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Purification and characterization of α- and β-benincasins, arginine/glutamate-rich peptides with translation-inhibiting activity from wax gourd seeds

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Abstract

Two peptides, with a molecular mass of about 11 kDa and an N-terminal sequence abundant in arginine and glutamine residues, were isolated from wax gourd seeds. The isolation protocol included affinity chromatography on Affi-gel blue gel, ion-exchange chromatography on Mono-S and gel filtration on Superdex 75. The peptides, designated α- and β-benincasins, inhibited cell-free translation in a rabbit reticulocyte lysate system with an IC_{50} of 20 and 320 pM, respectively. α-Benincasin exhibited weak antifungal activity toward *Coprinus comatus* and *Physalospora piricola* but not toward *Mycosphaerella arachidicola*.
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Keywords: Arginine/glutamate-rich peptides; Seeds; Wax gourd; Purification

1. Introduction

The seeds of cucurbitaceous plants constitute a source of proteins including ribosome inactivating proteins (RIPs) [1,4,10,17–19], ribonucleases [4,15], lectins [18], α-amylase inhibitors [22] and trypsin inhibitors [6,16]. There is evidence suggesting that these proteins play a defensive role in plants. Among these proteins, RIPs have formed a popular subject of research. RIPs manifest a range of activities encompassing antifungal, antiviral, antiproliferative, antitumor and immunomodulatory activities [1,2,10–12,14,17,19]. There are different types of RIPs. Type 1 RIPs consist of a single chain with a molecular mass of about 30 kDa. Type 2 RIPs represent a combination of a Type 1 RIP and a lectin, yielding a molecular mass of about 60 kDa [1]. Small RIPs with a molecular mass of about 10 kDa are by comparison much less well known and have been isolated only from bitter gourd seeds [13] and sponge gourd seeds [5]. Structural information about small RIPs is meager. In addition to the aforementioned RIPs, arginine/glutamate-rich polypeptides (AGRPs) have been reported from sponge gourd seeds [7,8]. Their relationship to small RIPs is unknown though they demonstrate translation-inhibiting activities [7,8,21].

Taking into account the paucity of literature pertaining to small RIPs and AGRPs, we undertook the present investigation to isolate small proteins from wax gourd seeds. The N-terminal sequences of these small proteins disclose that they are AGRPs but not fragments of Type 1 RIPs. In addition, these AGRPs manifest translation-inhibiting activity.

2. Materials and methods

2.1. Materials

Seeds were purchased from a local shop. Affi-gel blue gel was purchased from Bio-Rad, DEAE-cellulose was from Sigma Chemical Co. (St. Louis, MO), 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, USA). All other chemicals used were of reagent grade.

2.2. Isolation of proteins

Fresh seeds collected from wax gourds (*Benincasa hispida* var. *dong-gua*) were obtained locally. A crude extract was obtained by homogenizing the seeds in 10 mM Tris–HCl (pH 7.2), centrifuging the suspension and removing the

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supernatant. The crude extract was passed through a 0.22 μm syringe filter (Nalgene) to remove any particulates. The clear filtrate was applied to a 1.5 × 20-cm Econo-column (Bio-Rad) containing 100 ml of Affi-gel blue gel previously equilibrated with the starting buffer, 10 mM Tris–HCl (pH 7.2). The column was washed with the same buffer to elute unadsorbed proteins until the absorbance at 280 nm was less than 0.05, and then adsorbed protein was eluted with 1.5 M NaCl in the starting buffer. Fractions of 6 ml were collected at a flow rate of 1 ml/min. Protein peaks monitored by A280 were collected, and the fractions were assayed for translation-inhibitory activity. The adsorbed protein solution was then dialyzed extensively against distilled water for 36 h at 40 °C with four changes of water and then lyophilized.

The lyophilized powder was dissolved in 10 mM NH_4OAc buffer (pH 4.6) and applied onto a 1 ml FPLC Mono-S (HR 5/5) column, which had been pre-equilibrated with the same buffer, through an FPLC (Pharmacia AKTA Purifier) system. After unadsorbed proteins were eluted with the same buffer, a linear NaCl gradient from 0 to 1 M in the starting buffer was applied to elute adsorbed proteins at a flow rate of 1 ml/min. Finally, the fractions were dialyzed and lyophilized. They were dissolved in 0.1 M NH_4HCO_3 (pH 9), and applied individually to an FPLC Superdex 75 column (Amersham Pharmacia Biotech), which had been pre-equilibrated with 0.1 M NH_4HCO_3 buffer (pH 9) and was then eluted at a flow rate of 0.4 ml/min. The purified products were designated α- and β-benincasins.

2.3. 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 masses of α- and β-benincasins were determined by comparison of their electrophoretic mobility with those of molecular mass marker proteins from Amersham Pharmacia Biotech.

2.4. Amino acid sequence analysis

The N-terminal amino acid sequences of α- and β-benincasins were analyzed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett-Packard 1000A protein sequencer equipped with an HPLC system [10].

2.5. Assay for cell-free translation-inhibitory activity

Alpha- and β-benincasins were tested for this 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 [10] was used. Different amounts of the test sample in 10 μl were 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.

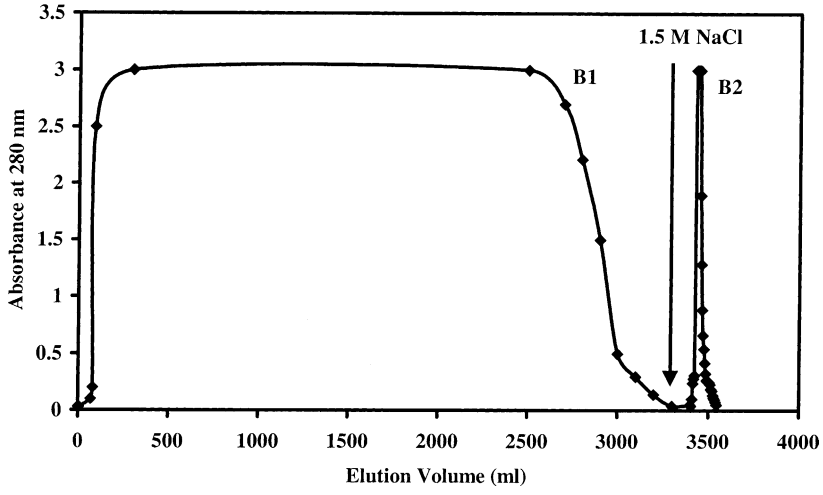

Fig. 1. Elution profile of the crude extract of wax gourd (*B. hispida* var. *dong-gua*) seeds from an Affi-gel blue gel column previously equilibrated with the starting buffer (10 mM Tris–HCl, pH 7.2). The column was initially washed with starting buffer and subsequently eluted with 1.5 M NaCl in the starting buffer.

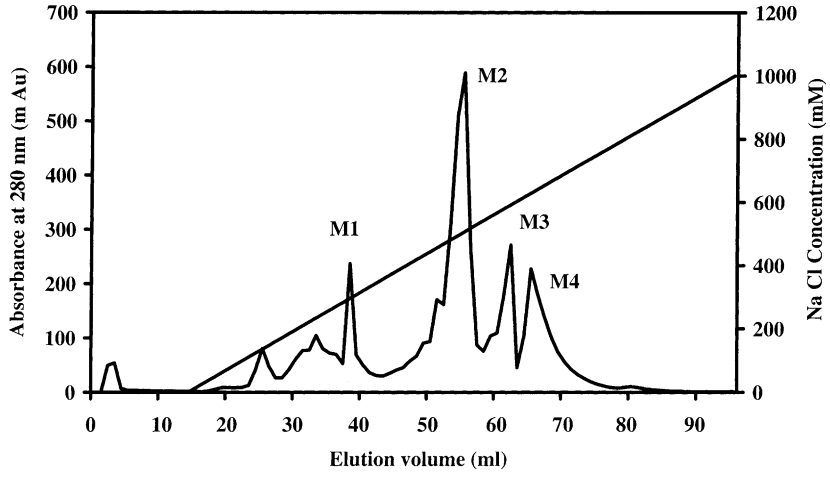

Fig. 2. Elution profile of wax gourd fraction B2 from a Mono-S column previously equilibrated with the starting buffer (10 mM NH_4OAc, pH 4.6). The column was washed initially with the starting buffer and subsequently eluted with 0–1 M NaCl in the starting buffer.

2.6. Assay of antifungal activity

The chromatographic fractions at various stages of purification were tested for antifungal activity after lyophilization. The assay for antifungal activity toward *Mycosphaerella arachidicola*. *Physalospora piricola* and *Coprinus comatus* was carried out in 100 mm × 15 mm Petri plates containing 10 ml of potato dextrose agar. These fungal species have been shown to be sensitive to a variety of antifungal proteins. 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 (10 μl containing 20 μg or 100 μg) of a solution of α-benincasin 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 zones of inhibition around disks containing samples with antifungal activity [20].

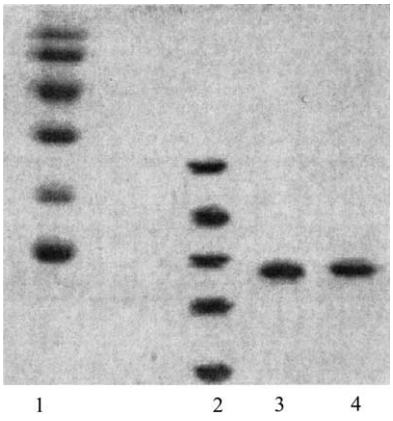

Fig. 3. SDS–PAGE of wax gourd fractions from the Superdex 75 column. Lane 1 shows Pharmacia molecular mass markers, from top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa). Lane 2 shows Pharmacia molecular mass markers, from top downward: triose phosphate isomerase (26.7 kDa), myoglobin (16.9 kDa), α-lactalbumin (14.4 kDa), aprotinin (6.5 kDa) and oxidized insulin b chain (3.5 kDa). Lanes 3 and 4 show α-and β-benincasins, respectively.

2.7. Assay for N-glycosidase activity

The assay was conducted as detailed by Endo and Tsurugi [3] and Lam et al. [10]. The test peptide was incubated at 37 °C with rabbit reticulocyte lysate in 25 mM KCl, 5 mM $MgCl_2$, 25 mM Tris–HCl, pH 7.6, for 30 min. The reaction was terminated by addition of SDS to a final concentration of 0.5%. Total rRNA was extracted with phenol/chloroform, recovered by ethanol precipitation, and allowed to react with

Table 1
Comparison of N-terminal sequences of α- and β-benincasins with AGRPs from *Luffa cylindrica* seeds

Protein	Amino acid sequence
α-Benincasin	**R**DW**ERREFERRQ**NEL**RREQEQRREE**LL
β-Benincasin	**R**DW**RR·EQERRQERR**
5 k-AGRP	**ER**GPDW**RKEQQRRRRREQQRE**HRG
6.5 k-AGRP	P**R**GSP**RTEYE**AC**R**V**R**C**Q**VA**E**HGV**ERQ**
12.5 k-AGRP	
a-chain	**EPRRGEE**AFD**E**CC**RE**L**R**NVD
b-chain	**EGRQQR**C**RQ**MRA**RQE**L**R**SC**E**
14 k-AGRP	
a-chain	AV**E**HL**EE**CC**RQ**L**R**GM**E**DPC**R**
b-chain	**ER**PWL**QR**C**RQQ**I**Q**GP**R**L**R**HCE

Arginine (R), glutamine (E) and glutamate (Q) are indicated in bold. The sequences of AGRPs are obtained from Ishihara et al. [7].

Table 2
A summary of purification of benincasins from wax gourd

Fraction	Protein (mg[a])	IC_{50} (U[b])	Specific activity (U × 10^6/mg)	Total activity (U × 10^6)	Yield (%)
Crude extract	1427	74	13,514	19,283,784	100
B2	68	10	100,000	6,800,000	35.263
M3	1.75	0.4	2,500,000	4,375,000	22.687
M4	1.5	3	333,333	500,000	2.593
α-Benincasin	0.4	0.22	4,545,455	1,818,182	9.429
β-Benincasin	0.6	3.4	294,118	176,471	0.915

[a] Protein obtained from 100 g of starting material.
[b] IC_{50} is expressed in U. One U is defined as the amount of protein (ng/ml) inhibiting protein synthesis by 50%.

1 M aniline/0.8 M acetic acid (pH 4.5) for 5 min at 60 °C prior to electrophoresis in 1% agarose gel containing 3.7% formaldehyde for 1.5 h at a constant voltage of 60 V. The gel was stained in ethidium bromide (0.5 μg/ml) and destained with distilled water. The RNA bands were visualized on a UV transilluminator and photographed with Polaroid 667 instant film.

3. Results

3.1. Purification

Affinity chromatography of a crude extract of wax gourd seeds (IC_{50} of cell-free translation inhibition = 74 ng/ml) on Affi-gel blue gel yielded a large unadsorbed peak (B1) and a sharp adsorbed peak (B2) (Fig. 1). Fraction B2 from Affi-gel blue gel with higher cell-free protein synthesis-inhibitory activity (IC_{50} = 10 ng/ml) than fraction B1 (IC_{50} of inhibition = 750 ng/ml) was chosen for further purification. B2 was fractionated into four major peaks M1–M4 upon FPLC using an FPLC Mono-S column (Fig. 2). The IC_{50} values of M1–M4 in the cell-free translation-inhibition assays were, respectively, 4000, 2800, 0.4 and 3 ng/ml. Fractions M3 and M4 with high cell-free activities gave a single peak each in FPLC-gel filtration on Superdex 75 named α- and β-benincasins, respectively (data not shown). Their molecular masses were determined to be 12 kDa each in both gel filtration and SDS–PAGE (Fig. 3).

3.2. N-terminal sequence analysis

The N-terminal sequences of purified α- and β-benincasins showed considerable homology to other AGRPs (Table 1).

3.3. Biological activities

Alpha- and β-benincasins displayed very strong protein synthesis-inhibitory activity, with an IC_{50} of 20 and 320 pM, respectively (Fig. 4). In the *N*-glycosidase assay, a band similar to the Endo's band [10] was obtained (data not shown). α-Benincasin displayed species-specific albeit weak antifungal activity (Fig. 5a and b). Mycelial growth

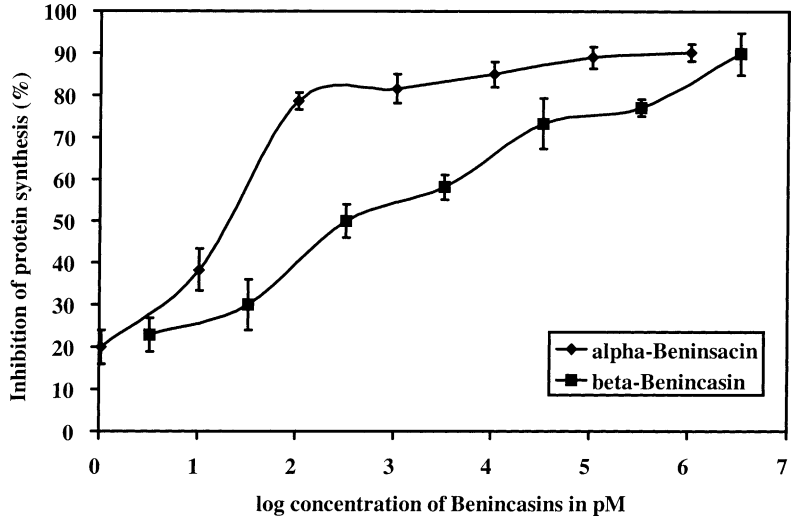

Fig. 4. Protein synthesis-inhibitory activity of α- and β-benincasins (data represent means ± S.D. $n = 3$) (values of 1 and 2 on the horizontal axis indicate 10 pM and 100 oM, respectively).

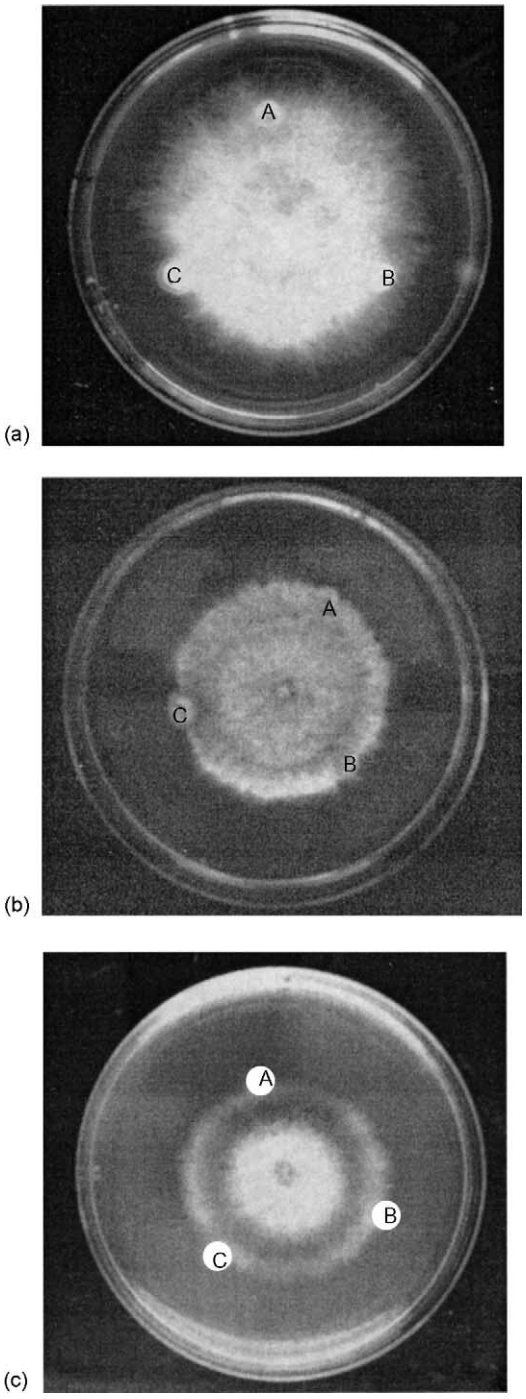

Fig. 5. (a) Inhibitory activity of α-benincasin towards *C. comatus*: (A) 0.1 M NH_4OAc (pH 5.5), 10 μl; (B) 20 μg α-benincasin in 10 μl 0.1 M NH_4OAc (pH 5.5) and (C) 100 μg α-benincasin in 10 μl 0.1 M NH_4OAc (pH 5.5). (b) Inhibitory activity of α-benincasin towards *P. piricola*: (A) 0.1 M NH_4OAc (pH 5.5), 10 μl; (B) 20 μg α-benincasin in 10 μl 0.1 M NH_4OAc (pH 5.5) and (C) 100 μg α-benincasin in 10 μl 0.1 M NH_4OAc (pH 5.5). (c) Lack of inhibitory activity of α-benincasin towards *M. arachidicola*: (A) 0.1 M NH_4OAc (pH 5.5), 10 μl; (B) 20 μg α-benincasin in 10 μl 0.1 M NH_4OAc (pH 5.5) and (C) 100 μg α-benincasin in 10 μl 0.1 M NH_4OAc (pH 5.5).

in *C. comatus* and *P. piricola* (Fig. 5a and b) but not that in *M. arachidicola* (Fig. 5c) was inhibited.

3.4. Yields and specific activities

The purification of benincasins from wax gourd seeds is summarized in Table 2. Approximately 0.4 mg α-benincasin and 0.6 mg β-benincasin were obtained from 100 g starting material. Activity was monitored at each step by determining the inhibition of protein synthesis in the rabbit reticulocyte system. The specific activity of the peptide was increased from 13,514 U × 10^6/mg of the crude extract to 4,545,455 U × 10^6/mg for α-benincasin and 294,118 U/mg for β-benincasin after the finals gel filtration step. The purity of the peptide at different stages of purification was analyzed by SDS–PAGE and subsequently by Coomassie blue staining. The crude extract contained a heterogeneous assembly of proteins, from which contaminating proteins were removed as the peptides passed through different types of chromatographic columns. Finally, α- and β-benincasins with molecular masses of 11 and 10.6 kDa, respectively, were obtained.

4. Discussion

The peptides isolated from wax gourd seeds, designated α- and β-benincasins, can be considered as AGRPs because of their rich content of arginine, glutamate and glutamine residues in their N-terminal sequences, just like AGRPs from the sponge gourd [7,8]. The small RIP from the sponge gourd is also characterized by a high content of these residues as reflected in the amino acid composition [5]. Both α- and β-benincasins manifest potent translation-inhibiting activity, but α-benincasin shows only slight antifungal activity. The IC_{50} values for their cell-free translation-inhibiting activity, 20 pM for α-benincasin and 320 pM for β-benincasin, are in the range of values for Type 1 RIPs [1]. The lower translation-inhibitory potency of β-benincasin compared to that of α-benincasin despite marked similarity in N-terminal sequence and molecular mass, may be due to possible differences further along the sequence. The 9-kDa AGRP from sponge gourd, and the small RIPs luffin-S and AGRP from sponge gourd, and the small RIPs luffin-S and γ-momorcharin, inhibit cell-free translation with an IC_{50} of 140, 0.34 and 55 nM, respectively [5,13,21]. The weak antifungal activity of α-benincasin is similar to a similar observation on RIPs which demonstrate weaker antifungal activity than antifungal proteins like chitinases [11,14]. The molecular masses of benincasins (∼10 kDa) are much lower than those of Type 1 RIPs (25–32 kDa) [1]. Benincasins, like the RIPs, are adsorbed on Affi-gel blue gel [10] and Mono-S.

This study furnishes evidence for the presence of peptides with cell-free translation-inhibiting and antifungal activities in the wax gourd seeds. The wax gourd peptides are similar

to small RIPs like luffin-S [5] in that they are AGRPs with an inhibitory action on cell-free translation.

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