The suggested method makes it possible to effectively isolate homogeneous preparations of 7S and 11S globulins from broad beans and pea seeds. It is characterized by high yield and makes these proteins available for extensive research on concentrated protein systems.

LITERATURE CITED

Casey, R.; Domoney, C. The biochemical genetics of pea seed proteins. *Kulturphlanze* **1984**, *32*, 99–108.

Claesson, S.; Moring-Claesson, I. Ultracentrifugation. In *A Laboratory Manual of Analytical Methods of Protein Chemistry*; Alexander, P., Block, R. J., Eds.; Pergamon Press: London, 1961.

Danielsson, C. E. Seed globulins of gramineae and leguminosae. *Biochem. J.* **1949**, *44*, 387–400.

Derbyshire, E.; Wright, D. J.; Boulter, D. Legumin and vicilin, storage proteins of legume seeds. *Phytochemistry* **1976**, *15*, 3–24.

Gwiazda, S.; Schwenke, K. D.; Rutkowski, A. Isolation and partial characterization of protein from pea. *Nahrung* **1980**, *24*, 939–950.

Mori, T.; Utsumi, S. Purification and properties of storage proteins of broad bean. *Agric. Biol. Chem.* **1979**, *43*, 577–583.

Osborne, T. B.; Campbell, G. F. Proteids of the pea. *J. Am. Chem. Soc.* **1898a**, *20*, 348–362.

Osborne, T. B.; Campbell, G. F. Proteids of the horse bean (*Vicia faba* L.). *J. Am. Chem. Soc.* **1898b**, *20*, 393–405.

Osborne, T. B.; Campbell, G. F. The proteids of the pea, lentil, horse bean and vetch. *J. Am. Chem. Soc.* **1898c**, *20*, 410–428.

Perrin, C. H. Rapid modified procedure for determination of Kjeldahl nitrogen. *Anal. Chem.* **1953**, *25* (5), 968–971.

Reisfeld, B. A.; Lewis, U. J.; Williams, D. E. Disk electrophoresis on basic proteins and peptides on polyacrylamide gels. *Nature* **1962**, *195*, 281–283.

Schlesier, B. A gel electrophoretic study on the polypeptide structure of vicilin. *Kulturphlanze* **1984**, *32*, 223–225.

Scholz, G.; Richter, J.; Manteuffel, R. Studies on seed globulins from legumes. 1. Separation. *Biochem. Physiol. Pflanz.* **1974**, *166*, 163–171.

Varfolomeeva, E. P.; Danilenko, A. N.; Bikbov, T. M.; Grinberg, V. Ya.; Tolstogusov, V. B. The reological properties of diluted solutions of 11S globulin isolated from soybeans by using selective thermal denaturation of 2S and 7S globulins. *Nahrung* **1985**, *28*, 503–515.

Wright, D. J.; Boulter, D. Purification and subunit structure of legumin of *Vicia faba* L. (Broad bean). *Biochem. J.* **1974**, *141*, 413–418.

Received for review June 13, 1988. Revised manuscript received April 17, 1989. Accepted June 30, 1989.

Characterization of Pure Proanthocyanidins Isolated from the Hulls of Faba Beans

Laila Hussein,*,† Mohy Abdel Fattah,‡ and Ezzildin Salem‡

Department of Nutrition, National Research Center, Dokki, Cairo, Egypt, and Department of Chemistry, Faculty of Science, Suez Canal University, Ismaileya, Egypt

Extraction of the hulls of faba beans with 70% aqueous acetone containing 2.8 mmol/L ascorbic acid proved to be a very efficient solvent system for the extraction of phenols. Treatment of the concentrated extract with ethyl acetate and liquefied phenol and batch adsorption on Sephadex LH-20 freed the isolated crude proanthocyanidins (PA) from small molecular weight UV-absorbing and protein contaminants. Gel filtration of the purified extract on Sephadex LH-20 yielded pure PA and showed homogeneous band during the elution at 275.2 nm (maximum). The overall yield was 12%; the preparation contained six flavan-3-ol units/molecules.

The hulls of faba beans are rich in proanthocyanidins (condensed tannins) (Martin-Tanguy et al., 1977; Cansfield et al., 1980; Griffiths and Moseley, 1980; Hewitt and Ford, 1982; Cabrera and Martin, 1986), yielding anthocyanidins after hydrolysis with strong mineral acid (Freudenberg and Weinges, 1958). These phenolic compounds, PA, were found to inhibit many enzymes in vitro, including digestible enzymes such as trypsin and α-amylase (Griffiths and Moseley, 1980).

In humans, the consumption of diets based on faba beans rich in PA was found to reduce the digestibility of protein (Hussein and Abbas, 1985a) and sulfur-containing amino acids (Hussein and Mottawei, 1985b).

Only few reports dealt, however, with the extraction, purification, isolation, and characterization of the PA.

The present work deals with the purification and characterization of the PA isolated from the hulls of faba beans.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were reagent grade. The origins to all the listed chemicals were Pharmacia (Uppsala, Sweden) and E. Merck (Darmstadt, West Germany).

The seeds Giza 3 were from the Department of Legumes of the Egyptian Ministry of Agriculture.

Extractions. The hulls (seed coats) were removed from the beans with a pair of scissors, ground in an electric mill, and extracted in one of the following solvents to which was added 2.8 mmol/L ascorbic acid as an antioxidant: (1) water; (2) 10% acetic acid; (3) 1% HCl in MeOH; (4) 10% tartaric acid in methanol; (5) 70% aqueous acetone, at a ratio of 1:20 (w/v). After filtration, the residue was reblended four more times with 150 mL of the same solvent system, and the combined filtrates were

† National Research Center.
‡ Suez Canal University.

0021-8561/90/1438-0095$02.50/0

concentrated in vaccuo and analyzed for total phenol content. *Caution*: Extractions with 75% acetone in a high-speed blender can be inflammable.

Isolation of PA. The procedure followed those described by Hagerman and Butler (1980). Beans were extracted with 70% aqueous acetone. The concentrated aqueous solution was extracted twice with ethyl acetate to remove small molecular weight contaminants. The water layer containing the crude PA was mixed with Sephadex LH-20 slurry (2 g/10 mL of EtOH) for 30 min, followed by successive washing with EtOH to remove UV-absorbing contaminants. The PA was then eluted by successive washings with 50% aqueous acetone. After removal of acetone in vacuo, the aqueous concentrate was extracted three times with liquefied phenol to dissociate the protein–PA complex, followed by extraction with diethyl ether to remove residual phenol.

The aqueous phase containing the partially purified PA was evaporated and redissolved in ethanol (120 mg/mL). A 5-mL aliquot was applied to the top of a Sephadex LH-20 column (2.5 × 36 cm) packed and equilibrated with ethanol. When a stable base line was reached, the mobile phase was changed to 50% aqueous acetone at a flow rate of 72 mL/h. Five-milliliter fractions were collected, and the eluates were monitored at 400 and 540 nm. Each fraction was lyophilized, weighed, and kept in an air-tight container in the dark in a cold place for subsequent analysis.

Preparation of Derivatives of PA. *Acetylation.* The reaction was carried out at −30 °C for 48 h by mixing 1 mg of PA with 6 mL of pyridine–acetic anhydride (5:1, v/v). The reaction was terminated by dilution with excess water, followed by lyophilization, subsequent washing, and freeze-drying (Thompson et al., 1972).

Methylation. The PA (1 mg) was mixed with freshly prepared cold diazomethane in ethyl ether under standard experimental conditions (Furns et al., 1978). The reaction was allowed to proceed for 2 h at 0 °C and for a further 38 h at −30 °C, followed by subsequent washing and lyophilization.

Analytical Methods. Total phenols were determined by the Folin–Denis method using tannic acid as a standard (AOAC, 1984).

Protein-precipitable phenols were measured by the method of Hagerman and Butler (1978). The proanthocyanidins were analyzed by colorimetry after hydrolysis to anthocyanidins (anthocyanidin formation assay) in BuOH–HCl (Gupta and Haslam, 1979).

The absorption spectrum of the formed anthocyanidins was scanned between 400 and 600 nm.

The tannin specific activity of PA was determined by dividing the protein-precipitating capacity to the total content of phenolic groups in the sample (Butler, 1982). In the present work, the total phenolic groups were assayed by the Folin–Denis method according to the AOAC method (1984).

The degree of polymerization was estimated as described by Butler et al. (1982). The vanillin reaction was performed in glacial acetic acid, and the degree of polymerization was determined by dividing the $E\%$ 510 nm of catechin by the $E\%$ 510 of PA.

Spectrophotometry. Absorption spectra of pure untreated, acylated, and methylated PA were obtained. Solutions containing 1 mg/100 mL of methanol were scanned on a Shimadzu UV 240 scanning spectrophotometer. The spectra were scanned between 260 and 400 nm against methanol reference. PA solutions treated with sodium methoxide, aluminum chloride, and boric acid (Mabry et al., 1970) were analyzed similarly.

Paper and Thin-Layer Chromatography. The chromatographically pure PA fractions were separated on precoated silica gel plates (0.25-mm thickness) with fluorescent indicator and the solvent systems butanol–acetic acid–water (4:1:5) (BAW) for the first dimension and butanol–ethyl methyl ketone–acetic acid–water (2:5:1:2) (BEAW) for the second (Cansfield et al., 1980).

The anthocyanidin pigments were separated on Whatman 3MM filter paper with use of toluene–acetone–formic acid (6:6:1) (Lea, 1978). The mixtures of acetylated PA were separated on a silica gel plate and developed with toluene–acetone (8:2). For methylated PA the same plates were used with chloroform–methanol (99.5:0.5) (Thompson et al., 1972). The TLC spots were observed in UV light or sprayed with ferric chloride and potassium ferricyanide reagent (Kirby and White, 1954).

The protein content of the lyophilized products was measured by micro-Kjeldahl analysis (Markham, 1942).

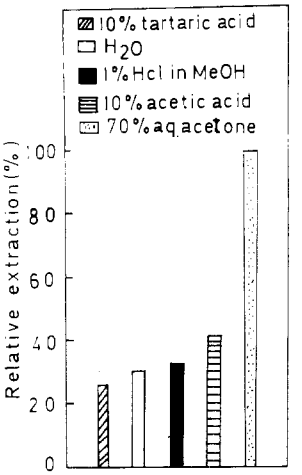

Figure 1. Efficiency of five solvents in extracting total phenols from faba beans.

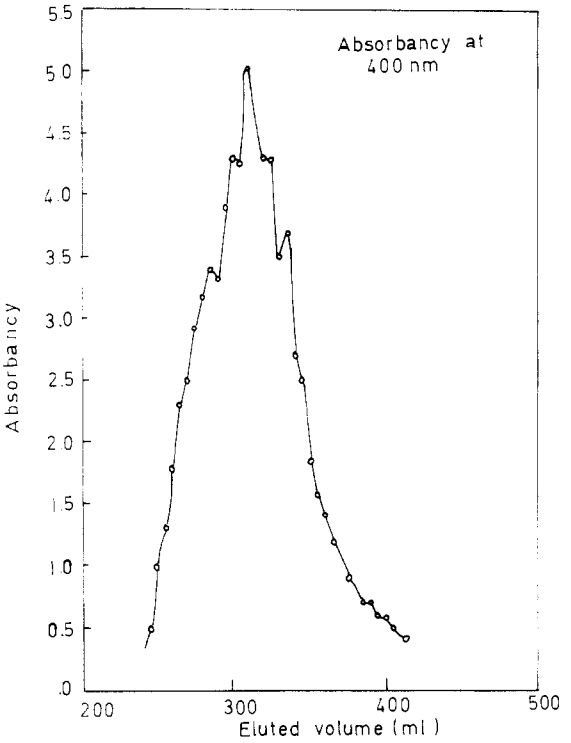

Figure 2. Elution profile from Sephadex LH-20 column.

RESULTS AND DISCUSSION

Extractions. The concentration of total phenols was highest when the hulls were extracted five times with 70% aqueous acetone in the presence of 2.8 mmol/L ascorbic acid at a hull to solvent ratio of 1:30 (w/v) compared to respective values obtained with other solvents (Figure 1).

Figure 2 illustrates the elution pattern of PA from a Sephadex LH-20 column, which was eluted as one homogeneous peak at an elution volume of 320 mL. The tannin specific activity of PA collected between 300 and 330 mL was unity.

Table I presents typical purification steps and percent recovery of PA. Percentage recoveries of 56, 45, 19, and 12% PA relative to the original total phenol content

Table I. Purification of Tannin (PA) from the Hulls (100 g) of Faba Beans

purification step	protein-precipitating phenols g/100 g	protein-precipitating phenols %	protein, g/100 g
crude extract	6.24	100.0	7.38
acetate extraction	3.50	56.1	1.05
Sephadex LH-20 batch absorption	2.82	45.2	0.22
liquefied phenol extraction	1.17	18.8	ND[a]
gel filtration on Sephadex LH-20 column	0.75	12.0	0.03

[a] ND = not determined.

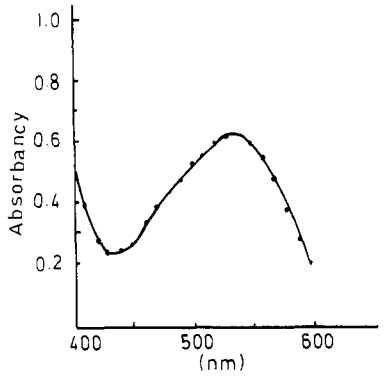

Figure 3. Spectra of anthocyanidins formed by acid hydrolysis of PA in 1-butanol.

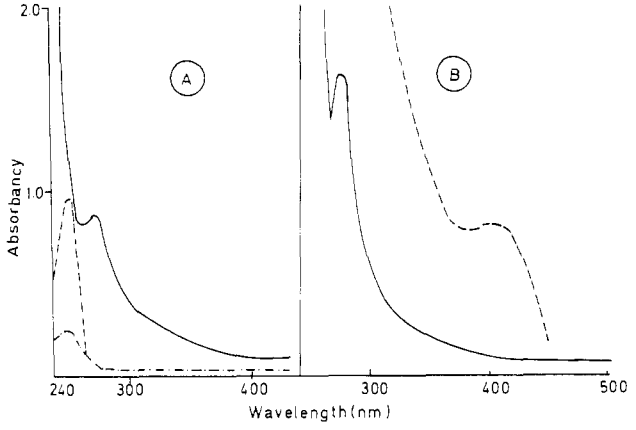

Figure 4. UV spectra: (a) PA, methyl-PA, and acetyl-PA; (b) PA, NaOMe-treated PA.

of the hulls were obtained after the extraction with ethyl acetate, batch adsorption using Sephadex LH-20, extraction with liquefied phenol, and chromatographic separation, respectively.

The protein content changed from 7.38% in the crude PA solution to 0.03% after extraction with liquefied phenol.

The maximum of absorption of the anthocyanidin production assay was 545 nm for PA eluted between 300 and 330 mL (Figure 3). In the less purified fractions obtained at the right-hand side of the curve (elution volumes more than 350 mL), absorption maxima in BuOH–HCl reaction varied between 540 and 550 nm. This is due to the relative amounts of anthocyanidins (delphenidin to cyanidin ratio) formed during the acid hydrolysis.

Ultraviolet Spectrum. The methanol spectra of the chromatographically pure lyophilized PA exhibited one major band at 275.2 nm and a second negligible band at 375.2 nm. The UV spectra of the methylated freeze-dried proanthocyanidins showed a maximum at 254 nm with a hypochromic shift of 21 nm. The acetylated compound behaved similarly (Figure 4A).

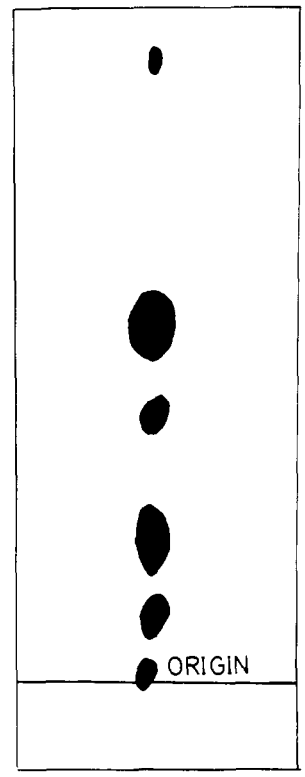

Figure 5. PC chromatogram of anthocyanidins formed by acid hydrolysis of PA. Conditions: Whatman No. 3 paper; solvent system, toluene–acetone–formic acid (6:6:1); visualized with ferric chloride–potassium ferricyanide.

The addition of sodium methylate in methanol produced a bathochromic shift of 13 nm (275–288 nm) (Figure 4B) and an increase in the UV absorbancy. The addition of $AlCl_3$ in the absence and in the presence of HCl to methanolic PA did not change the UV spectrum.

Treating the PA with boric acid led to minor shift of 2 nm. Furthermore, the intensity of the absorption was reduced by 51.3% of the corresponding absorbancy obtained with methanol spectrum.

Estimation of the degree of polymerization gave a value of six flavan-3-ol units per oligomeric tannin molecule. Paper chromatographic separation of the cyanidins revealed the presence of six distinct spots, presenting further evidence for the presence of monomeric and oligomeric flavan-ols up to six units.

The results also reveal the absence of flavan-4-ol from the purified PA. Pure faba bean PA gave six distinct spots after separation on TLC, when the solvent system of Lea (1978) was used (toluene–acetone–formic acid, 60:60:10). Similarly, paper chromatography of the anthocyanidin pigments gave six distinct spots; the slowest did not move from the origin (Figure 5).

The acetylation of PA was almost instant, since 1 h after starting the reaction, the acetylated PA derivative migrated fast with R_f 0.9 in toluene–acetone (8:2). However, a residual UV spot remained at the start, suggesting that the acetylation was not 100%.

The methylated derivative migrated similarly fast with R_f 0.79 in chloroform–methanol (99.5:0.5), and no residual UV spots remained at the start, indicating 100% methylation.

Cold distilled water (Cansfield et al., 1980) and methanol containing ascorbic acid (10 mmol/L) (Hagerman and Butler, 1980) were used as solvent systems in the

extraction of PA from faba and common beans, respectively. In the present work, 70% aqueous acetone containing 2.8 mmol/L ascorbic acid was found to be superior to the other two solvent systems. In this regard, it had been reported that acetone-based solvents give higher yields with highly polymerized flavanoid from fruits and cereal grains (Foo and Porter, 1981; Brandan and Foo, 1982).

Treatment of the crude extract with liquefied phenol successfully ruptured the PA–protein complex, resulting in PA preparation with negligible protein content (0.03%), which is much lower than the figure of 2% reported for sorghum tannin subjected to the same treatments (Hagerman and Butler, 1980). The overall yield of purified PA obtained in the present work is 12%, which is higher than the figure of 7% reported for common bean tannins extracted with methanol and purified by similar methods (Hagerman and Butler, 1980). Other purification techniques of PA extracted from the hulls of faba beans gave two fractions after Sephadex LH-20 with four main components (Cansfield et al., 1980). In the present work six flavan-3-ol units were found in the chain length of oligomeric material per molecule of pure faba bean PA. Similar values of six to seven flavan-3-ol units per molecule had been reported for the chain length in the PA purified from sorghum (Gupta and Haslam, 1979), and values of 4.8 to 5.1 were also reported for other sorghum varieties (Asquith et al., 1983).

The ultraviolet absorption maximum (275.2 nm) for methanolic faba beans PA corresponds to band II of the benzoyl ring of flavanone. Isoflavones had their major band II between 245 and 270 nm, whereby all flavones and flavanols possessed a prominent absorption maximum for band I close to 370 nm. Both methylation and acetylation of the faba bean tannins led to hypochromic shift of 21 nm (275–254 nm). This hypochromic shift after methylation of PA reflects the presence of a 3-, 5-, or 4′-hydroxyl group on the flavone nucleus (Mabry et al., 1970). The bathochromic shift (275–288 nm) observed in the UV spectrum of sodium methoxide treated faba bean PA indicates the presence of a free OH group on position 3 or 4′, since the strong basicity of sodium methoxide ionizes all the hydroxyl groups on the flavonoid nucleus (Mabry et al., 1970).

The material with R_f 0 that appeared on the chromatograms of the cyanidins (Figure 5) is believed to be phlobaphenes, previously described as a product of the side reaction occurring during the acid hydrolysis of PA (Gupta and Haslam, 1979).

ACKNOWLEDGMENT

This work was financially supported by a grant from the International Center for Agricultural Research in the Dry Areas (ICARDA) Nile Valley Project on faba beans, to whom we are grateful. We are grateful to Prof. Dr. Ali Abdou Ismail, Head, Laboratory of Microbial Genetics, for providing laboratory facilities for UV scanning, and we thank Prof. L. Butler, Department of Biochemistry, University of Purdue, for advice.

LITERATURE CITED

AOAC. *Official Methods of Analysis*, 14th ed.; AOAC: Washington, DC, 1984.

Asquith, T. N.; Izuno, C. C.; Butler, L. G. *J. Agric. Food Chem.* **1983**, *31*, 1299–1303.

Brandan, M. J.; Foo, L. Y. *Phytochemistry* **1982**, *21*, 2953–2957.

Butler, L. G. In *Proceedings, International Symposium on Sorghum Grain Quality*, Patencheru, India; International Crops Research Institute for the Semi-Arid Tropics: India, 1982.

Butler, L. G.; Price, M. L.; Brotherton, J. E. *J. Agric. Food. Chem.* **1982**, *30*, 1087–1089.

Cabrera, A.; Martin, A. *J. Agric. Sci.* **1986**, *106*, 377–382.

Cansfield, P. E.; Marquardt, R.; Campbell, L. *J. Sci. Food Agric.* **1980**, *31*, 802–812.

Foo, L. Y.; Porter, F. *J. Sci. Food Agric.* **1981**, *32*, 711–716.

Freudenberg, K.; Weinges, K. *Fortschr. Chem. Org. Naturst.* **1958**, *XVI*, 1.

Furns, B. S.; Hannoford, A. J.; Rogers, V.; Smith, P. W.; Tatehell, A. R. *Vogels Textbook of Practical Organic Chemistry*, 4th ed.; Longman: Birmingham, AL, 1978.

Griffiths, D. W.; Moseley, G. *J. Sci. Food Agric.* **1980**, *31*, 255–259.

Gupta, R. K.; Haslam, E. In *Polyphenols in Cereals and Legumes*; Hulse, J., Ed.; IDRC: Canada, 1979.

Hagerman, A. E.; Butler, L. G. *J. Agric. Food Chem.* **1978**, *26*, 809–812.

Hagerman, A. E.; Butler, L. G. *J. Agric. Food Chem.* **1980**, *28*, 947–952.

Hewitt, D.; Ford, J. E. *Proc. Nutr. Soc.* **1980**, *41*, 7–17.

Hussein, L.; Abbas, H. *Nutr. Rep. Int.* **1985a**, *31*, 67–81.

Hussein, L.; Mottawei, H. *Nutr. Rep. Int.* **1985b**, *32*, 1241–1247.

Kirby, K. S.; White, T. *J. Soc. Leather Trades' Chem.* **1954**, *38*, 215–222.

Lea, A. G. *J. Sci. Food Agric.* **1978**, *29*, 471–475.

Mabry, T. J.; Markham, K. R.; Thomas, M. B. In *The Systematic Identification of Flavonoids*; Springer Verlag: Berlin, 1970.

Martin-Tanguy, J.; Guillaume, J.; Kossa, A. *J. Sci. Food Agric.* **1977**, *28*, 757–765.

Markham, R. *Biochem. J.* **1942**, *36*, 511–512.

Thompson, R. S.; Jacques, D.; Haslam, E.; Tanner, R. J. *J. Chem. Soc., Perkin Trans. 1* **1972**, *3*, 1387–1399.

Received for review February 16, 1988. Revised manuscript received January 25, 1989. Accepted August 4, 1989.