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Kunitz-Type Serine Protease Inhibitor from Potato (*Solanum tuberosum* L. cv. Jopung)

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An antifungal protein, AFP-J, was purified from tubers of the potato (*Solanum tuberosum* cv. L Jopung) by various chromatographic columns. AFP-J strongly inhibited yeast fungal strains, including *Candida albicans*, *Trichosporon beigelii*, and *Saccharomyces cerevisiae*, whereas it exhibited no activity against crop fungal pathogens. Automated Edman degradation determined the partial N-terminal sequence of AFP-J to be NH_2-Leu-Pro-Ser-Asp-Ala-Thr-Leu-Val-Leu-Asp-Gln-Thr-Gly-Lys-G lu-Leu-Asp-Ala-Arg-Leu-. The partially sequence had 83% homology with a serine protease inhibitor belonging to the Kunitz family, and the protein inhibited chymotrypsin, pepsin, and trypsin. Mass spectrometry showed that its molecular mass was 13 500.5 Da. This protease inhibitor suppressed over 50% the proteolytic activity at 400 μg/mL. These results suggest that AFP-J is an excellent candidate as a lead compound for the development of novel antiinfective agents.

KEYWORDS: Antifungal protein J (AFP-J); potato; serine protease inhibitor; Kunitz family

INTRODUCTION

Plants and animals are in close contact with diverse bacteria and fungi, but only in rare cases does this association result in the development of disease, mostly because of the existence of antimicrobial defense systems. Although these defense mechanisms vary considerably among different types of organisms, such as the lack of an adaptive immune response in plants, recent evidence suggests that defense strategies of plants and animals share common features, including the use of a wide variety of small antimicrobial proteins as effector molecules of nonspecific or innate immunity (*1*−*2*). Plants produce several types of proteins that mediate defense against pathogens and invading organisms, including ribosome-inactivating proteins (*3*), lectins (*4*), protease inhibitors (*5*), and antifungal proteins (*6*).

Protease inhibitors are ubiquitously abundant in tubers and plant seeds (*7*). Several gene families of protease inhibitors have been characterized in higher plants, especially the serine protease inhibitors from Leguminosae, Solanaceae, and Graminae (*8*). Protease inhibitors in plants are generally considered to act as storage proteins (nitrogen source) and as a defense mechanism (*9*). They have recently received renewed interest because of their ability to potently inhibit carcinogenesis in a wide variety of in vivo and in vitro systems (*10*).

Here, we describe the purification and characterization of antifungal protein J (AFP-J) from potato tubers. AFP-J has inhibitory activity against human pathogenic fungal strains and shows sequence homology with a protease inhibitor that is produced by potato tubers upon fungal attack (*11*). Finally, we characterized the protease inhibitor activity of AFP-J and determined its molecular mass by mass spectrometry.

MATERIALS AND METHODS

Potato Tubers. Potato tubers (*Solanum tuberosum* L. cv. Jopung) were obtained from the Natural Institute of Highland Agriculture (Kangwon-do, Korea) and were stored at 4 °C in the dark at a relative humidity of 60% for up to 6 months to use in experiments.

Purification and Characterization of AFP-J. Potato tubers were first soaked in distilled water for a few hours and then ground to a fine powder in a coffee grinder, and protein extraction buffer [50 mM Tris-HCl, pH 7.5, 10 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 1% dimethyl sulfoxide (DMSO), and 0.1% β-mercaptoethanol] was added. The supernatant was then separated by chromatography on a Sephacryl S-100 gel-filtration column (2.5 × 95 cm) in 50 mM ammonium bicarbonate buffer (pH 8.0), followed by fast protein liquid chromatography (FPLC) on a Superdex 200 prep-grade column with the same buffer. The purity and molecular weight of the fraction with antifungal activity were estimated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on a 15% polyacrylamide gel according to the method of Laemmli and Favre (*12*).

Fungal Strains. *Saccharomyces cerevisiae* (KCTC 7296), *Trichosporon beigelii* (KCTC 7707), *Candida albicans* (TIMM 1768), *Colletotrichum gloeosporioides* (KACC 40003), *Colletotrichum coccodes* (KACC 40803), and *Didymella bryoniae* (KACC 40669) were obtained from the Korean Collection for Type Cultures (KCTC), Teikyo University Institute of Medical Mycology (TIMM), and Korean Agricultural Culture Collection (KACC).

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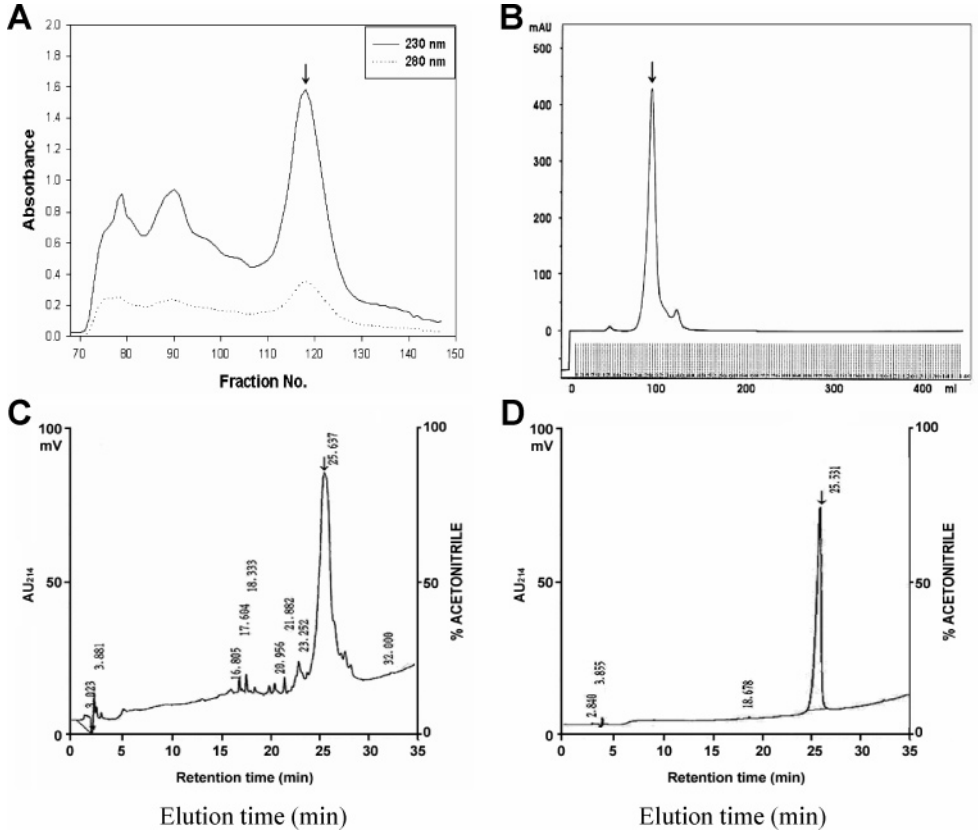

Figure 1. Elution profile of AFP-J. (**A**) Sephacryl S-100 column chromatography. Proteins were eluted with 50 mM ammonium bicarbonate buffer (pH 8.0). Fractions with antifungal activity were pooled and concentrated. (**B**) A 5-mg sample of the concentrated, pooled protein was separated by FPLC on a Superdex 200 prep grade column (flow rate = 0.5 mL/min; fraction size = 3 mL). (**C**) The fraction containing the major retained peak was separated by reversed-phase HPLC on a C_{18} column. (**D**) The purified antifungal protein was again subjected to reversed-phase HPLC on a C_{18} column. In panels **C** and **D**, the proteins were eluted with a linear gradient (2% increase/min) of acetonitrile (10–95%) containing 0.1% trifluoroacetic acid at a flow rate of 1 mL/min, and the effluent was monitored at 230 nm.

Assay for Antifungal Activity. Microdilution assays to establish minimal inhibition concentration (MIC) values of AFP-J were performed. *S. cerevisiae*, *T. beigelii*, and *C. albicans* were grown at 28 °C in YPD (2% dextrose, 1% peptone, and 0.5% yeast extract, pH 5.5) for 3 h. Cell densities were counted with a hemocytometer. The fungal cells (2×10^3/well) were seeded on the wells of a flat-bottom 96-well microtiter plate (Greiner, Nurtingen, Germany) containing YPD (100 μL/well). Serial dilutions of AFP-J solution were added to each well, and the cell suspension was incubated at 28 °C for 24 h. Ten microliters of a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution (5 mg/mL) was added to each well, and the plates were incubated at 37 °C for 4 h (*13*). The absorbance at 570 nm was measured on an Emax microtiter plate reader (Molecular Devices). All assays were performed in triplicate. To visualize the fungicidal effect, morphological changes were examined by phase-contrast light microscopy on an Eclipse TE300 microscope (Nikon, Japan).

The assay for antifungal activity against *C. gloeosporioides*, *C. coccodes*, and *D. bryoniae* was carried out in 100 × 15 mm Petri dishes containing YPD. After the mycelial colony had developed, sterile blank paper disks (8 mm diameter) were placed 5 mm away from the leading edge of the mycelial colony. An aliquot of the test sample in MES buffer (20 mM, pH 6.0) was added to each disk, and the plates were incubated at 28 °C for 72 h. Antifungal activity was shown as a clear zone of growth inhibition.

Preparation of Human Red Blood Cells and Assay of Hemolytic Activity. Human red blood cells were centrifuged and washed three times with phosphate-buffered saline (PBS; 35 mM phosphate buffer with 0.15 M NaCl, pH 7.0). The hemolytic activities of the AFP-J and melittin (positive control) were evaluated by measuring the release of hemoglobin from fresh human erythrocytes. Aliquots (100 μl) of an 8% suspension of red blood cells were transferred to 96-well plates, and hemolysis was determined by measuring the absorbance at 414 nm on an Emax plate reader. No hemolysis (0%) and full hemolysis (100%) were determined in PBS and 0.1% Triton X-100, respectively. The percent hemolysis was calculated from the following equation: % hemolysis = [(Abs_{414nm} with peptide solution − Abs_{414nm} in PBS)/(Abs_{414nm} with 0.1% Triton-X 100 − Abs_{414nm} in PBS)] × 100.

Mass Spectrometry. MALDI-MS (matrix-assisted laser desorption ionization mass spectrometry) analysis was performed in the linear mode on a Voyager DE RP instrument (PerSeptive Biosystems, Framingham, MA) as described by Pouvreau et al. (*14*).

N-Terminal Amino Acid Sequencing. For protein sequencing, AFP-J was separated by SDS−PAGE on a 15% polyacrylamide gel in the presence of 2-mercaptoethanol and then transferred onto a poly(vinylidene difluoride) membrane (Bio-Rad, Hercules, CA). The protein band was identified by staining with Coomassie brilliant blue, destained with 10% acetic acid, and then excised. The N-terminal amino acid sequence was determined from the excised band by the Sequence Centre of Korea Basic Science Institute (Seoul, Korea).

Measurement of Trypsin, Pepsin, and Chymotrypsin Inhibitory Activities. A portion of the inhibitor was incubated for 5 min at 25 °C with 25 mg of trypsin, pepsin, or chymotrypsin in 100 mL of 50 mM Tris-HCl buffer (pH 8.0) containing 200 mM $CaCl_2$. Residual trypsin, pepsin, or chymotrypsin activity was determined by adding 300 mL of 1% casein (w/v) and incubating at 25 °C. The reaction was terminated after 15 min by adding 1 mL of cold 5% trichloroacetic acid. The reaction mixtures were centrifuged for 20 min at 32000*g*. The absorbance of the supernatant was determined at 280 nm.

RESULTS AND DISCUSSION

Chromatography of the potato tuber extract on Sephacryl S-100 yielded two unadsorbed peaks and one large adsorbed

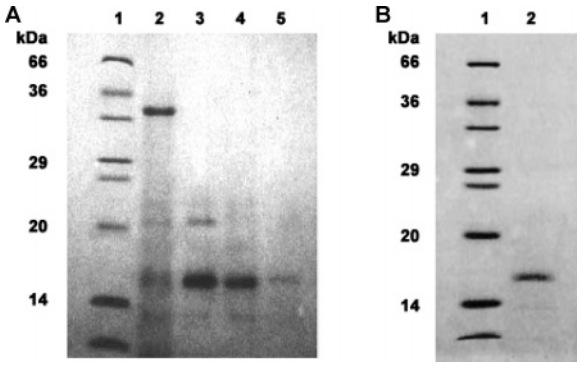

Figure 2. SDS–PAGE of purified AFP-J from potato tubers (*Solanum tuberosum* L. cv. Jopung) at various steps. (**A**) Lane 1, molecular size marker; lane 2, total protein; lane 3, after Sephacryl S-100 gel-filtration column chromatography; lane 4, after Superdex 200 prep-grade FPLC column chromatography; lane 5, after C_{18} reversed-phase HPLC (AFP-J). (**B**) Lane 1, molecular size markers; lane 2, purified AFP-J.

peak with antifungal activity (**Figure 1A**). The large adsorbed peak was then fractionated by FPLC on a Superdex 200 prep-grade column. This produced a large adsorbed peak and several small unadsorbed peaks (**Figure 1B**). The large absorbed peak, which we refer to as AFP-J, was then purified by two steps of C_{18} reversd-phase high-performance liquid chromatography (HPLC) (**Figure 1C,D**). The large adsorbed peak contained a single protein with an approximate molecular mass of 15 kDa on the basis of SDS−PAGE (**Figure 2**).

We next examined the antifungal activity of AFP-J against various human and plant pathogenic fungi using MTT and paper disk assays (**Table 1**). AFP-J had potent antifungal activity against the human pathogenic fungi *C. albicans*, *T. beigelli*, and *S. cerevisiae*. As shown in **Figure 3**, light microscopy confirmed

Table 1. Antifungal Activities of Purified Protein against Fungal Strains[a]

	fungal strains	MIC (μg/mL)
human pathogenic	T. beigelii	6.25
	S. cerevisiae	6.25
	C. albicans	6.25
plant pathogenic	C. gloeosporioides	>100
	C. coccodes	>100
	D. bryoniae	>100

[a] Microdilution assays to establish minimal inhibition concentration (MIC) values of AFP-J were performed. The fungal strains were grown at 30 °C in YPD medium. The fungal cells were inoculated on the wells of a 96-well microtiter plate of YPD medium at a density of 2×10^3 cells (100 μL/well). The antifungal effects were detected by MTT assay. The turbidity of each well was measured at 570 nm by use of a microtiter ELISA reader.

Table 2. Hemolytic Activities of Antimicrobial Protein AFP-J[a]

	% hemolysis							
peptide	25 μg	12.5 μg	6.25 μg	3.13 μg	1.56 μg	0.78 μg	0.39 μg	0.19 μg
AFP-J	0	0	0	0	0	0	0	0
melittin	100	100	100	95	93	31	0	0

[a] The hemolytic activities of AFP-J and melittin (positive control) were evaluated by measuring the release of hemoglobin from fresh human erythrocytes. No hemolysis (0%) and full hemolysis (100%) were determined in PBS and 0.1% Triton X-100, respectively. The percent hemolysis was calculated from the following equation: % hemolysis = $[(Abs_{414nm}$ with peptide solution $- Abs_{414nm}$ in PBS)/$(Abs_{414nm}$ with 0.1% Triton-X 100 $- Abs_{414nm}$ in PBS)$] \times 100$.

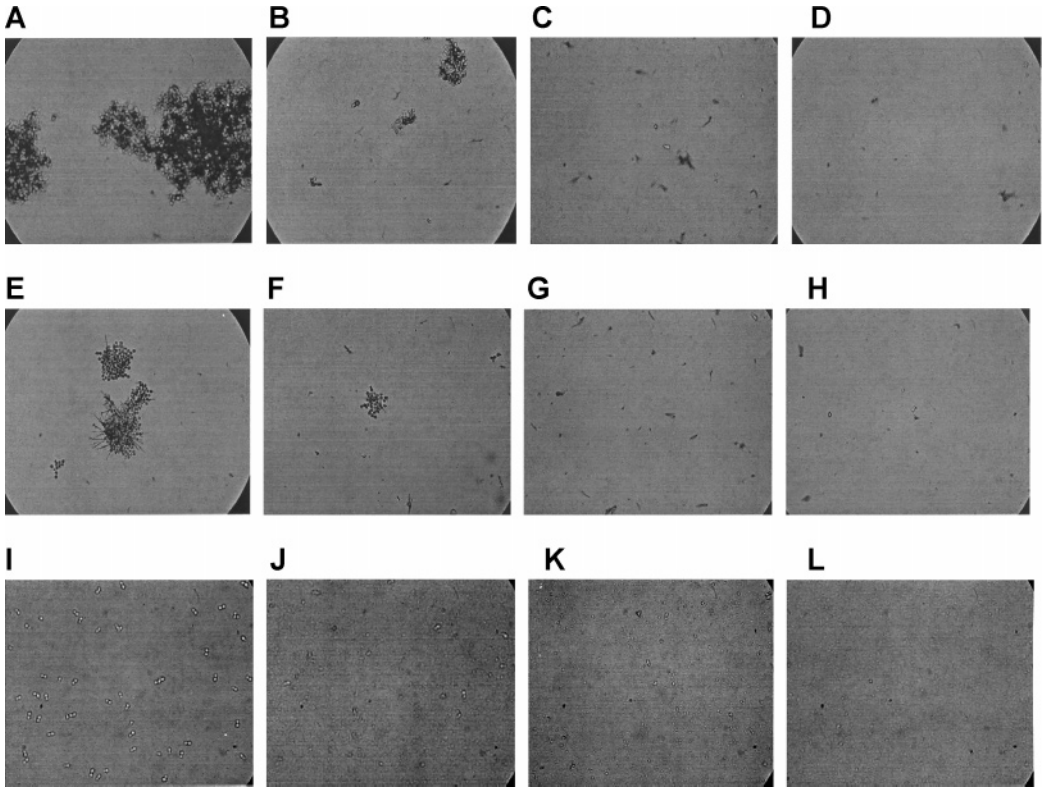

Figure 3. Antifungal activity of purified proteins against various fungal strains. Yeast cells were suspended at approximately 2×10^3/mL in YPD. Dilutions of the peptide were added, and reaction mixture was incubated for 24 h at 30 °C. Top row: *T. beigelii* cells were treated with 0 (**A**), 1.56 (**B**), 6.25 (**C**), or 25 (**D**) μg of AFP-J. Middle row: *S. cerevisiae* cells were treated with 0 (**E**), 1.56 (**F**), 6.25 (**G**), or 25 (**H**) μg of AFP-J. Bottom row: no fungal cells in the presence of 0 (**I**), 1.56 (**J**), 6.25 (**K**), or 25 (**L**) μg of AFP-J.

Figure 4. Alignment of partial amino acid sequences of AFP-J from potato tuber with a Kunitz-type protease inhibitor and a serine protease inhibitor.

that AFP-J strongly inhibited the growth of *C. albicans*, *T. beigelli*, and *S. cerevisiae* but that it did not have a noticeable inhibitory effect on the plant pathogenic fungi *C. gloeosporioides*, *C. coccodes*, and *D. bryoniae* (**Table 1**).

We further examined the cytotoxicity of AFP-J against mammalian cells by measuring the lysis of human erythrocytes. AFP-J showed no hemolytic activity, whereas melittin, used as a positive control, was strongly hemolytic (**Table 2**). These results demonstrated that AFP-J has a remarkable antifungal activity against human pathogenic fungi but no hemolytic activity.

AFP-J had a partial N-terminal amino acid sequence of NH_2-Leu-Pro-Ser-Asp-Ala-Thr-Leu-Val-Leu-Asp-Gln-Thr-Gly-Lys-Glu-Leu-Asp-Ala-Arg-Leu-. Using this sequence for a homology search, we found that it was 83% identical to potato serine protease inhibitor (**Figure 4**), which was purified and sequenced by Valueva et al. (*15*) and is a dimeric protein belonging to the Kunitz superfamily of protease inhibitors. It therefore appears that AFP-J from *S. tuberosum* L. cv. Jopung is also a member of the Kunitz family.

The relative molecular weight of AFP-J was 13 500.50, directly determined by MALDI-MS (**Figure 5**).

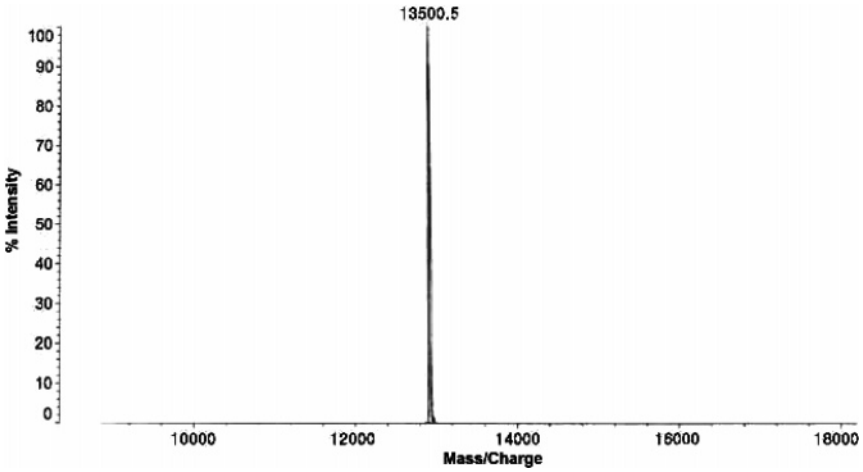

Figure 5. MALDI-MS (matrix-assisted laser desorption ionization mass spectrometry) determination of AFP-J molecular mass.

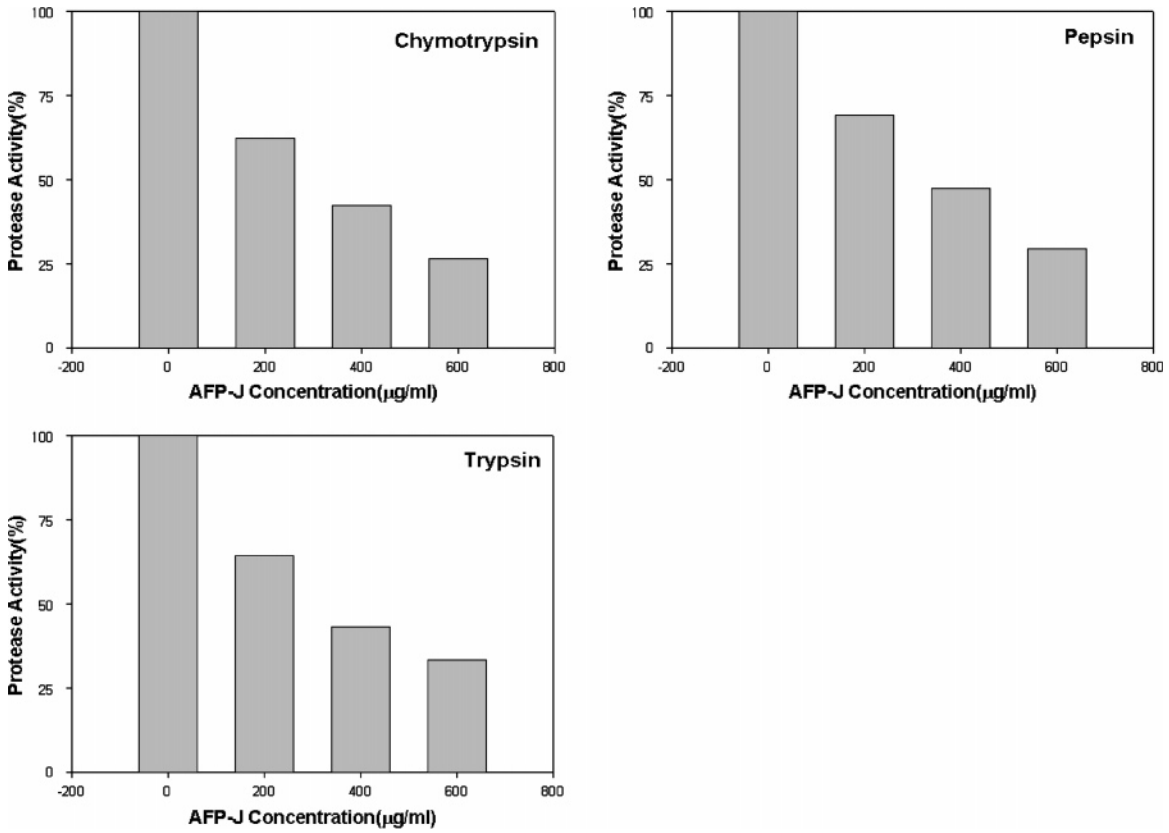

Figure 6. Inhibition of selected proteases by AFP-J. Chymotrypsin, pepsin, and trypsin were assayed in the presence of 0, 200, 400, or 600 μg/mL purified AFP-J. Results are shown as the percentage of protease activity in the absence of AFP-J.

We tested AFP-J for its ability to inhibit chymotrypsin, pepsin, and trypsin. A typical result is presented in **Figure 6**, showing that AFP-J dose-dependently inhibited all three serine proteases. Collectively, these results show that the potent antifungal protein, AFP-J, is a serine protease inhibitor.

The protease inhibitor must be nontoxic to humans and must be capable of inhibiting each of the major intestinal proteases, including pancreatic trypsin, α-chymotrypsin, and elastase, in order to be useful as a drug candidate. Several potentially nontoxic protease inhibitors, mostly of bacterial or plant origin, for example, from barley seeds, cabbage leaves, and *Streptomyces*, have been purified and are commercially available in preventing protease-induced perianal dermatitis (*16*).

A 2S-albumin-like protease inhibitor from barley seeds was reported to have some antifungal activity (*17*). This protein acted synergistically with thionins to permeabilize fungal membranes (*18*). Trypsin and chymotrypsin inhibitors from cabbage leaves have also been shown to induce the leakage of intracellular contents from susceptible fungal species (*19*). In addition, a 14-kDa trypsin inhibitor from corn has been reported to retard the growth of *Aspergillus flavus.* This inhibitor was present at high concentrations in *Aspergillus*-resistant genotypes and is either at low levels or absent in *Aspergillus*-susceptible genotypes (*20*). Chen et al. (*21*) demonstrated that the resistance of certain corn genotypes to fungal infection might be related to the ability of a trypsin inhibitor to decrease the production and activity of fungal α-amylase, consequently reducing the availability of simple sugars for fungal growth. Similarly, an alkaline serine proteinase inhibitor from barley (*22*), a 28-kDa alkaline protease inhibitor from *Streptomyces* (*23*), a 24-kDa cysteine protease inhibitor from pearl millet (*24*), and the HIV-1 protease inhibitors (*25*) have been shown to exhibit antifungal activity. Antileukoprotease, a protease inhibitor in mucosal surfaces, including those of the respiratory and genital tracts, exerts antifungal activity (*26*) and may present a new therapeutic option against fungal infections in these tissues (*27*). Finally, potato tuber proteins have been shown to efficiently inhibit human fecal proteases, and these could be useful in the treatment of perianal dermatitis (*16*).

AFP-J has potent antifungal activity against human fungal pathogens. In this study, we examined the effect on *C. albicans*, which is the most common cause of oral, esophageal, vaginal, and urinary (*28*) candidiasis, and *T. beigelli*, which is found in soil and occasionally is part of the normal flora in human skin (*29*). In immunocompromised individuals, *T. beigelli* can cause white piedra, an innocuous superficial hair shaft infection, and although rare, it can cause disseminated trichosporonosis in these individuals (*29*). Therefore, like others in this class of protease inhibitors, AFP-J may have potential therapeutic use.

In summary, we isolated AFP-J from the potato tuber (*S. tuberosum* L. cv. Jopung). AFP-J inhibits the growth of human pathogenic fungi cells but has no hemolytic activity. Because of this potent antifungal activity and lack of activity against eukaryotic cells, AFP-J may be a useful candidate for development as a therapeutic antibiotic.

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