Protein Accumulation and Protein Quality of Bengal Gram (*Cicer arietinum*) Cotyledons during Development

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Variable rates of accumulation of protein fractions of Bengal gram were observed during development of cotyledons. Most of the protein was synthesized between the 25- and 35-day stage. Three types of globulins (α, β, and γ) were synthesized during development. The proportion of these varied during development, but α-globulin was a dominant fraction throughout. the concentration of free amino acids was highest at the 25-day stage. The decrease in free amino acids during later stages was accompanied by a rapid accumulation of protein at the 35-day stage. Arginine and cystine predominated in free amino acids at maturity. The protein quality of developing cotyledons determined in N-balance experiments with growing rats was hardly affected by the stage of development.

Bengal gram (*Cicer artietinum*) is an important food crop in India and it ranks next to rice and wheat. Since it is rich in lysine (Chatterjee et al., 1977), it occupies an important place in supplementation of cereal-rich Indian diets in alleviating protein malnutrition (Gupta et al., 1979). Amino acid analyses showed that sulfur-containing amino acids and threonine are the most limiting amino acids in chickpea (Kaul and Gassi, 1971; Peruanskii, 1974). It has been further shown by chemical tests (Belew and Eaker, 1976) that Bengal gram proteins have lower digestibility. In soybeans quantitative and qualitative changes in protein during seed development have been studied (Hill and Breidenback, 1974). In maize (Gupta et al., 1977) postranslocation modification of storage protein has been shown to decrease protein quality, by losses of the limiting amino acid lysine. However, information about the protein quality of developing Bengal gram cotyledon is lacking. Therefore, in the present study chemical and biological evaluations for the nutritive quality of developing Bengal gram cotyledons have been carried out.

EXPERIMENTAL PROCEDURES

Sample Preparation. Bengal gram (*Cicer arietinum*) was grown on the Indian Agricultural Research Institute New Delhi farm. The flowers were tagged when fully open. This has been taken as the date of flowering. The pods were harvested at 15, 25, 35, and 45 days after flowering. The grain matured at 45 days after flowering. Cotyledons, collected in the cold from seeds after removal of testae and plant axes, were used immediately for analysis or stored in liquid nitrogen until use. Crude protein content ($N \times 6.25$) was determined by micro-Kjeldahl method (A.O.A.C., 1965).

Protein Fractionation. Cotyledons were lyophilized and ground to a fine powder (100 mesh) and the protein fractionation was done by the method described by Millerd (1972). Cotyledons were defatted and albumin was extracted with water. After complete removal of albumin, globulin was extracted with 10% NaCl solution until almost complete extraction of globulin as seen by the absorbance at 280 nm. All the pooled globulin fractions were brought to 85% ammonium sulfate saturation and allowed to stand for 1 h in the cold in order to precipitate globulins. Precipitated globulins were dissolved in 0.15 M phosphate buffer, pH 7.0 (buffer I), and dialyzed against 0.2 M sodium acetate buffer, pH 4.8 (buffer II), for 18 h with six changes in the cold (4 °C) to precipitate α-globulin. The precipitate obtained on centrifugation was dissolved in 10% NaCl and reprecipitated by adding ammonium sulfate to 85% saturation. The α-globulin fraction was dissolved in buffer I and dialyzed against buffer II, and finally the precipitated α-globulin collected by centrifugation was dissolved in buffer I.

Supernatant obtained after sedimenting α-globulin was dialyzed against the deionized water for 24 h with 10 changes of deionized water to precipitate β-globulin. The β-globulin obtained was dissolved in a small volume of buffer II and reprecipitated as above by dialysis and then dissolved in buffer II. From the supernatant obtained after sedimentation of β-globulin, γ-globulin was precipitated by adding ammonium sulfate to 50% saturation. Precipitate of γ-globulin was collected by centrifugation and dissolved in water. γ-Globulin was reprecipitated by adding ammonium sulfate to 50% saturation. The precipitate was collected and dissolved in water.

Amino Acid Analysis. Total free amino acids were extracted with boiling ethanol and estimated as described by Lee and Takahashi (1966). Protein hydrolysis and subsequent amino acids analyses were performed according to the method described by Hansen and Eggum (1973). *S*-Amino acids were determined as methionine sulfone and cysteic acid by oxidation of freeze-dried plant material for 15 min with performic acid at 50 °C according to Weidner and Eggum (1966). Tryptophan was estimated after hydrolysis with 5 N NaOH and 5.5 N $Ba(OH)_2$ in glass flasks heated, under reflux, on electric hot plates, as described by Eggum (1968).

N-Balance Study. Nitrogen balance of each sample was performed with five Wistar male rats (~70 g weight each) by the method of Eggum (1973) in a room maintained at 25 ± 1 °C as described by Lodha et al. (1976). Each rat received 10 g of dry matter containing 150 mg of N daily. The rats were arranged in groups of five in such a way that group means did not differ much. Each rat was housed in a perspex cage. The total experimental period of 9 days consisted of 4 days of preliminary feeding followed by a 5-days balance period in which pooled feces and pooled urine for each rat were analyzed for nitrogen.

Formulas used for the calculation of true digestibility, biological value, and other quality characters were described by Gupta et al. (1979).

RESULTS AND DISCUSSION

Dry weight, protein, and free amino acid contents of developing Bengal gram cotyledons are presented in Table I. Fresh weight of the cotyledons increased during development until the 35-day stage and then decreased at

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Table I. Fresh Weight, Dry Weight, Protein, and Free Amino Acids in Developing Cotyledons of Bengal Gram[c]

days after flowering	fresh wt, mg/Cot[a]	dry wt, mg/Cot	free AA (Leu equiv), μg/Cot	protein mg/Cot	protein %[b]
15	21.6	3.83	21	0.78	20.25
25	46.7	12.10	57	2.50	20.62
35	105.6	57.86	14	14.63	25.38
45	70.0	68.25	9	18.12	26.55

[a] Cot = cotyledon. [b] Values are on a dry weight basis. [c] Values given in the table are an average of duplicate analyses which agreed closely.

maturity. Dry weight of the cotyledons increased throughout during development. The accumulation of dry weight was maximum between the 25- and 35-day stage. The initiation of seed maturation was indicated by the onset of desiccation as observed by a decrease in the fresh weight after the 35-day stage.

Free amino acid content was maximum at the 25-day stage and decreased considerably thereafter. On the other hand, protein accumulation was slower up to the 25-day stage as only 14% of the total cotyledon protein was deposited during this period, while in a much shorter period of 10 days from the 25- to 35-day stage ~67% of the total cotyledon protein was deposited. From 35 days to maturity ~19% of the total protein was deposited. The decrease in free amino acid after the 25-day stage is mainly due to rapid synthesis of storage protein in the cotyledons. The protein percent also showed a simultaneous increase with the increase in dry weight from the 25- to 35-day stage. This indicates a higher rate of protein accumulation compared to the increase in dry weight. Since the rate of protein accumulation varied during cotyledon development, proteins were fractionated into albumin and globulin. Globulin was further fractionated into α-, β-, and γ-globulin (Table II).

At maturity albumin accounted for 17.7% of the total protein, while globulin accounted for 72.0% and the residue fraction after globulin extraction accounted for 10.3% of total protein (Table II). This indicates that globulin is the major storage protein in Bengal gram cotyledons. Globulin has been shown to be the major storage protein in *Vicia faba* (Boulter and Davies, 1968) and *Pisum sativum* (Danielsson, 1949).

On comparison of α-, β-, and γ-globulin content per cotyledon, it was seen that α-globulin increased about sevenfold from the 15- to 25-day stage while β- and γ-globulins increased about fivefold each during the same period. From the 25- to 35-day stage α-, β-, and γ-globulin increased 12-, 3-, and 20-fold, respectively. From the 35- to 45-day stage, both α- and β-globulins increased by ~50% while γ-globulin increased by 4.5-fold. γ-Globulin is known to be rich in methionine (Millerd, 1975). It has been shown that from day 25 to maturity most of the α-globulin was deposited. The overall decrease in methionine and lysine during later stages of cotyledon development was mainly due to the change in the synthesis of different types of globulin. α-Globulin which is poor in methionine was mainly deposited from day 25 to maturity. Since α-globulin accounts for 84% of the total globulin fraction at maturity, it results in an overall reduction in the protein quality of Bengal gram protein. Therefore, for improvement of the protein quality, efforts will have to be directed in identifying the genotypes which have higher content of γ-globulin.

Free amino acid composition from developing cotyledons is shown in Table III. The results showed marked changes in various free amino acids. Differences observed in free amino acids indicated a differential supply of various amino acids in the free amino acid pool. From the 25- to 35-day stage, the maximum amount of storage protein was deposited, and this resulted in a decrease of almost all the free amino acids during this period. However, the increase in serine and cystine up to the 35-day stage indicated that these amino acids were synthesized or were translocated into the cotyledons at a much faster rate than the rate of their utilization in protein synthesis. Serine and cystine concentration in protein did not show much difference during this period (Table IV). From day 35 to maturity, the concentration of most of the free amino acids decreased markedly except for tyrosine.

The changes in concentration of free amino acids observed may be due to protein synthesis and also due to differences in translocation and interconversion of amino acids. The results further indicate that different amino acids were supplied at different rates during cotyledon development. The amino acid composition of protein hydrolysates (g/16 g of N) from developing cotyledons is shown in Table IV. Lysine, alanine, and methionine decreased while glutamic acid, phenylalanine, and aspartic acid increased during cotyledon development. Proline, glycine, valine, histidine, and tryptophan did not show much change during cotyledon development. Arginine increased appreciably from the 35- to 45-day stage.

Total amino acid content per 100 cotyledons during cotyledon development is shown in Table V. The quantity of most amino acids increased considerably from the 25- to 35-day stage. Lysine, methionine, and alanine did not increase in direct proportion to the increase in protein during this period. This mainly resulted in the decrease in the proportion of these amino acids when expressed on 16 g of N (Table IV).

The protein quality index of cotyledon protein at different stages of development calculated from the FAO/WHO (1973) reference protein pattern is shown in Table VI. The protein quality index of developing Bengal gram calculated for infants, preschool children, and adults gives an idea of the nutritional suitability of the Bengal gram. It is the ratio expressed in percentage of the ideal protein required per kilogram per day to the protein in a sample which would satisfy the total requirement of the most limiting essential amino acid. According to the requirement suggested by FAO/WHO (1973), threonine became

Table II. Concentration of Albumin and Globulin in Developing Cotyledons[b]

days after flowering	albumin g/100 g of flour	albumin mg/Cot	globulin g/100 g of flour	globulin mg/Cot	globulin fractions α g/100 g of Glob[a]	α μg/Cot	β g/100 g of Glob	β μg/Cot	γ g/100 g of Glob	γ μg/Cot
15	2.30	0.10	4.10	0.18	55.45	87.0	43.63	68.5	0.91	1.4
25	2.12	0.26	2.96	0.97	64.62	623.4	34.59	333.8	0.77	7.5
35	3.56	2.36	14.78	9.72	87.24	743.0	10.99	940.0	1.76	150.0
45	4.30	3.11	17.48	13.05	84.15	10620.0	10.50	1330.0	5.34	670.0

[a] Glob = globulin. [b] Values given in the table are an average of duplicate analyses which agreed closely.

Table III. Concentration of Free Amino Acids in Developing Cotyledons

	concn, mg/100 cotyledons, at days after flowering of			
	15	25	35	45
aspartic acid	1.04	1.65	0.88	0.18
threonine	0.76	1.77	0.31	0.06
serine	0.88	1.02	1.41	0.11
glutamic acid	5.80	20.98	5.15	0.52
proline	0.01	0.23	0.16	0.03
glycine	0.05	0.42	0.14	0.04
alanine	1.68	3.45	0.98	0.43
valine	0.92	2.06	0.44	0.04
isoleucine	0.15	0.90	0.23	0.14
leucine	0.15	0.95	0.20	0.11
tyrosine	0.07	0.78	0.14	0.18
phenylalanine	0.14	0.84	0.25	0.03
lysine	0.35	0.90	0.73	0.13
histidine	0.20	0.51	0.27	0.06
arginine	1.48	7.29	4.42	2.30
methionine	0.11	0.05	0.05	0.01
cystine	0.01	1.56	2.41	1.79

Table IV. Concentration of Protein-Bound Amino Acids in Developing Cotyledons

	concn, g/16 g of N, at days after flowering of			
	15	25	35	45
aspartic acid	10.77	11.08	12.49	12.79
threonine	4.37	3.80	4.13	3.50
serine	4.46	4.61	4.95	4.84
glutamic acid	13.17	15.59	17.97	18.46
proline	4.18	4.36	4.56	4.17
glycine	3.90	4.09	4.25	4.19
alanine	5.26	5.14	4.74	4.63
valine	5.70	5.15	5.26	5.10
isoleucine	4.62	4.68	5.11	4.89
leucine	7.55	7.83	8.55	8.38
tyrosine	3.36	3.25	3.66	2.29
phenylalanine	4.77	5.59	6.37	6.52
lysine	7.09	6.11	5.50	5.95
histidine	2.44	2.56	2.56	2.91
ammonia	1.54	1.46	1.38	1.21
arginine	11.04	11.42	9.66	12.97
methionine	1.69	1.42	1.38	1.37
cystine	1.45	1.65	1.89	1.80
tryptophan	1.24	1.11	1.08	1.09

Table V. Concentration of Protein-Bound Amino Acids in Developing Cotyledons

	concn, mg/100 cotyledons, at days after flowering of			
	15	25	35	45
aspartic acid	6.71	24.00	131.53	179.80
threonine	2.73	8.23	43.49	49.20
serine	2.78	9.97	52.17	68.00
glutamic acid	8.21	33.74	189.27	259.40
proline	2.60	9.43	48.03	58.40
glycine	3.43	8.84	44.79	58.80
alanine	3.28	11.12	41.84	64.60
valine	3.55	11.15	55.41	71.60
isoleucine	2.88	10.12	55.86	68.60
leucine	4.70	16.54	90.10	116.40
tyrosine	2.09	7.03	38.58	32.10
phenylalanine	2.97	12.09	67.06	91.60
lysine	5.65	15.61	71.20	102.78
histidine	1.52	5.54	26.93	40.80
ammonia	0.96	3.17	14.50	16.80
arginine	6.88	23.72	101.75	182.26
methionine	1.35	4.27	21.88	27.80
cystine	0.92	3.58	19.94	24.60
tryptophan	0.99	2.84	113.98	17.40

the first limiting amino acid in mature cotyledons as well as in cotyledons at the 25- and 35-day stage.

Table VI. Protein Quality Index[a] of Developing Bengal Gram Cotyledons According to FAO/WHO (1973) Requirement for Different Age Groups

	protein quality index at days after flowering of			
	15	25	35	45
infant	94	87	94	75
preschool children	101	95	102	83
preschool children plus 30% extra allowance	77	71	77	62
adult	150	150	163	150

[a] Protein quality index = [requirement of protein ($N \times 6.25$) for age]/(amount of test protein to satisfy requirement of the most limiting amino acid of subjects of the same age) × 100. Protein requirement: infants, 1.85 g of ideal protein kg^{-1} day^{-1}; preschool children, 0.9 g of ideal protein kg^{-1} day^{-1}; adult, 0.55 g of ideal protein kg^{-1} day^{-1}. Threonine in ideal protein, 40 mg/g of protein. Sulfur amino acids in ideal protein, 35 mg/g of protein.

Table VII. True Protein Digestibility (td), Biological Value (bv), Net Protein Utilization (npu), and Utilizable Protein (up) in Developing Seeds of Bengal Gram[a]

days after flowering	td		bv		npu		up	
	%	s	%	s	%	s	%	s
15	86.1	2.3	69.3	2.5	59.6	1.7	13.8	0.3
25	84.9	1.9	66.7	1.8	56.6	1.8	13.0	0.2
35	86.2	2.5	69.8	0.9	59.8	2.4	14.3	0.4
45	88.6	1.6	67.8	1.7	60.1	1.1	16.3	0.5

[a] The values reported are an average of five experimental rats for each sample.

In cotyledons at the 15-day stage, sulfur-containing amino acids became the first limiting amino acids. Cotyledons at the 35-day stage showed a protein quality index at 102% for preschool children, indicting the best protein quality. Protein quality of immature cotyledons was superior to that of mature cotyledons. The cotyledons at the 15-day stage showed almost an ideal amino acid composition required for the preschool children.

Results of the nutritive evaluation by rat feeding experiments are presented in Table VII. True digestibility (td) ranged from 85 to 89%. These values are lower than the td for cereal grains like maize (Lodha et al., 1976) and rice (Eggum et al., 1977). The biological value (bv) was more or less similar at different stages of development. The bv for Bengal gram protein is in general higher than the bv for rice (Eggum et al., 1977). Net protein utilization (npu) which is the product of bv and td did not show much difference at different stages. Utilizable protein (up) was highest at maturity where the protein content was highest. At the 25-day stage it was slightly lower compared to the other stages.

The differences in protein quality of cotyledons at different stages of development observed in Bengal gram were narrower compared to the differences observed for developing maize grain (Gupta et al., 1978). Although in grain legumes sulfur-containing amino acids have been found to be limiting (Kaul and Gassi, 1971; Peruanskii, 1974), in Bengal gram threonine was observed to be the first limiting amino acid when compared with the FAO/WHO (1973) reference protein. Therefore, for improvement in the protein quality of Bengal gram, efforts should be directed toward increasing the threonine content and in identifying the genotypes which have higher content of γ-globulin.

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Effect of Treating *Candida utilis* with Acid or Alkali, To Remove Nucleic Acids, on the Quality of the Protein

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Dried *Candida utilis* cells were variously extracted with acidic and alkaline solutions to maximize the removal of nucleic acids but to minimize the damage to proteins. Response surface methodology procedures were used to examine effects of yeast concentration, time, temperature, and pH on protein yield, nucleic acid removal, available lysine content, lysinoalanine formation, racemization, and amino acid destruction. At increased cell concentrations, time, temperature, and pH, alkaline extractions were more efficient in removing nucleic acids but caused decreased availability of lysine, amino acid destruction, increased racemization, and formation of lysinoalanine. A 0% nucleic acid content, predicted for an alkaline isolate, would cause extensive damage to proteins. However, an isolate was obtained that could be consumed by an adult to essentially meet the daily recommended protein requirement without risking the danger of elevated uric acid levels. Low available lysine was the most serious detectable defect in the acid-extracted products.

At present single-cell protein (SCP) is mainly used for animal feed. The economics of yeast as a source of human protein have been discussed (Cartwright, 1958), but the use of yeast proteins in human food on a large scale has been limited by its high nucleic acid (NA) content. Nucleic acid is metabolized into uric acid, but man, unlike the lower forms of life, has no uricase enzyme to convert uric acid into allantoin which is soluble and easily excreted. Thus, high levels of uric acid can crystallize in tissues and organs to cause gout, stones in the urinary tract, and tophi in the soft tissues. Daily intake of NA should not exceed 2 g because higher quantities increase the uric acid content of blood plasma to abnormal levels (Edozien et al., 1970).

Rogozhin et al. (1970) used various concentrations of sodium hydroxide to extract protein from disintegrated yeast cells to obtain a product that was 96% protein and contained less than 2% NA. Ayukawa (1971) heated SCP in anhydrous liquid ammonia to obtain a product with increased protein content, improved odor, color, taste, and texture, and decreased NA content. Hedenskog and Ebbinghaus (1972) used alkaline conditions to extract proteins from SCP and reduced their NA content to 1–2% but observed that the availability of lysine was reduced after extraction at pH 12. Vananuvat and Kinsella (1975) homogenized a cell suspension in sodium hydroxide solution in the presence of glass beads to reduce the NA content of the cells to less than 5%. Zee and Simard (1975) reduced the NA content of *Rhodotorula glutinis* from 6.5 to 1.5% by heating the cells at 100 °C for 40 min at pH 11.0. Viikari and Linko (1977) reported that the NA content of *Paecilomyces varioti* was easily reduced from the initial 9% to less than 2% with diluted sodium hydroxide solutions. Newell et al. (1975) developed processes to prepare isolated protein products from yeast with reduced NA content. Sinskey and Tannenbaum (1975) have reviewed some methods for reducing the NA content of SCP using exogenous and endogenous enzymes.

Tumura et al. (1972) dehydrated wet yeast cells with methanol and esterified the proteins with methanolic hydrochloric acid and observed that the RNA was almost completely removed by the esterification process. Akin and Chao (1972) reduced the NA content of *Candida utilis* cells from 10.5 to 3.3% by treating the cells with phos-

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