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Purification of allivin, a novel antifungal protein from bulbs of the round-cloved garlic

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Abstract

A novel antifungal protein, designated allivin, was isolated from bulbs of the round-cloved garlic *Allium sativum var. round clove* with a procedure involving ion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, ion exchange chromatography on CM-Sepharose and FPLC-gel filtration on Superdex 75. Allivin possessed an N-terminal sequence demonstrating very little similarity to sequences of *Allium sativum* chitinases and ribosome inactivating proteins. Allivin exhibited a molecular weight of 13 kDa in gel filtration and SDS-polyacrylamide gel electrophoresis. It displayed antifungal activity against *Botrytis cinerea*, *Mycosphaerella arachidicola* and *Physalospora piricola*. It inhibited translation in a cell-free rabbit reticulocyte system with an IC_{50} of 1.6 μM.

Introduction

Protease inhibitors (1, 2), lectins (3), ribosome inactivating proteins (3–5), ribonucleases (6), α-amylase inhibitors (7) and antifungal proteins (8–17) are proteins which play a defensive role physiologically. Antifungal proteins are structurally diverse, consisting of chitinases (8, 10), glucanases (10), thaumatin-like proteins (9), cyclophilin-like proteins (11), miraculin-like proteins (12), protease inhibitors (13), ribosome inactivating proteins (14, 15), lectins (16, 17), antifungal peptides (18, 19), embryo-abundant protein (20) and others.

A vast amount of research activities has focused on garlic. Its broad spectrum of activities includes antimicrobial, anti-helminthic, anti-protozoal, antifungal, antibacterial, insecticidal, antitumor, antiarthritic, hypolipidemic and hypoglycemic properties (21, 22). Allicin, alliin (22, 23) and a lectin (24) have been isolated from garlic.

Two chitinases (CHITAS1 and CHITAS2) have been isolated from garlic (*Allium sativum*)

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bulbs (25). Their N-terminal sequences resemble chitinases APC-Dr, APC-D and APC-F isolated from the leek *Allium porrum* (26). Their molecular weights exceed 30 kDa. An antifungal protein with 93 amino acids, designated Ace-AMP1, has been isolated from onion (*Allium cepa*) seeds (27). We report herein isolation of a 13kDa antifungal protein, with N-terminal sequence exhibiting very little similarity to the aforementioned chitinases, from bulbs of a variety of garlic bearing only a single, round clove. This variety of garlic is also popular in the Orient.

Materials and methods

Materials

Round-cloved garlic (*Allium sativum var. round clove S.Y. Hu*) bulbs which differ from common garlic bulbs in possessing a single spherical clove instead of multiple cloves in each bulb, were purchased from a local shop. Affi-gel blue gel was purchased from Bio-Rad, and DEAE-cellulose was from Sigma, and CM-Sepharose and Superdex 75 HR 10/30 column were from Amersham Pharmacia Biotech. Chemicals for sequence analysis were obtained from Hewlett Packard (Palo Alto, CA, U.S.A.). All other chemicals used were of reagent grade.

Isolation of allivin

The bulbs (1.2 kg) were homogenized in 10 mM Tris-HCl (pH 7.2) at 4°C. The extract was applied to a column of DEAE-cellulose which had previously been equilibrated with and then eluted with 10 mM Tris-HCl buffer (pH 7.2). After elution of the unadsorbed peak D1 with antifungal activity, the adsorbed peaks D2 and D3 were obtained by elution with 150 mM and 1M NaCl respectively in the same buffer. D1 was next chromatographed on a column of Affi-gel blue gel, which had previously been equilibrated and eluted with 10 mM Tris-HCl buffer (pH 7.2). The unadsorbed fraction B1 was eluted in the same buffer. The adsorbed fraction B2, in which antifungal activity was concentrated, was eluted with 1.5 M NaCl in the Tris-HCl buffer, dialyzed and then further purified by ion exchange chromatography on a column of CM-Sepharose in 10 mM NH_4OAc buffer (pH 4.5). Unadsorbed materials were eluted in the buffer. Adsorbed proteins were desorbed using a linear (0 – 1 M) NaCl gradient. The adsorbed peak (CM3), in which the antifungal activity resided, was subjected to FPLC-gel filtration on a Superdex 75 HR 10/30 column in 200 mM NH_4HCO_3 (pH 8.6). The second (SU2) peak eluted represented the purified protein designated allivin.

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

It was conducted according to the method of Laemmli and Favre (28). After electrophoresis the gel was stained with Coomassie Brilliant Blue. The molecular weight of allivin was determined by comparison of its electrophoretic mobility with those of molecular weight marker proteins from Amersham Pharmacia Biotech.

Amino acid sequence analysis

The N-terminal amino acid sequence of allivin (about 500 picomoles) was analyzed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett

Packard 1000A protein sequencer equipped with an HPLC system. The initial and repetitive yields of the sequencing experiments exceeded 95% and 90% respectively.

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 (5, 29) was used. Allivin 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 containing 5.2 μg rabbit muscle creatine kinase (250 units/mg). 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.

Assay of antifungal activity

The assay for antifungal activity toward *Botrytis cinerea*, *Mycosphaerella arachidicola* and *Physalospora piricola* was carried out in 100 × 15 mm petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, at a distance of 0.5 cm away from the rim of the mycelial colony were placed sterile blank paper disks (0.625 cm in diameter). An aliquot of a solution of allivin was added to a disk. The plates were incubated at 23°C for 72 hours until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity (30).

Results

The seed extract was fractionated on DEAE-cellulose to yield an unadsorbed fraction (D1) and an adsorbed fraction (D2). When D1 was in turn applied on Affi-gel blue gel, an unadsorbed peak (B1) and an adsorbed peak (B2) were obtained (data not shown). B2 was chromatographed on CM-Sepharose to yield a small unadsorbed peak CM1 and two adsorbed peaks of approximately equal size, CM2 and CM3 (Fig. 1). Only CM3 exhibited antifungal activity. CM3 was separated into two peaks of nearly the same size, SU1 and SU2, upon gel filtration on Superdex 75. Only SU2 manifested antifungal activity. It possessed antifungal activity and a molecular weight of 13 kDa (Fig. 2). The purified protein was designated allivin. In SDS-PAGE it appeared as one band with a molecular weight of 13 kDa (Fig. 3). The yield of allivin was 10 mg/kg seeds. Allivin exhibited a small extent of similarity in sequence to *Allium* chitinases (Table 1) and to ribosome inactivating proteins (Table 2). Allivin inhibited cell-free translation with an IC_{50} of 1.6 μM.

Allivin was active against *Botrytis cinerea*, *Mycosphaerella arachidicola* and *Physalospora piricola* (Figs. 4–6). Allivin did not induce hemagglutination (data not shown).

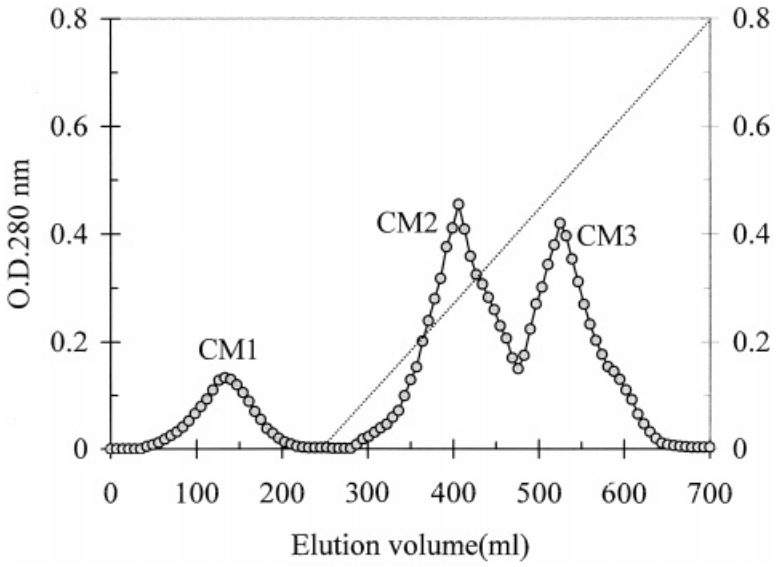

Fig. 1. CM-Sepharose ion exchange chromatography of a fraction of garlic bulb extract unadsorbed on DEAE-cellulose and subsequently adsorbed on Affi-gel blue gel. Column dimensions: 2.5 × 20 cm. Starting buffer: 10 mM NH_4OAc (pH 4.5). The slanting dotted line across the chromatogram indicates application of a 0–1M NaCl concentration gradient in the starting buffer. CM3 with antifungal activity was obtained with a yield of 1 mg.

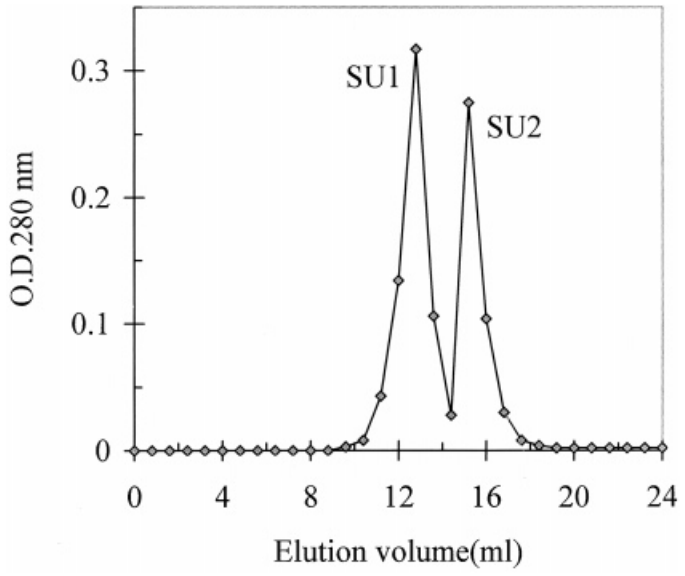

Fig. 2. FPLC-gel filtration of CM3 on Superdex 75. Flow rate: 0.4 ml/min. Fraction size: 0.8 ml. Fraction SU2 was obtained with a yield of 12 mg.

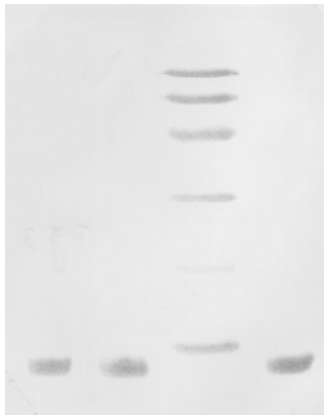

Fig. 3. SDS-PAGE results. From the left, lanes 1, 2 and 4: allivin. Lane 3: molecular weight markers, from top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa).

Discussion

Antifungal proteins have been isolated from the garlic (25), leek (26) and chive (31). These proteins are very similar to each other in sequence (being chitinase-like) and in molecular weight (all over 30 kDa). The antifungal protein isolated from the round-cloved variety of garlic is unique in its N-terminal sequence and low molecular weight (13 kDa). Most of the *Allium* antifungal proteins previously isolated are chitinases but allivin bears little or no resemblance to chitinases and Ace-AMP1 in sequence. Despite these differences, allivin could be isolated using a protocol which had been used successfully to purify clive antifungal protein (31) as well as other antifungal proteins. All of the antifungal proteins isolated thus far employing this purification procedure were unadsorbed on DEAE-cellulose and adsorbed on CM-Sepharose and Affi-gel blue gel (8, 11, 12, 20, 30, 31). On the other hand, leek and

Table 1
N-terminal sequence of allivin in comparison with chitinases of various *Allium* species

Allivin	DTFSDAGSFLDRGLAKSDDDAARRQFQPNNRYFTGGNKGAQGVVDGHHGL
CHITAS1	QQCGSQAGGALCSNRLCCSKFGYCGSTDPYCGTGCQSQCGGGGGGGGRGG
CHITAS2	QQCGSQGSGALCSNGLCCSQYGYCGNGGPYCGTGCQSQCGGPGGGSSGVAS
APC-Dr	EQCGRQAGGALCPGGLC
APC-D	EQCGRQA
APC-F	EQCGRQAGGAL
Ace-AMP1	QNICPRVNRIVTPCVAYGLGRAPIAPCCRALNDLRFVNTRNLRRAACRC
ATC	EQHGSQAGGALHPGXLHYSKYGGYGGTTPDYYGDGQQ

Residues identical to corresponding residues in allivin are underlined. Sequences of *Allium sativum* chitinases CHITAS1 and CHITAS2 are from reference 25 those of *Allium porrum* chitinase isoforms APC-Dr, -D and –F are from reference 26, that of *Allium cepa* antimicrobial protien Ace-AMP1 is from reference 27. The residues of allivin were identified in a single experiment, and that of *Allium tuberosum* chitinase (ATC) is from reference 31.

Table 2
Comparison of N-terminal amino acid sequence of allivin with ribosome inactivating proteins

Allivin	D·TF··SDA··GS···FL·D·R·G·L
RIPIriHo1.A2	IETVQERVTGTTRQSYSAELQTLRTR·L
α-Momorcharin	DVSFRLSGADPRSYGMFIKDLRNA·L
Trichosanthin	DVSFRLSGATSSSYGVFISNLRKA·L
Bryodin	DVSFRLSGATTTSYGVFIKNLREA·L
Luffin-a	DVRFSLSGSSSTSYSKFIGDL
Momorcochin	DVTFSLLGANTKSYAAFITNFRKD·V
Ricin-A	IINFTTAGATVQSYTNFIRAVR·GRL
Abrin-A	KFSTEGATSQSYKQFIEALR·ERL

Residues identical to corresponding residues in allivin are underlined. RIP Iri Hol. A2 is RIP A2 from *Iris hollandica* bulbs. Its sequence is from reference 34. Sequences of other RIPs are from references 5 and 33.

garlic chitinases were purified with the use of a chitin column, a mannose-Sepharose column, a phenyl-Sepharose column and a Mono Q column or a Mono S column (25, 26).

Allivin exhibits some degree of similarity in N-terminal sequence to ribosome inactivating proteins. Out of the eleven invariant or quasi-invariant residues in ribosome inactivating proteins, namely, D^1, V^2, F^4, G^8, A^9, S^{13}, Y^{14}, F^{17}, I^{18}, R^{22} and L^{25}, all except V^2, G^8, Y^{14} and I^{18} are retained in allivin.

Allivin is endowed with a translation-inhibitory activity (IC_{50} = 1.6 μM) which is more potent than those of other previously reported antifungal proteins including the leguminous proteins sativin (12) (IC_{50} = 14 μM), mungin (11) (IC_{50} = 25 μM), dolichin (8) (IC_{50} = 32 μM), and cowpea α- and β-antifungal proteins (32) (IC_{50} = 11 μM and 6.5 μM respectively). This may be attributed to the sequence similarity of allivin to ribosome inactivating

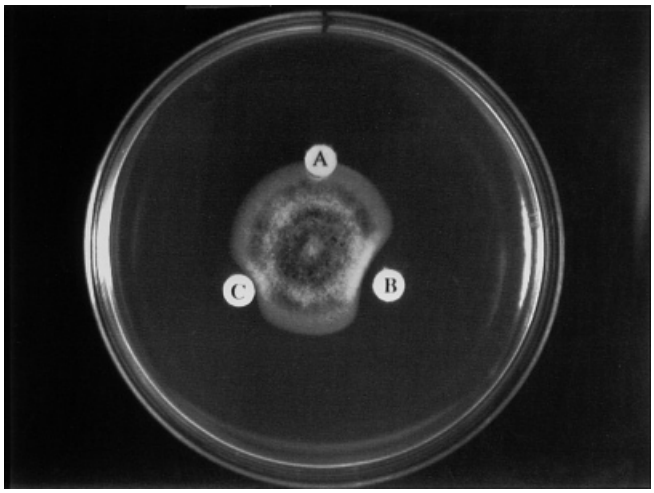

Fig. 4. Antifungal activity of allivin toward *Mycosphaerella arachidicola* (A) Control: 15 μl 0.1M MES buffer (pH 6) (B) 30 μg allivin in 15 μl MES buffer and (C) 6 μg allivin in 15 μl MES buffer.

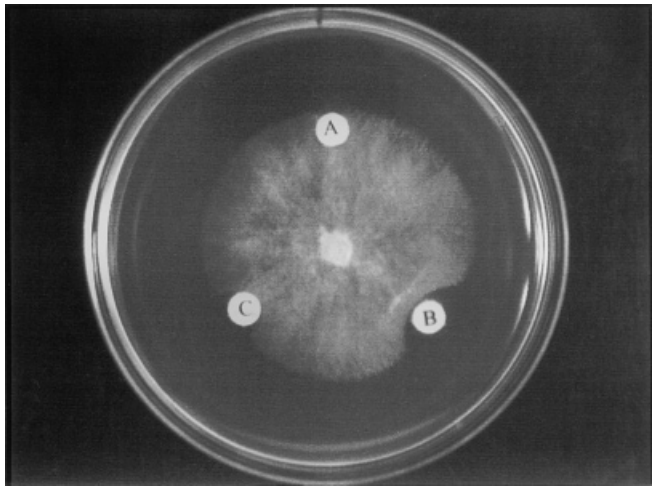

Fig. 5. Antifungal activity of allivin toward *Botrytis cinerea* (A) Control: 15 μl 0.1M MES buffer (pH 6) (B) 30 μg allivin in 15 μl MES buffer and (C) 6 μg allivin in 15 μl MES buffer.

proteins which inhibit translation in the rabbit reticulocyte lysate system with an IC_{50} in the picomolar and nanomolar ranges (33).

The antifungal activity of allivin toward *Botrytis cinerea*, *Mycosphaerella ardchidicola* and *Physalospora piricola* was more active than that of antifungal proteins such as mungin (11) and sativin (12).

Two varieties of the pea *Pisum sativum* have been studied. The variety *arvense Poir* elaborates a ribosome inactivating protein designated pisavin (5) which is similar in N-terminal

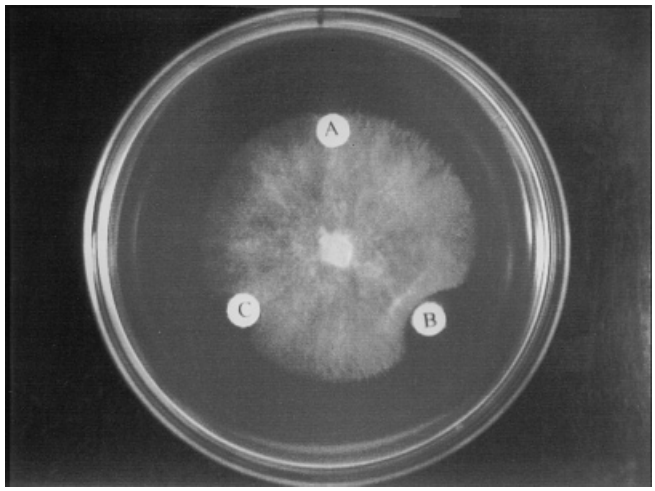

Fig. 6. Antifungal activity of allivin toward *Physalospora piricola* (A) Control: 15 μl 0.1M MES buffer (pH 6) (B) 30 μg allivin in 15 μl MES buffer and (C) 6 μg allivin in 15 μl MES buffer.

sequence to the antifungal protein sativin from the variety *macrocarpon* (11). Sativin has a much larger molecular weight and a much lower translation-inhibiting protency compared with pisavin (5, 11). The round-cloved variety of *Allium sativum* examined in this study produces an antifungal protein different in molecular weight and N-terminal sequence from the multiple-cloved variety. The above findings suggest that different varieties of the same species may produce different proteins.

The isolation of cysteine-rich chitinases from *Allium sativum* (25) and *Allium porrum* (26), a cysteine-deficient chitinase-like antifungal protein from *Allium tuberosum* (31) and a structurally unique antifungal protein from *Allium sativum var. round clove* in this study, indicate that structurally different antifungal proteins are synthesized by members of Alliaceae.

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