

Purification and Characterization Studies of Cadmium-Binding Proteins from the American Oyster, *Crassostrea virginica*

by Bruce A. Fowler,* David W. Engel,[†] and Marius Brouwer[‡]

The previously reported low molecular weight cadmium-binding protein (CdBP) from the American oyster, *Crassostrea virginica*, has been further purified and characterized by improved technical methods. The internal organ distribution of the protein within the oyster and effects of life cycle/season on CdBP production also have been evaluated. CdBP isolated by extended ion-exchange gradients or double ion-exchange chromatography followed by HPLC analysis possesses an electrophoretic R_f of about 0.7 and contains relatively little Zn, as previously reported. Cysteine, lysine, and glycine are the dominant amino acids. When ion-exchange columns are developed with NaCl gradients, the aromatic residues tryptophan, tyrosine, and phenylalanine are found to be present, but these may be largely removed depending upon whether the protein is denatured and carboxymethylated prior to analysis. The ultraviolet absorption spectrum of CdBP also was variable, with 250/280 nm ratios ranging from 17:1 immediately after ion-exchange chromatography to 2:1 following concentration procedures. Internal organ distribution studies showed that the visceral mass contained most of the Cd present with lesser amounts in the gills and mantle. In contrast with mammals, CdBP accounts for only about 30% of the total cell Cd burden in these tissues. Cu displacement of Cd from the protein is a particular problem during the summer spawning season and appears to stem from altered Cu metabolism during this period. Relative oyster dormancy during the winter also reduces CdBP production in response to Cd, and the protein is obtained most readily during the fall and spring. In summary, CdBP shares both similarities and differences with MT, and the magnitude of these parameters depends greatly on both the biology of the oyster and technical procedures used to isolate/characterize the protein.

Introduction

Previous studies from a number of laboratories (1-10) have demonstrated the presence of cadmium-binding proteins (CdBP) in marine molluscs with properties that are both similar to and different from mammalian metallothionein (MT) (11,12). As previously noted (11), basic research on nonmammalian metal-binding proteins is of great potential value in elucidating not only the evolutionary pathway(s) of MT but also in gaining insight into its as yet unknown normal biological function. In addition, since these proteins permit marine food organisms such as oysters to bioaccumulate toxic metals such as cadmium, they are also of practical interest with respect to

dietary human exposure to these metals.

In a first report (1), the American oyster, *Crassostrea virginica*, was found to produce a low molecular metal-binding protein in response to cadmium exposure. This protein, which was isolated via Sephadex G-75 column chromatography followed by DEAE-anion-exchange chromatography using an NaCl step gradient, was found to have a lower cysteine content than MT and contain aromatic amino acids. This protein was found to bind mainly Cd with moderate amounts of Cu and relatively little Zn. The present studies were undertaken to purify this CdBP further by using newer and improved protein purification methods and to assess the effects of these various methods and protein handling procedures on the properties of this molecule.

Materials and Methods

Treatment of Oysters

American oysters (*Crassostrea virginica*) were collected near Beaufort, NC, and exposed to 0.2 ppm Cd

*Laboratory of Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709.

[†]National Marine Fisheries Service, SEFC, Beaufort Laboratory, Beaufort, NC 28516.

[‡]Duke University Marine Laboratory/Marine Biomedical Center, Beaufort, NC 28516.

as CdCl_2 for 28 or 56 days, respectively, in a flowing seawater system operated at 20°C and a salinity of $>30\%$. The oysters were fed a mixed algae suspension twice per week, and the cadmium concentration of the seawater was monitored by atomic absorption spectrophotometry. At the end of the exposure period, the oysters were placed in clean flowing seawater for 72 hr. The animals were removed from their shells and processed as previously described (1) or flash-frozen in liquid N_2 followed by storage at -70°C .

Organ Distribution of Cd, Cu, and Zn and Percentage of These Metals in the Cytosolic Fraction

In order to gain insight into the total organ uptake and effects of Cd following prolonged treatment, shell liquor, gill, mantle, visceral mass, and muscle were separated/dissected, dried at 100°C , and oxidized in concentrated HNO_3 . Concentrations of Cd, Cu, and Zn were determined by atomic absorption spectroscopy. Concentrations of these metals present in the cytosolic fraction also were measured in order to determine if there were any marked changes in distribution with time.

Isolation of Oyster CdBP

Fresh or frozen oysters partially thawed at room temperature were removed from their shells and homogenized for 30 sec, to 1 min, using a Brinkman polytron homogenizer, with an equal volume of 0.06 M or 0.01 M Tris-HCl buffer (pH 7.9 or 8.6, respectively) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), with or without 0.1 mM β -mercaptoethanol to prevent SH group oxidations. N_2 gassed buffers were used in some preparations to maintain anaerobic conditions. The homogenate was centrifuged at $27,000g$ for 15 min, and the resulting supernatant was heat-treated at 60°C for 10 min. After cooling in an ice bath for 1 hr, the sample was centrifuged at $27,000g$ for 15 min and the supernatant fraction stored in aliquots at -70°C . In order to determine whether the heat-treatment step had any effect on the isolation of CdBP, some oyster preparations were homogenized and centrifuged at $105,000g$ to obtain a clear supernatant without the heat treatment step.

Initial purification of the CdBP was accomplished by Sephadex G-75 (Pharmacia) chromatography of the heat-treated supernatant. This, as well as subsequent procedures in the isolation process, was carried out at 2°C . The Sephadex G-75 column (5.0×50 cm) was eluted with 0.06 M or 0.01 M Tris-HCl buffer (pH 7.9 or 8.6), and fractions were monitored for Cd and for absorbance at 280 and 254 nm by using a Perkin-Elmer Model 305B atomic absorption spectrophotometer and ISCO Type 6 flow-through UV monitor, respectively. The peak Cd fractions corresponding to the CdBP described by Ridlington and Fowler (1) were pooled and further purified by either single or double DEAE-ion-exchange chromatography

with DEAE-Sephadex A-25 (Pharmacia). The DEAE columns were equilibrated with 0.01 M Tris-HCl (pH 7.8 or 8.6), and the sample was eluted by using either a linear 0 to 0.15 M NaCl gradient in 0.01 M Tris-HCl (pH 7.8 or 8.6) or a Tris-HCl gradient 60 to 200 mM. A single, sharp Cd peak eluting at 0.08 M NaCl was collected, further analyzed by high-performance liquid chromatography (HPLC), and desalted under 60 psi nitrogen pressure by using Amicon cells with YM2 membranes.

Polyacrylamide Gel Electrophoresis

Samples of oyster CdBP obtained following DEAE-ion-exchange chromatography were analyzed for homogeneity by polyacrylamide gel electrophoresis according to the procedure of Davis et al. (9). Gels were stained either with Coomassie Blue or by the silver stain method using Bio-Rad silver stain kits.

Ultraviolet Absorption Spectral Studies

The ultraviolet absorption spectra of oyster CdBP were measured by using a Gilford Model 2600 UV/visible spectrophotometer under a variety of protein concentrations and conditions.

Amino Acid Analyses

Amino acid composition studies were performed on oyster CdBP isolated via a number of procedures in three different laboratories where these analyses are performed routinely by both carboxymethylation and a performic acid oxidation procedure (13) for determination of cysteine.

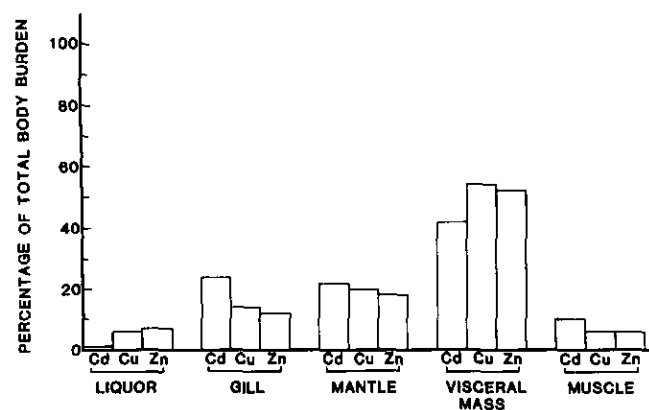


FIGURE 1. Results of total metal analyses for Cd, Cu, and Zn in shell liquor, gill, mantle, visceral mass, and muscle of oysters treated with Cd at 0.2 ppm in seawater for 28 days. Percentages for each tissue were calculated from total body burden of metal in individual animals. Each point is a mean of five oysters.

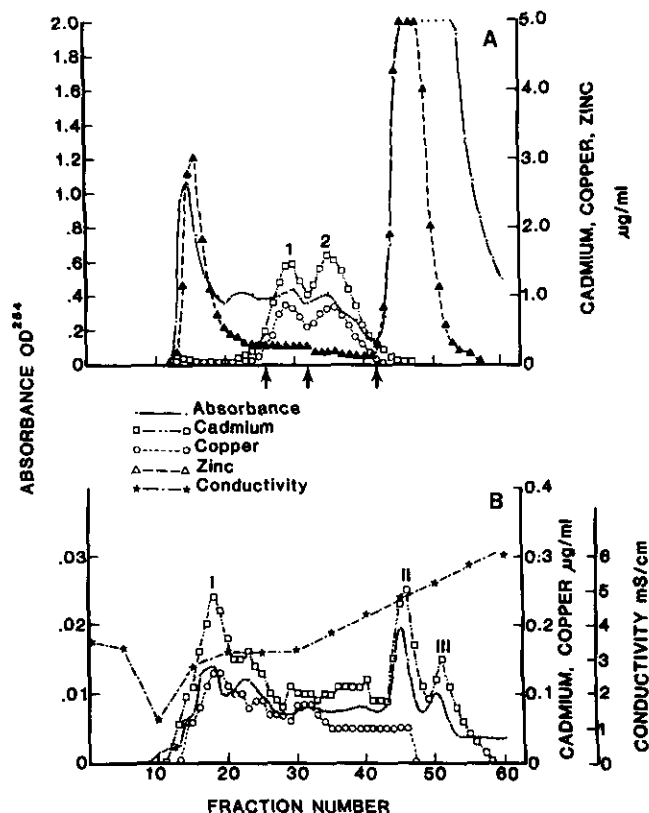


FIGURE 2. Gel filtration and ion exchange elution profiles of oyster cytosol prepared by using heat treatment: (A) Sephadex G-75 elution profile of oyster cytosol; (B) DEAE Sephadex ion-exchange elution profile of G-75 peak (2) eluted with a gradient generated from 500 mL 60 mM Tris and 500 mL 200 mM Tris, both pH 7.9.

Results

The results of total metal analyses for Cd, Cu, and Zn in various oyster compartments/organs are shown in Figure 1. The visceral mass showed the highest fractional content of these metals, followed by the gill, mantle, muscle, and shell liquor, respectively.

Sephadex G-75 column chromatography of heat-treated or nonheat-treated cytosol fractions (Fig. 2A) resulted in virtually identical chromatographic profiles with the characteristic doublet CdBP peaks (1 and 2) as reported in the original publication (1) on this protein. Note that in this particular preparation, these two CdBP peaks (with estimated molecular masses of 24,000 and 10,000 daltons, respectively) are also associated with coincident peaks of 254 nm and Cu. DEAE anion-exchange chromatography of the 10,000 dalton peak (peak 2) showed apparent differences in the CdBP peak patterns when the columns were eluted with either a continuous Tris-HCl gradient (Fig. 2B) or NaCl gradient (Fig. 3A). Using either gradient, a peak of variable height (peak I) was observed in the wash prior to beginning the gradients. When the Tris-HCl gradient was utilized (Fig. 2B), doublet peaks (II and III) were usually observed about three-quarters of the way through the gradient, with peak II consistently larger than peak III. In contrast, when the NaCl gradient was employed (Fig. 3A), a doublet peak was sometimes observed but always with peak III greater than peak II. DEAE rechromatography of peak III peak using the NaCl gradient gave a single, sharp peak (Fig. 3B). Both peaks II and III, using either gradient, gave coincident absorbances at 254 nm (data not shown) and when the NaCl gradient was applied, a

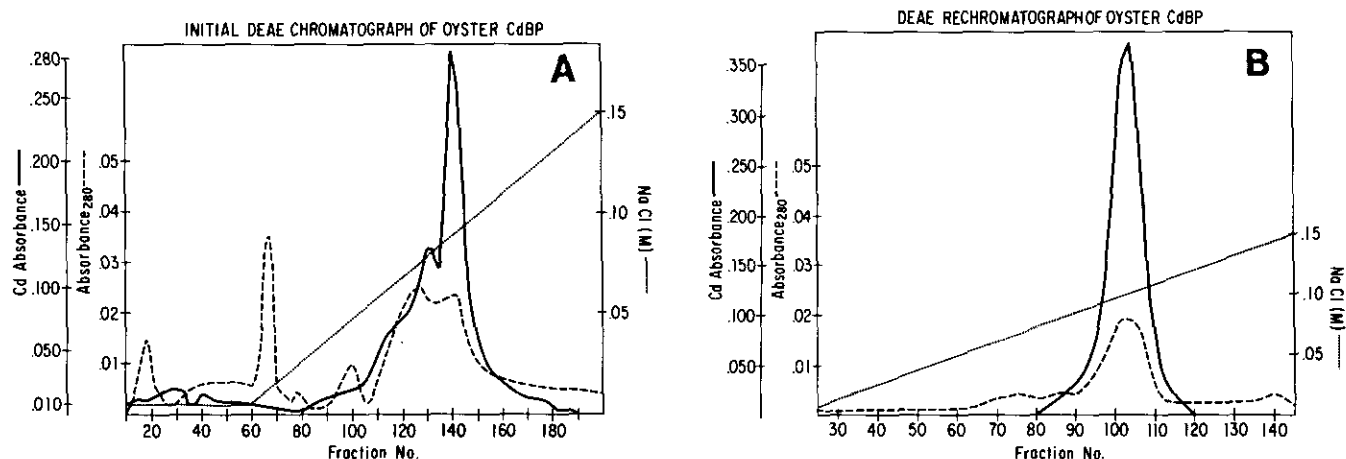


FIGURE 3. Heat-treated oyster supernatant eluted with a 0-0.15 M NaCl gradient in 0.01 M Tris HCl buffer (pH 8.6): (A) DEAE A-25 column chromatography showing the relative positions and dimensions of the various peaks. Note that the relative magnitude of peaks II and III is reversed in comparison with the Tris gradient; (B) rechromatography of peak III using the same gradient system as Fig. 3A showing a single peak with coincident 280 nm absorbance.

coincident absorbance at 280 nm was also evident through both anion-exchange chromatographic runs (Fig. 3). Copper, when present, was primarily associated with peak II (Fig. 2B), and this was strongly influenced by the season of the year (see below).

Further purification and desalting of the peak III CdBP eluted from the NaCl gradient was conducted by gel-permeation HPLC (Fig. 4) and clearly showed that the Cd 254 nm and 280 nm absorbances were coincident. Denaturation of the protein via dropping the pH to 2 or 6 M urea followed by Sephadex G-25 column chromatography (Fig. 5) showed 50% displacement of the 280 nm absorbance into the "free" region of the elution volume. Replicate studies performed with 6 M guanidine-HCl followed by gel-permeation HPLC analysis (Fig. 6) gave better resolution to this effect and clearly demonstrated the removal of about 50% of the 280 nm material into the "free" region of the elution volume, indicating that the 280 nm absorbant material being removed is of a very low molecular weight. The remaining 280 nm absorbant material may be removed by carboxymethylation of the protein (B. A. Fowler, unpublished observations). The importance of this phenomenon to spectral and amino acid composition studies is discussed below.

Electrophoresis

An electrophoretic preparation of purified CdBP following double anion-exchange chromatography by the NaCl gradient method is shown in Figure 7. As reported in the original publication on this protein (1), the R_f value for the major band is greater than 0.4 or 0.6. Values usually reported for mammalian MT are about 0.8 (range

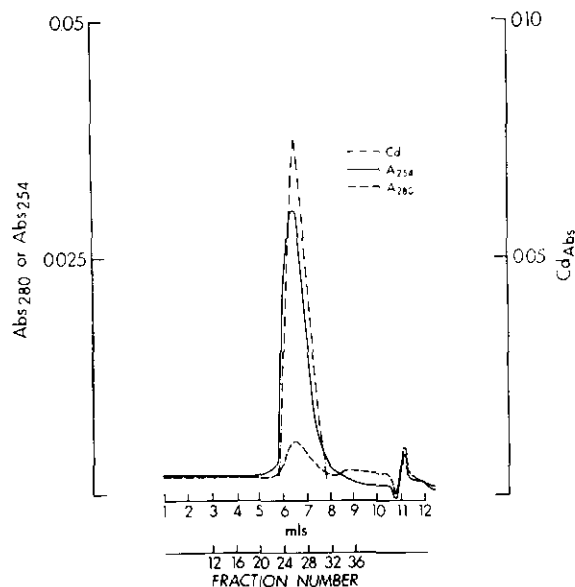


FIGURE 4. HPLC gel-permeation chromatographic analysis of CdBP from a second DEAE column eluted with an NaCl gradient showing coincident Cd (254 and 280 nm) absorbances.

0.65–0.80). Freezing without cryoprotectants or prolonged storage of the concentrated protein will result in denaturation and the production of aggregates. The denatured material usually migrates faster than the major band. Aggregated proteins also may be partially broken up by high concentrations of reducing agents (dithiothreitol or β -mercaptoethanol). SDS-gel electrophoresis of purified CdBP results in formation of aggregates with estimated molecular weights of up to 70,000 daltons even after carboxymethylation of the freshly isolated protein.

Spectral Studies

The ultraviolet absorption spectra of CdBP also were found to vary greatly depending upon the isolation procedure used and state of denaturation of the protein. An ultraviolet absorption spectrum with a maximum 250/280 nm ratio of 17:1 may be obtained in the dilute protein solution of the peak tube from the initial DEAE chromatography. It is also clear that denaturation of CdBP during the carboxymethylation

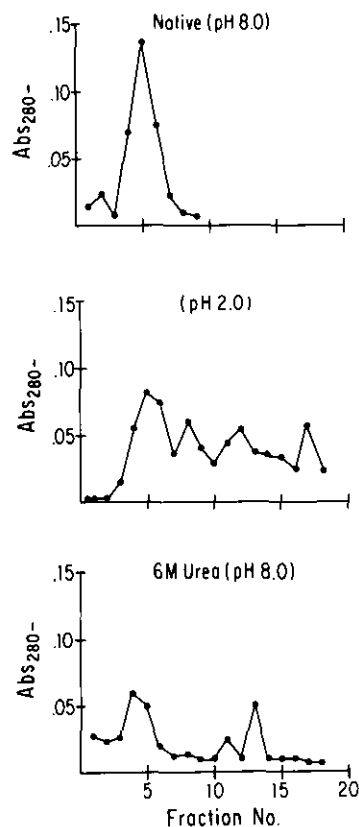


FIGURE 5. G-25 column chromatography of purified native CdBP following denaturation with acid to pH 2.0 or 6M urea, showing displacement of about 50% of the 280 nm absorbance into the "free" region of the column.

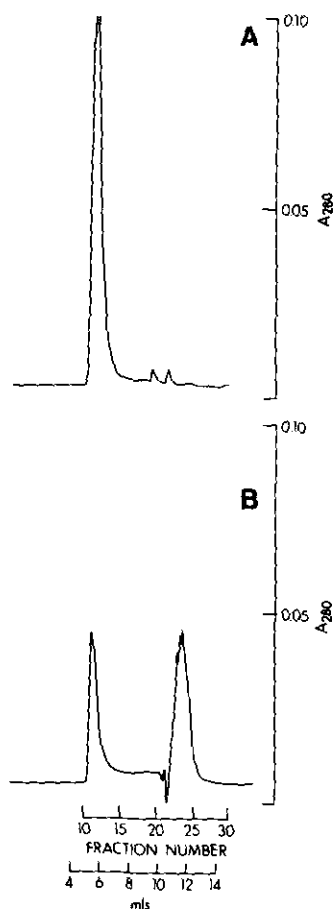


FIGURE 6. HPLC gel-permeation chromatographic analysis of purified CdBP: (A) before and (B) after denaturation with 6M guanidinium hydrochloride, showing with greater resolution the movement of about 50% of the 280 nm absorbance into the free region of the column.

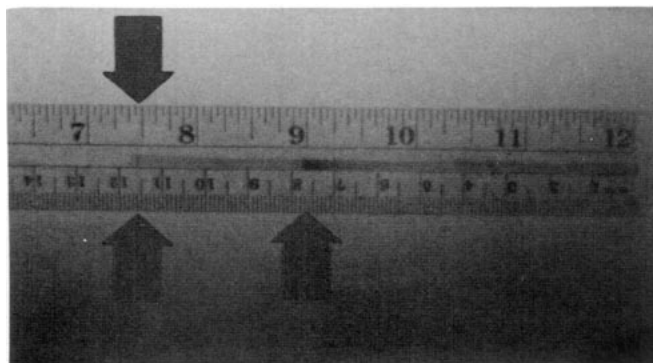


FIGURE 7. Nondenaturing gel electrophoresis of purified CdBP showing and R_f value of 0.8 (arrow).

matogram. Concentration of this solution by using an Amicon cell usually reduces the 250/280 nm ratio to about 4 to 5:1, with loss of the Cd-S chromophore, and cross-linking between or within protein chains. A spectrum

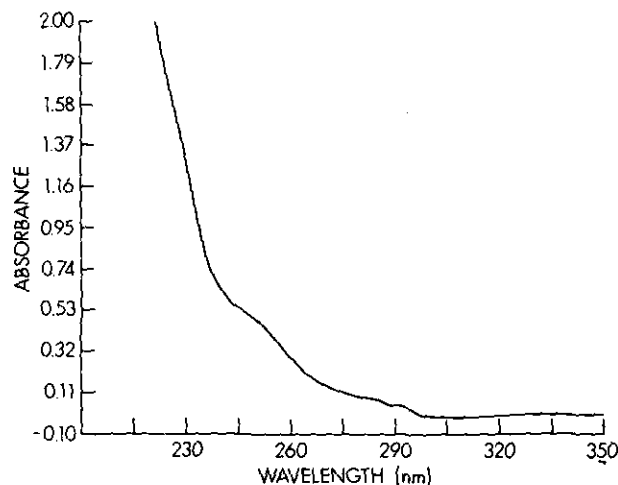


FIGURE 8. Ultraviolet absorption spectrum of purified CdBP following 10-fold concentration in an Amicon. The 250/280 nm ratio is 4:1.

with a 250/280 nm ratio of 4 to 5:1 is hence most typical of concentrated solutions after concentration procedures (Fig. 8). Finally, if the protein is concentrated, desalted, and frozen prior to spectral analysis, there is usually a reduction of the 250/280 nm ratio to about 2:1 as previously reported (1). The 250/280 nm ratio also may be reduced greatly by lowering the pH to 2.0, which releases the Cd from the protein, resulting in loss of the Cd-S chromophore in a manner similar to mammalian MT.

Amino Acid Analyses

The results of amino acid analyses performed on purified oyster CdBP in three different laboratories (A, B, and C) showed variable results, with cysteine values ranging between 15 and 24.8% and aromatic residue values ranging between 0.5 and 5.2% for individual aromatic amino acids. Laboratories A and B, which were given aliquots of the same sample isolated by NaCl-eluted DEAE-ion-exchange chromatography and which utilized the performic acid oxidation method (13), reported cysteine values of 18.4 and 15.0%, respectively. Laboratory A reported phenylalanine values of 5.1%, while laboratory B reported phenylalanine values of 2.1%. Tyrosine was not determined by laboratory A, while laboratory B reported tyrosine values of 1.5%. Laboratory C, which was given a sample isolated by using a Tris-Cl gradient followed by denaturation/carboxymethylation, reported cysteine values of 24.8% and phenylalanine and tyrosine values of 0.6 and 0.5%, respectively. The other amino acids determined showed less variability between the three laboratories. At present, it is unclear how much of this variability for cysteine and the aromatic amino acids is due to possible nonhomogeneity of the samples prepared by the different methods described above or technical preparative differences in the laboratories perform-

procedure would remove most of the 280 nm-absorbant (aromatic) material, thus decreasing these values and increasing the relative cysteine content. Studies are currently in progress to elucidate the nature of these inter-laboratory differences in amino acid composition for CdBP, and results will be reported in future publications.

Effects of Season and Physiological Status on Oyster Production of CdBP

It should be noted that, unlike mammals, oysters and many other marine invertebrates undergo marked seasonal changes in physiological status which are usually related to water temperature and reproductive cycle. Over the past two years, it has become increasingly clear that these cyclic changes have a profound effect on both the amounts of CdBP produced and Cu displacement of Cd from the proteins (unpublished data). A general summary of these effects is presented in Table 1. In winter, there is relatively little CdBP produced by the oysters compared with other seasons, apparently due to decreased water temperatures and attendant metabolic rates. In the spring, with increased water temperatures and metabolic rates, CdBP production increases, but Cu levels in the purified protein are still relatively low. During the summer months, CdBP production is high, but Cu displacement of Cd from the protein is also greatly increased. The presence of Cu and CdBP is probably due to changes in oyster Cu metabolism associated with gonadal development. Following spawning, CdBP production continues to be high in response to Cd exposure, but Cu content of the protein is again reduced. These seasonal changes are important because the presence of Cu on the CdBP greatly complicates purification of the protein cysteine determinations due to Cu catalyzed SH group oxidation. Variable amounts of Cu on the protein could hence greatly contribute to the variability in amino acid composition for cysteine discussed above. Utilization of reducing agents, such as β -mercaptoethanol to prevent SH group oxidation, appears to have both positive and negative effects. The presence of β -mercaptoethanol may actually increase the presence of Cu on the protein by

facilitating metal-metal exchange and thus alter native metal distribution patterns.

Discussion

The results of the studies presented in this paper are intended as a summary of our current knowledge with respect to the isolation and characterization of oyster CdBP. Biological factors and technical procedures that may influence these data also are discussed since it is quite clear that in this nonmammalian species, such factors are of great importance to obtaining proper results. The potential scientific value of oyster CdBP stems not only from its probable phylogenetic/evolutionary structural relationship to MT in higher organisms but also in gaining basic insight into the as yet unknown normal function of MT and similar proteins in biological systems.

Sephadex G-75 column elution profiles of oyster CdBP in the present studies are identical to those previously reported (1) and neither heat treatment nor centrifugation at 105,000g produced significant differences in the profile. Application of continuous NaCl or Tris-HCl gradients for elution of CdBP from DEAE-anion-exchange columns greatly improved resolution of the CdBP peak(s) from these columns in comparison with the step gradient employed in the original publication (1) on this protein. The general position of the peak(s) in these gradients was similar; however, to that originally reported with the step gradient.

Qualitative differences between the NaCl and Tris-HCl gradients were noted, however, both with respect to the apparent relative heights of the peak eluted but also with respect to the spectral properties of these peaks. In general, the 250/280 nm ratios of CdBP may range from 17:1 to 2:1, depending upon which gradient is used and whether crosslinking of the protein chains result in the loss of Cd-S chromophores and whether aromatic residues associated with the CdBP complex have been removed by preparative procedures and/or denaturation of the protein. The main point is that the ultraviolet absorption properties of the isolated protein are quite variable and depend greatly upon isolation and handling techniques.

Table 1. General effects of season/life cycle on production of CdBP from the American oyster (*Crassostrea virginica*).

Season	Physiological status of oyster	Effect on CdBP
Winter (December-March)	Relatively dormant (Low pumping rates and metabolism)	Production of CdBP
Spring (Mid-March to early June)	Pre-spawning (Pumping rates and metabolism)	Production of CdBP Absence of Cu displacement
Summer June-August)	Spawning (Pumping rates, gonadal development)	Production of CdBP/Cu dis- placement effects
Fall (September-early December)	Post-spawning (Pumping and metabolism to sustain winter)	Production of CdBP Absence of Cu displacement

This is in decided contrast to the relatively stable spectral characteristics of mammalian MT (18).

The presence of the low molecular weight 280 nm-absorbant material (F-fragments) from CdBP is important, since it has a marked influence on both spectral and amino acid analyses of the protein. These fragments may be viewed in two general ways. First, they may represent contaminating amino acids, oligopeptides, and/or some unidentified 280 nm-absorbant material which becomes closely associated with the protein before or during the isolation process and essentially interferes with the homogeneity of nondenatured protein preparations. On the other hand, these fragments clearly copurify with CdBP if procedures which do not denature the protein are employed. The association between reduction of ordered protein structure and removal of the fragments from the protein is intriguing, since it could suggest that these fragments are in some way involved in the maintenance of this structure and are, in fact, necessary to the protein. It should be noted that, for certain enzymes such as pyruvate kinase, a noncovalently bound valine has been shown (19) to be essential for activity of the enzyme. It is interesting to hypothesize that something similar could be operating for oyster CdBP and perhaps other metal-binding proteins where variability in amino acid analyses has occurred.

Another marked difference between oyster CdBP and mammalian MT concerns the electrophoretic mobility of these proteins on nondenaturing gels. As previously reported (1), the electrophoretic mobility of CdBP is greater than that of mammalian MT, with usual R_f values of about 0.7 (range 0.65–0.80) instead of 0.4 or 0.6 for MT. Part of this variability of oyster CdBP electrophoretic mobility appears to stem from variations in degree of metal saturation. Attempts at SDS-gel electrophoresis resulted in formation of aggregates with molecular masses up to 70,000 daltons being formed. To date, various procedures designed to block these effects have proven ineffective.

The effects of season and physiological status of the oyster on production/isolation of CdBP cannot be understated. For nonmammalian organisms whose biology is not under internal homeostatic control, seasonal and biological cycles in the organism may have profound effects on both the production and metal-binding characteristics of proteins such as CdBP. For example, there appears to be an increase in both Cu body burden and Cu binding to CdBP during the spawning cycle. Such changes in the Cd/Cu ratios on the protein create difficulties during the purification of these molecules.

In conclusion, the results of the present studies using refined preparative procedures generally confirm and extend results of the original report (1) on oyster CdBP with respect to heat stability, chromatographic and electrophoretic characteristics, and relative metal composition. These new studies also indicate a higher cysteine content than previously reported and a variability in aromatic amino acid composition and spectral properties as a function of the method utilized. The apparent copuri-

fication of noncovalently bound, 280 nm-absorbant, aromatic residues with the CdBP under conditions which maintain a partially ordered structure is particularly interesting since it would provide a reasonable explanation for observed variability in amino acid analyses for these amino acids. The question of whether these aromatics are contaminants or essential to maintenance of CdBP secondary structure remains to be resolved.

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