Solubilization and Characterization of Fenugreek Seed Proteins

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Ninety percent of fenugreek proteins are located in cotyledons plus embryo(s) (C + E) that were separated from seed coat plus endosperm by a new method based on the differences of their density. Over 70% of C + E proteins are soluble in water, 5% in NaCl (0.86 M), and 7% in NaOH (0.05 M). A treatment of C + E with 2-propanol at 78 °C in order to remove saponins for steroid preparations diminishes sharply the solubility of proteins in water; the removal of saponins at room temperature has little subsequent effect. The initial pH of a solution has little effect on protein solubility, except extreme values, between pH 1–4 and over pH 11. The electrophoretic compositions of albumins, globulins, and glutelins are very heterogeneous and dependent on solubilization conditions. The reduction of proteins with mercaptoethanol modifies considerably the electrophoretic patterns (PAGE–SDS) of albumins and globulins. According to their amino acid composition, the nutritional value of fenugreek proteins is similar to that of other legume proteins.

Fenugreek (*Trigonella foenum graecum* L., Papilionaceae–Leguminosae) is an annual pulse plant. Its growing area includes the mediterranean countries, the middle East, and India, where it is traditionally used as a condiment or for food and in folk medicine as an anabolism.

Fenugreek contains a high level of saponins whose aglycons sapogenins can be used for the synthesis of steroids (Sauvaire and Baccou, 1978a,b). Its protein content is also very high, between 25 and 30%. The lysine content in fenugreek proteins is comparable to that in the soybean proteins. The nutritional value of proteins determined on rats has been satisfactory (Sauvaire et al., 1976). For all these reasons, fenugreek seems to be an interesting plant, especially in the countries of its natural growing area, which are dependent on protein import.

Compared to the other seeds, very few research works have been carried out on fenugreek proteins. Actually, some of these works concern only incidently fenugreek proteins (Dudman and Millerd, 1975; Ladizinsky, 1979; Yacoub and Aboul-Ela, 1979); the few others are neither exhaustive nor carried out in the conditions comparable to ours (Wunschendorff, 1919; Rafik El-Mahdy and El-Sebaiy, 1982).

Considering the potential use of fenugreek as a source of both steroids and proteins, besides the fractional solubilization of proteins and their electrophoretic characterization, the influence of various parameters (saponins, temperature, pH) have been investigated. A special emphasis has been made on the influence of the heat treatment on the proteins since in industrial conditions the removal of saponins could be more interesting at higher temperatures (Paris et al., 1977).

MATERIALS AND METHODS

Two cultivars of fenugreek (*T. foenum graecum* L.), Cocipa and Gouka (grown at the Experimental Station of the University of Montpellier, harvested in 1976 and 1979, respectively), were used for all the analyses; the percent of germination was identical for the two cultivars, from 98 to 100.

Preparation of Samples. Mature seeds had been ground in two stages. At the first stage an experimental Miag mill (MLI-204) was used at the position no. 4. At this position, seeds are only coarsely ground. Thus the coarse particles obtained were put into a solution containing trifluorotrichloroethane (Flugène 113, Rhône Poulenc) and hexane, 77:23 v/v. In this solution, the different histological parts of the seed can be separated according to their density. In the case of fenugreek, seed coat or hull and endosperm are separated from cotyledons and embryo (C + E), which, being of lower density, are concentrated in the upper part of the solution. Seed coats and endosperm are at the bottom and are separated from C + E by a liquid interphase containing only fine particles. The separated seed parts are dried at room temperature and then ground into a fine powder by using a bowl mill; the particles passed through a sieve of mesh size 70. In our study we have investigated only the mixture of cotyledons and embryo(s) since they contain the major part of proteins.

All samples were defatted with hexane by using a Soxhlet apparatus. The extraction time was 12 h at the temperature of 42 °C.

From part of the samples, steroidal saponins were also extracted: they were solubilized either with 2-propanol–water, 70:30 v/v, at 78 °C by using a Soxhlet apparatus or with 2-propanol–water, 87:13 v/v, at 20 °C. In the latter case, six successive extractions were performed; each time samples were vigorously stirred by a special laboratory-made shaker and then filtered. Samples were dried at room temperature. At the moisture content of 9%, they were sieved again with a sieve of mesh size 70.

Defatted samples were labeled as C + E flour + S, Cocipa, or Gouka and C + E flour – S, Cocipa, or Gouka when saponins were removed from them.

Protein Extraction. The protocol is outlined in Figure 1. Four different solvents, distilled water, 5% NaCl (0.86 M), 70% ethanol, and 0.05 NaOH, were used in sequence in the solvent to meal ratio of 20:1 mL/g.

Samples in polypropylene centrifuge tubes were stirred for 20 min at 20 °C with an Ultra-Thurrax apparatus at the position 23/100 or shaken vigorously twice for 10 min with a special laboratory-made apparatus. The extracts were centrifuged at 25000*g* for 20 min and the supernatants filtered (Whatman No. 1). For each solvent, three successive extractions were carried out.

For further characterization of the proteins by electrophoresis supernatants were dialyzed against distilled water (ratio 1:30 v/v at 4 °C for 48 h; distilled water was renewed every 12 h) and freeze-dried.

Protein Determination. Both the micro-Kjeldhal ($N \times 6.25$) and the Lowry method (Lowry et al., 1951) (bovine serum albumin as a protein standard) were used. In the case of the Lowry method, proteins were previously precipitated from each supernatant by using trichloracetic

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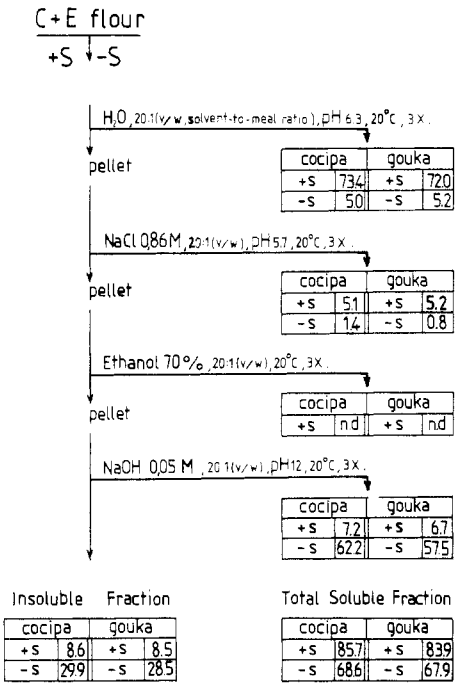

Figure 1. Protocol used to fractionate fenugreek proteins (cultivars Cocipa and Gouka) by their solubility and the percentage of total proteins ($N \times 6.25$) in each solvent (mean values obtained for three separate samples). C + E flour + S: defatted cotyledons and embryo(s) containing saponins. C + E flour − S: defatted C + E and saponins removed in the Soxhlet apparatus with 2-propanol–water, 70:30 v/v, at 78 °C, 9 h. 3× indicates that the given extraction was repeated 3 times on the pellet. The percentage of total protein content was determined in the combined supernatants. The pH of each supernatant was determined. n.d. = not detectable.

acid, final concentration 10%, 12 h of contact at 4 °C. After centrifugation, protein pellets were redissolved in 1 N NaOH. All the determinations were duplicated or triplicated. The mean coefficient of variation of analysis for nitrogen by the micro-Kjeldahl method is equal to 3.4% (n = 29).

Amino Acid Analysis. Freeze-dried proteins (30 mg/sample) are hydrolyzed under nitrogen with 10 mL of 6 N HCl at 110 °C for 24 h. The amounts of amino acids determined by using a Durrum D 500 automatic apparatus (laboratory of G.E.R.D.A.T., Montpellier). Cysteine–cystine contents were estimated after performic acid oxydation. Tryptophan has not been determined.

Electrophoretic Characterization of Proteins (PAGE-SDS). Polyacrylamide vertical gel electrophoresis was performed under the conditions described by Payne and Corfield (1979). Freeze-dried protein samples were dissolved in 0.05 M Tris-HCl, pH 6.8, in the ratio of 1 mg:0.1 mL; the buffer contained 2% of SDS and 10% glycerol. The reduction of proteins was carried out just before electrophoresis; for it 5% of mercaptoethanol was added to the protein solutions and the samples were heated at 97 °C for 3 min. A total of 0.050 mL of protein solutions was applied to the gels of 200 × 200 × 3 mm. The running gels of 17.5% of acrylamide were overlaid with 3% stacking gels. Running buffer was 0.025 M Tris-HCl–0.19 M glycine, pH 8.3, with 0.1% SDS. Pyronine was used as a tracer; the electrophoretic runs were stopped when the tracer entered into the buffer solution in the lower buffer container. When 12 mA at the tension of 50–60 V was applied, the migrations lasted about 16 h.

Proteins were stained according to Caldwell and Kasarda (1978). Gels were put for 16 h into a solution of trichloroacetic acid, 12%, containing 0.02% of Coomassie Brilliant Blue R 250 that should have been previously dissolved in ethanol. Gels were distained in distilled water.

Table I. Protein Content in the Different Parts of the Fenugreek Seed ($N \times 6.25$, % Dry Weight)

cultivars	whole seed	seed coat + endosperm	cotyledons + embryo(s) (C + E) defatted (+S)	cotyledons + embryo(s) (C + E) defatted and saponins removed (−S)
Cocipa	30.8	6.6	60.1	76.3
Gouka	28.2	6.2	56.2	72.0

In order to evaluate the molecular weights (M_r) of fenugreek proteins, the following standard proteins were used: phosphorylase b (M_r 94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400).

Effect of pH on the Solubility of Proteins and Saponins of Fenugreek. A total of 0.5 g of C + E flour + S (cv. Gouka) was put in contact with 10 mL of solution whose pH value had been adjusted from 1 to 12 with HCl or NaOH. After 10 min of vigorous shaking and a centrifugation of 20 min at 25000g, the supernatants were analyzed for their nitrogen content by the micro-Kjeldahl method and for the saponin content (see below), and their final pH values were also determined. Statistical analyses were performed at a 5% level of probability.

Determination of Steroid Saponins. The saponin content of the different samples was determined according to the method of Baccou et al. (1977). In the case of the protein fraction, previous to the spectrophotometric analyses, saponins were solubilized with 2-propanol. The mean coefficient of variation of analysis equals 4.1% (n = 28).

RESULTS AND DISCUSSION

Nitrogen Content of Different Seed Parts. Results are given in the Table I. As it can be seen, most of the proteins are concentrated in the cotyledons plus embryo(s) (C + E) fraction. It should be emphasize that in the case of fenugreek, C + E represents approximatively 50% of the seed weight. The fraction endosperm plus seed coat, that represents also about 50% of the seed weight, is very poor in protein (6–7%) and it contains a high level of the galactomannan type of gum (36%), which has a disturbing effect on protein solubilization.

For these reasons, as we have already mentioned above, the fraction endosperm plus seed coat was discarded and all our further studies have been carried out only on the seed fractions containing C + E.

Protein Solubilization. The classification of seed proteins according to their solubility was developed by Osborne (1924), distinguishing four different fractions: albumins (water soluble), globulins (soluble in salt solutions), prolamins (alcohol soluble), and glutelins (partially soluble in dilute NaOH).

While this classification is very suitable in the case of cereals, legume seed proteins do not have well-defined solubility fractions. However, we have adopted this solubilization procedure for this preliminary study on fenugreek since it is a widely used one. The results of sequential extractions with the four different solvents are presented on Figure 1 and given as percent of the total protein content.

Most of the proteins in fenugreek are soluble in water; for the two cultivars, Cocipa and Gouka, the albumin content of C + E flour + S is 73.4% and 72.0%, respec-

tively. In both cases, the salt-soluble fraction is between 5% and 6%. No measurable amount of ethanol-soluble protein could be obtained. The amounts of the NaOH-soluble fraction were also very similar for the two cultivars: 7.2% and 6.7%, respectively.

In legumes, storage proteins are of the globulin type (Millerd, 1975). In fenugreek, considering the high level of albumins, we can suppose that these proteins can have the role of storage proteins too. Similar suggestions have been made by Youle and Huang (1978) in the case of castor bean. It is also possible that part of the globulins was solubilized with distilled water (Brohult and Sandegren, 1954), hence the low amount of globulins found. This solubilization of globulins together with the albumins can be explained by the naturally present salts in the flour that are solubilized in the water, as well as by the effect of pH (Cerletti et al., 1978). However, as we will see later, the electrophoretic composition of albumins and globulins is very different.

It is interesting to note the very important variation of the data in the literature concerning the amount of albumins for the same legume species. Thus, the reported values for the albumin content of soybean vary from 10% (Boulter, 1977) to 69–76% (Sosulski, 1977; Hu and Esen, 1981) and from 21% (Boulter, 1977) to 80–85% (Reichert and Mac-Kensie, 1982) for the albumins of pea.

It is unlikely that such a large range exists in the albumin content of legumes. The discrepancies in the published data are more likely due to methods of extraction of albumins and their subsequent separation from globulins (Bhatty, 1982).

According to the most of the authors, legume seeds do not contain prolamins or only a very low percentage (Boulter, 1977; Sosulski, 1977). Our results on fenugreek confirm these observations. Though with regards to the prolamins our results do not agree with those of Rafik El-Mahdy and El-Sebaiy (1982), who found 6.3% prolamins in fenugreek. However, in general, their results are not comparable to ours since they used the whole seed in their experiments.

The amount of NaOH-soluble proteins in fenugreek is similar to that found in soybean (Boulter, 1977; Sosulski, 1977; Hu and Esen, 1981) and in pea (Boulter, 1977; Reichert and Mac-Kenzie, 1982) and it is lower than that in beans (Marquez and Lajolo, 1981).

Influence of the Removal of Saponins on the Extractability of Proteins. The influence of the removal of saponins on the subsequent protein extractability has also been investigated. As it can be seen in Figure 1, the distribution of the protein fractions is quite different when saponins are previously extracted. Only a little bit more than 5% of albumin and 5% globulin could be obtained. NaOH-soluble proteins became the most important fraction (62.2% and 57.5%). Moreover, a high amount of protein still remained even after the treatment with NaOH in the residue (29.9% for Cocipa –S) and 28.5% for Gouka –S). Therefore, it seemed to be interesting to investigate the reasons why proteins, especially albumins, became inextractable. For this we had to elucidate the influence of the extraction conditions of saponins on protein solubility and to determine whether the presence of saponins by itself would have a favorable effect on the solubility of fenugreek proteins. This latter hypothesis is supported by the results concerning the solubility of wheat proteins in the presence of detergents (Kobrehel and Bushuk, 1978; Kobrehel, 1980).

Saponins were removed with 2-propanol–water at 78 °C as described under Materials and Methods. The results of protein solubility reported in Figure 1 for Cocipa –S and Gouka –S were obtained following that treatment of the samples.

Table II. Influence of the Heat Treatment with 2-Propanol and the Presence of Saponins on the Solubility of Fenugreek Proteins in Water (Percentage of Solubilized Proteins of the Total Protein Content)

Cocipa (C + E flour)			Gouka (C + E flour)		
	–S			–S	
+S heated[a]	non-heated[b]	–S heated[c]	+S heated[a]	non-heated[b]	–S heated[c]
18.0	61.7	5.0	16.0	57.8	5.2

[a] C + E flour is put in contact with 2-propanol-water, 87:13 v/v, at 78 °C for 9 h. These proportions were chosen in order to obtain the azeotropic compositions of 2-propanol-water (vapor phase) when the extraction is carried out in a Soxhlet apparatus using the proportion of 70:30 at 78 °C as for –S heated. After evaporation of the solvent under vacuum at room temperature, the solubilization of proteins followed with distilled water, the solvent to meal ratio was 20:1 v/w, and extractions were repeated 3 times. [b] Instead of using a Soxhlet apparatus, saponins were extracted with 2-propanol, 87%, at room temperature, performing six successive extractions (no more saponin left in the C + E flour), and then proteins were solubilized with water in the conditions as above. [c] Saponins are removed with 2-propanol-water, 70:30 v/v, at 78 °C for 9 h with a Soxhlet apparatus.

In further experiments saponins were removed at 20 °C (see Materials and Methods) prior to the solubilization of albumins. Simultaneously, samples were heated at 78 °C for 9 h at the presence of 2-propanol without removing the saponins from them. This treatment was also followed by the extraction of albumins. Results of these experiments are presented in Table II.

The use of lower temperature for the extraction of saponins has subsequently increased considerably the solubility of albumins; however, it still remained lower than in the presence of saponins. The comparison of samples, which have been submitted to a heat treatment, confirmed that the sharp decrease of albumin solubility is due to the heat treatment. It has also shown that, even after a heat treatment, the solubility of albumins is somewhat higher in the presence of saponins.

All these results (Figure 1 and Table II) seem to indicate that the decrease of albumins' solubility after the removal of saponins is mainly due to the heat treatment. The importance of heat treatment on legume protein solubility is reported by different authors (Sosulski, 1977; Clatterbuck et al., 1980). Our results suggest also some interactions between proteins and saponins. These points will be developed later in the discussion on the electrophoretic patterns of the samples. It can be mentioned that according to Sosulski (1977), 2-propanol, the solvent used for the extraction of saponins, has an insolubilizing effect on proteins. The interactions between proteins and detergents have been shown in the case of wheat proteins (Kobrehel, 1980). It can also be noted here that according to our determinations, the saponin extract contains from 6% to 7% of nitrogen of the total nitrogen content of C + E flour, but the whole amount of nitrogen extracted with saponins is nonproteic nitrogen; they are not precipitated with trichloroacetic acid.

Influence of the pH on the Solubility of Proteins and Saponins. Figure 2 shows the variation of protein solubility according to the pH values. The shape of the curve suggests a rather important heterogeneity of fenugreek protein composition.

Table III. Amino Acid Composition of Fenugreek Seed Protein Fractions, cv. Gouka[a]

amino acids	whole meal C + E	C + E flour + S					C + E flour − S (78 °C, 9 h)				C + E flour − S (20 °C),
		H_2O soluble	H_2O[b] soluble	NaCl soluble	NaOH soluble	pellet	H_2O soluble	NaCl soluble	NaOH soluble	pellet	H_2O soluble
Asp	11.9	11.9	14.5	11.1	10.0	9.6	11.1	11.5	12.7	10.1	13.2
Thr	4.2	3.8	3.2	4.8	4.7	6.1	4.0	4.2	3.8	6.2	3.5
Ser	6.8	6.2	7.0	7.3	6.5	7.2	6.1	6.9	7.0	6.1	7.0
Glu	16.6	15.5	22.3	13.8	15.7	11.0	21.5	18.4	17.8	10.1	19.2
Pro	5.6	5.6	4.1	6.3	6.1	7.3	5.6	4.0	4.7	5.7	4.5
Gly	8.8	6.9	3.8	8.8	9.4	12.4	8.9	10.0	5.4	9.5	6.8
Ala	5.8	8.0	3.1	6.7	7.5	8.5	6.9	6.0	5.3	9.5	5.1
Val	4.4	5.3	1.0	5.3	5.2	5.5	2.4	3.3	5.7	6.8	3.6
Cys	0.8	1.9	2.5	0.9	0.3	0.5	1.2	1.4	0.6	0.5	1.3
Met	1.0	1.2	1.1	0.9	0.9	1.4	0.7	0.8	0.7	1.4	0.5
Ile	4.9	5.3	4.6	4.5	4.7	4.5	4.1	4.5	5.4	5.2	5.3
Leu	7.5	8.3	5.6	7.3	7.4	7.7	5.3	5.8	7.7	8.7	7.1
Tyr	2.2	2.3	5.1	2.8	2.7	4.2	1.9	2.4	2.6	3.5	2.5
Phe	3.5	3.9	6.1	4.9	4.2	4.5	2.9	3.3	4.3	4.8	4.5
His	2.2	2.4	2.0	2.5	2.6	2.7	2.3	2.6	2.2	1.9	1.5
Lys	6.1	5.7	5.7	4.9	3.9	4.2	6.7	6.6	5.3	5.2	5.5
Arg	7.7	5.8	8.3	7.2	8.2	2.7	8.4	8.3	8.8	4.8	8.9

[a] Results are expressed as molar percent of the protein recovered. [b] Proteins solubilized with water at room temperature after heat treatment of the C + E flour + S (see details in the text and Table II).

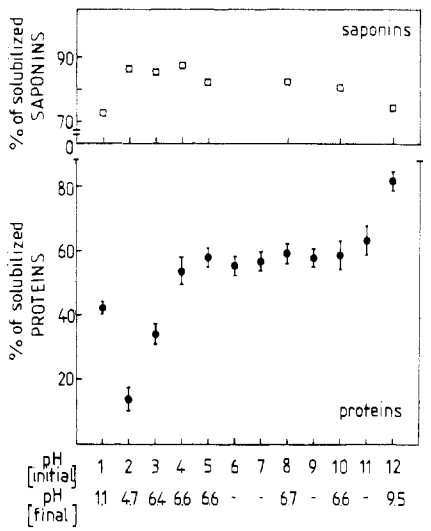

Figure 2. Effect of pH on the solubility of proteins and saponins of fenugreek.

The lowest solubility has been found at pH 2; the isoelectric point of most of the proteins should therefore be very low. However, it should be noted that the final pH value, after the solubilization of the proteins, is 4.7 instead of 2. Between pH 4.5 and pH 10 (initial pH values) the protein solubility is practically constant and relatively high, close to 55%. At very high pH values, the solubility increases sharply as the consequence of protein denaturation. It should be emphasized that the solubility curve of fenugreek proteins according to the pH is quite different in comparison to the solubility curves of other legumes (Sosulski, 1977; Sathe and Salunkhe, 1981).

As it is shown on Figure 2, most of the saponins (over 80%) are solubilized independently of the pH value. A slight decrease of saponins' solubility can be noticed at pH 1 and pH 12 (initial values) where 73% and 75% of the total saponins were solubilized, respectively. It can be mentioned that in the case of sequential extractions (Figure 1), all the saponins are removed with the albumins.

Amino Acid Analyses. For the amino acid compositions only the different protein fractions of the C + E flour + S and the C + E flour − S of the cultivar Gouka were analyzed. Results are presented in Table III.

The main amino acids for all the fractions are glutamic acid, aspartic acid, and arginine. Especially the concentration of glutamic acid and arginine is much higher in the soluble fraction than in the pellet. The level of leucine is also higher in the protein fractions than the level of other amino acids.

When comparing protein fractions, it is interesting to note the higher amount of cysteine–cystine in the water-soluble fractions and it is independent of the previous conditions to the solubilization. Similar results were reported by Meyer (1971) in the case of soybean, by Boulter and Derbyshire (1971) in the case of pea, and by Singh and Jambunathan (1982) for the chickpea; however, bean proteins seem not to have these characteristics (Hang et al., 1980; Sathe et al., 1981). The level of lysine tends to be also a bit higher in the water- and salt-soluble fractions than in the other ones. Actually, the level of most of the amino acids varies slightly according to the protein fractions.

Hydroxyisoleucine is present only in the C + E flour + S as a free amino acid (Fowden et al., 1973) and therefore discarded from the protein fractions during the dialyses.

In general, our results are comparable to those found for other legumes and the amino acid composition of the water- and salt-soluble fractions seems to be characteristic of the storage proteins of the legumes (Derbyshire et al., 1976) and is similar to the amino acid composition of the soybean protein 11S (Hu and Esen, 1982).

As an estimate of protein quality, the chemical scores (without tryptophan) were determined by the amino acid scoring pattern suggested by the joint FAO/WHO expert committee (1973) (Table IV).

Though this score may not give all the necessary information concerning the nutritional value of a protein, it gives very useful indications (Wolzak et al., 1981). Thus, Table IV shows that for the cystine–cysteine content, water-soluble protein fractions are well balanced but they are slightly deficient in threonine and valine. There are only small variations according to the extraction conditions. It is important to notice that the limiting amino acids are not the same in the C + E flour and in the different protein fractions.

Electrophoretic Analyses (PAGE–SDS). We have no knowledge of any electrophoretic study on fenugreek protein fractions; therefore, we could not make any com-

Table IV. Amino Acid Scores[a] of Fenugreek Protein Fractions, cv. Gouka, Based on the Reference Pattern of Amino Acids

amino acids	whole meal C + E	C + E flour + S					C + E flour − S (78 °C, 9 h)				C + E flour − S (20 °C), H_2O soluble
		H_2O soluble	H_2O[b] soluble	NaCl soluble	NaOH soluble	pellet	H_2O soluble	NaCl soluble	NaOH soluble	pellet	
Thr	87.5	85	67.5	110	110	145	90	95	85	145	77.5
Val	74	94	16	96	94	102	42	58	100	126	62
Cys + Met	71.4	140	154.3	77.1	48.6	77.1	82.8	102.8	51.4	74.3	85.7
Ile	115	132.5	107.5	115	120	117.5	102.5	110	132.5	135	130
Leu	98.6	118.6	74.3	104.3	107.1	114.3	75.7	82.8	108.6	127.1	98.6
Tyr + Phe	115	131.6	230	168.3	151.7	200	103.3	121.7	145	186.7	148.3
Lys	112.7	114.5	107.3	100	80	89.1	136.4	132.7	105.4	109.1	109.1

[a] Value for each amino acid is expressed as a percentage of the reference (FAO/WHO, 1973). [b] Proteins solubilized with water at room temperature after heat treatment of the C + E flour + S (see details in the text).

parison between our results and previous works.

According to our experiments, the number of protein units in fenugreek as shown by the electrophoretic patterns is very high; over 30 individual protein band can be seen in the two protein groups albumin and globulin and somewhat less in the glutelin. In each group, the M_r of protein units ranges from around 10 000 to over 100 000. However, the segregation of the proteins according to their M_r varies from one protein fraction to the other. Some qualitative and quantitative differencies could have been observed in the protein composition between cultivars when proteins were both nonreduced or reduced with mercaptoethanol. The reduction of proteins by itself modifies differently the electrophoretic patterns of different protein groups. It seems also that fenugreek proteins are highly sensitive to the heat treatment as well as to the presence of saponins.

Influence of the Reduction of Proteins. Figure 3 shows the electrophoretic patterns of both reduced and nonreduced albumins, globulins, and glutelins of Gouka. Very important differences can be noticed in the case of albumins and globulins; unlike them, the patterns of reduced and nonreduced glutelins are quite similar, which would indicate a very low amount or the nonexistence of S–S bonds in the glutelin fractions. Actually, the analyses of amino acids have shown a very low amount of cysteic acid; cystine and cysteine have not been determined separately.

It is quite surprising that albumins of both cultivars show more high M_r protein units in the reduced than in the nonreduced form. The explanation for it—since the distribution of proteins in PAGE–SDS gels is according to their M_r—might be the transformation of globular proteins into nonglobular ones through the reduction of intracatenary S–S bonds and, thus, unfolded proteins would show apparently high M_r values.

In fact, the thus obtained albumins have the highest amount of cystine content (2% mol). Relatively few albumin fractions seem to be identical when comparing them before and after reduction and even those having similar mobility differ in general in their intensity. Accordingly, we can hypothesize that most of these albumins contain intramolecular S–S bonds that are disrupted under the action of a reducing agent.

It should be also noted that proteins shown in Figure 3 were obtained from C + E flour containing saponins. Albumins extracted from these samples, even after dialysis, still contain 14% of saponins (w/w).

The presence of saponins and the heat treatment of C + E flour in order to remove saponins by using 2-propanol at 78 °C are factors that seem to influence the electrophoretic composition of both reduced and nonreduced albumins.

Globulins have more fractions than albumins with similar electrophoretic mobility before and after reduction. They have more high M_r fractions in nonreduced form, suggesting that some globulins might be composed of two or more polypeptide chains linked to each other through intercatenary S–S bonds.

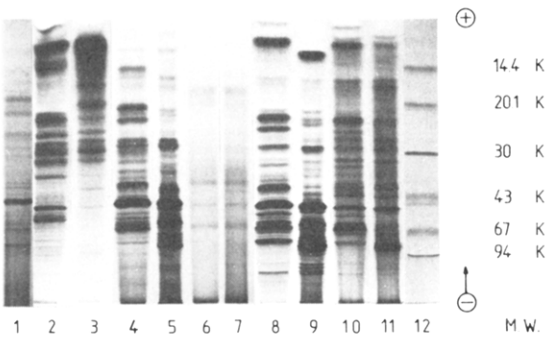

Figure 3. Vertical PAGE–SDS of reduced and nonreduced proteins from fenugreek, cv. Gouka. 1, protein soluble with SDS, 2, reduced albumins; 3, nonreduced albumins; 4, reduced globulins; 5, nonreduced globulins; 6, reduced glutelins; 7, nonreduced glutelins; 8, reduced albumins from C + E flour − S nonheated; 9, nonreduced albumins from C + E flour − S nonheated; 10, reduced albumins from C + E flour − S heated; 11, nonreduced albumins from C + E flour − S heated; 12, standard proteins. Concentration of proteins soluble with SDS applied to the gel: 0.6 mg in 0.1 mL of solution. A total of 1 mg of freeze-dried albumins, globulins, and glutelin was solubilized in 0.2 mL of buffer for 2, 3, 4, and 5, 1 mg in 0.1 mL for 6 and 7, and 1 mg in 0.15 mL for 8, 9, 10, and 11.

Varietal Differences between Protein Fractions. As we have seen, there are not quantitative differences between the two cultivars concerning the amounts of protein fractions solubilized. Lots of similarity could be noticed also between the electrophoretic compositions of their protein fractions both in their reduced and in their nonreduced form, though albumins and globulins have shown some dissimilarities.

Influence of Saponins and Heat Treatment with 2-Propanol on the Protein Composition. Figure 3 shows also the combining effect of the presence of saponins and 2-propanol (used to remove saponins either at room temperature or at 78 °C) on the electrophoretic compositions of both reduced and nonreduced albumins.

When we compare these electrophoretic patterns we should keep in mind that from nontreated C + E flour 72%, after the removal of saponins at room temperature 57–58%, and when saponins were previously removed with 2-propanol at 78 °C only 5.2% of albumins could have been solubilized. In any case, the composition of these differently obtained albumins shows very important dissimilarities. When saponins are removed at room temperature, the decrease of albumin recovered in comparison to the

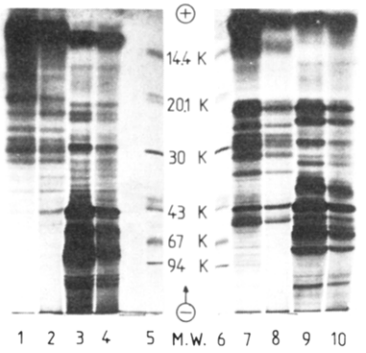

Figure 4. Vertical PAGE–SDS of proteins from fenugreek, cv. Gouka. Effect of the saponins on the electrophoretic compositions of albumins. 1, nonreduced albumins from C + E flour + S (containing 14% w/w saponins); 2, as (1) after the removal of the saponins from the freeze-dried proteins at room temperature; 3, nonreduced albumins from C + E flour – S nonheated (does not contain saponins); 4, as (3) but 1 mg of partially purified saponin is added to these albumins, in the ratio of 1:1, solubilized in water, and maintained in contact for 24 h and then the solution is applied to the gel; 5 and 6, standard proteins; 7, 8, 9, and 10, as samples 1, 2, 3, and 4, respectively, after reduction. Concentration of proteins applied to the gel:1 mg in 0.1 mL of buffer for 1, 2, 3, 7, 8, and 9 and 1 mg in 0.15 mL for 4 and 10.

albumins recovered from nontreated C + E flour is less than 15%; nevertheless, the electrophoretic patterns are not comparable any more neither for reduced nor for unreduced albumins.

In one hand, when saponins are present, the apparent M_r of most of the albumins is less than 35 000, on the other hand, albumins solubilized from saponin-free C + E flour seem to be composed mostly of polypeptides having their M_r over 35 000. Further investigations will be necessary in order to understand such an important modification. Besides the presence of saponins and the heat treatment, 2-propanol by itself may have an influence on the albumins of fenugreek.

Wolf (1976) postulated that in the case of soybean proteins, saponins are tightly bound to proteins. Our electrophoretic study might support this suggestion for fenugreek proteins too; however, the solubilization of proteins at different pHs has not given further confirmation for it.

In an attempt to understand the specific influence of saponins on the protein composition of fenugreek, on one hand, saponins were removed from the albumins extracted from nontreated C + E flour + S (which contain 14% w/w of saponins, first sample on Figure 4) and on the other hand, purified saponins were added to albumins extracted from saponin free C + E flour (second sample on Figure 4) and maintained in contact for 24 h. The electrophoretic patterns of these samples both in their nonreduced and in their reduced form are presented in Figure 4. Both the removal and the addition of saponins to the albumins cause some modifications in the electrophoretic patterns, suggesting selective affinities of saponins to some proteins. However, these experiments could not give the explanation for the differences that we can observe between the samples on the Figure 3.

The fact that in these experiments a purified fraction of saponin (not containing all the saponins of the C + E flour) was added to the albumins obtained from saponin-free C + E flour could be one of the reasons why their addition resulted only in relatively minor modifications of the electrophoretic composition of albumins. It seems that, according to the treatment of the flour with the solvents, irreversible changes are occurring in the composition of the albumins. However, it should be noted that these are not random modifications in the composition of albumins since separate experiments have reproduced similar results.

Characteristics of Protein Fractions. The electrophoretic composition of protein fractions according to their solubility shows important differences both in reduced and in nonreduced form (see Figure 3). It is interesting to notice that the electrophoretic pattern of albumins solubilized from saponin-free C + E flour (treatment with 2-propanol at room temperature) has more similarities with the electrophoretic patterns of globulins than with albumins containing saponins. Accordingly, the composition of protein fractions is highly dependent on the previous treatment of the samples.

The streakiness of glutelins (Figure 3) should be due to the denaturing effect of NaOH. The insolubility of these proteins can be explained by the presence of strong secondary interactions between them, hydrophobic or others, though, according to their amino acid composition, they would not be more hydrophobic than albumins and globulins. However, the amino acid composition does not give information on the surface hydrophobicity, which determines greatly the solubility of proteins.

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LITERATURE CITED

Baccou, J. C.; Lambert, F.; Sauvaire, Y. *Analyst (London)* **1977**, *102*, 458–465.
Bhatty, R. S. *J. Agric. Food Chem.* **1982**, *30*, 620–622.
Boulter, D. In "Protein Quality from Leguminous Crops"; Commission of the European Communities, Coordination of Agricultural Research: Luxembourg, 1977; EUR 5686 EN, pp 11–47.
Boulter, D.; Derbyshire, E. In "Chemotaxonomy of the Leguminosae"; Harborne, J. B.; Boulter, D.; Turner, B. L., Eds.; Academic Press: London and New York, 1971; Chapter 8, pp 285–308.
Brohult, S.; Sandegren, E. In "The Proteins"; Neurath, H.; Bailey, K., Eds.; Academic Press: New York, 1954; Chapter 18, pp 487–512.
Caldwell, K. A.; Kasarda, D. D. *Theor. Appl. Genet.* **1978**, *52*, 273–280.
Cerletti, P.; Fumagalli, A.; Venturin, D. *J. Food Sci.* **1978**, *43*, 1409–1411.
Clatterbuck, K. L.; Kehrberg, N. L.; Marable, N. L. *J. Food Sci.* **1980**, *45*, 931–935.
Derbyshire, E.; Wright, D. J.; Boulter, D. *Phytochemistry* **1976**, *15*, 3–24.
Dudman, W. F.; Millerd, A. *Biochem. Syst. Ecol.* **1975**, *3*, 25–33.
FAO/WHO "Energy and Protein Requirements. Report of a joint FAO/WHO ad hoc Expert Committee on Energy and Protein Requirements"; WHO: Geneva, 1973.
Fowden, L.; Pratt, H. M.; Smith, A. *Phytochemistry* **1973**, *12*, 1707–1711.
Hang, Y. D.; Steinkraus, K. N.; Hackler, L. R. *J. Food Sci.* **1980**, *45*, 388–389.
Hu, B.; Esen, A. *J. Agric. Food Chem.* **1981**, *29*, 497–501.
Hu, B.; Esen, A. *J. Agric. Food Chem.* **1982**, *30*, 21–25.
Kobrehel, K. *Ann. Technol. Agric.* **1980**, *29*, 125–132.
Kobrehel, K.; Bushuk, W. *Cereal Chem.* **1978**, *55*, 1060–1064.
Ladizinsky, G. *Plant Syst. Evol.* **1979**, *133*, 87–95.
Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–290.
Marquez, U. M. L.; Lajolo, F. M. *J. Agric. Food Chem.* **1981**, *29*, 1068–1074.
Meyer, E. W. *J. Am. Oil Chem.* **1971**, *48*, 484–488.
Millerd, A. *Annu. Rev. Plant Physiol.* **1975**, *26*, 53–72.
Osborne, T. B. "The Vegetable Proteins", 2nd ed.; Longmans, Green and Co.: London, 1924.

Paris, N.; Sauvaire, Y.; Baccou, J. C. Fr. Demande 2 315 508, 1977 (Appl. 75/20 274, 1975).
Payne, P. I.; Corfield, K. G. *Planta* **1979**, *145*, 83–88.
Rafik El-Mahdy, A.; El-Sebaiy, L. A. *Food Chem.* **1982**, *8*, 253–262.
Reichert, R. D.; Mac-Kensie, S. L. *J. Agric. Food Chem.* **1982**, *30*, 312–317.
Sathe, S. K.; Iyer, V.; Salunkhe, D. K. *J. Food Sci.* **1981**, *47*, 8–15.
Sathe, S. K.; Salunkhe, D. K. *J. Food Sci.* **1981**, *46*, 82–87.
Sauvaire, Y.; Baccou, J. C. *Lloydia* **1978a**, *41*, 247–256.
Sauvaire, Y.; Baccou, J. C. *Lloydia* **1978b**, *41*, 588–596.
Sauvaire, Y.; Baccou, J. C.; Besançon, P. *Nutr. Rep. Int.* **1976**, *14*, 527–537.
Singh, U.; Jambunathan, R. *Qual. Plant.—Plant Foods Hum. Nutr.* **1982**, *31*, 347–354.
Sosulski, F. W. In "Food colloids"; Graham, H. D., Ed.; Avi Publishing Co.: Westport, CT, 1977; Chapter 3, pp 152–206.
Wolf, W. J. In "Advances in Cereal Science and Technology"; Pomeranz, Y., Ed.; American Association of Cereal Chemists: St. Paul, MN, 1976; Chapter 6, pp 325–377.
Wolzak, A.; Elias, L. G.; Bressani, R. *J. Agric Food Chem.* **1981**, *29*, 1063–1068.
Wunschendorff, M. H. E. *J. Pharm. Chim.* **1919**, *20*, 86–88.
Yacoub, J. J.; Aboul-Ela, M. M. *Tex. J. Sci.* **1979**, *31*, 333–341.
Youle, R. J.; Huang, A. H. C. *Plant Physiol.* **1978**, *61*, 13–16.

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Amino Acid Composition of Sorghum Grains As Influenced by Grain Maturity, Genotype, and Nitrogen Fertilization

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Field experiments were conducted in 1977 and 1978 using three sorghum genotypes, namely, RCFA × L. 187 (a hybrid), L.187, and SK 5912, and four nitrogen rates (0, 35, 70, and 140 kg of N/ha). Nitrogen application generally increased total amino acids, g/16 g of N, but consistently decreased total essential amino acids of grains. Essential amino acid concentration significantly (P = 0.05) increased with grain maturity in both years. Genotype influenced grain amino acid composition with genotypes ranking in the orders of SK 5912 > RCFA X L.187 > L.187 for valine and SK 5912 > L.187 > RCFA × L.187 for methionine. Inverse relationships were evident between grain protein and lysine and methionine and between the latter two and leaf nitrate reductase activity.

All cereals tend to have an imbalance of the essential amino acids in that they are deficient in three (lysine, tryptophan, and methionine) of the eight amino acids considered indispensable for monogastrics (Breteler, 1976; Frey, 1973), yet the protein in cereals is, for most people, the major part of their protein intake, especially in the developing countries (Munck, 1975). Furthermore, of all cereals, sorghum [*Sorghum bicolor* (L.) Moench] has the lowest biological value because it has very low levels of lysine, tryptophan, and possibly arginine (Eggum, 1973; Sikka and Johari, 1979).

Nitrogen levels supplied to sand culture as solutions of nitrate in concentrations ranging from 0.2 to 16 mequiv/L have been shown to increase the proportions of certain amino acids in sesame (Mitchell et al., 1976). Reports have also indicated that the amino acid composition of grain is probably under genetic control (Nelson et al., 1965; Frey, 1973).

Although the work of Rhodes and Mathers (1974) on barley [*Hordeum vulgare* (L.)] indicated that amino acid composition of grains is affected by grain maturity, there is, however, little information on the amino acid composition of sorghum grain at various stages of grain development. The study reported here was, therefore, undertaken to investigate the pattern of amino acid composition of sorghum grain at different stages of grain development as affected by genotype and soil nitrogen level. The correlations between leaf nitrate reductase activity and grain protein at harvest and lysine and methionine were also determined. The three genotypes employed in this study are being used in cooperative research on grains for human consumption in the West African semiarid regions.

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MATERIALS AND METHODS

Three sorghum genotypes, namely, RCFA × L.187 (a hybrid), L.187, and SK 5912 were field grown on a sandy loam soil at the Agronomy Farm of the Institute for Agricultural Research, Ahmadu Bello University, Samaru, Zaria (11°11′ N and 7°38′ E), Nigeria.

The field received blanket applications of 89.6 kg/ha super phosphate and muriate of potash. These were broadcast and disked in at seed-bed preparation. Seeds of the three genotypes were sown in June on 90-cm ridges at 60 cm apart, giving a plant population of 37 000/ha at two plants per stand with uniform plot sizes of 9.28 m by 7.1 m. Four levels of nitrogen, 0, 35, 70, and 140 kg of N/ha, were applied as calcium ammonium nitrate according to AERLS Recommended Practices No. 2 (1976, 1978). The experiments carried out in 1977 and 1978 were a completely randomized block design with treatments replicated 4 times.

Nitrate reductase activity (NRA) of the fourth leaf was measured at 5, 7, 9, 11, 13, and 15 weeks after planting by using a combination of the in vivo methods of Klepper et al. (1971) and Radin (1973).

Grain protein and amino acid composition of the grains were determined at 10, 17, 24, and 67 (at harvest) days after anthesis by the Kjeldahl method and by using a multisample analyzer, Model TSM (Technicon), respectively. The hydrolysis of the amino acids was by 6 N hydrochloric acid in which the tryptophan was destroyed. The readings for cystine were so low that they were dis-

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