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Purification and characterization of a chitinase-like antifungal protein from black turtle bean with stimulatory effect on nitric oxide production by macrophages

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Abstract

An antifungal protein was isolated from black turtle bean, *Phaseolus vulgaris* cv. 'Black Turtle'. The purified protein displayed an N-terminal amino acid sequence with 60–80% homology to chitinases. The isolated protein specifically inhibited two species of fungi, namely *Fusarium oxysporum* and *Mycosphaerella arachidicola*, among several phytopathogenic fungi tested. Its antifungal activity was retained after incubation at 60°C for 15 minutes, diminished after exposure to 80°C, and was completely abrogated after treatment at 100°C. The antifungal protein had higher thermostability than the antifungal protein mollisin from chestnut. It stimulated nitric oxide production by murine peritoneal macrophages, but exerted neither mitogenic nor cytotoxic activity on murine splenocytes, activities that may be related to allergic responses.

Keywords: black turtle beans; chitinase-like antifungal protein; macrophage; nitric oxide production.

Introduction

A variety of antifungal proteins and peptides, involved in plant defense and belonging to the family of pathogenesis-related proteins, has been isolated from leguminous plants. The list comprises cyclophilin-like proteins (Ye and Ng, 2002), chitinases and chitinase-like proteins (Vogelsang and Barz, 1993; Ye et al., 2000), glucanases (Vogelsang and Barz, 1993), thaumatin-like proteins (Chu and Ng, 2003), and trypsin inhibitors (Ye et al., 2001). The aforementioned types of antifungal proteins may exert different potencies toward different fungal species (Ye et al., 2000, 2001; Ye and Ng, 2002; Chu and Ng, 2003)

The black turtle bean used in this study is a common dietary component, well known as a source of nutrients and proteins. The seed coat of *Phaseolus vulgaris* contains a variety of compounds with antioxidant activity (Beninger and Hosfield, 2003). Diets containing extracts of *Phaseolus vulgaris* seeds were reported to have hypoglycemic and hypolipidemic effects. The blood glucose level was also significantly reduced in both diabetic and normal rats fed on an aqueous extract of *Phaseolus vulgaris* seeds (Pari and Venkateswaran, 2003).

In this study, a chitinase-like antifungal protein was isolated from a commonly consumed legume, the black turtle bean. Its biochemical characteristics, antifungal and immunomodulatory effects were investigated and compared with other antifungal proteins where appropriate.

Results

A large unadsorbed peak (DE1) with antifungal activity and a smaller adsorbed peak (DE2) devoid of antifungal activity were obtained when the bean extract was subjected to ion exchange chromatography on DEAE-cellulose. DE1 was fractionated by affinity chromatography on Affi-gel blue gel, yielding a large unadsorbed peak (BG1) without antifungal activity and a very small adsorbed peak (BG2) with antifungal activity. BG2 was further resolved by ion exchange chromatography on CM-cellulose, yielding a small inactive unadsorbed peak (CM1) and two adsorbed peaks (CM2 and CM3). CM2 was much smaller than CM3 and was the only peak with antifungal activity. CM2 yielded essentially three peaks upon FPLC on a ceramic hydroxyapaptite column, the second of which (HA2) possessed antifungal activity, while the first (HA1) and third (HA3) peaks were devoid of activity. Fraction HA2 was further subjected to gel filtration by FPLC on a Superdex 75 HR 10/30 column and a major peak (SU) was then eluted (Figure 1). The yields of the various chromatographic fractions throughout the purification procedure from 200 g of black turtle bean were: 8450 mg of crude extract; 5850 mg of fraction DE1; 498 mg of fraction BG2; 69 mg of fraction CM2; 3.7 mg of fraction HA2; and 2.7 mg of peak SU from Superdex 75, representing purified antifungal protein. The molecular mass of purified protein (peak SU) was 28 kDa, as determined by both sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2) and gel filtration. The N-terminal sequence of the purified protein in peak SU is presented in comparison with other plant antifungal proteins in Table 1. Sequence similarities are noted. The antifungal activity of the purified protein toward various fungal species is shown in Figure 3. The antifungal activity of the isolated protein was fully preserved after heating at up to 60°C for 15 minutes, declined after incubation at 80°C for 10 minutes, and was indiscernible after boiling for 15 minutes (Figure 4). The concentration for 50% inhibition (IC_{50}) values of the antifungal activity of purified black turtle bean antifungal protein against *M. arachidicola* and *F. oxysporum* were 18.3 μM and 35.6 μM, respectively. The inhibitory activity of the purified protein toward *M. arachidicola* was more potent than that toward *F. oxysporum*. The isolated anti-

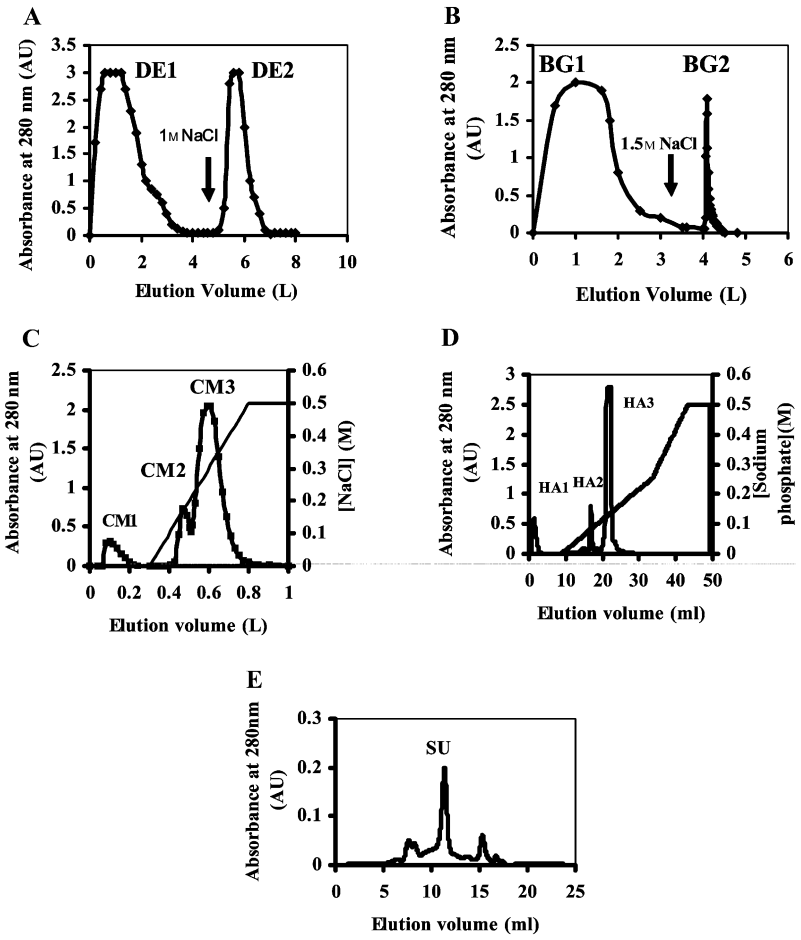

Figure 1 Purification of the antifungal protein from black turtle beans.
(A) Chromatography of the crude bean extract on a DEAE-cellulose column (flow rate, 4.5 ml/min; column size, 5×15 cm). (B) Affinity chromatography of the unadsorbed fraction DE1 from the DEAE-cellulose column on an Affi-gel blue gel column (flow rate, 3.8 ml/min; column size, 2.5×18 cm). (C) Ion exchange chromatography of fraction BG2 from Affi-gel blue gel column on a CM-cellulose column (flow rate, 4 ml/min; column size, 2.5×18 cm). (D) Chromatography of fraction CM2 from the CM-cellulose column on an FPLC-ceramic hydroxyapaptite column (flow rate, 2 ml/min; column size, 1×6.4 cm). (E) Gel filtration of fraction HA2 from the ceramic hydroxyapatite column on an FPLC-Superdex 75 column. The major peak SU was the purified antifungal protein. The column was calibrated and its elution volume of 11 ml corresponded to a molecular mass of 28 kDa (flow rate, 0.4 ml/min; column size, 1×30 cm).

fungal protein in the concentration range of 0–50 μM did not elicit either mitogenic or cytotoxic activity on murine splenocytes *in vitro* (data not shown). However, the isolated black turtle bean antifungal protein augmented nitric oxide (NO) production by murine peritoneal macrophages in a dose-dependent manner (Figure 5). The stimulation of nitric oxide production was reduced after the addition of dexamethasone and N-ω-nitro-L-arginine methyl ester (L-NAME) to the macrophages. However, the nitric oxide level remained when the purified protein was pretreated with polymyxin B (Table 2).

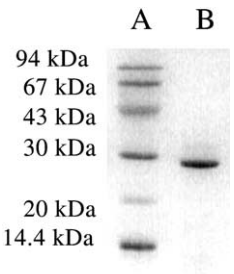

Figure 2 SDS-PAGE analysis of peak SU.
Molecular mass markers of lane A from top to bottom: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa).

Discussion

The antifungal protein isolated from black turtle beans showed certain resemblances in chromatographic behavior to its counterparts from other seeds, for example, mollisin from chestnuts (Chu and Ng, 2003) and chrysancorin from garland chrysanthemum seeds (Wang et al., 2001). It was not adsorbed by DEAE-cellulose but adsorbed on CM-cellulose, Affi-gel blue gel, and hydroxyapatite resin, which has previously not been commonly deployed in purifying antifungal proteins. The sequence of its first 19 N-terminal amino acid residues showed some similarity to chitinases from various plant species, for instance, field bean (Ye et al., 2000), potato (Gozia et al., 1993) and tobacco (Ponstein et al., 1994). The chitinase-like protein isolated was judged to be an antifungal protein by its potent inhibitory activity on mycelial growth. Many other plant defense proteins, such

Table 1 N-Terminal sequence of black turtle bean antifungal protein compared with other antifungal proteins.

Protein	Residue number	N-terminal amino acid sequence	Residue number	Identity (%)	Total number of residues
Turtle bean antifungal protein, *Phaseolus vulgaris* cv. Black Turtle	1	GPVGSVISASLFEQLIFHRN	20	100	
Chitinase, *Musa acuminate* (CAC81812)	74	GSVGSIISSSLFEQMLKHRN	93	70	317
Cotyledonous yieldin-like protein, *Vigna unguiculata* (BAC53632)	30	VGSVIGASLFDQLLKHRN	47	70	269
Chitinase precursor, *Canavalia ensiformis* (CAA07413)	30	VGSVIDASLFDQLLKHRN	47	77	270
Chain A, structure of the chitinase from Jack bean (1DXJ_A)	2	VGSVIDASLFDQLLKHRN	19	77	242
Chitinase class I, rice, *Oryza sativa* (CAA82850)	70	GSVASIISPSLFDQMLLHRN	89	60	308
Endochitinase, wheat, *Triticum aestivum* (CAA53626)	79	VSSIISQSLFDQMLLHRN	96	65	320
Class I chitinase potato, *Solanum tuberosum* (AAF25602)	73	GPSGGDIGDVISNSMFDQLLMHRN	96	54	329
Chitinase-like thermal hysteresis protein, *Solanum dulcamara* (AAP32201)	18	GSVISNSMFDQMLKHRN	34	64	267
Acidic class I chitinase, maize (T03026)	74	VASIIPESLFNQMLLHRN	91	55	318
Endochitinase precursor, *Humulus lupulus* (AAD34596)	76	VSSVISSALFEEMLKHRN	93	61	316
Basic chitinase, tobacco, *Nicotiana tabacum* (AAB23374)	80	GSIISSSMFDQMLKHRN	96	58	328
Chitinase, tomato, *Lycopersicon esculentum* (CAA78845)	73	GGVISNSMFDQMLNHRN	89	58	322

Identical corresponding amino acid residues are underlined. The BLAST accession numbers of the sequences are indicated in brackets.

as thaumatin-like proteins (Chu and Ng, 2003) and trypsin inhibitors (Ye et al., 2001), were also reported to possess antifungal activity. The protein isolated exhibited specific inhibition of fungal growth. Among the five kinds of phytopathogenic fungi tested, it demonstrated potent antifungal activity on *Fusarium oxysporum* and *Mycosphaerella arachidicola*, but was inactive on *Botrytis cinerea*, *Rhizoctonia solani* and *Physalospora piricola*. On the contrary, dolichin, a chitinase-like protein from the field bean *Dolichos lablab* (Ye et al., 2000), and pisumin from sugar snap (Ye and Ng, 2003), inhibited the growth of *R. solani*, which was not affected by the antifungal protein from black turtle beans. The IC_{50} values of the antifungal activity of the latter against *M. arachidicola* and *F. oxysporum* were 18.3 and 35.6 μM, respectively. Its antifungal activity against *M. arachidicola* was more pronounced than that against *F. oxysporum*. The protein isolated showed higher resistance to heat than mollisin, the antifungal activity of which was attenuated following incubation at 60°C for 15 minutes (Chu and Ng, 2003).

Leguminous proteins have been investigated for possible immunomodulatory effects. The antifungal protein isolated in the concentration range of 0–50 μM elicited neither mitogenic nor cytotoxic activity on murine splenocytes *in vitro*. Regarding other legume antifungal proteins, fabin demonstrated mitogenic activity on murine splenocytes (Ng and Ye, 2003) whereas mungin from mung bean displayed anti-mitogenic activity (Ye and Ng, 2000). However, the black turtle bean antifungal protein isolated augmented nitric oxide production by murine peritoneal macrophages in a dose-dependent manner. Polymyxin B, which binds to endotoxin and neutralizes its effect, did not significantly lower NO production induced by the purified protein in macrophages, suggesting that the purified protein was not contaminated with endotoxin. Dexamethasone and L-NAME, which inhibit the induction and activity of inducible nitric oxide synthase (iNOS), respectively (Rees et al., 1989; Di Rosa et al., 1990), were found to attenuate NO production induced by the purified protein in mouse peritoneal macrophages, indicating that increased expression and activity of iNOS were the underlying mechanism. Further studies on iNOS expression in mouse peritoneal macrophages and the pathway involved are in progress.

NO, the reactive free radical synthesized by iNOS in macrophages on exposure to cytokines and endotoxin,

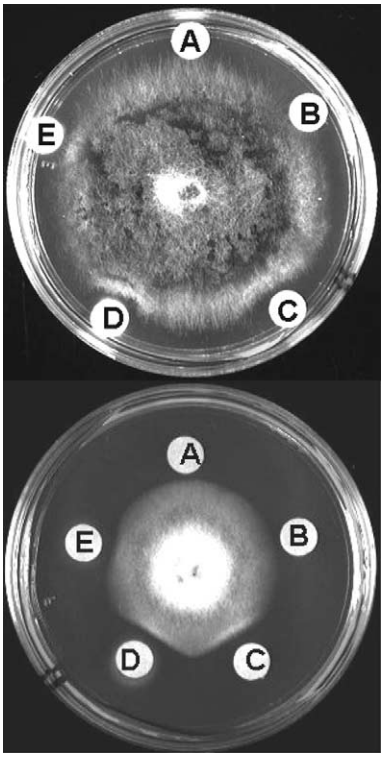

Figure 3 Antifungal activity of the purified protein toward various fungal species.
The purified protein inhibited mycelial growth in two fungi, *Fusarium oxysporum* (top) and *Mycosphaerella arachidicola* (bottom). The samples applied to the paper disks A, B, C, D and E were control (20 mM Tris-HCl, pH 7.3), 200 μg of bovine serum albumin, 200 μg of mollisin from chestnut, 200 μg of antifungal protein, and 40 μg of antifungal protein, respectively. The protein was dissolved in the same buffer used for the control.

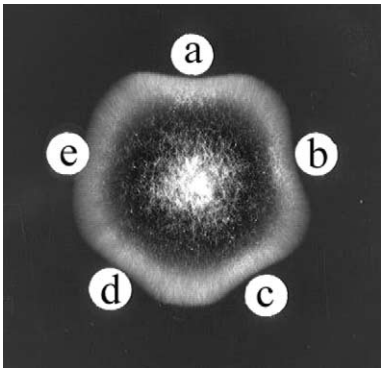

Figure 4 Thermostability of the antifungal activity of the purified protein toward *Mycosphaerella arachidicola*.
A solution of the protein was subjected to different temperatures for 10 minutes. The same amount (100 μg) of heat-treated antifungal protein was added to each respective paper disk: (a) 20; (b) 40; (c) 60; (d) 80; and (e) 100°C.

takes part in the host defense of the innate immune system. The production of NO by macrophages elicits various important immune responses, ranging from anti-microbial activity, anti-tumor activity, anti-inflammatory effect, and differentiation of T helper cells to modulation of the production of cytokines, chemokines and growth factors (Bogdan, 2001).

Legume isoflavones from *Sophora* species attenuated the stimulatory effect of lipopolysaccharide (LPS) on nitric oxide production by the macrophage cell line RAW 262.4 (Tashiro et al., 2002). Nitric oxide production from macrophages is believed to be an allergic response of the immune system. House-dust mite fecal pellets, containing the major house mite allergens Der p1 and Der p2, have been found to augment nitric oxide release from an alveolar macrophage cell line (Peake et al., 2003), which indicated that the induced NO production by macrophages reflects an allergic response of the immune system.

Allergy caused by beans has become more prevalent due to the increasing consumption of beans caused by the rising awareness of a healthier diet. This study provides an insight into the immunological activity of the protein isolated from common edible beans. In plant seeds, some proteins are responsible for food allergy, causing food intolerance and the IgE-mediated response when patients consume those kinds of food. The mechanism involves the epitopes of allergens that would cause the cross-linking of IgE on the surface of mast cells in the epithelium of gastrointestinal tract, resulting in an inflammatory response. Many food allergens have been isolated and characterized, and some chitinases are designated as allergens, for example, chitinase (Prs a1) from avocado (Diaz-Perales et al., 2003), endochitinase 4A from grape (Pastorello et al., 2003), and chestnut chitinase with a hevein-like domain (Diaz-Perales et al., 1998). Whether the purified black turtle bean antifungal protein possesses allergenic activity remains to be elucidated.

Materials and methods

Materials

Black turtle beans (*Phaseolus vulgaris* cv. 'Black Turtle') from the United States, which were used in this study, were purchased from a local vendor. They were authenticated by Dr. Shiuying Hu, Honorary Professor of Chinese Medicine, The Chinese University of Hong Kong. A voucher (no. PVBT01) was deposited in Room 302, Department of Biochemistry, The Chinese University of Hong Kong. Affi-gel blue gel was purchased from Bio-Rad (Hercules, CA, USA), DEAE-cellulose was obtained from Sigma (St. Louis, MO, USA), Superdex 75 HR 10/30 was from Amersham Biosciences (Uppsala, Sweden), and the Hydroxyapaptite column was from Bio-Rad. Chemicals for sequence analysis were obtained from Hewlett Packard (Palo Alto, CA, USA). All other chemicals used were of reagent grade.

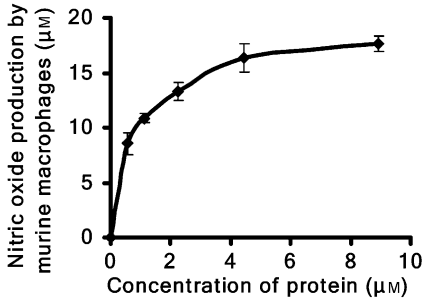

Figure 5 Stimulatory effect of the purified antifungal protein on nitric oxide production by murine peritoneal macrophages.
The purified protein has a similar effect as lipopolysaccharide (LPS) (n=3, values are mean±SD).

Table 2 Effect of black turtle bean antifungal protein on nitric oxide production by mouse peritoneal macrophages.

Treatment	Nitric oxide production (μM)
Black turtle bean antifungal protein (=sample)	8.54±1.27
Cells pretreated with dexamethasone 2 h before exposure to the sample	1.96±0.08
Cells treated with dexamethasone 12 h after exposure to the sample	4.91±0.73
Cells treated with L-NAME 10 min before exposure to the sample	2.14±0.08
Cells incubated with polymyxin B-pretreated sample	8.4±2.09
Cells incubated with LPS	16.29±3.02
Cells incubated with polymyxin B-pretreated LPS	2.03±0.35

Concentration of black turtle bean antifungal protein used, 0.56 μM; LPS, 1 μg/ml; dexamethasone, 10 μM; L-NAME, 300 μM; polymyxin B, 1 μg/ml (n=3, mean±SD).

Protein isolation

A 200-g sample of the seeds was homogenized in 0.8 l of 10 mM Tris-HCl buffer (pH 7.3) using a Waring blender. After centrifugation of the homogenate at 13 000 *g* for 30 minutes at 4°C, the supernatant obtained was applied to a DEAE-cellulose (Sigma) column (5×15 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.3) and was eluted with the same buffer. After unadsorbed proteins had been collected by eluting the column with the same buffer, adsorbed proteins were desorbed by including 1 M sodium chloride (NaCl) in the eluting buffer. The unadsorbed fraction (DE1) was immediately chromatographed on an Affi-gel blue gel column (2.5×18 cm) (Bio-Rad) in 10 mM Tris-HCl buffer (pH 7.3). Unadsorbed proteins were eluted with the same buffer while adsorbed proteins were eluted using the same buffer with 1.5 M NaCl added. The adsorbed fraction (BG2) was dialyzed against 10 mM ammonium acetate (NH_4OAc) buffer (pH 4.5) before chromatography on a CM-cellulose column (2.5×18 cm) (Sigma). After removal of unadsorbed proteins (CM1), the column was eluted with a linear concentration gradient of 0–0.5 M NaCl in 10 mM NH_4OAc buffer (pH 4.5). The first adsorbed peak (CM2) was then dialyzed prior to chromatography on a ceramic hydroxyapaptite column (1×6.4 cm) (Bio-Rad) in 5 mM sodium phosphate buffer (pH 6.8), which was eluted with a gradient of 0–0.5 M sodium phosphate, by fast protein liquid chromatography (FPLC) using an AKTA Purifier (Amersham Biosciences). The first adsorbed peak obtained (HA2) was subjected to gel filtration by FPLC on a Superdex 75 HR 10/30 column (1×30 cm) (Amersham Biosciences) in 0.15 M ammonium bicarbonate (NH_4HCO_3) buffer (pH 8.9), whereby the purified antifungal protein was obtained.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the method of Laemmli and Favre (1973). After electrophoresis the gel was stained with Coomassie Brilliant Blue (Sigma). The molecular mass of the black turtle bean antifungal protein was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins from Amersham Biosciences.

Amino acid sequence analysis

The N-terminal amino acid sequence of black turtle bean antifungal protein was analyzed by means of automated Edman degradation using a Hewlett Packard 1000A protein sequencer equipped with a high performance liquid chromatography system (Ye et al., 2000).

Assay of antifungal activity

The assay for antifungal activity toward various fungal species was carried out on 100×15-mm Petri plates containing 10 ml of potato dextrose agar (Sigma). After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (15 μl) of the purified protein was added to a disk. The plates were incubated at 23°C for 72 h until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity (Chu and Ng, 2003). Mollisin from chestnut *Castanea mollissima* (Chu and Ng, 2003) was used as a positive control in this assay.

To determine the IC_{50} value for the antifungal activity, six doses of the purified 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 Petri dishes of 60 mm in diameter. After the agar had cooled, a small amount of mycelia, the same amount to each dish, was added. Buffer only without the purified 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 (Chu and Ng, 2003). The IC_{50} value of the antifungal activity is the concentration producing 50% reduction in the area of the mycelial colony.

Assay of nitric oxide production by murine peritoneal macrophages

The assay for the ability to stimulate production of nitric oxide by mouse macrophages was conducted as described by Wang et al. (2001). Peritoneal macrophages were collected from mice 3 days after eliciting by 3% thioglycollate medium (Becton Dickinson, Le Pont de Claix, France). The cells were washed, counted, and resuspended in RPMI medium without phenol red, 10% fetal bovine serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Cells (2×10^5 cells/well per 200 μl) were allowed to adhere onto the surface of the wells of a 96-well culture plate for 1 h before incubation with the antifungal protein for 24 h. The amount of nitric oxide in the culture medium was determined by a colorimetric method using sodium nitrite ($NaNO_2$) as the standard (Liu et al., 1993). In the assay, a 100-μl aliquot of cell-free culture medium from each culture well was allowed to react with 50 μl of Griess reagent (1% sulfanilamide in 5% H_3PO_4/0.1% naphthalene-ethylenediamine dihydrochloride) for 10 min before the absorbance was read at 540 nm using a microplate reader (Bio-Rad 3550). Lipopolysaccharide from *Escherichia coli* (Sigma) was used as a positive control in this assay.

Effects of dexamethasone and L-NAME on the nitric oxide production induced by the black turtle bean antifungal protein

Murine peritoneal macrophages were treated with dexamethasone (10 μM) (Sigma), 2 h before or 12 h after treatment with the black turtle bean antifungal protein. Macrophages were treated with L-NAME hydrochloride (Sigma) at a final concentra-

tion of 300 μM for 10 min and then the purified protein was added to the cells. The nitric oxide level was determined, after 24 h of treatment with the purified protein, using Griess reagent by the method described in the assay of NO production.

Neutralization of endotoxin by polymyxin B

The purified protein was pretreated with polymyxin B sulfate (Sigma) at a concentration of 1 μg/ml for 30 min before incubation with macrophages. The nitric oxide level was determined after 24-h treatment of cells with the purified protein using Griess reagent by the method described in the assay of NO production.

Assay of mitogenic activity

The purified protein was tested in this assay, since some antifungal proteins such as chrysancorin demonstrate activity in this assay. The procedure was described by Wang et al. (2001). Concanavalin A from Jack beans (Sigma) was used as a positive control in this assay.

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