QUANTIFICATION OF METALLOTHIONEINS IN MARINE INVERTEBRATES

USING DIFFERENTIAL PULSE POLAROGRAPHY

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Summary

Metallothioneins (MT) are sulphydryl-rich, small molecular weight proteins which form complexes with heavy metals such as cadmium, copper, mercury and zinc. Functions attributed to MT include detoxification, storage and regulation of metals. Their induction may signify exposure to excessive concentrations of metal ions in cells. Consequently, the potential value of these relatively specific biochemical indicators of metal contamination would seem to be obvious. To date however, the full value of MT as a monitoring tool has rarely been demonstrated, partly due to difficulties in determining protein concentrations.

In this study differential pulse polarography has been used, together with the Brdicka electrolyte, to measure directly the levels of MT in marine organisms. The quantitative sulphydryl-specific polarographic procedure described, is based on the linear relationship between the height of the second of two polarographic waves (following cobalt reduction) and the protein concentration.

At room temperature, using the operating conditions described, the linear detection range was 5 – 300 μg MT l^{-1}, based on rabbit liver MT standard.

Levels of MT-like proteins are reported for a variety of marine molluscs, collected at sites in Portugal and the United Kingdom, and range from 1.69 – 21.3 mg/g (dry weight), depending on the species and tissue analysed. The technique is clearly sensitive enough to be of value in future environmental effects assessments.

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INTRODUCTION

Metallothioneins (MT) are cysteine rich (~30%), low molecular weight proteins which form a complex with heavy metals such as cadmium, copper, mercury and zinc.

The induction of such proteins within living organisms is often thought to occur as a result of exposure to environments contaminated with heavy metals. Thus in addition to their involvement in the normal metabolism of metals, MT and similar proteins may also sequester metals which might otherwise bind to sensitive cellular sites and exert toxic effects (1). Proposals have been put forward to use MT as specific indicators for the early detection of detrimental effects of certain metals and consequently to use them in marine pollution monitoring. Hence it is essential that the biological functions of MT and their quantification in tissues are determined.

A variety of methods have been employed in the isolation and purification of MT, mainly for the purpose of characterization. These include UV spectrophotometry, various types of chromatography (gel permeation, ion exchange, affinity and reverse phase), salt and organic solvent precipitation and electrophoresis.

The use of polarography for the estimation of thiolic proteins was first described by Brdicka (2). Subsequently the method was modified by Palecek & Pechan (3) by the use of differential pulse polarography (DPP) and later adapted for the specific analysis of MT (4, 5). Recent modifications (6) have

improved instrument response and detection limits.

The basis for the quantification of MT lies in the linear relationship between the protein concentration and the second of the two waves, designated 'A' and 'B', which follow the cobalt reduction wave (3, 5, 6). Wave 'A' (~ -1.35 V versus Ag/AgCl) cannot be quantified accurately because it is influenced by the cathodic side of the cobalt reduction wave, while wave 'B' is better resolved (~-1.47 V versus Ag/AgCl).

The object of the present study was to design a sensitive method for quantifying MT in marine invertebrates, using differential pulse polarography.

MATERIAL AND METHODS

Polarographic conditions and reagents·

A differential pulse polarographic assay for MT was accomplished using a PARC Model 174A analyser, a PARC/EG&G Model 303 static mercury drop electrode (SMDE) and a flat-bed X-Y recorder.

The three electrode system consisted of a bevelled capillary working electrode, a platinium counter electrode and a Ag/AgCl reference electrode.

Capillary electrodes were cleaned by repeatedly washing with hydrochloric acid (1M), and deionized water. After drying (60 °C) they were silanized using $(CH_3)_2SiCl_2$ (2%) in 1,1,1-trichloroethane for half an hour and dried (60 °C) overnight. Instrument settings listed in table I are similar to those

described by Thompson & Cosson (6).

The Brdicka supporting electrolyte was prepared according to the methods of Palecek & Pechan (3) and Imber & Thompson (7) and contained 1.0 M NH_4Cl, 1.0 M NH_4OH and 2.0 mM of $[Co(NH_3)_6]Cl_3$. Hexamminecobalt (III) chloride (BDH-GPR) was used without purification. The electrolyte was prepared weekly and stored at 4 °C when not in use.

Triton X-100 (SIGMA) was used at a concentration of 2.5 x 10^{-2} % (v/v) to suppress secondary maxima and minima and to eliminate baseline noise, as recommended by Thompson and Cosson(6).

Protein standards were of rabbit liver metallothionein, MT-I (SIGMA). The working standard solution was 10.0 mg/l, prepared in deionized water.

TABLE I

Instrument settings for DPP assay for Metallothioneins

PARC Model 174	PARC/EG&G Model 303(SMDE)
Amplitude modulation: 50 mV	Mode: DME
Potential scan: -1.42 V to -1.62 V	Drop size: Medium
Range: 2 mv s^{-1}	
Direction: Negative	Purge time: 2-4 min
Range: 1.5 V	
Current range: 5 μA	
Operation mode: Differential pulse	
Output offset: Off	
Drop time: 1 s	
Initial potential: -1.42 V	
Display direction: Positive	
Low pass filter: Off	

Ten milliliters of electrolyte were dispensed directly to the cell, together with 100 μl of Triton X-100 and aliquots of standard/sample. The cell was then purged for 2 minutes with purified N_2 prior to analysis.

Field Samples

Levels of MT were determined in a variety of molluscs (gastropods and bivalves) collected from sites in the United Kingdom and Portugal. Preparation of cytosolic extracts from 6-12 pooled individuals was similar to that described by Langston & Zhou (8). Interfering high molecular weight proteins were removed by heat denaturation (80 °C for 10 min) followed by high speed centrifugation (28,000 x g). Twenty-five microlitre aliquots of the supernatant, containing the heat stable MT, were taken for analysis.

Quantification of MT was accomplished by using the standard addition method.

RESULTS AND DISCUSSION

The basis for the determination of thiolic proteins (including MT) in the described procedure lies in the linear relationship between the concentration of protein and the second of the two waves following the cobalt reduction wave (3, 5, 6).

A complex of cobalt(II) ions with the protein molecules plays a decisive role in the two wave polarographic effect. A further condition necessary is the presence of sulphydryl or dissulphidic groups in the protein molecule. Brdicka *et al.* (9)

established that proteins not containing these groups are polarographically inactive, and, among the natural amino acids, only cystine and cysteine show waves of similar nature in buffered cobalt solutions.

The height and shape of the polarographic doublewave of MT varies with the concentration of all the individual components of the buffered cobalt solution. The following points summarize our efforts to establish the best suitable electrolyte concentration and conditions for the estimation of relatively low concentrations of these proteins:

1) As reported by Brdicka et al. (9) we found that the height of the double protein wave increases with increasing concentrations of hexamminecobalt(III) ions.

2) In agreement with other authors (3, 6) we found in the polarogram the presence of a maximum of the first kind on the limiting current of the cobalt. Triton X-100 was used to try to remove this maximum and to find the conditions under which the wave given by the protein would be better resolved.

3) Satisfactory results were obtained by maintaining the buffered solution concentration of NH_4Cl/NH_4OH to 1M, adjusting the cobalt concentration to 2.0 mM, and by the addition of a relatively small amount of Triton X-100 (100 μl).

4) Capillary cleanliness and silanization was shown to be fundamental for good reproductibility of the method.

A typical polarographic scan of a sample of *Ruditapes* (=*Venerupis*) *decussatus*, showing the two separate protein waves following the cobalt reduction wave, is shown in fig. 1. All the

potentials reported are relative to the Ag/AgCl reference electrode.

The polarographic response was also shown to be independent of metal content (Cd, Cu or Zn) of the protein, although a certain shift of the cobalt reduction wave towards more positive potentials can be detected when excess metal is added.
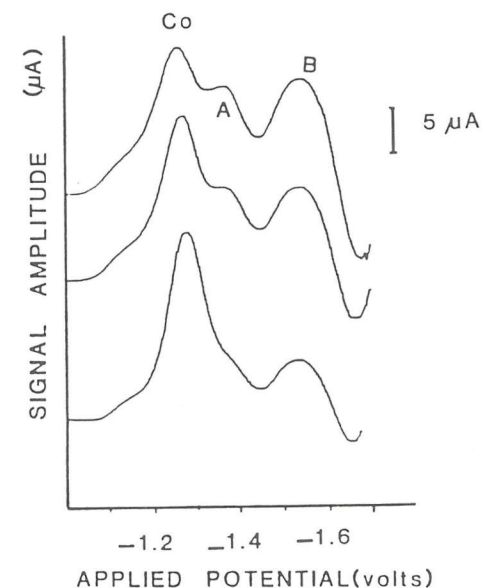

Fig. 1. Differential pulse polarograms of the Brdicka electrolyte containing 50, 100, 150 μl of cytosol of *R. decussatus*; obtained using static mercury drop electrode. Co, 'A' and 'B' denotes the reduction of the cobalt (Co) and the protein (A, B), respectively.

A MT calibration curve (fig. 2) generated at 20 °C, was linear over the range 5 μg/l to 300 μg/l (r=0.9985, P< 0.001);

this is equivalent to 0.77 nM to 46.15 nM, based on an average molecular weight for MT of 6500 (10). Data points represent the means of three or more replicate determinations. The detection limit (defined as 3 times the blank std. dev.) of 5 μg/l obtained in the present study (at 5 μA full scale) represents some improvement over that previously reported by Thompson & Cosson (6) and could be enhanced further by carrying out analysis at lower temperature.
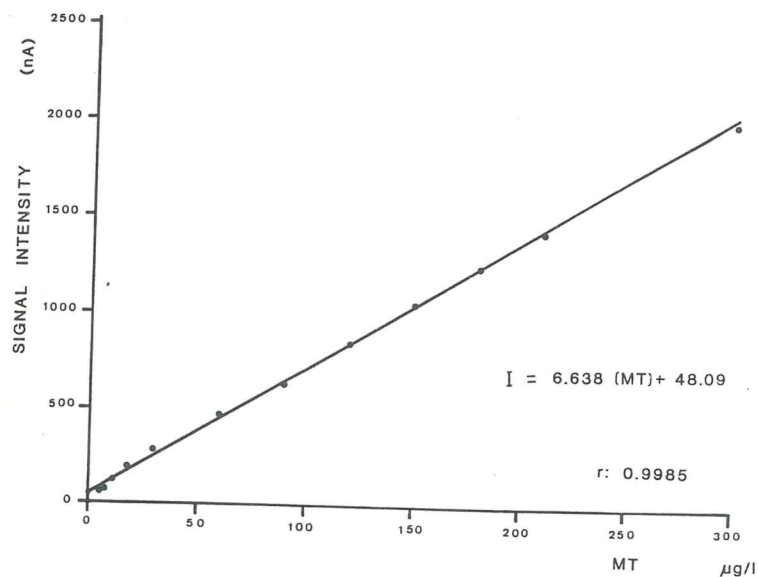

Fig. 2. Metallothionein (MT) calibration (Signal intensity, I, against concentration of Rabbit liver MT) using static mercury drop electrode, at room temperature. Linear regression equation and coefficient are indicated.

$$I = 6.638 \, (MT) + 48.09$$

$$r: 0.9985$$

Using the conditions described here, only a few microlitres of cytosol are needed for MT assay in many of the

molluscs sampled. Figure 3 shows polarograms of the 'B' wave of 100 μl aliquots of the cytosol from a sample of *Ruditapes decussatus*. The polarograms represent scans before and after the supernatant was subjected to heat denaturation at 80 °C for 10 min. Interferences from high molecular weight sulphydryl-containing proteins, such as albumins, can be removed by heat denaturation and centrifugation (or chromatography) prior to analysis. Low molecular weight sulphydryl-containing compounds, such as gluthatione, produce weak signals which do not interfere significantly with the detection or quantification of MT.
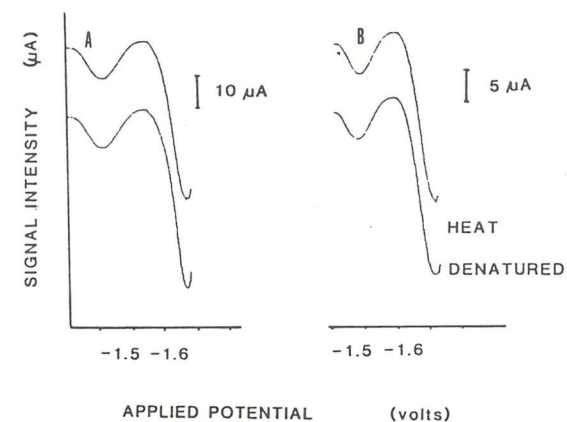

Fig. 3. Duplicate 'B' wave scans of solution containing 100 μl of *R. decussatus* (Ria Formosa - Portugal) homogenate supernatant before (A) and after (B) heat treatment. Note change of scales.

Table II summarizes the data obtained for MT using this

method in different species and tissues of marine invertebrates. Samples were collected in the Ria Formosa lagoon (Portugal) and at sites in South West England (United Kingdom). Protein concentrations range from 1.69 - 21.3 mg/g (dry weight).

TABLE II

Metallothionein levels in different marine invertebrates

Species	Size mm	Tissue	MT mg/g*	Site
Cerastoderma edule	30	Whole animal	4.55	Ria Formosa, Portugal
Donax vitatus	32	Whole animal	6.37	"
Littorina littorea	20	Digestive gland	11.93	Plymouth, U.K.
	20	Remaining tissues	2.55	"
Littorina saxatilis	13	Digestive gland	15.45	Minehead, U.K.
Mytilus edulis	58	Whole animal	2.43	Whitsand bay, U.K.
	70	Digestive gland	8.04	"
Nucella lapillus	26	Digestive gland	5.29	Plymouth, U.K.
	26	Remaining tissues	1.95	"
Patella vulgata	35	Digestive gland	21.30	"
	35	Remaining tissues	1.69	"
Ruditapes decussatus	20	Whole animal	4.29	Ria Formosa, Portugal
	37	Whole animal	6.34	"

* dry-weight

Although levels of MT-like proteins, measured using differential pulse polarography, have been reported for livers of perch (11), rainbow trout (10, 12, 13) and salmon (14), the only data reported in the literature, to date, for marine invertebrates are for crab hepatopancreas (5) and the digestive gland of oysters, *Crassostrea gigas*, which ranged between 5.02 - 7.18 g kg^{-1} (7).

In the absence of purified invertebrate proteins, MT concentrations in the present study are expressed as equivalents of rabbit liver MT. Since Thompson & Cosson (6) showed that there is a linear relationship between the polarographic response and the number of cysteine residues in the molecule of the protein, a degree of error in quantification (in terms of absolute concentrations) might be introduced due to inter-specific variations of the MT thiolic content. Thus although the procedure described here can be recommended for MT induction studies within species, clearly some caution should be exercised when comparing levels of protein in different organisms. Thompson & Cosson (6) estimated however that this error would not be greater than a factor of two for marine species.

Comparison of the pulse polarographic determination of MT with other commonly used methods (15) shows that its high specificity represents the most sensitive physicochemical method for direct quantification, currently available.

In our view this method will be an important asset for clarifying the primary function of metallothionein-like proteins, and in determining responses in relation to heavy

metal contamination in the marine environment.

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