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A new family of small (5 kDa) protein inhibitors of insect α-amylases from seeds or sorghum (*Sorghum bicolor* (L) Moench) have sequence homologies with wheat γ-purothionins

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Three isoinhibitors of locust and cockroach gut α-amylases were purified from seeds of sorghum by saline extraction, precipitation with ammonium sulphate, affinity chromatography on Red-Sepharose and preparative RP-HPLC on Vydac C_{18}. The complete primary structures were determined by automatic degradation of the intact reduced and *S*-alkylated proteins, and by manual DABITC/PITC microsequencing of peptides obtained from enzyme digests. The inhibitors consist of 47 (SIα-1) or 48 (SIα-2, SIα-3) amino acids, and are the smallest plant inhibitors of α-amylase currently known. The sequences of the three isoinhibitors exhibit between 38% and 87% identity among themselves and also have homology (32–81%) with the γ-purothionins recently isolated from wheat endosperm.

Sorghum α-amylase inhibitor; Amino acid sequence; Homology with γ-purothionin

1. INTRODUCTION

Interest in the plant protein inhibitors of enzymes has been stimulated by the recent finding of several new families (reviewed in [1,2]) and the discovery that a number of them have somewhat surprising sequence homologies with other proteins and enzymes. For example, a bifunctional inhibitor of trypsin and α-amylase from maize seeds has strong sequence similarities with the sweet protein thaumatin, virus-induced pathogenesis-related (PR) proteins [3] and plant proteins induced by salt- [4] and water-stress [5]. An inhibitor of locust gut α-amylase from seeds of *Coix lachryma-jobi* which exhibited sequence similarity with various endochitinases was subsequently found to possess this enzyme activity [6]. Similarly when a protein from barley seeds [7] with homology to the α-amylase inhibitor 2 from ragi (*Eleusine coracana*) seeds [8] was also observed to have similarity to various phospholipid transfer proteins [9], later assays confirmed that it possessed this function [10]. Also the further discovery that an amylase inhibitor from beans has the same structure as lectin (agglutinin)-like proteins [11] tends to confirm the suspicion that many of these apparently defensive inhibitor proteins may have other as yet unsuspected functions.

In this paper we report the characterization of three homologous sulphur-rich inhibitors of locust and cockroach gut α-amylases from seeds of sorghum (*Sorghum bicolor*) which are currently the smallest known plant inhibitors of α-amylase. These inhibitors have strong sequence similarities with the γ-purothionins recently isolated from wheat endosperm [12] which in turn are related to various toxic plant thionins that modify membrane permeability [13] and inhibit in vitro protein synthesis in cell-free systems [14].

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Abbreviations: RP-HPLC, reverse phase high performance liquid chromatography; DABITC, 4-*N*,*N*-dimethylaminoazobenzene-4′-isothiocyanate; PITC, phenyl isothiocyanate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid

2. MATERIALS AND METHODS

2.1. *Extraction and purification*

Seeds of sorghum (*Sorghum bicolor* (L) Moench, cv. French red) were obtained from Dr J. Cecil (Overseas Development Natural Resources Institute, Culham, Abingdon). 330 g of milled seed was defatted with acetone and extracted for 12 h at 4°C with 0.1 M HCl in 0.1 M NaCl (1.3 liters). After centrifugation (10000×*g*, 25 min, 4°C) the pH of the supernatant was adjusted to 7.0 with 1 M NaOH. The proteins which precipitated were removed by centrifugation, and ammonium sulphate was added to the supernatant to give a 60% saturation. The proteins which precipitated were collected by centrifugation, dissolved in 30 ml of 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl and dialyzed against the same buffer for 24 h. Insoluble proteins were removed by centrifugation and the solution applied to a column (2.5×20 cm) of Red-Sepharose CL-6B equilibrated in the same buffer. The column was washed with the equilibrating buffer until the A_{280} reached zero when the proteins bound to the column were eluted with 0.05 M Tris-HCl, pH 7.0, containing 3

M NaCl. The eluate was dialyzed against distilled water and lyophilized.

The proteins eluted from the Red-Sepharose column (40 mg) were dissolved in 1.5 ml of 6 M guanidine HCl in 0.1% aqueous TFA and applied to a preparative RP-HPLC column (22 mm × 25 cm) of Vydac C_{18} (218 TP 1022, Technicol Ltd) which was eluted with a gradient of acetonitrile in 0.1% aqueous TFA. The individual peaks containing α-amylase inhibitors were rechromatographed on an analytical RP-HPLC column (4.6 mm × 25 cm) of Vydac C_{18} (218 TP 54, Technicol Ltd).

2.2. *α-Amylase assays*

α-Amylases from human saliva, porcine pancreas, *Aspergillus oryzae*, *Bacillus* spp. and barley were purchased from Sigma Chem. Co. Crude extracts of the α-amylases from the guts of mature larvae (nymphs) of the African migratory locust (*Locusta migratoria migratorioides*) and adult cockroaches (*Periplaneta americana*) were prepared by homogenizing the dissected whole guts in 0.025 M Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$. Lipids were removed by extraction (×3) with chloroform. The activity of all α-amylases was determined by using the method of Bernfeld [15].

2.3. *Electrophoresis*

SDS-PAGE was carried out using a modified Laemmli system with 20% gels [16]. Isoelectric focussing on polyacrylamide gels containing ampholines covering the pH range 3.0–10.0 was as described in [17].

2.4. *Molecular weight estimations*

The molecular weights of the purified amylase inhibitors were also measured using the plasma desorption time of flight method in the Biolon 20 Biopolymer mass analyzer (Applied Biosystems Ltd).

2.5. *Reduction and S-alkylation*

Samples used for enzymatic digestions were reduced and *S*-carboxymethylated as described in [18]. Samples used for automatic sequencing were *S*-pyridylethylated on the sequencer glass fibre discs using 4-vinylpyridine and tributylphosphine in the vapour-phase as described in [19].

2.6. *Amino acid analysis*

Samples of the reduced and *S*-carboxymethylated proteins and the peptides derived from them were hydrolyzed with 5.6 M HCl containing 0.02% (v/v) cresol at 108°C for 24 h. The amino acids in the hydrolyzates were derivatized with PITC and analyzed by HPLC using the Waters Pico-Tag method [20].

2.7. *Sequence determination*

The native and *S*-alkylated proteins (5 nmol) were sequenced using a model 477A automatic pulsed liquid phase protein sequencer (Applied Biosystems Ltd) employing a standard Edman degradation sequenator programme.

Samples (2 mg) of the *S*-carboxylmethylated proteins were digested separately with trypsin, chymotrypsin and thermolysin as described in [21]. Peptides obtained from these digests were purified by RP-HPLC on analytical columns of Vydac C_{18} using gradients of acetonitrile in 0.1% (v/v) aqueous TFA. The sequences of the peptides were determined using the manual DABITC/PITC double coupling method [22].

3. RESULTS AND DISCUSSION

Affinity chromatography on Red-Sepharose (procion red HE-3B ligand) has proved to be a useful step in the purification of protein inhibitors of α-amylases from a number of cereal seeds [1,6]. The inhibitors of α-amylases obtained from sorghum extracts by this method (Fig. 1) were further fractionated by preparative RP-HPLC. This yielded five major peaks of inhibitory activity (Fig. 2). Peaks 4 and 5 contained proteins with M_r of 13 000 whose amino acid sequences (unpublished results) showed that they clearly belonged to the cereal superfamily of amylase/proteinase inhibitors [1,2].

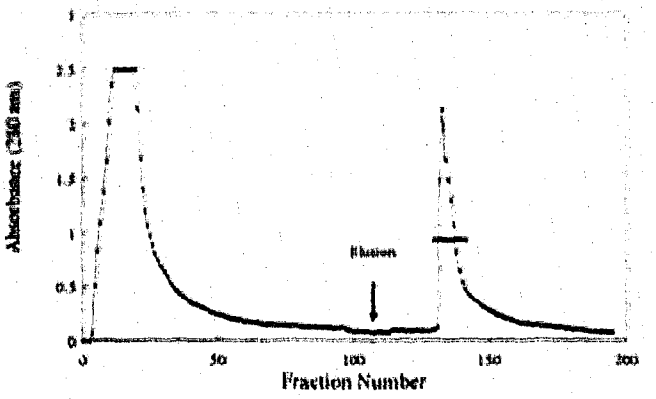

Fig. 1. Affinity chromatography of 60% ammonium sulphate precipitate from extract of sorghum seeds on Red-Sepharose CL-6B. Details as described in section 2.1. The arrow indicates the start of the elution with buffer containing 3 M NaCl. The fractions containing inhibitors of α-amylase which were collected are shown by the bar.

Peaks 1–3 contained proteins of about 5 kDa (by SDS-PAGE, not shown) contaminated with other proteins in low amounts. Rechromatography of these peaks on analytical RP-HPLC columns (not shown) yielded homogeneous components which were called S1α1, S1α2 and S1α3.

All three components strongly inhibited the α-amylases from the guts of locust and cockroach, showed weak inhibition of the enzyme from *Aspergillus*

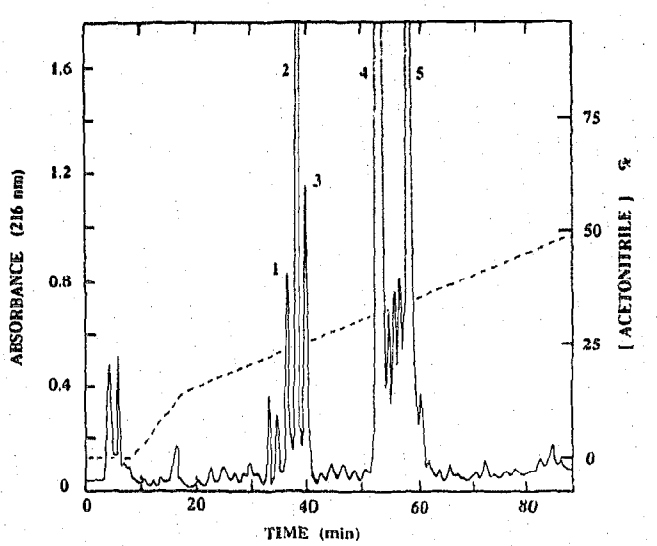

Fig. 2. Preparative RP-HPLC separation of sorghum α-amylase inhibitors obtained from affinity chromatography on Red-Sepharose. Chromatography was performed as described in section 2.1. The dotted line indicates the gradient of acetonitrile concentration in 0.1% TFA. The numbered peaks (1–5) contained inhibitors of α-amylase.

oryzae and human saliva and failed to inhibit the α-amylases from porcine pancreas, barley and *Bacillus* spp. This spectrum of inhibitory activity towards α-amylases from different sources is very typical of many such proteins previously isolated from cereal seeds [2].

The amino acid sequences of the inhibitors S1α1, S1α2 and S1α3 determined by both automated and manual sequencing methods are shown in Fig. 3. The sequences were in good agreement with the results of amino acid analyses, and the M_r values of 5367, 5308 and 5382 calculated from the sequences corresponded well with the estimates of 5200–5300 made from SDS-PAGE and plasma desorption mass spectroscopy. As far as we are aware these are the smallest known α-amylase inhibitors obtained so far from a plant source.

The sequences of all three inhibitors are notable for their high contents of cysteine residues (8 in each) and basic amino acids. They failed to focus when subjected to isoelectric focussing on gels containing ampholines in the pH range 3.0–10.0 which suggests that their p*I* values are above 9.5.

The three inhibitors are clearly homologous (Fig. 4), with the greatest identity (87%) occurring between S1α2 and S1α3, whilst S1α1 has only 42% identity with S1α2 and 38% with S1α3. No significant homologies were found when the sorghum proteins S1α1, 2 and 3 were compared with the other known families of plant inhibitors of enzymes [1,2] by the computer FASTP programme [23]. However these sorghum inhibitors of insect α-amylases are strikingly similar in their amino acid sequences to the γ-purothionins recently isolated from wheat endosperm [12] with identities in the range 32–83%. The wheat γ-purothionins in turn show close resemblance to the α- and β-purothionins and hordothionins from wheat and barley [24–28] and the plant toxins crambin [29], pyrularia [30] and viscotoxin [31]. This group of cysteine-rich thionins, which are known to be toxic to insect larvae [32] are thought to form a part of the plant defence mechanisms against pests and pathogens. Previous workers have demonstrated that they modify membrane permeability [13], inhibit cell-free protein synthesis [14] and inhibit ribonucleotide reductases by competing for reducing equivalents from thioredoxin [33].

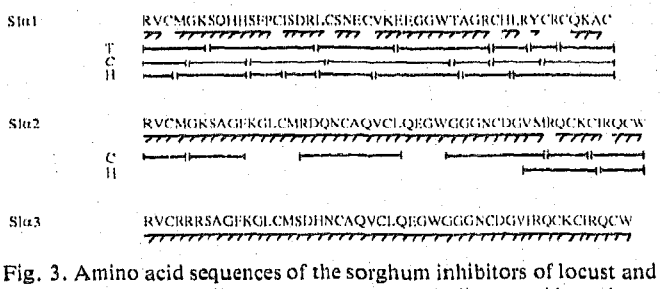

Fig. 3. Amino acid sequences of the sorghum inhibitors of locust and cockroach gut α-amylases. Arrows (—↑—) indicate residues determined by automatic degradation of native (S1α1) or *S*-pyridylethylated (S1α2 and S1α3) protein samples. Solid lines indicate peptides obtained from digestions with trypsin (T), chymotrypsin (C) and thermolysin (H) which were sequenced by the manual DABITC/PITC method.

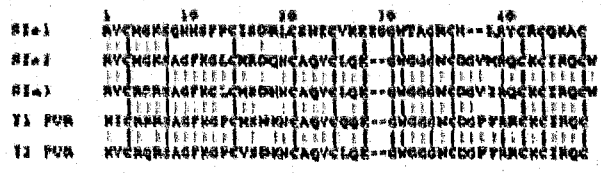

Fig. 4. Comparison of amino acid sequences of sorghum inhibitors (SI 1, 2, 3) of insect gut α-amylases and γ1 and γ2 purothionins from wheat endosperm [12]. -, deletions inserted in sequences to maximize homology.

The high degree of homology between the γ-purothionins and the sorghum inhibitors of insect α-amylases reported in the present work, suggests that the other thionins should perhaps be examined for their possible effects on α-amylases, particularly from insects. It is of interest to note that other workers have reported a weak inhibition of wheat α-amylase by the wheat purothionins, although only at relatively high concentrations [34]. Conversely we are currently investigating the possible multifunctional effects of the sorghum proteins on membranes and on in vitro protein synthesis.

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