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Distribution of chitinases and characterization of two chitinolytic enzymes from one-year-old Korean Ginseng (*Panax ginseng* C.A. Meyer) roots

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We report the tissue-specific distribution of chitinolytic activity in Korean ginseng root and characterize two 31-kDa chitinolytic enzymes. These two enzymes (SBF1 and SBF2) were purified 70- and 81-fold with yields of 0.75 and 1.25%, respectively, and exhibited optimal pH and temperature ranges of 5.0-5.5 and 40-50°C. With [^{3}H]-chitin as a substrate, K_m and V_{max} values of SBF1 were 4.6 mM and 220 mmol/mg-protein/h, respectively, while those of SBF2 were 7.14 mM and 287 mmol/mg-protein/h. The purified enzymes showed markedly less activity with *p*-nitrophenyl-*N*-acetylglucosaminide and fluorescent 4-methylumbelliferyl glycosides of D-*N*-acetylglucosamine oligomers than with [^{3}H]-chitin. End-product inhibition of both enzymes demonstrated that both are endochitinases with different *N*-acetylglucosaminidase activity. Furthermore, the NH_2-terminal sequence of SBF1 showed a high degree of homology with other plant chitinases whereas the NH_2-terminal amino acid of SBF2 was blocked. [BMB reports 2010; 43(11): 726-731]

INTRODUCTION

Panax ginseng is renowned for its beneficial effects. Ginsenosides isolated from *P. ginseng* reportedly serve as functional ligands of glucocorticoid receptors (1), regulate the induction of tyrosine amino transferase gene transcription (2), and potentiate NO-mediated neurogenic vasodilatation (3). In addition, oligopeptides of *Panax* bind metals (4) and have a somnogenic effect (5). *P. ginseng* induces cell-proliferation (6), stimulates immune response (7) and DNA and protein synthesis (8), and inhibits human platelet activity (9). It may also be useful for its hypoglycemic (10) and anti-lipolytic (11) effects.

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Although the potential roles of *P. ginseng* in human health have been well studied, less is known about the chitinases found in its roots (12, 13). Chitinases are important components of pathogenesis-related (PR) proteins that are induced by pathogenic attack. Investigation of these enzymes could result in the development of effective methods for preventing fungal invasion. In particular, root rot diseases caused by *Fusaria*, *Botrytus*, and *Cylindrocarpon* (14) result in devastating losses of cultivated ginseng. The potential for controlling these diseases rests in part on a better understanding of natural protection phenomena such as the role of chitinases. This paper reports the distribution of chitinolytic activity in rhizomes and main and lateral roots of 1-year-old ginseng seedlings, and describes the biochemical properties of two 31-kDa chitinolytic enzymes.

RESULTS AND DISCUSSION

Chitinolytic activity within ginseng roots

The ginseng root is composed of the rhizome, main roots, and lateral roots, and each of which was examined separately. As shown in Supplemental Table 1, the lateral roots had the highest chitinase activity (139 mmol/mg protein/h), followed by main roots (103 mmol/mg protein/h) and rhizomes (88 mmol/mg protein/h). Activity band staining after SDS-PAGE of crude extracts revealed three types with molecular weights of 43, 31, and 29 kDa and varying degrees of activity (Supplemental Fig. 1). In the main roots, the 31-kDa type was the primary chitinase with the other two being minor constituents. However, both the 31-kDa and 29-kDa types were major constituents of the lateral roots and rhizome.

Purification of two 31-kDa chitinolytic enzymes

As shown in Fig. 1A, a stepwise gradient of pH was used to isolate two major active fractions (QF1 and QF2) accounting for 52% and 25% of the total activity, respectively. As shown in Fig. 1B, one unbound fraction (SF1) containing 21% of the total activity and three bound fractions (SBF1, 2, and 3) comprising 30, 21, and 9% of the total activity, respectively, were obtained. SF1 and SBF3 exhibited single activity bands at 43

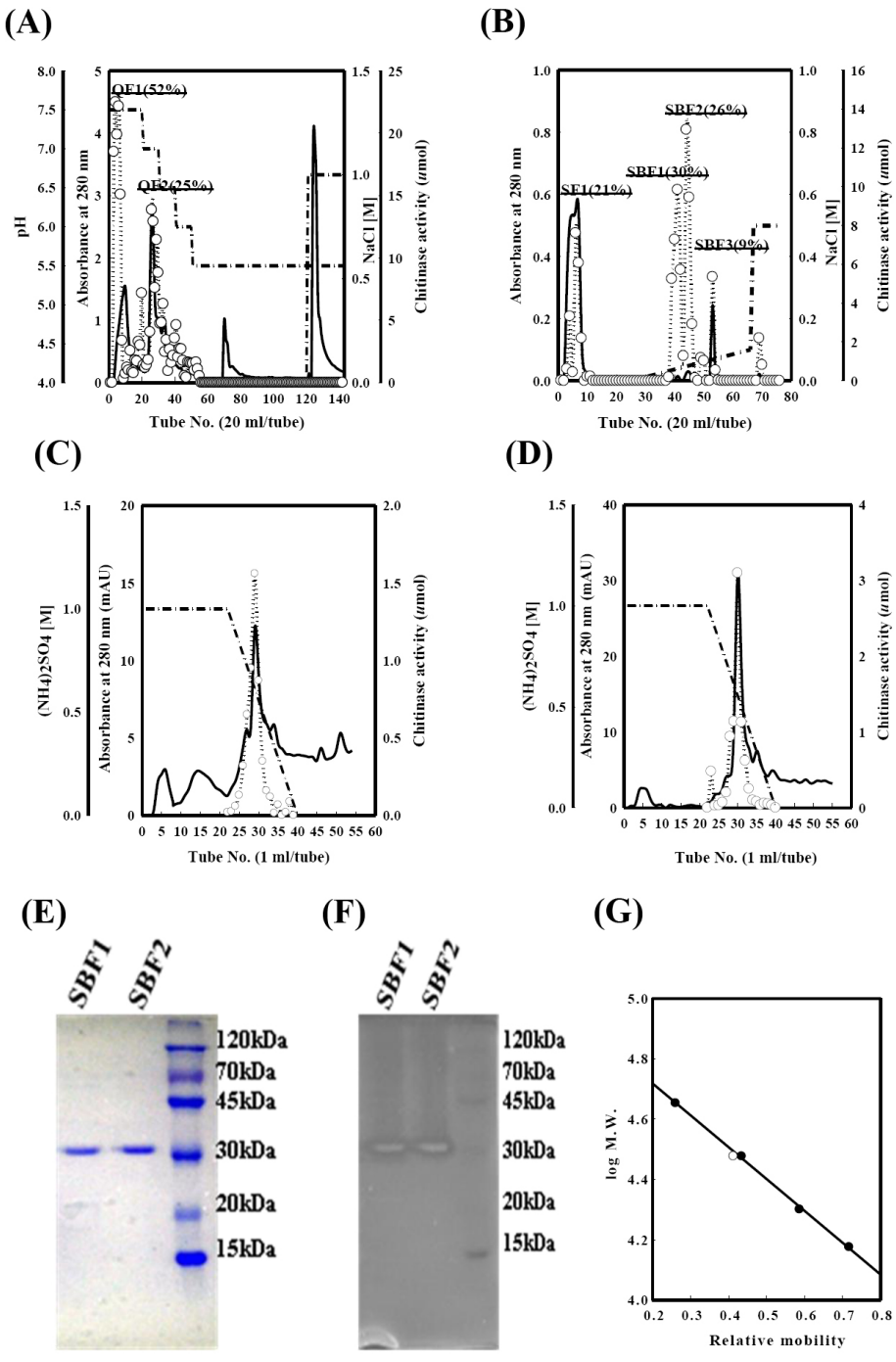

Fig. 1. Chromatographic purification of the two 31-kDa chitinolytic enzymes; the number in parentheses is the percentage of the total activity exhibited by each enzyme: (A) Anion exchange chromatogram (Source-Q, 2.6 x 15 cm), (B) Cation exchange chromatogram of QF1 (Source-S, 2.6 × 15 cm), (C) Hydrophobic interaction chromatogram of SBF1 (Phenyl Sepharose HP, 1 ml), (D) Hydrophobic interaction chromatogram of SBF2 (Phenyl Sepharose HP, 1 ml), (E) SDS-PAGE pattern of purified SBF-1 and SBF-2, (F) Chitinolytic activity staining of purified SBF-1 and SBF-2, (G) MW determination of each enzyme. (———) absorbance at 280 nm, (—○—○—) chitinase activity, (—•—•—) pH/salt gradient.

kDa and 29 kDa, respectively (data not shown). In contrast, SBF1 and SBF2 shared the same activity band at 31 kDa. Further purification of SBF1 and SBF2 on an HIC column resulted in a single major active fraction for each (Figs. 1C, D). The two purified enzymes (SBF1 and SBF2) showed a single band by SDS-PAGE corresponding to a molecular weight of 31 kDa (Fig. 1E), which correlated positively with the aforementioned activity band shown in Supplemental Fig. 1. The HIC fraction of SBF1 was purified 70-fold with a yield of 0.75% and that of SBF2 was purified 81-fold with a yield of

1.25%. These results are summarized in Table 1.

Enzymatic properties of purified SBF1 and SBF2

As shown in Fig. 2A, SBF1 and SBF2 exhibited linear activities for 30 and 60 min, respectively, before reaching a plateau. The optimal pH values for SBF1 and SBF2 were 5.5 and 5.0, respectively (Fig. 2B). SBF2 exhibited a second optimum at pH 3 with 78% of the activity observed at pH 5.0. Similarly, SBF2 had two temperature optima, with activity peaking at 40°C and 60°C (Fig. 2C). The activity of SBF1, however, peaked at 50°C. K_m and V_{max} for SBF1 were 4.6 mM and 220 mmol/mg- protein/h, respectively, and for SBF2 were 7.14 mM and 287 mmol/mg-protein/h (Fig. 2D).

Table 1. Purification of SBF1 and SBF2 from main roots of one-year-old Korean ginseng roots. [^{3}H]-chitin was used as substrate. Four hundred and eighty eight dpm correspond to 5 nmol of chitin

Treatment	Protein (mg)	Activity (μmol/h)	Specific activity (μmol/mg/h)	Recovery (%)	Purification (fold)
Crude	1,290	95.7	0.074	100	1
Source-Q	63	65.72	1.04	68.7	14
Source-S					
SBF1	6.85	16	2.30	17.0	31
SBF2	4.20	16	3.90	17.0	53
HIC					
SBF1	0.14	0.72	5.14	0.75	70
SBF2	0.20	1.20	6.00	1.25	81

Substrate specificity of SBF1 and SBF2

Although both enzymes hydrolyzed *p*-nitrophenyl-*N*-acetylglucosaminide, the activity of SBF1 remained higher than that of SBF2. The most significant difference was observed after 180 min of incubation, when SBF1 activity was 10 times that of SBF2 (Fig. 3A). To further characterize these enzymes, SBF1 and SBF2 were challenged with 4MU-$(GlcNAc)_{1-4}$. While both SBF1 and SBF2 were highly active with the monomer of 4MU-GluNAc, the activity of SBF1 was 10 times higher than that of SBF2 after 40 min of incubation (Figs. 3B, C), when hydrolysis of the monomer was at its highest. Hydrolysis of the tetramer was also significant with further incubation resulting in feedback inhibition. Enzymatic activity toward dimeric and trimeric forms of the substrate was relatively poor. As shown in Supplemental Table 2, both enzymes hydrolyzed [^{3}H]-chitin more efficiently than artificial substrates. These results indicate that SBF1 and SBF2 are endochitinases with different *N*-acetylglucosaminidase activities.

Amino sequence identification of SBF1 and SBF2

As shown in Supplemental Fig. 2A, the NH_2-terminal sequence of SBF1 was highly homologous (85-100%) with that of other plant chitinases (X represents either a cystein residue or a modified amino acid) while that of SBF2 was blocked. Interestingly, SBF1 exhibited 100% homology with the chitinase of *Medicago sativa* and a small chitinase-like anti-fungal protein from *Panax notoginseng* (Sanchi ginseng) roots, which has a molecular

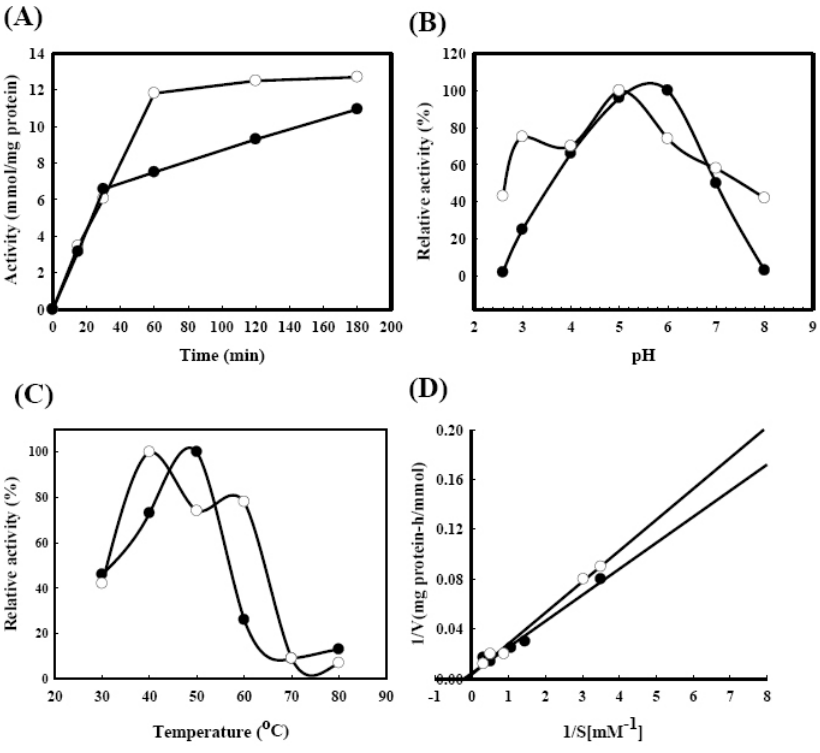

Fig. 2. Effects of (A) Incubation time, (B) pH, and (C) Temperature on the chitinolytic activity of purified SBF1 (●) and SBF2 (○). (D) Lineweaver-Burk plot shows the hydrolysis of [^{3}H]- chitin by SBF1 (●) and SBF2 (○).

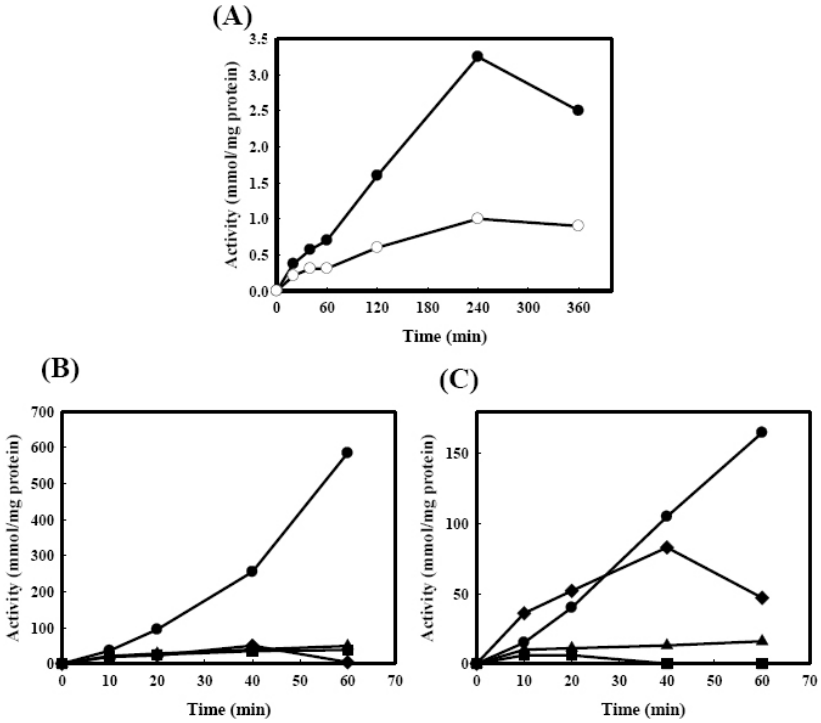

Fig. 3. Hydrolysis of two artificial substrates by SBF1 (●) and SBF2 (○). Hydrolysis of (A) *p*-nitrophenyl-*N*-acetylglucosaminide and fluorescent 4-MU derivatives of N-acetylglucosamine and its oligomers by (B) SBF1 and (C) SBF2: (—●—) 4MU-GlcNAc, (—■—) 4MU- $(GlcNAc)_2$, (—▲—) 4MU-$(GlcNAc)_3$, (—◆—) 4MU-$(GlcNAc)_4$.

weight of 15 kDa (12). Because all of the chitinases listed in Supplemental Fig. 2A are reportedly class I chitinases, and the chitinase of *Panax notoginseng* is not classified, it is reasonable to assume that SBF1 is a class I chitinase. Recently, the chitinase I gene was identified from 4-year-old Korean ginseng (13). Comparing this sequence with our NH_2-terminal results suggests that the first 20 amino acid residues deduced from the gene (13) represent a signal peptide. SignalP 3.0 software (15) predicted a high enzyme cut priority between amino acids 20 and 21 (http://www.cbs.dtu.dk/services/SignalP/; data not shown). The LC-MS/MS spectra of SBF1 and SBF2 shown in Supplemental Fig. 2B are similar to the 34.9-kDa chitinase in ginseng root (13) with high coverage (14% and 19%, respectively). As shown in Supplemental Fig. 2C, MS data of SBF2 indicated modification of cysteine, methionine and asparagine/glutamine. These data do not explain the observed differences between the two isozymes as indicated by chromatographic and enzymatic data described herein (Figs. 2, 3). Therefore, the post-translational modification patterns of these two enzymes are currently being investigated.

MATERIALS AND METHODS

Materials

One-year old Korean ginseng roots (*Panax ginseng* C.A. Meyer) were harvested from experimental field. All packing materials of the chromatography and prepacked columns were purchased from GE Healthcare (www.gebio.co.kr). [^{3}H]-acetic anhydride (500 mCi/mmol) was purchased from Amersham (England) and all the other chemicals were obtained from Merck (Germany) or Sigma (USA) and were of analytical grade.

Extraction and purification

Rhizomes, main and lateral roots were separated and minced with Ace-homogenizer. Chitinases were extracted as previously described (16). The protein extracts were centrifuged at 3,000 g for 10 min and then at 20,000 g for 20 min. The freeze-dried extracts were re-dissolved in 0.02 M Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer before the anion exchanger chromatography. Source-Q anion exchange column (2.6 × 15 cm) was eluted with a step-wise gradient of 50 mM phosphate buffer, pH 7.5, 7.0, 6.5 and 6.0, 50 mM acetate buffer, pH 5.5. The unbound fraction (QF1) was applied on a Source-S column previously equilibrated with 20 mM Tris-HCl (pH 7.5). The column was amply washed with the same buffer and eluted with a linear gradient of NaCl from 0.0 M to 1.0 M. After dissolved in pH 7.5 50 mM Tris-HCl buffer containing 1.0 M $(NH_4)_2SO_4$, the active fractions (SBF1 and SBF2) were separately loaded on Phenyl-Sepharose HP column (1 mL) and eluted with a linear gradient of $(NH_4)_2SO_4$ from 1.0 M to 0.0 M.

Electrophoresis and detection of enzyme activity

SDS-PAGE was performed using the method of Laemmli (17) in the presence of 0.01% glycol chitin, and the bands of enzyme activity were detected with Calcofluor White M2R under

UV light after treating the gel with Triton X-100 (18).

Enzyme assays

Chitinase activity was measured using [^{3}H]-chitin as previously described (19). [^{3}H]-chitin was suspended in the extraction buffer (1 mg/ml, w/v), and 100-μl aliquots were removed during continuous agitation (488 dpm was equivalent to 5 nmol of dry chitin). The radioactivity was quantified with a β-scintillation counter (Tricarb 1600, Packard). For kinetic studies, 35,000 dpm to 1,570,000 dpm of [^{3}H]-chitin were added to 100 μl purified enzyme solution (2.0 μg protein in 0.05 M citrate-phosphate buffer, pH 4.0). The final volume was adjusted to 1 ml. After a 60-min incubation period at 40°C and pH 4.0, the reaction was stopped by boiling at 100°C for 10 min (20). K_m and V_{max} were expressed as mM and mmol/mg-protein/hr, respectively.

Substrate specificity

β-*N*-acetylglucosaminidase assays were carried out with *p*-nitrophenyl-β-*N*-acetylglucosaminide in citrate-phosphate buffer (0.05 M, pH 4.5) (21). The fluorogenic substrates were 4-methylumbelliferyl (4-MU) glycosides of N-acetylglucosamine (GlcNAc) oligosaccharides [4MU-GlcNAc, 4MU-$(GlcNAc)_2$, 4MU-$(GlcNAc)_3$, and 4MU-$(GlcNAc)_4$]. Assays were performed using black 96-well microtitre plates (22). Fluorescence was monitored (excitation 355 nm, emission 460 nm) using a Fluoroskan II Microtitre plate fluorometer. The media were added individually to the wells of a microtitre plate, containing 80 μl McIlvaine buffer (pH 4.0, 0.1 M) and 5 μl of the appropriate fluorogenic substrate at a concentration of 0.8 mM. The plate was placed in a shaking incubator at 40°C and reading was recorded over a 60 min period. The reaction was terminated by addition of 100 μl of 1 M glycine/NaOH (pH 10.6) and fluorescence was read 10 min later

Determination of amino acid sequence and LC-MS/MS

The purified enzyme was blotted on a PVDF membrane. Automated Edman degradation was performed with protein/peptide sequencer 471A (Applied Biosystem, USA) according to the method described by the manufacturer. Protein spots from SDS-PAGE analysis were digested in-gel with sequencing grade modified trypsin (Promega, USA). LC-MS/MS were performed using reversed phase capillary HPLC directly coupled to a Finnigan LCQ ion trap mass spectrometer. The output peak list files were used to query either MSDB or NCBI using the MASCOT program (http://www.matrixscience.com).

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