ELSEVIER

Journal of Chromatography B, 685 (1996) 176–180

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

High-performance liquid chromatographic determination of the imino acids (opines) *meso*-alanopine and D-strombine in muscle extract of invertebrates.

Ben Sandee[a,*], Cor A. Schipper[a], Richard H.M. Eertman[b]

[a]*National Institute for Coastal and Marine Management, RIKZ, P.O. Box 8039, 4330 EA Middelburg, Netherlands*
[b]*Netherlands Institute of Ecology, Centre for Estuarine and Coastal Ecology, Yerseke, Netherlands*

Received 24 January 1996; revised 14 March 1996; accepted 14 March 1996

Abstract

A sensitive HPLC method is presented for the determination of the imino acids alanopine and strombine, anaerobic metabolites that are formed in muscle tissue of several species of invertebrates. The separation of alanopine and strombine was achieved using the Alltech OA 2000 cation-exchange column. The analysis of the two opines does not require any complicated derivatization and can be performed in a pH neutralized sulphuric acid solution. The sensitivity of this method is in the range of 100 pmol to at least 10 nmol for both investigated opines. For the first time opines were demonstrated in the bivalves *Macoma balthica* and *Cerastoderma edule*.

Keywords: Imino acid; Opines; Alanopine; Strombine

1. Introduction

Opines, or iminocarboxylic acids, are important end-products of anaerobic metabolism in a range of invertebrates, including poriferans, annelids and molluscs [1,2]. Opines are synthesized by a number of different NAD^+/NADH-dependent pyruvate oxidoreductases, the so-called opine dehydrogenases, enzymes that are involved in the maintenance of the cytoplasmic redox balance during anaerobiosis. Strombine [*N*-(carboxymethyl)-(D)-alanine], alanopine [*meso*-*N*-(1-carboxyethyl)alanine], and octopine [*Nα*-(D-1-carboxyethyl)-L-arginine] are the result of the reductive condensation of pyruvate with respectively the amino acids glycine, alanine and arginine. Species specific differences exist in opine formation, regarding both the types of opines that are formed and the respective concentrations of each opine. The mussel *Mytilus edulis* for instance is known to produce strombine, alanopine and octopine, of which strombine occurs in the highest concentration [3]. Besides their role as end-products of anaerobic metabolism, little is known about the function of opines in invertebrates. It has been suggested that opines could have a function as fish attractants [4] or cryoprotectants [5] in bivalves. When studying the anaerobic metabolism of invertebrate species in detail or the specific functions of opines in general, analytical techniques are necessary for the determination of opines that are sensitive, reliable and preferably cost- and time-efficient.

Various methods have been used in the past for the

*Corresponding author.

0378-4347/96/$15.00
PII S0378-4347(96)00142-9

determination of strombine and alanopine in tissue extracts. The enzymatic assays that are often used [6,7] do not discriminate between alanopine and strombine, and can only determine the sum of both compounds. Additionally, the opine dehydrogenases have to be purified from tissue extracts, as they are not commercially available, and relatively large sample volumes are needed for the assay. Other methods include paper electrophoresis [8,9], gas chromatography [10], HPLC with the use of derivatization [11,12] and isotachophoresis [13]. Although each of these methods has certain advantages, they also have disadvantages. Some methods are elaborate, others lack sensitivity or require a complicated derivatization. Research into the anaerobic metabolism of invertebrates would benefit from a simple method for the detection of the opines strombine and alanopine. Siegmund and Grieshaber [14] gave a stimulus to opine research by developing a simple HPLC method not requiring any derivatization. However, these authors prepared the HPLC column themselves and the cation-exchange resin used is no longer available. In the present study we describe a similar technique for the determination of strombine and alanopine using an alternative cation-exchange column (Alltech OA 2000). We will demonstrate that strombine and alanopine can be quantified conveniently in a number of invertebrates.

2. Experimental

2.1. Materials

The imino acids (opines) strombine and alanopine are not commercially available and were kindly provided by Dr A. de Zwaan (Netherlands Institute of Ecology, Centre for Estuarine and Coastal Ecology) and Prof. dr M. Grieshaber (Heinrich Heine Universität, Düsseldorf, Germany) respectively. Alanopine and strombine were synthesized following the procedure of de Zwaan and Zurburg [13]. Potassium carbonate was purchased from Sigma (St. Louis, MO, USA), perchloric acid from Baker, and analytical grade sulphuric acid from Merck. All solutions were prepared using distilled water which was purified in a Milli-Q Reagent Water System (Millipore, Bedford, MA, USA).

2.2. HPLC system

Separation of the opines was performed on a Spectra-Physics (TSP) SpectraSYSTEM, consisting of a P4000 pump, a SN4000 controller and a AS3000 autosampler equipped with a Rheodyne injector. An IBM 486DX/2 computer was employed for data processing and storage. The opines were separated on an Alltech OA-2000 column (100×6.5 mm I.D.) fitted with a OA-1000 guard column, both packed with a strong cation-exchange resin. During the test period analyses were performed using a range of eluent concentrations ($2.5 \cdot 10^{-5}$–$5.0 \cdot 10^{-3}$ M) and temperatures (≥45°C) to achieve an optimal separation of the two opines within relatively short time. Optimal conditions for analysis were found to be: eluent concentration $6.0 \cdot 10^{-5}$ M sulphuric acid (degassed by vacuum); flow-rate 0.7 ml min^{-1}; column temperature 74°C. Alanopine and strombine were monitored with conductivity detection (Waters Model 431 conductivity detector), as both compounds have low UV absorption. Prior to analysis the column was equilibrated with eluent for a minimum of 4 h. Sample volumes ranged from 10 to 40 μl. After prolonged use the column may become contaminated and has to be washed with the eluent.

2.3. Experimental animals

The bivalves *Mytilus edulis* and *Crassostrea gigas*, as well as the gastropod *Littorina littorea* were collected from the shore of the Oosterschelde basin. The lugworm *Arenicola marina* was collected from the intertidal flat of this sea arm. The bivalves *Cerastoderma edule* and *Macoma balthica* were collected from the shore of the Wadden Sea. The blood clam *Scapharca inaequivalvis* was dredged in the Adriatic Sea off the Italian coast. The metabolism of opines was stimulated in the experimental animals by exposing them to anoxia. This was achieved by placing the animals in N_2-gassed seawater for 24 h, according to the method described by De Zwaan et al. [15].

2.4. Preparation of tissue extracts

The posterior adductor muscle (PAM) of the investigated bivalves, the foot muscle of the gas-

tropod and the body wall musculature of the lugworm were excised after 24 h exposure to N_2-gassed seawater, and immediately frozen in liquid nitrogen. The frozen muscles were stored at −80°C until further use. Before preparing extracts the frozen muscles were first lyophilized for 48 h (wet weight/dry weight ratio ≈4.6). The dry muscle tissue of individual specimens was then powdered, weighed and homogenized in a thirty-fold volume of 7% (v/v) perchloric acid (PCA). As the adductor muscles of *C. edule* and *M. balthica* were very small, three and four muscles respectively were pooled for opine analysis. The powdered tissues were homogenized using an electrically driven Potter–Elvehjem teflon homogenizer. The homogenate was centrifuged at 20 000 *g* for 15 min, precipitating the proteins. For the determination of the opines strombine and alanopine, a 500-μl volume of the supernatant was adjusted to approximately pH 7 with 313 μl of 1 *M* K_2CO_3. The $KClO_4$ formed was removed by a second centrifugation. The supernatant was used for the determination of the opines.

3. Results and discussion

HPLC separation of the opines alanopine and strombine without complicated derivatization or elaborate tissue preparation could be performed satisfactorily using the Alltech OA-2000 cation-exchange column. The separation of alanopine and strombine standards is shown in Fig. 1A. Alanopine and strombine gave single, fully separated, peaks with comparable peak areas. During the extensive testing of the HPLC column a distortion of the respective peak shapes was never observed, indicating that interference by unknown compounds was unlikely. The separation of alanopine and strombine can be performed with high accuracy. The variation in retention times is less than 0.6% for each compound (Table 1). The detection limit for the two compounds (signal-to-noise ratio 3) just exceeds the 100 pmol level. At signal-to-noise ratios ≥9 (≈300 pmol) the reproducibility of the determination was ±4.5% or better (S.D.). Highly significant correlations (r>0.99) exist between the concentration of alanopine and strombine and peak area and peak height. The separation of the two opines in an extract

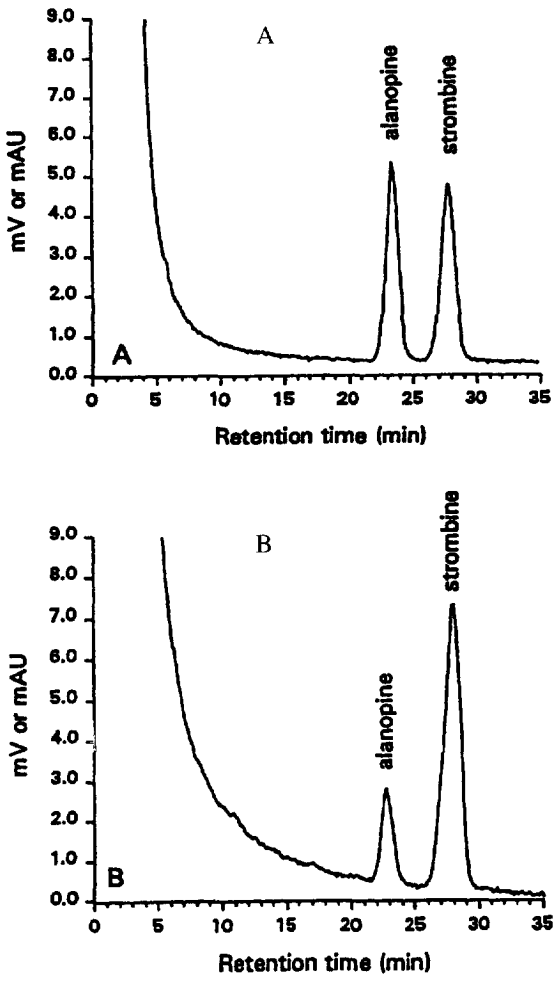

Fig. 1. Separation profile of alanopine and strombine on the Alltech OA 2000 cation-exchange column with conductivity detection. (A) Chromatogram of alanopine and strombine standards (both 2.5 nmol). (B) Chromatogram of alanopine (1.1 nmol) and strombine (4.5 nmol) in the posterior adductor muscle of the mussel *Mytilus edulis* after 24 h exposure to anoxia.

of the posterior adductor muscle of the mussel *M. edulis* after 24 h exposure to anoxia is shown in Fig. 1B. The retention times of the two compounds did not exceed the ranges established for the standards. Standard additions of alanopine and strombine standards (0.5 and 1.0 nmol of each compound) resulted in almost full recovery of the two compounds (>95%). In agreement with data from literature [16] the formation of strombine exceeds that of alanopine in *M. edulis*. Besides the two opines no other

Table 1
Retention times, detection limits and linear regression ranges of opine standards

Compound	n	Retention time (min)	Detection limit (pmol)	Linear regression range
Alanopine	10	23.26±0.12	105	105 pmol–10 nmol
Strombine	10	27.71±0.16	115	115 pmol–10 nmol

For HPLC conditions see Section 2.2.

compounds with comparable retention times were detectable. In addition to opines a range of volatile fatty acids are formed by mussels and other marine invertebrates during anoxia. These metabolites pass through the Alltech column within a few minutes under the given HPLC conditions. Separation of these compounds on a cation-exchange column requires a more concentrated eluent [17].

Table 2 shows the concentrations of alanopine and strombine in muscle extracts of various marine invertebrates after 24 h of anoxia. The retention times and elution profiles of alanopine and strombine in the tissue extracts of the various species were comparable to the values and profile of *M. edulis* as shown in Fig. 1B. The blood clam *S. inaequivalvis* is known to produce both α- and ß-alanopine [9,18]. However, only a single peak was present on the chromatogram indicating that both isomers behave identically on this cation-exchange column. The concentrations of the two opines show large variations between species and the alanopine to strombine ratio also differs between species. The concentrations that were estimated with this HPLC method agree well with the concentrations that were reported in literature [1,14]. Grieshaber and Kreutzer were unable to detect alanopine or strombine in the cockle *Cerastoderma (Cardium) taberculatum* with the enzymatic method, although this species did posses the necessary opine dehydrogenase enzyme activity. With the present method strombine could be detected in the related species *C. edule*. The sensitivity of this HPLC method enables the detection of alanopine and strombine in the muscle tissues of species that were far too small for the enzymatic method to be used. Concentrations of the two opines in the minute muscle tissue of *M. balthica* have not been reported before. The HPLC method developed by Sato et al. [12] also showed high sensitivity, but requires an elaborate precolumn derivatization with phenylisothiocyanate (PITC). PITC derivatization has the advantage that other, less common, acetic imino acids (tauropine and β-alanopine) can also be determined in tissue extracts. However, as a result of the derivatization procedure α-alanopine appeared as two separate peaks rather than as a single peak. Our method of sample preparation is very similar to the initial steps of sample preparation used by Sato et al. [12]. Whereas our extract is ready for HPLC analysis after the second centrifugation, Sato et al. still had to complete various procedures (elution of extract from Dowex column, concentrating of extract to appropriate volume and finally PITC derivatization). Besides the fact that these procedures are very time consuming, they may also introduce a larger degree

Table 2
Concentration of alanopine and strombine in various species of marine invertebrates after 24 h of anoxia

Species	Average tissue weight per sample (mg DW)[a]	Alanopine (μmol g^{-1} DW)	Strombine (μmol g^{-1} DW)
Mytilus edulis	50	1.7	4.4
Crassostrea gigas	56	0.2	2.7
Littorina littorea	52	7.9	2.1
Cerastoderma edule	33	<0.15	3.3
Macoma balthica	5	34.2	35.6
Scapharca inaequivalvis	562	0.2	0.5
Arenicola marina	89	3.3	32.9

[a] Amount of tissue processed during initial sample preparation; DW=dry weight.

of error into the analysis. Our procedure for sample preparation is simple and short and by processing ten subsamples we were able to estimate the margin of error as a result of handling at 2.1% (S.D.), well within the accuracy of the method. For the determination of α-alanopine and strombine, two of the most common opines in invertebrates, the method presented in this paper has proven to be both sensitive and convenient.

Acknowledgments

The authors wish to thank Prof. dr M. Grieshaber and Dr A. de Zwaan for kindly providing the alanopine and strombine standards. The authors are grateful to Dr W. Zurburg for his useful advice, and to Ir B. Timmermans for collecting two bivalve species along the Wadden Sea shore. This publication is registered as number 2006 at the Netherlands Institute of Ecology, Centre for Estuarine and Coastal Ecology.

References

[1] M.K. Grieshaber and U. Kreutzer, Zool. Beitr. N.F., 30 (1987) 205.
[2] D.R. Livingstone, A. de Zwaan, M. Leopold and E. Marteijn, Biochem. System. Ecol., 11 (1983) 415.
[3] W. Zurburg, A. De Bont and A. De Zwaan, Mytilus edulis L. Mol. Physiol., 2 (1982) 135.
[4] A.W. Sangster, S.E. Thomas and N.L. Tingling, Tetrahedron, 31 (1975) 1135.
[5] S.H. Loomis, J.F. Carpenter and J.H. Crowe, Mytilus edulis. Biochim. Biophys. Acta, 943 (1988) 113.
[6] A. De Zwaan, P. Cortesi and O. Cattani, Sci. Total Environ., 171 (1995) 121.
[7] A. Demers and H. Guderley, Mar. Biol., 118 (1994) 115.
[8] M. Sato, Y. Sato and Y. Tsuchiya, Bull. Jpn. Soc. Sci. Fish., 48 (1982) 1411.
[9] M. Sato, M. Takahara, N. Kanno, Y. Sato and W.R. Ellington, Comp. Biochem. Physiol., 88B (1987) 803.
[10] K.B. Storey, D.C. Miller, W.C. Plaxton and J.M. Storey, Anal. Biochem., 125 (1982) 50.
[11] G.B. Fiore, C.V. Nichitta and W.R. Ellington, Anal. Biochem., 139 (1984) 413.
[12] M. Sato, S. Suzuki, Y. Yasuda, H. Kawauchi, M. Kanno and Y. Sato, Anal. Biochem., 174 (1988) 623.
[13] A. De Zwaan and W. Zurburg, Mar. Biol. Lett., 2 (1981) 179.
[14] B. Siegmund and M.K. Grieshaber, Hoppe-Seyler's Z. Physiol. Chem., 364 (1983) 807.
[15] A. De Zwaan, P. Cortesi, G. van den Thillart, J. Roos and K.B. Storey, Mar. Biol., 111 (1991) 343.
[16] U. Kreutzer, B.R. Siegmund and M.K. Grieshaber, J. Comp. Physiol., 159 (1989) 617.
[17] C. Womersley, L. Drinkwater and J.H. Crowe, J. Chromatogr., 318 (1985) 112.
[18] A. De Zwaan, P. Cortesi, O. Cattani, G. Isani and G. Vitali, Biol. Mar., Suppl. Notiziario SIBM, 1 (1993) 91.