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New Antimicrobial Activity from Korean Radish Seeds (*Raphanus sativus L.*)

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Abstract To isolate antifungal substances from Korean radish (*Raphanus Sativus L.*) seeds, various purification techniques such as DE52 cellulose anion exchange, SP-Sephadex C-25 cation exchange, and Sephadex G-50 gel filtration chromatographies were used. The molecular masses of two purified *R. sativus* antifungal proteins (RAPs) were estimated to be about 6.1 kDa (RAP-1) and 6.2 kDa (RAP-2) by SDS-PAGE, and 5.8 kDa (RAP-1) and 6.2 kDa (RAP-2) by a gel filtration chromatography, respectively. Purified proteins RAP-1 and 2 clearly exhibited different growth inhibitory activities against other microorganisms like *Candida albicans and Saccharomyces cerevisiae*. Although they have similar molecular masses, both RAP-1 and 2 proteins are not identical because their microbial inhibitory actions were different. Therefore, RAP-1 could be a new antifungal protein when compared with the antifungal activities of 2S albumins, Rs-AFPs, Mj-AMPs, chitinase, glucanase, permatin, and ribosome inactivating proteins, all of which are antifungal proteins of plants.

Key words: Korean radish seeds, *Raphanus sativus L.*, *Botrytis cenerea*, *R. sativus* antifungal proteins (RAPs)

Gray mold rot is caused by *Botrytis cenerea*, which is a wildly distributed plant pathogen and the most damaging disease known among green house plants of the world. To overcome plant fungal pathogens, many kinds of chemical drugs have been used in many countries for a long time. As we know, chemical drugs cause not only environmental pollution but they are also harmful to humankind as well. Because of the undesirable side-effects of chemical drugs, many biologists have been intensively studying biological control against plant fungal pathogens.

The inducible antimicrobial substances from plants and many microorganisms such as hydrolytic enzymes and ribosome-inactivating proteins have been thoroughly studied [1, 2, 3, 6, 11, 13, 16, 19]. Plant seeds exhibited a natural substrate which is rich in microorganisms. Because of the environmental facts of seeds, plant seeds have gained effective defence systems against microorganisms. Some of the defending substances and antimicrobial peptides present in many plant species are called plant defensin. Plant defensins are small (45–54 aa) and have a complex structure stabilized by many disulfide-linked cystines [8]. They are structurally similar to insect and mammalian defensins and are nontoxic to either mammalian or plant cells [12, 14, 15]. For these reasons, plant defensin is considered as a promising agent of biological control against plant pathogens.

We have been studying the biological control agents against insect and microbe pathogens [9, 10]. In this report, we describe the isolation and purification of two defensin-like antifungal proteins derived from Korean radish seeds. Interestingly, we found that the protein had a characteristic in antifungal activity, different from other known defensins of seeds. Therefore, we suggest that the purified antifungal protein, named RAP-1, may be an another defensin of radish seeds.

Microorganisms and Growth Conditions

Botrytis cenerea, *Candida albicans*, and *Saccharomyces cerevisiae* were used in this study. *B. cenerea* (KACC 40574) was grown in potato dextrose (PD) agar, and spores were harvested and stored as previously described [21]. *C. albicans* (KACC 30050) and *S. cerevisiae* (KCTC 7919) were precultured overnight in YM (0.3% yeast extract, 0.3% malto extract, 1.0% glucose) and YPD (2.0% bactopeptone, 1.0% yeast extract, 2.0% glucose) mediums at 30°C, respectively.

Assay for Antifungal and Anti-Yeast Activities

For detection of antifungal activity, a disk assay was used [17]. One-hundred μl of *B. cenerea* spore suspension was inoculated into 5 ml of PD agar and poured over the plate. Then, disks soaked with test samples were loaded onto the

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solidified PDA agar surface. After incubation for 72 h at 24°C, the plates were checked for inhibition zone. Inhibition was regarded as a positive if the width of the clear zone around the test sample of the soaked disk was 5 mm or larger. For an anti-yeast activity test against *C. albicans* and *S. cerevisiae*, 100 µl of overnight precultures of the strains were used.

Purification of RAP-1

As presented in Fig. 1, 100 g sample of Korean radish seeds (Nongwoo Bio Co., Suwon, Korea) was homogenized in 1.5-l of 50 mM Tris-HCl buffer (pH 8.3) and allowed to stand for 2 h at 4°C. The homogenate was filtered with a gauze and the filtrate was centrifuged at 10,000 rpm for 20 min at 4°C. Then, the resulting supernantant was treated with ammonium sulfate (0-60% saturation) and centrifuged. The precipitate obtained was completely dialyzed against 10 mM Tris-HCl buffer (pH 8.3) at 4°C and lyophilized. To purify active proteins from lyophilized crude protein powder, DE52 cellulose (Whatman Ltd., Maidstone, U.K.) chromatography was performed with 10 mM Tris-HCl buffer (pH 7.5) as an efflux buffer and also using a linear gradient of sodium chloride (0-0.3 M) in the same buffer. The major part of antifungal activity from the anion exchanger column was recovered in the DE52 nonadsorbed fractions (Fig. 1). However, the adsorbed fraction did not exhibit the activity. The DE52 nonadsorbed fractions represented 14.2% (about 280 mg) of the lyophilized crude protein powder (Table 1). On the Sephadex G-50 SF (Pharmacia Co., Uppsala, Sweden) gel filtration chromatography by using Tris-HCl buffer, 140 mg of the DE52 nonadsorbed fractions gave three peaks (Fig. 1). The first peak did not exhibit any antifungal activity, while the last peak had some activity. Then these third fractions were further re-chromatographed on the same Sephadex G-50 column. As shown in Fig. 2A, a single peak was obtained that was named, 'purified RAP-1'. The total amount of RAP-1 was about 6.1% (about 18.0 mg) of the DE52 nonadsorbed fractions.

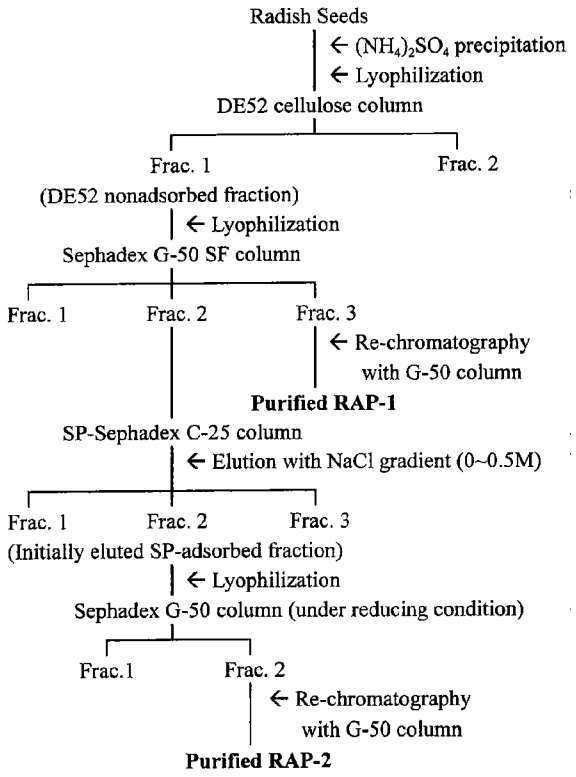

Fig. 1. Purification of antifungal active proteins (RAP-1 and 2) from Korean radish seeds.

Table 1. The amounts of recovered active fractions from purification steps.

Purification steps	Recovered amounts (mg)
Seeds	100,000
Lyophilized crude protein powder	2,100
DE52 nonadsorbed fraction	280
Purified RAP-1	18
Purified RAP-2	9

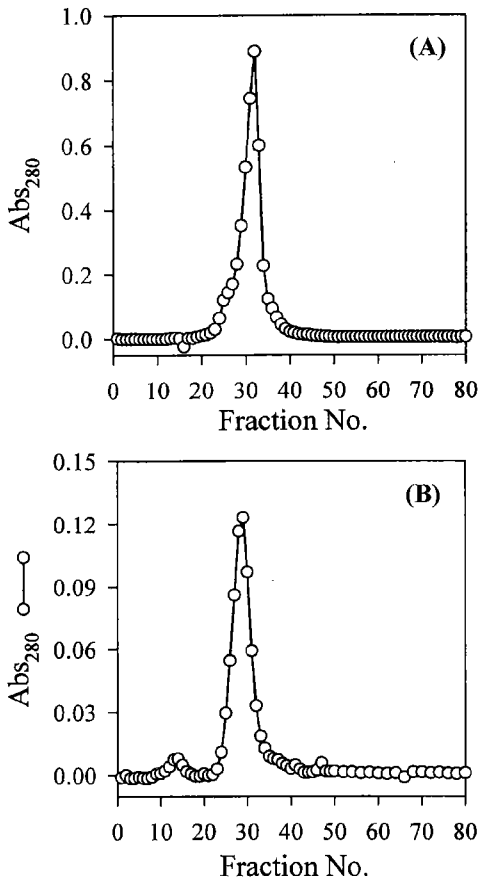

Fig. 2. Chromatograms on (A) Sephadex G-50 column of purified RAP-1 protein and (B) same Sephadex G-50 column of purified RAP-2 protein.

In SDS-PAGE, purified RAP-1 migrated as a single band, shown by Coomassie Brillant Blue R250, and its molecular mass was estimated to be 6.1 kDa (Fig. 3A, lane 2). On the other hand, the molecular mass of the fraction by gel filtration chromatography was about 5.8 kDa (Fig. 3B).

Purification of RAP-2

Among the fractions separated from the above Sephadex G-50 gel filtration chromatography, the fractions containing the second peak also exhibited a weak antifungal activity. To purify another antifungal protein from second fractions, SP-Sephadex C-25 (Pharmacia Co., Uppsala, Sweden) chromatography was performed. One-hundred mg of the fraction solution was applied to a SP-Sephadx C-25 column using 50 mM Tris-acetate buffer (pH 4.8) as an efflux buffer and also using a linear gradient of sodium chloride (0~0.5 M) in the same buffer. The second fractions gave three protein peaks (Fig. 1) and major antifungal activity was recovered in the SP-adsorbed fractions initially eluted by sodium chloride gradient. According to the analysis of SDS-PAGE, the initially eluted SP-adsorbed fractions consisted of two protein bands, and their molecular masses were estimated to be 13 kDa and 6 kDa, respectively (data not shown). To isolate it further, the SP-adsorbed fractions were then subjected to gel filtraton on Sephadex G-50 SF column by using Tris-HCl buffer (pH 8.3) as an elution buffer. Only one peak was obtained which indicated that this protein may be a dimer structure. Therefore, gel filtration chromatography in the presence of β-mercaptoethanol in an elution buffer was further performed. The SP-adsorbed fractions gave two peaks (Fig. 1) and the former peak did not exhibit any antifungal activity, while the latter showed some activity (data not shown). The latter fractions were re-chromatographed on the same Sephadex G-50 column without β-mercaptoethanol and a single peak was obtained as shown in Fig. 2B and named as purified RAP-2. The amount of the 'purified RAP-2' was about 3.2% (about 9.0 mg) of the DE52 nonadsorbed fractions.

On SDS-PAGE, the molecular mass of purified RAP-2 was estimated to be 6.2 kDa (Fig. 3A, lane 3), and it was also 6.2 kDa (Fig. 3B) by gel filtration chromatography.

Anti-Yeast Activities of Purified RAP-1 and 2

As presented in Table 2, growth-inhibitory activities of purified protein RAP-1 and 2 on both yeast strains *C. albicans* and *S. cerevisiae* were measured. The purified RAP-1 exhibited clear activity on both strains, while RAP-2 did not.

In summary, our preliminary study suggested that Korean radish seeds have an antifungal activity against *B. cenerea*, most likely due to the presence of a certain proteinaceous substance with molecular mass of over 3.5 kDa. The protein was different from low molecular antifungal compounds of less than 1,000 Da from radish juice. Thus, by chromatographic separation and by an assay for growth inhibition of *B. cenerea*, two antifungal proteins from Korean radish seeds were purified. Both purified RAP-1 and 2 proteins showed very similar molecular mass on both SDS-PAGE and gel filtration chromatography. However, they were guite different in protein structure, since RAP-2 could be isolated from the SP-adsorbed fraction only in the presence of β-mercaptoethanol and it could be a heterodimer consisting of 13 kDa and 6 kDa bands, as indicated by SDS-PAGE (data not shown).

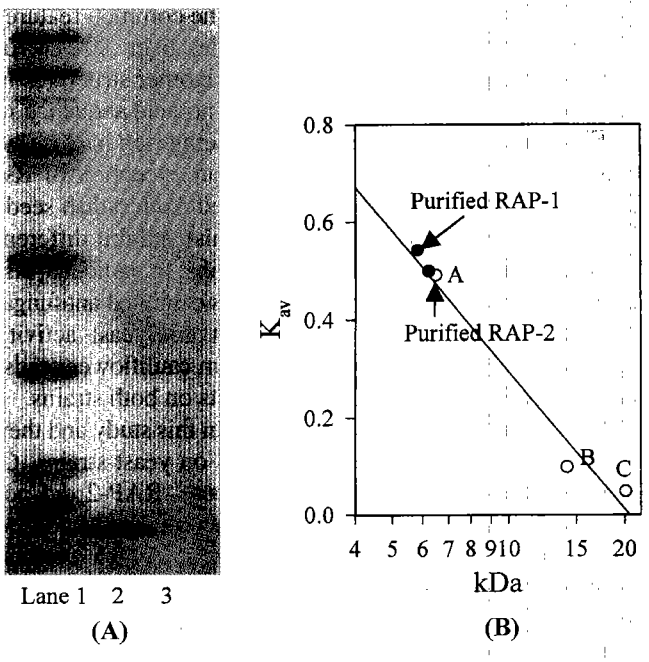

Fig. 3. Molecular weight estimation of purified RAP-1 and 2 proteins.
(A) 12.5% SDS-PAGE; Lane 1, molecular weight standard markers (phosphorylase b (94 kDa), bovine serum album (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (14.4 kDa), aprotinin (6.5 kDa), and insulin (5 kda)); Lane 2, purified RAP-1; Lane 3, purified RAP-2. (B) Gel filtration chromatography. Molecular weight markers; A, aprotinin (6.5 kDa); B, α-lactalbumin (14.2 kDa); C, trypsin inhibitor (20.1 kDa).

There are many proteins in seeds with antifungal activities which include chitinase [16], β-1,3-glucanase [13], permatin [22], thionin [3, 8], ribosome-inactivating proteins (RIPs) [12, 15, 16], AMPs [4, 5], 2S albumins [20, 21], Rs-AFPs

Table 2. Antifungal and anti-yeast activities of purified RAP-1 and 2[a].

Microorganisms	RAP-1	RAP-2
Candida albicans	+	-
Saccharomyces cerevisiae	+	NC[b]
Botrytis cenerea	+	+

[a]For the antimicrobial test, the purified samples were tested at the concentration of 5 mg/disk, respectively.
[b]NC, not checked.

[21], PAFPs [18], Dolichin [23], etc. The common features of these proteins are, as an example, over 20 kDa of molecular masses in RIPs, dimer or oligomer structures in chitinase and glucanase, a similar amino acid sequence in PAFPs and AMPs, or whether or not being rich in cysteine content. However, all of them, with an exception of Rs-AFP and 2S albumin, are not originated from radish seeds but from other seeds. These proteins also exhibit different activities, when tested on microbial strains. In particular, Rs-AFPs and 2S albumin from radish seeds exhibited antifungal activities against *B. cenerea* but not anti-yeast activity against *S. cerevisiae*, while AMPs (from cauliflower seeds) and β-purothionin showed active effects on both strains.

RAP-1 and 2 proteins were purified in this study and they were found to have opposite activities on yeast strains, *C. albicans* and *S. cerevisiae*. Among these, RAP-2 did not show any anti-yeast activity and this was in accordance with that of Rs-AFPs and 2S albumin, which are the only antifungal proteins isolated from the same radish seeds [21]. In addition, RAP-2 (the original RAP-2 was estimated to be a heterodimer protein, as mentioned above) was similar to 2S albumin, which consists of subunits of 10 kDa and 4 kDa as a heterodimer protein. Although a more detailed study like amino acid analysis is required, these observations suggested that RAP-2 is similar to 2S albumin. On the other hand, RAP-1 was found to be different from any other proteins. In fact, the protein in the native form had a monomer structure and anti-yeast activity in contrast to those of Rs-AFPs and 2S albumin of radish seeds. These facts are very similar to thionin of antifungal proteins as reported previously. In particular, β-purothionin with molecular mass of 6 kDa, one of the thionin-like analogues, showed a similarity to RAP-1, but the existence of that protein in radish seeds has not been reported. Also, thionins containing its analogues are known to exhibit very different activities depending on microbials tested [7]. Thus, it is not clear whether or not the RAP-1 purified in this study has structural and biological similarities to thionins. Therefore, to compare the presently purified RAPs to other well-known antifungal proteins, especially 2S albumin and thionins, more detailed characterization of RAP-1 and RAP-2 proteins are needed.

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