Proc. Indian natn. Sci. Acad. **B48** No. 1 pp 92–96 (1982)

Qualitative Studies on Proteins from the Crystalline Style of *Donax cuneatus*

N BHARATHI and K RAMALINGAM
Zoology Department, University of Madras, Madras 600005

(Received 16 September 1981; after revision 11 January 1982)

Chromatographic analysis of the free and protein-bound amino acids of the style of *Donax cuneatus*, an intertidal bivalve of the sandy shore, was made. In addition, proteins of the style of *Donax cuneatus* were analysed using disc electrophoresis. The consistency of the style and the nature of the structural protein of this extracellular organ is explained with the help of this qualitative study. The protein pattern of the style clearly indicates a taxonomic relationship with other bivalves.

Key Words: *Donax cuneatus*, **Crystalline Style, Amino acids, Chromatography, Protein-Pattern, Electrophoresis**

Introduction

The bivalve crystalline style is a concentrically laminated hyaline rod, composed largely of mucoprotein (Bailey & Worboys 1960, Kristensen 1972). It resides in a caecum, the style sac which is open to the stomach (Yonge 1926). The end of the crystalline style which is exposed to the stomach softens and sols, releasing a variety of digestive enzymes into the stomach (Yonge 1926). The function of the style has been considered by many workers and now it is generally agreed, that it secretes digestive enzymes (Bailey & Worboys 1960). A variation in the consistency of the style of different species of bivalves has been reported, the difference being correlated with the rapidity with which the structure underwent dissolution (Yonge 1926, Morton 1951). It was found that the styles of unionidae, *Mylitus* and *Ostrea* are soft and gelatinous, whereas the styles of *Cardium*, *Solen*, *Donax* and *Pholax* are much firmer (Morton 1951, 1952). Such differences in the hardness of the style could be due to the nature of the structural protein. The difference in the protein composition of the styles of different bivalves is evidenced by the difference in their amino acid composition. Extracellular matrices among invertebrates exhibit a wide structural diversity and chemical complexity. Various mechanisms of hardening of the structural proteins have been postulated (Brown 1950). Hence the present study was undertaken with a view to understand the chemical nature of the structural protein of the crystalline style of *Donax cuneatus*. The results are compared with the reports already available on the free

and protein-bound amino acids of the style in other bivalves. Moreover, a close correlation between the protein patterns of style and taxonomic relationships of bivalves (Bedford & Reid 1969) has been noted.

Materials and Methods

Donax cuneatus was collected from the lower intertidal regions of the sandy shores of Madras coast during hightide and midtide levels, as it has been reported that the style dissolves during the lowtide period (Morton 1952). Crystalline styles, usually 15 mm long, were removed from animals ranging in size from 38 mm to 42 mm. Free and protein-bound amino acids were determined by ascending paper chromatographic technique (Smith 1976). Several styles were pooled and homogenized with distilled water in a glass homogenizer for the determination of free amino acids. It was centrifuged after addition of twice the volume of alcohol. The supernatant liquid was treated with thrice the volume of chloroform and centrifuged. The acqueous layer containing the free amino acids was used for analysis. For the analysis of bound amino acids, the styles were homogenised with 6N HCl and hydrolysed for 20 hr at 100°C. The HCl was evaporated over a steam bath and subsequently evaporated thrice with water. Finally a few drops of ammonia were added to remove the traces of HCl. The thin film of amino acid hydrochlorides was dissolved in 10% isopropanol. This was spotted for chromatographic analysis of protein-bound amino acids (Ersser & Smith 1976). Standard amino acids were run along with samples as control. The solvent mixture used was Butanol: Acetic acid: Water in the ratio of 4:1:1. Developing reagent used was 0.2% ninhydrin in acetone.

Electrophoresis

To study the protein pattern of the crystalline style of *Donax cuneatus*, polyacrylamide gel electrophoresis was carried out as described by Davis (1964): 5% gel was used as supporting medium, Tris-HCl at *p*H 8.9 as gel buffer; and Tris-glycine at *p*H 8.3 as tank buffer. Samples containing 300–400 μg protein, were directly applied on top of the running gel. Proteins from the crystalline styles were extracted by grinding the crystalline style in 20% sucrose in distilled water (10% w/v) and centrifuging at 3000 g for 10 min. The supernatant was immediately analysed by polyacrylamide gel electrophoresis. The gels were stained in 1% amido black in 7% acetic acid in water (v/v). The gels were scanned in *Joyce Loebel* recording densitometer.

Results

The crystalline style of *Donax cuneatus* contains about ten free amino acids. They include two sulphur-containing amino acids, namely cystine and cysteine, two aromatic amino acids namely tyrosine and phenylalanine and two basic amino acids, histidine and arginine. Besides these amino acids, threonine, alanine, valine and leucine are present. Interestingly enough both the acidic amino acids are wanting. The results of the bound amino acids are of interest in that the style contains about fifteen amino acids (table 1). Electrophoretically, the style is heterogenous, showing six distinct bands, as seen from the zymogram (figures 1 & 2).

Discussion

The style contains acid mucopolysaccharide protein complex (Bailey & Worboys 1960, Kristensen 1972) of which the

Figure 1 Protein pattern of the crystalline style of *Donax cuneatus*

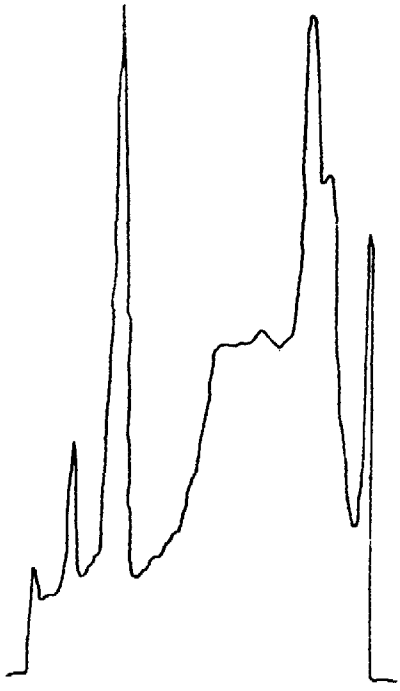

Figure 2 Zymogram showing the protein pattern of the crystalline style of *Donax cuneatus*

Table 1 *Chromatographic analysis of free and protein-bound amino acids from the crystalline style of* Donax cuneatus

Amino acids	Samples (free)	Samples (protein-bound)
Cystine	+ +	+ +
Cysteine	+	+ +
Lysine	—	—
Histidine	+ + +	+ +
Arginine	+ +	+ +
Glycine	—	—
Aspartic acid	—	—
Serine	—	+
Glutamic acid	—	+
Threonine	+ +	+
Alanine	+ +	+
Tyrosine	+ + +	+ +
Tryptophan	—	—
Methionine	—	+
Valine	+	+ +
Phenylalanine	+ +	—
Isoleucine	—	+
Leucine	+ +	+ +
Hydroxyproline	—	+ +
Proline	—	+ +

— = Negative; + = Positive; + + = Very positive; + + + = Intensely positive

protein component is rich in aromatic amino acids. Several authors have investigated the amino acid composition of the style of bivalves, with a view to derive the chemical composition of the style (Hashimoto et al. 1954, 1955, Venugopalan 1956, Swaminathan 1958). The observed pattern of free amino acids in *D. cuneatus* is in general accordance with the earlier reports though there are some interesting variations. A comparison of the pattern of free amino acids of the style of *Donax* with *Mactra subcataria* and *Sanguinolaria diptor* indicates that cystine, alanine, tyrosine and phenylalanine are common to the style of these three species. However, a high proportion of tyrosine, cystine, methionine and tryptophan was reported in some marine bivalves (Bailey & Worboys 1960). Ten free amino acids are reported in the style of *Mactra subcataria* (Hashimoto et al. 1954, 1955), thirteen in *Sanguinolaria diptor* (Venugopalan 1956) and eight in *Telescopium telescopium* (Swaminathan 1958). We

found ten free amino acids in *D. cuneatus*. Thus, the composition of free amino acids in the style varies in different bivalves.

Our results show the presence of 15 protein-bound amino acids in *D. cuneatus* as against 18 in *Sanguinolaria diptor* (Venugopalan 1956). While ornithine, asparagine, aspartic acid, and lysine are present in the style protein of *S. diptor*, they are absent in the style of *D. cuneatus*. On the other hand the amino acids cysteine, threonine and hydroxyproline which are absent in *S. diptor* have been detected in the crystalline style of *Donax cuneatus*. The presence of hydroxyproline and proline in the style of *D. cuneatus* is suggestive of collagenous type of protein (Brown 1950). Yet another feature of interest in the style protein of *Donax cuneatus* is the presence of the amino acid cystine. Therefore, it is possible that S–S crosslink in the style protein renders hardness (Brown 1950). The possible presence of more than one protein component in the styles of different species of bivalves has already been reported (Bailey & Worboys 1960, Bedford & Reid 1969). In addition, there is the possibility of stabilization by quinone tanning [as already established by Pryor (1940) and Brown (1950)] since enzyme phenoloxidase has been reported in the crystalline style of a few bivalves (Berkley 1935, Johansson 1945) and correspondingly a high concentration of aromatic amino acids is seen in *Donax cuneatus* as in the case of other bivalves (Bailey & Worboys 1960).

Electrophoretic analysis reveals the presence of six protein fractions and it compares with the style protein pattern of other bivalves already reported by Bedford and Reid (1969). The notable common feature is the dense protein band about half way down the gel. Thus the protein pattern of the crystalline style of *Donax cuneatus* shows a distinct taxonomic relationship with other bivalves.

It can be concluded that the free and protein-bound amino acids vary in different bivalves and correspondingly a difference in the protein pattern of the style is noted. Different modes of stabilization of the structural protein of the style have been discussed in relation to the qualitative analysis. The difference in the protein pattern could possibly be the reason for the rigidity of the style in different bivalves.

Acknowledgement

The author (N B) is thankful to the Council of Scientific and Industrial Research, New Delhi, for financial assistance.

References

Bailey K and Worboys B D 1960 The lamellibranch crystalline style; *Biochem. J.* **76** 487–491

Bedford J J and Reid M S 1969 Gel electrophoresis of proteins in the crystalline style of certain mollusca; *Comp. Biochem. Physiol.* **29** 659–664

Berkley C 1935 The chemical composition of the crystalline style and gastric shield with some new observations on the occurrence of the style oxidase; *Biol. Bull.* **68** 107–114

Brown C H 1950 A review of the methods available for the determination of the types of forces stabilizing structural proteins in animals; *Quart. J. micros. Sci.* **91** 331–339

Davis B J 1964 Disc electrophoresis II Method and application to human serum proteins; *Ann. N.Y. Acad. Sci* **121** 404–427

Ersser R S and Smith I 1976 Amino acids and related compounds; in *Chromatographic and Electrophoretic Techniques* Vol I (4th edn.) pp 75–108 eds I Smith and J W T Seakins (London: William Heinemann Medical Books Ltd)

Hashimoto Y, Sato T and Miyamoto K 1954 Studies on the crystalline style of molluscs. I. Chemical constituents of the style of *Mactra subcataria* Reeve; *Bull. Jap. Soc. Sci. Fish.* **20** 406–410

——and—— 1955 Studies on the crystalline style of molluscs. II, Chemical constituents of the style of *Mactra subcataria*; *Bull. Jap. Soc. Sci. Fish.* **21** 352–356

Johansson J 1945 Ueber Oxydasen and Peroxydasen im Kristallstiel der Muscheln; *Ark. Zool.* **36A** 13 1–7

Kristensen J H 1972 Structure and function of crystalline styles of bivalves; *Ophelia* **10** 91–108

Morton J E 1951 The ecology and the digestive system of the Struthiolariidae (Gastropoda); *Quart. J. microsc. Sci.* **92** 1–25

——1952 The role of the crystalline style; *Proc. malac. Soc. Lond.* **29** 85–92

Pryor M G M 1940 On the hardening of the Ootheca of *Blatta orientalis; Proc. R. Soc.* **B128** 378–393

Smith I 1976 Paper Chromatographic Apparatus and Techniques; in *Chromatographic and Electrophoretic Techniques* Vol. I (4th edn) pp 18–39 eds I Smith and J W T Seakins (London: William Heinemann Medical Books Ltd.)

Swaminathan S 1958 The amino acids of the Crystalline style of *Telescopium telescopium* Linn. (Gastropoda — Prosobranchiata); *J. Zool. Soc. India* **10** 150–154

Venugopalan V K 1956 The amino acids of the crystalline style of *Sanguinolaria diptor; J. Anim. Morph. Physiol.* **3** 109–113

Yonge C M 1926 The disappearance of the crystalline style; *Nature, Lond.* **117** 691