Amino Acids, Peptides, and Proteins of Irish Moss, *Chondrus crispus**

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The insolubility of algal proteins has made their separation and purification difficult so that, at present, only crude products have been studied (1–5). Phycocyanin and phycoerythrin from *Ceramium rubrum* and other species of red algae, are exceptions. A few simple peptides have been detected in several species of marine algae (6, 7). In *Fucus vesiculosus*, free amino acids accounted for 10 per cent of the total nitrogen, simple peptides for 7 to 8 per cent, and volatile bases for 2 to 3 per cent (8). The nitrogenous compounds in algae include proteins at 60 to 70 per cent, and other forms, such as free amino acids, peptides, organic bases, etc., at 10 to 33 per cent (9).

Combined citrulline and ornithine have been detected in the red alga, *Chondrus crispus* (9). This unusual finding deserved further investigation. Attempts to fractionate the amino acids, peptides, and proteins of *Chondrus* are described in this report. Ornithine and citrulline have been established as occurring both in the free state and in simple and complex peptides but not in the insoluble protein. This protein is now shown to have a distribution of amino acids very similar to that in other algae.

EXPERIMENTAL

Specimens of *C. crispus* were collected in bulk locally, in March or April, dried in a current of air at 30° in about 3 hours, crushed, and passed through a Wiley mill and screen of 100 mesh. This material had a moisture content of about 10 per cent and was preserved in this form. The extraction of nitrogenous material from this preparation was followed by the micro-Kjeldahl procedure or by the quantitative biuret reaction (10) on aliquots clarified when necessary by centrifuging at 18,000 r.p.m. for a few minutes in the multispeed attachment of the refrigerated International centrifuge. Dialysis was carried out in cellophane tubing at 4° with mechanical stirring of the internal fluid.

Procedure I

A portion of the powdered plant was suspended in a solution of 0.1 per cent sodium borate in 10 per cent sodium chloride, pH 8.4, and extracted for several hours with occasional mixing in a Servall Omni-Mixer at 4°. The insoluble residue was separated by centrifugation and the extraction was repeated with fresh buffer until only a trace of nitrogen was obtained in the solvent. The Potter-Elvehjem homogenizer was found to be of no use in this extraction because of the leather-like character of the particles. The first extract usually contained about 50 per cent of the total nitrogen in the powder, the second 15 per cent, and the remainder 5 per cent or less.

The combined saline borate extracts were stirred mechanically at 4°, and an equal volume of an anhydrous mixture of ethanol-ether (4:1) was added slowly. A gelatinous precipitate of carrageenin formed and was removed by centrifugation after 1 hour. It carried down 10 to 16 per cent of the soluble nitrogen.

The supernatant fluid was distilled *in vacuo* at 35° to the appearance of solid sodium chloride. The concentrate was adjusted to pH 5.1 and a small precipitate was removed. The filtrate was dialyzed until free from chloride. The percentage of dialyzable nitrogen was 75 to 96 of that in solution.

Nondiffusable Portion of Saline-Borate Extract—The fluid in the dialyzer was divided into two portions. One was made half saturated with ammonium sulfate at pH 4.8 without any precipitation. The concentration was raised to full saturation and a flocculent precipitate formed. It was recovered by centrifugation.

Another portion was concentrated *in vacuo* to a small volume, clarified by centrifuging at 13,000 r.p.m., and examined in the ultracentrifuge and by paper chromatography. This nondialyzable, soluble fraction showed polydispersity and slow sedimentation in the ultracentrifuge. This suggested the presence of peptides. Five spots appeared in one-dimensional paper chromatograms with Whatman No. 4 paper, butanol-acetic acid as solvent (11), and a ninhydrin reagent as spray (12). Two faint spots with higher R_F values were seen on chromatograms with maximal loading. On two-dimensional chromatograms with butanol-acetic acid as developing solvent in both directions, six to eight spots in the lower R_F range and one or two spots in the higher R_F range appeared. After hydrolysis with hydrochloric acid at 105° and removal of the acid by evaporation *in vacuo*, the residue was chromatographed in two dimensions with 80 per cent phenol in an atmosphere of 0.3 per cent ammonia and with butanol-acetic acid. The acid hydrolysate contained citrulline, ornithine and all of the amino acids previously encountered in combined form in *Chondrus*. In addition, there was an unknown faint spot near serine.

Insoluble Residue—The residue after extraction with borate buffer contained 40 per cent protein (protein N × 6.25). After repeated extraction with boiling water to remove residual κ-carrageenin, the insoluble material was recovered in the centrifuge at 2000 r.p.m. The gelatinous green residue was suspended in a mixture of absolute ethanol and ether (4:1) and stirred mechanically overnight. It was centrifuged, treated repeatedly with absolute acetone, and finally dried *in vacuo* to constant weight. This material contained 8.57 per cent of nitrogen, equivalent to 53.5 per cent of protein.

The distribution of nitrogen in these extracts is shown in Table

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TABLE I

Distribution of nitrogen in fractionation of Chondrus (Procedure I)

Fraction	Nitrogen	
	mg.	%
Dry plant powder (6.7 gm.)	344	100
Soluble in borate buffer, pH 8.4	(267)*	(78)
Precipitated with carrageenin by ethanol	31	9.0
Insoluble in NaCl (saturated), pH 5.1	91	5.6
Dialyzed	198	57.5
Insoluble in $(NH_4)_2SO_4$ (saturated), pH 4.8	9	2.6
Extracted by hot water	16	4.7
Insoluble residue	53	15.4
Totals	326	94.8

* Figures in parentheses are not included in totals.

I and the content of amino acids in the insoluble residue is shown in Table II as Residue 1.

Procedure II

The same initial treatment was used on a fresh collection of *Chondrus*. 10 gm. of the powder were extracted with 75 per cent ethanol for 24 hours at 4° in a Burrell Wrist-Action shaker. A green solution was obtained by centrifuging at 1500 r.p.m. at 0°. The extraction was repeated with fresh solvent.

The green alcoholic extract was concentrated *in vacuo* at 40° to incipient precipitation, clarified by adding a little ethanol, and extracted with ether which removed the green pigment. The ethereal extract contained a negligible amount of nitrogen. The slightly yellowish aqueous solution was dialyzed against water for 24 hours, and 72 per cent of the nitrogen passed through the membrane. The fluid in the dialyzer was concentrated *in vacuo* at 40° almost to dryness. It was diluted with water to 10 ml., clarified by centrifugation at 15,000 r.p.m., and examined chromatographically. The results were similar to those obtained with the nondialyzable portion from the saline-borate extraction described above under Procedure I.

The insoluble material was extracted successively with four lots of borate buffer at 0° with continuous mechanical stirring. The combined extracts were dialyzed against distilled water until free from chloride; 77 per cent of the nitrogen in solution was dialyzable. The distribution to this point is shown in Table III. To the fluid in the dialyzer was added an equal volume of the ethanol-ether mixture without any precipitation of carrageenin. The organic solvents were removed by distillation *in vacuo* at 40°. The aqueous solution was acidified with dilute hydrochloric acid to pH 2.5, diluted to 1500 ml. and treated with a 1 per cent solution of cetyl-trimethyl-ammonium bromide in an attempt to remove carrageenin by the method of Scott (13). Only a small, flocculent precipitate was obtained and this was discarded. The excess of quaternary salt was removed by adding 1 per cent potassium iodide until no further precipitate formed. The crystalline precipitate was removed by centrifugation. The supernatant fluid was made alkaline to pH 8.7 and repeatedly extracted with chloroform. The aqueous layer was concentrated *in vacuo* at 40° to small volume, made to 25 ml. with water, clarified in the centrifuge at 15,000 r.p.m., and examined chromatographically; the results were similar to those for the nondialyzable ethanolic extract.

The distribution of amino acids in the insoluble residue at this stage is shown in Table II as Residue 2-A. Since the insoluble residue still contained 51 per cent of the nitrogen in the original powder, more drastic efforts were made to extract it. The residue was treated for 15 hours with 100 ml. of a 2 M solution of

TABLE II

Distribution of amino acids in insoluble residues of extraction of Chondrus (amino acid N as percentage of total N in hydrolysate)

Amino acid	Whole plant*	Insoluble residue†				Ratio, insoluble Residue 1 to whole plant	*Chlorella vulgaris*‡
		1	2-A	2-B	2-C		
Alanine	3.8	7.5	7.0	6.8	7.2	2.0	7.7
Arginine	33.6	16.9	16.8	14.4	17.4	0.5	15.8
Aspartic acid	3.8	7.1	6.8	7.3	6.6	1.9	6.4
Citrulline	5.8	0	0	0	0		0
Cystine							0.2
Glutamic acid	4.1	5.9	6.0	5.6	6.2	1.4	7.8
Glycine	3.5	6.1	5.7	5.8	6.2	1.7	6.2
Histidine	0.9	2.9	2.8		0.6	3.2	3.3
Isoleucine	1.8	3.5	3.3	3.4	3.5	1.9	3.5
Leucine	2.9	5.8	5.5	5.3	5.8	2.0	6.1
Lysine	4.9	8.4	6.7	7.6	7.8	1.7	10.2
Methionine	0.5	1.0	0.9	0.5	0.6	2.0	1.4
Ornithine	7.1	0	0	0	0		0
Phenylalanine	1.5	3.1	3.0	3.2	3.1	2.1	2.8
Proline	1.9	4.0	4.2	4.1	4.5	2.1	5.8
Serine	2.2	4.1	4.2	3.9	4.1	1.9	3.3
Threonine	2.2	4.1	4.1	4.1	4.3	1.9	2.9
Tyrosine	1.0	2.3	2.3	1.4	2.1	2.4	2.8
Valine	2.7	5.0	5.1	4.8	5.2	1.9	5.5
Amide N	10.1	9.4	12.3	14.4	11.9	1	6.1
Total	94.3	97.4	96.7	92.6	97.1		97.8
Total N§	3.42	8.57	8.12	7.37	4.16		11.5
Protein (N × 6.25)	21.4	53.5	50.7	46.1	26.0		71.8

* Whole plant, after extraction with 75 per cent ethanol.

† Insoluble Residue 1, after saline-borate extraction; 2-A, after extraction with 75 per cent ethanol and saline-borate; 2-B, after extraction with a quaternary salt, urea, Versenate (ethylenediaminetetraacetate), and sodium lauryl sulfonate; and 2-C, after multiple freezing and thawing and extraction with 0.2 per cent sodium hydroxide.

‡ Fowden (4).

§ Figures for total N represent that amount present in the particular preparation as percentage of the dry matter and from which the content of protein was calculated.

TABLE III

Distribution of nitrogen in fractionation of Chondrus (Procedure II)

Fraction	Nitrogen		
	Amount	Distribution	Dialyzable
	mg.	%	%*
Dry plant powder (10 gm.)	440	100	
Ether extraction	0.7	0.2	
Ethanol extraction	80	18	72
Borate extraction	137	31	77
Insoluble residue (by difference)	222	51	

* Per cent of N in the particular extract prior to dialysis.

urea. The mixture was separated in the centrifuge at 1500 r.p.m. and the supernatant fluid was examined for protein by the biuret reaction. The amount dissolved was neligible. The insoluble, washed residue was extracted twice with a 0.1 per cent solution of disodium ethylenediaminetetraacetate in 1 per cent sodium chloride at pH 8.2. A negligible amount of protein was dissolved by this treatment. The residue was repeatedly washed with distilled water. It was then mixed with a 2 M solution of potassium thiocyanate without extracting any protein. The residue was again washed with water and extracted unsuccessfully with a dilute solution of sodium lauryl sulfate at pH 5.0 according to Stainsby *et al.* (14). An aliquot at this point was extracted with acetone and dried *in vacuo*. It contained 45 per cent of protein and was examined chromatographically after acid hydrolysis. The results are shown in Table II as Residue 2-B.

On the assumption that the residual protein might be firmly held within the chloroplasts, the moist, washed residue was frozen and thawed about 30 times. A negligible amount of nitrogen could then be dissolved in water. The residue was next extracted with 0.05 N sodium hydroxide with mechanical shaking. This dissolved 25 mg. of the calculated 200 mg. of nitrogen remaining. Finally, the residue was neutralized with 0.05 N hydrochloric acid, washed free from chloride, dehydrated with absolute ethanol and ether, and dried *in vacuo*. The final dry powder still contained 26 per cent of protein. This material was examined chromatographically after hydrolysis and the results are shown in Table II as Residue 2-C.

TABLE IV

Distribution of amino acids in various extracts of Chondrus (amino acid N as percentage of total N in solution or hydrolysate)

Amino acid	Whole plant*	Ethanolic extract (1955)		Ethanolic extract (1956)
		Before hydrolysis	After hydrolysis	Before hydrolysis
Alanine	3.8	0.5	0.6	0.9
Arginine	33.6	16.0	26.9	15.8
Aspartic acid	3.8	0.5	0.5	0.2
Citrulline	5.8	8.7	4	7.2
Glutamic acid	4.1	2.2	2	2.2
Glycine	3.5	0.2	2.0	0.2
Histidine	0.9			
Isoleucine	1.8	0.1	0.1	0.2
Leucine	2.9	0.1	0.1	0.1
Lysine	4.9			
Methionine	0.5	0.1	0.1	0.1
Ornithine	7.1	0.1	13.0	0.2
Phenylalanine	1.5	0.8	0.8	1.1
Proline	1.9			
Serine	2.2	0.8	0.4	0.4
Taurine		2.6	2.7	3.0
Threonine	2.2	0.1	0.3	0.2
Tyrosine	1.0			
Valine	2.7		0.1	0.2
Amide N	10.1	2.8	16.9	2.2
Total	94.3	35.6	70.5	34.2
Amino N		32	66	

* After extraction with 75 per cent ethanol.

Attempted Liberation of Protein by Carrageenase

Since only about 50 and 78 per cent of the original N in the plant can be extracted by 75 per cent ethanol and borate buffer, an attempt was made to dissolve more by previous digestion with κ-carrageenase. This was based on the assumption that protein might be held in an insoluble form in combination with the acidic sulfated polysaccharide, carrageenin. The residue, after extraction with borate buffer, was suspended in 0.034 M phosphate buffer, pH 7.1, and a solution of bacterial κ-carrageenase was added (15). Toluene was used as preservative. The mixture was stirred mechanically for 24 hours at 23° and centrifuged, and the residue was treated with fresh solutions of buffer and enzyme. The combined solutions contained 21 per cent of the nitrogen originally present in the plant, after correction for N in the enzymic preparation. Hydrolysis of the κ-carrageenin present thus liberates an appreciable portion of the nitrogen.

Analyses of Amino Acids in Various Preparations

Ethanolic Extracts—A portion of ethanolic extract, obtained as described under Procedure II but before dialysis, was evaporated to dryness *in vacuo*. The residue was dissolved in water to contain about 6 mg. of N per ml. It was clarified by centrifuging at 18,000 r.p.m. A portion of the solution was hydrolyzed with an equal volume of concentrated hydrochloric acid by boiling under reflux for 40 hours. Excess acid was removed by repeated distillation *in vacuo*. The residue was dissolved in water as above, adjusted to pH 4.5 with silver oxide, and centrifuged until clear. Aliquots were analyzed quantitatively by the technique of ion exchange (16) and qualitatively by two-dimensional chromatography on paper (17). The distribution of amino acids is shown in Table IV. A number of unknown ninhydrin-positive components were observed and three unidentified peaks appeared on the effluent curve from the long column of Dowex 50 resin. They were not altered by hydrolysis, but a further component, as yet unidentified, appeared just before the glycine.

It is thus evident that a number of free amino acids, including ornithine, are present in *Chondrus* and that arginine, glycine, threonine, ornithine, and possibly the unknown component are present as peptides, soluble in 75 per cent ethanol. Part of the ornithine was probably present originally as citrulline, as explained below. The free amino acids constituted only 36 per cent of the total N present and it is surprising that only 35 per cent was added after acid hydrolysis. Thus only 70.5 per cent of the nitrogen present in the ethanolic extract is accounted for as amino acids, peptides, and ammonia. The amino nitrogen in the extract would be 18 per cent of the total N by calculation from the free amino acids determined. It would be 48 per cent in the hydrolysate on a similar basis. The amino N found was 32 and 66 per cent, respectively, by microformol titration. The difference can probably be explained by the presence of free amino groups in the peptides before hydrolysis and of unknowns, for which there is evidence from chromatography.

Attempts to identify the unknown components in the ethanolic extract by two-dimensional chromatography were unsuccessful because of distortion of the normal patterns. Circular paper chromatography (18) gave clear patterns, but resolution of the mixture was not sufficient to permit identification of all amino acids present.

Since the presence of free citrulline was indicated by the dis-

tortion of the peak in the usual position of glutamic acid, a sequence of separations on resin columns followed by paper chromatography was tried. An aliquot of the ethanolic extract was passed through a 100 cm. column of Dowex 50. The desired peaks were located in the effluent and the fractions which comprised the peak were combined and desalted on a column of Dowex 2-X10 (19). The salt-free fractions of the effluent were collected in a 50 ml. centrifuge tube, previously coated with a silicone preparation (Desicote, Beckman). The liquid was evaporated on a water bath in a current of warm air. The tube was placed in a desiccator and a high vacuum was maintained for several hours with an oil pump. The residue was dissolved in 0.1 or 0.2 ml. of water and chromatographed on paper in two dimensions. The peak in the position of glutamic acid was thus resolved into three components, citrulline, glutamic acid, and one which gave a faint spot slightly to one side and ahead of the latter in the phenol direction. This spot gave no color with Ehrlich's reagent. The concentration of citrulline was at least equal to that of glutamic acid. In attempting to confirm the presence of citrulline, it was discovered that added citrulline was partially converted to ornithine under the usual conditions of hydrolysis with hydrochloric acid. Repetitions of the conditions of hydrolysis with pure citrulline indicated a conversion of citrulline to ornithine of 75 per cent or more. This result confirms the observations of Miettinen and Virtanen (20).

Another peak which occurred at 38 ml. on the effluent curve from the resin column was identified as taurine and found to give an R_F value on paper which corresponded to it. The presence of taurine was further confirmed by the coincidence of added taurine with the spot in question. The substance also gave an intense red spot under ultraviolet radiation when the paper was dipped in an acetone solution of *o*-phthalaldehyde and then in alcoholic potassium hydroxide (21).

A fresh ethanolic extract was passed through resin columns and reexamined with the modified ninhydrin reagent of Moore and Stein (22). This reagent was not satisfactory for the present purpose and was modified by omission of hydrindantin and the addition of stannous chloride in the proportion of the earlier reagent (23). The blanks were low and relatively constant. The extract contained 24 per cent of the total N of the sample of *Chondrus*. The results were essentially the same as previously found and are shown in Table IV. The incomplete separation of the peaks of citrulline and glutamic acid was resolved by calculation as proposed by Stein and Moore (24).

Insoluble Protein—The residual and highly insoluble protein, after prolonged extraction as described above, remained essentially the same in its distribution of amino acids. As a result of extraction with saline-borate buffer, the values for most amino acids were doubled, all citrulline and ornithine was removed, and the value for arginine was reduced by half, as shown in Table II. These figures are a better picture of the composition of the basic protein of *Chondrus* than are those of the whole plant after ethanolic extraction. Figures for the latter in Table II differ from those published previously (9) only in minor degree, except for appreciably higher levels of arginine, citrulline, lysine, and ornithine. The total recovery was 94 per cent of the nitrogen in the hydrolysate as against 79 per cent.

DISCUSSION

The data of Table IV lead to certain definite conclusions. It is apparent that traces of most of the common amino acids were extracted from *Chondrus* by 75 per cent ethanol and must have occurred free in the plant as collected in the Spring. Arginine, citrulline, glutamic acid, and taurine were each present as more than 1 per cent of the nitrogen in solution. About 75 per cent of the ethanolic extract was dialyzable. Arginine, citrulline, ornithine, glycine, and glutamic acid were present as simple peptides, soluble in ethanol. This conclusion is supported by previous findings of alanine, arginine, and glutamic acid in algal peptides (6, 7). Free ornithine and acetylornithine have been reported in several plants (20, 26, 27). Ornithine is known to occur in two natural peptides, tyrocidine (28), and bacitracin A (29). The tendency to conversion of citrulline to ornithine and ammonia in 20 per cent hydrochloric acid at 110° introduces some uncertainty as to the figures for these amino acids in the plant. Some spots on the two-dimensional chromatograms and three peaks on the effluent curve from the long resin column remain unidentified.

The occurrence of free taurine in *Chondrus* is not unusual since it has been detected in several algae (30, 31, 25).

Saline borate buffer of pH 8.4 proved to be an effective solvent, and on one occasion dissolved as much as 78 per cent of the plant nitrogen. The dissolution of only traces of protein, however, is noteworthy. The extraction of much larger quantities of the plant will be required to characterize the proteins which are precipitable with saturated salt solutions.

Except for the level of arginine, the composition of the residual protein resembles the analyses for the proteins of *Rhodymenia palmata* and *Ulva lactuca* previously published (9). The resemblance to the protein of the unicellular green alga, *Chlorella vulgaris*, is even closer (4) and may indicate a fundamental relationship or basic pattern in the protein structure of all algae.

SUMMARY

The dried red alga, *Chondrus crispus*, has been extracted with aqueous ethanol and the extract has been shown to contain many common amino acids and especially arginine, citrulline, glutamic acid, and taurine. The presence of simple peptides which contained arginine, citrulline, glycine, threonine, and ornithine was demonstrated chromatographically. Several components remain unidentified.

Extraction of the dried plant with a saline-borate solution of pH 8.4 dissolved about 70 per cent of the total nitrogen in the plant, of which about 80 per cent was dialyzable. Complex peptides were demonstrable in the ultracentrifuge and on paper chromatograms. Traces of albumin and globulin-like proteins were detected.

About 25 per cent of the protein nitrogen was held firmly in the insoluble residue and could not be dissolved by such reagents as urea, sodium ethylenediaminetetraacetate, potassium thiocyanate, or sodium lauryl sulfate. Such residues contained more than 50 per cent of protein. The distribution of amino acids in them indicated the absence of citrulline and ornithine, a high content of arginine, and marked similarity to other algal proteins.

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