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Novel Antifungal Peptides from Ceylon Spinach Seeds

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Two novel antifungal peptides, designated α- and β-basrubrins, respectively, were isolated from seeds of the Ceylon spinach *Basella rubra*. The purification procedure involved saline extraction, $(NH_4)_2SO_4$ precipitation, ion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, ion exchange chromatography on CM-cellulose and FPLC-gel filtration on Superdex peptide column. α- and β-basrubrins exhibited a molecular weight of 4.3 and 5 kDa, respectively. They inhibited translation in a rabbit reticulocyte system with an IC_{50} value of 400 and 100 nM, respectively. α- and β-basrubrin inhibited HIV-1 reverse transcriptase by (79.4 ± 7.8)% and (54.6 ± 3.6)%, respectively, at a concentration of 400 μM, and (10.56 ± 0.92)% and (2.12 ± 0.81)%, respectively, at a concentration of 40 μM. Both α- and β-basrubrins exerted potent antifungal activity toward *Botrytis cinerea*, *Mycosphaerella arachidicola*, and *Fusarium oxysporum*. © 2001 Academic Press

Key Words: **antifungal peptides; Ceylon spinach; seeds**

A diversity of bioactive compounds are elaborated by seeds. They include lectins with anti-insect (1), antifungal (2), antitumor and immunomodulatory activities (3), protease inhibitors with antitumor and anti-insect activities (4, 5), arcelin and α-amylase inhibitors with anti-insect activity (6, 7), ribosome inactivating proteins with immunomodulatory, antiviral, antitumor and translation-inhibitory activities (8) and antifungal proteins (9). All of these proteins play a significant role in defense.

Antifungal proteins can be divided into different types comprising thaumatin-like proteins (10), chitinases (11), glucanases (11), cyclophilin-like proteins (12), miraculin-like proteins (9), embryo-abundant protein (13), and antifungal peptides (14). The objective of the present study was to isolate and characterize proteinaceous components with antifungal activity from the seeds of the vegetable *Basella rubra*.

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MATERIALS AND METHODS

Isolation. Ceylon spinach (*Basella rubra*) seeds (500 g) purchased from a local vendor were extracted with saline. Following centrifugation proteins were precipitated from the supernatant by addition of $(NH_4)_2SO_4$ to 30–80% saturation. The precipitate was dissolved and then dialyzed against 10 mM Tris–HCl (pH 7.2) before loading on a DEAE-cellulose (5 × 10 cm) column previously equilibrated with 10 mM Tris–HCl buffer (pH 7.2). The column was then eluted, initially with the Tris–HCl buffer, and then with 150 mM and 1 M NaCl in the Tris–HCl buffer to yield fractions D1, D2, and D3, respectively. D1, the only fraction with antifungal activity, was then subjected to affinity chromatography on a column of Affi-gel blue gel (2.5 × 20 cm). After elution of unadsorbed materials (B1) devoid of antifungal activity with 10 mM Tris–HCl (pH 7.2), adsorbed proteins (B2) with antifungal activity were eluted with 1.5 M NaCl in the Tris–HCl buffer. After dialysis, fraction B2 was subjected to ion exchange chromatography on a 1.5 × 15 cm column of CM-Sepharose in 10 mM NH_4OAc (pH 4.5). Fraction B2 was fractionated into an unadsorbed peak CM1 (eluted by 10 mM NH_4OAc, pH 4.5) and three adsorbed peaks CM2, CM3 and CM4 eluted by a linear concentration (0–1 M) gradient of NaCl in the NH_4OAc buffer. CM3 and CM4 were then chromatographed by fast protein liquid chromatography (FPLC) on a Superdex peptide column in 0.2 M NH_4HCO_3 (pH 8.8). CM3 yielded three peaks CM3SU1, CM3SU2 and CM3SU3. CM4 yielded three peaks CM4SU1, CM4SU2 and CM4SU3. The column had previously been calibrated with molecular weight markers including bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), ribonuclease (13.7 kDa) and cytidine (0.246 kDa). The purified proteins CM3SU2 and CM4SU3 were designated α-basrubrin and β-basrubrin, respectively.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). It was conducted according to the method of Laemmli and Favre (15) using 18% gel. After electrophoresis the gel was stained with Coomassie brilliant blue. The molecular weights of α- and β-basrubrins were determined by comparison of their electrophoretic mobilities with those of molecular weight marker proteins from Amersham Pharmacia Biotech.

Amino acid sequence analysis. The N-terminal amino acid sequences of α- and β-basrubrins were analyzed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett–Packard 1000A protein sequencer equipped with an HPLC system.

Assay of antifungal activity. The assay for antifungal activity toward various fungal species was carried out in 100 × 15 mm petri plates containing 10 ml of potato dextrose agar. At a distance of 0.5 cm away from the rim of the mycelial colony were placed sterile

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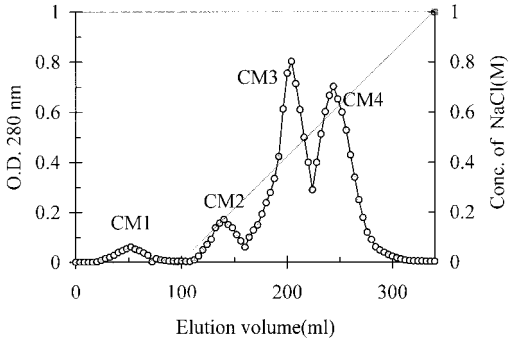

FIG. 1. Ion exchange chromatograph of fraction B2 from 500 g seeds on a CM-Sepharose CL-6B column (2.5 × 15 cm) in 10 mM NH_4OAc buffer (pH 4.5). The slanting dotted line across the chromatogram represents the linear concentration gradient (0–1 M) of NaCl used to elute the adsorbed peaks CM2, CM3, and CM4. The yields of CM1, CM2, CM3, and CM4 were 2.5, 4.3, 24.7, and 19.4 mg, respectively.

blank paper disks (0.625 cm in diameter). An aliquot (10 μl) of the test sample 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.

Assay for cell-free translation-inhibitory activity. Rabbit reticulocyte lysate was prepared from the blood of rabbits rendered anemic by phenylhydrazine injections. An assay based on the rabbit reticulocyte lysate system was used. α- or β-basrubrin (10 μl) was added to 10 μl of hot 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 (16).

Assay for HIV-1 reverse transcriptase inhibitory activity. Reverse transcriptase activity was measured by ELISA as described by Collins *et al.* (17) using a nonradioactive kit from Boehringer Mannheim (Germany). The inhibition assay was performed as described in the protocol included with the kit, except that each well contained 2 ng recombinant HIV-1 reverse transcriptase in a total reaction volume of 60 μl.

RESULTS

After saline extraction, $(NH_4)_2SO_4$ precipitation, and dialysis, the crude protein preparation from Ceylon spinach seeds was chromatographed on DEAE-cellulose to yield an unadsorbed fraction (D1) and two adsorbed fractions (D2 and D3). Only D1 possessed antifungal activity. D1 was fractionated on Affi-gel blue gel into a broad unadsorbed fraction B1 and a sharp adsorbed fraction B2 (data not shown). Antifungal activity resided only in B2. Upon ion exchange chromatography on CM-Sepharose, B2 was fractionated into a small unadsorbed peak CM1, a small adsorbed peak CM2 and two large adsorbed peaks of about the same size, CM3 and CM4 (Fig. 1). Subsequent gel filtration of CM3 on a Superdex peptide column yielded three peaks CM3SU1, CM3SU2 and CM3SU3. The purified antifungal protein in CM3SU2 designated α-basrubrin exhibited a molecular weight of 4.3 kDa (Fig. 2). CM4 was resolved by FPLC-gel filtration on the Superdex peptide column into two small peaks CM4SU1 and CM4SU2 and a large peak CM4SU3. The purified antifungal protein in CM4SU3 was designated β-basrubin (Fig. 3). It appeared as a single band with a molecular weight of 5.0 kDa in SDS–PAGE (Fig. 4). The N-terminal sequences of α- and β-basrubrins are presented in Tables 1 and 2,

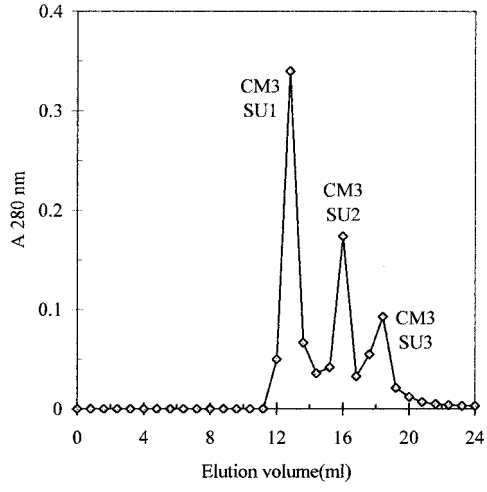

FIG. 2. FPLC-gel filtration of fraction CM3 (24.7 mg) on a Superdex peptide column in 0.2 M NH_3HCO_3 buffer (pH 8.8). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml. The yield of CM3SU2 (α-basrabrin) was 2.5 mg.

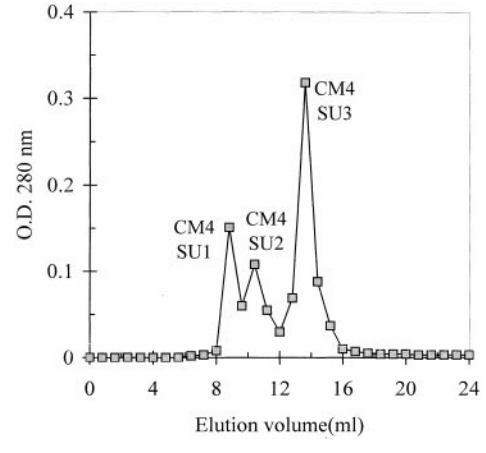

FIG. 3. FPLC-gel filtration of fraction CM4 (19.4 mg) on a Superdex peptide column in 0.2 M NH_3HCO_3 buffer (pH 8.8). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml. The yield of CM4SU3 (β-basrubrin) was 7.1 mg.

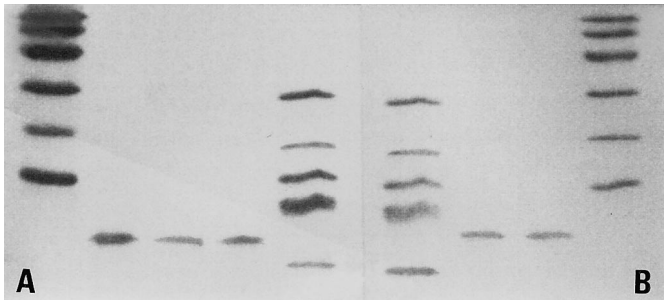

FIG. 4. SDS–PAGE of α- and β-basrubrin. Left lane of A and right lane of B: Pharmacia molecular weight markers, from top downward, phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa). Middle lanes of A represent α-basrubrin and middle lanes of B represent β-basrubrin. Right lane of A and left lane of B: Pharmacia molecular weight markers, from top downward, 26.625, 16.95, 14.437, 6.512, and 3.496 kDa.

respectively. α-Basrubrin and β-basrubrin inhibited cell-free translation with an IC_{50} of 400 and 100 nM, respectively (Table 3). α-Basrubrin and β-basrubrin respectively brought about (79.4 $\pm$ 7.8)% and (54.6 $\pm$ 3.6)% inhibition of HIV-1 reverse transcriptase at 400 μM and (10.6 $\pm$ 0.9)% and (2.1 $\pm$ 0.8)% at 40 μM.

α-Basrubrin exhibited potent antifungal activity toward *Botrytis cinerea* and *Fusarium oxysporum* and some antifungal activity toward *Mycosphaerella arachidicola* (Figs. 4–7). β-Basrubrin was stronger than α-basrubrin in its antifungal activity toward *Mycosphaerella arachidicola* but similar to α-basrubrin in its antifungal potency against *Botrytis cinerea* and *Fusarium oxysporum* (Figs. 8–10). Neither α- nor β-basrubrin exerted antifungal activity toward *Coprinus comatus* and *Rhizoctonia solani.*

DISCUSSION

The majority of the antifungal proteins known to date have a molecular weight exceeding 10 kDa. However, some are below 10 kDa in molecular weight. The antifungal peptides in this category include cysteine protease inhibitors from pearl millet seeds (18), a peptide designated GAFP from *Ginkgo biloba* leaves (14), hypogin with sequence similarity to peanut allergen Ara H1 from peanuts (19), and a novel peptide from cowpea (20). α- and β-basrubrins are novel antifungal peptides isolated in the present investigation. The presence of more than one antifungal peptide in Ceylon spinach seeds is in line with similar reports on cowpea (20) and barley (21).

The antifungal potency of α- and β-basrubrins is higher than that of leguminous antifungal proteins when tested using a similar assay system (9, 12, 19, 20, 23). The translation-inhibiting activities of α- and β-basrubrins are also higher than those of previously reported antifungal proteins. The IC_{50} values for α- and β-basrubrins are in the nanomolar range while those for previously reported antifungal proteins are in the micromolar range. The higher antifungal potency of the basrubrins may be related to their higher translation-inhibiting activities. It has been documented that ribosome inactivating proteins inhibit protein synthesis in fungi and exert antifungal effects (21).

Like the previously reported antifungal proteins from cowpea (20) and hypogin from peanut (19), α- and β-basrubrins manifest an inhibitory activity toward HIV-1 reverse transcriptase. The potencies of basrubrins are similar to those of some natural products (22).

TABLE 2

N-Terminal Sequence of β-Basrubrin (Results of BLAST Search)

	Residue		Residue
β-Basrubrin	1	KIMAKPSKFYEQLRGR	15
Chitinase A from *Streptomyces lividans*	533	KPSKTYPDLRG	543

Note. Identical amino acids are underlined. K is residue number 533 in chitinase A from *Streptomyces lividans.*

TABLE 1

N-Terminal Sequence of α-Basrubrin (Results of BLAST Search)

	Length	Residue		Residue
α-Basrubrin		1	GADFQECMKEHSQKQHQHQG	20
DNA binding protein from *Arabidopsis thaliana*	56	9	FQQ·LQIHSQKQQQQQ	23
Nucleic acid-binding protein from yeast	153	79	QKQHQHQ	85
G-box binding factor from *Dictyostelium discoideu*	708	225	QHSQPQQQHQ	234
Chitin synthase from *Aspergillus nidulans* and *Emericella nidulans*	189	7	CMKEHSQK	14
α-Basrubrin		1	GADFQECMKENS··QKQH··QHQ	20
Zinc finger protein from *Homo sapiens*	354	318	EC··EKSFVQKQHLLQHQ	333

Note. Identical amino acid residues are underlined. DNA-binding protein from *Arabidopsis thaliana* has 56 amino acid residues and F is its 9th residue.

TABLE 3

Ability of α- and β-Basrubrins to Inhibit Translation in Rabbit Reticulocyte Lysate

	Concn. (μM)	[^{3}H]Leucine incorporation (cpm, mean ± SD, n = 3)	% Inhibition (mean ± SD, n = 3)
CM3SU2	0	21630 ± 815	0
(α-basrubrin)	0.005	20721 ± 720	4.26 ± 3.37
	0.05	16549 ± 824	23.80 ± 3.86
	0.5	9449 ± 383	57.05 ± 1.80
	5	3850 ± 915	83.27 ± 0.43
	50	473 ± 33	99.08 ± 0.15
CM4SU3	0	21630 ± 815	0
(β-basrubrin)	0.005	16924 ± 329	22.04 ± 1.54
	0.05	12789 ± 616	41.41 ± 2.89
	0.5	6454 ± 282	71.08 ± 1.32
	5	2989 ± 36	87.31 ± 0.16
	50	324 ± 11.5	99.78 ± 0.05

Note. The nonspecific binding in this assay for translation-inhibiting activity in a rabbit reticulocyte assay system was 279 ± 22 cpm. The IC_{50} values of α- was β-basrubrin were 400 and 100 nM, respectively.

α- and β-basrubrins exert a potent suppressive action on mycelial growth in *B. cinerea, M. arachidicola* and *F. oxysporum*. Antifungal proteins including dolichin (23), sativin (9), mungin (12) and hypogin (19) display a weaker antifungal activity on *F. oxysporum*.

The chromatographic procedure employed in the present investigation to isolate α- and β-basrubrins has also been utilized to purify other antifungal proteins such as sativin (9), dolichin (23), mungin (12), and α- and β-antifungal proteins from cowpea (20). These antifungal proteins are all unadsorbed on DEAE-ion exchangers and adsorbed on CM-ion exchangers and the like as well as Affi-gel blue gel.

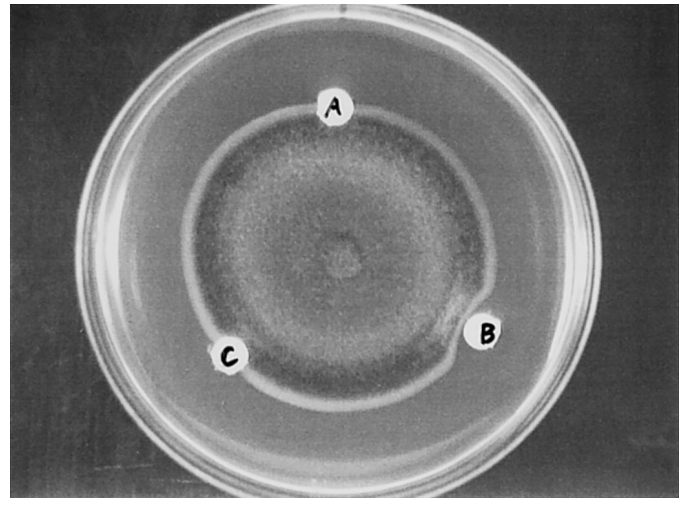

FIG. 6. Inhibitory activity of CM3SU2 toward *Mycosphaerella arachidicola*. (A) Control: 10 μl 0.1 M Mes buffer (pH 6); (B) 16 μg CM3SU2 in 10 μl 0.1 M Mes buffer (pH 6); (C) 3.2 μg CM3SU2 in 10 μl 0.1 M Mes buffer (pH 6).

Both α- and β-basrubrins are peptides characterized by an abundance of basic amino acids. The N-terminal of α-basrubrin is rich in histidine, glutamine and lysine. Arginine and lysine residues are present in the N-terminal sequence of β-basrubrin. Noteworthy is the observation that α-basrubrin has an N-terminal sequence analogous to those of DNA-binding protein and zine finger protein. Probably an interaction between the proteins and DNA would ultimately lead to an antifungal effect. This is reminiscent of the interaction of a steroid hormone receptor with DNA culminating in

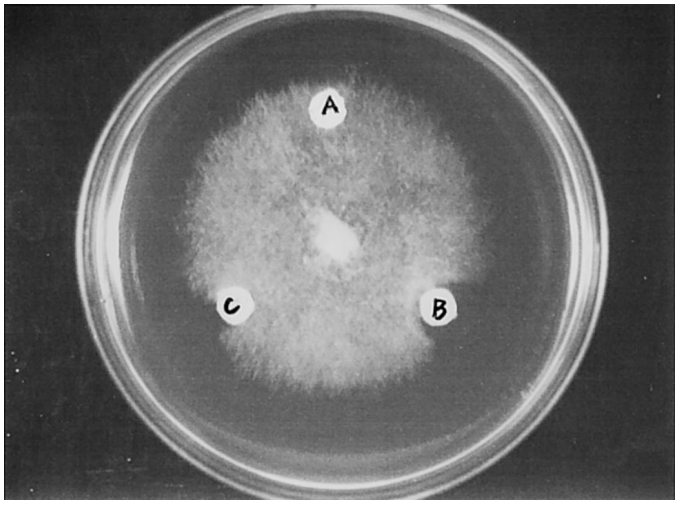

FIG. 5. Inhibitory activity of CM3SU2 toward *Botrytis cinerea*. (A) Control: 10 μl 0.1 M Mes buffer (pH 6); (B) 16 μg CM3SU2 in 10 μl 0.1 M Mes buffer (pH 6); (C) 3.2 μg CM3SU2 in 10 μl 0.1 M Mes buffer (pH 6).

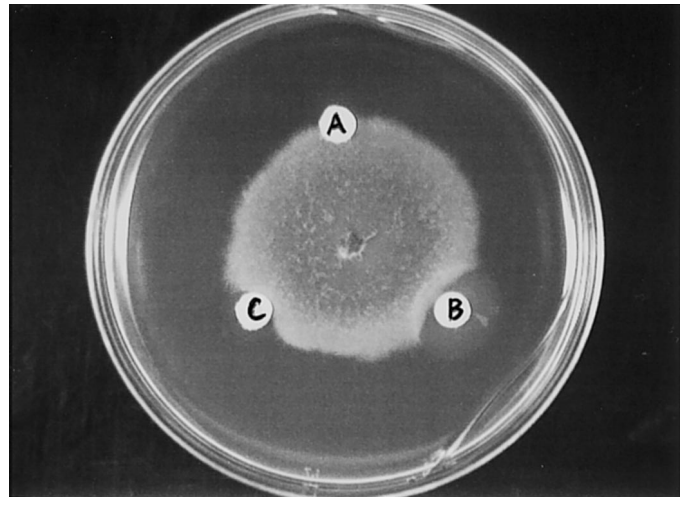

FIG. 7. Inhibitory activity of CM3SU2 toward *Fusarium oxysporum*. (A) Control: 10 μl 0.1 M Mes buffer (pH 6); (B) 16 μg CM3SU2 in 10 μl 0.1 M Mes buffer (pH 6); (C) 3.2 μg CM3SU2 in 10 μl 0.1 M Mes buffer (pH 6).

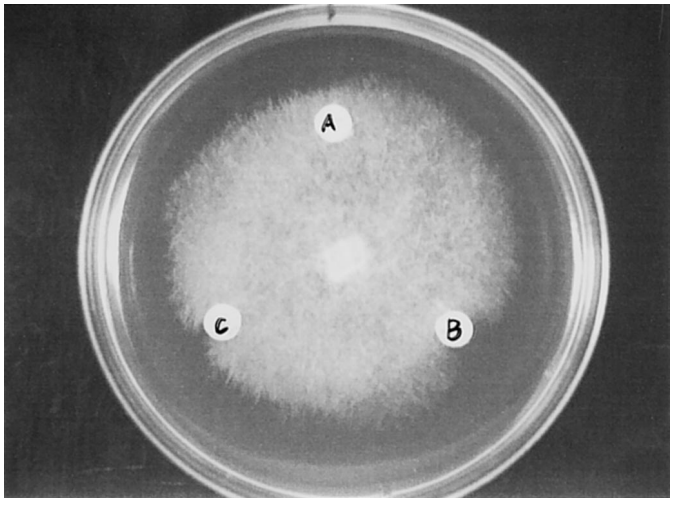

FIG. 8. Inhibitory activity of CM4SU3 toward *Botrytis cinerea*. (A) Control: 10 μl 0.1 M Mes buffer (pH 6); (B) 16 μg CM3SU3 in 10 μl 0.1 M Mes buffer (pH 6); (C) 3.2 μg CM3SU3 in 10 μl 0.1 M Mes buffer (pH 6).

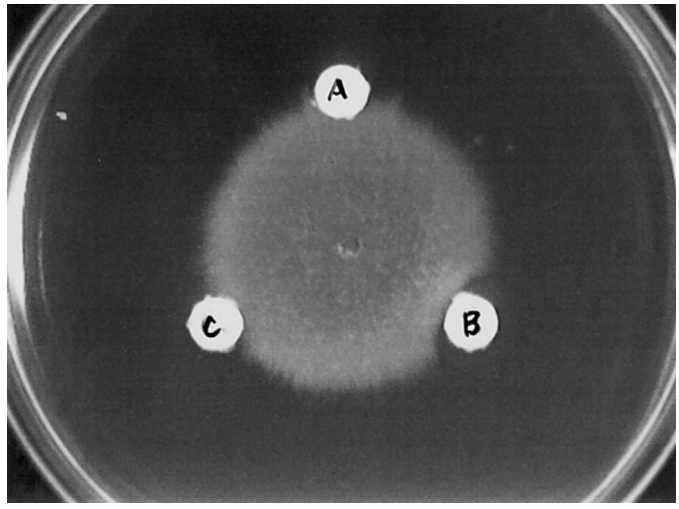

FIG. 10. Inhibitory activity of CM4SU3 toward *Fusarium oxysporum*. (A) Control: 10 μl 0.1 M Mes buffer (pH 6); (B) 16 μg CM3SU3 in 10 μl 0.1 M Mes buffer (pH 6); (C) 3.2 μg CM3SU3 in 10 μl 0.1 M Mes buffer (pH 6).

expression of the physiological effect of the steroid hormone. DNA binding proteins are implicated in translation (24). The N-terminal sequence of α-basrubrin is similar to G-box binding factor which has DNA binding activity (25). Another interesting finding is that α-basrubrin resembles chitin synthase in a portion of the N-terminal sequence. Sometimes a part of a molecule may produce an effect antagonistic to that of the intact molecule. For instance, activin and inhibin have opposite effects on secretion of follicle stimulating hormone from the pituitary. Activin is formed from a dimer of the β-subunit of inhibin (26).

The N-terminal sequence of β-basrubrin demonstrates some similarity to the C-terminal sequence of chitinase A. Maybe this structural feature endows β-basrubrin with antifungal activity.

In summary, antifungal peptides but not antifungal proteins could be isolated from Ceylon spinach seeds.

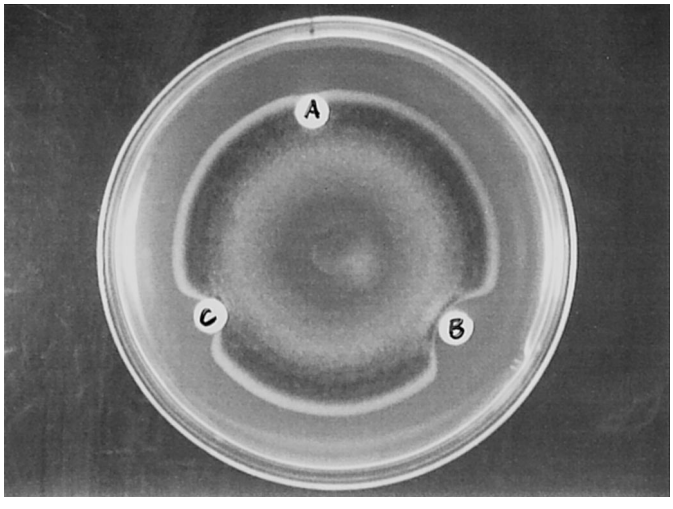

FIG. 9. Inhibitory activity of CM4SU3 toward *Mycosphaerella arachidicola*. (A) Control: 10 μl 0.1 M Mes buffer (pH 6); (B) 16 μg CM3SU3 in 10 μl 0.1 M Mes buffer (pH 6); (C) 3.2 μg CM3SU4 in 10 μl 0.1 M Mes buffer (pH 6).

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