CLVII. PROTEINS AND OTHER NITROGENOUS CONSTITUENTS OF WATER MELON SEEDS (*CITRULLUS VULGARIS*)

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THIS study was undertaken for the following reasons: (i) The seeds of the water melon are of some nutritional importance as they form an article of diet in certain parts of India. (ii) They are a potent source of urease [Damodaran & Sivaramakrishnan, 1937] and a study of the individual proteins present is of obvious interest in view of the suggested protein nature of this enzyme [Sumner, 1932]. From the point of view of their use in urea determinations it is also necessary to ascertain the presence among the non-protein nitrogenous constituents of the urea-producing bases arginine and canavanine. (iii) Citrulline was isolated by Wada [1930] from water melon fruits and the possibility existed that this interesting amino-acid might be present, free or combined, in the seeds.

The investigation followed conventional lines except that some experiments were carried out to ascertain the nature of the non-protein and non-extractable nitrogenous material.

The chief protein of the seed was readily isolated in crystalline condition from the NaCl extract in yields representing about 27 % of the weight of the fat-free seed meal. A glutelin was prepared in the usual way from the alkaline extract and amounted to 0.9 % of the meal. In the preparation of the glutelin, innovations suggested recently for cereals [Jones & Csonka, 1927; 1928], based upon the separation of globulin and glutelin from an alkaline extract containing both by fractional precipitation with ammonium sulphate, were tried without success, only mixtures of the two proteins being obtained by this method. It was found that the water melon globulin had an isoelectric point of pH 6.5 and that it was precipitated from a solution in 0.2 % NaOH by ammonium sulphate in a concentration as low as 0.0125 of saturation; thus the two criteria suggested by Jones & Csonka for the differentiation of globulin from glutelin do not appear to be of general validity.

Of the nitrogen left in the seed meal after removal of material soluble in water and NaCl only a fraction (16.5 %) was extractable with 0.2 % NaOH. On digesting the residue after NaCl extraction with pepsin it was found that nearly 90 % of the nitrogen went into solution, indicating that the unextracted material was in any case protein in nature. Further information about this fraction was obtained by submitting to acid hydrolysis the residue after NaCl extraction as well as the residue after treatment with NaOH. A large amount of humin material was naturally formed. But on analysing the two filtrates by the nitrogen distribution method it was found that the results were sufficiently in agreement with each other and with those for the isolated glutelin to justify the conclusion that the nitrogenous matter unextractable by NaOH was of the same nature as the isolated glutelin.

Neither the globulin nor the glutelin possessed any urease activity. But the aqueous extracts of the seeds contained small quantities of a protein which could be precipitated at about pH 4.5 and which in the crude state showed strong urease activity. On account of the difficulty experienced in obtaining purified preparations with constant activity further study of this protein had to be reserved for a separate investigation. The non-protein-N of the aqueous extract was found to consist mainly of complex material (proteoses and peptones), simple substances not precipitable by phosphotungstic acid accounting only for a small fraction of the total N extractable by water. The amount of free arginine present was extremely small (5 mg. in 100 g. of fat-free meal) and therefore incapable of causing significant error in urea determinations in which water melon seeds are used as the source of urease.

EXPERIMENTAL

1. Preparation of material

Analysed according to the standard methods of the Association of Official and Agricultural Chemists, the seed contained on the average 5.5 % moisture, 3.8 % ash, 5.5 % of ether-extractable fat and 5.4 % of N. For all extractions the defatted meal was used. The shell-free seeds were ground up in an end-runner to a fine pasty mass, shaken up five or six times with acetone, washed with light petroleum at the pump and dried in a current of air at room temperature. This material contained 11.8 % moisture and 11.73 % N (on dry weight).

2. Nitrogen partition in different protein solvents

The defatted material was exhaustively extracted with water, 10 % NaCl and 0.2 % NaOH in succession. Precipitation with trichloroacetic acid was used for distinguishing between protein and non-protein-N in the extracts (Table I).

Table I. N partition in different solvents

	Total N in extract (as % of N of seed meal)	Non-protein-N (as % of total N of extract)
Water	12.64	50.15
10 % NaCl	77.91	5.98
0.2 % NaOH	1.56	—
Residue	7.83	—

3. Aqueous extract

(a) *Fractionation according to Wasteneys and Borsook.* A water extract of the meal (containing about 0.7 g. N per 100 ml.) was analysed according to the scheme of fractionation of Wasteneys & Borsook [1924] for incomplete protein hydrolysates (Table II). Instead of the "sub-peptone" fraction of these authors, which was obtained by precipitation of the filtrate from tannic acid treatment with alcohol, material precipitable by phosphotungstic acid in the presence of 5 % H₂SO₄ was determined.

The residual filtrate, containing presumably amino-acids (other than basic) and simple peptides not precipitable by phosphotungstic acid, gave positive reactions with ninhydrin, Folin's phenol reagent (tyrosine and tryptophan) and Folin's uric acid reagent (cystine). Tests for citrulline (dimethylamino-benzaldehyde and vanillin hydrochloric acid [Wada, 1930]), canavanine (irradiated nitroprusside [Kitagawa & Yamada, 1932]), and tyrosine (Millon's reaction) were negative.

Table II. *Nature of the nitrogenous constituents in the aqueous extract*

Form	Reagent	Nitrogen (as % of total N in extract)
Protein	Trichloroacetic acid	49.86
Metaprotein	Adjustment to pH 6	Nil
Proteose	Sodium sulphate	28.05
Peptone	Tannic acid	8.93
Basic	Phosphotungstic acid	0.63
Residual	—	12.52

In the filtrate from the tannic acid precipitate arginine was determined by the method of Klein & Taubock [1932]. Excess tannin was removed by means of lead and the latter by means of H_2S . The quantity of arginine found corresponded to 4.88 mg. per 100 g. of seed meal. The solution gave no tests for citrulline or canavanine.

(b) *N distribution after hydrolysis.* The aqueous extract was concentrated *in vacuo* to contain about 600 mg. N per 100 ml. It was refluxed for 16 hr. with 2 vol. of conc. HCl and the main forms of N determined (Table III).

Table III. *Nitrogen distribution on the hydrolysed aqueous extract*

	% of total N
Amide-N	8.46
Humin-N	0.52
Basic amino-N	15.66
Basic non-amino-N	20.81
Monoamino-N	47.95
Non-amino-N	5.45

4. Sodium chloride extract

(a) *Preparation of the globulin.* The defatted meal, after thorough water extraction, was extracted twice with warm 10 % NaCl (5 ml. for each g. of seed meal) by shaking for 2 hr. each time and the combined extracts filtered through paper pulp. The clear filtrate which had a pH of 6.5 was diluted with vigorous stirring with 15 times the volume of distilled water at 40° when immediate precipitation of the protein took place. After allowing to settle overnight in the ice chest the precipitate was separated by centrifuging, redissolved in the minimum amount of 10 % NaCl at 40°, filtered through pulp and reprecipitated by dilution as before. The protein was separated on the centrifuge, washed free from chloride using ice-cold distilled water saturated with CO_2 for the first two washings and graded strengths of acetone afterwards, finally dehydrated with pure acetone and dried at room temperature. The twice precipitated sample which represented 27 % by weight of the defatted material contained 18.6 % N on moisture-free basis and was already pure. Further treatment by dissolving in NaCl and reprecipitation by dilution effected no change in the nitrogen content. The pure protein had an ash content of 0.07 % and analysed as follows: C, 48.12; H, 6.06; N, 18.60; S, 1.02; O, 26.20 %. Molisch's test and the test for phosphorus were negative.

(b) *Crystallization of the globulin.* The protein separating out from the NaCl solution on dilution was in the form of spheroids. Dialysis also yielded a similar product. Crystalline preparations were however readily obtained by the following method: 10 g. of the defatted material were added with vigorous stirring to 160 ml. of 2 % NaCl at 60° and immediately filtered by suction. The protein which began to separate in the filter flask was redissolved by warming to 50° in a water bath, the solution again filtered rapidly, placed in a water bath

at 50° and allowed to cool down slowly to room temperature and then left overnight in the ice chest. The globulin which separated out was composed wholly of microscopic octahedral crystals.

(c) *Nitrogen distribution.* The Van Slyke procedure was followed with the following modifications:

(i) Amide-N was determined by distillation for 4½ min. in the Parnas-Wagner apparatus.

(ii) The dicarboxylic acid-N was estimated according to Damodaran [1931].

(iii) The precipitate of the bases with phosphotungstic acid was dissolved in Winterstein's acetone-water mixture (4:3 vol.) and the phosphotungstic acid removed by means of baryta. No solubility correction was applied to the values for the constituents of this fraction.

(iv) Arginine was determined in the solution of the bases, after removal of acetone by distillation and of Ba by H₂SO₄, by the colorimetric method of Klein & Taubock [1932]. Arginine was also determined in a separate hydrolysate without treatment with phosphotungstic acid by three independent methods, viz. by colorimetry [Klein & Taubock, 1932], by precipitation with flavianic acid [Kossel & Gross, 1924] and by means of arginase [Hunter & Dauphinee, 1930]. All three methods yielded concordant results and the average was in excess of the value obtained from the phosphotungstic acid precipitate by an amount that could be accounted for by the solubility factor for arginine phosphotungstate as given by Van Slyke.

(v) Histidine was estimated in the basic fraction both by the usual calculation from the non-amino-N and by a colorimetric method [Hanke & Koessler, 1920]. The value obtained by the two methods agreed within the limits of experimental error.

(vi) No cystine or sulphur was found in the solution of the bases [cf. Damodaran & Sivaswamy, 1936]. Cystine was estimated in a separate hydrolysate by the method of Folin & Marenzi [1929], as modified by Tompsett [1931], and also by the Sullivan method, as modified by Prunty [1933], pure cystine being used as standard in both cases.

In addition to the amino-acids included in the usual Van Slyke scheme tyrosine and tryptophan in the protein were determined according to Folin & Marenzi [1929]. The results are given in Table IV.

Table IV. *Analysis of the globulin*

	% of total N	
	N distribution	Separate determinations
Amide	8.09	—
Humin	0.42	—
Dicarboxylic acid	17.11	—
Arginine	28.03	30.52*; 30.05†; 29.95‡
Histidine	1.47; 1.42§	—
Cystine	(Not found in the P.T.A. precipitate)	0.78 ; 0.76¶
Lysine	5.23	—
Monoamino-monocarboxylic acid	35.71	—
Non-amino	4.81	—
Tyrosine	—	1.92
Tryptophan	—	1.37

* Klein & Taubock [1932].

† Hunter & Dauphinee [1930].

|| Tompsett [1931].

‡ Kossel & Gross [1924].

§ Hanke & Koessler [1920].

¶ Prunty [1933].

5. *Sodium hydroxide extract*

(a) *Preparation of the glutelin.* 200 g. of the defatted seed meal were extracted with water and NaCl solution successively until no more protein was removed, as shown by the absence of turbidity in the final extracts on the addition of trichloroacetic acid. The residue (39 g. containing 5.66% N by dry weight), after being washed free from NaCl with water, was extracted eight times by shaking with 0.2% NaOH, 50 ml. lots being used for each extraction which lasted an hour. At the end of each extraction the liquid was centrifuged and the centrifugate filtered through paper pulp. To the clear filtrate 0.2% HCl was slowly added with stirring till the pH of the solution was 5.9 (chlorophenol red), which was found by trial on a number of samples to be the point of maximum precipitation.

The absence of any precipitate or turbidity on adjusting the pH to 6.5 (the isoelectric point of the globulin) can be taken to prove that no globulin was present in the solution. Each extract was brought to pH 5.9 with acid immediately after filtration in order to prevent the protein being left in long contact with alkali. The precipitates were combined, redissolved in the minimum amount of dilute alkali and reprecipitated after filtration as before; this process was repeated three times more, a sample being washed and dried for analysis after each precipitation. The five times-precipitated sample was used for elementary analysis and the three times-precipitated one for nitrogen distribution. The nitrogen content (% of dry weight) after successive precipitations was 14.95, 15.02, 15.11, 15.15 and 15.15 respectively, the values being unaffected after the third precipitation.

The protein did not exhibit any tendency to disperse in the water used for washing it free from chloride. It was dehydrated with acetone and dried at room temperature under a fan. The yield of the glutelin was 1.76 g. It gave a negative Molisch test but contained phosphorus. The five times-precipitated sample after air-drying had a moisture content of 11.51% and 0.59% ash (calculated on the dry weight of protein). After drying *in vacuo* over P₂O₅ at 100° it analysed as follows: C, 47.80; H, 5.98; N, 15.15; S, 0.98; P, 0.12; O, 29.97%. Sulphur was determined by two methods, by fusion with sodium peroxide and by Pirie's method [1932], the values obtained being 0.95 and 0.98%. Phosphorus was determined by the colorimetric method of Fiske & Subbarow [1925] on a sample of the protein which was extracted with methyl alcohol, acetone, light petroleum and finally ether.

Table V. *Analysis of the glutelin*

	% of total N	
	N distribution	Separate determinations
Amide	7.26	—
Humin	2.06	—
Dicarboxylic acid	13.15	—
Arginine	14.85*	15.18*
Histidine	4.41	—
Cystine	0.17†	2.85‡; 2.75†
Lysine	4.73	—
Monoamino monocarboxylic acid	45.43	—
Non-amino	7.89	—
Tyrosine	—	1.63
Tryptophan	—	1.08

* Klein & Taubock [1932].

† Prunty [1933].

‡ Tompsett [1931].

(b) *Nitrogen distribution.* The procedure adopted was the same as in the case of the globulin with slight differences. 1 g. samples of the protein were taken for hydrolysis instead of 3 g., and all volumes and reagents correspondingly reduced. The precipitate of the bases with phosphotungstic acid was dissolved in NaOH and the phosphotungstic acid removed by means of baryta. Arginine determinations carried out in this solution and in a separate hydrolysate by the method of Klein & Taubock gave values which did not differ appreciably from each other.

6. Unextracted nitrogenous material

(a) *Action of pepsin.* As only one-sixth of the nitrogen of the residue after NaCl extraction was extractable by dilute alkali the action of pepsin on this residue was tried to ascertain the nature of the nitrogenous material present. 5 g. of the residue after sodium chloride extraction were ground intimately with 0.05 *N* HCl, and made up to 250 ml. with 0.05 *N* HCl containing 0.25 g. of pepsin. After standing at 37° for one week the digest was filtered, the insoluble material washed free from chloride, and the filtrate and washings analysed for nitrogen content. 89.31 % of the nitrogen in the residue was found to have gone into solution under the action of pepsin. The insoluble material weighed 1.4 g., representing 5.98 % of the weight of seed meal, and contained 1.83 % N (on dry weight).

(b) *Hydrolysis of the residues.* As the digestion with pepsin also failed to bring the whole of the nitrogen into solution, it was thought desirable to analyse the residues after NaCl extraction and after extraction with NaOH, and compare the values with those obtained with the glutelin. The results are given in Table VI.

Table VI. *Analysis of the residues before and after extraction of the glutelin*

	% of total N	
	Residue after NaCl extraction	Residue after NaOH extraction
Amide	7.30	7.43
Humin	3.41	3.43
Arginine	14.98*	14.75*
Histidine	4.11	4.49
Cystine	(Not found in the P.T.A. precipitate)	
Lysine	4.94	4.67
Monoamino	57.40	57.96
Non-amino	7.45	7.06
Tyrosine	1.67	1.74
Tryptophan	1.00	1.004
Cystine	2.98†; 2.71‡	2.92†; 2.70‡

* Klein & Taubock [1932].

† Tompsett [1931].

‡ Prunty [1933].

SUMMARY

1. The nitrogen of water melon seeds is shown to be made up of: glutelin, 9.4 % (extractable 1.6 %, not extractable 7.8 %); globulin, 73.2 %; water-soluble protein, 6.3 %; proteoses, 3.5 %; peptones, 1.1 %; material precipitable by phosphotungstic acid, 0.1 %; simpler substances not precipitable by phosphotungstic acid, 1.6 %.

2. The globulin and the glutelin have been isolated in pure, the former in crystalline condition, and the most important amino-acids in the two proteins determined. Independent determinations of arginine and histidine have borne

out the accuracy of the values for these amino-acids obtained by the nitrogen distribution method.

3. The seeds do not contain canavanine or citrulline, either free or combined, and only traces of free arginine.

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