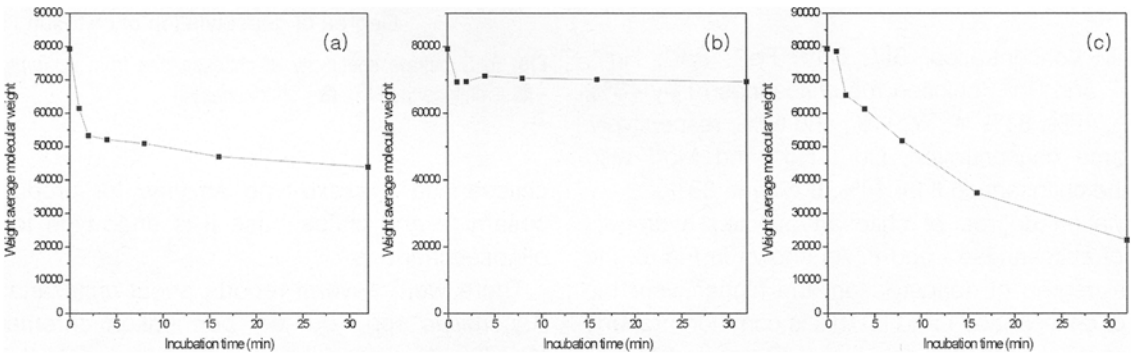

Fig. 8. GPC results of enzyme hydrolysis products of *A. fumigatus* KB-1 chitosanases. (a) chitosanase I and II, (b) chitosanase I, (c) chitosanase II.

tive control of chitosanase I and II activities are required to produce specific hydolyzed prodcuts of chitosan. In addition to HPLC, GPC analysis might be required to characterize the molecular distribution of chitosanase reaction products.

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