Peptide Mapping of Vertebrate and Invertebrate Metallothioneins

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Metallothioneins are an ubiquitous class of low M_r cysteine-rich proteins, binding unusually high amounts of heavy metal ions such as Zn, Cu, Cd, Hg [1]. Those isolated from mammalian species display very substantial polymorphism. In human tissue at least eleven genes or pseudogenes have been detected [2]. As MT† biosynthesis is induced by a variety of cellular factors (for a review, see ref. 3) it is of interest to study the pattern of MTs expressed in various tissues or at different stages of development. In a recent paper the resolution of six MT isoforms from human hepatic tissue by HPLC has been reported [4]. To further characterize purified MT isoforms a rapid and sensitive peptide mapping procedure would be desirable. In this report we describe a technique which is based on HPLC analysis of fragments obtained by tryptic cleavage of *S*-carboxamidomethylated thioneins. This procedure is equally suited for the analysis of MTs both from vertebrate and invertebrate sources. Hence, it is expected to foster our understanding of the functions and evolution of this unique family of proteins.

Experimental

Protein Isolation

Fetal bovine MTs were isolated from livers as described previously [5]. The two isoproteins both contained Cu and Zn with a stoichiometry of 3:4. The Cd containing isoprotein MT-1 from the marine crab *Scylla serrata* was provided by R. Olafson, Canada [6].

Protein Modification

The metal-free derivatives of the different MTs were prepared by incubation in 0.5 M HCl, 6 M GdnHCl and 10 mM EDTA (2 h, 20 °C) and subsequent gel filtration on Sephadex G-25 in 10 mM HCl. Thioneins were modified by *S*-carboxamidomethylation as described [7].

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†The abbreviations used are: MT, metallothionein; HPLC, high pressure liquid chromatography; T, trypsin.

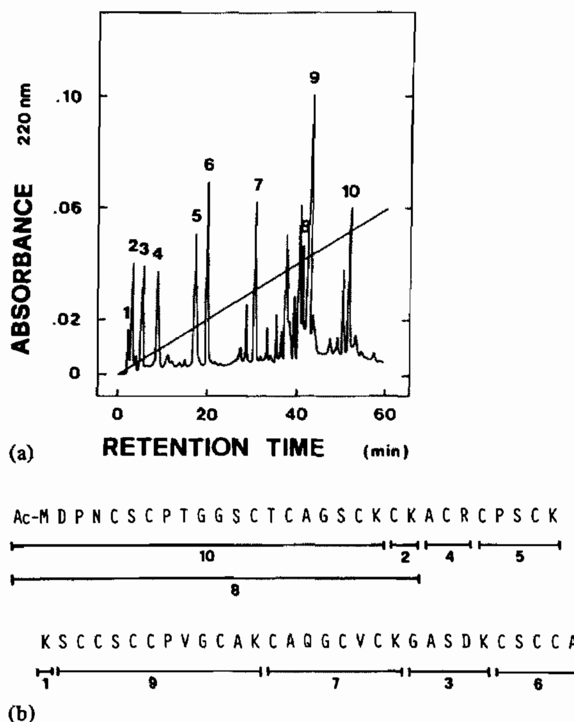

Fig. 1. HPLC profile of the tryptic peptides of *S*-carboxamidomethylated bovine fetal liver MT-1 (a) and the alignment of the fragments in the amino acid sequence of the protein (b) [5]. HPLC was performed in buffer system I using a linear gradient of buffer B (0.5%/min). See experimental section for details.

Tryptic Digestion

The *S*-carboxamidomethylated thioneins were digested with trypsin in 0.1 M ammonium bicarbonate, pH 8.5 using 2% per weight of trypsin for 1 h at 37 °C. A second portion of trypsin was added after 1 h and the mixture further incubated for 3 h at 37 °C followed by lyophilization.

Peptide Separation

Peptides were purified by HPLC on a Lichrosorb RP-18 column (0.46 X 25 cm). Two buffer systems were used. Buffer system I [8] was buffer A, 0.01 sodium perchlorate in 0.1% phosphoric acid, pH 2.1, and B, as A except 60% (*v*/*v*) in acetonitrile. Buffer system II [9] was buffer A, 50 mM ammonium acetate, pH 6.0, and B, as A except 60% (*v*/*v*) in acetonitrile. Chromatography was carried out using a linear gradient of buffer B (see Figs. 1 and 2 for details) at 20 °C with a flow rate of 1 ml/min. Absorbance was recorded at 220 nm.

Amino Acid Analysis

Samples were hydrolyzed for 22 h at 110 °C in vacuum-sealed glass tubes and analyzed with a Dur-

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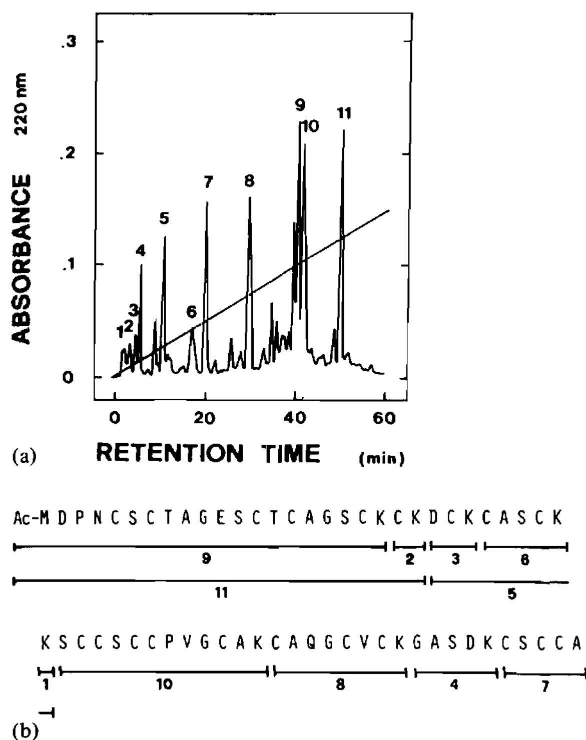

Fig. 2. HPLC profile of the tryptic peptides of *S*-carboxamidomethylated bovine fetal liver MT-2 (a) and the alignment of the fragments in the amino acid sequence of the protein (b) [5]. HPLC was performed in buffer system I using a linear gradient of buffer B (0.5%/min). See experimental section for details.

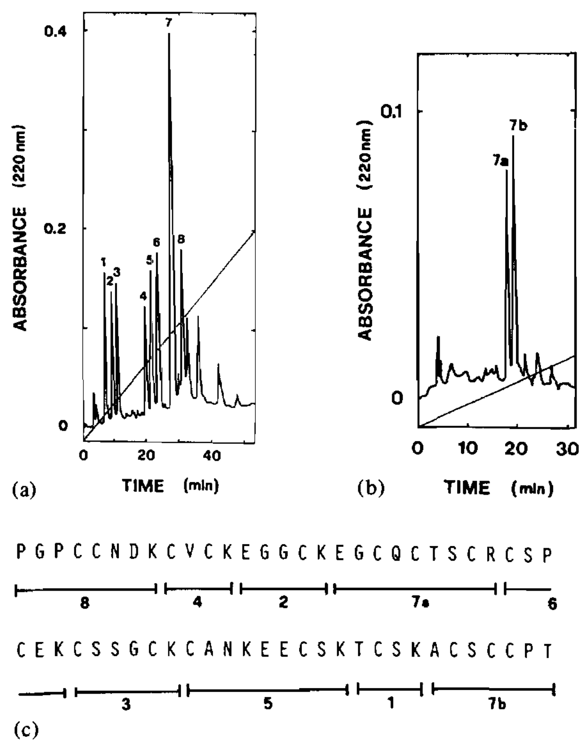

Fig. 3. HPLC profile of the tryptic peptides of *S*-carboxamidomethylated *Scylla serrata* MT-2 (a, b) and the alignment of the fragments in the amino acid sequence of the protein (c) [10]. The initial separation of the peptides was performed in buffer system I (a), peak 7 was rechromatographed in buffer system II (b). In both cases linear gradients of buffer B (0.5%/min) were used. See experimental section for details.

rum D-500 amino acid analyzer. Values given in the Tables are corrected for the partial loss of serine and threonine during the hydrolytic procedure.

Results and Discussion

In Figs. 1 and 2 are shown the alignments of the tryptic peptides of *S*-carboxamidomethylated bovine

TABLE I. Amino Acid Compositions of the Tryptic Peptides from *S*-Carboxamidomethylated *Scylla serrata* MT-1

Amino acid	T1	T2	T3	T4	T5	T6	T7a	T7b	T8
Lys	1.1(1)	1.1(1)	1.1(1)	1.1(1)	2.0(2)	1.1(1)			1.0(1)
Arg							1.0(1)		
Asp					1.1(1)				2.0(2)
Thr	1.0(1)						1.0(1)	1.0(1)	
Ser	0.9(1)		1.9(2)		0.9(1)	1.0(1)	0.9(1)	1.0(1)	
Glu		1.0(1)			2.0(2)	1.1(1)	2.1(2)		
Pro						1.0(1)		1.0(1)	2.1(2)
Gly		2.0(2)	1.0(1)				1.0(1)		0.9(1)
Ala					1.1(1)			1.0(1)	
Val				0.9(1)					
Cys(Ca)	1.0(1)	1.0(1)	1.9(2)	2.0(2)	2.1(2)	1.9(2)	3.0(3)	3.0(3)	1.9(2)
Total residues[a]	(4)	(5)	(6)	(4)	(9)	(6)	(9)	(7)	(8)

[a]Residues per molecule. Integral values in parentheses obtained from sequence analysis [10].

fetal liver MT-1 (Fig. 1b) and MT-2 (Fig. 2b) and the corresponding HPLC profiles (Figs. 1a and 2a). The alignments are based on the observation that the sequence positions of the basic residues of all mammalian MTs sequenced so far are higly conserved [1] and on the amino acid compositions and partial amino acid sequence analysis of the peptides (data not shown). From both isoproteins, 9 major tryptic peptides were isolated in good yields. Under the enzymic digestion conditions used, some overlapping peptides were also obtained. As shown in Figs. 1 and 2, MT-1 yielded one, MT-2 furnished 2 overlap fragments.

To further demonstrate the general value of this technique, an isoform of an invertebrate MT (MT-1 of the crab *Scylla serrata*) was analyzed. The HPLC profile of the tryptic peptide mixture is shown in Fig. 3a. At low pH (buffer system I, see experimental section), with the exception of peak 7, all peptides were obtained in pure form and could be readily aligned according to the previously established amino acid sequence [10] as shown in Fig. 3c. The peptide mixture in peak 7 was further resolved by rechromatography at pH 6.0 (buffer system II, see experimental section) (Fig. 3b). The amino acid compositions are listed in Table I.

Taken together the data convincingly demonstrate, that this peptide mapping technique is far superior to previously used ones (such as ion-exchange chromatography, paper chromatography, paper electrophoresis) both in terms of resolution, speed and sensitivity. Because MTs are characterized by an exceptionally high amount of cysteine ($\geqslant$30%), the commonly used sulfhydryl modifications (*S*-pyridylethylation, *S*-aminoethylation, performic acid oxidation and *S*-carboxymethylation) lead to either highly hydrophobic or highly charged derivatives. However, *S*-carboxamidomethylation used in this procedure yields a product devoid of these undesirable side-effects. Not surprisingly, the peptide mixtures originating from the *S*-carboxamidomethylated MT derivatives can be effectively separated by HPLC both at pH 2.1 and pH 6.0.

An application of this method to follow the reconstitution of rabbit liver MTs was recently published [11]. In view of the simplicity and the sensitivity of the methods described this procedure should be of general value to characterize purified MT isoforms.

Acknowledgments

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