Phytochemistry, Vol. 28, No. 11, pp. 3019–3026, 1989.
Printed in Great Britain.

0031–9422/89 $3.00 + 0.00

TRYPSIN INHIBITORS FROM *COLOCASIA ESCULENTA*, *ALOCASIA MACRORRHIZA* AND *CYRTOSPERMA CHAMISSONIS*

BRENDON C. HAMMER,* DENIS C. SHAW and J. HOWARD BRADBURY†‡

Chemistry Department, Faculty of Science and Protein Chemistry Group, John Curtin School of Medical Research, Australian National University, Canberra, ACT, 2600, Australia

(*Received in revised form* 15 *May* 1989)

Key Word Index—*Colocasia esculenta*; *Alocasia macrorrhiza*; *Cyrtosperma chamissonis*; Araceae; taro; trypsin inhibitor; protein evolution.

Abstract—Trypsin inhibitors from three edible aroids of the family Araceae, viz. taro (*Colocasia esculenta* var. *esculenta*), giant taro (*Alocasia macrorrhiza*) and giant swamp taro (*Cyrtosperma chamissonis*), obtained from the Pacific region, were isolated by affinity chromatography and purified by gel filtration. The M_r of these inhibitors, as determined by gel filtration were 35 000–38 000, but were *ca* 20 000 by SDS gradient PAGE. A time course of heating in SDS showed a ready dissociation of the native protein into subunits of equal size. Further experiments showed that there were no disulfide bonds between these subunits. A single *N*-terminal sequence was found for each inhibitor showing that the two subunits had similar primary structure. Each of the *N*-terminal sequences showed homology with that of soybean trypsin inhibitor. To our knowledge, this finding follows only one other example of a Kunitz family inhibitor being located in a monocotyledonous, rather than dicotyledonous, plant species, and indicates that the ancestral gene from which Kunitz family inhibitors originate predates the evolutionary divergence of flowering plants into monocotyledons and dicotyledons.

INTRODUCTION

There have been many reports over more than 40 years on the isolation and characterization of protein proteinase inhibitors from plant tissues [1, 2], yet it is only recently that interest has been shown in plant species outside the families Leguminosae, Gramineae and Solanaceae. The need to study other plant families becomes apparent when it is realised that these inhibitors are important not only in protein chemistry [1, 3, 4] but also in agricultural anthropology [5], human and animal nutrition [2, 6–8], medicine [9–11], plant physiology and phytochemistry [4, 12, 13] and plant taxonomy [14].

Here we report research on trypsin inhibitors from three plant species in the family Araceae; taro (*Colocasia esculenta* var. *esculenta*; CE), giant taro (*Alocasia macrorrhiza*; AM) and giant swamp taro (*Cyrtosperma chamissonis*; CC). A study of these inhibitors is important because the three species described, together with taro *Xanthosoma sagittifolium*, are estimated to form the staple diet of about 200 million people living in the tropics [15–17]. The first work in this field was by Ryan *et al.* [18] who reported a high level of antichymotryptic activity in corms of the taro variety *C. esculenta* var. *antiquorum* but none from var. *esculenta*. However Sumathi and Pattabiraman [19] found very little antichymotryptic activity from corms of var. *antiquorum* while observing a high level of antitryptic activity. More detailed studies have subsequently been made on trypsin inhibitors from var. *antiquorum* [20–22] and from AM [23]. *Xanthosoma sagittifolium* has been found to be free of both antitryptic and antichymotryptic activity [24]. This paper reports work in a series of studies undertaken on the trypsin inhibitors present in CE, AM and CC and describes their isolation and characterization as members of the Kunitz soybean trypsin inhibitor family [25].

RESULTS

M_r *by gel filtration*

The elution profiles obtained at the gel filtration step using Sephadex G-100 for each species of trypsin inhibitor show two separated peaks, one at the void volume ($K_{av} = 0.0$) and another between $K_{av} = 0.2$ and 0.3. The K_{av} values for AM, CE and CC are 0.238, 0.273 and 0.265 respectively. In each case the void volume material consisted mainly of carbohydrate and had a slight antitryptic activity.

In a separate experiment, gel-filtration of each of the same starting materials on Sephadex G-200 gave three chromatograms similar to those obtained on Sephadex G-100. For AM, two eluent fractions were then taken corresponding to the peaks which had $K_{av} = 0.0$ and 0.238 on Sephadex G-100. The separate fractions were then left to stand for one day after which they were dialysed, lyophilized and individually rechromatographed as before. Each fraction then chromatographed to give a single peak at the same elution volume to which it had previously run, indicating that there had been no equilibrium

*Present address: Chemistry Department, University College, University of New South Wales, Australian Defence Force Academy, Campbell, ACT, 2600, Australia.

†Present address: Botany Department, Australian National University, Canberra, ACT, 2600, Australia.

‡Author to whom correspondence should be addressed.

between the constituents previously isolated. Similar results were subsequently obtained for CE and CC [24].

The M_r of each trypsin inhibitor was determined by calibration of the Sephadex G-100 column using a series of nine protein mass markers from lysozyme (M_r 17200) to bovine serum albumin (M_r 66000). A plot of K_{av} vs ln (M_r) gave a smooth curve [24] from which the apparent M_r values of the trypsin inhibitors from AM, CE and CC were found to be 37400, 35200 and 35900 respectively.

Gradient PAGE of SDS denatured protein fractions

The six fractions obtained by gel filtration on Sephadex G-200 were dissolved in the PAGE solution and dissociated as indicated below (see PAGE). The results of the experiment shown in Fig. 1 indicate that each product gave a single, major band except for lanes 7 and 9 (corresponding to minor fraction A from CE) which gave two faint bands after a loading of 100 μg.

It was clear that for each aroid the inhibitor activity in both the major (peak B) and minor (peak A) fractions was probably due to a similar or identical protein. The minor peak A fractions stained strongly for carbohydrate in the high M_r region of the gel, suggesting that they were mainly composed of polysaccharide material with some trypsin inhibitor (see above). A graph of R_f vs ln (M_r) for the calibration proteins used in lane of Fig. 1 gave a smooth curve [24]. Under the denaturing conditions of the experiment, the M_r values corresponding to the strongest band observed in those lanes where major gel filtration fractions were run were 19500, 21800 and 21800 for trypsin inhibitors from AM, CE and CC respectively. The major peak B fractions did not stain for carbohydrate. Lane 14, when scanned on the left hand side, gave a standard curve almost identical to that for lane 1. Where double banding occurred, R_f values were averaged.

Dimer to monomer conversion of inhibitors

As the M_r values of the undenatured trypsin inhibitors estimated by gel filtration were roughly twice those obtained for the respective denatured proteins, it was considered useful to study their behaviour on heating in SDS using gradient PAGE. In Fig. 2 there is a band in lane 2 (weaker in lane 3) and in lane 7 in the region of M_r values indicated for the trypsin inhibitors of CE and CC by gel filtration. As for lanes 2 and 7 the samples were unheated and lane 3 was heated only for 5 sec, it follows that these bands correspond to undenatured protein. A new, strong band in the position previously observed in Fig. 1 for denatured trypsin inhibitor occurred in lanes 3–6 and 8–11, while the higher M_r bands seen in lanes 2, 3 and 7 disappeared. This shows that on heating in SDS the trypsin inhibitors of CE and CC dissociate into two polypeptide chains of the same, or very similar, M_r. It is unlikely that the two chains are covalently linked by a disulfide bond, because bond scission would not be expected to occur at 100° and pH 6.9 in less than 5 sec [26–28], the condition for which dissociation was observed in the case of the trypsin inhibitor from CC.

Trypsin inhibitor from AM gave a similar result to that obtained for inhibitors from CE and CC, viz. that the inhibitor from AM is composed of two discrete polypeptide chains having very similar or identical M_r values, which dissociate with SDS denaturation. However, the heating time required at 100° for complete dissociation of the dimer from AM was greater than 15 min (lanes 7 and 8) rather than the few sec. required for dissociation of the dimer of CE and CC.

Gel filtration of urea denatured inhibitor from AM

In order to check whether the trypsin inhibitor from AM may have contained disulphide bonds linking the

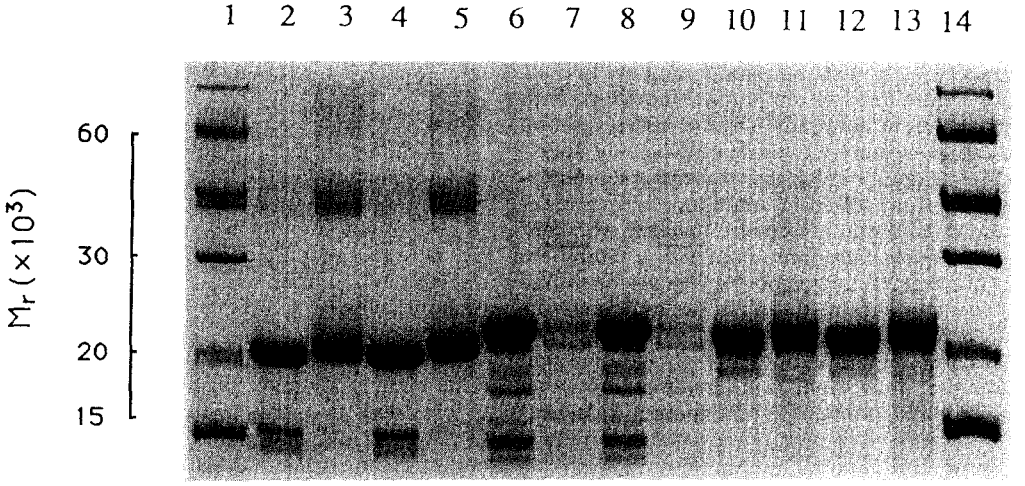

Fig. 1. The result of an SDS/gradient PAGE experiment on fractions from Sephadex G-200 gel filtration. Lines 1 and 14 are M_r standards. For the major fractions, peaks B, duplicate lanes 2 and 4 are for AM, lanes 6 and 8 are for CE, and lanes 10 and 12 are for CC. For the minor fractions, peaks A, duplicate lanes 3 and 5 are for AM, lanes 7 and 9 are for CE, and lanes 11 and 13 are for CC. For the major fractions 10–12 μg of dialysed, lyophilized and pretreated material was applied, for the minor fractions 75–100 μg of similarly processed material was applied.

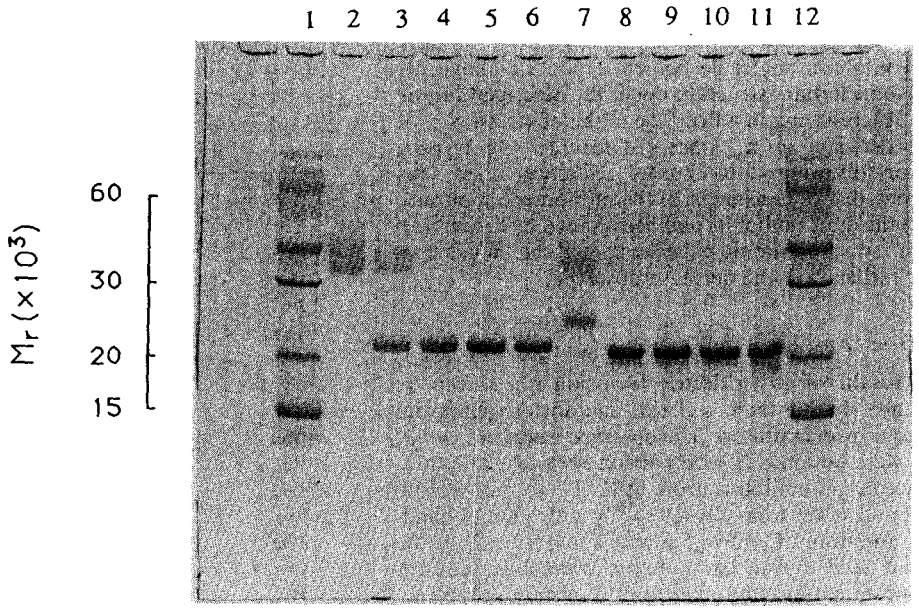

Fig. 2. A gradient PAGE experiment run using samples of progressively SDS denatured trypsin inhibitors from CE (lanes 2 to 6) and CC (lanes 7 to 11). Prior to the run, inhibitors were heated at 100° in a mixture of buffer 3 (73%), glycerol (25%) and SDS (2.0%) for 0, 5, 10, 20 and 60 sec for the respective consecutive lanes in 2 through to 6, and 7 through to 11. Lanes 1 and 12 were M_r standards.

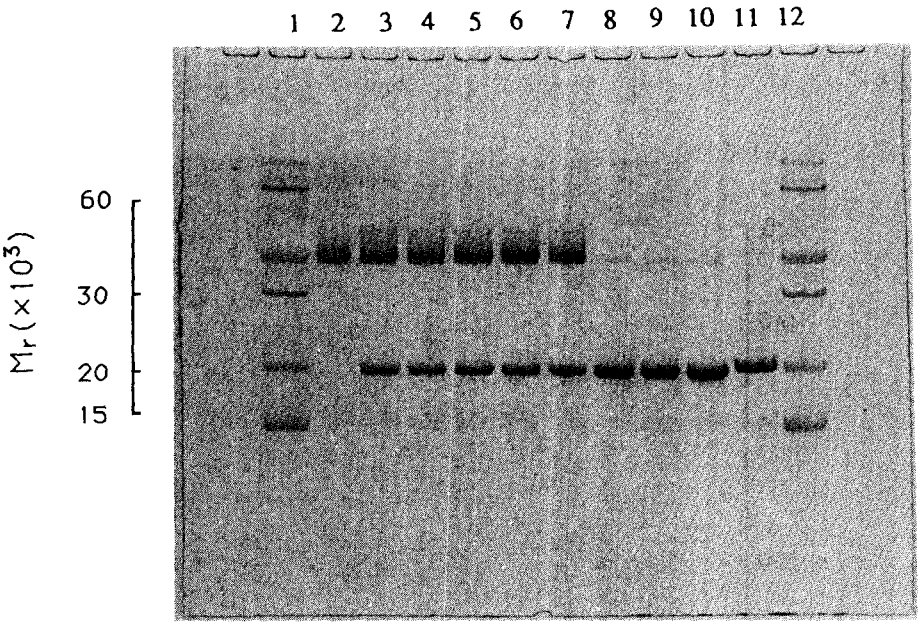

Fig. 3. A gradient PAGE experiment run using samples of progressively SDS-denatured trypsin inhibitor from AM. Preparation was as for Fig. 2 for lanes 2 to 10, with respective heating times of 0, 5, 7.5, 10, 12.5, 15, 30, 45 and 90 min. Lane 11 was for inhibitor heated for 10 min in a solvent modified by addition of β-mercaptoethanol (5%). Lanes 1 and 12 were M_r standards.

two subunits, gel filtration on Sephadex G-200 using a 9 M urea eluent was carried out on the trypsin inhibitor both before and after reduction with excess dithiothreitol [29]. In each case a single, symmetrical peak was obtained and K_{av} values of 0.39 and 0.47 were recorded for the reduced and unreduced inhibitor respectively. As a control, a parallel experiment was conducted using the same column and conditions with soybean trypsin inhibitor, a single chain protein of M_r 20 100, with two internal disulphide bonds [30]. Here single, symmetrical peaks were again obtained with K_{av} values of 0.33 and 0.53 for the reduced and unreduced inhibitor respectively.

Clearly, the dithiothreitol reduction of internal disulphide bonds allowed further expansion of the soybean trypsin inhibitor molecule (which would still have retained some tertiary structure under the described conditions [31], resulting in a decrease in the observed K_{av}. A similar decrease in K_{av} observed for the AM trypsin inhibitor following dithiothreitol reduction must also have been due to expansion of the monomeric molecule since, if the dimer subunits had been joined by disulphide bond(s), reduction should have brought about an increase in K_{av}, rather than the decrease observed.

Isoelectric focussing

The results of an isoelectric focussing run shown in Fig. 4 indicate that there is charge microheterogeneity in all three trypsin inhibitors. The observed isoelectric point ranges were obtained by comparison with p*I* standards. They were, for inhibitors from AM, 4.7 to 5.0, with a major band at 4.9; from CE, 4.9 to 5.3, with a major band at 5.17; and from CC, 4.5 to 5.5, with a major band at 4.8. The acid used during the isolation could have caused some deamidation and contributed to the observed microheterogeneity.

Amino acid compositions of inhibitors from AM, CE and CC

Amino acid analyses were carried out on each of the AM, CE and CC trypsin inhibitors and the results

Table 1. Amino acid compositions (residues per molecule) of trypsin inhibitors from CE, AM and CC

	Trypsin inhibitors from CE	AM	CC
Asp	25.1	19.0	19.3
Thr	9.6	11.9	10.4
Ser	18.1	13.5	21.1
Glu	21.0	17.4	19.2
Pro	15.6	15.3	16.2
Gly	15.4	21.0	22.1
Ala	17.1	12.7	17.0
Cys/2	2.2	3.0	3.3
Val	14.0	11.9	10.4
Met	1.4	2.9	1.2
Ile	6.9	5.4	4.5
Leu	18.1	14.4	16.2
Tyr	4.5	5.2	7.9
Phe	6.5	8.1	11.0
Lys	3.5	5.7	4.6
His	2.4	2.2	3.8
Arg	15.8	9.0	9.2
Trp	1	1	1
Total residues	197	180	198
Monomer M_r	22000	19500	22000

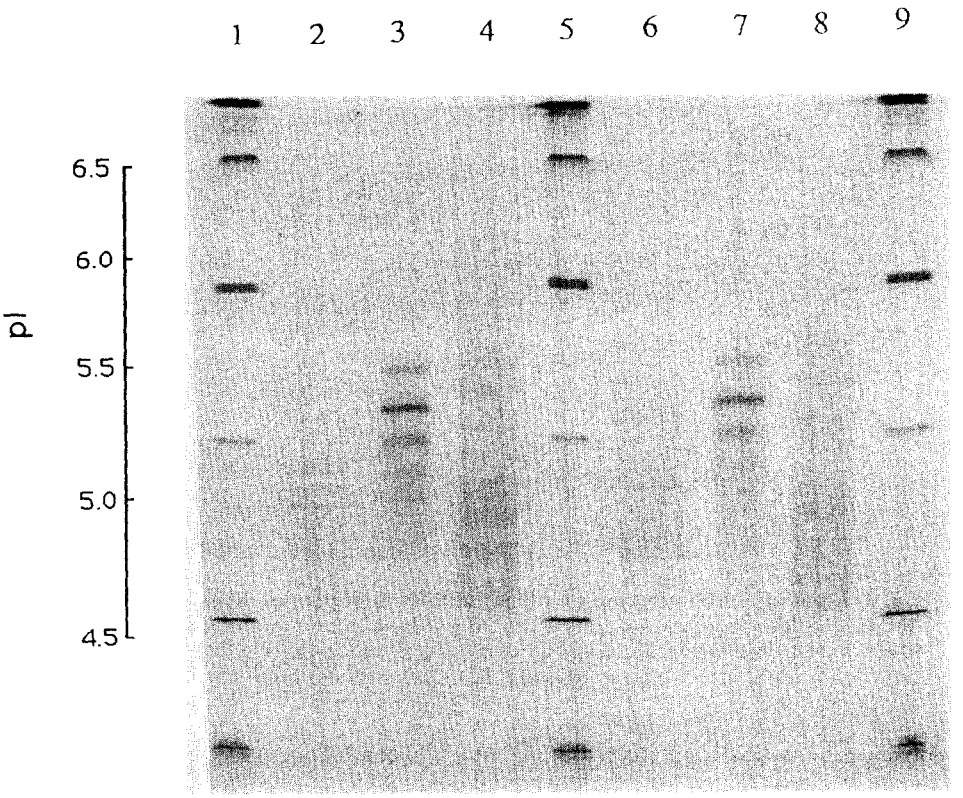

Fig. 4. Isoelectric focussing chromatogram in the pH range 4 to 7 with trypsin inhibitors of AM, CE and CC loaded in lanes 2 and 6, 3 and 7, 4 and 8 respectively. Lanes 1, 5 and 9 contained p*I* standards. The dark bands at the top and bottom of the chromatogram represent the lines of the cathode and anode respectively.

Table 2. The *N*-terminal sequences of trypsin inhibitors from *A. macrorrhiza* (AMTI), *C. esculenta* (CETI), *C. chamissonis* (CCTI) and some other serine proteinase inhibitors

AMTI			Thr	Asn	**Pro**	**Val**	**Leu**	**Asp**	Val	**Asp**	**Gly**	**Asn**
CETI			Ala	Asp	Ala	**Val**	**Leu**	**Asp**	Val	**Asp**	**Gly**	**Asn**
CCTI			Ala	Asn	**Pro**	**Val**	**Leu**	**Asp**	Val	**Asp**	**Gly**	**Asn**
STI*				Asp	Phe	**Val**	**Leu**	**Asp**	Asn	Glu	**Gly**	**Asn**
WTI†				Glu	**Pro**	Leu	**Leu**	**Asp**	Ser	Glu	**Gly**	Glu
ATI‡				Lys	Gly	Leu	**Leu**	**Asp**	Ser	**Asp**	**Gly**	Asp
AII§				Lys	Glu	Leu	**Leu**	**Asp**	Ala	**Asp**	**Gly**	Asp
BII§				Asn	Leu	Leu	**Leu**	**Asp**	Thr	**Asp**	**Gly**	**Asn**
DE-3α‖					Val	Leu	**Leu**	**Asp**	Gly	‖ ‖	**Gly**	Gln
DE-3β¶					Val	Leu	**Leu**	**Asp**	Gly	Asn	**Gly**	Glu
BASI**	Ala	Asp	Pro	Pro	**Pro**	**Val**	His	**Asp**	Thr	**Asp**	**Gly**	His
RPI††		Ala	Pro	Pro	**Pro**	**Val**	Tyr	Asn	Thr	Gln	**Gly**	His
API‡‡				Asp	**Pro**	**Val**	Val	**Asp**	Ser	**Asp**	**Gly**	Asp
APTI§§				Arg	Glu	Leu	**Leu**	**Asp**	Val	**Asp**	**Gly**	**Asn**

*Soybean trypsin inhibitor [30].
†Common to two trypsin inhibitors from *Psophocarpus tetragonolobus* [33].
‡Common to two trypsin inhibitors from *Acacia elata* [34].
§One of four similar inhibitors from *Albizzia julibrissin* [35].
‖One of two similar inhibitors from *Erythrina lattissima* [36].
¶One of four similar inhibitors from *Erythrina caffra* [37].
**A subtilisin/amylase inhibitor from *Hordeum vulgare* [38].
††A subtilisin inhibitor from *Oryza sativa* [35, 39].
‡‡One of two trypsin inhibitors from *Sagittaria sagittifolia* [40].
§§One of eight similar trypsin inhibitors from *Adenanthera pavonia* [32].
‖ ‖Asn/Val. (for BASI and RPI see references cited for further evidence of homology).

calculated on the basis of the monomer M_r of each inhibitor, are shown in Table 1. Tryptophan was destroyed during acid hydrolysis; however, the existence of one tryptophan residue in each inhibitor could be inferred from subsequent peptide mapping work, where only a single tryptophan containing peptide was observed in each case [24].

N-*terminal sequences of inhibitors*

The sequences of 10 residues from the *N*-terminus of trypsin inhibitors from AM, CE and CC are given in Table 2 together with data taken from the literature for some other proteinase inhibitors of plant origin. Homology is indicated by bold residues. It has been reported that, under certain conditions, free proteinase inhibitor released through the dissociation of an enzyme inhibitor complex may consist of a significant fraction of molecules having two *N*-termini due to enzymic cleavage at the reactive site [32]. Because our purification of trypsin inhibitors involved the use of affinity chromatography on a trypsin-Sepharose column, it is noteworthy that only a single *N*-terminal sequence was observed in each case. This observation also served to confirm that the relatively rapid method of purification adopted gave a homogeneous product.

DISCUSSION

Aqueous extracts from corms of CE, AM and CC have each been shown to contain a single major trypsin inhibitor. While gel filtration in a benign solvent gave M_r values of about 35 000 to 38 000 for these proteins, SDS denaturation followed by gradient polyacrylamide gel electrophoresis indicated that each native inhibitor was composed of two subunits of about M_r 20 000. Further experiments showed that the association of these subunits did not occur through disulphide bonds.

In earlier work, it was shown that the trypsin inhibitor from AM had an M_r of about 32 000 by gel filtration in 5 M urea [23]. This M_r is consistent with only partial dissociation of the inhibitor dimer, a reasonable explanation, since the urea concentration used is unlikely to have fully denatured the protein [31, 41]. Further evidence in support of this explanation may be drawn from the results of a later study on a trypsin inhibitor from taro *C. esculenta* var. *antiquorum*, for which an M_r of 40 000 was found using the same methodology [20]. Subsequent work on this taro variety showed the presence of several trypsin inhibitor monomers of M_r 20 000 which were derived by the denaturation of a native protein of M_r 40 000 [21, 22].

The two monomeric units that are produced by denaturation of the native trypsin inhibitor dimers have about the same M_r values, but the question arises of whether or not they are identical. Only one *N*-terminal sequence was obtained from each of the trypsin inhibitor dimeric molecules (See Table 2), showing that within each inhibitor the monomer units are likely to have similar sequences. In the case of the trypsin inhibitor from AM, evidence of the identity of the two monomers has been recently obtained through full sequencing of the monomer molecule [42].

Homology to soybean trypsin inhibitor

On grounds of sequence homology, Laskowski *et al.* [25] proposed the existence of at least four evolutionarily

distinct families of plant serine endoproteinase inhibitors. Recent work on species in Cucurbitaceae [43–46] and Gramineae [47, 48] has confirmed the existence of two further families. The six firmly established serine endoproteinase inhibitor families are then the Bowman–Birk family, the Kunitz soybean trypsin inhibitor family, the Potato I family, the Potato II family, the Cucurbitaceae family and the Gramineae family.

The three tyrpsin inhibitors from CE, AM and CC show close *N*-terminal homology to soybean trypsin inhibitor and have similar subunit M_r values which indicates that they belong to the Kunitz family, a finding which may have important implications for plant taxonomy. With the exception of the inhibitors described here, which are from the plant family Araceae, and a trypsin inhibitor from *Sagittaria sagittifolia* [40], from the plant family Alismataceae, all members of the Kunitz family characterized to date have been found in species from the dicotyledenous plant family Leguminosae. Araceae and Alismataceae families are monocotyledenous, which indicates that the ancestral protein from which Kunitz family inhibitors have evolved, predates the evolutionary divergence of the flowering plants (Angiospermae) into monocotyledons and dicotyledons about 200 million years ago [49].

Currently, major difficulties exist in developing a phylogenetic classification of plants in Angiospermae based purely upon physiological characteristics as the fossil record is very incomplete [50–52]. For this reason Boulter [53] has suggested that 'chemotaxonomic' data, specifically that involving the relative homology of proteins, may prove indispensable in the construction of a sound phylogeny. The work reported here, together with that of Chi *et al.* [40] on *Sagittaria sagittifolia*, suggests that Kunitz type inhibitors may be widespread among both monocotyledenous and dicotyledenous species, and hence may prove well suited for chemotaxonomy at the evolutionary level in question. Some early evidence of their taxonomic potential is already apparent in the consistent sequence differences found between Kunitz inhibitors isolated from species belonging to different Leguminosae subfamilies [32, 34].

EXPERIMENTAL

Materials. Bovine serum albumin of M_r 66 000 [54] was obtained from Nutritional Biochemical Corp, deoxyribonuclease 1, M_r 31 000 [55], lysozyme, M_r 17 200, ovalbumin, M_r 43 500 and pepsin, M_r 35 500 [56] were all from Worthington, and peroxidase, M_r 40 000 [55], protease, alkaline, (subtilisin BPN), M_r 28 000 [57] and trypsin, M_r 23 746 [58] were obtained from Sigma [24]. Soybean trypsin inhibitor was prepared by rechromatographing type 1-S soybean trypsin inhibitor from Sigma on Sephadex G-50 fine using buffer 1 (see below) as the mobile phase. The major, higher M_r fraction was collected, dialysed against dist. H_2O, and lyophilized. The product gave a single band at 20 000 when electrophoresed using gradient, PAGE SDS/β-mercaptoethanol; reported M_r was 20 100 [30].

Buffer solutions. 'Buffer 1' was prepared by dissolving 4.845 g Tris and 1.47 g $CaCl_2 \cdot 2H_2O$ in 1 l H_2O and adjusting to pH 8.10 by addition of HCl. 'Buffer 2' was 400 g Tris dissolved in 2.5 l H_2O and the pH was adjusted to 8.50 by addition of HCl. 'Buffer 3' was 0.0250 M KH_2PO_4 and Na_2HPO_4 in H_2O, pH 6.87. 'Buffer 4' was 0.025 M KH_2PO_4, 0.025 M Na_2HPO_4, 9.0 M urea in H_2O and the pH was adjusted to 7.0 using conc NaOH.

Isolation of inhibitors. Corms were sent by airfreight from the South Pacific as follows: taro (*C. esculenta* var. *esculenta*) cv Samoa green from Fiji, giant taro (*A. macrorrhiza*) cv Fui from Western Samoa and giant swamp taro (*C. chamissonis*) cv Ikaraoi green from Kiribati. They were peeled and the sliced fresh corm tissue was dried at 40° in a convection oven [8]. Tissue powder (about 200 g) was homogenized in 200 ml of buffer 1 in a blender at 20° and the suspension centrifuged for 90 min at 7500 *g* and 5° in a Servall refrigerated centrifuge. The supernatant soln was loaded on a column (i.d. 15 mm, length 280 mm) which contained trypsin-Sepharose 4B [59] and washed with buffer 1. The inhibitor bound to the affinity column and was decoupled by elution with 0.2 M HCl/0.5 M KCl [34]. The inhibitor concentrate was dialysed against water using dialysis tubing with 6000–8000 M_r cutoff (Spectrum Medical Industries, U.S.A.) and the solution lyophilized to yield crude inhibitor. The yield of inhibitor was much greater from AM than from CE and CC tissues [24] and was limited by the size of the affinity column used to about 50 mg per saturated column. Gel filtration on Sephadex G-100 using buffer 1 at 20° and a calibrated column (i.d. 25 mm, length 380 mm) gave a single peak in the M_r range 30 000–50 000 for each plant species studied. In each case the eluent under this peak was dialysed and lyophilized to give inhibitor which was homogeneous when subjected to SDS/PAGE and *N*-terminal sequencing (see below). M_r was estimated by gel filtration [60]. The parameter K_{av} was calculated by the equation

$$K_{av} = (V_e - V_o)/(V_t - V_o),$$

where V_e = elution volume, V_o = void volume determined using Blue Dextran 2000 and V_t = total bed vol. determined using sodium chromate.

Polyacrylamide gel electrophoresis (PAGE). Polyacrylamide gradient gels (180 × 140 × 2.7 mm, 10–20% acrylamide linear gradient, SDS 0.05%) were prepared, loaded and run at 15° as described in ref. [61]. The sample concn was about 5 mg/ml dissolved in buffer 2 (69%), glycerol (25%), β-mercaptoethanol (5%), sodium dodecyl sulphate (SDS) (1%) which also contained Bromophenol Blue (30 μg/ml) as a marker. Samples were electrophoresed after heat denaturation at 105° for 15 min in sealed glass tubes. Staining for protein was with Coomassie Brilliant Blue R-250 [61] and for carbohydrate by the method of ref. [62]. Relative molecular mass standards were obtained from Pharmacia's 'Low Molecular Weight Calibration Kit; viz α-lactalbumin (14 400), soybean trypsin inhibitor (20 100), carbonic anhydrase (30 000), ovalbumin (43 000), albumin (67 000) and phosphorylase B (94 000).

Isoelectric focussing. Isoelectric focussing gels (115 × 230 × 1 mm, total acrylamide 7.5%, *N,N'*-methylene bisacrylamide 4%, pH 4 to 7 using Pharmalyte ampholytes) were prefocussed (30 W constant for 40 min, across 115 mm), the samples loaded, and run (25 W constant for 2.5 hr) on a Pharmacia 'Flat Bed Apparatus' FBE-300 at 10°. Other conditions were according to the manufacturers instructions [61]. Gels were fixed in aq. trichloroacetic acid (10%) and 5-sulphosalicylic acid (5%) and were then equilibrated in 20% HOAc–EtOH (1:1) for 2 hr. They were stained for 1.5 hr with Coomassie Brilliant Blue R-250.

Amino acid analyses. Samples for amino acid analysis were hydrolysed in evacuated glass tubes using 6 M HCl for 22 hr at 110° [63]. Analyses were by the method of ref. [64] using a Beckman Model 120C amino acid analyser, modified to give nmol sensitivity and single column operation. Tryptophan was estimated by inspection of peptide maps [24].

N-Terminal sequences. *N*-Terminal sequences were determined via automatic Edman degradation in a Beckman Model 890 M sequencer using a 0.1 M Quadrol program. Amino acids

were identified by use of a Hewlett-Packard 1084A HPLC fitted with a reverse phase 'Ultrasphere' C18 ODS column and a Model 165 variable wavelength detector, from Altex Scientific Co., U.S.A. The mobile phase was a NaOAc acetonitrile gradient (NaOAc 0.2 M, pH 5.1/acetonitrile, 16–42%) [65].

Acknowledgements—We wish to thank co-workers in the ACIAR/ANU Program on Nutrition of Tropical Root Crops in the South Pacific for help and advice and in particular Dr J. E. Wilson (Western Samoa), Messrs P. Sivan (Fiji) and B. Ratieta (Kiribati) for provision of root crops. We thank Mr K. McAndrew for assistance with amino acid analyses. Dr G. Persley (ACIAR, Fiji) and Dr G. V. H. Jackson (UNDP/SPC, Fiji) are thanked for continued support of this work.

REFERENCES

1. Laskowski, M. and Kato, I. (1980) *Annu. Rev. Biochem.* **50**, 593.
2. Liener, I. E. and Kakade, M. L. (1980) in *Toxic Constituents of Plant Foodstuffs* (Liener, I. E., ed.) 2nd Edn, p. 7. Academic Press, New York.
3. Laskowski, M. and Sealock, R. W., (1971) in *The Enzymes* (Boyer, P. D., ed.) 3rd Edn, Vol. 3, p. 375. Academic Press, New York.
4. Richardson, M. (1977) *Phytochemistry* **16**, 159.
5. Hymowitz, T. and Kaizuma, N. (1981) *Econ. Botany* **35**, 10.
6. Liener, I. E. (1983) in *Chemistry and Biochemistry of Legumes* (Arora, S. K., ed.), p. 217. Edward Arnold, London.
7. Burns, R. A. (1987) *J. Food Protection* **50**, 161.
8. Bradbury, J. H. and Holloway, W. D. (1988) *Chemistry of Tropical Root Crops: Significance for Nutrition and Agriculture in the Pacific.* Australian Centre for International Agricultural Research, Canberra, Australia.
9. Shumacher, G. F. B., Swartout, J. R. and Zuspan, F. P. (1971) in *Proc. Intern. Res. Conf. Proteinase Inhibitors, Munich*, 1970, (Fritz, H. and Tschesche, H., eds). Walter de Gruyter, New York.
10. Barton, P. G. and Yin, T. Y. (1973) in *Metabolic Inhibitors* (Hochster, R. M., Kates, M. and Quastel, J. H. eds), Vol. 4, p. 215. Academic Press, New York.
11. Bradbury, J. H., Baines, J., Hammer, B., Anders, M. and Miller, J. S. (1984) *J. Agric. Food Chem.* **32**, 469.
12. Ryan, C. A. (1973) *Annu. Rev. Plant Physiol.* **24**, 173.
13. Ryan, C. A. (1981) in *Proteins and Nucleic Acids* (Marcus, A., ed.), Vol. 6, p. 351. Academic Press, New York.
14. Weder, J. K. P. (1981) in *Advances in Legume Systematics*, (Polhill, R. M. and Raven, P. H., eds), Vol. 2, Pt 1. Royal Botanic Gardens, Kew, England.
15. Plucknett, D. L. (1976) in *Evolution of Crop Plants* (Simmonds, N. W., ed.), p. 10. Longman, London.
16. Chan, H. T. (ed.) (1983) *Handbook of Tropical Foods*, Preface. Marcel Dekker, New York.
17. Chandra, S, (ed.) (1984) *Edible Aroids*, Preface. Clarendon Press, Oxford.
18. Ryan, C. A., Huisman, O. O. and van Denburgh, R. W. (1968) *Plant Physiol.* **43**, 589.
19. Sumathi, S. and Pattabiraman, T. N. (1975) *Indian J. Biochem. Biophys.* **12**, 383.
20. Sumathi, S. and Pattabiraman, T. N. (1979) *Biochim. Biophys. Acta.* **566**, 115.
21. Ogata, F. and Makisumi, S. (1984) *J. Biochem. (Tokyo)* **96**, 1565.
22. Ogata, F. and Makisumi, S. (1985) *J. Biochem. (Tokyo)* **97**, 589.
23. Sumathi, S. and Pattabiraman, T. N. (1977) *Biochem. Biophys. Acta* **485**, 167.
24. Hammer, B. C. (1987) Ph.D. Thesis, Australian National University, Canberra, Australia.
25. Laskowski, M., Kato, I. and Kohr, W. J. (1978) in *Versatility of Proteins* (Li, C. H., ed.), p. 307. Academic Press, New York.
26. Anderson, L. O. and Berg, G. (1969) *Biochim. Biophys. Acta.* **192**, 534.
27. Donovan, J. W. and White, T. M. (1971) *Biochemistry* **10**, 32.
28. Friedman, M. (1973) *The Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides and Proteins.* Pergamon Press, Oxford.
29. Cleland, W. W. (1964) *Biochemistry* **3**, 480.
30. Koide, T. and Ikenaka, T. (1973) *Eur. J. Biochem.* **32**, 417.
31. Edelhoch, H. and Steiner, R. F. (1963) *J. Biol. Chem.* **238**, 931.
32. Richardson, M., Campos, F. A. P., Xavier-Filho, J., Macedo, M. L. R., Maia, G. M. C. and Yarwood, A. (1986) *Biochim. Biophys. Acta.* **872**, 134.
33. Yamamoto, M., Hara, S. and Ikenaka, T. (1983) *J. Biochem. (Tokyo)* **94**, 849.
34. Kortt, A. A. and Jermyn, M. A. (1981) *Eur. J. Biochem.* **115**, 551.
35. Odani, S., Ono, T. and Ikenaka, T. (1979) *J. Biochem. (Tokyo)* **86**, 1795.
36. Joubert, F. J., Carlsson, F. H. H. and Haylett, T. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 531.
37. Joubert, F. J. (1982) *Int. J. Biochem.* **14**, 187.
38. Hejgaard, J., Svendson, I. and Mundy, J. (1983) *Carlsberg Res. Commun.* **48**, 91.
39. Kato, I., Tominaga, N. and Kihara, F. (1972) in *Proc. 23rd Conf. of Protein Structure Maebashi* (Iwai, K., ed.), p. 53.
40. Chi, C. W., Zhu, D. X., Lin, N. Q., Xu, L. X., Tan, F. L. and Wang, L. X. (1985) *Biol. Chem. Hoppe-Seyler* **366**, 879.
41. White, A., Handler, P., Smith, E. L., Hill, R. L. and Lehman, I. R. (1978) *Principles of Biochemistry*, 6th Edn. McGraw-Hill, Kogakusha, Tokyo.
42. Argall, M. E. (1987) Honours Thesis, Australian National University.
43. Polanowski, A., Wilusz, T., Nienavtowicz, B., Cieslar, E., Slominska, A. and Nowak, K. (1980) *Acta. Biochim. Pol.* **27**, 371.
44. Hojima, Y., Pierce, J. V. and Pisano, J. J. (1982) *Biochemistry* **21**, 3741.
45. Wilusz, T., Wieczorek, M., Polanowski, A., Denton, A., Cook, J. and Laskowski, M. (1983) *Hoppe-Seyler's Z. Physiol. Chem.* **364**, 93.
46. Joubert, F. J. (1964) *Phytochemistry* **23**, 1401.
47. Campos, F. A. P. and Richardson, M. (1983) *FEBS Letters* **152**, 300.
48. Shewry, P. R., Lafiandra, D., Salcedo, G., Aragoncillo, C., Garcia-Olmedo, F., Lew, J. L. E., Dietler, M. D. and Kasarda, D. D. (1984) *FEBS Letters* **175**, 359.
49. Ramshaw, J. A. M., Richardson, D. L., Meatyard, B. T., Brown, R. H., Richardson, M., Thompson, E. W. and Boulter, D. (1972) *New Phytol.* **71**, 773.
50. Tortora, G. J., Cicero, D. R. and Parish, H. I. (1970) *Plant Form and Function.* Collier–Macmillan, London.
51. Heywood, V. H. (ed.) (1978) *Flowering Plants of the World.* Oxford University Press, Oxford.
52. Jensen, W. A. and Salisbury, F. B. (1984) *Botany* 2nd Edn. Wadsworth, Belmont, California.
53. Boulter, D. (1973) in *Chemistry in Botanical Classification*, (Bendz, G. and Santesson, J., eds), p. 211. Academic Press, New York.
54. Peters, T., (1975) in *The Plasma Proteins* (Putnam, F. W., ed.). Vol. I, p. 133. Academic Press, New York.

55. Decker, L. A. (1977) *Worthington Enzyme Manual* (Decker, L. A., ed.). Worthington Biochemical Corp., Freehold, New Jersey.
56. Smith, M. H. (1968) in *Handbook of Biochemistry* (Sober, H. A., ed.), p. C-3. CRC Press, Cleveland, Ohio.
57. Tsuru, D. (1973) in *Handbook of Microbiology* (Laskin, A. I. and Lechevalier, H. A., eds), Vol. 3, p. 593. CRC Press, Cleveland, Ohio.
58. Rick, W., (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.). 2nd Ed, Vol. 2, p. 1013. Academic Press, New York.
59. March, S. C., Parikh, I. and Cautrecasas, P. (1974) *Anal. Biochem.* **60**, 149.
60. Pharmacia Fine Chemicals (1982) *Gel Filtration, Theory and Practice* p. 30. Pharmacia Fine Chemicals AB, Uppsala.
61. Pharmacia Fine Chemicals (1983) *Polyacrylamide Gel Electrophoresis, Laboratory Techniques* revised Edn, p. 44. Pharmacia Fine Chemicals AB, Uppsala.
62. Glossmann, H. and Neville, D. M. (1971) *J. Biol. Chem.* **246**, 6339.
63. Crestfield, A. M., Moore, S. and Stein, H. (1963) *J. Biol. Chem.* **238**, 622.
64. Spackman, D. H., Stein, W. H. and Moore, S. (1958) *Anal. Chem.* **30**, 1190.
65. Zimmerman, C. L. and Pisano, J. J. (1977) *Methods Enzymol.* **57**, 45.