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Amino Acids and Peptide in Seven Species of Marine Green Algae

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(Figs. 1 – 2; Tables 1 – 4)

Many data have been collected so far on amino acid composition of marine algal extracts from the food chemical and biochemical point of views[1, 2]. However number of marine algae species, which have not been examined yet, is still considerable. And the amino acid patterns of the seaweeds seem to be not established. We tried out therefore a systematic survey on the amino acid composition before and after hydrolysis in extracts from 7 marine green algae: by name *Ulva pertusa, Enteromorpha linza, Cladophora densa, Caulerpa racemosa, Chlorodesmis comosa, Codium adhaerens* and *Codium fragile*. Among these, *Ulva pertusa* was newly found to contain a dipeptide, L-arginyl-L-glutamine, in a considerable amount. This peptide was first isolated from a fresh-water alga *Cladophora* sp. by MAKISUMI[3], but has not been detected in marine algae by this time.

The present paper deals with the amino acid composition of extracts of 7 kinds of green seaweed and with the isolation of L-arginyl-L-glutamine from *Ulva pertusa*. The distribution of aminosulfonic acids in these algae was examined at the same time.

EXPERIMENTAL AND RESULTS

Materials. The materials of *Ulva pertusa, Enteromorpha linza, Cladophora densa* and *Codium fragile* were collected at the intertidal region of Hiroshima City; *Caulerpa racemosa, Chlorodesmis comosa* and *Codium adhaerens* were collected at the beach near Ibusuki, Kagoshima Prefecture between April and December 1975, as shown in Table 1. The seaweeds from Hiroshima were put into ethanol as soon as possible after collection, and those from Kagoshima were brought to our laboratory, frozen with dry-ice and treated with ethanol.

The amino acid composition of the extracts. The fresh specimens were kept in 3 volumes of ethanol for 1 week at a room temperature on occasional stirring, respectively. The obtained ethanolic extracts were condensed under reduced pressure in order to remove ethanol and were defatted with diethylether. The cleared solutions obtained were used for estimation of amino acids by means of a Hitachi liquid chromatography 034 type apparatus and further analysis. A part of the solution was hydrolyzed with 6 *N* HCl in the usual manner. After the excess hydrochloric acid was removed by evapo-

Table 1. Compsitions of amino acids in the extracts of some marine green algae (N μg in 1 g of fresh algae)

Species	*Ulva pertusa*		*Enteromorpha linza*		*Cladophora densa*		*Caulerpa racemosa*		*Chlorodesmis comosa*		*Codium adhaerens*		*Codium fragile*	
Locality	Hiroshima		Hiroshima		Hiroshima		Kagoshima		Kagoshima		Kagoshima		Hiroshima	
Date of sampling	May 1975		Dec. 1975		Dec. 1975		June 1975		June 1975		June 1975		May 1975	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Alanine	13.6	25.9	12.0	15.0	10.8	24.6	9.17	8.82	4.38	11.28	9.02	10.49	5.80	6.25
Arginine	4.88	89.5	+	35.1	−	+	−	2.35	−	0.62	−	3.08	+	1.97
Arginylglutamine	110.0	−	35.0	−	−	−	−	−	−	−	−	−	−	−
Asparagine	−	−	−	−	−	−	1.47	−	0.25	−	1.06	−	1.02	−
Aspartic acid	+	11.0	1.30	7.55	8.28	11.9	1.36	5.31	0.34	2.10	5.42	9.16	6.55	8.37
Cysteic acid	−	−	−	−	12.6	10.4	6.72	6.92	1.12	0.78	+	0.83	−	−
Glutamic acid	4.14	38.9	25.1	52.0	50.7	46.0	7.67	16.9	7.83	17.98	12.14	20.89	8.20	17.10
Glutamine	1.34	−	−	−	−	−	8.09	−	6.57	−	2.67	−	9.66	−
Glycine	8.30	19.6	2.52	5.10	4.79	8.10	18.62	22.79	2.45	6.01	10.37	15.60	−	1.34
Hydroxyproline	−	−	−	−	−	−	+	+	−	−	−	−	−	−
Isoleucine	1.14	4.80	+	+	−	−	0.66	1.96	−	0.31	0.81	1.69	+	+
Leucine	2.62	8.17	+	+	−	−	0.46	2.35	−	0.32	−	1.76	+	+
Lysine	2.06	9.60	−	+	−	−	−	−	−	−	−	2.46	+	1.46
Methionine	+	1.51	−	−	−	−	−	−	−	−	−	−	−	−
Phenylalanine	+	3.61	−	−	−	−	+	+	−	−	−	+	−	−
Proline	7.03	10.9	31.7	35.0	27.9	36.6	+	1.90	−	1.23	1.20	3.64	+	+
Serine	1.62	6.97	+	2.47	+	+	2.74	4.09	−	1.97	2.63	5.04	+	0.78
Taurine	13.6	12.2	9.38	12.8	25.2	33.4	8.01	8.26	3.91	6.55	5.31	5.07	+	+
Threonine	7.88	12.8	−	0.94	+	+	1.36	2.02	−	1.11	1.08	1.96	+	0.63
Tyrosine	+	2.77	−	−	−	−	−	−	−	−	−	−	−	−
Valine	3.14	9.86	+	+	+	+	1.88	2.95	+	1.16	1.88	2.93	+	0.56
Ammonia	5.61	46.7	5.82	40.8	41.0	106.0	16.38	55.93	29.22	66.96	16.04	57.88	17.90	26.80
Total	186.96	314.79	122.82	206.76	181.27	277.00	84.59	142.55	56.07	118.38	69.63	142.48	49.13	65.26
Total N of the extract	569		−		−		286		219		−		94.0	

A, before hydrolysis; B, after hydrolysis.

ration, the residue was dissolved in water and then applied to amino acid analysis. The amino acid compositions before and after hydrolysis are shown in Table 1, in which the concentration of each constituent is expressed as nitrogen μg in 1 g of fresh fronds. And the values expressed for taurine are calculated from the sum of the peaks appearing near the taurine peak as taurine equivalent.

In *Ulva pertusa*, a large characteristical peak appeared between ammonia and arginine, similarly as L-citrullinyl-L-arginine appeared in a red alga *Grateloupia turuturu*[4,5]. This compound was identified as L-arginyl-L-glutamine as described below. This peptide was predominant and accounted for about 20 % of total nitrogen of the extract. After hydrolysis, this compound disappeared completely and was replaced by arginine and glutamic acid. Similar results were obtained in the same alga collected at Manabeshima, Okayama Prefecture. Arginylglutamine was detected also in *Enteromorpha linza* in a minor quantity yet not at all in the other 5 green algae. *Enteromorpha linza* was considerably rich in proline together with arginylglutamine. In *Cladophora densa,* the levels of glutamic acid and proline were remarkably high. No arginylglutamine was detected in this alga, although a fresh-water alga belonging to the same genus had been reported to contain that component[3]. The level of glycine was considerably high in *Caulerpa racemosa* and *Codium adhaerens,* especially in the former. *Codium fragile* and *Chlorodesmis comosa* seemed to contain a relatively limited quantity of amino acid nitrogen. But glutamic acid and glutamine were predominant in these 2 algae.

Aminosulfonic acids. The distribution of aminosulfonic acids in the above mentioned seaweeds was examined by paper chromatography in the following way: The extract of each alga was passed through a column of Dowex 50-X8 in H^+ form. The effluent and washings were put together and were passed through a column of Dowex 1-X8 in OH^- form. From the column rinsed with water, the adsorbed fraction was eluted out with 4 % acetic acid. The eluate which was positive to ninhydrin reaction was collected and concentrated in order to remove the acetic acid. The residue was then dissolved in water and submitted to paper chromatography and further purification. Aminosulfonic acids were developed on Toyo-roshi No. 50 with a mixture of phenol and water (4:1) in ascending. As detecting reagents, 0.2 % ninhydrin butanol saturated with

Table 2. Aminosulfonic acids detected in seven species of green algae

Compounds*	Tau	MMT	D-Cys	HT
Ulva pertusa			+	
Enteromorpha linza			+	
Cladophora densa			+	+
Chlorodesmis comosa	+	+		
Caulerpa racemosa	+			
Codium adhaerens	+	+		
Codium fragile	+			

* Tau, taurine; MMT, N-monomethyltaurine; D-Cys, D-cysteinolic acid; HT, homotaurine.

water and KIO_4–Nessler's reagent were used. The aminosulfonic acids detected are shown in Table 2.

Taurine was found in 4 species, namely *Caulerpa racemosa, Chlorodesmis comosa, Codium adhaerens* and *C. fragile.* D-Cysteinolic acid was detected in *Ulva pertusa* and *Enteromorpha linza* as reported formerly by ITO[6] . In *Cladophora densa,* the presence of homotaurine was observed in considerable quantity together with D-cysteinolic acid. The former compound had been isolated first from a red alga *Grateloupia livida* by the authors of this study[7] . N-Monomethyltaurine was detected in *Chlorodesmis comosa* and *Codium adhaerens* but was not confirmed by isolation.

From 1.3 kg of fresh *Cladophora densa,* homotaurine and D-cysteinolic acid were isolated by preparative paper chromatography in yields of 140 mg and 100 mg, respectively, using a mixture of phenol and water as developing solvent. These compounds were identical with the isolated specimens from *Grateloupia livida*[7] and *Ulva pertusa*[6] in paper chromatography and IR spectra, respectively.

Isolation and identification of L-Arginyl-L-glutamine from *Ulva pertusa* The extract from 330 g of a fresh sample of *Ulva pertusa* was poured onto a column of Dowex 50-X8 in H^+ form. From the column rinsed with water, the adsorbed substances were eluted out with 2 *N* NH_4OH. The fractions containing ninhydrin positive compounds were combined and concentrated to remove excess ammonia. The resulting solution was then passed through a column of Dowex 50-X8 in NH_4^+ form. After washing the column with water, the adsorbed basic substances were eluted out with 2 *N* NH_4OH. The basic fraction obtained was again passed through a column (2 x 60 cm) of Dowex 50W-X4 (NH_4^+ form, 100-200 mesh) after removal of ammonia by evaporation. The adsorbed compounds were eluted with 0.15 *N* NH_4OH. Each 10 m*l* portion of eluate was collected and checked by paper chromatography using ninhydrin and Sakaguchi's reagents for color developing. After 230 m*l* of eluate had been collected, arginylglutamine began to emerge from the column in its pure state judged by paper chromatography between 250 and 400 m*l* portion. The fractions revealing only one spot of arginylglutamine were combined and concentrated in order to remove ammonia. The resulting solution was neutralized with acetic acid. This solution gave colorless crystals when left in an ice-box on addition of 2 volumes of ethanol. Recrystallization from aqueous ethanol gave prisms; m.p. 187°C (uncorrected). Ref.[3] , m.p. 183°C. Yield 100 mg. *Anal.* Found : C, 42.03; H, 7.17; N, 22.85 %. Calcd. for $C_{11}H_{22}N_6O_4 \cdot CH_3COOH$: C, 43.08; H, 7.23; N, 23.19 %. $[\alpha]_{589}^{25}$ = +21.2° (c = 1 in water). Ref.[3] , $[\alpha]_D^{20}$ = + 16.9° (c = 2 in water). This preparation was positive to ninhydrin and Sakaguchi's reactions and showed only one spot in paper chromatography and thin-layer chromatography. R*f* values of this compound are summarized in Table 3. IR spectrum is shown in Fig. 1.

Hydrolysis products were determined with an amino acid analyzer. One μmole of this compound gave 0.99 μmole of glutamic acid, 0.97 μmole of arginine and 0.88 μmole of ammonia when hydrolyzed with 6 *N* HCl at 110°C for 18 hr in a sealed tube. After

Table 3. R*f* values of L-arginyl-L-glutamine and its related compounds

	A	B	C	D	E
Isolated L-arginyl-L-glutamine	0.30	0.17	0.20	0.37	0.26
Hydrolysis product in 1 *N* HCl	0.30	0.22	0.25	0.35	0.32
Synthesized L-arginyl-L-glutamic acid	0.30	0.22	0.25	0.35	0.32
Arginine	0.24	0.21	0.23	0.29	0.28
Glutamine	0.27	0.18	0.29	0.52	0.28
Glutamic acid	0.35	0.25	0.44	0.35	0.34

A, B, C, D . . . Thin-layer chromatography using cellulose powder. E . . . Paper chromatography using Toyo-roshi No. 50 in descending. Solvent system. . . . A, 1-butanol-acetic acid-water (4 : 1 : 2). B, 1-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2). C, *tert*-butanol-formic acid-water (70 : 15 : 15). E, 1-butanol-acetic acid-water (4 : 1 : 2).

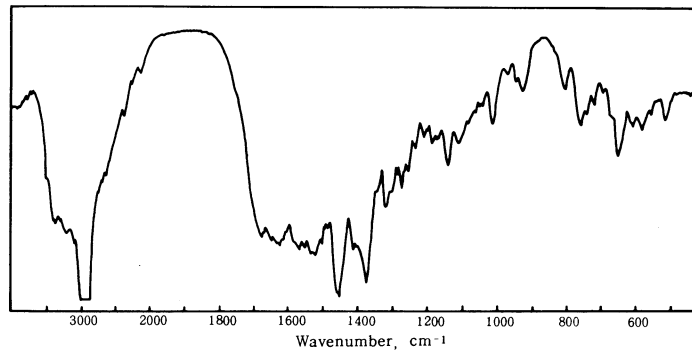


Fig. 1. IR spectrum of isolated peptide (Nujol).

hydrolysis in 1 *N* HCl at 100°C for 1.5 hr, it afforded 0.97 μmole of ammonia and 0.99 μmole of unidentified compound, which appeared at the top of the basic fraction in the 10 cm column for basic amino acid analysis and was proven to be arginylglutamic acid.

Dinitrophenyl derivative of this compound was prepared by the method of SANGER[8]. Hydrolysis products of DNP-derivative in 6 *N* HCl at 105°C for 18 hr were identified as DNP-arginine and glutamic acid by thin-layer chromatography, of which R*f* values

Table 4. R*f* values of DNP-peptide and its hydrolysis products in thin-layer chromatography

	A	B	C	D
DNP-peptide	0.32	0.70	–	–
Hydrolysate-I	0.52	0.75	–	–
DNP-arginine	0.52	0.75	–	–
Hydrolysate-II	–	–	0.13	0.32
Glutamic acid	–	–	0.13	0.32

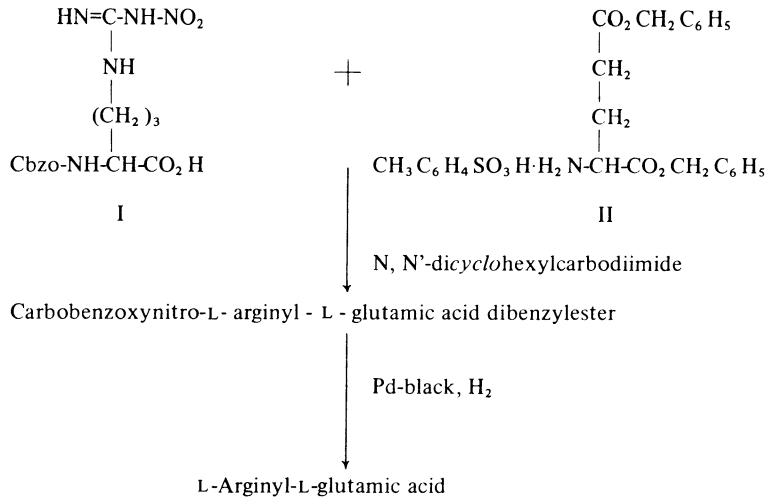
I, DNP fraction; II, amino acid fraction.
A, 1-butanol-aqueous ammonia (4 : 1), Silica Gel G.
B. 1-propanol-aqueous ammonia (7 : 3), Silica Gel G.
C. 1-butanol-acetic acid-water (4 : 1 : 2), Avicel SF.
D. 1-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2), Avicel SF.

are shown in Table 4.

Since the above described results pointed to a presence of glutamine as a constituent, its identification was attempted. One hundred mg of the peptide acetate was dissolved in 20 m*l* of phosphate buffer (pH 7.8) and to this solution 50 mg of mixed peptidase from hog intestine purchased from Seikagakukogyo was added. The mixture was kept at 37°C for 24 hr. After incubation, the protein was precipitated from the digest with sufficient basic lead acetate. Excess lead was removed with sulfuric acid in the usual manner. The obtained solution was passed through a column of Dowex 50-X8 in H^+ form. From the column rinsed with water, the adsorbed substance was eluted with 2 *N* NH_4OH and ninhydrin positive fraction was collected. After removal of ammonia by evaporation, the solution was passed through a column of Dowex 50-X8 in NH_4^+ form. The effluent and washings were combined and concentrated to a small volume. This solution on addition of ethanol gave crystals when kept in an ice-box. Recrystallization from aqueous ethanol afforded 33 mg of crystals, m.p. 185°C. In IR spectrum, this compound showed a good agreement with L-glutamine.

Two hundred mg of the peptide acetate was hydrolyzed in 1 *N* HCl at 100°C for 1.5 hr. After removal of the excess hydrochloric acid by evaporation, the hydrolysis products were put on a column (0.9 x 20 cm) of Dowex 50W-X4 (NH_4^+ form, 100–200 mesh). From the column rinsed with water, the adsorbed compound was eluted out with 0.15 *N* NH_4OH. After 20 m*l* of eluate had been collected, the subsequent 20 m*l* portion revealed only one spot with ninhydrin reagent in paper chromatography. This portion was concentrated to remove the ammonia. The solution gave colorless crystals when treated with ethanol. Recrystallization from aqueous ethanol afforded plates ; m.p. 205°C. Ref.[3], m.p. 205°C. Yield 140 mg. *Anal.* Found : C 41.20 ; H, 6.96; N, 21.88 %. Calcd. for $C_{11}H_{21}N_5O_5 \cdot H_2O$: C, 41.11; H, 7.22; N, 21.80 %. $[\alpha]_{589}^{25}$ = + 20.9° (c = 1 in water). Ref.[3], $[\alpha]_D^{17}$ = + 20.5° (c = 2 in water). This compound showed positive reaction with ninhydrin and Sakaguchi's reagents similar to the original peptide. After hydrolysis in 6 *N* HCl at 110°C for 18 hr in a sealed tube, it gave equimolar arginine and glutamic acid which were evaluated by means of an amino acid analyzer.

For comparison, L-arginyl-L-glutamic acid was synthesized by the method of IZUMIYA and MAKISUMI[9] as follows. Using the carbodiimide method, 2.1 g of carbobenzoxynitro-L-arginine (I) was condensed with 3.0 g of L-glutamic acid dibenzylester-*p*-toluene sulfonate (II). After crystallization from a mixture of methanol, diethylether and petroleum ether, crystals of carbobenzoxynitro-L-arginyl-L-glutamic acid dibenzylester was secured. Yield 2.11 g (53 %). Two grams of this compound was then dissolved in a mixture of methanol, acetic acid and water and subjected to catalytic hydrogenation over palladium black for a period of 10 hr. After removal of the catalyst, the filtrate was concentrated to dryness under reduced pressure. Recrystallization from aqueous ethanol gave plates of L-arginyl-L-glutamic acid monohydrate. Yield 930 mg (78 %). M.p. 202°C. Ref.[9], 201–202°C. $[\alpha]_{589}^{25}$ = + 20.2° (c = 1 in water). Ref.[9], $[\alpha]_D^{25}$ = + 20.9° (c = 2 in water). *Anal.* Found : C, 41.04; H, 6.91; N, 21.81 %. Calcd. for



$C_{11}H_{21}N_5O_5 \cdot H_2O$: C, 41.11; H, 7.22; N, 21.80 %. Depression of the melting point was not observed on admixture with specimen derived from natural peptide. The synthesized specimen was identical with the derived one in thin-layer chromatography and in the IR spectrum as shown in Fig. 2. These results clearly indicated that the isolated peptide is L-arginyl-L-glutamine.

The occurrence of arginylglutamine in the extract of *Enteromorpha linza* was confirmed by thin-layer chromatography.

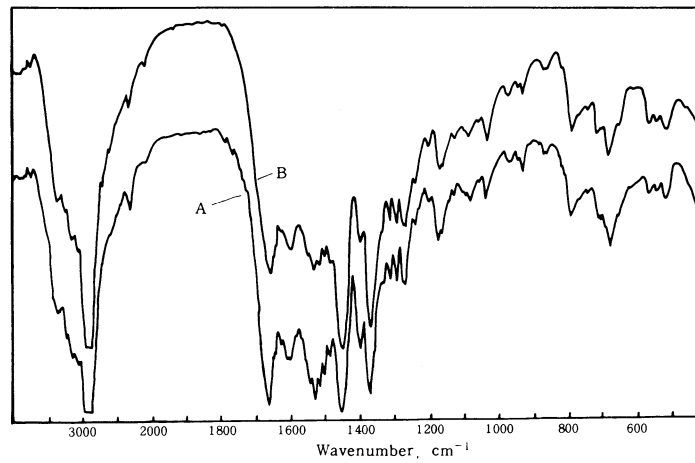


Fig. 2. IR spectra of isolated (A) and synthesized (B) L-arginyl-L-glutamic acid (Nujol).

DISCUSSION

The occurrence of L-arginyl-L-glutamine in the extract of a green seaweed *Ulva pertusa* was thus established. Although this peptide had been before isolated from a fresh-water alga *Cladophora* sp. by MAKISUMI[3], this is the first time now that it has been detected in marine algae too. Besides in *Ulva pertusa*, it was detected also in *Entero-*

morpha linza but in smaller quantity. Its presence however was not observed in *Cladophora densa*, which belongs to the same genus of alga reported by MAKISUMI. This may be attributable to the difference in circumstances or species.

As shown in Table 1, amino acids and ammonia nitrogen increased to nearly twofold after hydrolysis in all the species examined here. As this was due in a considerable part to the increment of the ammonia fraction, it seems necessary to re-examine the constituents of the ammonia fraction. In *Ulva pertusa*, L-arginyl-L-glutamine took up about 60 % of the amino acids and ammonia nitrogen before hydrolysis and the change in the amino acid composition during the hydrolysis was clearly explained by its isolation.

As for the aminosulfonic acids, taurine was detected in 4 species, D-cysteinolic acid in 3, N-monomethyltaurine in 2 and homotaurine in 1, respectively, in total 7 species of green algae examined here. Homotaurine and D-cysteinolic acid were isolated from *Cladophora densa*, and the former compound was detected in green algae for the first time. Co-existence of these compounds in this alga may point to a certain metabolic relation between them.

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SUMMARY

The amino acid compositions of 7 marine green algal extracts were examined by means of an amino acid analyzer. *Ulva pertusa* was for the first time found to contain a dipeptide, L-arginyl-L-glutamine, in a large quantity. This peptide was detected also in *Enteromorpha linza* but not in the other five species. Glutamic acid and glutamine were relatively predominant in *Codium fragile*, *C. adhaerens* and *Chlorodesmis comosa*. In *Caulerpa racemosa*, the level of glycine was remarkably high. Glycine and proline were predominant in *Cladophora densa*.

Aminosulfonic acids in these algae were examined by paper chromatography. Taurine was detected in 4 species, D-cysteinolic acid in 3, N-monomethyltaurine in 2, and homotaurine in 1, respectively. Occurrence of homotaurine in *Cladophora densa* was also established.

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数種緑藻の遊離アミノ酸とペプチッド

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7種の海産緑藻のエキスの遊離アミノ酸組成を調べた。その結果，アナアオサ (*Ulva pertusa*) にジペプチッド，L-arginyl-L-glutamine が著量に存在することを認めた。このペプチッドはイオン交換樹脂カラムクロマトグラフィーにより単離され，加水分解生成物の同定および合成によりその構造が確認された。アナアオサでは L-arginyl-L-glutamine は全エキス窒素の約20%を占め，主成分をなしていた。さらにウスバアオノリ (*Enteromorpha linza*) にも検出されたが，外の5種には認められなかった。ウスバアオノリでは arginyl-glutamine とともに proline の含量が比較的高かった。この外，アサミドリシオグサ (*Cladophora densa*) では glycine と proline，マユハキモ (*Chlorodesmis comosa*)，ハイミル (*Codium adhaerens*) およびミル (*Codium fragile*) では glutamic acid と glutamine，スリコギイワヅタ (*Caulerpa racemosa*) では glycine の含量がそれぞれ高かった。

またこれら緑藻におけるアミノスルフォン酸の分布をペーパークロマトグラフィーにより調べた。その結果，taurine を4種の海藻に，D-cysteinolic acid を3種に，N-monomethyltaurine を2種に，homotaurine を1種にそれぞれ検出した。このうちアサミドリシオグサでは homotaurine を分離して確認した。