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Talanta 66 (2005) 244–248
Talanta
www.elsevier.com/locate/talanta

Capillary electrophoretic analysis of dimethylsulfoniopropionate in sugarcane and marine algal extracts

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Received 13 August 2004; received in revised form 19 November 2004; accepted 19 November 2004
Available online 4 January 2005

Abstract

A simple and high-resolution analytical method for the determination of dimethylsulfoniopropionate (DMSP) in sugarcane and marine algae is described. Effective extraction of DMSP from plant samples was also investigated using organic solvents, 5% perchloric acid or deionised water. To increase the sensitivity, DMSP in the extracts was first converted to a phenacyl ester, and the reaction mixture was applied directly to capillary electrophoresis without any pretreatment. Water extraction followed by esterification in a pH 4 reaction buffer was found most suitable for the measurement of alkaline-labile DMSP. This method was applied to the determination of DMSP levels in marine algae samples collected from the seashore of Nagasaki, Japan. An increase in DMSP content in *Ulva pertusa* in the winter period was observed.

Keywords: Capillary electrophoresis; Dimethylsulfoniopropionate; Marine algae; Sugarcane

1. Introduction

Dimethylsulfoniopropionate (DMSP) is a tertiary sulphonium compound found in a wide range of marine algae and in only a few flowering plants such as *Spartina* spp., *Wollastonia biflord* and sugarcane [1–3]. In marine algal studies, DMSP is perhaps best known as the major biological precursor of dimethylsulphide (DMS), the most important volatile organic sulfur compound involved in the global cycling of sulphur, which affects global climate change [4,5]. Secondly, because of its zwitter-ionic nature, DMSP appears to act as an osmoprotectant, also termed a compatible solute, to maintain turgor pressure inside plant cells against outer osmotic pressure caused by the high salinity of seawater [6,7]. In the higher plants, on the other hand, DMSP has been detected in high concentrations in only a few genera as described above, and its function as a compatible solute remains unclear. The reason for this unclearness on the function of DMSP was elucidated through some experiments which suggest that, unlike other compatible solutes such as betaines, which are generally accumulated in response to increased salinity, DMSP does not appear to be induced by variations in external salt concentrations [3,8,9]. Moreover, the biosynthetic pathways of DMSP in marine algae and in terrestrial plants are different [2,10]. Therefore, it is necessary to further our understanding of the behaviour of DMSP levels in response to various adverse environmental conditions, including not only salinity but also drought, high temperature and a deficiency of nitrogen or other nutrients.

In order to further illuminate these alterations of DMSP in plant samples, the compound must be determined with high resolution, sensitivity, rapidity and reliability. A few methods are available for the quantitation of DMSP. One of the typical measurements is an indirect method using GC, which is based on the measurement of the DMS released when DMSP is treated with alkali [11,12]. To overcome this

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doi:10.1016/j.talanta.2004.11.019

shortage of methods, the HPLC method was originally developed by Gorham et al. and modified by Colmer et al. [13,14]. The HPLC method, however, requires preliminary purification using ion-exchange columns; otherwise, good resolution cannot be obtained for a real sample detected at a UV short wavelength such as 194 nm, where many organic compounds have absorption. In the last few years, the commonly used methods have been NMR [15] and FAB-MS [16]. Neither of these methods is entirely satisfactory since neither uses standard laboratory equipment and both methods are costly. Recently, capillary electrophoresis (CE) has become a powerful analytical method for many compounds, even in complex materials, as a high-performance separation technique using more than 100,000 theoretical plates [17]. Another advantage of CE is that the amount of sample required for the analysis is very small, several nanolitres of sample being enough for one run, which is suitable for studies at a cellular level, even for a single plant cell [18,19]. However, CE has a major limitation; i.e., the very short optical pathlength for on-capillary UV detection causes poor sensitivity. This limitation could be overcome by introducing UV-absorbing chromophore to the compound [20].

Our recently developed low-pH CE analytical technique has provided a powerful means of identifying and quantifying betaines, the quaternary ammonium compounds famous as compatible solutes [21,22]. The main aim of this study was to apply low-pH CE to determine DMSP in plant samples and to develop a simple, rapid and high-resolution method using an ultraviolet-absorbing ester to increase sensitivity. The present report also describes improvements in the extraction procedure and the esterification condition for omitting any further pretreatment and for decreasing the decomposition of DMSP. The levels of DMSP in actual sugarcane and marine algae samples are discussed.

2. Experimental

2.1. Instrumentation

All separations were performed with the capillary electrophoresis system CAPI-3300 (Otsuka Electronics, Osaka, Japan) equipped with a photodiode array detector and monitored at 254 nm. A conventional straight uncoated fused-silica capillary (total length, 80 cm; effective length, 68 cm; and internal diameter, 50 μm) was used. A constant voltage of 25 kV was applied for the separations at 25 °C. Sample solutions were siphoned for 60 s in the hydrostatic mode (height, 25 mm). In order to maintain the run-to-run reproducibility, the capillary had to be conditioned between runs by flushing for 2 min each with 0.2 M sodium hydroxide and deionised water and then for 3 min with the electrolyte buffer. A JNM-A500 ^{1}H NMR (JEOL, Tokyo, Japan) was used to confirm the DMS-phenacyl ester.

2.2. Reagents and electrolyte solution

All chemicals were of analytical grade. DMSP was obtained from Tokyo Kasei (Tokyo, Japan). *p*-Bromophenacyl bromide (PBB) and 18-crown-6 were from Wako Pure Chemical Industries (Osaka, Japan). Deionised water was purified by passing distilled water through a Milli-Q system (Millipore, MA, USA).

Working standard solutions of DMSP were prepared daily by diluting a 10 mM stock standard solution with deionised water. PBB was dissolved in acetonitrile to make a 2% (w/v) solution. As an operating electrolyte solution, 100 mM sodium dihydrogenphosphate was used. The pH value was adjusted to 3.0 with 100 mM phosphoric acid. The solution was prepared daily and filtered through a 0.45 μm disk filter (Iwaki Glass Co. Ltd., Chiba, Japan) before use.

2.3. Sample collection and pretreatment

Extracts of sugarcane (*Saccharum officinarum* L.) leaves and marine algae were used for DMSP analysis by CE. Sugarcanes were collected from two farms, Tamagusuku and Sashiki, in Okinawa during December 2001. They were transported to the laboratory by cool-express and then kept frozen. Marine algae were collected from two sites, Nagayo and Teguma, located on the seashore of Nagasaki from October 2002 to January 2003 and were also stored in a freezer.

Three extraction methods were compared: (a) methanol: chloroform:water (12:5:3, v/v), (b) cold 5% (w/v) perchloric acid and (c) water only. All operations were run in an ice bath. The sample (usually 0.5 g) was frozen in a mortar with liquid nitrogen and crushed with a pestle, and 10 mL of cold extract solution was added. The mixture was centrifuged at 1500 × *g* for 15 min. The supernatant was transferred, and the residue was again extracted. Supernatants were combined and used for esterification. For method (a), the organic solvents were removed by evaporation in a rotary evaporator under vacuum at 30 °C, and for (b), the extract pH was adjusted to 4.0 by slowly adding 1.0 M potassium carbonate as described by Fan et al. [23].

2.4. Sample esterification

To a glass tube, 0.2 mL of the standard or sample extract was transferred, and then 0.1 mL of a reaction buffer composed of 100 mM potassium dihydrogenphosphate: 18-crown-6 (1 mg/mL in acetonitrile) = 1:2 (v/v) was added. The pH of the reaction buffer was adjusted to 4.0 by adding 100 mM phosphoric acid. The tube was shaken, and 0.6 mL of PBB solution was added. The tube was capped, heated at 70 °C for 40 min and cooled. The reaction mixture was filtered through a 0.45 μm disk filter and then applied directly to CE. To avoid the evaporation of the organic solvent, every sample vial was capped with a rubber septum (Otsuka Electronics).

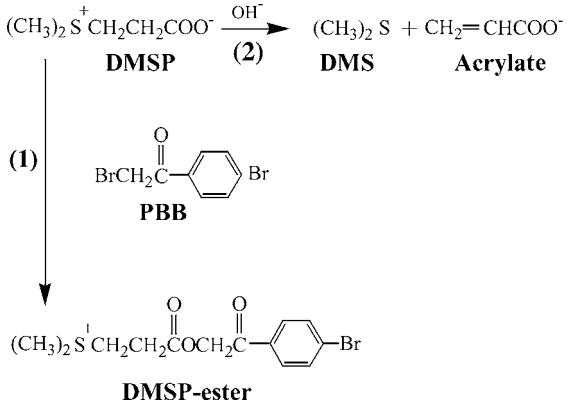

Fig. 1. Chemical structure of dimethylsulfoniopropionate (DMSP) and its decomposition: DMS: dimethylsulphide; PBB: *p*-bromophenacyl bromide.

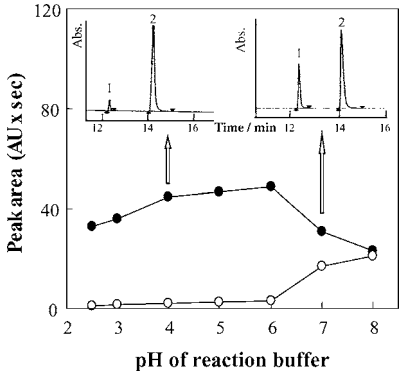

Fig. 2. Effect of reaction buffer pHs on the esterification of DMSP: (●) DMSP-ester; (○) DMS derivative. Inset: (left) electropherogram at pH 4; (right) at pH 7. Reaction and electrophoresis conditions are described in Section 2.

3. Results and discussion

3.1. CE analysis of DMSP-ester

As DMSP has no strong absorbance within the UV range, it was esterified with PBB as shown in Scheme 1 of Fig. 1 to form a *p*-bromophenacyl ester (DMSP-ester), with the absorption maximum at 262 nm according to the method of Gorham et al. [13]. To avoid the decomposition of DMSP to DMS and acrylate under an alkaline condition (Scheme 2 of Fig. 1), the pH of the reaction buffer (phosphate) was changed from 2.5 to 8, and the products were analysed by CE as shown in Fig. 2. Two peaks appeared in the electropherograms (Fig. 2 inset). The major peak 2 (●) was a DMSP-ester, and a stable yield was observed between pH 4 and 6. The small peak 1 (○), which appeared prior to the main peak 2, was a by-product. It was assigned as the phenacyl ester of DMS $(CH_3)_2S^+CH_2COC_6H_4Br$. The structure of the by-product was confirmed both by ^1H NMR of the authentic specimen which was synthesized from DMS and PBB in the same buffer condition as that used in the DMSP-ester preparation and by the coincidence of the migration time of the specimen by CE (data not shown). A decrease of peak 2 together with an increase of peak 1 above pH 6 indicated that the decomposition of DMSP increased as the pH was elevated. DMSP decomposition could be controlled to under 5% as expressed by the peak area ratio of peak 1 to peak 2 at pH 4.

In the present study, esterification was done in the buffer pH 4, and the reaction mixture was directly introduced into the capillary to determine DMSP-ester at 254 nm. Good linearity was shown in the concentration range from 0.02 to 5.0 mM with a 0.005 mM detection limit (S/N = 3). The repeatability ($n = 3$) of the migration time was less than 0.64%, and that of the peak area was less than 2.1%. Under a low pH of around 3, where the electroosmotic flow was negligible, cationic DMSP-ester migrated electrophoretically, and a run was completed within 15 min. A phosphate electrolyte concentration of 100 mM at pH 3.0 was found suitable for analysis.

For comparison, direct measurement of DMSP at 190 nm was done, however, it gave a poorer detection limit of 0.25 mM. Esterification in the present method gave 50-fold higher sensitivity.

3.2. Comparison of three extraction procedures

Three extraction media were compared for the extraction of DMSP in leaf tissue of sugarcane and in *Ulva pertusa*, i.e. (a) methanol:chloroform:water (12:5:3, v/v), (b) cold 5% (w/v) perchloric acid or (c) deionised water only. As shown in Table 1, procedures (a) and (c) gave similar results, whereas (b) showed exceptionally low values. In case (b), the low values could be caused by potassium carbonate, which was used to neutralise perchloric acid. As Gorham et al. pointed out,

Table 1
Comparison of the extraction procedure for the determination of DMSP in sugarcane (*S. officinarum*) and *Ulva pertusa*

Extraction procedure	μmol/g fresh weight		
	S. officinarum[a]	*S. officinarum*[b]	*U. pertusa*[c]
(a) Methanol:chloroform:water	6.49 ± 0.29	6.29 ± 0.44	9.23 ± 0.13
(b) 5% perchloric acid	1.08 ± 0.08	1.40 ± 0.37	2.57 ± 0.67
(c) Water	6.18 ± 0.29	6.77 ± 0.53	9.43 ± 0.41

Values given are means of three separate replicates ± standard error.
[a] Collected from Tamagusuku.
[b] Collected from Sashiki.
[c] Collected in October 2002.

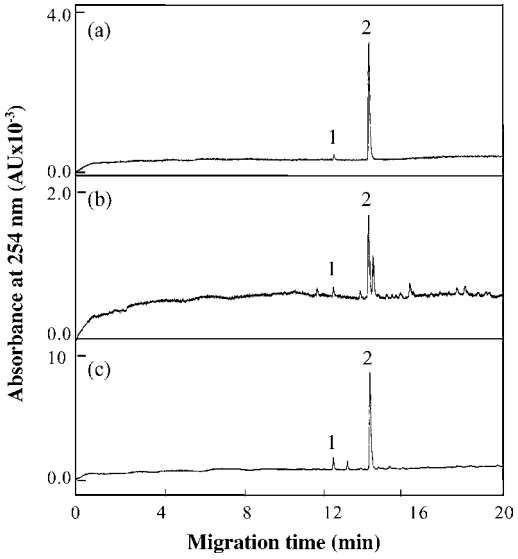

Fig. 3. Electropherograms of DMSP standard solution (a), a tissue extract of sugarcane (b) and *Ulva pertusa* (c) after esterification, detected at 254 nm. Other conditions are described in Section 2.

carbonate interferes with the reaction of DMSP with PBB to give a lower DMSP-ester yield (see Section 2.3) [13]. In the case of organic solvent extraction (a), which has been often used in plant analysis because of the efficient elimination of chlorophylls, DMSP was extracted in a methanol-water layer and esterified quantitatively. However, removal of a trace amount of chloroform remaining in the methanol–water layer was necessary as chloroform interferes with the esterification reaction. Some time-consuming processes to remove the solvent were unavoidable in this solvent system.

Lastly, the water extraction procedure (c) was selected in this study. The typical electropherograms of sugarcane and *Ulva pertusa* are shown in Fig. 3. Extraction was undertaken under a weak acidic condition, from pH 5.6 to 5.9, to keep DMSP stable. Decomposition of DMSP through whole procedures from extraction to esterification was best suppressed, as shown in Fig. 3, where the by-product peak 1 appeared minimal. In Fig. 3b, the major peak which appeared at ca. 13.7 min was assigned to DMSP-ester by the standard addition, while the smaller peak closely appeared at ca. 13.9 min was unknown.

To confirm the recovery of DMSP by this method, 20.0 μmol of standard DMSP was added to the extract of fresh *Ulva pertusa* (1.00 g). The average value of three separate runs was 36.9 ± 1.7 μmol. As the DMSP content of the weed was 17.8 ± 0.2 μmol/g, the recovery of DMSP was 95.5%, indicating the method was reliable.

As shown in Table 1, the average content of DMSP in the leaf tissues of sugarcane was 6.48 μmol/g fresh weight (6.18 and 6.77 in Table 1). On a dry basis, it was 14.9 μmol/g dry weight, the value being within the range of the previously reported levels for mature leaves of sugarcane (9.0–22.1 μmol/g dry weight) [8].

3.3. Determination of DMSP in marine algae

DMSP levels in 13 species of marine algae collected from the seashore of Nagasaki were determined, and the results are summarised in Table 2. DMSP was detected from *U. pertusa*, *C. fragile*, *H. fusiformis* and *S. thunbergi*, but not others. The average content in the green alga *U. pertusa* was 33.5 μmol/g dry weight, which was from 3.2- to 58.8-fold higher than in other three algae. Similar results were reported previously for *Ulvaceae* (10.5–53.0 μmol/g dry weight). Another green alga, *C. fragile*, also showed a high DMSP content as 10.4 μmol/g dry weight, similar to the reported values in *Codiaceae* (9.0–30.6 μmol/g dry weight) [24]. However, DMSP contents in brown and red algae are remarkably lower than the contents reported for green algae.

Changes of DMSP content in *U. pertusa* were monitored by this analytical method in the winter of 2002 as illustrated in Fig. 4. *U. pertusa* samples were collected approximately monthly, but the sampling at the Teguma site (○) was not conducted after November 2002 because of very poor growth of the species. On the other hand, a remarkable increase in DMSP content was observed in *U. pertusa* collected from

Table 2
DMSP contents of marine algae collected from the seashore of Nagasaki, Japan[a] (μmol/g dry weight)

Nagayo		Teguma	
Species	Content	Species	Content
Ulva pertusa	36.4 ± 3.1	*Ulva pertusa*	30.6 ± 3.5
Codium fragile	10.4 ± 0.1	*Hizikia fusiformis*[b]	0.76 ± 0.15
Sargassum thunbergii	0.57 ± 0.02	*Gelidium elegans*	<dl
Actinotrichia fragilis	<dl	*Sargassum horneri*	<dl
Dictyota dichotoma	<dl	*Sargassum thunbergii*	<dl
Lomentaria catenata[c]	<dl	*Ishige okamurae*	n.d.
Hypnea flexcaulis	n.d.		
Grateloupia filicina[c]	n.d.		
Sargassum hemiphyllum[b]	n.d.		

Values given are means of three separate replicates ± standard error. dl: detection limit; n.d.: not detected.
[a] Samples were collected in October 2002.
[b] Collected in November.
[c] Collected in December.

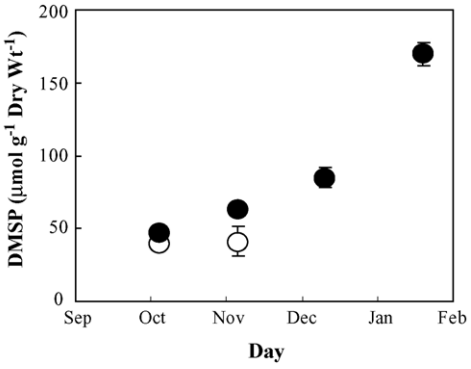

Fig. 4. DMSP contents of *Ulva pertusa* collected from Nagayo (●) and Teguma (○) from October 2002 to January 2003.

Nagayo (●), which is located in the river mouth and in the deepest part of Omura Bay, which is almost enclosed. To our knowledge, this is the first report as monitored by CE that DMSP in natural *U. pertusa* increased along with its growth. The reason for the increase in DMSP content is now under investigation in relation to the osmotic adaptation under cell development.

4. Conclusion

Low-pH capillary electrophoresis was developed as a simple, rapid and high-resolution method for determining DMSP in higher plant and algal samples. Even though the method involves esterification processes, it is free from complicated pretreatments required in other analytical processes. Above all, the sensitivity of DMSP-ester increased 50-fold more than direct detection of DMSP itself by CE at 190 nm. Moreover, the extraction of DMSP with water only is simpler than that using organic solvents or strong inorganic acids. The developed method was applied to natural samples, and clear electropherograms were obtained, indicating the method is suitable for routine assay because the procedure is simple and time is saved in sample handling.

Acknowledgements

A part of this work was supported both by the Mishima Kaiun Memorial Foundation 2003 and by a Grant-in-Aid for Scientific Research (B) (No. 15380225) from the Ministry of Education, Science, Sports and Culture of Japan. The authors would also like to express thanks to Dr. M. Iima, Nagasaki University, for marine algae classification and Ms. Y. Ikehara, Okinawa Fermentation Technology Co. Ltd., for providing sugarcane samples.

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