CHAPTER 1.6

Intracrystalline Molecules from Brachiopod Shells

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Key words: Brachiopod, intracrystalline, protein primary sequence, amino acid analysis.

INTRODUCTION

Shells are composed of both organic and inorganic constituents. It is believed that the organic compounds have important functions at several stages during the formation of biominerals. In brachiopod shells the disposition of inorganic biominerals and their enclosing organic sheaths have been thoroughly investigated using both scanning and transmission electron microscopy but little is known about the biochemistry of the intracrystalline molecules i.e. those enclosed within the inorganic portion. Such information is crucial for an understanding of biominerals if, as has been suggested, these compounds (i) induce crystal nucleation by providing a surface for precipitation, (ii) form compartments that determine the shape and volume of the biocrystal and (iii) determine the pattern of growth in the mineral phase in what is termed 'matrix mediated mineralisation' [1].

This study presents the first details of the organic intracrystalline components from the shell of the articulate brachiopod, Neothyris lenticularis (Deshayes). The shell of N. lenticularis is composed of numerous long calcite fibres from which the proteins studied here have been extracted. Although this protocol has disadvantages in that it is very time-consuming and the great proportion of the shell is discarded, the major advantage of such a strategy is that it avoids the possibility of including extraneous molecules both from contaminating organisms, such as bacteria, which may infest the organic sheaths of shell calcite fibres and from human finger tips during collection and preparation.

Partial N-terminal sequence and amino acid analyses of two shell proteins are presented here alongside SDS PAGE and hplc analyses of the intracrystalline molecules of N. lenticularis.

MATERIALS AND METHODS

Extraction of Shell Proteins

Shells of living N. lenticularis were collected from Stewart Island, New Zealand and killed by dehydration. The shells were cleaned thoroughly and incubated for 2 h at 22°C in an aqueous solution of bleach (5% v/v) to destroy the organic sheath and any possible bacterial contamination. The shells were then powdered in a ceramic pestle and mortar before incubating overnight at 4°C in an aqueous solution of bleach (1% v/v). The bleach was removed by repeated washes with Milli Q™ water followed by centrifugation (8 g.h). The precipitate was washed until no bleach could be detected (typically ten 2 l washes) and then lyophilised and EDTA (20% w/v), pH 11 added in the ratio of 23 ml to 1 g shell. The entire mixture was agitated at 4°C for 72 h or until the inorganic phase had dissolved. Following centrifugation (20 g.h) the supernatant was concentrated and the EDTA removed using the Millipore Minifiltr™ tangential flow system. The preparation was further concentrated in a minicon static concentrator (Amicon) with a 10 kDa cut-off membrane.
Separation of Proteins by hplc

An aliquot of concentrated shell extract was applied to a reverse-phase Aquapore™ RP-300 narrow bore (2.1 mm diameter x 30 mm length) column in trifluoroacetic acid (0.1% v/v) at a flow rate of 0.1 ml/min. After 5 min, a 40 min linear gradient of 0 to 70 % (v/v) acetonitrile, in 0.1% (v/v) trifluoroacetic acid, was applied to fractionate the shell proteins. The eluate was monitored at 280 and 214 nm.

Separation of Proteins by SDS PAGE

Small gels (9 cm x 7 cm) of 0.75 mm thickness containing 15% polyacrylamide were prepared according to the method of Schagger and Van Jagow [2]. Glycine, which is used in most SDS PAGE systems is here replaced by tricine. Samples for electrophoresis were heated at 100°C for 4 min in an equal volume of sample buffer containing final concentrations of 0.15 M Tris / HCl, pH 6.8, 0.2 M 2-mercaptoethanol, 0.1% (w/v) SDS, 30% (v/v) glycerol and 0.0002% (w/v) of the tracking dye, bromophenol blue. Molecular weight standards; bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and a-lactalbumin (14.2 kDa) were included on every gel. Electrophoresis of samples in the small gel system required a constant voltage of 100 V for 2 h. Following electrophoresis, proteins were either fixed in the gel and visualised using Coomassie Brilliant Blue-R or electroblotted onto ProBlott™ membrane (Applied Biosystems).

Electroblotting of Proteins

Following SDS PAGE, the proteins in the gel were transferred to ProBlott™ membrane. The transfer was performed in transfer buffer (10 mM CAPS buffer, pH 11, 10% (v/v) methanol) in a Bio-Rad Trans Blot cell. A constant voltage of 50 V for 0.5 h moved the proteins from the gel towards the membrane. Coomassie Blue staining was used to reveal the protein bands on the ProBlott™ membrane.

Amino-Terminal Sequence Determination and Amino Acid Analysis

Automatic Edman degradation was carried out on the stained bands using a pulsed liquid protein sequencer (Applied Biosystems 477A). Bands were also loaded onto the 420-H amino acid analyser with automatic hydrolysis (Applied Biosystems) to determine the overall amino acid composition. Amino acid analysis of the hplc fractions was employed to identify the major proteins by comparing with the analyses from the homogeneous electroblotted proteins. Stained ProBlott™ membrane with no protein attached and all buffer solutions employed were analysed to determine the background level of amino acids present.