

# STUDIES IN THE PROTEINS OF INDIAN FOODSTUFFS.

## PART VI. THE GLOBULINS OF THE COWPEA

(*Vigna catieng* WALP.)

By (Miss) K. Bhagvat.

The cowpea, one of the well-known major pulses, is grown in all tropical countries, usually as a subordinate crop. In Iraq, it is grown as an intercalary crop among the date palms. In Burma and Malay States, it is raised as a catch crop in rubber plantations. *Dolichos sesquipedalis*, *Vigna catieng* and *Vigna sirenensis* are the three well-known varieties of this pulse, the last of which is extensively cultivated in the United States and in South Europe. The variety with long pods (*Vigna catieng*) is raised as a vegetable and is used as a substitute for French beans. The green leaves of the cowpea are found to contain 0.3 per cent. nitrogen and are used in Cuba as a green manure and forage. The value of the cowpea plant as fodder is considered high especially when it is used in combination with *Soja* (*Soja biopida* Menckh). Separate statistics regarding the acreage of its cultivation and the quantity of its production are not available. In India, the pulse is consumed by the poorer and middle classes.

In a study of the proteids of the cowpea, Osborne and Campbell (*J. Amer. Chem. Soc.*, 1897, 19, 494) adopted methods of dilutions and dialysis and succeeded in obtaining "Vignin" as the predominant protein of the pulse. They also showed the presence of two other globulins, a phaseolin not precipitated from its solution in 10 per cent. sodium chloride by appreciable quantities of acetic acid and a third globulin, highly soluble in very dilute salt solutions, completely precipitable only by dialysis against alcohol. Osborne and Harris (*J. Amer. Chem. Soc.*, 1903, 25, 323) determined the Hausmann distribution of nitrogen in vignin and later Osborne and Hoyle (*Amer. J. physiol.*, 1908, 22, 362) in estimating the individual amino acids by Fischer's esterification method were able to account for only 59-67 per cent. of the total nitrogen. Niyogi, Narayana and Desai (*Ind. J. Med. Res.*, 1931-32, 19, 859) isolated the total globulins from the pulse, conducted a Van Slyke analysis of the protein and determined its nutritive value by feeding experiments. At 10 per cent. level of intake, they found that the proteins of the cowpea possess a high biological value.

While Osborne indicated the presence of three distinct proteins differing from each other in certain properties as solubility and coagulability, the fractions have not been subjected to any detailed chemical

analysis. Niyogi, Narayana and Desai have experimented only with the total globulins and have not made any attempts at a purification of the proteins employing the more recent methods like electro dialysis. In view of the high nutritive value ascribed to this pulse, it appeared desirable to extend the investigation with a view to purify and fractionate the total globulins into individual proteins.

### EXPERIMENTAL.

The seeds (*Vigna catiang*) were sun-dried and coarsely powdered along with the husk which could not be removed. Table I gives the results of a proximate analysis of the powder, carried out according to the methods recommended by the A.O.A.C. (1930).

TABLE I.

(Percentages on the weight of the seed meal)

Moisture	Ether extractives	Total N	Crude protein (N×6.25)	Ash	Crude fibre	Carbohydrates	
						Soluble	Insoluble
11.03	2.68	4.16	26.63	2.92	2.64	9.07	39.97

A qualitative test indicated the presence of vitamin A in the ether extract and the ash, on analysis, showed the presence of copper. Table II gives the results of the ash analysis, expressed as percentages on the weight of gram.

TABLE II.

Ash	SiO <sub>2</sub>	Ca	Fe	Cu	P
2.92	0.05	0.22	0.013	0.004	0.93

Iron was estimated by Elvehjem's method (*J. Biol. Chem.*, 1930, 86, 463) and copper by Biazzo's method as modified by Elvehjem and Lindow (*J. Biol. Chem.*, 1929, 81, 435).

The calorific value of the pulse as determined by the Bomb Calorimeter amounts to 4371.6 Cal./g.

*Distribution of Nitrogen.*—With a view to determining the various forms of nitrogen in the seed, the meal (200 g.) was extracted repeatedly with 4 per cent. saline. The combined extracts were passed

through a filter, made up to a known volume by 4 per cent. saline and nitrogen determined in an aliquot of the extract. The residue was subsequently extracted with 70 per cent. alcohol and later with 0.1 per cent. sodium hydroxide. Total nitrogen was determined in each of the extracts. The results are given in Table III.

TABLE III.

Saline soln.	Percentages of total nitrogen extracted by		
	70 per cent. alcohol	0.1 per cent. NaOH	Residue by difference
68.0	1.8	23.45	6.75

*Extraction of the globulins.*—With the object of determining the optimum conditions of extraction of the seed globulins, the meal (20 g.) was extracted with 250 c.c. of various concentrations of salt solution for 1 hour at the room temperature (25°). The nitrogen in an aliquot of the filtrate was then determined by Kjeldahl. Results are given in Table IV, which shows that 4 per cent. sodium chloride extracts the maximum percentage of nitrogen from the seeds. A study of the time factor involved in the extraction (see Table V) shows that the maximum amount can be extracted in 15 minutes.

TABLE IV.

<i>Salt concentration per cent.</i> ...	0.0	1.0	2.0	3.0	3.5	4.0	5.0	10.0	15.0
Percentage of total N extracted ..	19.8	51.0	55.4	57.2	57.8	58.8	55.4	55.4	54.3

TABLE V.

<i>Period of extraction in mins.</i>	..	15	30	45	60	120
Percentage of Nitrogen extracted ..	..	61.9	60.6	59.7	59.7	58.8

It was observed that in presence of the meal, the saline extract gradually darkens on standing for 3 or 4 days. If the saline extract is aerated, the darkening is accelerated, but a pronounced acceleration is secured by the addition of tyrosinase but not tyrosine. This confirms

the suspicion that tyrosine or related compounds are enzymatically split off during the extraction, the oxidation of which appears to be greatly facilitated by the added tyrosinase.

*Preparation of the globulins.*—The total globulins were prepared by four different methods:—(1) electro-dialysis; (2) hydrodialysis through parchment membranes; (3) dilution; and (4) saturation with ammonium sulphate. During the course of electro-dialysis, it was observed that the protein migrated towards the anode while the colouring matter got deposited on the cathode membrane. The globulins prepared by electro-dialysis gave the best preparation so far as colour was concerned. When the diffusate was free from chlorides the suspension of globulins was centrifuged, washed successively with water, alcohol and ether and the preparation (A) dried *in vacuo* over sulphuric acid. Further dialysis of the centrifugate in cellophane bags against distilled water gave a further precipitate which was too small for analysis.

Another batch of the saline extract was dialysed in parchment bags against distilled water. The precipitate was redissolved in saline solution and redialysed. The precipitates thus obtained were washed on the centrifuge successively with water, alcohol and finally with ether. The preparation (B) thus obtained was dried *in vacuo* over sulphuric acid.

The third preparation of the globulin (C) was obtained by diluting 2 litres of the saline extract with 12 litres of distilled water. To facilitate the precipitation of the fine suspension, carbon dioxide was passed through the liquid. The precipitate was redissolved in 4 per cent.

TABLE VI.

	Preparation obtained by			
	Electro-dialysis (A)	Dialysis (B)	Dilution (C)	Saturation with ammonium sulphate (D)
Yield (per cent. on the weight of the meal) .. .. .	13.8	4.1	5.1	4.0
Moisture .. .. .	8.76	9.32	8.84	10.13
Ash .. .. .	1.45	0.32	0.46	1.00
Total Nitrogen (ash and moisture free) .. .. .	16.36	14.70	15.82	17.02

saline and dialysed against distilled water. The precipitate was washed on the centrifuge with alcohol, acetone and ether and dried *in vacuo* over sulphuric acid.

The fourth preparation (D) was obtained by saturating 1,500 c.c. of the saline extract with finely ground ammonium sulphate. The precipitate thus obtained was filtered off and dissolved in water, the adhering saturated solution of ammonium sulphate being sufficient to reprecipitate the precipitate. The solution was filtered and the clear filtrate dialysed against distilled water until the diffusate showed a negative test for ammonia with Nessler's reagent. The precipitate was washed on the centrifuge successively with alcohol, acetone and ether and finally dried *in vacuo* over sulphuric acid.

The yields of the various preparations together with their contents of moisture, ash and total nitrogen are given in Table VI.

All the four preparations were analysed by the method of Van Slyke (*J. Biol. Chem.*, 1911, 10, 15) as modified by Plimmer and Rosedale (*Biochem. J.*, 1925, 19, 1004), as also Plimmer and Lowndes (*Biochem. J.*, 1927, 21, 247). Tyrosine and tryptophane were directly determined by the method of Folin and Marenzi (*J. Biol. Chem.*, 1929, 83, 89) and an independent estimation of cystine in all the preparations was carried out by the method of Folin and Marenzi (*J. Biol. Chem.*, 1929, 83, 103).

The results in Table VII are in general agreement with the values characteristic of vegetable globulins and there appears to be nothing particularly noteworthy with regard to its composition.

It is, however, interesting to note that there is a great variation in the cystine content, the preparation (D) having a value about six times as high as that of (B) which has the lowest value in the series, suggesting the probable presence of two or more fractions rich in cystine.

*Fractionation of globulins.*—The saline extract was saturated with ammonium sulphate, the precipitate centrifuged off and reprecipitated in saline solution and filtered. The filtrate containing the total globulins was treated with calculated quantities of finely ground ammonium sulphate raising the concentration of the salt, in stages, to  $1/3$ ,  $1/2$  and full saturation. At each stage, the precipitate obtained was centrifuged off, reprecipitated with saline and dialysed in cellophane bags against distilled water till the diffusate was free from any trace of ammonia. The contents of the bag was then centrifuged, the precipitate washed successively with distilled water, alcohol, acetone and ether and finally dried *in vacuo* over sulphuric acid. The three fractions, thus obtained, have been analysed and the results tabulated in Tables VIII and IX.

It will be observed from the table that the cystine content of the three fractions progressively decreases, the third fraction containing

TABLE VII.

*Distribution of Nitrogen (as percentages of the total nitrogen)*

Form of Nitrogen	Preparations			
	A	B	C	D
Melanin .. .. .	2.09	1.45	1.40	1.81
Amide .. .. .	10.99	11.17	11.2	10.50
<i>Basic—</i>				
Arginine .. .. .	11.09	11.57	10.09	13.43
Histidine .. .. .	2.53	3.65	2.85	4.37
Cystine .. .. .	0.44	0.57	0.57	0.80
Lysine .. .. .	7.39	6.91	5.31	2.40 (?)
<i>Non-basic—</i>				
Amino .. .. .	63.54	64.47	67.79	65.92
Non-amino .. .. .	1.20	0.99	0.73	0.78
	99.87	100.78	99.94	100.01
<i>Independent estimations of Individual amino acids—</i>				
Cystine .. .. .	1.39	0.34	0.76	1.82
Tyrosine .. .. .	2.67	4.75	4.80	4.24
Tryptophane .. .. .	0.59	0.55	0.51	0.53

TABLE VIII.

Fraction	Yield per cent. on the wt. of the meal	Moisture	Ash	Total Nitrogen (ash and moisture free)
I	0.4	11.9	0.69	15.33
II	1.6	11.4	0.50	14.84
III	0.5	8.3	1.20	16.48

TABLE IX.

*Distribution of Nitrogen (as percentages of the total nitrogen)*

Form of Nitrogen	Fractions		
	I	II	III
Melanin .. .. .	1.60	1.29	0.89
Amide .. .. .	9.88	10.80	10.75
<i>Basic—</i>			
Arginine .. .. .	12.25	9.40	9.61
Histidine .. .. .	2.79	3.90	4.30
Cystine .. .. .	0.69	0.25	0.04
Lysine .. .. .	7.09	5.16	15.09
<i>Non-basic—</i>			
Amino .. .. .	63.96	66.93	58.32
Non-amino .. .. .	1.17	1.23	2.36
	99.43	99.26	101.36
<i>Direct estimation of Individual amino acids—</i>			
Cystine .. .. .	0.76	0.33	0.12
Tyrosine .. .. .	3.79	2.97	2.60
Tryptophane .. .. .	0.50	0.33	0.38

only a sixth of the quantity characterising fraction I which is the richest not only with regard to cystine but also with respect to the other three essential amino acids, arginine, tyrosine and tryptophane. It may, therefore, be concluded that a definite fractionation of the total globulins of cowpea has been achieved by progressive saturations with ammonium sulphate.

Further confirmation of the existence of several fractions was obtained by a determination of the temperatures of coagulation, each fraction having an optimum range of temperature.

The total globulins obtained by dialysis of the saline extract was reprecipitated in 4 per cent. saline solution and filtered. The clear filtrate

was heated in a bath whose temperature was gradually raised at the rate of about  $0.5^{\circ}$  per minute. The temperature at which the slight turbidity or opalescence appeared was noted and maintained for  $\frac{1}{2}$  hour to facilitate complete coagulation of the particles. The appearance of the turbidity could be sharply observed against dark background with a diagonally oriented illumination.

After the coagulation of the particles, the solution was filtered off and the filtrate subjected to heating at higher temperatures, the same experimental procedure being adopted. The turbidity which appeared at the next higher temperature indicated the presence of another fraction and it was thus possible to show the existence of several fractions in the total globulins of the cowpea. Table X gives the results of a study of the coagulation temperatures of the various fractions together with their yields from a litre of 4 per cent. saline solution of the globulins.

TABLE X.

Fraction	A	B	C	D	E
Temperature of coagulation ..	52-53°	71-72°	78-79°	88-89°	95-96°
Yield (in g.) ..	0.2	1.4	1.0	7.0	6.55

It is clear from the table that the cowpea consists of a greater proportion of the fraction coagulating at higher temperatures. This is in harmony with the findings of Osborne who has indicated the presence of difficultly coagulable globulins. A detailed physico-chemical study of these fractions will form the subject of a future communication.

### SUMMARY.

1. The total globulins of the cowpea (*Vigna catiung*) have been isolated by four different methods, electro dialysis, hydrodialysis, dilution and saturation with ammonium sulphate. A nitrogen distribution of the four preparations, according to the method of Van Slyke and an independent determination of some of the essential amino acids, have been carried out. The results definitely indicate the presence of two or more fractions rich in cystine.

2. The globulins of the cowpea have been fractionated by saturating the saline solution with ammonium sulphate to different degrees and the three fractions thus obtained have been found to be distinctly different from one another as revealed by the content of their essential



amino acids. The first fraction is the richest not only with regard to its cystine content but also with respect to the other three essential amino acids, arginine, tyrosine and tryptophane.

3. Further confirmation of the existence of the several fractions has been obtained by a determination of the temperatures of coagulation each of the fractions precipitating within a definite range of temperature.

4. The greater proportion of globulins of the cowpea appears to be characterised by its high thermostability.

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*Department of Biochemistry,  
Indian Institute of Science,  
Bangalore.*

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