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CHEMICAL STUDIES ON MARINE ALGAE

XIII. Isolation of Crystal *L*-Citrulline from *Chondrus ocellatus* and *Rhodoglossum pulchrum*

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Introduction

L-Citrulline was first encountered as a constituent of the juice of the watermelon (Koga & Odaki¹⁾; Wada²⁾), and it is well known at present as an intermediate of the Krebs ornithine cycle.

As to the preparation of citrulline, though various methods such as alkali degradation of arginine³⁾, and synthesis from ornithine copper salt,⁴⁾ etc., have been described, no preparation from natural sources has ever been attempted.

The present authors have already reported the existence of citrulline in the ethanol extract of one of the red algae, *Porphyra tenera*⁵⁾. In *Chondrus crispus*, citrulline was detected by Smith and Young⁶⁾, not only in free state but also in simple and complex peptides. It was also detected by L. Fowden⁷⁾ in the fresh water alga *Chlorella vulgaris*.

The below described experiments revealed that both of *Chondrus ocellatus* and *Rhodoglossum pulchrum* contain considerable amounts of citrulline in free state. Attempts were made to isolate citrulline from the ethanol extract of these algae by the use of the technique of displacement chromatography.

Experimental

PREPARATION OF THE EXTRACT *Chondrus ocellatus* collected at Nanaehama, Hakodate in Jan. 1958 was washed with water and dried on the steam bath. Three kg of this sample were extracted with 20 liters of 70 % ethanol for two weeks in the room temperature and the residue was filtered off. The extract was evaporated to small bulk *in vacuo* at 40° to remove ethanol, and then shaken twice with ether to remove the pigments such as chlorophyll. The aqueous layer, diluted to two liters, was shaken for an hour with 75g of charcoal treated with acetic acid to absorb remaining colouring matter (Partridge, 1949⁸⁾). The extract was filtered through a Buchner's funnel and the charcoal was washed thoroughly with four liters of water. By this treatment about six liters of colourless extract were obtained.

FIRST FRACTIONATION OF THE EXTRACT The algal extract contained inorganic salts in high concentration, so it was necessary to remove them before fractionation. After that it was passed through three Dowex 50 X-4 H⁺ columns (resin vol. 1200 ml each) washed with water; thus the amino acids were displaced by 0.2 N NH₃ without

fractionation. Amino acids fraction was next added to the three tier column system of Dowex 50 H⁺ (200, 60, 10 ml), washed with water and then fractionated with 0.2 N NH₃ into 75 fraction 30 ml each (Fig. 1).

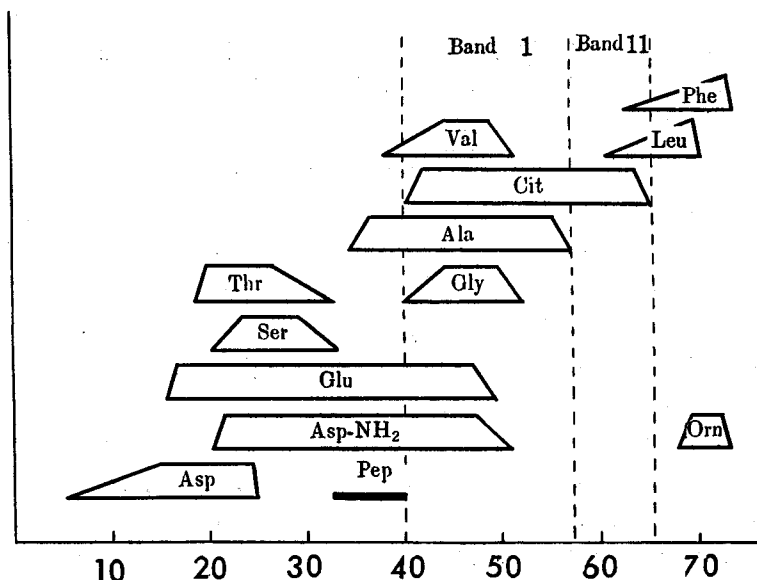


Fig. 1. Primary fractionation of the free amino acids occurring in *Chondrus ocellatus*. Three tier column system of Dowex 50 X-4 H⁺ (200, 60 and 10 ml) was used.

Each fraction was developed by a paper chromatography. All paper chromatograms were run on Tōyō filter paper No. 50 and the solvents used were ; (1) N-butanol-acetic acid-water (4:2:1), (2) phenol saturated with water, (3) phenol saturated with ammonia solution and (4) 70 % propanol.

The amino acids found were aspartic acid, glutamic acid, threonine, serine, asparagine, citrulline, glycine, alanine, valine, leucines, ornithine, phenylalanine and a faint spot of peptide (Rf value in N-butanol-acetic acid-water, 0.06, in phenol-water, 0.11). Proline, methionine, glutamine, histidine, tryptophan and tyrosine were not detected.

ISOLATION OF CITRULLINE Citrulline was eluted on the fraction from 40 to 66. The portion which eluted with asparagine, glutamic acid, glycine, alanine and valine was combined (band 1 in Fig. 1). The portion which eluted with leucines (band 11 in Fig. 1) was next added to the two tier column system of Dowex 50 H⁺ (30 and 10 ml) and displaced with 0.1 N NH₃ into 40 fractions of nine ml each (Fig. 2).

In Fig. 2, band 1 was still contaminated with slight alanine, so this portion was added to band 1 in Fig. 1. Citrulline was obtained in pure state from band 11. Band 111 was contaminated with leucines but the attempts failed to remove these amino acids through the small column of Dowex 50.

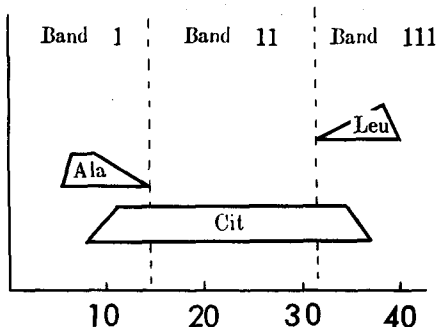


Fig. 2. Secondary fractionation of the acids of band 11 in Fig. 1 by means of two tier column system of Dowex 50 (resin vol. 30 and 10 ml).

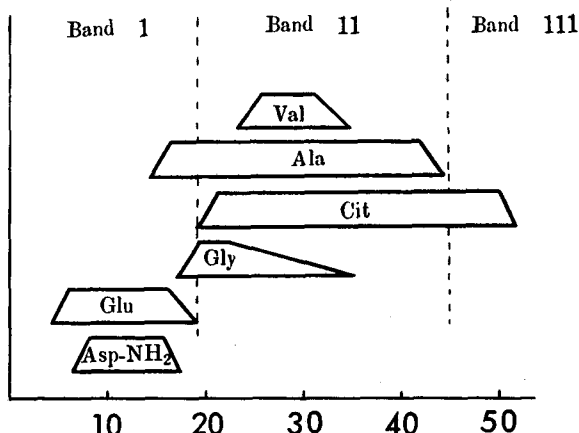


Fig. 3. Secondary fractionation of the acids of band 1 in Fig. 1 and band 1 in Fig. 2 by means of three tier column system of Dowex 50 (resin vol. 100, 30 and 10 ml).

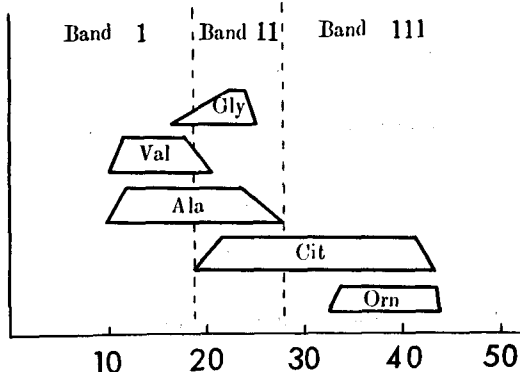


Fig. 4. Fractionation of the acids of band 11 in Fig. 3 with Dowex 2 X-4 OH. Two tier column system of 40 and 20 ml was used.

Band 1 in Fig. 1 and Fig. 2 were combined, added to the three tier column system of Dowex 50 (100, 30 and 10 ml) and fractionated by 0.2 N NH_3 into 50 fraction 20 ml each (Fig. 3). Glutamic acid and asparagine could be removed from the elution area of citrulline but it was still contaminated with alanine, glycine and valine. Further attempts to fractionate citrulline from these amino acids through the Dowex 50 columns were unsuccessful.

The efficient separation of citrulline was

obtained by the use of the strong basic resins, Dowex 2 X-4 ($-\text{OH}^-$). This portion (band 11 in Fig. 3) was added to the two tier column of Dowex 2 (40 and 20 ml) and was displaced by 0.1 N HCl to 43 fractions of five ml each (Fig. 4).

It should be noted that the new faint ninhydrin reactive spot appeared from 33 to 43 ml. Being quite in agreement with ornithine on paper chromatograms, degradation of citrulline must have occurred during the passage through the column.

Citrulline eluted with other amino acids (band 11 in Fig. 4) was combined and fractionated again through Dowex 2 columns. Citrulline fractions obtained with Dowex 2 had to be used to remove ornithine and for this purpose refractionation through Dowex 50 column was most efficient.

Citrulline solution, which can be obtained everywhere, combined,

concentrated *in vacuo*, was crystallized by addition of ethanol. Citrulline was recrystallized four times from aqueous ethanol. Five g of white crystal citrulline was obtained from three kg of dried *Chondrus ocellatus* (Fig. 5).

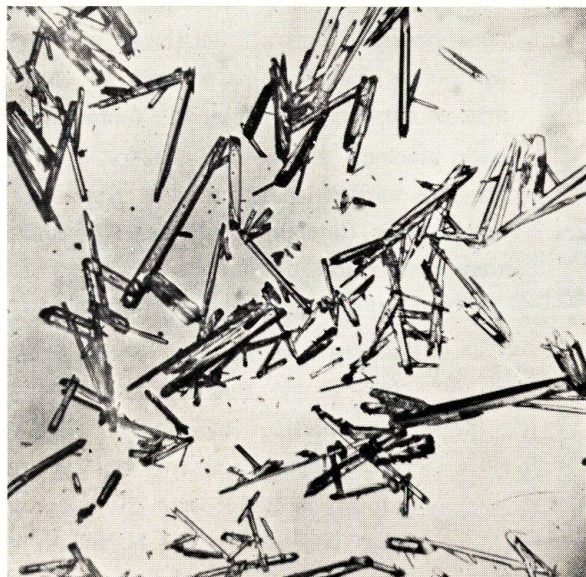


Fig. 5. Crystals of *L*-Citrulline obtained from *Chondrus ocellatus*

ISOLATION OF CITRULLINE FROM RHODOGLOSSUM PULCHRUM.....

One kg of dried sample (harvested in Jan. 1958) was extracted with 70 % ethanol and desalted by the method described above. The amino acid fraction was absorbed on the two tier column system of Dowex 50, X-4 H⁺ 100 and 30 ml; after having been washed with water, it was fractionated into 75 fractions of 13 ml each. The figure obtained was essentially the same as for *Chondrus ocellatus*. Citrulline was detected from 43 to 60 fraction; these fractions were combined and converted into

copper salt by addition of copper carbonate on a boiling water bath. Boiling was continued for one hour to make the reaction complete. Copper citrulline was immediately precipitated and the darkish solution of the copper salt was kept in a refrigerator overnight. The precipitate was collected by filtration, suspended in water, and decomposed with H₂S. After removal of copper sulfide by filtration, free amino acids solution was developed on the paper chromatography. Leucines, alanine and glycine were detected as impurities.

The solution was next run through the Dowex 2 column and fractionated with 0.1 N HCl. At last 1.3 g of crystal citrulline was obtained.

IDENTIFICATION AND PROPERTIES OF CITRULLINE..... The crystal thus obtained was dried *in vacuo* over P₂O₅ at 100°. The yield was five g from three kg of dried matter of *C. ocellatus* and 1.3 g from one kg of dried *R. pulchrum*.

Melting point was 219–222° and the copper salt decomposed at 256°. This value quite agreed with that of citrulline copper salt synthesized from arginine by the method of Fox³⁾. As for the melting point of citrulline 219.5° (Gornall & Hunter)⁴⁾, 226° (Wada)¹⁸⁾, 220–222° (Ackerman)⁹⁾, 220–221° (Kurtz)¹⁰⁾ have been reported.

The specific rotation was found to be $[\alpha]_D^{18} = +3.7$ (H₂O, C=5), whereas Gornall & Hunter⁴⁾ gave $[\alpha]_D^{22} = +3.5$ (H₂O, C=5) for citrulline synthesized from L-ornithine.

Molecular weight estimated by cryoscopic method gave 173 (calculated 175.19).

The result of elementary analysis was

	C	H	O	N
Estimated	41.09 %	7.46 %	27.91 %	23.54 %
Calculated	41.13	7.48	27.41	23.98

as $C_6H_{13}O_3N_3$

The infrared spectrum of this crystal is shown in Fig. 6.

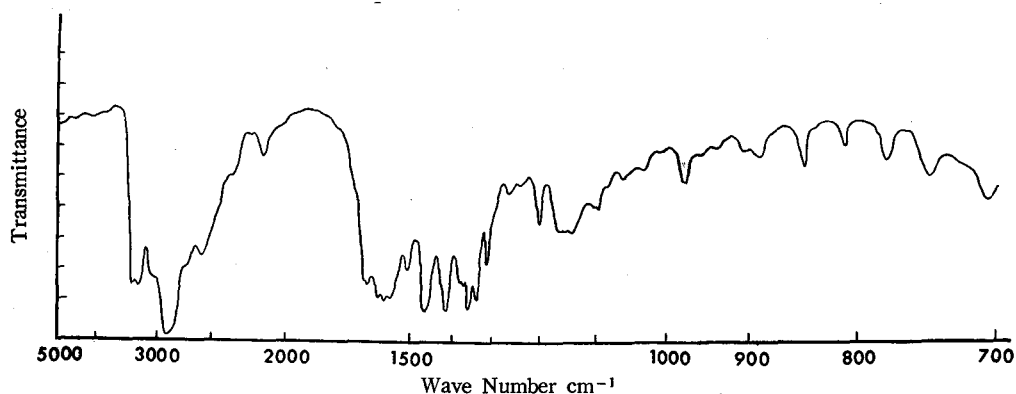


Fig. 6. Infrared spectrum of the crystal obtained from *Chondrus ocellatus*.

This solution gives a red color in reaction with Fearon's reagent¹¹⁾ and yellow with Ehrlich's reagent.⁷⁾

Though unstability of this compound to a mineral acid has been reported in a few papers^{6,12)}, behavior to acid hydrolysis was estimated in a pure solution.

One-tenth gram of citrulline was hydrolysed with 6 N HCl at 100° in a sealed tube for 24 hrs. Excess hydrochloric acid was removed by repeated drying in a vacuum alkaline desiccator. Hydrolysate was made up to 25 ml with water and 0.005 ml portion was chromatographed. After color development with ninhydrin, each spot was cut off, transferred to the test tube for the full ninhydrin color development under the controlled condition of Moore and Stein¹³⁾. Mole ratio of citrulline and ornithine was 0.35 and 0.18 mM respectively (93 % recovery of the original compound). Therefore 34 % of citrulline was decomposed to ornithine by acid hydrolysis.

Application of weak acid hydrolysis for the amide nitrogen¹⁴⁾ revealed 28 % degradation of this compound.

Discussion

Owing to the high concentration of the inorganic salts in the extracts, it was necessary to remove them before primary fractionation. In the present experiments, 10 or 15 times of resin as much as usual which absorbs all the amino acids in the extract was necessary

for the desaltation. Though such a great deal of resin was required, no loss of the common amino acids was encountered during this procedure. Only taurine and a certain faint spot (Rf in butanol-acetic acid-water 0.32 and Rf in phenol-water 0.27) were detected in both extracts in the nonabsorbable fraction of Dowex 50 columns, but these amino acids were not absorbable by the sulfonic polystyren resins. The unknown spot described above also gave a bright brown color on the paper chromatograms when sprayed with 2 % p-nitrobenzoyl-chloride in benzol and successively with pyridine, so that this spot is thought to be a N-CH₃-taurine. This amino acid has been detected in the red algae *Ptilota pectinata* and *Porphyra umbilicauris* by Lindberg.¹⁶⁾

As the algal extract contains a small amount of monoamino monocarboxylic acids, isolation of citrulline was not very complicated. But the removal of the alanine, glycine and valine was not satisfactory on the Dowex 50 columns. Though the degradation of citrulline may occur, Dowex 2 gave a good result. Conversion to copper salt also gave a good result; the material being still contaminated with slight impurities, fractionation by Dowex 2 column was necessary. Citrulline fraction, obtained on the Dowex 2 apparatus, was always accompanied by a slight degradation product and refractionation with Dowex 50 was required. If Dowex 2 resin itself catalytically decomposes citrulline, the by-product must come out from the top of the elution, because ornithine is produced in this case as a degradation product and on the basic resin, it is proper to think that ornithine, which is more basic, appears before of citrulline. In spite of these considerations, ornithine was detected later than expected in effluent, so that it seems likely that this result is due to the hydrochloric acid used for the displacement agent. The use of a suitable displacement agent instead of 0.1 N HCl may prevent the degradation of citrulline.

Smith and Young⁶⁾ detected paper chromatographically an unknown spot on the effluent fraction of glutamic acid and citrulline by the method of Stein and Moore¹⁷⁾ in the ethanol extract of *Chondrus crispus*. Same experiments were carried out. Free amino acids, extracted quantitatively with 75 % ethanol, were passed through 100 cm column of Dowex 50; the fraction which comprises of citrulline and glutamic acid was chromatographed with three kinds of solvent system as mentioned above, after desalting with a small column of Dowex 50 H⁺; no other spot but citrulline and glutamic acid can be detected. so that, an unknown spot reported by the above authors may reasonably be presumed to be ornithine produced during the passage of Dowex 2 X-10.

Being unstable to the mineral acid, about 34 % of this compound was decomposed quantitatively to ornithine under the usual conditions of acid hydrolysis and 28 % even by the weak acid hydrolysis to the amide nitrogen.

Summary

L-Citrulline was isolated by the use of ion exchange resin from red algae, *Chondrus*

ocellatus and *Rhodoglossum pulchrum*. The yield was 1.7 and 1.3 g per kg dried matter respectively.

Both extracts were proven to have very similar amino acid composition and there were detected in the extracts: aspartic acid, glutamic acid, threonine, serine, asparagine, glycine, alanine, valine, leucines, phenylalanine, ornithine and a faint spot of peptide. In the nonabsorbable fraction of Dowex 50, taurine and a faint spot, probably N-CH₃-taurine, were detected.

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