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Characterization of an Antimicrobial Chitinase Purified from the Grapefruit Extract

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자몽 추출물로부터 분리된 항균성 Chitinase의 특성

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ABSTRACT : An antimicrobial chitinase was purified from grapefruit extract and its properties were characterized. The chitinase was purified with a single step chromatography on regenerated chitin affinity gel column. The molecular weight of the purified chitinase was 29 kDa. The grapefruit extract contained the chitinase protein more than 50% of its total soluble proteins measured by coomassie stained protein bands. When the purified chitinase was incubated with polymers of N-acetylglucosamine (NAG), such as mycelia of *Fusarium oxysporum* and swollen chitin, they were degraded to oligosaccharides, and the oligosaccharides were then further hydrolyzed by the same enzyme to monomer and dimer of NAG. This result suggests that the chitinase contained both endo- and exo- chitinase activities. The chitinase was stable to heat and pH treatment; its activity was not diminished by the heat treatment upto 70°C for 1 hr, and it showed a pH stability in the range of pH 4.0 to 12.0.

***Key words* : Grapefruit, chitinase, antifungal activity.**

The accumulation of several pathogen-related (PR) proteins upon infection with fungi, bacteria, or viruses has been described in many plant species. PR proteins share a number of characteristics such as relatively low molecular weights, accumulation in the apoplast, high resistance to proteolytic enzymes, and extreme isoelectric points (6, 23). Of these PR proteins, the two glucan hydrolyzing enzymes of chitinases and β-1,3 glucanases, have received much attention in recent years, particularly with regard to their putative involvement in the defense response of higher plants to potential pathogens (1, 2). Since the major components of cell walls in many phytopathogenic fungi and bacteria are composed of chitin and β-1,3 glucan (9), it is presumed that the coordinated accumulation of chitinases and β-1,3 glucanases in plant cells is a part of a multicomponent defense response, aimed at degrading the cell walls of many pathogens. Several chitinases in plants have been found to possess the degrading activity of fungal cell wall (3, 11, 24). It is also reported that fungal growth is inhibited by bean chitinase-I and combinations of chitinase and glucanase from pea *in vitro* (7, 8, 14).

Grapefruit (*Citrus paradisi*) has a broad antimicrobial activity against many pathogenic fungi and bacteria and it has been used for a natural preservative in foods, cosmetics, and beverages (5, 16). Although it has been permitted to use as a natural preservative, the antimicrobial substance in grapefruit extract has not been identified. In this study, we describe the purification and the characterization of the purified chitinase from grapefruit extract.

MATERIALS AND METHODS

Chemicals. Electrophoresis chemicals, AG501-X8 resin (20~50 mesh), molecular weight standards

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and dye reagents for protein assay were purchased from Bio-Rad (California, U.S.A). Practical grade crab shell, chitosan powder, glycol chitin, calcofluor white M2R and triton X-100 were obtained from Sigma Co (St Louis, U.S.A). Fungal mycelia of *Fusarium oxysporum* after incubating for 72 hrs was used as a substrate for the chitinolytic enzyme. All other reagents were of the highest purity available.

Activity assay of a chitinase. For the colorimetric assay of chitinase, the reaction mixture in an eppendorf tube containing 700 μl of 0.5% swollen chitin in 50 mM sodium phosphate buffer (pH 6.6) and 700 μl of the diluted enzyme was incubated at 40℃ for 30 min with shaking at scale 2 of vortex-genie II mixer (Scientific Inc.). After immediately cooling the reaction mixture at 0℃, reducing sugar was monitored for 1 ml of supernatant by dinitrosalicylic acid (DNS) by using a standard curve for N-acetylglucosamine (NAG). Enzyme treated at 100℃ for 30 min was used as a control.

Affinity chromatography on regenerated chitin column. Grapefruit (200 g) was homogenized with 800 ml of an ice-cold 50 mM potassium phosphate buffer (pH 6.6) containing 1 mM EDTA, 1 mM EGTA, and 1 mM DTT. After centrifugation of the crude extract of grapefruit juice at 10,000 g for 30 min, the supernatant was adjusted to 80 % saturation with solid ammonium sulfate, and the precipitated protein was obtained. It was dissolved in 100 ml of homogenizing buffer and applied to a pre-equilibrated regenerated chitin column (4×10 cm) at a flow rate of 1.0 ml/min. The column was washed with 300 ml of 20 mM sodium formate buffer (pH 3.3) followed by 300 ml of 20 mM Tris-Cl buffer (pH 8.4). After washing the column, the chitinase was eluted with 300 ml of 50 mM sodium phosphate-NaOH buffer (pH 12.0). The pH of the eluate was immediately adjusted to 7.0 with formic acid. The pooled active fraction was washed in a Centricon microconcentrator (Amicon Co.) with a salt-free buffer (50 mM sodium phosphate buffer, pH 6.6), concentrated to 5 mg pro/ml, and stored at −20 ℃. The regenerated chitin used in this study was prepared by the acetylation of chitosan as described by Molano *et al* (17).

Detection of a chitinase activity on SDS polyacrylamide gel. Electrophoresis was done on a gel containing 0.01% (w/v) glycol chitin and 0.1% sodium dodecyl sulfate. After electrophoresis, gels were incubated for 2 hrs at 37 ℃ with reciprocal shaking in 50 mM sodium phosphate buffer (pH 6.6), containing 1% (v/v) triton X-100 which was purified through a mixed bed deionizing resin (AG 501-X8). Gels were then stained with 0.01% (w/v) calcofluor white M2R in 500 mM Tris-Cl (pH 8.9) and destained as described by Trude and Asselin (22). The lytic zones were photographed under the UV-transilluminator (Bio-rad).

Analysis of the reaction products by HPLC. High performance liquid chromatography was performed to analyze the reaction products which were produced by the grapefruit chitinase by using the substrate of swollen chitin and fungal mycellia of *F. oxysporum*. A Gilson 303 HPLC equipped with a 131 refractive index detector was used for the analysis. A Cosmosil-NH_2 column (4.6×150 mm, Supelco Co.), a mobile phase of 73% acetonitrile and water 73 : 27, v/v) and a flow rate of 1.0 ml per min were used.

Determination of thermal and pH stability. The purified chitinase was pre-incubated at various ranges of temperatures (30∼100℃) for 1 hr and then the residual activity was measured. The pH stability of the chitinase was measured by pre-incubation of the enzyme solution to the solutions of 20 mM glycine-HCl buffer (pH 2∼3), 20 mM citric acid phosphate buffer (pH 4∼5), 20 mM sodium-phosphate buffer (pH 6∼8), 20 mM carbonate buffer (pH 9∼11) and 20 mM sodium phosphate-NaOH buffer (pH 12∼13). After adjusting the pHs of the enzyme solution to 7.0, the chitinase activity was assayed with swollen chitin as a substrate or its residual activity was stained on active staining SDS-polyacrylamide gel.

Protein determination. Protein concentration was measured by Bradford' method (4) by using a bovine serum albumin as a standard protein.

RESULTS

Purification of a chitinase from grapefruit. To study an antimicrobial chitinase activity from grapefruit extract, we purified a chitinase from grapefruit by using the chitin affinity column chromatography. The supernatant of grapefruit extract was precipitated with 80% saturated ammonium sulfate. Ammoniumn sulfate removed the viscous polysaccharides in the grapefruit extract which interfered

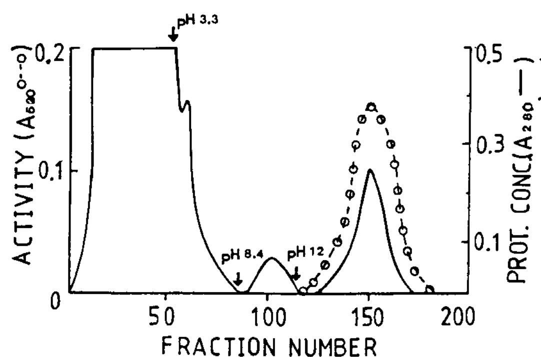

Fig. 1. Affinity chromatography on regenerated chitin column. The column (4×10 cm) was washed with 20 mM sodium formate buffer (pH 3.3) followed by Tris-Cl buffer (pH 8.4). The chitinase was eluted by 50 mM sodium phosphate-NaOH buffer (pH 12.0). The arrows indicate the pH changes of buffer. Chitinase activity was marked as a circle (O-O), and the protein concentration was denoted as a line (-----).

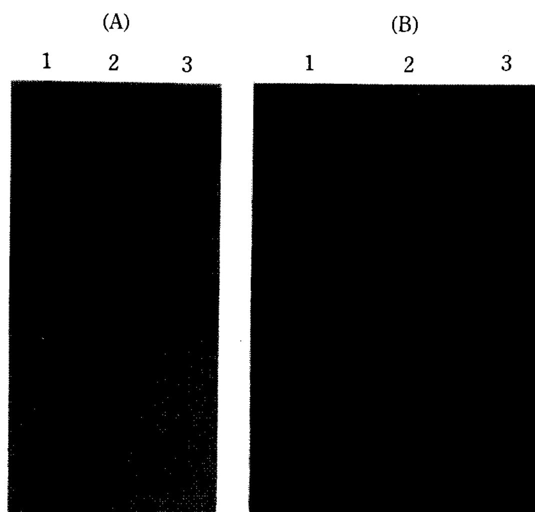

Fig. 2. SDS-polyacrylamide gel electrophoresis of chitinases pooled during the purification steps (A; Coomassie blue staining) and the chitinase activity staining in a gel containing 0.01% (w/v) glycol chitin after removal of SDS (B; active staining) 1 : Crude extract of grapefruit, 2 : 80% ammonium sulfate precipitates from the soluble extract of grapefruit, 3 : Proteins eluted from the chitin column by 50 mM sodium phosphate-NaOH buffer, pH 12.0.

chromatographic procedures. Since the binding force of the chitinase with the regenerated chitin was strong, the dialysis was not necessary to remove ammonium sulfate before loading the enzyme solution to chitin column. Proteins were not bound to regenerated chitin column in the presence of ammonium sulfate, and thus much impure proteins could be removed by this chromatographic step as shown in Fig. 1. After complete washing the non-specifically bound proteins from the regenerated chitin column with 20 mM sodium formate buffer (pH 3.3) and 20 mM Tris-Cl buffer (pH 8.4), the chitinase was eluted by 50 mM sodium phosphate-NaOH buffer (pH 12.0). A single protein peak was obtained and it was correlated with the profile of chitinase activity. Each active fraction was immediately neutralized with formic acid, concentrated by amicon concentrator (YM-10, Amicon Co.) and stored at −70℃ until use.

SDS-PAGE and active staining the chitinase. The active fractions of chitinase enzyme obtained from each purification step, were analyzed on a 12% SDS polyacrylamide gel and the protein bands were stained with coomassie brilliant blue. As shown in Fig. 2-A, a single protein band was detected and its molecular weight was estimated to be 29 kDa. To confirm the chitinase activity of the 29 kDa protein had the chitinase activity, the same proteins were tested for their activity on active staining SDS-polyacrylamide gel containing 0.01% glycol chitin as a substrate (Fig. 2-B). Since the chitinase protein can be renatured on SDS-polyacrylamide gel after removal of SDS (21), it can hydrolyze its substrate, glycol chitin, on SDS-polyacrylamide gel. As can be expected, a single chitin hydrolytic band was clearly generated by the renatured chitinase enzyme on the active staining gel. When we compared mobility of the chitin hydrolytic band with the standard molecular weight marker proteins, its molecular weight was shown as exactly the same as that of protein band appeared in Fig. 2-A. This result indicates that the 29 kDa protein band purified from the chitin column chromatography had a chitin hydrolytic activity on SDS-polyacrylamide gel containing 0.01% glycol chitin. It is very remarkable that the amount of the chitinase enzyme protein consisted more than 50% of the total soluble proteins in crude extract of grapefruit, based on coomassie stained protein bands as shown in Fig. 2-A (lane 1).

Degradation of a fungal cell wall by the chitinase. To study the effect of a grapefruit chitinase on the hydrolysis of fungal mycelia, it was incubated

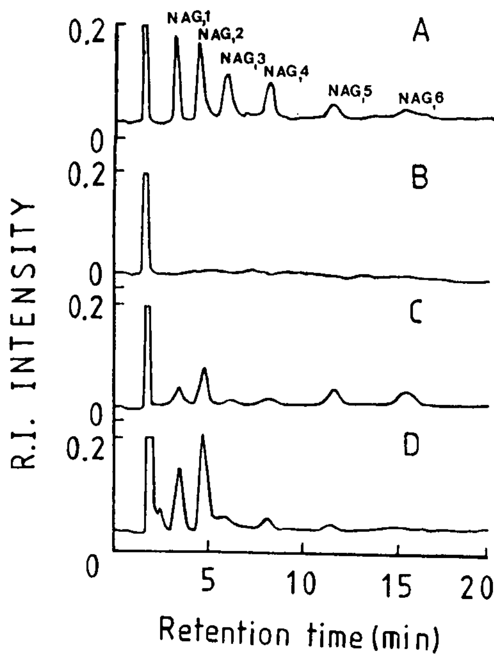

Fig. 3. HPLC analysis of the reaction mixture produced by the grapefruit chitinase using the swollen chitin as a substrate. Mixture of NAG oligomers was used as a standard material (A). The supernatants of the reaction products obtained from the incubation of the grapefruit chitinase with the swollen chitin for 0 min (B), 2 hr (C), and 5 hr (D).

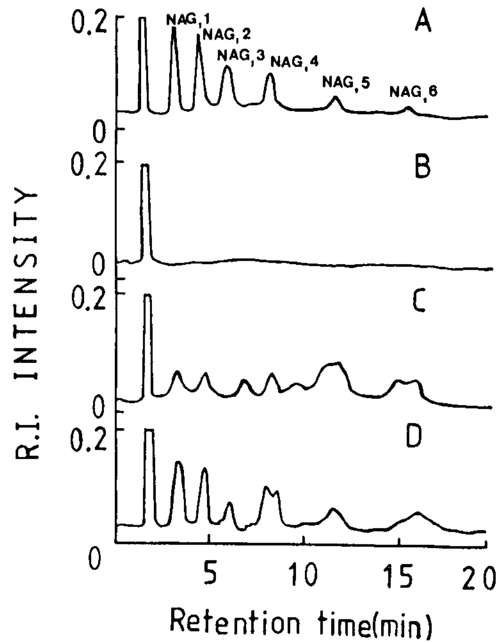

Fig. 4. HPLC analysis of the reaction mixture produced by the grapefruit chitinase by using the mycelia of *Fusarium oxysporum* as a substrate. Mixture of NAG oligomers were used as a standard material (A). The supernatants of the reaction products obtained from the incubation of the grapefruit chitinase with the mycellium of *Fusarium oxysporum* for 0 min (B), 2 hr (C) and 5 hr (D).

with the swollen chitin and a fungal cell wall isolated from *F. oxysporum* as substrates. When the reaction product of the swollen chitin was analyzed by HPLC, the chitinase degraded the substrates to monomer and oligomer of NAGs. Small amounts of NAG oligomers were formed at the begining of the reaction and the amounts of monomer and dimer of NAGs were gradually increased with the incubation time (Fig. 3). When the fungal cell wall, of which major components are known as chitin and β-1,3 glucan, was hydrolyzed with the purified chitinase, it was cleaved to its constituent as same patterns as that of swollen chitin hydrolysis (Fig. 4). The major products of fungal cell wall hydrolysis were also monomer and dimer of NAGs. As shown in Fig. 4, the grapefruit chitinase yielded more complexed products of which retention times were not correlated with those of standard NAG oligomers. This result might be due to the complexity of the fungal cell wall components compared to chitin. These results indicate that the grapefruit chitinase had the dual chitin hydrolytic activities of endo- and exo-chitinase activities. Therefore, it hydrolyzed the chitin substrate to oligosaccharides at first, and the latter was further hydrolyzed to monomer and dimer of NAG by the same enzyme slowly. However, the rate of chitin hydrolysis by grapefruit chitinase might be too slow to explain the antimicrobial activities of grapefruit extract against many pathogens. Certain other critical factors might be coped with the attacks of various pathogens *in vivo*.

Stability of the chitinase from grapefruit. The purified enzyme was treated with various ranges of pHs and temperatures. The chitinase was stable to heat treatment; its activity was not changed by heat treatment upto 70℃ as shown in Fig. 5. However, when the temperature was raised to 100℃, about 90% of total activity was diminished within 30 min. This result was confirmed by the active staining in SDS-polyacrylamide gel as well as the measurement of chitinase activity by using the swollen chi-

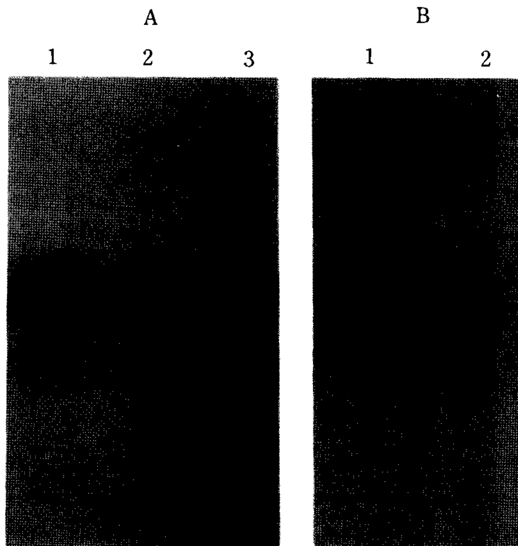

Fig. 5. Thermal stability of the purified grapefruit chitinase. The enzyme solution was pre-incubated at 70℃ for 0 min (A-1), 30 min (A-2), 60 min (A-3) and 100℃ for 10 min (B-1), and 30 min (B-2). The residual chitinase activity was measured by active staining on SDS-polyacrylamide gel.

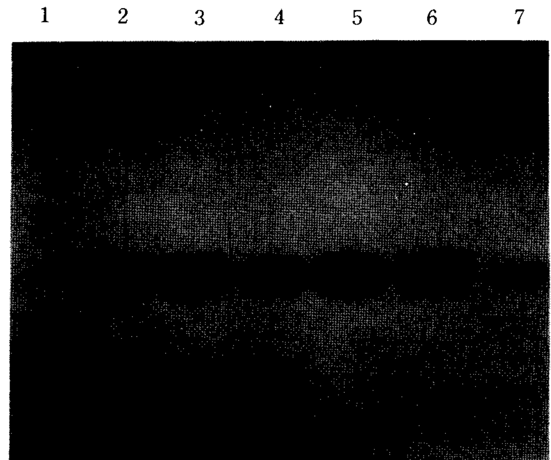

Fig. 6. pH stability of the purified grapefruit chitinase. Enzyme solution was pre-incubated for 1 hr at room temperature in various pH buffers (1: pH 2.0, 2: pH 4.0, 3: pH 6.0, 4: pH 8.0, 5: pH 10.0, 6: pH 12.0, 7: pH 14.0) described in 'Materials and Methods'. The residual chitinase activity was measured by active staining on SDS-polyacrylamide gel.

tin as a substrate. The chitinase was highly stable to broad pH changes of enzyme solution as shown in Fig. 6. Its activity was not changed in the range of pH 4.0 to 12.0. The chitinase activity was decreased at pH 14.0 and no activity was detected below pH 2.0. The chitinase was more stable in alkaline than in acidic solution.

DISCUSSION

Pathogen attack and elicitor treatment dramatically increase the production of many PR proteins in plants (12). Of these PR proteins, chitinases and β-1,3 glucanases have supposed to play a critical role in direct defence systems, because they have been shown to be capable of attacking cell walls of fungi and bacteria (13). The chitinases are excreted across the cell wall and accumulated in intercellular spaces, which are the common sites of attack by many plant pathogens (20). There are many reports for the possible role of plant chitinases in plant defenses. In cucumber, chitinase is induced in response to viral, bacterial and fungal infections (15). In tomato, *Verticillium* or *Fusarium* wilt induced an increase in β-1,3 glucanase and chitinase activity (25). These enzyme activities were always higher in the susceptible plants, whereas more fungal biomass was present than in resistant ones (10, 19). In *Verticillium*-infected tomato plants, the increase in β-1,3 glucanase and chitinase activity coincided with a leveling off and subsequent reduction of the amount of viable pathogen in the tissue (18).

Although the compound representing the antimicrobial activities in grapefruit extract has not been identified, it has been well known that the grapefruit extract showed a strong antimicrobial activity against various phytopathogens (5). To investigate a chitinase role in the antifungal activity of grapefruit extract, we purified a chitinase from the grapefruit extract and found that the grapefruit contained the chitinase protein more than 50% of the total soluble proteins. The grapefruit chitinase is also quite stable to pH and temperature changes. When we incubated the purified chitinase with a fungal mycelium of *F. oxysporum*, it slowly degraded the fungal cell wall into monomer and dimer of NAGs. These results indicates that the grapefruit chitinase may play an important role in its antimicrobial activities. However, certain other factors should be necessary to cope with the pathogen attack, because the hydrolyzing rate of grapefruit chitinase to fungal mycelia is too slow to explain the whole antimicrobial acti-

vities.

요 약

본 실험에서는 자몽즙으로부터 chitinase를 순수분리하고 이들의 항균특성을 연구하였다. 단백질을 전기영동한 후 염색된 단백질의 농도로부터 측정하였을때, chitinase 단백질이 자몽즙 가용성 단백질의 약 반 이상을 차지하고 있다. 자몽즙의 chitinase를 chitin affinity 크로마토 그래피법을 이용하여 순수분리하였으며, 순수분리된 chitinase의 분자량을 전기영동방법에 의하여 측정하여 본 결과, 분자량은 29 kDa이었다. 자몽즙 chitinase를 NAG의 중합체인 swollen chitin 이나 chitin으로 구성된 *Fusarium oxysporum*의 균사체와 반응시킨 후 반응생성물을 분석하여 본 결과, 반응초기에 곰팡이의 chitin 폴리머를 분해하여 NAG의 oligomer을 생성시켰으며 반응시간이 경과함에 따라 NAG oligomers들은 NAG의 monomer와 dimer로 분해됨으로써 자몽 chitinase가 exo-와 endo- chitinase의 활성을 동시에 지니고 있음을 알 수 있었다. 자몽즙으로부터 분리한 chitinase는 열과 산도 변화에 대단히 안정한 성질을 나타냄으로써 70℃에서 1시간 동안 열처리하여도 활성의 변화가 거의 나타나지 않았으며, pH 4.0에서 12.0까지의 넓은 범위에서도 안정한 상태를 나타내었다. 본 실험의 결과, 자몽즙 chitinase는 병원균으로부터 자신을 방어하는 과정에 중요한 역할을 할 것으로 생각되었다.

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