Effect of Proteases on Arachin, Conarachin I, and Conarachin II from Peanut (*Arachis hypogaea* L.)†

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Arachin, conarachin II, and conarachin I were isolated from defatted peanut (*Arachis hypogaea* L.) meal in a homogeneous form using a method of preferential ammonium sulfate precipitation combined with gel filtration. The homogeneities of the protein fractions were established by sedimentation velocity, polyacrylamide gel electrophoresis, gel filtration, and DEAE-cellulose chromatography. Enzymatic hydrolysis of protein fractions was determined with pepsin, trypsin, and chymotrypsin. The extent of hydrolysis with pepsin was in the order conarachin I > conarachin II > total protein > casein > arachin. With trypsin the extent was in the order conarachin I > casein > total protein > arachin > conarachin II. With α-chymotrypsin the order was conarachin II > total protein > conarachin I > arachin > casein. Addition of aflatoxin B_1 to protein fractions and denatured hemoglobin reduced the extent and rate of hydrolysis.

INTRODUCTION

Among the various sources of plant proteins considered as food ingredients, peanut seeds have excellent potential because of their high protein content and quality. The oil-free meal contains nearly 50–60% protein of good nutritional quality. Arachin (14S), conarachin II (8S), and conarachin I (2S) are the major protein fractions which make up nearly 75% of the peanut total proteins (Prakash and Rao, 1986).

The storage proteins of peanut have been extensively studied by several investigators (Jones and Horn, 1930; Tombs, 1965; Neucere, 1969; Dawson, 1971; Shetty and Rao, 1974; Cherry, 1977; Yamada *et al.*, 1980; Shokraii *et al.*, 1985). Although several attempts have been made to isolate protein fractions in a homogeneous form, a modified method for isolation of all three protein fractions is desirable. In this investigation a method has been developed to isolate the three protein fractions of peanut, namely arachin, conarachin II, and conarachin I, in a homogeneous form.

In vitro enzymatic hydrolysis of plant preoteins provides information on their *in vivo* digestibility. Also, the *in vitro* hydrolysis of proteins with appropriate enzymes may indicate certain biochemical and structural features that affect their susceptibility *in vivo* to proteolytic enzymes.

Aflatoxins are a family of closely related heterocyclic compounds produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins are highly toxic and carcinogenic compounds which contaminate cereals, legumes, and oilseeds when improperly stored. Among this group of toxins, aflatoxin B_1 has been found to be one of the most potent environmental carcinogens. Almost all of the available epidemiological evidence strongly implicates aflatoxin B_1 in primary liver cancer (Goldblatt, 1977).

Generally, peanuts are contaminated by aflatoxin due to improper storage. During protein isolation the majority of aflatoxins originally present in the peanut meal is precipitated with the protein fractions (Sreedhara and Subramanian, 1981). Various findings reported in the literature indicate that seed proteins are less attacked by mammalian digestive endopeptidases and are frequently less digestible than animal proteins (Kakade, 1974; Lynch *et al.*, 1977a,b; Romero and Ryan, 1978). Though resistance to hydrolysis of seed proteins by proteases has been well documented (Tasneem and Prakash, 1989), work on the effectiveness and the order of hydrolysis of peanut protein fractions with different proteolytic enzymes is not available in depth. Also, the effect of aflatoxin B_1 on the extent and rate of hydrolysis of homogeneous peanut protein fractions is not clear.

The objective of the present investigation is to see the order of effectiveness of the different proteases on the pure protein fractions of peanut and the effect of aflatoxin B_1 on the extent and rate of hydrolysis.

MATERIALS AND METHODS

Authenticated seeds of peanut, variety TMV-2, were obtained from the Karnataka State Seeds Corp. Ltd., Mysore, India.

Pepsin, trypsin, α-chymotrypsin, and aflatoxin B_1 were obtained from Sigma Chemical Co. Casein was from E. Merck and hemoglobin from Worthington Biochemical Co. Sepharose 6B and Sephadex G-100 were obtained from Pharmacia Fine Chemicals.

Preparation of Defatted Peanut Meal. Peanuts were decorticated and the seeds flaked. The flakes were extracted with *n*-hexane five or six times using a flake to solvent ratio of 1:1 (w/v) such that the final fat content was less than 1%. The defatted flakes were air-dried and powdered to obtain 150-μm flour.

Extraction and Fractionation of Peanut Proteins. Total proteins were extracted by stirring defatted flour for 1 h with 0.01 M phosphate buffer, pH 7.9, containing 0.5 M NaCl (extraction buffer, EB) using a meal to solvent ratio of 1:10 (w/v). The pH of the extract was 7.9. Insoluble residue was removed by centrifugation at 6000 rpm for 30 min at 27 °C. The total protein extract was fractionated with ammonium sulfate to arachin and conarachins. Conarachins were further fractionated into conarachins II and I on a Sephadex G-100 column using the EB (Figure 1).

The protein contents in all of the fractions were quantitated according to the Lowry *et al.* (1951) method after dialysis against the EB.

Protein Concentration. The concentration of protein in solution was determined by measuring the absorbance of the

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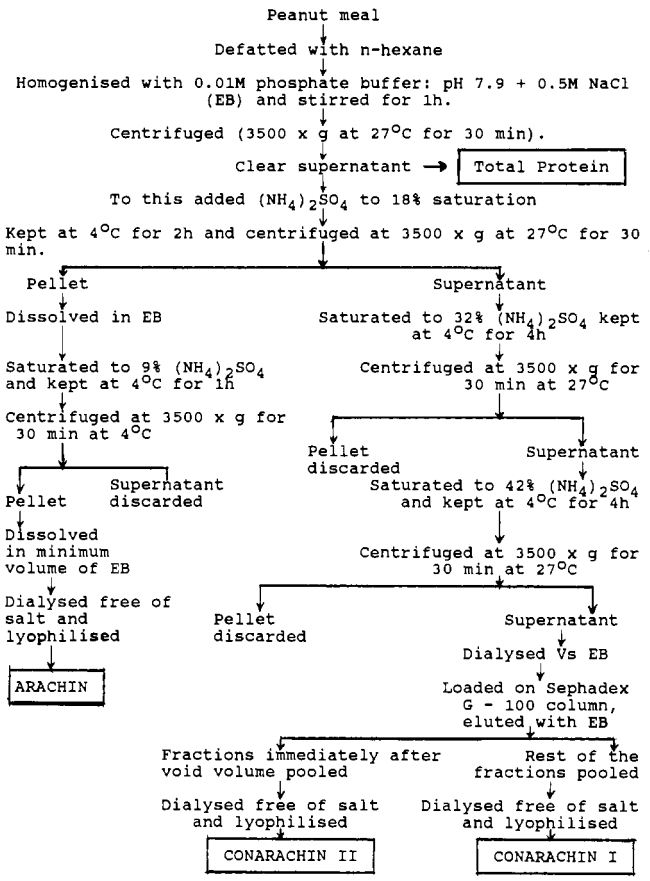

Figure 1. Schematic flow sheet of extraction of peanut protein and its fractionation.

protein solution in a Shimadzu UV-150-02 spectrophotometer. The nitrogen content per unit weight of dry protein was determined by the Kjeldahl method and correlated with absorbance at 280 nm. The values ($E^{1\%}_{1cm}$) obtained for total protein, arachin, conarachin II, and conarachin I were 16.0 ± 0.05, 8.0 ± 0.05, 6.3 ± 0.05, and 6.0 ± 0.05, respectively.

Sedimentation Velocity. Sedimentation velocity experiments were performed at 27 °C at 59 780 rpm in an analytical ultracentrifuge equipped with standard schlieren optics. A 1% protein solution in EB was used. Photographs were taken at regular intervals of time. The plates were read in a Abbe comparator adopted to read the ultracentrifuge plates and $s_{20,w}$ values calculated by standard procedure (Schachman, 1959). The percentage of the fractions was calculated by enlarged tracings of the ultracentrifuge pattern with a Gaertner M-2000 microcomparator attached with to a Gaertner X–Y axes digital reader.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out using 7.5% acrylamide gel with 0.4% methylenebis(acrylamide) as the cross-linking agent in 8.5 × 0.6 cm gel tubes. Protein samples (50–100 μg) were loaded on the gel. Electrophoresis was performed in 0.01 M phosphate buffer, pH 7.9, at a constant current of 3 mA/gel for 90 min, at which time the bromophenol blue dye had moved to the bottom of the gel. The gels were stained with 0.5% amido black and destained by diffusion with 7.5% acetic acid.

Protein Subunits and Molecular Weights. The number of subunits and their molecular weights of the peanut protein fractions were determined following the procedure of Weber and Osborn (1969). A 10% acrylamide gel with 0.27% methylenebis-(acrylamide) as the cross-linking agent in 8.5 × 0.6 cm tubes was used. Protein (20–50 μg) was loaded on the gel, and electrophoresis was conducted for 7 h at a constant current of 8 mA/gel, by which time the indicator dye, bromophenol blue, had moved almost to the bottom of the gel. Standard proteins (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin) were used as molecular weight markers. Gels were stained in 0.05% Coomassie brillant blue R-250 and destained by diffusion first in methanol–acetic acid–water (3:1:6 v/v/v) and then in 2-propanol–acetic acid–water (1.25:1:7.75 v/v/v). The relative mobilities of the protein bands were measured, and the molecular weights of the subunits were calculated from the standard calibration curve of mobility vs the log molecular weight of protein.

Gel Filtration. Sepharose 6B-100 gel in EB was gravity packed into a glass column of 2.0 × 100 cm. Approximately 75 mg of the protein in the above buffer was loaded onto the column and was eluted with the same buffer. Three-milliliter fractions were collected in an automatic fraction collector at a flow rate of 20 mL/h, and the absorbance of the fractions was measured at 280 nm in a Shimadzu UV-150-02 spectrophotometer.

Ion-Exchange Chromatography. DEAE-cellulose after regeneration by the procedure of Peterson (1970) was equilibrated with 0.01 M phosphate buffer, pH 7.9, and packed into a 2.5 × 22 cm column under pressure. Approximately 75 mg of the protein was loaded on the column and eluted with a 0–0.8 M linear gradient of NaCl in the same buffer at a flow rate of 20 mL/h. Fractions of 3.5 mL were collected, and the absorbance was monitored at 280 nm. Chloride ion in the fractions was estimated by the conductometric method in a Consort K 220 microcomputer conductometer using 0.01 M KCl at 25 °C for standardizing the instrument.

Ultraviolet Absorption Spectra. The absorption spectra of the peanut protein fractions in EB were recorded in a Shimadzu UV-150-02 spectrophotometer in the range 400–230 nm in a 1-cm quartz cell at 27 °C.

Fluorescence Emission Spectra. Fluorescence emission spectra of protein fractions were recorded in the range 300–400 nm after excitation at 280 nm using a Shimadzu RF-5000 spectrofluorophotometer at 27 °C.

Nitrogen Determination. Nitrogen content in the defatted meal and in all of the protein fractions was determined by the micro-Kjeldahl method (AOAC, 1984). A factor of 6.25 was used to convert the percentage nitrogen to protein content.

Carbohydrate Estimation. Carbohydrate content of the protein fractions was determined by the phenol–sulfuric acid procedure (Montgomery, 1961).

Phosphorus Estimation. Phosphorus content of the protein fractions was estimated by the procedure of Taussky and Shorr (1953).

Amino Acid Analysis. Amino acid analysis of peanut protein fractions was carried out in a LKB Alpha Plus II amino acid analyzer following the standard procedure of Spackman *et al.* (1958). Protein fractions were hydrolyzed using 6 N HCl at 110 °C for 22 h. Tryptophan was estimated separately according to the method of Spande and Witkop (1967).

Aflatoxin–Protein Fraction Binding. The following procedure was used to prepare aflatoxin protein complex. A 1% protein solution was mixed with 1 × 10^{-5} M aflatoxin B_1 in 0.1 M borate buffer, pH 8.1, containing 1 × 10^{-4} M $CaCl_2$. The mixture was incubated at 37 °C for 2 h.

Hydrolysis of Protein Fractions with Proteolytic Enzymes. For hydrolysis with pepsin, the substrate concentrations were 1% (w/v). The pH was adjusted to 2.0 with 0.1 M HCl. The assay mixture contained 1.0 mL of the substrate solution and 1.0 mL of the enzyme solution containing 50 μg/mL of freshly prepared pepsin in 0.1 M HCl to give an enzyme to substrate ratio of 1:200 (Bhagya *et al.*, 1992).

For hydrolysis with trypsin, the same substrate concentrations were used, but the pH was adjusted to 8.0 with 0.05 M Tris-HCl buffer containing 0.01 M $CaCl_2$ (Bhatty, 1988). The trypsin concentration was 250 μg/mL. The enzyme to substrate ratio was 1:40.

For hydrolysis with chymotrypsin the same substrate concentration was added to 0.1 M borate buffer, pH 8.1, containing 1 × 10^{-4} M $CaCl_2$ (Tasneem and Prakash, 1989). The enzyme (200 μg/mL) was added to the above mixture to give an enzyme substrate ratio of 1:50. All assays were conducted at 37 °C. Enzyme activity was stopped by adding 2 mL of 20% trichloroacetic acid. After the hydrolysis, the samples were centrifuged at 6000 rpm for 15 min at 27 °C and the absorbance of the supernatant was recorded at 280 nm. Appropriate blanks were run with each assay. Casein/denatured hemoglobin was used as a standard.

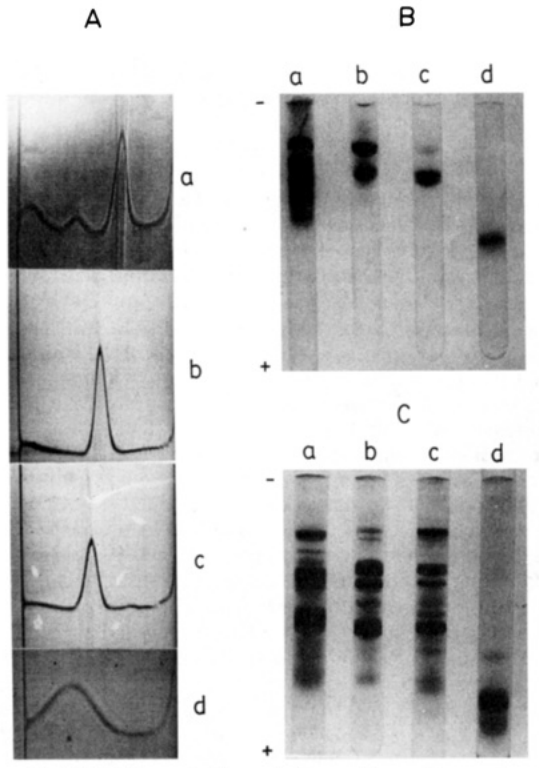

Figure 2. (A) Sedimentation velocity pattern of peanut protein fractions: (a) total protein; (b) arachin; (c) conarachin II; (d) conarachin I. (B) Polyacrylamide gel electrophoresis of peanut protein fractions in 0.01 M phosphate buffer, pH 7.9: (a) total protein; (b) arachin; (c) conarachin II; (d) conarachin I. (C) SDS–PAGE pattern of peanut protein fractions: (a) total protein; (b) arachin; (c) conarachin II; (d) conarachin I.

RESULTS AND DISCUSSION

The present work has shown that various protein fractions of peanut can be isolated from fat-free meal by extracting with 0.01 M phosphate buffer, pH 7.9, containing 0.5 M NaCl at a 1:10 (w/v) ratio. Using this extract as a starting material, pure arachin and conarachins II and I can be prepared by using a series of simple steps involving differential solubility, ammonium sulfate precipitation, and dialysis to alter salt concentrations (Figure 1).

By this method, nearly 33% was extracted as total protein from 100 g of defatted meal. The yields of arachin, conarachin II, and conarachin I were 28%, 5%, and 0.3–0.5%, respectively, calculated as percentage of defatted meal.

Sedimentation Velocity. The sedimentation velocity patterns of peanut protein fractions are shown in Figure 2A. The total protein (Figure 2Aa) gave three components having sedimentation values of 11.7 (arachin), 7 (conarachin II) and 1.6 S (conarachin I), constituting approximately 66%, 13%, and 16%, respectively. The sedimentation velocity patterns of arachin, conarachin II, and conarachin I (Figure 2Ab–d) all showed a single symmetrical peak with $s_{20,w}$ values of 11.8, 6.4, and 1.5 S, respectively. As to arachin, the purified preparations were not homogeneous but contained two components in the physicochemical analyses. Johnson and Shooter (1950) showed that the sedimentation pattern of arachin at a low ionic strength exhibited two components (9 and 14.6 S) but a single component (14.6 S) at high ionic strength. In the present study single symmetrical peaks were obtained for all of the peanut protein fractions in 0.01 M phosphate buffer, pH 7.9, containing 0.5 M NaCl, i.e., high ionic strength.

Polyacrylamide Gel Electrophoresis. All of the protein fractions gave single bands on the gel except arachin. The band at the top (Figure 2Bb) which did not penetrate the gel was due to well-established arachin polymer, and the other two bands of almost equal intensity were due to well-established arachin "monomer" and "dimer" (Johnson and Shooter, 1950; Tombs, 1965; Dawson, 1971; Yamada *et al.*, 1979). Tombs (1965) and Yotsuhashi and Shibasaki (1973) have also reported two components on polyacrylamide gel electrophoresis of arachin, and they suggested that these components would correspond to the 9S and 14.6S components of Johnson and Shooter (1950), respectively.

Protein Subunits and Their Molecular Weights. The subunit composition of the peanut protein fractions analyzed by SDS–polyacrylamide gel electrophoresis in the presence of a disulfide reducing agent indicated eight bands (Figure 2Ca) for total protein of molecular weights between 15 800 and 66 100. The predominant subunits had molecular weights of 66 100, 50 100, 43 600, 33 100, and 22 400.

Arachin gave seven bands (Figure 2Cb) of molecular weights from 15 800 to 72 400. Six of them were prominent and had molecular weights of 72 400, 60 300, 39 800, 33 100, 26 900, and 21 900. Tombs and Lowe (1967) observed the subunit pattern of arachin to contain six major subunits and classified them into two groups of hydrophilic and hydrophobic subunits. Basha and Pancholy (1981) also demonstrated six molecular weight (between 15 500 and 68 000) classes of polypeptides in arachin. Proteins derived from different peanut species are known to show differences in their polypeptide composition and exhibit different behavior in the dissociation–association properties of the arachin molecule (Basha, 1979). Location and season also had a major effect on the arachin monomer and dimer (Basha, 1992). It was also observed by Shokraii and Esen (1992) that when the disulfide bonds in arachin were reduced by 2-mercaptoethanol, a number of new bonds appeared after sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Conarachin II (Figure 2Cc) consisted of eight subunits having molecular weights 72 400, 39 800, 33 100, 26 900, 24 000, 21 900, 18 600, and 15 800. Basha and Pancholy (1981) have reported nine molecular weight (between 16 000 and 170 000) classes of polypeptides for conarachin II.

Conarachin I (Figure 2Cd), the low molecular weight fraction of peanut, had three subunits, and their molecular weight ranged from 12 000 to 18 200. Subunits with molecular weights of 12 000 and 13 200 were predominant.

Gel Filtration. Gel filtration of the total protein in Sepharose 6B-100 in EB also indicated three peaks (Figure 3a). The major peak, arachin, eluted between 150 and 200 mL and constituted the major protein fraction; and the peaks eluting between 215 and 240 mL and between 240 and 287 mL constituted conarachin II and conarachin I, respectively. The gel filtration pattern of arachin, conarachin II, and conarachin I gave single symmetrical peaks (Figure 3b–d).

Ion-Exchange Chromatography. DEAE-cellulose chromatography of peanut total protein in 0.01 M phosphate buffer, pH 7.9, showed three peaks. The major peak eluted at 0.24 M NaCl, while the other two peaks eluted at 0.06 and 0.14 M NaCl, respectively (Figure 4a). A turbid fraction was eluted near the void volume (between 0 and 50 mL). This may be attributed to nonspecific aggregates of peanut proteins (Prakash and Rao, 1986). Arachin (Figure 4b), conarachin II (Figure 4c), and conarachin I

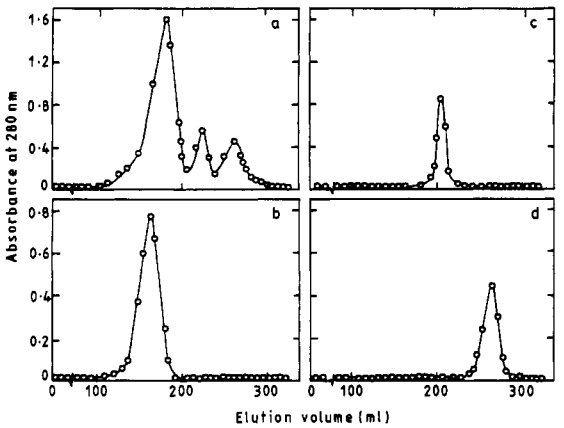

Figure 3. Gel filtration pattern of peanut protein fractions on Sepharose 6B-100 gel in 0.01 M phosphate buffer, pH 7.9, containing 0.5 M NaCl: (a) total protein; (b) arachin; (c) conarachin II; (d) conarachin I.

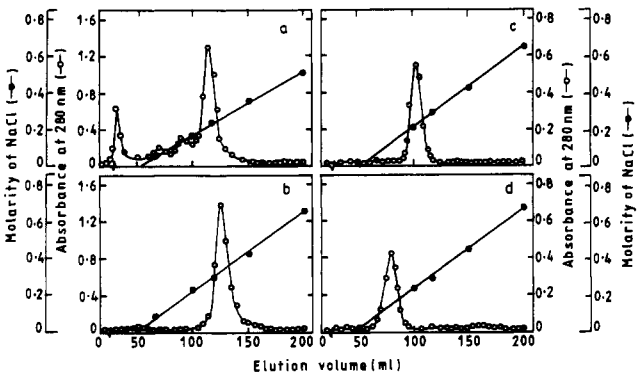

Figure 4. DEAE-cellulose chromatography pattern of peanut protein fractions in 0.01 M phosphate buffer, pH 7.9, and eluted with a linear gradient of NaCl from 0 to 0.8 M: (a) total protein; (b) arachin; (c) conarachin II; (d) conarachin I.

(Figure 4d) fractions eluted at 0.33, 0.23, and 0.15 M NaCl, respectively, the peaks being symmetrical.

Ultraviolet Absorption Spectra. The ultraviolet absorption spectra of peanut protein fractions showed an absorption maxima at 279 nm for total protein and arachin, 278 nm for conarachin II, and 269 nm for conarachin I.

Fluorescence Emission Spectra. The fluorescence emission spectra of peanut protein fractions such as total protein, arachin, conarachin II, and conarachin I showed maximum fluorescence intensity at 310, 320, 342, and 307 nm, respectively. The fluorescence emission of protein containing tryptophan and tyrosine residues is more characteristic of tryptophan residues, and the contribution of tyrosine is not predominant (Teale, 1960).

Nitrogen Determination. Fat-free defatted peanut meal had a nitrogen content of 9.84%, and the crude protein content was 61.50%. The nitrogen contents of total protein, arachin, conarachin II, and conarachin I were 13.99%, 14.69%, 15.64%, and 15.72%, respectively.

Carbohydrate Estimation. The data showed that all of these protein fractions of peanut contained sugars. The total protein contained 3.89% carbohydrate. Arachin and conarachin II and I fractions contained 0.57%, 0.64%, and 0.72% carbohydrate, respectively.

Phosphorus Estimation. The phosphorus content of the peanut protein fractions ranged from 0.03% to 0.19%. Total protein had 0.19%, whereas arachin, conarachin II, and conarachin I had 0.07%, 0.06%, and 0.03% phosphorus, respectively.

Amino Acid Analysis. The amino acid compositions of various protein fractions are shown in Table 1. Analysis

Table 1. Amino Acid Composition of Peanut Protein Fractions (Grams per 100 g of Protein)

amino acid	total protein	arachin	conarachin II	conarachin I
aspartic acid	12.91	12.29	12.68	7.95
threonine	2.50	2.68	2.97	3.06
serine	5.22	4.60	5.50	7.97
glutamic acid	23.01	21.43	22.24	8.28
proline	5.01	5.84	3.11	2.63
glycine	5.15	3.99	4.16	29.73
alanine	3.74	3.87	3.99	2.87
half-cystine	0.33	0.33	0.19	0.23
valine	3.65	4.77	5.73	3.47
methionine	0.52	0.71	0.78	0.34
isoleucine	2.83	3.61	4.12	2.40
leucine	6.25	6.42	7.22	4.26
tyrosine	4.12	4.13	2.38	5.69
phenylalanine	5.41	6.87	6.27	6.07
lysine	3.12	3.06	5.46	7.13
histidine	2.22	3.50	2.81	2.54
arginine	12.43	10.69	10.19	4.82
tryptophan[a]	1.59	1.21	0.91	0.59

[a] Tryptophan was determined according to the method of Spande and Witkop (1967).

Table 2. Bigelow Parameters[a] and Partial Specific Volume[b] Obtained from Amino Acid Composition of Peanut Protein Fractions

protein	NPS	C	P	HQ	$\bar{v}$ (mL/g)
total protein	0.412	0.587	0.553	780	0.708 ± 0.002
arachin	0.437	0.564	1.383	880	0.715 ± 0.001
conarachin II	0.420	0.580	1.457	800	0.718 ± 0.001
conarachin I	0.649	0.351	0.145	550	0.700 ± 0.002

[a] Bigelow (1967). [b] Cohn and Edsall (1943).

of total protein of peanut reflects low contents of half-cystine, methionine, tyrosine, and lysine and high contents of aspartic acid, glutamic acid, and arginine. The amino acid profile obtained in the present study is in good agreement with the amino acid composition reported by Basha and Cherry (1976). The amino acid compositions of arachin and conarachin II are also closely related to the values obtained by several workers (Tombs, 1965; Dawson, 1971; Neucere, 1969; Shetty and Rao, 1974). In general, levels of serine, glycine, and lysine were higher in conarachin I. The glycine content of conarachin I was 6 times higher than the amount of glycine present in the total protein. However, aspartic acid, proline, alanine, valine, isoleucine, and arginine were lower in conarachin I than the other protein fractions. The amino acid composition of conarachin I compares well with published data (Basha and Cherry, 1976).

The frequency of nonpolar side chains (NPS) calculated according to Waugh's definition (Waugh, 1954), charge (C), ratio of polar to nonpolar side-chain ratio (P), average hydrophobicity (HQ) calculated according to the method of Tanford (1961) and Bigelow (1967), and the partial specific volume ($\bar{v}$) calculated according to the method of Cohn and Edsall (1943) from the amino acid composition of peanut protein fractions are summarized in Table 2.

The results from the present investigation are the modified method of isolation of different peanut protein fractions, namely, arachin, conarachin II, and conarachin I. Studies on the various physicochemical properties and amino acid composition suggest that the protein fractions are homogeneous and can be isolated in large quantities by this procedure. This new information would pave the way for more developmental work in the area of seed proteins.

Cherry *et al.* (1973) examined the importance of the Virginia 56 R variety of peanut and provided information that may be potentially useful in the preparation of high-

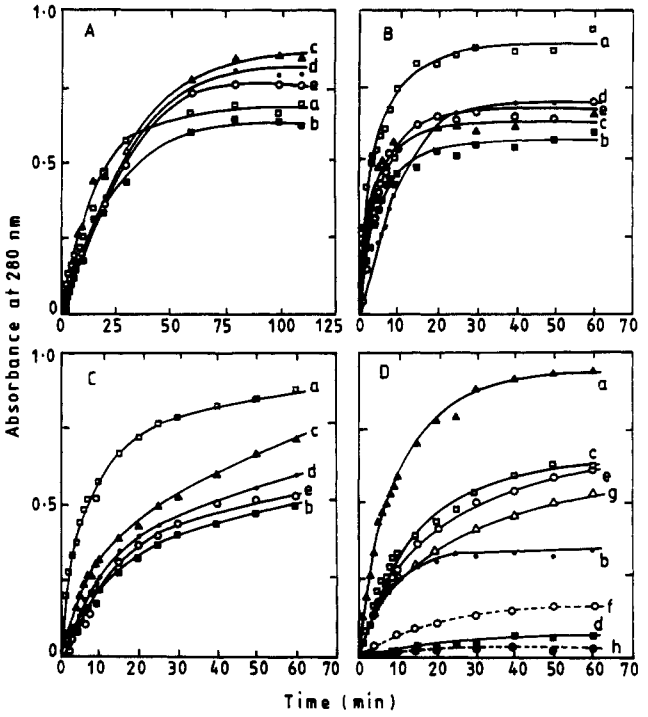

Figure 5. (A) Hydrolysis of peanut protein fractions by pepsin at pH 2.0: (a) casein; (b) total protein; (c) arachin; (d) conarachin II; (e) conarachin I. (B) Hydrolysis of peanut protein fractions by trypsin at pH 8.0: (a) casein; (b) total protein; (c) arachin; (d) conarachin II; (e) conarachin I. (C) Hydrolysis of peanut protein fractions by α-chymotrypsin at pH 8.1: (a) casein; (b) total protein; (c) arachin; (d) conarachin II; (e) conarachin I. (D) Hydrolysis of peanut protein–aflatoxin B_1 complex by α-chymotrypsin at pH 8.1: (a) hemoglobin; (b) hemoglobin–aflatoxin B_1 complex; (c) arachin; (d) arachin–aflatoxin B_1 complex; (e) conarachin II; (f) conarachin II–aflatoxin B_1 complex; (g) conarachin I; (h) conarachin I–aflatoxin B_1 complex.

quality protein concentrates and isolates. Basha (1991) showed that the time of harvest has a massive role in the accumulation pattern of sulfur-containing amino acids of peanut proteins. The polymorphic forms of arachin were due to differences in molecular size, net charge, and polypeptide composition of the native protein (Krishna and Mitra, 1987). The different classes of polypeptides available in arachin showed differences in their polypeptide composition (Basha, 1979).

Hydrolysis of Protein Fractions with Proteolytic Enzymes. The extent of hydrolysis of the peanut protein fractions by pepsin is shown in Figure 5A. The curves had a linear portion and a region of decreasing slope, which is characteristic of a protein hydrolysis curve. The different protein fractions of peanut were hydrolyzed at different rates. The extent of hydrolysis with pepsin was in the order

Con I > Con II > total protein > casein > arachin

The extent of hydrolysis of peanut protein fractions by trypsin is shown in Figure 5B. The extent of hydrolysis was in the order

Con I > casein > total protein > arachin > Con II

Hydrolysis of peanut protein fractions by α-chymotrypsin is shown in Figure 5C. The extent of hydrolysis was in the order

Con II > total protein > Con I > arachin > casein

In this study the enzyme to substrate ratios used varied from 1:40 to 1:200 by weight. Lynch *et al.* (1977a,b) used an enzyme to protein ratio of 1:100, Romero and Ryan (1978) a ratio of 1:10, and Boonvisut and Whitakar (1976) a ratio of 1:50 for pepsin and trypsin. Starch and sugars present in the protein fractions would interfere with proteolysis (Sharon, 1984), so it was also decided to determine the enzymatic hydrolysis by using pure protein fractions. The various protein fractions of peanut have different molecular weights and subunit structures, which may affect their hydrolysis by proteolytic enzymes. Romero and Ryan (1978) reported a much lower *in vitro* hydrolysis of a large molecular weight protein isolated from bean compared to hydrolysis of bovine serum albumin, a lower molecular weight protein. The difference in hydrolysis in this case was ascribed to structural constraints rather than to the size of the proteins, although many factors may influence enzymatic hydrolysis of proteins (Kakade, 1974). Krishna and Mitra (1987) have also observed that arachin monomer can undergo limited proteolysis during storage.

The hydrolysis of peanut protein fractions by α-chymotrypsin, along with a standard protein hemoglobin, and the effect of aflatoxin B_1 on the action of α-chymotrypsin are shown in Figure 5D. Considerabnle inhibition of α-chymotrypsin action was observed at an aflatoxin B_1 concentration of 1×10^{-5} M. The inhibition decreased with the increase in digestion time. The addition of aflatoxin B_1 to hemoglobin also affected its hydrolysis. The addition of aflatoxin B_1 at low concentration (1×10^{-5} M) to arachin decreased the reaction rate constant from 0.3453 to 0.1099 min^{-1}, which amounts to 68%.

In general, the lower proteolysis of seed proteins by the proteases could be due to the presence of ligand, the presence of protease inhibitors (peptides from hydrolysis), the presence of carbohydrate moieties in the protein molecule, or the specific structure or conformation of proteins (Tasneem and Prakash, 1989). Peanut proteins do not contain any protease inhibitors or intrinsic ligand, and their carbohydrate content is very low (0.57%); the conformation of the protein or the peptides released from hydrolysis or the added extrinsic ligand aflatoxin B_1 may be vital in offering resistance to chymotryptic attack.

The above studies strongly point to the fact that the different fractions of peanut protein undergo hydrolysis to different extents and at different rates with pepsin, trypsin, and α-chymotrypsin. The difference might also be arising due to the structural features of the individual fractions of peanut protein. The hydrolysis disrupts such ordered structures, resulting in smaller peptides, and might have feedback inhibition on the system. These results confirm that the presence of an external ligand such as aflatoxin B_1 with peanut protein fractions inhibits the action of α-chymotrypsin.

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