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Eryngin, a novel antifungal peptide from fruiting bodies of the edible mushroom *Pleurotus eryngii*

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Abstract

An antifungal peptide with a molecular mass of 10 kDa was isolated from fruiting bodies of the mushroom *Pleurotus eryngii*. The peptide, designated as eryngin, inhibited mycelial growth in *Fusarium oxysporum* and *Mycosphaerella arachidicola*. It was unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel and S-Sepharose. Its N-terminal sequence demonstrated some similarity to the antifungal protein from the mushroom *Lyophyllum shimeiji* and little resemblance to thaumatin and thaumatin-like proteins.

Keywords: Mushroom; Antifungal peptide; Fruiting bodies

1. Introduction

Living organisms produce a variety of molecules to protect themselves from environmental insults or pathogens. One family of these defense molecules is represented by antifungal proteins and peptides which have been isolated from animals [5,11,27], plants [1–4,6–10,12,14,17–40] and fungi [8,15]. Antifungal proteins and peptides can be classified into many different classes based on their structures and/or functions. The classes include thaumatin-like proteins [7,21,23,38], chitinases [1,10,14,17,24,37,39], glucanases [24], peroxidases [33], ribonucleases [20,25], protease inhibitors [4,12,35], miraculin-like proteins [40], ribosome inactivating proteins [15,18,22], cyclophilin-like proteins [30,32], embryo-abundant proteins [26], and lectins [2,6,36].

In contrast to the voluminous amount of literature on antifungal proteins and peptides of plant origin [1–4,6–10,12,14,17–40], there are only very few reports regarding mushroom antifungal proteins and peptides [8,15]. The intent of the present study was to isolate an antifungal peptide from the common edible mushroom *Pleurotus eryngii*.

2. Materials and methods

2.1. Isolation of antifungal peptide

Fresh fruiting bodies of the mushroom *P. eryngii* were obtained from a local vendor. They were homogenized in distilled water, and the homogenate was centrifuged (15 000 × *g* for 30 min). The resulting supernatant was chromatographed on a DEAE-cellulose (Sigma) column (5 cm × 20 cm) in 10 mM Tris–HCl buffer (pH 7.3). The unadsorbed fraction (D1) eluted with the Tris–HCl buffer was then passed through an Affi-gel blue gel (Bio-Rad) column (2.5 cm × 20 cm) in 10 mM Tris–HCl buffer (pH 7.3). After elution of unadsorbed proteins (B1) with the same buffer, adsorbed proteins (B2) were desorbed by inclusion of 1.5 M NaCl in the Tris–HCl buffer. Fraction B2 was dialyzed and then chromatographed on a column of S-Sepharose (Amersham Biosciences) (1.5 cm × 15 cm) in 10 mM NH_4OAc (pH 4.6). Following elution of unadsorbed proteins (S1) with the NH_4OAc buffer, adsorbed proteins were eluted as two peaks (S2 and S3) by addition of a linear salt concentration (0–1 M NaCl) in the NH_4OAc buffer. S3 was subsequently subjected to fast protein liquid chromatography on a Superdex 75 HR 10/30 gel filtration column (Amersham Biosciences) in 0.2 M NH_4HCO_3 (pH 8.5). The second peak obtained (SU2) represented purified antifungal peptide designated as eryngin. The molecular mass of eryngin was estimated from its elution volume com-

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pared with the elution volumes of molecular mass marker proteins from Amersham Biosciences.

2.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

It was conducted according to the method of Laemmli and Favre [13]. After electrophoresis the gel was stained with Coomassie Brilliant Blue. The molecular mass of eryngin was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins from Amersham Biosciences.

2.3. Amino acid sequence analysis

The N-terminal amino acid sequence of eryngin was analyzed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett-Packard 1000A protein sequencer equipped with a high performance liquid chromatography system [16].

2.4. Assay of antifungal activity

The assay for antifungal activity toward *Fusarium oxysporum* and *Physalospora piricola* was carried out in 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 at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (12 μl containing either 14.4 or 72 μg) of eryngin 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 [17].

To determine the IC_{50} value for the antifungal activity, three doses of eryngin were added separately to three aliquots each containing 4 ml potato dextrose agar at 45 °C, mixed rapidly and poured into three separate small petri dishes. After the agar had cooled down, a small amount of mycelia, the same amount to each plate, was added. Buffer without eryngin only 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 [28].

2.5. Assay for hemagglutinating activity

Eryngin was tested for this activity since some lectins exhibit antifungal activity. A serial two-fold dilution of a solution of eryngin in microtiter U-plates (50 μl) was mixed with 50 μl of a 20% suspension of rabbit erythrocytes in phosphate buffered saline (pH 7.2) at room temperature. The results were recorded after about 1 h when the blank had fully sedimented.

2.6. Assay of ribonuclease activity

The activity of eryngin toward yeast tRNA was assayed by determining the generation of acid-soluble, UV-absorbing species with the method of Ng and Wang [20]. Eryngin was incubated with 200 μg of yeast tRNA in 150 μg 100-mM MES buffer (pH 6.0) at 37 °C for 1 h. The reaction was terminated by introduction of 350 μl of ice-cold 3.4% (v/v) perchloric acid. After leaving on ice for 15 min, the sample was centrifuged (15 000 × *g*, 15 min) at 4 °C. The OD260 of the supernatant was read after appropriate dilution. One unit of enzymatic activity is defined as the amount of enzyme that brings about an increase in OD260 of 1 unit/min in the acid-soluble fraction per milliliter of reaction mixture under the specified condition.

3. Results

When the fruiting body extract was chromatographed on DEAE-cellulose, antifungal activity was located in the unadsorbed fraction D1, but not in the adsorbed fraction D2 (data not shown). The activity was retained in the adsorbed fraction B2 and not in the unadsorbed fraction B1 when D1 was fractionated on Affi-gel blue gel (data not shown). B2 was resolved on S-Sepharose into a small unadsorbed peak S1, and a larger adsorbed peak S2 devoid of antifungal activity. Antifungal activity resided in the most strongly adsorbed and the largest peak S3 (Fig. 1). S3 was separated by gel filtration on Superdex 75 into a larger peak SU1 and a smaller peak SU2 (Fig. 2). The latter peak designated as eryngin contained antifungal activity. It exhibited a single band with a molecular mass of 10 kDa in gel filtration (Fig. 2) and SDS–PAGE (Fig. 3). Its antifungal activity against *F. oxysporum* and *Mycosphaerella arachidicola* is illustrated in Figs. 4 and 5, respectively. The IC_{50} values for

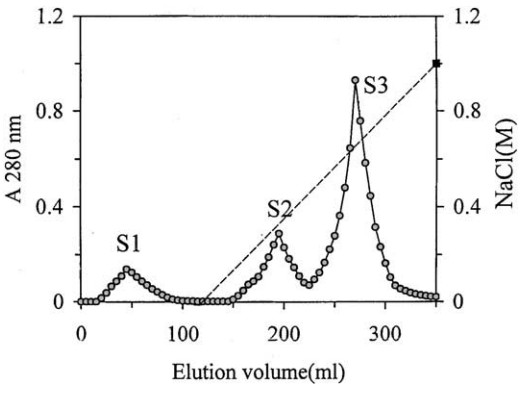

Fig. 1. Ion exchange chromatography on an S-Sepharose column (1.5 cm × 15 cm). Sample: fraction of fruiting body extract unadsorbed on DEAE-cellulose and subsequently adsorbed on Affi-gel blue gel. Buffer: 10 mM NH_4OAc (pH 4.6). Slanting dotted line across the chromatogram indicates linear NaCl concentration gradient (0–1 M) employed to elute the adsorbed fractions S2 and S3.

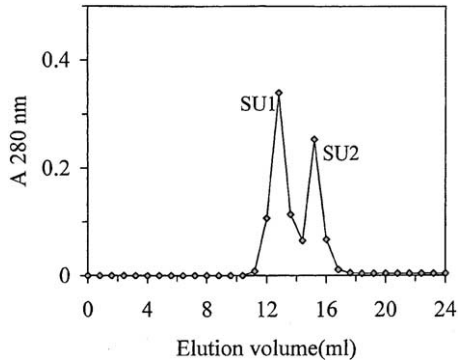

Fig. 2. Gel filtration on a Superdex HR 10/30 column by fast protein liquid chromatograpy. Sample: fraction S3 from S-Sepharose column chromatography. Buffer: 0.2 M NH_4HCO_3 (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml.

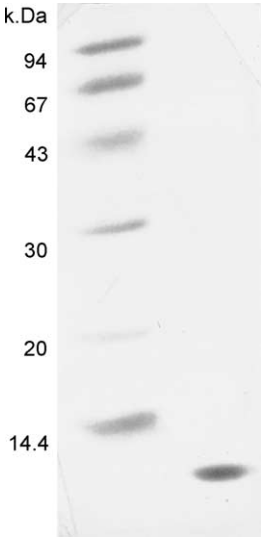

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of fraction SU2 from Superdex 75 column chromatography. Right lane: SU2 (12 μg). Left lane: molecular mass markers from Amersham Biosciences. From top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbmin (14.4 kDa).

Table 1
Comparison of N-terminal sequences of eryngin, *Lyophyllum* antifungal protein, thaumatin-like proteins and thaumatin

Eryngin	A · TRVVYC · N · · · · · RRSG · SVVGG · DDTVYYEG
Lyophyllum	AGTEIVTCYNAGTKVPR · GPSAXGGAIDFFN
Eryngin	ATR · VV · YC · NRRSG · ·SVVGGDDTVYYEG
Thaumatin (I)	ATFEIVNRCSYTVWAAASKGGGRQLNSGE
Chickpea TLP (22)	ANFEIVNNCPYTVWAAASPGGGRRLDRGQ
Barley TLP	ATFTVINKCQYTVWAAAVPAGGGQKLDA · GQ
Maize TLP	AVFTVVNQCPFTVWAA · SVPVGGGRQLNT · GE

(··): space created to maximize sequence similarity.
Identical corresponding amino acid residues are underlined.

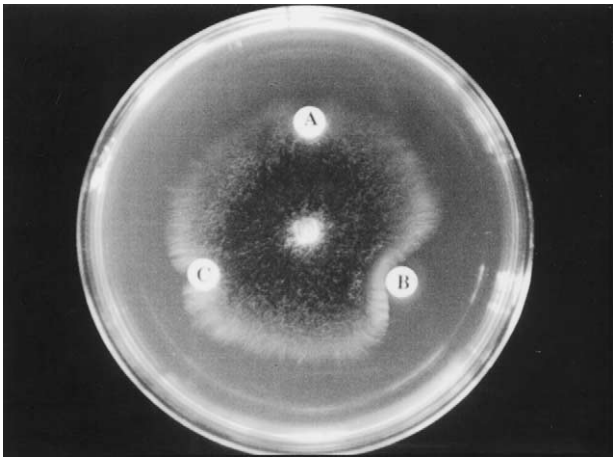

Fig. 4. Antifungal activity of eryngin toward *Fusarium oxysporum*: (A) control (12 μl 0.1-M MES buffer pH 6.5), (B) 72 μg eryngin in 12 μl MES buffer, (C) 14.4 μg eryngin in 12 μl MES buffer.

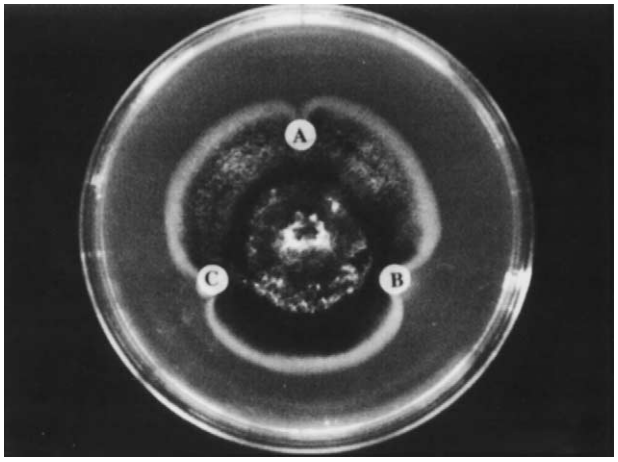

Fig. 5. Antifungal activity of eryngin toward *Mycosphaerella arachidicola*: (A) control (12 μl 0.1-M MES buffer, pH 6.5), (B) 72 μg eryngin in 12 μl MES buffer, (C) 14.4 μg eryngin in 12 μl MES buffer.

the antifungal effects were 1.35 ± 0.15 μM and 3.5 ± 0.4 μM ($n = 3$ determinations), respectively. Eryngin was devoid of lectin and ribonuclease activities when tested at 100 and 10 μg, respectively (data not shown). There was only slight resemblance of the N-terminal sequence of eryngin to those of *Lyophyllum* antifungal protein, thaumatin-like proteins and thaumatin (Table 1).

4. Discussion

The N-terminal sequence of eryngin manifests a small degree of resemblance to that of the antifungal protein from the mushroom *Lyophyllum shimeiji* [15], and only slight similarity to thaumatin and thaumatin-like proteins. However, thaumatin does not exhibit antifungal activity. The molecular mass of eryngin is much lower than those of *Lyophyllum*

antifungal protein [15], angiosperm thaumatin-like proteins and thaumatin [7,21,23,38].

Eryngin is adsorbed on Affi-gel blue gel and unadsorbed on DEAE-cellulose, like most of the antifungal proteins and peptides reported to date [25–28]. It is adsorbed on S-Sepharose comparable to the adsorption of *Lyophyllum* antifungal protein on CM-cellulose and CM-Sepharose [15].

Lyophyllum antifungal protein is active against *Mycospharella arachidicola* and *P. piricola*. Eryngin exerts an antifungal action against *F. oxysporum* and *M. arachidicola*. Eryngin is devoid of lectin and ribonuclease activities. It is known that some lectins [2,33] and ribonucleases [20,25] exhibit antifungal activity.

A ribosome inactivating protein and a lectin in addition to an antifungal protein can be isolated from the fruiting bodies of *L. shimeiji* [15]. In the case of *P. eryngii* no ribosome inactivating protein or lectin can be isolated.

In summary, the report of eryngin constitutes an addition to the scanty literature on mushroom antifungal proteins and peptides.

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