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Isolation of Allicepin, a Novel Antifungal Peptide from Onion (*Allium cepa*) Bulbs

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Abstract: From the bulbs of the onion *Allium cepa*, a novel antifungal peptide distinct from the antimicrobial peptide previously reported from onion seeds was isolated. The antifungal peptide, designated allicepin, was purified with a procedure that involved aqueous extraction, ion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel and FPLC-gel filtration on Superdex 75. Allicepin was unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel. The molecular weight of allicepin was estimated to be 10 K by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration on Superdex 75. Allicepin exerted an inhibitory activity on mycelial growth in several fungal species including *Botrytis cinerea*, *Fusarium oxysporum*, *Mycosphaerella arachidicola* and *Physalospora piricola*.

Keywords: onion; bulbs; antifungal peptide; isolation

INTRODUCTION

Antifungal proteins and peptides have been purified from a variety of *Allium* species including the onion *Allium cepa* [1], the leek *Allium porrum* [2], the garlic *Allium sativum* [3,4] and the chive *Allium tuberosum* [5]. The antifungal proteins from leek roots and outer leaf sheaths, garlic bulbs and leaves, and chive shoots are chitinases while the onion seed produces an antimicrobial peptide. In view of the observation that different tissues of the same organism may produce the same protein [6] or different proteins [7,8], the present investigation was undertaken to isolate the antifungal principle present in onion bulbs. The results disclose that the antifungal peptide purified is structurally distinct from the other *Allium* antifungal proteins and peptide.

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MATERIALS AND METHODS

Purification of Antifungal Peptide

Fresh onion bulbs with brown, dry outer scale leaves and white fleshy inner scale leaves (1.8 kg) were obtained from a local supermarket. A water extract was prepared by homogenizing the bulbs in distilled water, centrifuging the homogenate and collecting the supernatant. Tris-HCl buffer (pH 7.3) was added to the supernatant until the concentration of Tris reached 10 mM. The supernatant was then applied to a column of DEAE-cellulose (5 × 20 cm) in 10 mM Tris-HCl buffer (pH 7.3). The column was eluted with the same buffer to obtain unadsorbed proteins which formed fraction D1 and then with the same buffer containing 0.8 M NaCl to remove adsorbed proteins which formed fraction D2. Fraction D1 was next subjected to affinity chromatography on an Affi-gel blue gel column (2.5 × 20 cm). Unadsorbed proteins (fraction B1) were eluted with 10 mM Tris-HCl buffer (pH 7.3) while adsorbed proteins

(fraction B2) were eluted with 1.5 M NaCl in the 10 mM Tris-HCl buffer. Fraction B2 was dialysed, lyophilized and then further purified by fast protein liquid chromatography on a gel filtration Superdex 75 HR 10/30 column in 0.2 M NH_4HCO_3 buffer (pH 8.5). The second peak constituted purified antifungal protein which was designated allicepin.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It was conducted according to the method of Laemmli and Favre [9]. After electrophoresis the gel was stained with Coomassie Brilliant Blue. The molecular weight of allicepin was determined by comparison of its electrophoretic mobility with those of molecular weight marker proteins from Amersham Biosciences.

Amino acid sequence analysis. The *N*-terminal amino acid sequence of allicepin was analysed 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 [10].

Assay of antifungal activity. The assay for antifungal activity toward *Mycosphaerella arachidicola* and *Physalospora piricola* was carried out in 100 × 15 mm petri dishes 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 (15 µl) of allicepin was added to a disk. The dishes 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 [5].

To determine the IC_{50} value for the antifungal activity, three doses of allicepin 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 only without allicepin 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 [5].

RESULTS

The extract of onion bulbs was fractionated on DEAE-cellulose into an unadsorbed fraction D1 with antifungal activity and an adsorbed fraction D2 without activity. D1 was separated on Affi-gel blue gel into an unadsorbed peak B1 without antifungal activity and an adsorbed peak B2 with antifungal activity (Figure 1). B2 yielded, upon gel filtration on Superdex 75, two peaks SU1 and SU2 (Figure 2). Antifungal activity resided in the latter peak. SU2 exhibited a single band with a molecular weight of

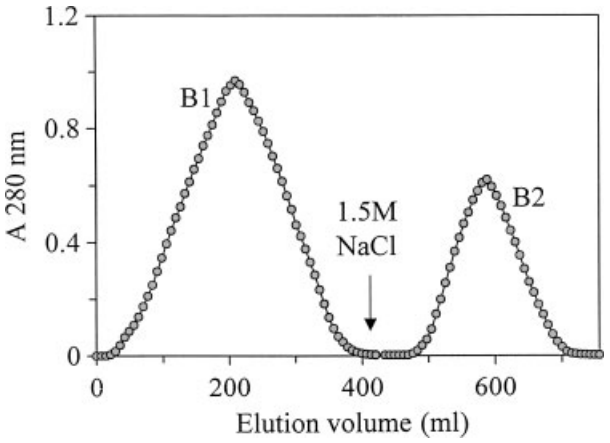

Figure 1 Affinity chromatography on Affi-gel blue gel. Column dimensions: 2.5 × 20 cm. Sample: Fraction D1 unadsorbed on DEAE-cellulose. Starting buffer used to elute unadsorbed fraction B1: 10 mM Tris-HCl buffer (pH 7.3). Buffer for elution of adsorbed fraction B2: 1.5 M NaCl in starting buffer.

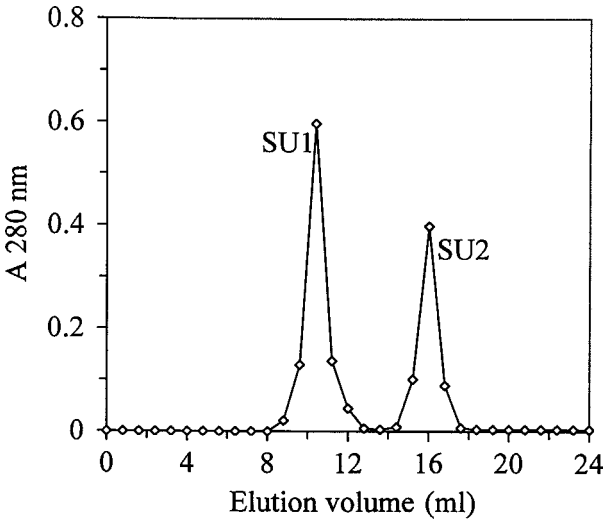

Figure 2 Fast protein liquid chromatography of fraction B2 on a Superdex 75 HR 10/30 column. Buffer: 0.2 M NH_4HCO_3 (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml. SU2 represented purified antifungal protein designated allicepin.

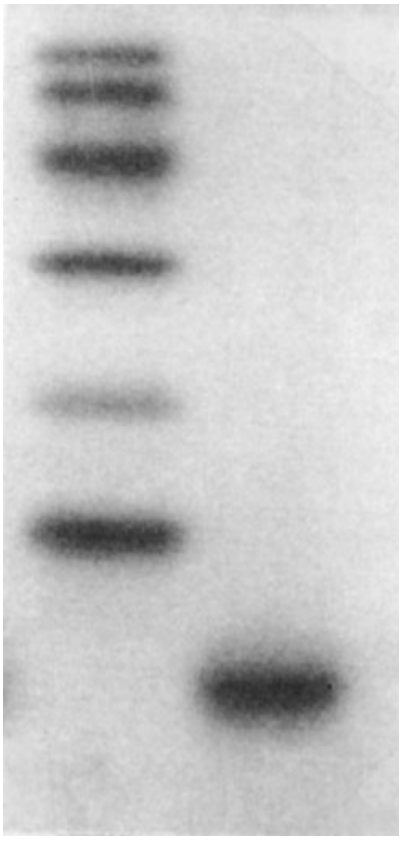

Figure 3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of allicepin. Left lane: Amersham Biosciences molecular weight standards. From top downward: phosphorylase b (94 K), bovine serum albumin (67 K), ovalbumin (43 K), carbonic anhydrase (20 K), soybean trypsin inhibitor (20 K) and α-lactalbumin (14.4 K). Right lane: allicepin.

10 K in SDS-PAGE (Figure 3). It possessed the *N*-terminal sequence YRCCELQ. Allicepin exerted an antifungal action against *Physalospora piricola* at 15 μg. Its antifungal activity against *Botrytis cinerea*, *Fusarium oxysporum* and *Mycosphaerella arachidica* was discernible only at 75 μg (Figures 4–7). The IC_{50} values of its antifungal activity against the four aforementioned fungal species were respectively 14.4 μM, 11.7 μM, 10.3 μM and 4.2 μM for *Botrytis cinerea*, *Fusarium oxysporum*, *Mycosphaerella arachidicola* and *Physalospora piricola*.

DISCUSSION

The *N*-terminal sequence of allicepin is distinct from the antimicrobial peptide Ace-AMP1 from onion seeds [1], the antifungal protein allivin from bulbs

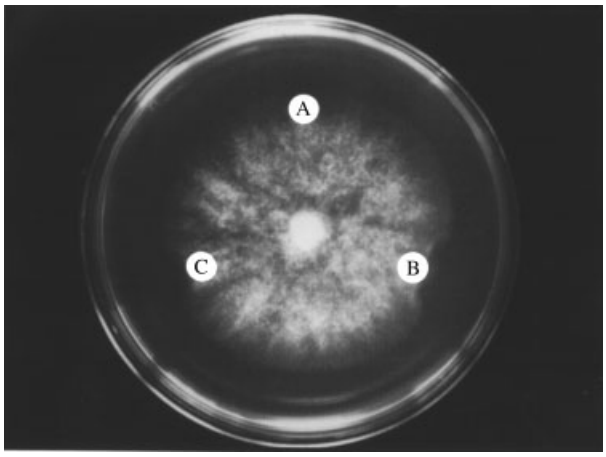

Figure 4 Antifungal activity of allicepin toward *Botrytis cinerea*. A: control (15 μl 50 mM MES buffer, pH 6.0). B: 75 μg allicepin in 15 μl MES buffer. C: 15 μg allicepin in 15 μl MES buffer.

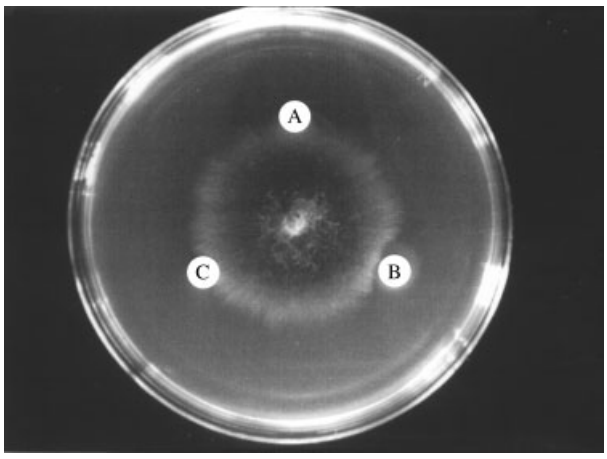

Figure 5 Antifungal activity of allicepin toward *Fusarium oxysporum*. A: control (15 μl 50 mM MES buffer, pH 6.0). B: 75 μg allicepin in 15 μl MES buffer. C: 15 μg allicepin in 15 μl MES buffer.

of the round-cloved garlic [4] and chitinases from *Allium sativum* [3], *Allium porrum* [2] and *Allium tuberosum* [5]. However, allicepin resembles the chitinases of the other *Allium* species in that they are all rich in cysteine residues.

The molecular weight of allicepin was similar to that of the antimicrobial peptide from onion seeds [1] and allivin from garlic bulbs [4], but much smaller than antifungal proteins from *Allium tuberosum* [5], *Allium porrum* [2] and *Allium sativum* [3] chitinases.

Allicepin was unadsorbed on DEAE-cellulose but adsorbed on Affi-gel blue gel, similar to that observed for other antifungal proteins and

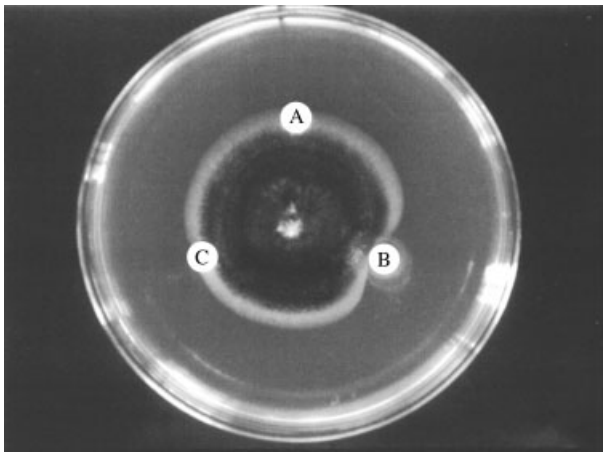

Figure 6 Antifungal activity of allicepin toward *Mycosphaerella arachidicola*. A: control (15 μl 50 mM MES buffer, pH 6.0). B: 75 μg allicepin in 15 μl MES buffer. C: 15 μg allicepin in 15 μl MES buffer.

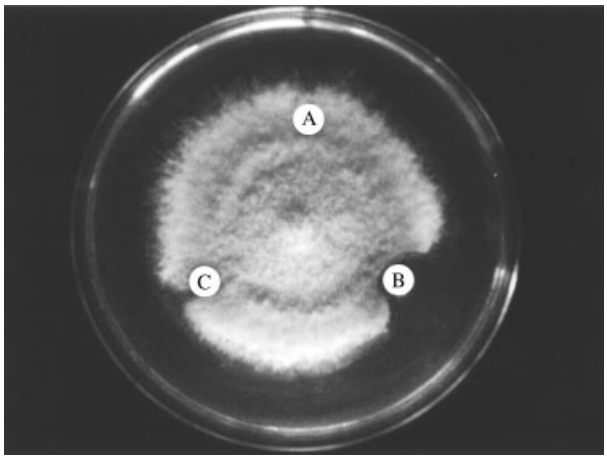

Figure 7 Antifungal activity of allicepin toward *Physalospora piricola*. A: control (15 μl 50 mM MES buffer, pH 6.0). B: 75 μg allicepin in 15 μl MES buffer. C: 15 μg allicepin in 15 μl MES buffer.

peptides [4,5,11,12]. The unadsorbed fraction with antifungal activity from the DEAE-cellulose column can be directly chromatographed on Affi-gel blue gel without the need for dialysis beforehand. On the other hand, *Allium porrum* and *Allium sativum* chitinases were purified using a chitin column, a mannose-Sepharose column, and a Mono Q or a Mono S column [2,3].

Like allivin [4] and *Allium tuberosum* chitinase [5], allicepin was active against a diversity of fungi including *Botrytis cinerea*, *Fusarium oxysporum* and *Mycosphaerella arachidicola*. It is noteworthy that *Allium porrum* chitinase exerted no antifungal activity against *Fusarium oxysporum* and *Botrytis cinerea* [2] while *Allium sativum* chitinase was characterized with regard to endochitinase but not antifungal activity [3].

Table 1 Comparison of *N*-terminal Sequence of Allicepin with Antifungal Proteins and Peptides from Various *Allium* species

Allicepin	YRCCELQ
Ace-AMP1	QNICPRVNRIV
CHITAS1	QQCGSQAGGAL
CHITAS2	QQCGSQGSGAL
Allivin	DTFSDAGSFLD
ATC	EQHGSQAGGAL
APC-Dr	EQCGRQAGGAL
APC-D	EQCGRQA
APC-F	EQCGRQAGGAL

Sequences of *Allium cepa* antimicrobial protein Ace-AMP1 is from reference [1]. Sequences of *Allium sativum* chitinases CHITAS1 and CHITAS2 are from reference [3] and those of *Allium porrum* chitinase isoforms APC-Dr, -D and -F are from reference [8]. The sequences of round-cloved antifungal protein from *Allium sativum* (allivin) and *Allium tuberosum* chitinase are from references [4] and [5], respectively.

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