Separation of Polythiolate Peptides and Metallopeptides by Covalent Affinity Chromatography

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Key Words

Covalent chromatography
Metallothionein
Quantitative isolation
Mechanism of polythiolate isolation

Summary

Metallothionein (MT) a low-molecular weight polythiolate metallopeptide was isolated quantitatively by covalent affinity chromatography on Sepharose DTNB support of our own synthesis. The protein was prepared from the vital organs of rats exposed to heavy metals (Hg, Cd).

Isolation of low-molecular weight polythiolate and thiolate proteins of a small number of SH-groups are reported. Changes in II and III order structure of the protein and aggregation resulting from chaotic formation of disulphide bridges were observed.

A mechanism of separation of polythiolate proteins by covalent chromatography, based on the presented data, is suggested.

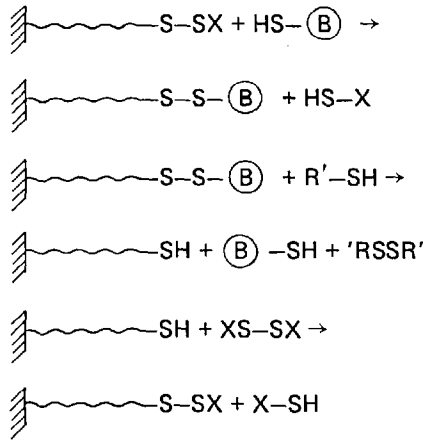
Introduction

Metallothionein (MT) was discovered in 1957 by Margoshès and Vallee [1]. It was characterized by Kägi and Vallee [2–5], as a protein of a low molecular weight of about 10000, when gel chromatography was used, and 6000–7000 when aminoacid analysis was employed.

Metallothionein is a polypeptide rich in SH-groups (33% of the content of amino acids is cisteine), of high metal content, of a characteristic amino acidic composition with no aromatic amino acids (no adsorption at 280nm), and a characteristic spectrum coming from metallothiolate chromophores.

The MT chain in an oxidized form has a radius ranging from 16.0–16.5Å [4]. Application of the methods of circular dichroism (CD), electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) allowed determination of the III order structure of the protein. The presence of two metallothiolate clusters (A) and (B), of different structures and a difference in affinity to individual metals was noticed [6–13]. Application of radioimmunological methods [14–18] allowed determination of MT in human urine and plasma in normal physiological states and after exposure to metals [19–22]. These show very good correlation with metal contents in physiological liquids and tissues ranging from 0.75–0.95 and are a good indicator of exposure to these metals. MT also plays an important part in the mechanism of detoxication of heavy metals.

Covalent chromatography is a technique of separation based on interactions between disulphide bridges, –S–S–, fixed on a support and SH-groups of the proteins being separated [23, 24]:



The method has often been used for separation of thiolate proteins of high molecular weights and small number of SH-groups. For isolation of MT the method was first applied in 1977 [25]. Then it was employed several times with varying results [16, 26–33], in spite of a high theoretical predisposition of MT to isolation by means of this technique.

The aim of the investigation was to apply covalent chromatography to quantitative isolation of MT for application in toxicological diagnosing. Based on the data obtained, a mechanism of separation of the proteins by means of the above mentioned method is suggested.

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Experimental

Pre-purified MT preparations were obtained by gel chromatography [34, 35] from the vital organs of experimental animals (female, white, Wistar rats) after exposure to heavy metals (Hg 1 mg/kg, 3 times weekly for 3 weeks, Cd 1 mg/kg, 3 times weekly for 3–5 weeks). Sepharose gel -[5,5'-dithiobis-(2-nitrobenzoate acid)] (Sepharose-DTNB) was synthesized according to a modified Lin and Foster's method [36], reaching substitutions 0.11 and 0.13 mM DTNB/g gel and other parameters similar to factory-made supports. The following preparations were used for the investigation: Hg-thionein (Hg-Th) [37], Cd-thionein Cd-Th) [38, 39] and apothionein (Apo-Th) [40]. Different methods of elution were employed. The fractions obtained were rechromatographed by gel chromatography.

In the investigations the following determinations were carried out: protein [41, 42], SH-groups [43], Hg – by the method of cold vapours [44], Cd by atomic absorptiometry (Beckman AAS Model 1248 with Massman Cuvette 1268 and Deuterium Lamp CNA 1275) [38, 39] and absorption UV/VIS (Beckman Acta C III).

Results and Discussion

Typical separations of Hg-Th and Cd-Th by covalent chromatography are presented in Fig. 1 and 2. Chromatograms obtained as a result of rechromatography of MT peaks unbound with the support and those bound with it are shown in Figs. 3 and 4.

Mean percentages of protein and metals for bound and unbound fractions, for different types of MT and different methods of elution are presented in Table I. Mean percentages of proteins and metals for individual MT fractions, after rechromatography by gel chromatography, are listed in Tables II and III.

For MT blocked with metals (Hg-Th, Cd-Th) the degree of binding was not very high, although the theoretical capacity of the support was not exceeded, it being 25–30% of retained protein. Similar results were obtained for high-molecular weight proteins blocked with Hg (Hg-HMW), where the mean efficiency of binding was also about 30%. A clear increase in binding efficiency was observed only for MT with unblocked SH-groups (Apo-Th) were it ranged from 45–50% of retained material (Table I). For Hg proteins (Hg-Th, Hg-HMW) most of the supported metal was in the fraction bound with the support, 60–65% of all the supported Hg (Table I). Its conversion into theoretical capacity of the protein indicated direct binding of Hg with the support. For Cd such effects were not observed and the fraction of the proteins bound with the support contained about 20% of the supported Cd. Rechromatography by gel chromatography of the fractions bound and unbound with the covalent chromatography support showed clearly the presence of 3 protein fractions. These were of masses above 70 000, in the range 33–42 000 and a monomer of the mass from 7000 to 9000 i.e. lower than the native mass of MT (Table II). The content of metals in the fractions was different (Table III). It proved impossible to determine SH-groups in the fractions by the Ellman

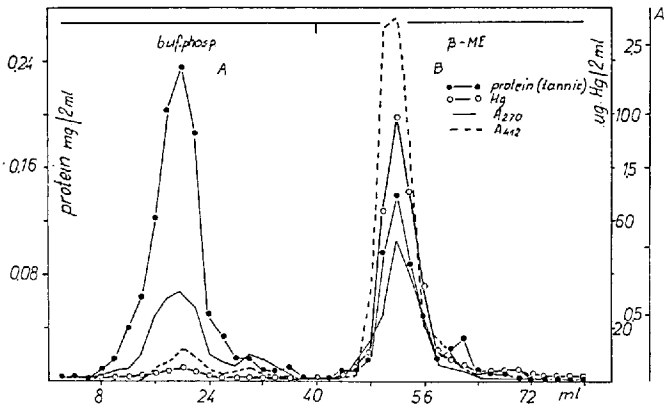


Fig. 1

Covalent chromatography on Sepharose-DTNB (11.0ml, 0.11mM DTNB/g Sepharose) of Hg-Th (two step elution: phosphate buffer – pH = 8.0, 0.05M (peak A), buffered 0.04M β-ME (peak B), flow rate 30ml/h, fractions of 2.0ml were collected); protein conc. (tannic method) – •——•, Hg. conc. – o——o, A_{270} – ——, A_{412} – – –

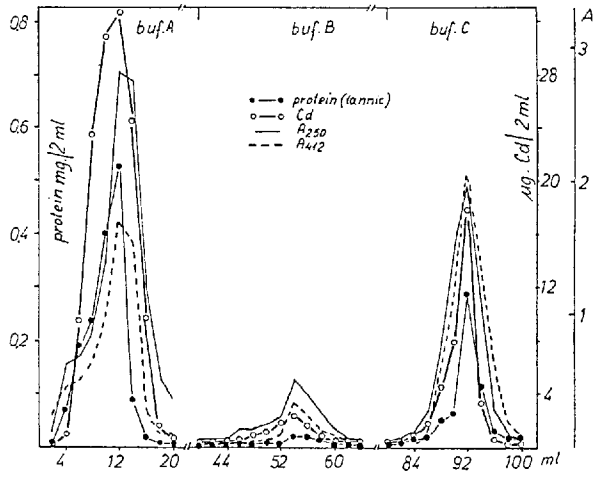


Fig. 2

Separation of Cd-Th by covalent chromatography on Sepharose-DTNB using three-step, elution (column Sepharose-DTNB 10.0ml, 0.11mM DTNB/g Sepharose, flow rate 30ml/h, fractions 2.0ml were collected);

I (A) pH = 4.0, 100mM CH_3COONa, 300mM NaCl, 1mM EDTA
II (B) pH = 8.0, 100mM Tris-HCl, 300mM NaCl, 1mM EDTA
III (C) pH = 8.0, 50mM Cys-SH in buffer B

•——• – protein conc. (tannic method), o——o – Cd. conc., – – – – A_{412}, —— – A_{250}

method although they were detected in the fractions of high-molecular proteins where they were present in much smaller amounts [37].

Comparative material concerning the efficiency of separation of polythiolate proteins is small. Only one paper [28] mentions such large amounts of unbound proteins. As the applied method of prepurification of proteins by gel chromatography [37–40] allows purification to 85–90% [45] the amount of balast proteins should range from 10–15%. If the unbound proteins were treated as the balast ones, the values would be 30–70% (Table I), contrary to the

Table I. Mean percentage concentration of protein and metal in fractions bound and unbound with covalent affinity chromatography support.

No	Protein	Steps of elution	Mean percentage concentration							
			Unbound fractions [A]				Bound fractions [B]			
			pH	Protein	Metal	μGr.At./μM	Protein	Metal	μGr.At./μM	pH
1.	[^{203}Hg]Hg-Th (*)	1	8.0	–	38.15	–	–	60.73	–	8.0
2.	[^{203}Hg]Hg-HMW (*)	1	8.0	–	37.45	–	–	61.11	–	8.0
3.	Hg-Th (**)	2	8.0	60.01	4.31	0.537	25.20	78.97	23.446	8.0
4.	Hg-HMW (***)	2	8.0	56.20	13.56	0.114	28.92	60.70	0.989	8.0
5.	Cd-Th (****)	2	9.0	57.94	70.62	5.367	29.52	21.35	3.122	8.0
6.	Cd-Tg (*****)	3	4.0	66.59	74.59	4.896	17.57	12.95	3.295	8.0
7.	Cd-Th (*****)	4	8.0	68.46	70.58	4.469	19.31	17.84	4.135	8.0
8.	Apo-Th	3	4.0	32.98	–	–	50.63	–	–	8.0
9.	Apo-Th	4	8.0	29.83	–	–	46.30	–	–	8.0

(*) – percentage concentration of counts (counts 1.0ml 10min)
(**) – initial concentration of Hg 7.482μGrAt/μM
(***) – initial concentration of Hg 0.471 μGrAt/μM
(****) – initial concentration of Cd 4.374μGrAt/μM
(*****) – initial concentration Cd 4.281 μGrAt/μM

Table II. Mean percentage of protein in individual fractions obtained from MT peaks rechromatographed by gel chromatography.

No	Protein	Steps of elution	Percentage concentration of protein							
			poliaggregate n > 7 M > 70 000		oligoaggregate n = 3–4 M = 30 000–40 000		monomer n = 1 M = 7000–9000		fraction M < M_{MT}	
			[A]	[B]	[A]	[B]	[A]	[B]	[A]	[B]
1.	[^{203}Hg]Hg-Th	1	–	–	–	–	–	–	–	–
2.	[^{203}Hg]Hg-HMW	1	–	–	–	–	–	–	–	–
3.	Hg-Th	2	27.61	6.67	4.45	2.27	14.27	15.30	3.13	0
4.	Hg-HMW	2	39.92	17.39	–	–	7.61	8.37	3.80	1.73
5.	Cd-Th	2	33.26	8.84	7.46	4.65	9.64	11.77	1.26	1.12
6.	Cd-Th	3	29.26	4.52	10.99	2.82	17.98	7.30	1.55	1.12
7.	Cd-Th	4	28.69	5.26	11.75	3.81	21.51	7.94	1.51	0.74
8.	Apo-Th	3	10.44	10.72	2.80	7.21	15.72	25.81	1.72	2.17
9.	Apo-Th	4	8.71	12.06	4.06	6.78	12.49	21.36	1.18	1.81

[A] – unbound fractions with the gel for covalent chromatography
[B] – bound fractions

Table III. Mean percentage of metals in individual fractions obtained from MT peaks rechromatographed by gel chromatography.

No	Protein	Steps of elution	Percentage concentration of metals							
			polyaggregate n > 7 M > 70 000		oligo-aggregate n = 3–4 M = 30 000 –40 000		monomer n = 1 M = 7000 –9000		complex M_m-TNB_n M = 1000–3000	
			[A]	[B]	[A]	[B]	[A]	[B]	[A]	[B]
1.	[^{203}Hg]Hg-Th (*)	1	–	6.53	–	0.08	–	0.93	–	86.49
2.	[^{203}Hg]Hg-HMW (*)	1	–	7.34	–	0.11	–	1.43	–	85.41
3.	Hg-Th	2	0.52	5.78	0.29	3.62	0.47	1.67	2.55	62.75
4.	Hg-HMW	2	0.43	19.77	0.05	0.25	1.17	3.63	10.34	32.51
5.	Cd-Th	2	2.93	3.74	2.17	2.39	10.94	3.14	39.99	10.38
6.	Cd-Th	3	0.28	2.15	0.32	1.42	1.56	1.98	72.08	6.12
7.	Cd-Th	4	0.61	1.69	0.33	2.41	1.91	2.89	65.93	8.24
8.	Apo-Th	3	–	–	–	–	–	–	–	–
9.	Apo-Th	4	–	–	–	–	–	–	–	–

(*) – percentage concentrations of counts
[A] – unbound fractions with the gel for covalent chromatography
[B] – bound fractions with the gel for covalent chromatography

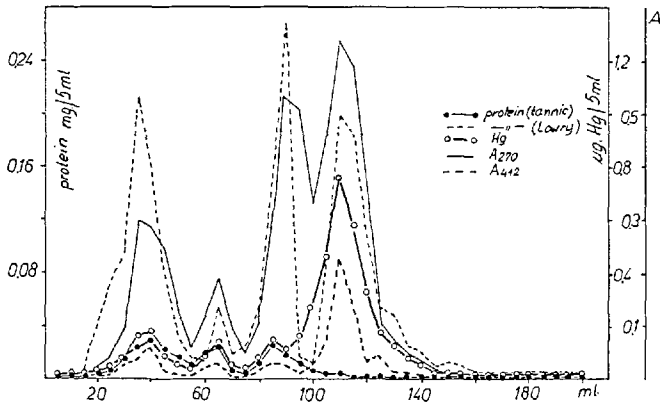


Fig. 3a

Sephadex G-75 gel chromatography of Hg-Th from Sepharose-DTNB (unbound fraction – peak A) (column 1.5 X 85cm – K 15/90 Pharmacia, 0.1 M, ammonium – formate buffer pH = 8.0, ionic strength μ = 0.1 flow rate 10ml/h, fractions 5.0ml were collected); •——• – protein conc. (tannic method), – – – – protein conc. (Lowry method), o——o – Hg. conc., —— – A_{270}, – – – – A_{412}

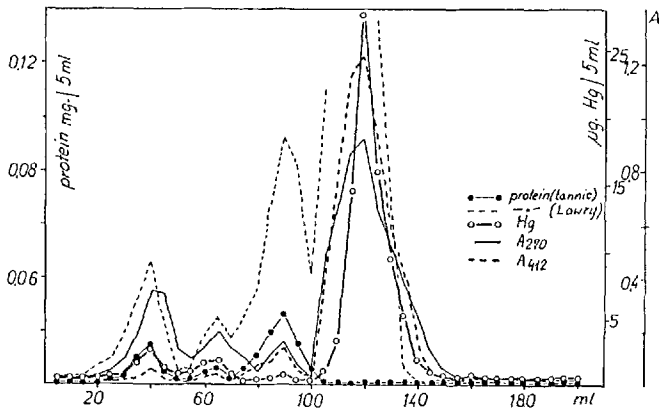


Fig. 3b

Sephadex G-75 gel chromatography of Hg-Th from Sepharose-DTNB (bound fraction – peak B) (column 1.5 X 85cm – K 15/90 Pharmacia, 0.1 M, ammonium – formate buffer pH = 8.0, ionic strength μ = 0.1 flow rate 10ml/h, fractions 5.0ml were collected); •——• – protein conc. (tannic method), – – – – protein conc. (Lowry method), o——o – Hg conc., —— – A_{270}, – – – – A_{412}

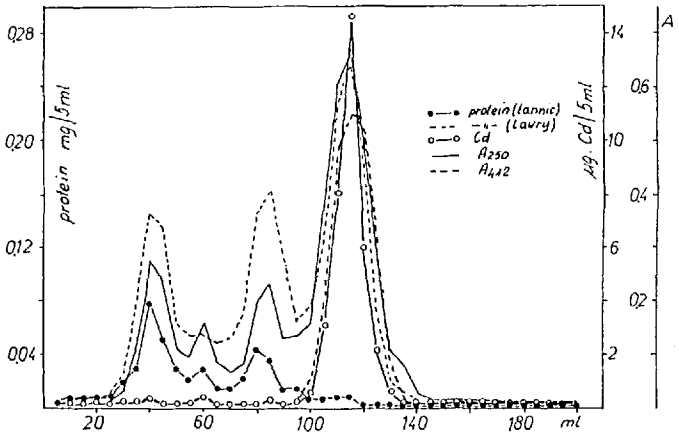


Fig. 4a

Sephadex G-75 gel chromatography of Cd-Th from Sepharose-DTNB (unbound fraction – peak I/B) (column 1.5 X 85cm – K 15/90, ionic strength μ = 0.1 flow rate 10ml/h, fractions 5.0ml were collected); •——• – protein conc. (tannic method, – – – – protein conc. (Lowry method), o——o – Cd conc., —— – A_{250}, – – – – A_{412}

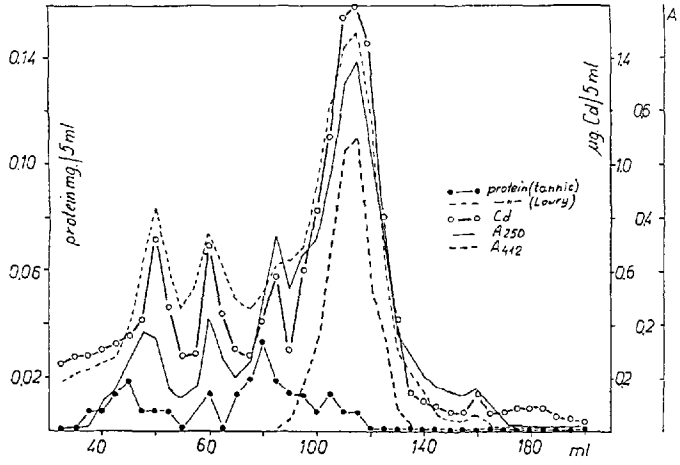


Fig. 4b

Sephadex G-75 gel chromatography of Cd-Th from Sepharose-DTNB (bound fraction – peak III) (column 1.5 X 85cm – K 15/90, ionic strength μ = 0.1 flow rate 10ml/h, fractions 5.0ml were collected) – •——• – protein conc. (tannic method), – – – – protein conc. (Lowry method), o——o – Cd conc., —— – A_{250}, – – – – A_{412}

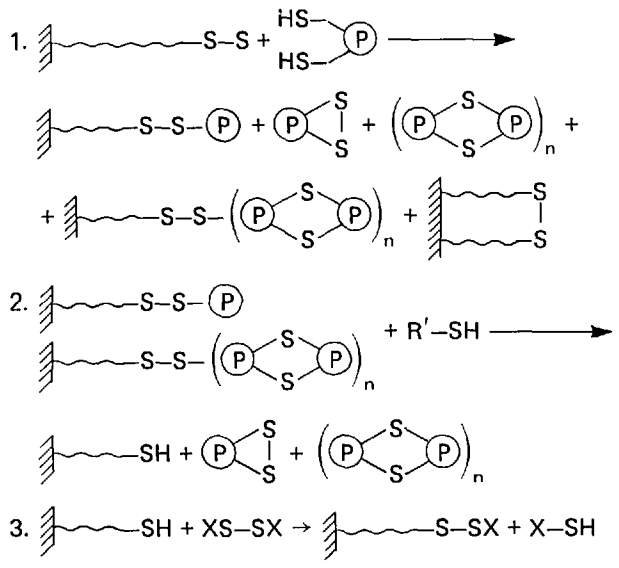
data usually obtained for preparations of that type [46–48]. Application of modified elution [39, 40] led to the conclusion that balast proteins contained only in peak I, according to a three-step Squibb and Cousins elution [25, 26, 29] form 1.5–5% of the supported protein. According to the same authors [26] the yield of recovery, estimated from the number of counts (^{35}S), is about 90% higher than that indicated by the results obtained in the paper. However, for copper proteins [28] the binding efficiency was only 11%. The appearance of polymeric and oligomeric MT fractions is reported only by Ryden and Deutsch [28], who separated Cu-Th and other low-molecular weight copper proteins rich in SH-groups. The authors suggest that SH-groups oxidizing to disulphide bridges in the presence of copper ions are responsible for formation of the aggregates. Similar results were obtained for Hg-Th, Cd-Th, and Apo-Th. Lower differences were observed in the number of MT chains contained in oligoaggregates (Table II). A very high metal loss was noticed (Tab. I, Tab. III). In the process of separation by covalent chromatography only 10–25% of supported metal is bound with the protein. Brenner and Mehra [32] mention the loss of Cd and Zn during chromatographic separation, from an initial value of 4–7 : 1 to 2.7 : 1 which

is 40–70% of the supported metal. According to Ryden and Deutsch [28] about 50% of the supported Cu dissociates from the bonds. The high loss in Hg strongly bound with protein is a surprise, since it is bound with mercaptide bonds normally unbeakable even at very low pH values. Another difficulty is a complete lack of any comparative data.

The problems with quantitative isolation of MT by covalent chromatography have already been reported [49, 50]. From the data it follows that the type of metal bound with the protein only slightly affects the efficiency of binding and thus separation, in spite of the different behaviours of Hg and Cd towards the chromatographic support (Table I).

For MT polythiolate protein a decisive factor affecting efficiency of separation was blocking of SH-groups in the protein with metals, or their unblocking, which was seen while comparing Hg-Th and Cd-Th with the data concerning Apo-Th (Tab. I). Another important factor affecting binding efficiency is the formation of high-molecular weight and low-molecular weight fractions of mass lower than that of monomer MT [37–40]. This fact is due to formation of disulphide bridges and, as a result, oligo- and polyaggregates. Intermolecular –S–S– bridges are responsible for combining individual MT chains into big aggregates. Intramolecular oxidation of SH-groups is the cause of a denser and more globular monomer. This results in decrease in the sige of the protein molecule and the Stokes radius, and, as a consequence, the apparent loss in mass, determined by gel chromatography, observed in experiments [37–40]. Oxidation of SH-groups results in a loss in reactive thiolate groups, capable of binding protein with a support, which is mainly responsible for low efficiency of separation of polythiolate proteins. The pH values of initial buffers (protein binding) only slightly influence the efficiency of quantitative separation, as can be seen in Table I. Another factor affecting the binding efficiency is the state of the support. For mercury proteins (Hg-Th, Hg-HMW) strong binding of Hg with the support was observed [37]. Support thus blocked becomes inactive though, in this case, the proteins may be bound through mercaptide bonds, as in chromatography on immobilized Hg compounds. Another reason may be oxidation of the support itself and formation of disulphide bridges between individual ligands. However, this fact is not likely to affect the efficiency in a considerable degree since for Apo-Th [40] an increase in binding efficiency is observed in spite of the presence of unblocked, reactive thiolate groups. The final cause of the decrease in effectiveness of MT binding is deterioration of the support, resulting in a loss of active ligands (binding, elution, regeneration) and a decrease of their concentration on the surface of the support. Investigations carried out for Cd-Th [39] showed that after about 55–60 cycles of work, the efficiency of binding decreases from 25–30% to about 4%.

The following mechanism is suggested for the covalent chromatography of polythiolate proteins [51].



but it does not take into consideration all the possible metal interactions, only reactions of SH-groups of the proteins with a chromatographic support, where oxidation of SH-groups of the protein is a permanent element of separation. Initially it was assumed that the differences between the masses determined by gel chromatography and other methods were due to a wrong assumption about the globular structure of this protein [52]. The cause was, however, different and connected with the properties of SH-groups. The existence of high-molecular weight fractions resulting from the separation of MT is reported by Webb and Etienne [53], and Bremner and Young [54]. Even mercaptoethanol protected SH-groups from oxidation [55]. Formation of –S–S– bridges was also observed in the case of Cd-Th [56], Hg-Th [57] and Zn-Th [58] where Zn^{++} ions underwent dissociation from mercaptide bonds.

For Cu-Th, uncontrolled oxidation of SH-groups to disulphide bridges was observed. This was shown by the presence of high-molecular fractions and shifts in EPR and CD spectra [59–62]. Bridging takes place in the presence of metal ions: Zn^{2+} [58], Cu^{+} [61, 63], as a result of deprotonation occurring under neutral conditions of pH [60, 64] and owing to autocatalytic properties of SH-groups. All these data seem to confirm the suggested mechanism of separation and formation of poly- and oligomeric fractions. Such data are missing from the existing literature data on covalent chromatography separation.

The investigations indicate that:

- during separation by covalent chromatography mercaptide bonds with Hg in polythiolate complexes (Hg-Th) are broken, and cadmium-thiolate clusters (Cd-Th) in MT are decomposed unblocking SH-groups, which leads to formation of apothionein,
- low binding efficiency of MT, 20–30%, without exceeding the theoretical capacity of the support, is due to the loss in reactive thiolate groups resulting from inter- or intramolecular oxidation of SH-groups to disulphide bridges, which is shown by the formation of high molec-

ular weight fractions and a decrease in the Stokes radius of a monomer and apparent decrease in molecular mass determined by gel chromatography,

- another cause of a low binding efficiency of the proteins blocked with Hg (Hg-Th, Hg-HMW) may be the blocking of the support by metal dissociating from mercaptide bonds of MT and oxidation of SH-groups of the support into unreactive disulphide bridges,
- an important factor influencing binding efficiency of proteins and polythiolate polypeptides with covalent chromatography support is blocking (or its absence) of protein SH-groups by metals,
- binding efficiency is 8–10 times lower when the concentration of ligand on the surface is reduced in the process of work.

Conclusions

1. Application of covalent affinity chromatography for isolation and separation of polythiolate peptides and metallopeptides results in changes in II and III order structure of the proteins and of its native character.
2. This form of chromatography can be used for the preparation of apoproteins, particularly from thiolate-mercury proteins, where mercaptide bonds are normally unbreakable even at low pH values.
3. The method can be used in toxicological diagnosing, as it selectively isolates Hg from the proteins characteristic of intoxication with this metal (Hg-thionein).
4. When covalent affinity chromatography is used to obtain biochemical or pharmacological preparations an examination of the homogeneity of the obtained preparation should be carried out.

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