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Antifungal Activity of Korean Radish (*Raphanus sativaus L*) Extracts Against Pathogenic Plant

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Abstract

A study of the anti-fungal properties in Korean radish was conducted using a variety of purification procedures such as Extrelut column, RP(Reverse Phase) C18 Column Chromatography, HPLC etc. to separate anti-fungal substances from Korean radish juices to test them against a common gray mold called *Botrytis cenerea*. Dialysis tube operation showed that these substances were presumably thermostable compounds with low molecular mass (less than 3.5 kDa). Differences of anti-fungal activities depending upon types of radishes used did not show any noticeable variation. The antifungals were presumably composed of more than 5 compounds. Among these, the most anti-fungal fraction was analyzed by HPLC in which one peak was obtained. Disease-affected plants were inoculated with 10mg of Extrelut fraction and results showed similar anti-fungal activity to pesticides suggesting possible usage of these substances as environmentally friendly antibiotics.

Key words – Antifungals, *Botrytis cenerea*, Korean radish juices, *Raphanus sativaus*

Introduction

Intensive agriculture has led to excessive use of synthetic organic pesticides, and consequently, environmental deterioration has occurred. Pesticide residues in agricultural products caused by improper use of pesticides are threatening humans health, soil and water ecosystems, and furthermore, reducing farm household income.

Hereupon, many environmental microbiologists as well as the author have worked on the development of environmentally friendly antibiotics, including microbial antibiotics[1,2,6,9,110,11,13,19,21].

The suburban Po-hang City areas local special products, Chinese chive (*Allium tuberosum* Rottler) and spinach (*Spinacia oleracea*) are subject to infection by gray mold disease. During the research aimed at biological control of *Botrytis cenerea*, we found anti-fungal activity of the Korean radish (*Raphanus sativaus L*) against gray mold. So we tried to isolate these anti-fungal substances. Radish has long been used as an ingredient for *kimchi* a type of traditional Korean food, and also has been used as folk medicine to treat influenza, asthma, indigestion, and eczema.

Anti-fungal proteins such as RAP1,2 (*Raphanus* Anti-fungal Protein) and Rs-AFP (*Raphanus sativus* Anti-fungal Protein) in radish seeds as well as many plants extracts have been reported in the previous studies[7,18,22,23] and have anti-microbial activity on some bacteria[12]. However to our knowledge, this study is the first report to show anti-fungal properties in radish juices.

Antifungals in plant seeds as well as radish seeds are reported as chitinase, β-1,3-glucanase, RIP (ribosome

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inactivating proteins), and zeamatin[3,4,5,8,15].

The structures of these hydroxylase and proteins called plant defensins are similar to those of mammalian defensins so that they are harmless to both animals and plants [14,16,17,20,].

The objective of this research is to isolate and purify anti-fungal substances against *Botrytis cenerea*. By conducting plant experiments, we evaluated the possible usage of these substances as antibiotics.

Materials and Methods

Radish samples and culture conditions

Radishes purchased for this research were grown in the highland region, Jookjang Myon, Pohang City, Kyungbuk Province. Samples were originally cultivated for ingredients of winter *kimchi* (Baekyang, Baekja, Baekwoon).

Radishes were washed and grated. The radish juices obtained were freeze-dried in a lyophilizer (Il Sin Eng. Co. Seoul. Korea) and preserved at -70℃ before use.

Botrytis cenerea strain KACC49574 was grown on PDA medium (Difco. Co. Detroit, USA) at 25℃ for 1 week and used after spore formation.

Evaluation of anti-fungal activity

Anti-fungal activity was evaluated by disk assay method[21]. Firstly, *B. cenerea* strain KACC49574 was incubated on PDA plate at 25℃ for 1 week. After spore formation, it was diluted in sterile water, centrifuged at 1,000 rpm for 1 min. In the supernatant, hyphae were removed and only spores were collected. Then the spores were added to sterile water, stored at 20℃ and used as required. The obtained spores (100㎕) were mixed on 0.7% soft agar and was added onto a PDA plate. Afterwards, a sterile 5㎜ paper disk (Whatman Ltd. Maidstone, UK) was placed on the PDA plate and samples were put under disk suction. After cultivation at 25℃, the samples anti fungal levels were determined.

Extraction and purification of anti-fungal substances

Less than 3.5 kDa molecular mass substances from lyophilized radish powders were gathered and concentrated by dialysis tube (Whatman Ltd. Maidstone, UK) and put into Extrelut column (Merck. Co. Darmstadt, Germany). Bound substances in the Extrelut column were eluted with chloroform:MeOH(80:20). After the activity test, RP (Reverse Phase) C18 Column Chromatography was carried out to obtain fractions. The fractions activities were tested via the foregoing method and the active regions were identified by HPLC analysis (Shimadz. Co. Tokyo, Japan) with MeOH solvent at 1㎖/min. under 254㎚ condition.

Activity test by plant experiment

Plant experiment was conducted with disease-affected Chinese chive grown in a greenhouse. The collected chives were stored in petri dishes and treated with the extract, Diethofencarb. Carbenda (Dongbang Agro. Co. Seoul, Korea); gray mold fungicide, and sterile water, respectively. The extracts activity was tested after 3 days incubation in the growth chamber under optimal condition for gray mold at 25℃ and 80% relative humidity.

Results and Discussion

Anti-fungal activities depending upon types and concentrations of radish juice extracts

Our research staff has already isolated and purified anti-fungal and anti-yeast substances in radish seeds[18]. Among these, we identified the substance RAP1 as a the key compound different from previously reported plant defensins[24,25]. Seeds contain anti-fungal substances, defensins to germinate in harsh subterranean environment.

Radish also contains anti-fungal substances to grow in the same environment. We conducted activity test of both outer and inner dialysates to compare activity differences between radish and radish seed. As shown in Table 1, seed extracts exhibited anti-fungal activity in the inner dialysate (more than 3.5 kDa) while radish juices exhibited high anti-fungal activity in the outer dialysate (less than 3.5 kDa). In the previous study, we have already reported that anti-fungal substances identified from radish seeds were about 60 kDa molecular mass proteins[18]. Therefore, it was suggested that anti-fungal substances in radish juices were low molecular compounds. A kind of defensins, anti-fungal substances in seeds were inactivated by heat treatment for about 30 min, meanwhile those in radish juices were not (Data unavailable). By summing up all these results, anti-fungal substances in radish juices were presumably thermostable compounds with low molecular mass. There were no differences of activities dependent upon types of radishes used.

RP (Reverse Phase) C18 Column Chromatography operation results

Lyophilized radish powders were passed through Extrelut column. To further purify the resulting fractions by their polarities, RP C18 Column Chromatography operation was done. A total 6 fractions were separated. They exhibited very high activities against gray mold (Fig. 1). Table 2 summarizes individual fractions activities and recovery rates. Fractions were mostly C18A with no activities and others showed very low recovery rates. Among these, C18B1 and C18B2 were presumed to be similar substances and showed the highest activities. Therefore, we concluded that these substances were composed of 5 compounds at least. The most active C18B1

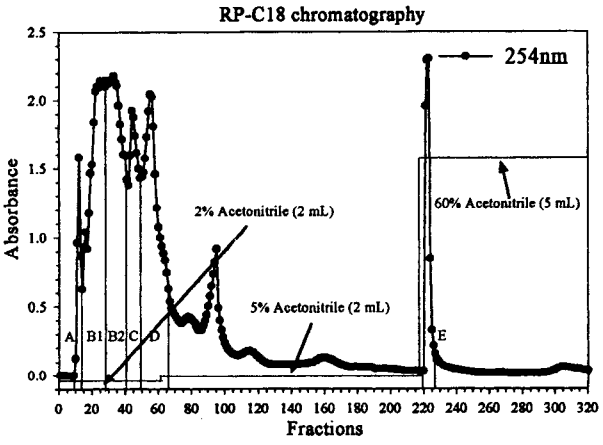
Table 1. Anti-fungal effects of the fractions by dialysis against the gray mold *Botrytis cinerea*

Treatment	Radish Species					
	Baekyang		Baekja		Baekwoon	
	Seed	Radish	Seed	Radish	Seed	Radish
MW over 3,500	+	-	++	-	++	-
MW below 3,500	-	++	-	++	-	++

*All samples received a dose equivalent to 25㎎/disk of crude juice extract and 12㎎/disk of seed extract, respectively.

*+,-: Diameter of inhibitory zone(-: no inhibition, +: under 12mm, ++: 13-16mm).

A



B

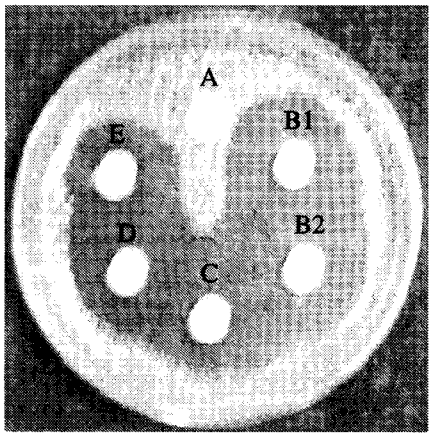


Fig. 1. Chromatogram pattern and Anti-fungal effect of each sub-fractions.
A: RP-C18 column chromatography of Extrelut extract. Each sub-fraction is identified on the graph.
B: Anti-fungal (against the target fungi: *Botrytis cenerea*) effect of RP-C18 colum chromatogram. sub-fraction as seen on the plate.

Table 2. Recoveries of sub-fractions from Extrelut extract using RP-C18 column chromatography and their anti-fungal effects against *Botrytis cenerea*

	Fractionated Amount(mg)	Recovery(%)	Antifungal Effect.
Extrelut ext.	110.00		+
A	87.74	62.7	-
B1	2.97	2.07	++++
B2	1.94	1.39	++++
C	1.11	0.79	+++
D	1.37	0.98	++
E	2.18	1.58	++

*All fractions were loaded at the dose of 1.1mg /disk.
*+, -: Diameter of inhibitory zone(-: no inhibition, +: under 12mm, ++: 13~16mm, +++: 17~18mm, ++++: above 18mm).

and C18B2, were analyzed by HPLC to separate and purify anti-fungal substances. Fig. 2 shows the analysis results of C18B1 and C18B2 fractions by HPLC. There were 4 peaks and the second peak was determined to be active.

On the basis of the results above, anti-fungal substances from radish juice were presumably active agent different from anti-fungal compounds from radish seeds that we previously reported. However, unlike seed anti-fungal juice related substances did not show any anti-yeast activity in any of our preliminary study on yeast.

Results of plant experiment

Due to the low recovery rates of the final separates, we freeze-dried extracts passed through Extrelut column and applied them extracts onto disease-affected plants. Gray mold disease-affected Chinese chives were treated with the active extract and a commercial fungicide, respectively; a comparison of the results is represented in Fig. 3. 10㎎ of active extract, sterile water, and fungicide (100-fold diluted) were sprayed on the chives and each treatment was incubated in growth chamber for 4 days. The fungicide and active substance treated dishes did not show further development of gray mold disease. However, vigorous development of the disease was observed in the sterile water treated dish.

Results suggest that possible usage of anti-fungal substances as antibiotics. We are under currently in the process of exploring different methods of extracting and purifying the anti-fungal agent in large quantities for

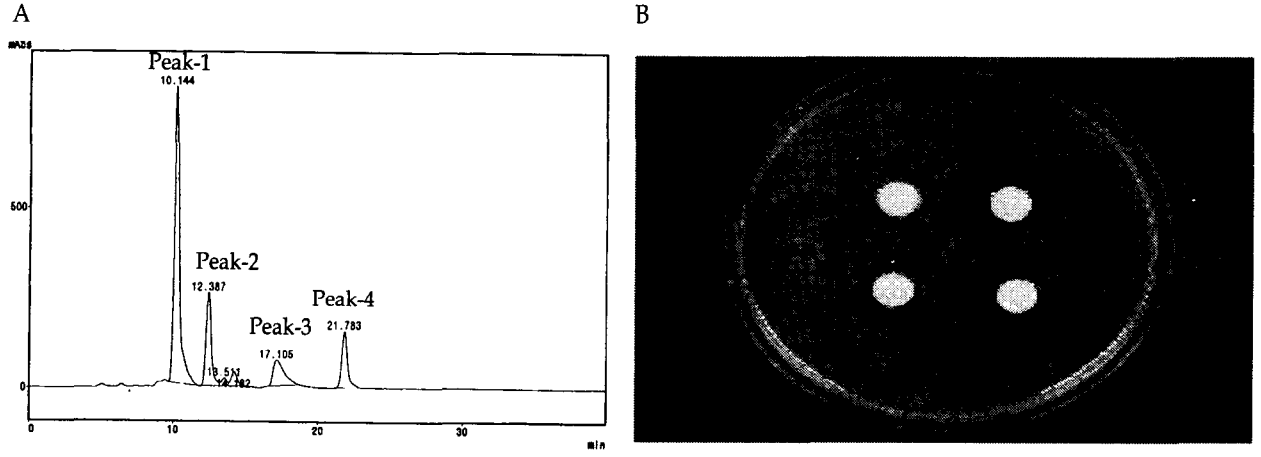


Fig. 2. HPLC elution pattern of the C18B antifungal compounds and its antifungal effect.
A: HPLC elution pattern.
*The elution was done with MeOH solvent at flow rate of 1.0 ml/min. under 254 nm condition.
B: Antifungal effect of each fractions.
*All fractions were applied at the dose of 0.1 mg/disk.

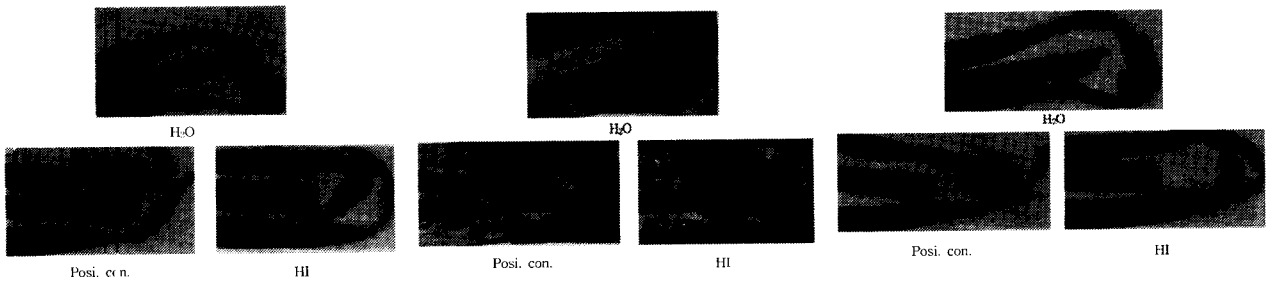


Fig. 3. Antifungal efficacy in the plant experiment.
*10 mg extracts through Extrelut column were sprayed on the disease-affected chive.
*Posi. Con.(positive control) slides show plants treated with Diethofencarb. Carbenda (gray mold fungicide)
*HI slides show plants treated with anti-fugal extracts through Extrelut column in the study.
*H_2O slides show plants treated with sterile water.

commercial purpose. We are also continuing a more in depth analysis of these unknown compounds.

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초록 : 한국산 무 추출물의 곰팡이 병균에 대한 항진균성

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국내산 무의 추출물을 여러가지 Column과 HPLC를 이용하여 잿빛곰팡이 병원균에대한 항 진균성 물질을 분리하였다. 투석튜브를 이용한 분리에서 이물질이 3.5kDa 이하의 저분자물질이며 또한 내열성 물질임을 확인하였다. 이물질들은 최소한 5종의 물질로 구성되어 있으며 이중 가장 항진균성이 강한 물질을 HPLC를 이용하여 순수 분리 하였으며 잿빛곰팡이병에 감염된 식물에 적용한결과 농약과 비슷한 효과를 나타내어 환경친화적인 항진균 물질로서 사용가능성을 확인하였다.