Characterization of Trypsin Inhibitor in Florunner Peanut Seeds *(Arachis hypogaea L.)*[1]

E. M. Ahmed* and J. A. Applewhite[2]

ABSTRACT

Florunner peanut seeds contained five trypsin isoinhibitors. Amino acid profiles of the trypsin inhibitors fraction showed high levels of aspartic acid, half-cystine and serine and low levels of histidine and tyrosine. The molecular weight of the inhibitor was 8.3 KDa.

The presence of multiforms of this inhibitor, its low molecular weight and the high amount of half-cystine indicate that peanut trypsin inhibitor is of the Bowman-Birk type.

Key Words: Antinutrients, Groundnuts, Arachis hypogaea L.

Proteinaceous inhibitors of trypsin (TI) have been found in various legume seeds, including those of the peanut. Peanut TI is localized in the albumin protein fraction (5). Trypsin inhibitors (TI) of legumes are generally composed of several protein components: lima beans contained six (8), navy beans contained five (1), soybeans contained eight (13), *Lathyrus sativus* contained five protein components (15), and winged beans contained eight (3). However, there have been few reported studies of TI in peanut seeds. Two inhibitors of molecular weight 17 KDa were found in peanuts and it was assumed that these were tetrammers of a subunit containing 48 amino acid residues (9). Tur-Sinai, *et al.* (19) have described a single inhibitor of molecular weight 7.5 KDa that inhibited equally well trypsin and chymotrypsin. Most recently two sets of anti-tryptic factors were separated into a total of five isoinhibitors which inhibited chymotrypsin to a larger extent than the trypsin (14). Their molecular weights ranged from 6.7 to 7.6 KDa. Amino acid analysis of the isomers showed high levels of cysteine.

The objective of this study was to characterize the trypsin inhibitor(s) present in Florunner peanut seeds.

Materials and Methods

Blanched Florunner peanut seeds were obtained from Pert Labs, Edenton NC. All chemicals used in this study were obtained from Sigma Chemical Co., St. Louis Mo., unless otherwise stated.

Extraction

The seeds were pressed in a hydraulic press at 30,000 kg of pressure per 0.122 sq. meter for thirty min to partially remove the oil and then were soaked in hexane for four hr to remove any remaining expressed oil distributed throughout the press cake. Two hundred g of the solvent free defatted seeds were suspended in 0.02 M HCl and stirred with magnetic stirrer for at least three hr at room temperature. The suspension was then centrifuged at 6000 g for 20 min at room temperature. The yellow supernatant was then filtered through Whatman #4 filter paper. The pH of the filtrate was then raised to 7.0 using concentrated NaOH and the mixture was again filtered through Whatman #4 filter paper. Ammonium sulfate was added to the filtrate to 70% saturation (at room temperature). The gray mixture was allowed to precipitate overnight at 3.5 C, centrifuged at 6000 g for twenty min and the supernatant was discarded. The gray precipitate was then desalted by either dialysis or by gel filtration chromatography on Sephadex G-15. Dialysis was performed for one day against magnetically stirred distilled water at room temperature with frequent changes. Dialysis tubing was obtained from Fisher Scientific Company (3.5 KDa cutoff). The salt-free solution was lyophilized in a Virtis Model 25 SRC bulk freeze dryer. The sample was poured into clean metal trays and frozen to -50 C. followed by sublimation of water at a shelf heat of 0 C and a vacuum of 100 microns. The yield was 1.6 mg of lyophilized TI obtained from 200 g defatted peanut seeds.

[1]Florida Agricultural Experiment Station Journal Series No. 8970.

[2]Professor and Chemist II Technician, Food Science and Human Nutrition Department, IFAS, University of Florida, Gainesville, FL 32611.

Analytical Methods

Protein was determined by the method of Bradford (2). The absorbance was meausured at 595 nm.

Trypsin inhibiting activity was measured by the test developed by Kakade and Rackis (11), which employs benzoyl-DL-arginine p-nitroaniline HCl (BAPA) as a substrate for trypsin. Trypsin from bovine pancreas (Type III) was used in this assay. Digestion of BAPA produced a yellow color due to the release of P-nitroanaline. The absorbance was measured at 410 nm.

Chromatography

Affinity chromatography was carried out on a trypsin-agarose column (U.S. Biochemicals). The column was equilibrated with a pH 8.0 buffer containing 0.05 M Tris, 0.1 M KCl, 0.02 M CaCl2 and 0.02 M NaN3. After sample introduction the column was washed with the equilibrating buffer until non-specific proteins were removed as indicated by the negative Bradford assay (2). The inhibitors were eluted with 0.2 M KCl, pH 2. This stage was monitored by measuring the anti-tryptic activity of each fraction (11). Inhibitor fractions were pooled, desalted, and freeze dried. The total protein of the lyophilate was measured and this material was used for the size exclusion chromatography amino acid analyses and electrophoretic analysis.

Size exclusion chromatography was performed on Sephadex G-50 and Sephacryl S-200. Mobile phases used on each column were: pH 2 HCL; pH 8.2, 0.01 M Tris buffer in 0.5 M NaCl; and pH 8.2, 0.01 M Tris buffer in 0.5 M NaCL with 0.01 M mercaptoethanol added (on the G-50 column only). The proteins were also chromatographed on the G-50 column in 6 M guanidine hydrochloride, 0.01 M mercaptoethanol (7). The columns were run at a flow rate of 0.5 to 1.0 mL per minute and the fractions were collected utilizing a fraction collector.

Amino acids analyses

HPLC analysis of amino acids were carried out on Perkin-Elmer liquid chromatograph (Series 400) equipped with pecosphere 5C C18 column, fluorescence detector model LS-1 and Laboratory Computing Integrator model LCI-100. The TI protein was hydrolyzed with 6 N HCl for 24 hr at 110 C. The HCl contained 2% thioglycolate to protect tryptophan (10) and 0.25 M dimethyl sulfoxide (DMSO) for the conversion of half-cystine to cysteic acid (17) during the hydrolysis. The hydrolyzed sample was lyophilized and resuspended in 1.0 mL of 0.1 N HCl, filtered and derivatized using 50 µl sample with 200 µL O-phthaldialdehyde-thiol (OPT) solution, incubated for 2 min at room temperature and immediately injected (20 µL) on column. The buffer used contained 2% tetrahydrofuran (THF) for the separation of threonine and glycine (10).

Proline was determined using Beckman single column amino acid analyzer (Beckman 119CL). Protein hydrolysis was for 4 hr in 6 N HCl at 145 C. The column used was 6 x 460 mm column packed with Beckman W-3H resin. Amino acids analysis was performed according to Beckman Application Notes No. 118/119 CL-AN001. Proline is expressed as % of total amino acids present in TI hydrolysate. Tryptophan was determined by the method of Edelhoch (6).

Electrophoresis

Electrophoresis in a non-denaturing system of 10% acrylamide and 0.264% N, N^{1}methylene-bis-acrylamide was carried out in 5 mm X 100 mm tubes according to the method described by the Sigma Technical Bulletin (16). Acrylamide and N,N'-Methylene-bis-Acrylamide were obtained from Bio-Rad. Electrophoresis was carried out at a constant current of two milliamps per tube until the marker dye was one cm from the end of the tube. Gels were fixed in a methanol:acetic acid: water solution (40:7:53, v/v/v) for two hr and stained overnight in a solution of Commassie Brilliant Blue R (0.5 g in 500 mL of fixative solution). The gels were destained by diffusion in a Bio-Rad Destainer (model 556).

Electrophoresis in a denaturing system was carried out in the presence of the anionic detergent sodium dodecyl sulfate and was used to determine the molecular weight of the proteins (17). The gels were 10% acrylamide and 1.25% N,N^1-methylene bis-acrylamide. Molecular weight markers for low molecular weight proteins were obtained from Sigma (MW-SDS-17). Electrophoresis was at constant current (one milliamp per tube) until the marker dye was one centimeter form the end of the gel. The gel was fixed for five hr in a trichloroacetic acid and 5-sulfo-salisylic acid solution (50 g and 15 g respectively in 500 mL water) and then stained overnight in a Comassie Brilliant Blue R solution (1.24 g in 500 mL of a solution containing 25 mL methanol and 37.5 mL of acetic acid). The gels were destained by diffusion against several changes of destaining solution (methanol: acetic acid 25:37.5 mL in 1000 mL water) at room temperature.

Results and Discussion

The TI eluted from the affinity column was desalted by dialysis and size exclusion to determine if loss of the low molecular weight compounds was occurring through the dialysis tubing. There was no difference in the results of either method.

The attempt to separate the TI on the size exclusion gel using 0.5 M NaCL at pH 2.0 or 8.0 did not result in the resolution of TI. A change to a more inert stationary phase (S-200 Sephacryl) was attempted, but did not resolve the TI protein fraction. Denaturing the protein fraction in 6.0 M guanidine hydrochloride, equilibrating the G-50 column with the 6.0 M solution, and using the guanidine hydrochloride as a mobile phase, five fractions, each exhibiting anti-tryptic activity, were resolved indicating the presence of five isoinhibitors (Fig 1).

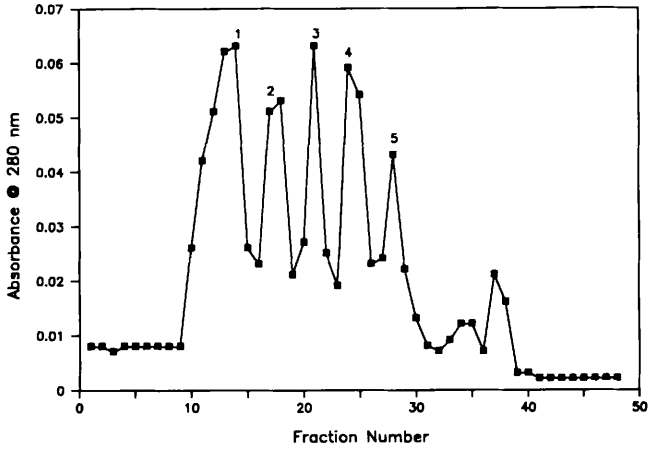

Fig. 1. Size exclusion chromatography. Sephadex G-50 column equilibrated with 6 M guanidine hydrochloride containing 0.01 M mercaptoethanol which was also used as a mobile phase. Peaks 1-5 represent fractions exhibiting antitryptic activity.

Non-denaturing gel electrophoresis of the TI also resulted in the resolution of five bands (Fig 2). The trypsin inhibitors consistently ran well ahead of the lowest molecular weight standard (alpha-lactoalbumin, bovine milk, MW 14,200) available for the non-denaturing gel electrophoresis. Denatured gel standards, with molecular weights ranging from 2.5 to 16.9 KDa, were used to determine the molecular weight of the isoinhibitors. The denatured trypsin isoinhibitors were too close to resolve on the gel, but the center of the band was at a molecular weight of 8.3 KDa (Fig 3).

Amino acid analysis of Florunner TI indicated high levels of aspartic acid, half-cystine and serine and low levels of histidine and tyrosine and an absence of tryptophan (Table 1). These findings agree with the characteristic amino acid composition of Bowman-Birk type inhibitors (20). Other characteristics of these inhibitors are their low molecular weight and the multiple forms of the inhibitor, isoinhibitors (20). This agrees with the results obtained in the present study. Amino acid analysis obtained in the present study contained more glycine, serine and valine but less half-cystine and

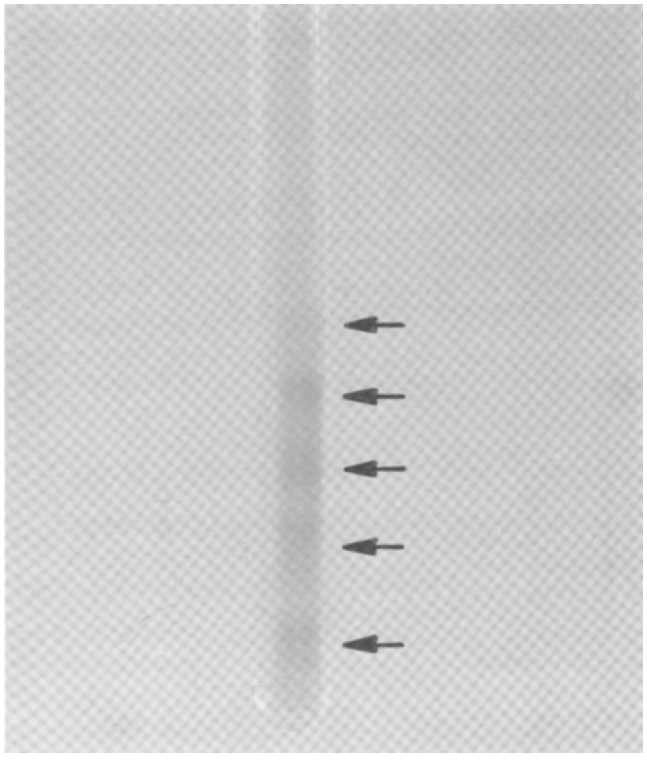

Fig. 2. Nondenatured gel electrophoresis of fraction recovered from affinity column.

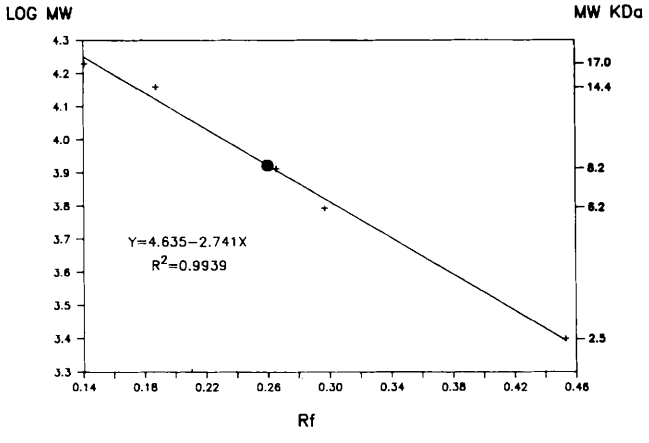

Fig. 3. Regression line of log molecular weights of denatured protein: + standards, ● peanut TI. Standards are: Myoglobin (polypeptide backbone) 16,950 kDa, myoglobin (fragment I + II) 14,400 kDa, myoglobin (fragment I) 8,160 kDa, myoglobin (fragment II) 6,210 kDa, and myoglobin (fragment III) 2,510 kDa.

arginine than those reported by other investigators (9,14,19) as shown in Table 2.

Calculations of the isoelectric point (pI) from the net electrical charges of specific amino acids at different pH's showed a value of 4.1 (Fig 4). This pI calculations from the reported amino acid analysis (19) indicated a value of 4.67 of the Indian peanuts, although the value reported was 8-9 (19). However, this value of 4.67 is in much closer agreement with that found in the present study and those calculated for the German and Japanese peanuts (9,14). Various proteins possess isoelectric points ranging from 1.9 to 11.0 (13).

Table 1. HPLC amino acid profile of TI (% of total) in Florunner peanut seeds.

Amino Acid	Mean % of Total[a]
Aspartic acid	11.80 ± 0.61
Threonine	7.64 ± 0.33
Serine	12.01 ± 1.28
Glutamic acid	8.61 ± 0.69
Glycine	9.04 ± 0.38
Alanine	7.12 ± 0.85
Valine	8.19 ± 0.67
Isoleucine	1.91 ± 0.14
Leucine	3.30 ± 0.46
Tyrosine	1.59 ± 0.83
Phenylalanine	3.93 ± 0.06
Lysine	4.04 ± 1.14
Histidine	0.81 ± 0.80
Arginine	5.95 ± 0.03
Cystine (half)[b]	14.68 ± 0.76
Methionine	0
Tryptophan	0
Proline[c]	8.19 ± 0.55

[a]Data presented as mean ± standard deviation (2 replicates.

[b]Half-cysteine determined as cysteic acid.

[c]Proline was determined using Beckman single column amino acid analyzer (Beckman 119CL).

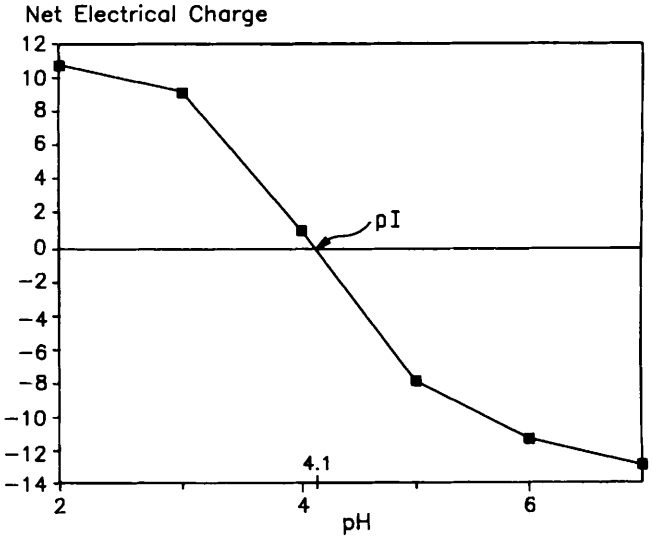

Fig. 4. Net electrical charges for aspartic acid, glutamic acid, lysine, histidine and arginine at different pH's. pI is the pH where the net charge = 0.

Results obtained in the present study (Table 2) agree to a great extent with those found by other workers (9,14,19). Peanut seed TI is of low MW, present in several forms (isoinhibitors), and its amino acid profile shows a high content of aspartic acid, half-cystine and serine and low levels of tyrosine. These properties classify TI in peanut seed as of Bowman-Birk type.

Acknowledgement

The authors acknowledge with gratitude the HPLC amino acid analysis by Dr. C. McGowan's Laboratory and the stimulating discussions during the progress of this research by Mr. E. R. Mason of the Food Science and Human Nutrition Department, University of Florida.

Table 2. Characterization of TI in peanut seeds grown in different parts of the world.

Location	Japan (10)[a]	Germany (8)[a]	India (16)[a]	US[b]
Amino acid[c]				
Aspartic acid	13	10	11	12
Threonine	8	10	9	8
Serine	8	6	7	12
Glutamic acid	6	8	8	9
Proline	9	12	9	8
Glycine	3	2	5	9
Alanine	5	4	4	7
Half cystine	22	21	19	15
Valine	6	6	7	8
Methionine	0	0	0	0
Isoleucine	0	0	0	2
Leucine	2	0	1	3
Tyrosine	2	2	1	2
Phenylalanine	3	4	3	4
Tryptophan	0	0	0	0
Lysine	2	2	3	4
Histidine	2	2	3	1
Arginine	9	8	9	6
Isoinhibitors	5	2	1	5
KDa	7.2	17.0[d]	7.6	8.3
pI	---	---	8-9	
pI calculated[e]	4.3	4.5	4.7	4.1

[a]Reference number

[b]Present study

[c]Values reported as % of total amino acids to the nearest integers

[d]Tetramer (TI complex)

[e]Calculated from % aspartic acid, glutamic acid, lysine, histidine and arginine of total amino acids and dissociation constants for each acid at pH's 2,3,4,5,6 and 7.

Literature Cited

1. Bowman, D. E. 1971. Isolation and properties of a proteinase inhibitor of navy beans. Arch. Biochem. Biophys. 144:541-548.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
3. Chan J. P. and B. O. deLumen. 1982. Properties of trypsin inhibitor from winged beans *(Psophocarpus tetragonobus)* seed isolated by affinity chromatography. J. Agric. Food. Chem. 30:42-46.
4. Cheftel, J. C., J. L. Cug and D. Lorient. 1986. Food Chemistry. O. R. Fennema, Ed. Marcel Dekker Inc. N.Y., pp. 249-253.
5. Cherry, J. P., J. M. Dechary and R. L. Ory. 1973. Gel electrophoresis analysis of peanut proteins and enzymes. I. Characterization of DEAE-celluose separated fractions. J. Agr. Food Chem. 21:652-655.
6. Edelhoch, E. 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. Biochem. 6:1948-1954.
7. Davison, P. F. 1968. Proteins in denaturing solvents: Gel exclusion studies. Science. 161:906-907.
8. Haynes R. and R. E. Feeney. 1967. Fractionation and properties of trypsin and chymotrpsin inhibitors from lima beans. J. Biol. Chem. 242:5378-5385.
9. Hochstrasser, Von K., K. Illchmann and E. Werde. 1969. Reindarsstellung der polyvalenten protease ninhibitoren aus *Arachus hypogaea.* Hoppe-Seyler. Z. Physiol. Chem. 350:929-932.
10. Joseph, M. H. and C. A. Marsden. 1986. Amino Acids and Small Peptides. Ch. 2 *In:* HPLC of Small Molecules. C. K. Lim, (ed). IRL Press, Washington, D.C.
11. Kakade, M. L., J. J. Rackis, J. J. McGhee and J. E. Puski. 1974. Determination of trypsin inhibitor activity of soy products: A collaborative analysis of an improved procedure. Cereal Chem. 51:376-382.
12. Leiner, I. E. and M. L. Kakade. 1969. Protease Inhibitors. In: Toxic Constituents in Plant Foodstuffs. Leiner, I. E., (Ed.) Academic Press, N.Y. p. 16.
13. Malamud, D. and J. W. Drysdale. 1978. Isoelectric points of proteins: A table. Anal. Bioch. 86:620-647.
14. Norioka, S., K. Omichi and T. Ikenaka. 1982. Purification and characterization of protease inhibitors form peanuts *(Arachis hypogaea).* J. Biochem. 91:1427-1434.
15. Roy, D. N. 1980. Trypsin inhibitor from *Lathurys sativus* seeds: Partial purification, separation of protein component, properties and characterization. J. Agric. Food Chem. 28:48-54.
16. Sigma Chemical Company. 1986. Nondenatured Protein Molecular Weight Marker Kit. Technical Bulletin No. MKR-137.
17. Sigma Chemical Company. 1986. SDS Molecular Weight Markers. Technical Bulletin No. MWS-877P.
18. Spencer, R. L. and F. Wold. 1969. A new convenient method for estimation of total cystine-cysteine in proteins. Anal. Biochem. 32:185-190.
19. Tur-Sanai A., Y. Y. Birk, A. Gertler and M. Rigbi. 1972. A basic trypsin and chymotrypsin-inhibitor from groundnuts *(Arachis hypogaea).* Biochem. Biophysica Acta. 263:666-672.
20. Wilson, K. A. 1981. The Structure, Function and Evaluation of Legume Proteinase Inhibitors. ch. 11. In "Antinutrients and Natural Toxicants in Foods." R. L. Ory, (Ed.). Food and Nutrition Press, Inc. Westport, Conn. 06880.

Accepted December 2, 1988