DOI:10.1021/jf8035337

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

# Isolation and Characterization of Juncin, an Antifungal Protein from Seeds of Japanese Takana (*Brassica juncea* Var. *integrifolia*)

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An 18.9 kDa antifungal protein designated juncin was isolated from seeds of the Japanese takana (*Brassica juncea* var. *integrifolia*). The purification protocol employed comprised anion-exchange chromatography on Q-Sepharose, affinity chromatography on Affi-gel blue gel, cation exchange chromatography on SP-Sepharose, and gel filtration on Superdex 75. Juncin was adsorbed on Affi-gel blue gel and SP-Sepharose but unadsorbed on Q-Sepharose. The protein exhibited antifungal activity toward the phytopathogens *Fusarium oxysporum*, *Helminthosporium maydis*, and *Mycosphaerella arachidicola* with $IC_{50}$ values of 13.5, 27, and 10 $\mu$M, respectively. It was devoid of mitogenic activity toward splenocytes and nitric oxide inducing activity toward macrophages. It inhibited the proliferation of hepatoma (HepG2) and breast cancer (MCF7) cells with $IC_{50}$ values of 5.6 and 6.4 $\mu$M, respecitvely, and the activity of HIV-1 reverse transcriptase with an $IC_{50}$ of 4.5 $\mu$M. Its N-terminal sequence differed from those of antifungal proteins that have been reported to date. Compared with *Brassica campestris* and *Brassica alboglabra* antifungal peptides, juncin exhibits a different molecular mass and N-terminal amino acid sequence but similar biological activities.

**KEYWORDS: *Brassica juncea*; seeds; purification; antifungal protein**

## INTRODUCTION

Plants produce a great diversity of defense proteins to protect themselves from predators such as insects and pathogens including fungi, viruses, and bacteria. These defense proteins comprise protease inhibitors (*1*), lectins (*2*), antifungal proteins (*3*), and antiviral proteins (*4*). Much effort has been dedicated to research on antifungal proteins because of the associated economic implications. Antifungal proteins are a large assemblage of structurally different proteins that comprise chitinases (*5*), chitinase-like proteins (*6*), chitin-binding proteins (*7*), glucanases (*8*), ribosome-inactivating proteins (*9*), protease inhibitors (*10*), defensins (*11*), defensin-like proteins (*12*), lipid transfer proteins (*13*), lectins (*5*), thaumatin-like proteins (*14*), embryo abundant protein-like proteins (*15*), cyclophilin-like proteins (*16*), miraculin-like proteins (*17*), peroxidases (*18*), and allergen-like proteins (*19*).

Most of the aforementioned antifungal proteins were isolated from seeds. Recently, an antifungal lipid transfer protein was purified and characterized from seeds of the vegetable *Brassica campestris* (*20*). It exhibited antiproliferative activity toward tumor cells and HIV-1 reverse transcriptase inhibitory activity (*21*). In view of the scant literature on *Brassica* antifungal proteins, the present study was initiated to ascertain if the seeds of another *Brassica* species, *B. juncea*, also could produce some antifungal protein.

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## MATERIALS AND METHODS

**Materials.** Seeds of *B. juncea* var. *integrifolia* were obtained from Kyuusyuu, Japan. The fungi used were provided by the Department of Microbiology, China Agricultural University, China. SP-Sepharose, Q-Sepharose, and Superdex 75 HR10/30 columns were from GE Healthcare (Hong Kong). Affi-gel blue gel was from Bio-Rad. Trizma base (98% purity), NaCl (99% purity), and $NH_4OAc$ (98% purity) were from Sigma Chemical Co., St. Louis, MO.

**Isolation of Antifungal Protein.** The seeds were homogenized in distilled water (8 mL/g), followed by centrifugation at 13000*g* at 4 °C. Tris-HCl buffer (pH 7.8) was added to the supernatant until the concentration of Tris attained 10 mM. The crude extract of *B. juncea* seeds was chromatographed on a 5 × 20 cm of Q-Sepharose in 10 mM Tris-HCl buffer (pH 7.8). Unadsorbed proteins (fraction Q1) were eluted with the same buffer. Adsorbed proteins were eluted with 10 mM Tris-HCl buffer (pH 7.8) containing 1 M NaCl. Fraction Q1 was applied on a 2.5 × 20 cm column of Affi-gel blue gel in 10 mM Tris-HCl buffer (pH 7.8). Unadsorbed proteins (fraction B1) were eluted with the same buffer. Adsorbed proteins (fraction B2) were eluted with 10 mM Tris-HCl buffer (pH 7.8) containing 0.2 M NaCl. Fraction B2 was subjected to ion exchange chromatography on a 2.5 × 30 cm column of SP-Sepharose, which had been equilibrated with and was then eluted with 10 mM $NH_4OAc$ buffer (pH 4.6). After unadsorbed proteins (fraction SP) had come off the column, the column was eluted with a linear gradient of 0−0.5 M NaCl formed by 390 mL of 10 mM $NH_4OAc$ buffer (pH 4.6)

 Published on Web 4/8/2009 

in one chamber of the gradient mixer to 390 mL of 10 mM $NH_4OAc$ buffer (pH 4.6) containing 0.5 M NaCl to yield fractions SP1−SP4. Fraction SP3 was dialyzed against water, lyophilized, redissolved, and subjected to a final purification step on a Superdex 75 HR 10/300 column in 0.02 M $NH_4HCO_3$ buffer (pH 8.5). The first peak constituted purified antifungal protein, which was designated juncin.

**Protein Determination.** Protein concentration was determined by the dye-binding method (Bio-Rad) using bovine serum albumin as a standard.

**Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE).** SDS-PAGE was conducted according to the method of Laemmli and Favre (*22*). After electrophoresis, the gel was stained with Coomassie Brilliant Blue (Bio-Rad). The molecular mass of the isolated antifungal protein was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins from GE Healthcare. SDS-PAGE markers from GE Healthcare included phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43k Da), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa).

**Mass Spectrometry.** Mass spectrometric (MS) analysis of juncin was performed on a Finnigan LCQ-MS, an instrument that essentially consists of an atmospheric pressure electrospray positive-ion source, attached to a triple-quadrupole mass analyzer. Juncin (100 pmol) was dissolved in water/methanol (50:50, v/v) containing 1% (v/v) acetic acid at a protein concentration of 5 mmol/L and then applied on the MS instrument. The *m*/*z* range covered was 1000−30000.

**N-Terminal Amino Acid Sequence Analysis.** The N-terminal amino acid sequence of the purified protein was performed by Edman degradation using a Hewlett-Packard amino acid sequencer (*23*).

**Assay of Antifungal Activity.** The assay of various chromatographic fractions and the purified antifungal protein for antifungal activity was executed using 100 mm × 15 mm Petri plates containing 10 mL of potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed around and at a distance of 1 cm away from the rim of the mycelial colony. An aliquot (8 μL containing 0.5 or 1 μg) of the purified protein in 20 mM PBS buffer (pH 6.0) was loaded on a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped peripheral disks containing the control (buffer) and had produced crescents of inhibition around disks containing samples with antifungal activity. The fungal species tested were *Fusarium oxysporum*, *Helminthosporium maydis*, and *Mycosphaerella arachidicola*.

To determine the $IC_{50}$ value for the antifungal activity of the isolated antifungal protein, three doses (10, 20, and 40 μM) of the protein were added separately to three aliquots each containing 4 mL of potato dextrose agar at 45 °C, mixed rapidly, and poured into three separate small Petri dishes. After the agar had cooled, a small amount of mycelia, the same amount by dry weight to each dish, was added. Buffer only without antifungal protein served as a control. After incubation at 23 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth determined from the following equation: % inhibition of growth = % reduction in area of colony = $(\text{area}_{\text{control}} - \text{area}_{\text{antifungal}}) \div \text{area}_{\text{control}} \times 100\%$, where $\text{area}_{\text{control}}$ = area of mycelial colony in the absence of antifungal protein and $\text{area}_{\text{antifungal}}$ = area of mycelial colony in the presence of antifungal protein. The concentration of the isolated antifungal protein that brought about 50% reduction in the area of mycelial colony is the $IC_{50}$ (*24*). Nystatin (Sigma 5010 USP units/mg) was used for comparison.

**Assay of Permeabilization of Hyphal Membrane.** This assay was performed by observing the uptake of SYTOX Green, a high-affinity nuclear stain that penetrates cells with compromised membranes as described by Thevissen et al. (*25*). Briefly, cultures of the yeast *Candida albicans* were grown in the presence or in the absence of juncin. SYTOX Green (Invitrogen) was added to the yeast cultures (various final concentrations up to 0.5 μM). After incubation for 10 min, yeast cells were observed under a fluorescence microscope (Nikon TE2000). Purple pole bean defensin was used as a positive control.

**Assay of Chitin Deposition at Hyphal Tips.** This assay was conducted as described by Moreno et al. (*26*) to observe chitin deposition at hyphal tips of *M. arachidicola*. Pregermination and incubation of fungal cultures were performed in 96-well microplates. Following incubation with juncin, Congo Red was added until a final concentration of 1 mM was reached. Fluorescence was examined 10 min later by confocal microscopy using an excitation wavelength of 543 nm and an emission wavelength of 560−635 nm. The tips of growing hyphae would not stain with Congo Red, whereas hyphal tips with inhibited growth would be stained. Purple pole bean defensin was used as a positive control.

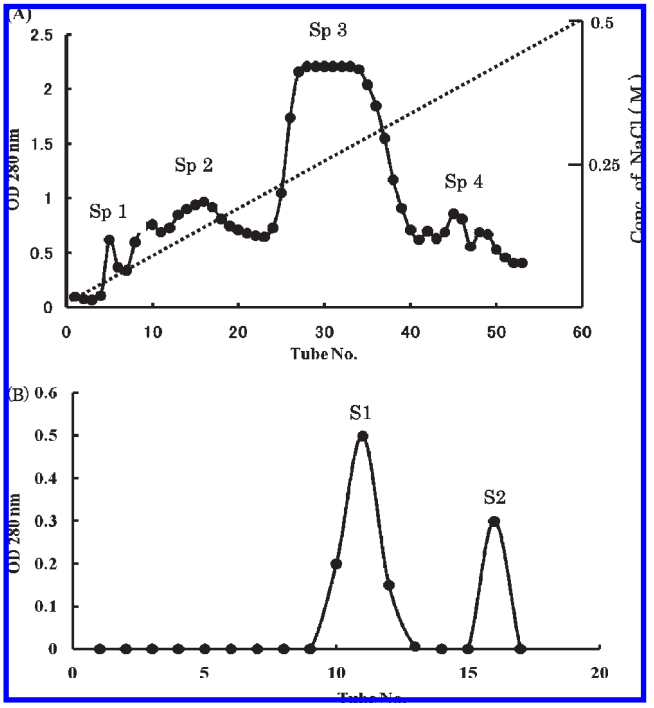


**Figure 1.** (**A**) Ion exchange chromatography on SP-Sepharose column. Sample was the fraction of seed extract unadsorbed on Q-Sepharose and subsequently adsorbed on Affi-gel blue gel. The slanting dotted line represents the linear NaCl concentration gradient used. Antifungal activity resided in SP3. Fraction size = 13 mL. (**B**) FPLC gel filtration of fraction SP3 on a Superdex 75 column. Antifungal activity resided in S1. Flow rate = 0.4 mL/min. Fraction size = 0.8 mL.

**Assay of Antiproliferative Activity on Tumor Cell Lines.** The assay was carried out in view of previous reports that some antifungal proteins have this activity (*3*). Breast cancer MCF-7 cells and hepatoma HepG2 cells were seeded to a well of a 96-well plate, followed by incubation for 24 h. Juncin was then added. After 72 h, 20 μL of a 5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added, and the plates were incubated for 4 h before centrifugation. The supernatant was removed, and dimethyl sulfoxide was added to dissolve the MTT−formazan at the bottom of the wells. After 10 min, the absorbance at 590 nm was measured (*3*). Doxorubicin was used for comparison.

**Assay for HIV-1 Reverse Transcriptase Inhibitory Activity.** The assay for HIV-1 reverse transcriptase inhibitory activity was carried out in view of previous reports that some antifungal proteins have this activity (*3*). It was conducted according to instructions supplied with the ELISA kit from Boehringer Mannheim (Germany). A fixed amount (4−6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of juncin was calculated as percent inhibition as compared to a control without the antifungal protein (*22*, *27*). *B. campestris* antifungal protein was used as a positive control.

**Assay of Mitogenic Activity on Mouse Spleen Cells and Assay of Nitric Oxide Inducing Activity toward Mouse Macrophages.** The assays were conducted as described by Wong et al. (*28*) and Wong and Ng (*12*) in view of the reports of these activities in some antifungal proteins (*28*, *29*). ConA was used a positive control in the mitogenic activity assay, and emperor banana lectin was used as a positive control in the nitric oxide induction assay.

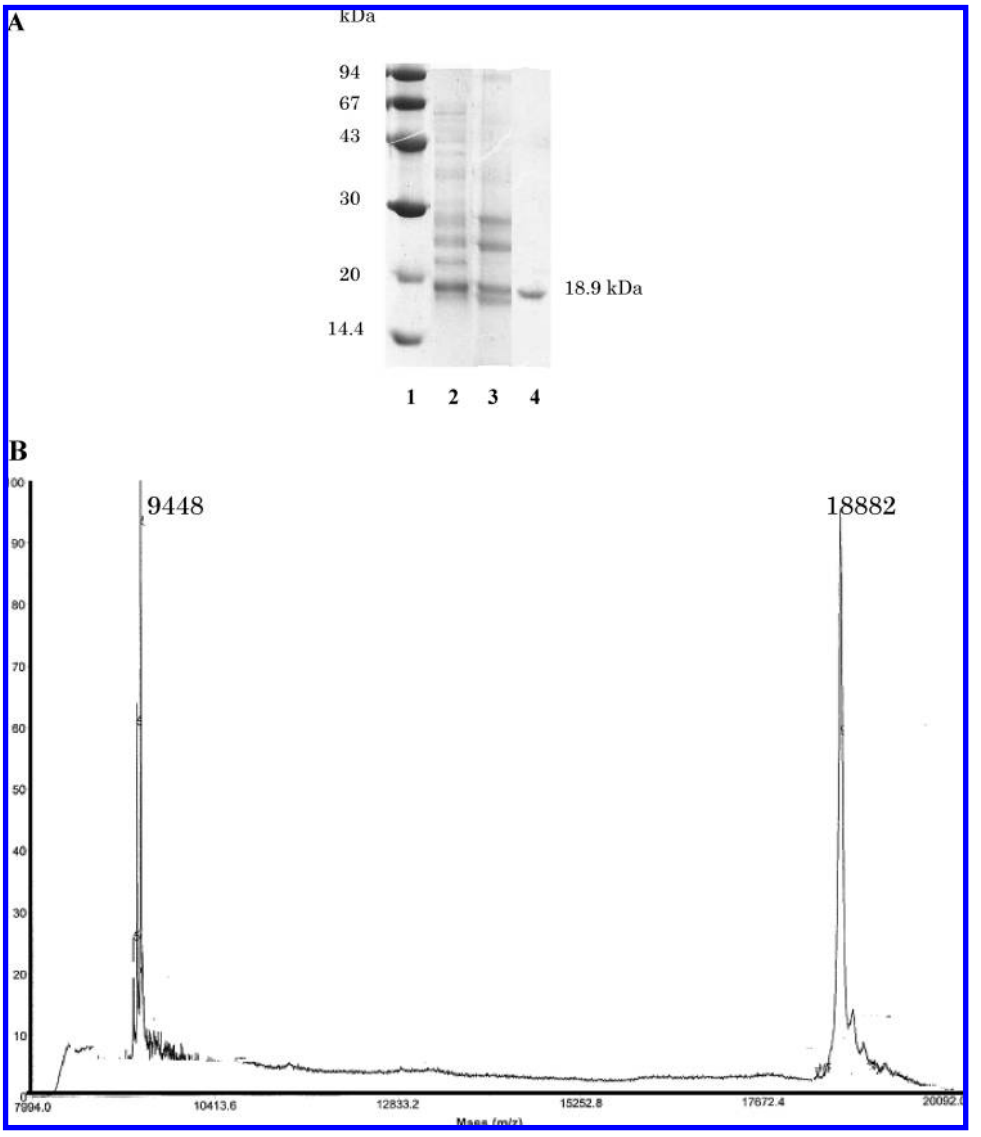


**Figure 2.** (**A**) SDS-PAGE results: lane 1, markers from GE Healthcare, which included phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa); lane 2, crude extract of *B. juncea* seeds; lane 3, fraction of seed extract unadsorbed on Q-Sepharose and subsequently adsorbed on Affi-gel blue gel and eluted from 0.2 M NaCl; lane 4, purified antifungal protein. (**B**) Mass spectrometry results showing that the molecular mass of *B. juncea* antifungal protein was 18.9 kDa.

**Assay of HIV-1 Integrase Inhibitory Activity.** The assay was conducted as described previously (*30*) in view of the reports of this activity in some antifungal proteins (*30*).

**Assay of Antifungal Activity on *C. albicans*.** *C. albicans* was incubated in 10 mL of potato medium in a thermal shaker for 6 h at 37 °C, and then 5 mL of this yeast suspension was transferred to 50 mL of potato medium and incubated for another 6 h to shift yeast growth to the midlogarithmic phase. Yeast suspension was then centrifuged at 2000*g* for 10 min, and the yeast pellet was washed and resuspended in normal saline. A total of $10^7$ yeast cells per milliliter was obtained by dilution guided by the optical density at 595 nm. The experiment was conducted in triplicate. The samples were then incubated in a shaker, and aliquots were obtained at 6 h, serially diluted with potato medium, and spread on agar plates. After incubation at 37 °C for 24 h, the colonies were counted. The number of yeast cells for each dilution was determined from the average colony counts for three plates.

## RESULTS AND DISCUSSION

The aqueous extract of *B. juncea* seeds (5500 mg/150 g of seeds) was first fractionated on Q-Sepharose. Antifungal activity resided in the fraction (1350 mg) unadsorbed on Q-Sepharose The adsorbed fraction was devoid of antifungal activity. The unadsorbed fraction was then resolved on Affi-gel blue gel into an unadsorbed fraction without antifungal activity and an adsorbed fraction (236 mg) with antifungal activity ($IC_{50}$ vs *M. arachidicola* = 7.7 ± 0.3 mg/mL, $n$ = 2). The latter fraction was chromatographed on SP-Sepharose to yield an unadsorbed fraction and a number of adsorbed fractions SP1−SP4 (**Figure 1A**). Antifungal activity ($IC_{50}$ = 4.1 ± 0.2 mg/mL, $n$ = 2) was detected only in the most prominent adsorbed fraction SP3 (123 mg). Fraction SP3 was resolved by gel filtration on a Superdex 75 column, resulting in two peaks (**Figure 1B**). The first peak (fraction S1, 12 mg) from the gel filtration column corresponding to a molecular mass of 18.9 kDa (**Figure 1B**) displayed a single 18.9 kDa band in SDS-PAGE (**Figure 2A**) and a molecular mass of 18.9 kDa in mass spectrometry (**Figure 2B**). The molecular mass of juncin falls in the range of molecular masses (from a few kilodaltons to >60 kDa) reported for other antifungal proteins (*3*, *6*, *9*, *11*, *16*, *17*, *21*, *22*, *24*, *27*). The chromatographic behavior of juncin on Affi-gel blue gel and ion exchangers is similar to that of other antifungal proteins (*3*, *6*, *9*, *11*, *16*, *17*, *21*, *22*, *24*, *27*, *34*) in that they are adsorbed on Affi-gel blue gel, unadsorbed on anion

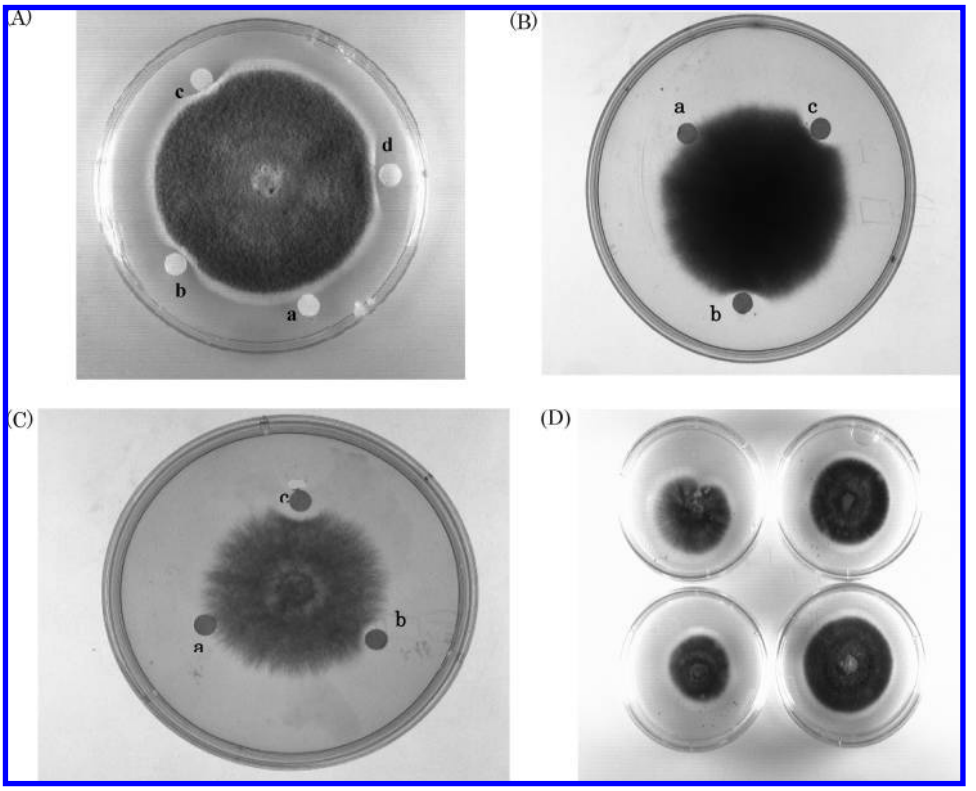


**Figure 3.** Inhibitory activity of antifungal protein isolated from *B. juncea* seeds toward (**A**) *M. arahidicola*, (**B**) *F. oxysporum*, and (**C**) *H. maydis*: (a) 10 $\mu$L of 20 mM Tris-HCl buffer; (b) 0.5 $\mu$g of antifungal protein in 10 $\mu$L of 20 mM Tris-HCl buffer (pH 7.2); (c) 1 $\mu$g of antifungal protein in 10 $\mu$L of 20 mM Tris-HCl buffer (pH 7.2); (d) 1.5 $\mu$g of antifungal protein in 10 $\mu$L of 20 mM Tris-HCl buffer (pH 7.2). (**D**) Determination of $IC_{50}$ value of antifungal activity of protein isolated from Japanese aso tanaka (*B. juncea* var. *integrifolia*) seeds toward *M. arachidicola*: lower right, control; upper right, 40 $\mu$M antifungal protein; upper left, 20 $\mu$M antifungal protein; lower left, 10 $\mu$M antifungal protein. $IC_{50}$ value was determined to be 10 $\mu$M.

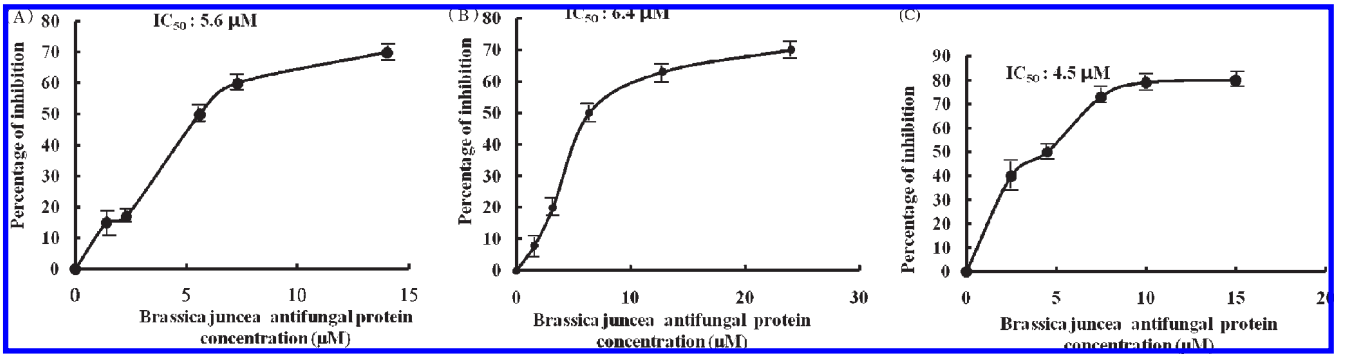


**Figure 4.** (**A**) Antiproliferative activity of *B. juncea* antifungal protein on HepG2 cell. Results are means ± SD ($n$ = 3). (**B**) Antiproliferative activity of *B. juncea* antifungal protein on MCF7 cells. Results are means ± SD ($n$ = 3). (**C**) HIV-1 reverse transcriptase inhibitory activity of *B. juncea* antifungal protein. Results are means ± SD ($n$ = 3).

exchangers, and adsorbed on cation exchangers. It inhibited mycelial growth in the fungi *M. arachidicola* (**Figure 3A**), *F. oxysporum* (**Figure 3B**), and *H. maydis* (**Figure 3C**), with $IC_{50}$ values of 10 $\mu$M (**Figure 3D**), 13.5 $\mu$M, and 27 $\mu$M, respectively (data not shown). In the assay, 5 $\mu$M purple pole bean defensin (positive control) could induce membrane permeabilization in *C. albicans* as evidenced by increased SYTOX Green when viewed under a fluorescence microscope. The hyphal tips of *M. arachidicola* were stained by Congo Red after treatment with 5 $\mu$M purple pole bean defensin, indicating inhibition of growth. However, antifungal protein isolated in this study yielded negative results, that is, no increase in uptake of SYTOX green and no difference in staining by Congo Red (data not shown). The mechanism of inhibition of fungal growth by juncin was probably by inhibition of protein synthesis in the fungus because some antifungal proteins have this activity (*16*).

The antifungal protein inhibited proliferation of Hep G2 (hepatoma) (**Figure 4A**) and MCF-7 (breast cancer) cells (**Figure 4B**) and the activity of HIV-1 reverse transcriptase (**Figure 4C**) with $IC_{50}$ values of 5.6, 6.4, and 4.5 $\mu$M, respectively. It was devoid of mitogenic activity toward mouse splenocytes, nitric oxide inducing activity toward macrophages and HIV-1 integrase inhibiting activity (data not shown). The yield of the antifungal protein was 12 mg from 150 g of seeds (**Table 1**). The protein did not resemble other antifungal proteins in N-terminal sequence. Instead, there was similarity to meprin (**Table 2**). The antifungal activities of nystatin toward *M. arahidicola*, *F. oxysporum*, and *H. maydis* were higer than those of the antifungal protein from *B. juncea* (**Table 3**). The number of *C. albicans* cells dropped from (110.6 ± 11.1) × $10^7$/mL in the negative control to (53.7 ± 3.2) × $10^7$/mL (mean ± SD, $n$ = 3) after treatment with juncin.

**Table 1.** Yields and Activities of Chromatographic Fractions Obtained during Purification of Antifungal Protein from *Brassica juncea* Seeds[a]

| fraction | total protein from 150 g of seeds (mg) | $IC_{50}$ of antifungal activity toward *M. arachidicola* (mg/mL) |
|---|---|---|
| extract | 5500 | 181 ± 4 |
| unadsorbed on Q-Sepharose | 1350 | not tested |
| adsorbed on Affi-gel blue gel | 236 | 7.7 ± 0.3 |
| Sp3 | 123 | 4 ± 0.2 |
| S1 | 12 | 0.2 ± 0.01 (=10 μM) |

[a] Data represent mean ± SD ($n$ = 2).

**Table 2.** N-Terminal Sequence of *Brassica juncea* Antifungal Protein in Comparison with Other Proteins (Results of BLAST Search)[a]

| | amino acid residue no. | | amino acid residue no. |
|---|---|---|---|
| *B. juncea* antifungal protein | 1 | GVEVTRELRSERPSGKIVTI | 20 |
| *B. campestris* antifungal peptide | 1 | ALSCGTVSGNLAACAGYV | 18 |
| meprin and TRAF homology domain-containing protein | 50 | —VTRELREERPSSKIVTI | 66 |
| *B. napus* trypsin inhibitor | 1 | SECLKEYGGDVGFGFCAPR | 19 |
| *B. alboglabra* antifungal peptide | 1 | PEGPFQGPKATKPGDLAXQT WGGWXGQTPKY | 31 |

[a] N-terminal sequences of *B. campestris* antifungal peptide and *B. alboglabra* antifungal peptide are from refs *20* and *34*.

**Table 3.** Comparison of Antifungal Activity between *Brassica juncea* Antifungal Protein and Nystatin[a]

| | *B. juncea* antifungal protein ($IC_{50}$, μM) | nystatin ($IC_{50}$, μM) |
|---|---|---|
| *Mycosphaerella arahidicola* | 10 ± 0.01 | 0.0025 ± 0.03 |
| *Fusarium oxysporum* | 13.5 ± 0.04 | 0.01 ± 0.05 |
| *Helminthosporium maydis* | 27 ± 0.05 | 0.02 ± 0.04 |

[a] Data represent mean ± SD ($n$ = 2).

From the seeds of *Brassica* species, napins and napin-like polypeptides have been purified (*23*, *31*−*33*). However, *B. campestris* lipid transfer protein (*21*) and *B. alboglabra* antifungal peptide (*34*) are the only *Brassica* antifungal proteins known to date. The results of the present investigation provide evidence for the production of an antifungal protein by another *Brassica* species. However, *B. juncea* antifungal protein (juncin) differs from its counterpart from *B. campestris* and *B. alboglabra* in many aspects. Juncin exhibits a molecular mass of 18.9 kDa, which is twice the molecular mass of *B. campestris* lipid transfer protein (*21*) and three times that of *B. alboglabra* antifungal peptide (*34*). The N-terminal sequence of juncin is homologous to a partial sequence of meprin and a TRAF homology domain-containing protein and distinctly different from that of *B. campestris* lipid transfer protein, which is strikingly similar to other lipid transfer proteins (*21*). *B. campestris* lipid transfer protein (*21*) and *B. alboglabra* antifungal peptide (*34*) inhibit the activity of HIV-1 reverse transcriptase and suppress proliferation of HepG2 hepatoma cells and MCF-7 breast cancer cells, all with an $IC_{50}$ values of $<6$ μM. Juncin exhibits these activities with $IC_{50}$ values of 4.5, 5.6, and 6.4 μM, respectively. Juncin, *B. campestris* lipid transfer protein (*21*), and *B. alboglabra* antifungal peptide (*34*) are devoid of HIV-1 integrase inhibitory activity. Juncin also lacks mitogenic and nitric oxide inducing activities. Because antifungal protein may or may not have HIV-1 reverse transcriptase inhibitory, antiproliferative, mitogenic, and nitric oxide inducing activities, it seems that there are different structural requirements for antifungal activity and the other aforementioned activities. A comparison of antifungal protein from *B. campestris* with juncin is presented in **Table 4**. The antifungal activity and antiproliferative activity of juncin toward MCF-7 cells are weaker than, but its antiproliferative activity toward HepG2 cells and HIV-1 reverse transcriptase inhibitory activity are similar to, those of *B. campestris* antifungal protein.

**Table 4.** Comparison of *Brassica juncea* Antifungal Protein with *Brassica campestris* Antifungal Protein Regarding Antifungal, Antiproliferative, and HIV-1 Reverse Transcriptase Inhibitory Activities

| | *B. juncea* antifungal protein ($IC_{50}$, μM) | *B. campestris* antifungal protein ($IC_{50}$, μM) |
|---|---|---|
| antifungal activity (toward *Fusarium oxysporum*) | 13.5 | 8.3 |
| antifungal activity (toward *Mycosphaerella arahidicola*) | 10 | 4.5 |
| antiproliferative activity on HepG2 cells | 5.6 | 5.8 |
| antiproliferative activity on MCF-7 cells | 6.4 | 1.6 |
| HIV-1 reverse transcriptase inhibitory activity | 4.5 | 4 |

Despite the aforementioned differences, juncin, *B. campestris* lipid transfer protein (*21*), and *B. alboglabra* antifungal peptide (*34*) inhibit mycelial growth in a number of fungal species including *F. oxysporum*, *M. arahidicola*, and a *Helminthosporium* species. Their chromatographic behaviors on Affi-gel blue gel, Q-Sepharose, and cationic exchangers are similar: they are adsorbed on Affi-gel blue gel and on cation exchanger and unadsorbed on Q-Sepharose.

When the results of the present study and a previous investigation (*21*) are taken together, the finding of two structurally disparate antifungal proteins produced by two closely related *Brassica* species is reminiscent of the situation in mungbean (*Phaseolus mungo*) protease inhibitor (*35*) and lipid transfer protein (*36*) and also in the circumstance of French bean (*Phaseolus vulgaris*) thaumatin-like protein (*37*) and peroxidase (*18*). These proteins all have antifungal activity. Similarly, *Allium cepa* (onion), *Allium sativum* (garlic), *Allium ascalonicum* (shallot), and *Allium tuberosum* (chive) produce structurally different antifungal proteins (*38*−*41*).

Some antifungal proteins can inhibit only one of several fungal species tested (*40*, *42*). It is noteworthy that juncin exerts a potent antifungal activity against all three fungal species studied. In summary, juncin is a potentially exploitable antifungal protein in view of its high antifungal potency. The mechanism of its antifungal activity awaits elucidation because, unlike plant defensins, it does not have membrane permeabilizing action on *C. albicans*. It does not affect chitin deposition in fungal hyphae either.

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**Received for Review November 14, 2008. Revised manuscript received February 20, 2009. Accepted March 27, 2009.**