Cryptogamie, Algol., 2004, 25 (2): 199-205

The occurrence of some betaines in five macroalgal species from the Moroccan Atlantic coast[1]

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(Received 20 October 2002, accepted 12 May 2003)

Abstract — The occurrence of glycine betaine and γ-aminobutyric acid betaine extracted from the macroalgal species *Ulva lactuca* (Ulvophyceae), *Cystoseira humilis, Fucus spiralis, Fucus vesiculosus* (Phaeophyceae) and *Gelidium sesquipedale* (Rhodophyceae), growing on the Atlantic Moroccan coast was investigated. Seaweed extracts analyzed by TLC showed that all the species contained glycine betaine, but γ-aminobutyric acid betaine was detected only in members of the classes Fucaceae and Gelidiaceae. Extracts of *Gelidium sesquipedale* contained glycine betaine, γ-amino butyric acid betaine and α-alanine betaine. Extracts of *Cystoseira humilis (myriophylloides)* contained glycine betaine only, while *Ulva lactuca* extract contained glycine betaine and α-alanine betaine. Within the Phaeophyceae, yields of these compounds were consistently higher in the Fucaceae than in Cystoseiraceae. High performance liquid chromatography using short wavelength ultra-violet radiation detection was found to be unsuitable for the analysis of betaines in seaweed extracts, even after partial purification by passage through ion-exchange columns. Analysis by ^{1}H NMR spectroscopic methods confirmed the presence of glycine betaine, γ-amino butyric acid betaine and α-alanine betaine in the seaweed extracts.

γ-amino butyric acid betaine / α-alanine betaine / ^{1}H NMR / marine seaweeds

Résumé — Présence de bétaïnes dans cinq espèces de macroalgues de la côte atlantique marocaine. La présence de la glycine betaïne et l'acide γ-aminobutyrique betaïne a été recherchée chez quelques espèces de macroalgues de la côte atlantique marocaine : *Ulva lactuca* (Ulvophyceae), *Cystoseira humilis, Fucus spiralis, Fucus vesiculosus* (Pheophyceae) et *Gelidium sesquipedale* (Rhodophyceae). Les extraits d'algue analysés par chromatographie sur couche mince ont montré que toutes les espèces contiennent de la glycine betaïne, alors que l'acide γ-aminobutyrique betaïne n'a été détecté dans les familles des Fucaceae et les Gelidiaceae. L'extrait de *Gelidium sesquipedale* renferme de la glycine betaïne, de l'acide γ-aminobutyrique betaïne et une α-alanine betaïne. L'extrait de *Cystoseira humilis (myriophylloides)* contient seulement de la glycine betaïne tandis que l'extrait d'*Ulva lactuca* contient de la glycine betaïne et une α-alanine betaïne. Parmi les Pheophyceae, les Fucaceae appraissent les plus riches en ces composés que les Cystoseiraceae. La détection des betaïnes dans les extraits d'algue, à l'aide de la chromatographie liquide à haute performance à des longueurs d'onde courtes (radiations ultra-violet) ne

1. For reasons beyond of our control, the present note could not be published last year. Sincere apologies are thus extended to the relevant authors.

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Communicating editor: John Beardall

donne pas de résultat satisfaisant même après purification des extraits par chromatographie sur colonne échangeuse d'ion. L'analyse spectroscopique par RMN du proton, a permis la détection de la glycine betaïne, l'acide γ-aminobutyrique betaïne et une α-alanine betaïne dans les extraits d'algue marine.

Acide γ-aminobutyrique betaïne / α-alanine betaïne / macroalgues marines / RMN du proton

INTRODUCTION

Marine algae have been collected and used as fertilizers and soil-conditioning agents in Europe for many years. However, their utilization has been restricted mainly to coastal areas because of the high costs involved in collection, drying, and transportation. Comparison of farmyard manure with an equivalent weight of fresh seaweed showed that the nitrogen content of both were similar, but the potassium level was higher and the phosphorus level lower in the seaweed (Chapman & Chapman, 1980). The activity of a cytokinin-like hormone in seaweeds was detected in seaweeds by the application of algal extracts on tomatoes (Moujahid, 1994).

Blunden *et al.* (1984) first suggested that seaweed extracts might contain compounds that behave like cytokinins. Betaines were suggested as likely compounds, particularly as it had been shown by Wheeler (1973) that glycine betaine and γ-amino butyric acid betaine have activity reminiscent of cytokinins in several growth tests, and that betaine, as well as others compounds, has been isolated from many of the species of brown algae used for the production of seaweed extracts (Blunden *et al.*, 1982, 1985). Glycine betaine and other betaines have been shown to be major cytoplasmic osmotica in certain higher plants and cyanobacteria adapted to salt and water stress, and glycine betaine has also been reported to have a role in frost resistance (Borakev & Ivanova, 1971). In view of the possible contribution of betaines to the beneficial effects produced by seaweed extracts, a number of commercially available products have been examined for their content of betaines, and different analytical methods have been developed and evaluated for the quantitative estimation of these compounds. These, when applied to winter wheat, were found to enhance both the water utilization efficiency and the grain yield of the treated plants (Bergmann & Eckert, 1984). Glycine betaine has also been shown to ameliorate salt stress in seedlings and to afford partial protection of some enzymes against salt inhibition (Pollard & Wyn Jones, 1979). In the work reported here, the distribution of these compounds in the more commonly available species of Ulvophyceae, Phaeophyceae and Rhodophyceae from the Moroccan Atlantic coast was studied.

MATERIAL AND METHODS

The algal species tested were collected on the predominantly rocky coast of Rabat, Morocco, between February and December 1999. The plant material was stored carefully in order to reduce contamination from extraneous material, and observed epibionts were removed prior to storage at –20°C until required for extraction. After collection, each alga was cut into pieces and, still fresh, 200 g was extracted with methanol in a Soxhlet apparatus for 8 h. The extract was concen-

trated under reduced pressure to remove the methanol and filtered to remove suspended material. The aqueous seaweed extract was added onto a column of Amberlite IR-120 resin (H^+ form).

After washing with water, the quaternary ammonium compounds were eluted from the column with 3N ammonia solution (Blunden, 1981). The eluate was evaporated to dryness under reduced pressure, the residue dissolved in water and examined by thin-layer chromatography using air-dried layers of silica gel G (Merck), 250 μ in thickness. Two-way TLC was employed using double development in methanol:water (50:50) in the first direction and methanol: chloroform: ammonia solution:water (50:50:12.5:10) in the second (Blunden *et al.*, 1985) Visualization of compounds was achieved by spraying the plate first with Dragendorff's reagent, followed by a saturated solution of sodium hydrogen sulfate (Blunden *et al.*, 1981) The estimation of the betaine content of seaweed extracts by high performance liquid chromatography was performed on the eluate obtained after passage of the seaweed extract through a column of Amberlite IR-120 (H^+ form). The eluate was evaporated to dryness under reduced pressure, and dissolved in 5 ml distilled water for high performance liquid chromatography evaluation. The high performance liquid chromatography system (Water Associates) consisted of a M 600 A pump, a valve injector fitted with a 2 ml sample loop and variable wavelength detector set at 195 nm, using a sensitivity of 0.1 AUFS. Separations were performed isocratically using a 250 * 4.6 mm I.D. Microbandpack C18 column and a mobile phase of 0.05 M potassium dihydrogen phosphate in methanol:water (5:95), pH 4.6, at a flow rate of 1 ml min^{-1} (Gorham, 1984). The extract reserved for analysis by ^1H NMR spectroscopy was dissolved in 0.5 ml deuterium oxide (DO_2) and the solution transferred to an ^1H NMR spectroscopic tube. As an external standard, a 58 mM solution of 3(trimethylsilyl)propionic acid-d4-sodium salt (DSS) in D_2O was sealed in a glass capillary and fitted into the ^1H NMR spectroscopic tube. The spectrum of the purified seaweed extract and DSS was produced using apparatus WH-270 MHz spectrometer with a 4 KHz sweep width. The heights of signals produced by the nine proton $N^+(CH_3)_3$ group of each betaine present were measured, as well as the signal produced by DSS (Fig. 1). The amount of each betaine present, in mg, was calculated from the following formula:

$$[\text{Height of } N^+(CH_3)_3 \text{ peak of betaine / Height of Si } (CH_3)_3 \text{ peak of DSS}] \times \text{“apparent” concentration of DSS (mM)} \times [\text{molecular weight of betaine / 1000}] \times \text{solvent volume (ml) / 200}$$

The DSS solution acting as external standard and contained in the inner concentric reference tube gave an integrated signal for the $(CH_3)_3$ protons 9.88 times less than that given by the signal of the same solution placed in the outer tube. Comparisons of concentrations in the inner and outer tubes have to be corrected by this factor. The actual concentration of DSS used in the reference tube was 58 mM. The “apparent” concentration in this case was therefore 58 / 9.88 = 5.87 mM. (Blunden, 1986).

RESULTS

The purified extracts of all the marine algal species showed, on two-way TLC, the presence of Dragendorff-positive components. The compounds identified are listed in Table I.

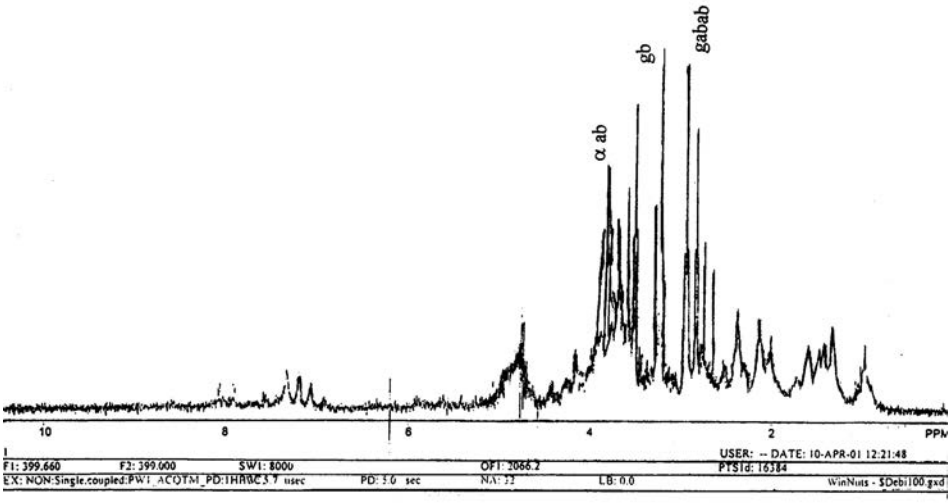

Fig.1. ^{1}H NMR spectrum of seaweed extract sample in DO_2 – gb: glycine betaine; gabab: γ-aminobutyric acid betaine; α-alanine betaine.

Table 1. Betaines extracted from some species of algae from the Moroccan Atlantic coast.

	Betaine compounds detected			
Species	*Glycine betaine*	*γ-aminobutyric acid betaine*	*α-alanine betaine*	*Other*
ULVOPHYCEAE				
Ulva lactuca	+	–	+	+
PHAEOPHYCEAE				
Cystoseira humilis	+	–	–	+
Fucus spiralis	++	++	–	+
Fucus vesiculosus	++	++	–	+
RHODOPHYCEAE				
Gelidium sesquipedale	+	+	+	+

++ Major components, + Minor components (based on color intensity of product spot on TLC);

These results show that all the species of marine algae collected on the Moroccan Atlantic coast contained glycine betaine. α-Alanine betaine was identified only in the green alga *Ulva lactuca*, and in the red alga *Gelidium sesquipedale*.

The amount of compounds isolated from three species of Phaeophyceae shows that *Fucus spiralis* and *Fucus vesiculosus* contained more glycine betaine and γ-amino butyric acid betaine than *Cystoseira humilis (myriophylloides)*. α-Alanine betaine was not detected in any of these species (Table 1).

To estimate the quantities of betaines present in the seaweed extracts, the high performance liquid chromatography procedure described by Gorham (1984) was used initially. When standards of the glycine betaine, γ-aminobutyric acid betaine and α-alanine betaine were injected the weights and peak areas produced by HPLC were proportional. When purified seaweed extracts were examined by high performance liquid chromatography, however, it was not possible to detect the betaines present. A proton magnetic resonance spectroscopic assay method for the estimation of betaine in seaweed extracts was therfore developed.

The nine large proton singlets were produced by the $N^+(CH_3)_3$ groups of betaines. By comparison of the peak height of $N^+(CH_3)_3$ signal produced in the ^1H NMR spectrum of the betaine with that of the peak produced in the same spectrum by the external standard (DSS) of known molarity, the concentration of the betaine present can be calculated. Using different known molecular weights of all the betaines to check the accuracy of the ^1H NMR spectroscopic method, the procedure was shown to be consistently reliable. ^1H NMR spectroscopy has been used previously for the estimation of glycine betaine and choline in foodstuffs. (Chastellain & Hirsbrunner, 1976).

As with the HPLC method of analysis, it was not possible to use unpurified seaweed extracts. However, the application of the ion-exchange extracts was found to be highly satisfactory and it was possible to calculate the contents of glycine betaine, γ-amino butyric acid betaine and α-alanine betaine. The difference between the chemical shifts of the $N^+(CH_3)_3$ signals of glycine betaine, γ-amino butyric acid betaine and α-alanine betaine is sufficient to estimate the relative proportions of each betaine in the extract by measurement of peak heights. The results obtained with different seaweed extracts are given in Table 2. Betaine contents varied from 0.02 to 0.07 mg/g fresh wt, with the two *Fucus* species showing the higher values of betaine concentration.

Other compounds were detected in all species studied in this work. These components are currently unidentified but will be the subject of future investigations. The presence or absence of the compounds in the samples of all the species examined did not appear to have any relationship to either the date of collection or the state of fertility of the plants (data not shown). However, this work showed that the extracts prepared from brown algae, and from the Fucaceae in particular, were of major importance, containing as they did a large amount of glycine betaine and γ-amino butyric acid betaine.

Table 2. Betaine content of seaweed extracts as determined by the ^1H NMR spectroscopic method. Values are means ± standard error (n = 3).

	Betaine contents (mg/g fresh weight)		
α-alanine betaine	glycine betaine	γ-aminobutyric acid betaine	α-alanine betaine
Ulva lactuca	0.02 ± 0.001	nd	0.02 ± 0.001
Cystoseira humilis	0.03 ± 0.001	nd	nd
Fucus spiralis	0.06 ± 0.002	0.07 ± 0.005	nd
Fucus vesiculosus	0.05 ± 0.001	0.06 ± 0.002	nd
Gelidium sesquipedale	0.02 ± 0.001	0.03 ± 0.001	0.02 ± 0.001

nd = not detected

All the species of marine algae tested yielded one or other (or several) of the betaines (glycine betaine, γ-amino butyric acid betaine and α-alanine betaine). It would appear therefore, that these components are a characteristic feature of individual marine algae. However, many of the others compounds appear to occur only infrequently. It is therefore of interest to report the occurrence of α-alanine betaine, as previously it had been recorded only for species of *Ulva lactuca* in the Chlorophyceae and *Gelidium sesquipedale* in the Rhodophyceae.

The results obtained in this study show there to be a high level of consistency in the composition of betaines found within different algal species. For example, glycine betaine has been isolated from all species of algae, but γ-amino butyric acid betaine was isolated only from *F. spiralis, F. vesiculosus* and *G. sesquipedale*; however, glycine betaine and γ-amino butyric acid betaine were major components of all the *Fucus* species studied.

DISCUSSION

Some seaweed extracts were analyzed by both HPLC and ^{1}H NMR spectroscopy. In the first part, purification by TLC gave similar results to those obtained by Konosu & Watanabe (1973) for the green alga *Ulva lactuca*, and for the red alga *Gelidium sesquipedale*, as also reported by Hori *et al.* (1979). Our results were also similar to those reported previously for these species collected from the UK coast (Blunden *et al.*, 1981). While *Ulva lactuca* extracts from Morocco were shown to contain glycine betaine and α-alanine betaine, these latter compounds were not detected in the same species collected from the Red Sea and UK coasts (Blunden, 1985), indicating bio-geographic differences. Betaines have been reported for most of the species of marine algae used in the manufacture of seaweed extracts (Blunden *et al.*, 1982). In some commercial seaweed extracts, for example, the concentration of glycine betaine varied from 2.3 to 31.6 mg/l, and that of γ-aminobutyric acid betaines from 5.4 to 27.2 mg/l (Blunden, 1986).

In the second part of the work reported here, it did not prove possible to detect the betaines by HPLC methods. Similar problems with HPLC detection of betaines in extracts were reported by Blunden (1986). The failure to detect betaines by HPLC can be explained by the intense UV absorption at 195 nm, due to the presence of interfering substances. In control experiments, when aqueous solutions containing only glycine betaine and γ-aminobutyric acid betaine were passed through an ion-exchange column in the same way as the seaweed extracts, UV-absorbing compounds were eluted from the column, which made the estimation of glycine betaine impossible. It was considered, therefore, that the HPLC method was not suitable for the estimation of betaine contents of seaweed extracts without their further purification. The procedure, described by Gorham (1984), which utilized strong anion and weak cation exchange resins, was not satisfactory for the seaweed extracts investigated.

The detection of considerable amounts of these compounds in seaweed extracts opens a new perspective as far as the growth regulation properties of this product are concerned. Wheeler (1973) has reported cytokinin-like activity of betaines in fertilizer products. The present results therefore show the potential for a marked effect of seaweed extract on the frost resistance of higher plants and

amelioration of salt stress in seedlings (Borakev & Ivanova, 1971). Betaines are certainly common compatible solutes affording partial protection of some enzymes against salt inhibition (Pollard & Wyn Jones, 1979).

Acknowledgement. We thank Dr. John Beardall for his help and advice in revising an earlier version of this paper.

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