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Peptides 24 (2003) 969–972
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Isolation of cucurmoschin, a novel antifungal peptide abundant in arginine, glutamate and glycine residues from black pumpkin seeds

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Received 3 April 2003; accepted 11 June 2003

Abstract

A novel antifungal peptide, with a molecular mass of 8 kDa in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and in gel filtration on Superdex 75 and designated cucurmoschin, was isolated from the seeds of the black pumpkin. The peptide was unadsorbed on DEAE-cellulose but adsorbed on Affi-gel blue gel. Cucurmoschin inhibited mycelial growth in the fungi *Botrytis cinerea*, *Fusarium oxysporum* and *Mycosphaerella oxysporum*. It inhibited translation in a cell-free rabbit reticulocyte lysate system with an IC_{50} of 1.2 μM. The N-terminal sequence of cucurmoschin was rich in arginine, glutamate and glycine residues.

Keywords: Antifungal peptide; Pumpkin; Seeds; Isolation

1. Introduction

The seeds of plants affiliated to the family of Cucurbitaceae produce a number of proteins and peptides including trypsin inhibitors [5], lectins [16], ribosome-inactivating proteins [1,16,24] and ribonucleases [4].

The intent of the present study was to isolate an antifungal peptide from seeds of *Cucurbita maxima* cv. black pumpkin. To date, a host of antifungal proteins and peptides are known. They can be categorized into lectins [24], thaumatin-like proteins [7], chitinases [11], glucanases [15], ribonucleases [18], deoxyribonucleases [20], embryo-abundant proteins [25], protease inhibitors [4,5,10], ribosome-inactivating proteins [12,13], miraculin-like proteins [25], cyclophilin-like proteins [21], arginine- and glutamate-rich proteins [19], and peptides [6,22].

The results revealed that the black pumpkin seeds produce an antifungal peptide with a molecular mass of 9 kDa. In line with the finding of antifungal activity in arginine- and glutamate-rich proteins [19] and glycine-rich proteins [2,3], the N-terminal of the antifungal peptide isolated from black pumpkin seeds is abundant in arginine, glutamate and glycine residues.

2. Materials and methods

2.1. Isolation of peptide

Seeds of the black pumpkin (*Cucurbita moschata* cv black pumpkin) (0.5 kg) were pulverized and then homogenized in water (4 ml/g). To the supernatant obtained after centrifugation, Tris–HCl buffer (pH 7.0) was added until the final concentration reached 10 mM. The extract was then chromatographed on a DEAE-cellulose column (5 cm × 20 cm). After elution of unadsorbed material with 10 mM Tris–HCl buffer (pH 7.2), adsorbed material was eluted with 1 M NaCl in 10 mM Tris–HCl buffer (pH 7.2). The unadsorbed fraction (D1) containing antifungal activity was then loaded on a column of Affi-gel blue gel (2.5 cm × 20 cm), which had been pre-equilibrated with and was eluted with 10 mM Tris–HCl buffer (pH 7.2). After removal of unadsorbed material without antifungal activity, adsorbed material was then eluted with a linear NaCl gradient (0–1 M) in the buffer. The adsorbed peak with antifungal activity was subjected to further purification on a Superdex 75 HR 10/30 column (Amersham Biosciences) in 0.2 M

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0196-9781/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0196-9781(03)00191-8

NH_4HCO_3 buffer (pH 8.5) at a flow rate of 0.4 ml/min. The purified antifugnal peptide was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and N-terminal sequencing as described below.

2.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

In order to estimate the molecular mass of the purified peptide, SDS–PAGE was performed as described by Laemmli and Favre [9]. Fast protein lipid chromatography (FPLC) on a Superdex 75 HR 10/30 column (Amersham Biosciences), which had been calibrated with molecular mass markers, was also conducted. The N-terminal sequence of the peptide was determined by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC system [10].

2.3. Assay for antifungal activity

In the assay for antifungal activity, sterile petri plates (100 mm × 15 mm) containing 10 ml potato dextrose agar were used. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 1 cm from the rim of the mycelial colony. A 25 μl aliquot of the test sample in 0.05 M MES buffer (pH 6.0) was applied to a disk. Incubation of the petri plate was carried out at 23 °C for 72 h until mycelial growth had surrounded peripheral disks containing the control and had generated crescents of inhibition around disks with antifungal samples. Three fungal species, *Botrytis cinerea*, *Fusarium oxysporum*, and *Mycosphaerella arachidicola* were examined in the assay [8,14].

2.4. Assay for translation-inhibiting activity

The purified antifungal peptide was assayed for cell-free translation-inhibiting activity in a rabbit reticulocyte lysate system as described by Lam et al. [10]. Rabbit reticulocyte lysate was prepared from the blood of rabbits rendered anemic by phenylhydrazine injections. The antifungal peptide (10 μl) was added to 10 μl of radioactive mixture (500 mM KCl, 5 mM $MgCl_2$, 130 mM phosphocreatine and 1 μCi [4,5-^{3}H]-leucine) and 30 μl working rabbit reticulocyte lysate containing 0.1 μM hemin and 5 μl creatine kinase. Incubation proceeded at 37 °C for 30 min before addition of 330 μl 1 M NaOH and 1.2% H_2O_2. Further incubation for 10 min allowed decolorization and tRNA digestion. An equal volume of the reaction mixture was then added to 40% trichloroacetic acid with 2% casein hydrolyzate in a 96-well plate to precipitate radioactively labeled protein. The precipitate was collected on a glass fiber Whatman GF/A filter, washed and dried with absolute alcohol passing through a cell harvester attached to a vacuum pump. The filter was suspended in scintillant and counted in an LS6500 Beckman liquid scintillation counter.

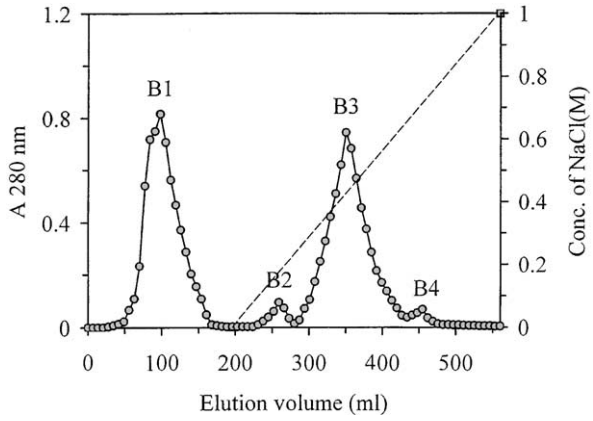

Fig. 1. Affinity chromatography of fraction of black pumpkin seed extract unadsorbed on DEAE-cellulose. Column: Affi-gel blue gel (2.5 cm × 20 cm). Starting buffer: 10 mM Tris–HCl (pH 7). Slanting line across right half of chromatogram indicates linear NaCl concentration gradient used to elute adsorbed proteins.

3. Results

Ion-exchange chromatography of the black pumpkin seed extract on DEAE-cellulose yielded an unadsorbed peak (D1), in which antifungal activity was concentrated, and an inactive adsorbed peak (data not shown). Subsequent affinity chromatography of D1 on Affi-gel blue gel resulted in a large inactive unadsorbed peak (B1) and a major adsorbed peak (B3), in which antifungal activity resided (Fig. 1). Gel filtration of B3 by FPLC on Superdex 75 gave rise to a small peak SU1 and two peaks of approximately equal size (SU2 and SU3) (Fig. 2). Only SU3 possessed antifungal activity. The molecular mass of SU3 (cucurmoschin) was estimated to be 9 kDa by both gel filtration (Fig. 2)

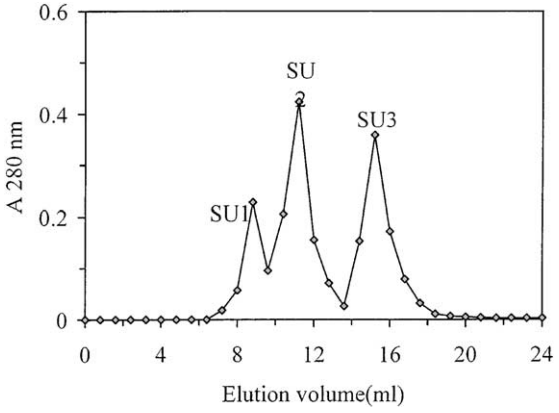

Fig. 2. Gel filtration of peak B3 by fast protein liquid chromatography on a Superdex 75 HR 10/30 column. Buffer: 0.2 M NH_4HCO_3 (pH 8.5); fraction size: 0.8 ml; flow rate: 0.4 ml/min. Antifungal activity resided in peak SU3. The elution times of the various molecular mass markers used to calibrate the column were as follows: alcohol dehydrogenase (150 kDa), 7.48 min; bovine serum albumin (67 kDa), 9.09 min; ovalbumin (43 kDa), 10.02 min; carbonic anhydrase (30 kDa), 11.55 min; ribonuclease A (13.7 kDa), 13.36 min; and insulin (6 kDa), 15.80 min.

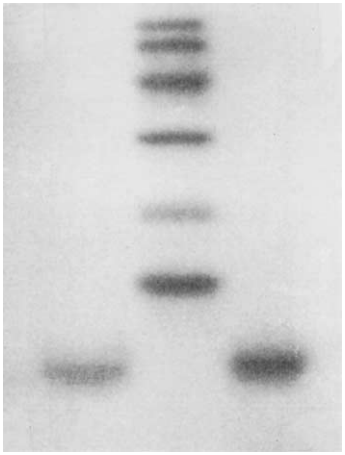

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Left and right lanes: SU3 (= cucurmoschin). Middle lane: molecular mass markers; from top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), α-lactalbumin (14.4 kDa).

and SDS–PAGE (Fig. 3). The sequence of cucurmoschin, PQRGEGGRAGNLLREEQEI demonstrated an abundance of glycine (G), arginine (R) and glutamate (E) residues. Antifungal activity of cucurmoschin against *B. cinerea*, *F. oxysporum* and *M. arachidicola* was evident at a dose of 375 μg. Slight inhibition was detected at 75 μg (Fig. 4). The antifungal peptide exerted an inhibitory effect on cell-free translation with an IC_{50} of 1.2 μM.

4. Discussion

Cucurmoschin was purified from an extract of black pumpkin seeds using a protocol which has proven efficacious for isolating other antifungal proteins [11,17–25]. Cucurmoschin was unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel like most of the previously reported antifungal proteins [11,17–25] (Table 1).

A wide range of molecular masses has been reported for antifungal proteins. Some are over 30 kDa in molecular mass, e.g. the miraculin-like protein from sugar snap legumes [25]. Some are only a few kilodaltons in molecular mass, for instance, antifungal peptides from *Cicer arietinum* [23]. Others have a molecular mass between 20 and 30 kDa, e.g. thaumatin-like protein from maize [7] or between 10 and 20 kDa, e.g. mungin from the mung bean

Table 1
A summary of purification of cucurmoschin from black pumpkin seeds

Fraction	Protein (mg)	IC_{50} value for translation-inhibiting activity (μM)
Crude extract	3100	64.2
D1	760	20.7
B3	207	5.6
SU3 (cucurmoschin)	50	1.2

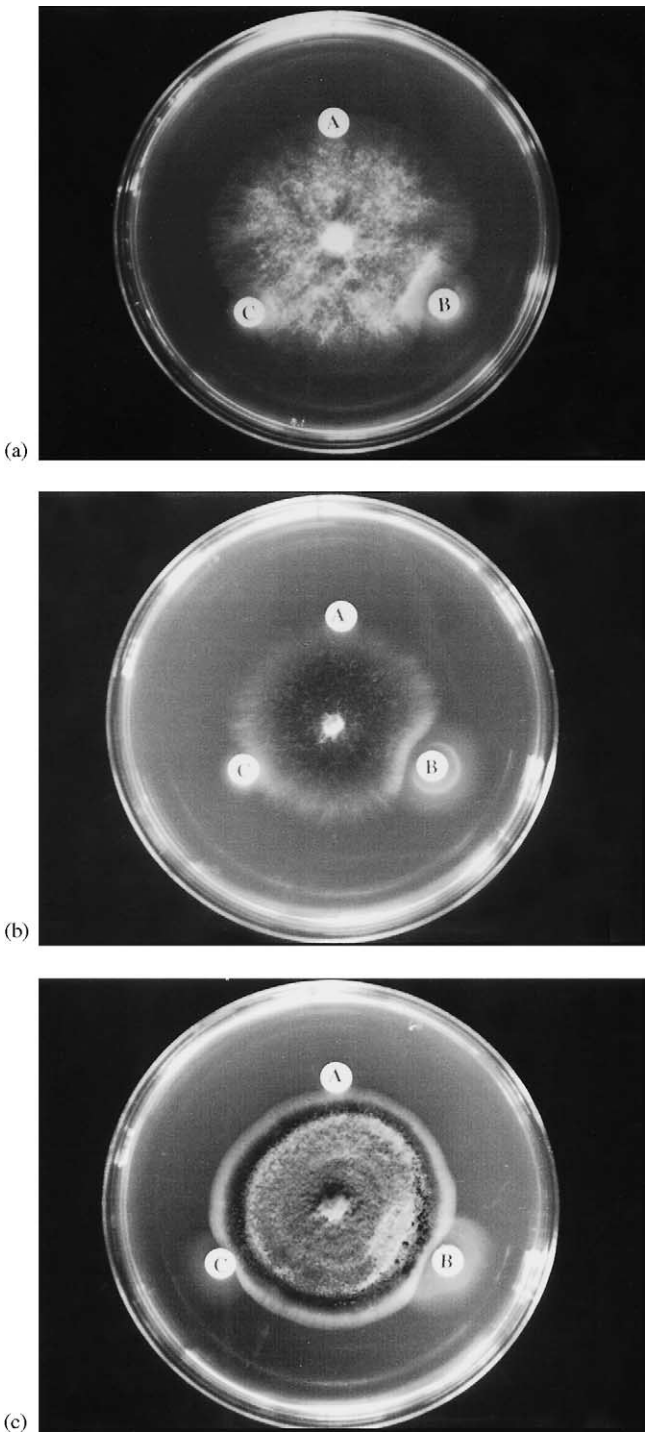

Fig. 4. (a) Antifungal activity of cucurmoschin toward *Botrytis cinerea*. (A) Control: 0.05 M MES buffer, pH 6 (25 μl); (B) 375 μg cucurmoschin in MES buffer; and (C) 75 μg cucurmoschin in MES buffer. (b) Antifungal activity of cucurmoschin toward *Fusarium oxysporum*. (A) Control: 0.05 M MES buffer, pH 6 (25 μl); (B) 375 μg cucurmoschin in MES buffer; and (C) 75 μg cucurmoschin in MES buffer. (c) Antifungal activity of cucurmoschin toward *Mycosphaerella arachidicola*. (A) Control: 0.05 M MES buffer, pH 6 (25 μl); (B) 375 μg cucurmoschin in MES buffer; and (C) 75 μg cucurmoschin in MES buffer.

[21]. Cucurmoschin possesses a molecular mass which is on the lower side, i.e. 8 kDa.

An antifungal protein with an arginine- and glutamate-rich N-terminal sequence has been isolated from lily bulbs [19]. Glycine-rich proteins have antimicrobial action [2,3]. The N-terminal sequence of cucurmoschin is abundant in arginine, glutamate and glycine.

Cucurmoschin manifested a translation-inhibitory activity ($IC_{50} = 1.2\,\mu M$) which was more potent than some of the antifungal proteins [11,17–25] and the antifungal peptides from red bean [22], pinto bean [22] and chickpea [23]. Ribosome-inactivating proteins inhibit translation in rabbit reticulocyte lysate with a much higher potency (IC_{50} in pM concentration) [1] and they also inactivate fungal ribosomes [13]. It is likely that the translation-inhibiting activity of antifungal proteins accounts for, at least partially, their antifungal effect.

Some antifungal proteins exert an antifungal activity on only one fungal species out of the several tested [20]. The majority display inhibitory activity against several fungal species [17,19]. Cucurmoschin was active against *B. cinerea*, *F. oxysporum* and *M. arachidicola*, similar to the observation on red bean [22], pinto bean [22] and chickpea [23]. Cucurmoschin exhibited a higher antifungal potency than cicerin from chickpea [23]. Its antifungal potency was similar to arietin from chickpea [23] but lower than the antifungal peptides from red bean [22] and pinto bean [22].

All in all, an antifungal peptide with a novel N-terminal sequence was purified from black pumpkin seeds.

Acknowledgments

The expert secretarial assistance of Miss Fion Yung is appreciated. The award of a direct grant from the Medicine Panel, CUHK Research Committee is gratefully acknowledged.

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