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Chelated mercury as a ligand in immobilized metal ion affinity chromatography of proteins

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Abstract

Chelation of mercuric ions by an iminodiacetate–Sepharose gel was evaluated. The retentive properties of iminodiacetate–Sepharose gel column was studied towards proteins varying the composition of eluting systems from 2-mercaptoethanol to NaCl and imidazole, determining also the extent of mercury leaching. It was demonstrated that chelated mercury contained free sites for interaction with proteins such as bromelain and recombinant human granulocyte colony stimulating factor from *E. coli*. The extraction of the latter by chromatography of its inclusion bodies solution on Hg(II)-loaded Sepharose–iminodiacetate gel was also evaluated. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Immobilized metal ion affinity chromatography (IMAC) of proteins, first introduced by Porath et al. [1] under the name of metal chelate chromatography, has become a well-established and versatile technique for both analytical and large scale separations. IMAC procedure is based on coordination of transition metal ions chelated by appropriately prepared adsorbent and electron–donor groups on a protein surface. Histidine, cysteine and tryptophan were originally suggested as the amino acids responsible for coordination with chelated metal ions [1]. Progressive studies on IMAC have shown that the selectivity of protein retardation is governed by the content and topography of metal-binding residues onto its surface. Among such, histidyl residues remain of special interest since as it has been clearly indicated [2–13], they are the predominant ligands affecting selective retention on metal-affinity columns. As the result of those studies, the principle of IMAC was further combined with protein engineering by the use of polyhistidine-containing affinity tails [14], or creating high-affinity metal coordination sites with the histidine residues introduced by site-directed mutagenesis of the existing protein [8,10,14–17]. This serves as a powerful tool for identification [18] and purification of recombinant proteins [14,17,19].

Retention studies of amino acids, peptides and peptide hormones on Me(II)–iminodiacetate (IDA)

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gels have revealed that cysteine and tryptophan follow histidine in displaying the highest contribution to the coordination with metal ions [20–22]. With regard to this, several attempts have also been made to evaluate the role of cysteine in IMAC of proteins, e.g. bovine serum albumin [7] on Ni(II) or Cu(II), protamines [23] on Zn(II), and synthetic peptides containing a cysteine residue but lacking histidine and tryptophan [24] on Cu(II) and Ni(II)-loaded supports, respectively. However, the direct coordination of free thiol group was not detected. Despite this, such interaction remains attractive since the free thiol group might be found in proteins, e.g. cysteine proteases, some representatives of recombinant proteins like β-interferon, interleukin-2 and others. In this respect, it was of interest to evaluate the interaction of proteins containing free SH group with mercuric ions chelated by the IDA gel, first of all paying attention to its further application for probing the accessibility of such a group on a protein surface.

Interaction of thiol proteins with immobilized Hg(II) ions has already been introduced to protein purification in the frame of so-called ''covalent chromatography'' through the use of affinity matrices containing immobilized organomercurial ligands such as mercuric(II) acetate, *p*-mercurianiline, *p*-aminophenylmercuric acetate and *p*-chlormercuribenzoate [25]. However, to date there are no available data on Hg^{2+} ions chelation by conventionally used chelating adsorbents, e.g. IDA gel used for the IMAC of proteins, particularly containing free thiol groups. The reason for this might be related to the fact that free thiol group may scavenge Hg(II) ions from its complex with IDA gel simply owing to the incomparably higher stability of the Hg–SH complex compared with a complex formed between Hg(II) ions and IDA. Obviously, in this case the IMAC might fail, as it was discussed by Hemdan et al. [5].

In this paper we report that at appropriately chosen conditions Hg^{2+} ions chelated by IDA gel may have a free residual site available for interaction with bromelain and human recombinant granulocyte colony stimulating factor (rhG-CSF), as a representative of recombinant proteins, which has a free unpaired cysteine residue. Chromatography of proteins was performed under selected eluting systems, containing 2-mercaptoethanol, imidazole and NaCl, by evaluating the recovery of proteins and the extent of mercury leakage depending on a type of eluent. The comparative interaction of rhG-CSF and its derivative, which possesses chemically modified unpaired cysteine and histidine residues with Hg(II) and Ni(II)-loaded IDA–Sepharose was studied and enabled to show, that histidine residues do not play an essential role in the protein binding on IDA gel charged with Hg^{2+} ions. The principal possibility of rhG-CSF extraction via chromatography of its inclusion bodies solution on Hg(II)–IDA gel column was also shown.

2. Experimental

2.1. Materials

Bromelain preparation (EC 3.4.22.32) as a lyophilized powder (Lot 55H1035) was purchased from Sigma (St. Louis, MO, USA), or Serva (Heidelberg, Germany) and recombinant methionyl granulocyte colony stimulating factor, human (rhG-CSF) was a product of Biotechna UAB (Vilnius, Lithuania). Sepharose CL-6B and desalting columns PD-10 were obtained from Pharmacia (Uppsala, Sweden). Iminodiacetic acid (IDA) and epichlorohydrin were obtained from Fluka (Basle, Switzerland). All other chemicals were of analytical or reagent grade and obtained from Merck (Darmstadt, Germany), Sigma or Serva.

2.2. Synthesis of IDA–Sepharose gels

Sepharose CL-6B-IDA gel was prepared according to Porath and Olin [26] by reaction of an epichlorohydrin-activated matrix with disodium iminodiacetate. Sepharose activation was controlled by the content of epoxide groups during the reaction of activated matrix with sodium thiosulfate [27]. The content of iminodiacetate groups was controlled by the gel capacity to adsorb Cu^{2+} ions. For this, a 1.0-ml volume of Cu^{2+}-loaded and appropriately prewashed gel was treated with 50 m*M* solution of EDTA and the amount of copper was measured by atomic absorption spectrometry. Usually, IDA gel

with a copper capacity of 38–42 μmol/ml was used for the IMAC experiments.

2.3. Preparation of Sepharose–IDA gels for chromatography

Metal-free IDA gel was prepared by its subsequent washing on the filter funnel with 50 m*M* solution of EDTA (10-fold volume to the adsorbent bed volume) deionized water, 1.0 m*M* HCl solution and water. The adsorbent was packed in a separate column in deionized water and rinsed with the 10-fold excess of buffer solution of the defined pH.

Hg(II)-loaded gel was prepared as follows: metal-free IDA gel, prewashed with 1.0 m*M* HCl and water, was usually loaded with at least ten bed volumes of freshly prepared 20 m*M* $HgCl_2$ solution in 10 m*M* MES (2-morpholinoethanesulfonic acid) buffer, pH 6.0, then washed with a 10-fold volume of 10 m*M* MES buffer, and finally equilibrated with the chromatographic buffer.

The determination of Hg^{2+} ions capacity was performed applying a 1.0-ml bed volume sample of Hg(II)-loaded gel. It was packed in a column, the excess and loosely bound mercuric ions were washed out with water and thereafter the chelated mercury was completely released from the bed by passage of 15 ml 50 m*M* EDTA solution. A 0.2-ml aliquot of EDTA eluate was treated with dithizone solution in chloroform and the extracted Hg(II)–dithizone complex was determined spectrophotometrically at 490 nm as described in [28]. The Hg(II)-loaded IDA adsorbents were found to contain 16.0–17.0 μmol of Hg^{2+}/ml.

2.4. Chromatography of proteins on metal-free and metal-loaded gels

The following 10 m*M* concentration buffers were used: MES for pH 5.0–6.0, HEPES {2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid} for pH 7.0, and 10 m*M* HEPES with the additives of selected agents, e.g. 2-mercaptoethanol (5–10 m*M*), imidazole (up to 0.3 *M*), sodium sulfate (0.15–0.25 *M*) or NaCl (1.0 *M*).

Chromatography of bromelain was performed onto 1.0 or 3.0 ml and of pure rhG-CSF solution onto a 3.0 ml bed volume of metal-free or metal-charged IDA gels (for details see legends to tables and figures). Non-retained protein was washed out passing 10 bed volumes of the initial chromatographic buffer; the linear gradient elution of adsorbed protein was performed with the selected eluting agents and finally the column was developed with 5–7 bed volumes of the solution containing a fixed concentration of the selected agent.

Samples of purified rhG-CSF (supplied as a formulated protein solution in 10 m*M* acetate, pH 4.0) were dialyzed against the respective buffer of chromatography, centrifuged and loaded onto Sepharose–IDA columns.

2.5. Preparation of rhG-CSF inclusion body solubilizate for chromatography

A 5-g amount (wet mass) of harvested *E. coli* cells was homogenized in 50 ml 0.1 *M* Tris–HCl, 5.0 m*M* EDTA buffer, pH 7.0, thereafter lysozyme (0.1%), Triton X-100 (0.1%), β-mercaptoethanol (100 m*M*) and phenylmethylsulfonyl fluoride (PMSF) (1 m*M*) were added, the cells were incubated for 15 min at 4°C and sonicated on ice for 5×1 min. After centrifugation the collected pellets of inclusion bodies (1 g, wet mass) were washed twice with a solution of 1.0 *M* NaCl, 0.1% Tween 80, and finally with water. A 0.25-g amount of prewashed inclusion bodies was stirred overnight at 4°C in 10 ml of 10 m*M* HEPES buffer, pH 7.0, then the pH of the suspension was adjusted with NaOH to 11.5, maintained for 2–3 min at ambient temperature, and the pH was readjusted to 7.0. The solution was centrifuged and the clear supernatant (8.8 –10.1 ml, with a protein concentrations of 2.1–2.85 mg/ml) was loaded onto a 3-ml bed volume of Sepharose–IDA–Hg(II) columns equilibrated with 10 m*M* HEPES, pH 7.0. The column was developed with buffer, and the adsorbed protein was eluted by eluents containing β-mercaptoethanol (up to 10 m*M*) or imidazole (50–300 m*M*).

2.6. Assay of proteins in eluates

The protein concentration in eluate fractions during the chromatographic run was determined spectrophotometrically at 595 nm by Bradford assay [29]. In addition, the eluates from Hg^{2+} ions-loaded gels were

analysed and the protein was identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [30].

The purity of rhG-CSF as well as its conformational state during isolation from inclusion bodies were analysed by reversed-phase HPLC similarly as described in [31], except that a C_{18} (Bio-Rad, Hi-Pore RP-318, 25×0.46 cm I.D.) reversed-phase column was used instead of the reversed-phase C_4 column. Mobile phase: solvent A 0.10% trifluoroacetic acid (TFA), solvent B 0.10% TFA, 90% CH_3CN. The chromatographic run was performed by a linear gradient of 50–100% B over 50 min at a flow-rate of 1.0 ml/min. Peaks were detected at 215 nm (Waters detector, Model 481). If necessary a sample of purified rhG-CSF was used as a reference.

2.7. Preparation of chemically modified rhG-CSF

2.7.1. S-Carboxamidation of unpaired Cys^{17}

This was carried out following the general methods described by Darby and Creighton [32]. The pH value of formulated rhG-CSF solution was adjusted to 8.1 by adding 1.5 ml of 0.42 *M* Tris–HCl buffer, pH 8.2, 1.0 m*M* EDTA to 1.0 ml protein sample (0.75 mg/ml). A 64-mg amount of iodoacetamide (crystalline solid) was added to the protein sample (molar ratio=9000:1), the reaction was proceeded for 2 h at 25°C and terminated by desalting over Sephadex G-25 PD 10 column equilibrated with the buffer used for the chromatographic experiments on metal-loaded IDA gels, e.g. 10 m*M* MES, pH 5.0 containing 0.13 *M* Na_2SO_4 or 10 m*M* HEPES pH 7.0. The completeness of S-carboxamidation was controlled by the Ellman assay for free thiol groups [32,33] and was calculated to be 97–98% using the extinction coefficient for the liberated nitrothiobenzoate, $\epsilon = 1.36 \cdot 10^4$ M^{-1} cm^{-1} at 412 nm [33].

2.7.2. Modification of His residues with diethyl pyrocarbonate (DEP)

The concentration of prepared DEP stock solution in absolute ethanol was determined reacting its 425–430-fold diluted aliquot (20 μl) with 10 m*M* imidazole solution in 0.1 *M* potassium phosphate buffer, pH 7.5, and the generation of difference absorbance at 230 nm against reference imidazole solution containing 20 μl aliquot of ethanol instead of DEP was measured. The concentration of carbethoxylated-His was calculated using a molar absorptivity of 3000 M^{-1} cm^{-1} [34]. Before each series of modifications, the sample of DEP stock was diluted with absolute ethanol to give it a concentration equal to 0.227 *M*. We have chosen the pH value for G-CSF modification at 5.90, as was done by Kita et al. [35]. This was selected to avoid the loss of the protein due to its tendency to precipitate after the adjustment of pH above its isoelectric point, p*I* 5.9–6.1 [36]. The pH of G-CSF solution (2.6 ml, 0.66 mg/ml) was adjusted to 5.90 by adding 2.6 ml 0.2 *M* potassium phosphate, pH 6.45. An equal volume (2.5 ml) of a protein solution was added to two spectrophotometer cells and the reaction was started by the addition of 10 μl 0.227 *M* DEP solution and 10 μl of absolute ethanol to the sample and reference cells, respectively. The modification reaction was performed at 15–16°C until the difference absorbance at 240 nm reached a value of 0.282 (20–25 min), which corresponds to the carbethoxylation of five His residues of G-CSF ($\epsilon = 3200$ M^{-1} cm^{-1} for carbethoxylated histidine was used [35]). The reaction was terminated by the addition 50 μl of 0.2 *M* imidazole, pH 5.9, to the sample mixture and desalting over Sephadex G-25 PD-10 column equilibrated with 10 m*M* HEPES buffer, pH 7.0. The Ellman assay for free thiol indicated, that under such conditions, 10–15% of SH-groups of G-CSF were modified. The absence of difference absorbance at 270 nm showed that the tyrosine residue was not modified [37].

3. Results and discussion

3.1. Chromatography of bromelain and purified rhG-CSF on Sepharose–IDA adsorbent charged with Hg^{2+} ions

The column of Sepharose–IDA gel charged with mercuric ions was evaluated for the relative extent of metal ion leaching under various concentrations of agents, anticipated for their further use as eluents of adsorbed proteins. Table 1 shows that among the agents tested, imidazole (up to 100 m*M*), 85 m*M* sodium phosphate and β-mercaptoethanol at a concentration <1 m*M* caused slight leaching (up to

Table 1
Leaching of Hg^{2+} ions from Sepharose–IDA–Hg(II) caused by various selected agent in 10 m*M* HEPES buffer, pH 7.0[a]

Agent	Leached Hg^{2+} ions (%)
1.0 m*M* imidazole	5
10 m*M* imidazole	5
100 m*M* imidazole	8
85 m*M* sodium phosphate	13
0.1 m*M* β-mercaptoethanol	10
1 and 10 m*M* β-mercaptoethanol	8 and 43
0.1 *M* NaCl	39
1.0 and 2.0 *M* NaCl	87 and 12
10 m*M* EDTA	88

[a] Separate columns containing 1.0 ml (bed volume) of Sepharose–IDA gel charged with mercuric ions in 10 m*M* MES buffer, pH 6.0 (metal ion content 16.85 μmol/ml), were prewashed with 15–16 ml of 10 m*M* HEPES buffer, pH 7.0 and 15–16 ml volume of selected agent. Eluates of each experiment were collected and the amount of released mercuric ions was determined photometrically.

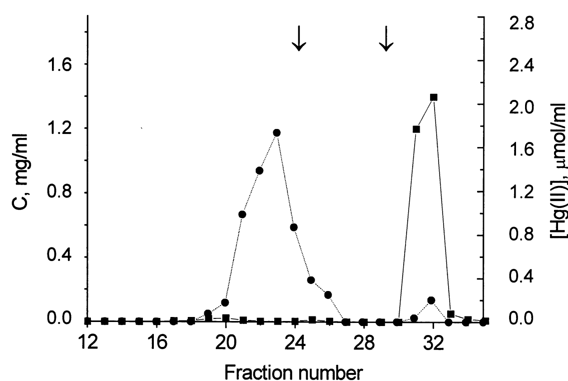

Fig. 1. Elution profile of bromelain from Sepharose–IDA–Hg(II) column. 1.0 ml (bed volume) of IDA gel charged with mercury (17 μmol/ml). Sample: 11.5 mg (0.6 mg/ml, 19.5 ml) of bromelain (Serva) in 10 m*M* HEPES, pH 7.0; flow-rate 10 ml/h, fraction volume 2.6 ml. The column with adsorbed protein was developed with a linear gradient to 5 m*M* of β-mercaptoethanol (fraction 12–24), further with buffer alone and buffer containing 1.0 *M* NaCl (arrows mark the position of eluent change). ■, protein concentration, mg/ml; ●, Hg^{2+}concentration, μmol/ml.

13%) of chelated mercury. Strong leaching was observed when the column was rinsed with buffer, pH 7.0, containing >1 m*M* β-mercaptoethanol, NaCl 0.1–1.0 *M* and 10 m*M* EDTA.

The retentive features of Sepharose–IDA gel and its complex with Hg(II) ions as well as eluting capability of selected eluents was evaluated towards sulfhydryl protease bromelain. Chromatography of bromelain on Hg(II)-charged IDA column was tested preliminary in 10 m*M* HEPES buffer, pH 7.0, without any salt additives. The elution of the adsorbed protein is shown in Fig. 1, and the recovery of bromelain from the column with respect to the varying types of eluent is summarized in Table 2. It is evident that the retention of this basic protein (p*I* of 9.55 [38]) onto Hg(II)-charged IDA–Sepharose occurred mainly via the ion–ion interaction and no function is revealed for chelated mercuric ion. It can be seen from Fig. 1, that the eluent containing β-mercaptoethanol to 5.0 m*M* only caused the release of mercury by ≈81%, while the adsorbed protein remained on the column and could be recovered under subsequent elution with a buffer containing 1.0 *M* NaCl. A separate gradient elution of adsorbed protein performed with NaCl to 1.0 *M*, or its introduction (at 1.0 *M*) into a buffer after a linear gradient elution had been performed to 100 m*M* of imidazole or sodium phosphate (Table 2) caused again desorption of the protein by 68–97%, and also the leaching of the major part (75–93%) of the mercury. Only the initial elution with the imidazole gradient enabled recovery of the minor part (18%) of adsorbed protein. The residual ion-exchange function of Hg(II)-charged IDA–Sepharose column towards bromelain may be completely suppressed by a moderate concentration of salts, e.g. sodium chloride, sodium or ammonium sulphate. The necessary salt concentration was selected performing the gradient elution of a protein retained by metal-free Sepharose–IDA-gel to 1.0 *M* of NaCl or to 0.25 *M* Na_2SO_4 (Fig. 2A) (in both cases a 99% yield of recovered protein was obtained). Further evaluation of the chromatographic behaviour of bromelain on the Sepharose–IDA adsorbent charged with Hg^{2+} ions was performed including 0.13 *M* of sodium sulphate into the initial buffer. The introduction of salts such as sodium chloride or ammonium sulphate, commonly used in the IMAC procedure for this purposes, has been avoided taking into account the strong affinity of Hg^{2+} ions to chloride anions [39] and a possible coordinating ability of ammonium ions, too. In the first case, strong leaching of chelated Hg(II) from the column might be expected, as evidenced in Table 1, in the second case, one can guess the competition between ammonium ions and

Table 2
Recovery of bromelain from Hg(II)-loaded Sepharose–IDA gel[a]

Exp. no.	Amount of adsorbed protein (mg/ml)	Eluent	Protein recovery (%)	Released Hg^{2+} ions (%)
1[b]	11.5	L.g[c]. to 5.0 m*M* of β-mercaptoethanol	2	81
		10 m*M* HEPES	–	9
		1.0 *M* NaCl	62	4
2	4.0	L.g. to 1.0 *M* of NaCl	96	75
3	5.3	L.g. to 100 m*M* of imidazole	18	
		10 m*M* HEPES	4	
		1 *M* NaCl	68	93
4	4.8	L.g. to 100 m*M* of sodium phosphate	3	–
		10 m*M* HEPES	–	–
		1 *M* NaCl	97	88

[a] 1.0 ml (bed volume) of IDA gel charged with mercury (17 μmol/ml) was developed with a protein solution in 10 m*M* HEPES buffer, pH 7.0. Unadsorbed protein was washed out by a buffer; elution of adsorbed protein with noted agents; flow-rate 10 ml/h.

[b] 11.5 mg (0.6 mg/ml, 19.5 ml) of bromelain (Serva) was loaded onto column, in other cases 4.0 – 6.1 mg (0.24–0.3 mg/ml, 16.6–21.2 ml) bromelain (Sigma) loads were used.

[c] L.g, linear gradient.

the donor-groups of proteins for the free coordination site of chelated mercuric ions. In the presence of 0.13 *M* Na_2SO_4, bromelain was also retained by Hg(II)–IDA–Sepharose, but contrary to the data in Table 2, its quantitative elution has been already caused by buffers containing β-mercaptoethanol, as evidenced in Fig. 2B and Table 3. These results indicate that mercury chelated by the IDA gel exhibited its ability to interact with the tested protein, probably via its residual coordination site. The fact that such interaction occurs in the presence of salt, e.g. Na_2SO_4 leads to the assumption, that the behaviour of mercuric ions chelated by IDA gel may parallel that of chelated metal ions commonly used in IMAC [22]. However, leaching of mercury chelated by the IDA gel also occurred when eluents containing agents with high affinity to Hg^{2+} ions, such as NaCl or β-mercaptoethanol were used (Fig. 1, Fig. 2B and Tables 2–3).

In addition to bromelain, the chromatographic behaviour of purified rhG-CSF was evaluated on metal-free and Hg(II)-charged IDA columns. The isoelectric point of this protein is 5.9–6.1 [36], therefore it was not retained at pH 7.0 on metal-free IDA gel, and all loaded protein (3.7 mg) was found in the breakthrough fractions. Its chromatography on Hg-loaded gel was performed in 10 m*M* HEPES buffer, pH 7.0 without Na_2SO_4. In this case, the sample of loaded protein (Table 3 and Fig. 2C) was completely retained by the column and eluted mainly (86%) by a buffer containing 5 m*M* of β-mercaptoethanol. Based on this, several attempts were made to evaluate the ability of Hg(II)-charged IDA–Sepharose to extract rhG-CSF from appropriately prepared solution of inclusion bodies that were isolated from *E. coli*. The procedures commonly used for solubilization of inclusion body in chaotropic agents, such as 8 *M* urea or 7 *M* guanidine hydrochloride (GdmCl) [40] seemed, in this case, not appropriate since a high urea concentration, and even more GdmCl might cause the discharge of Hg-loaded column. This is evident from the data on Hg^{2+} leaching presented in Table 1. To overcome this problem, the recently proposed method [41] for inclusion body solubilization by the use of detergent and a brief shift to pH 12 was used. A prepared solution of inclusion bodies was chromatographed on Hg-loaded IDA gels at pH 7.0. Figs. 3 and 4 show that the Hg-loaded column retained rhG-CSF, and part of it can be recovered from the column by the eluents containing imidazole (Fig. 3A) or β-mercaptoethanol (Fig. 3B). The electrophoretic pattern of protein samples drawn from the fractions of imidazole gradient eluates is shown in Fig. 4; Lines 5–8

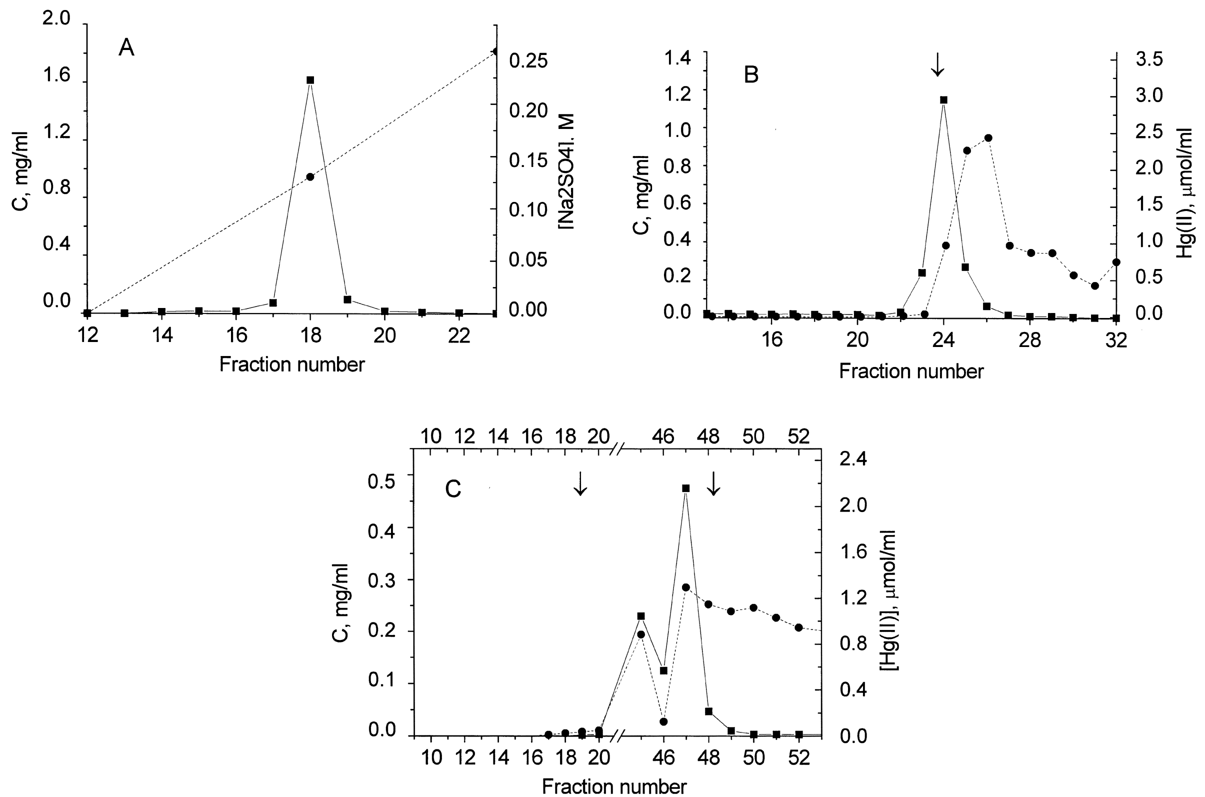

Fig. 2. Elution profiles of proteins from Sepharose–IDA columns. (A) 10.5 mg of bromelain (0.56 mg/ml, 18.8 ml) loaded onto 3 ml Sepharose–IDA column without metal ion: recovery of adsorbed protein (8.6 mg), linear gradient of Na_2SO_4. ■, Protein concentration, mg/ml; ●, sodium sulfate concentration, *M*. (B) A 19-mg amount of bromelain (1.04 mg/ml, 18.3 ml) or (C) 3.5 mg of rhG-CSF (0.4 mg/ml, 8.8 ml) loaded onto 3 ml (bed volume) of Sepharose–IDA–Hg(II) (16.7 μmol Hg^{2+}/ml) in 10 m*M* HEPES buffer, pH 7.0 for rh-G-CSF and 10 m*M* HEPES buffer containing 0.13 *M* Na_2SO_4 for bromelain; flow-rate 30 ml/h, fraction volume 3.5 ml. Recovery of adsorbed proteins: (B) linear gradient to 5 m*M* of β-mercaptoethanol (fraction 13–23), further, elution with 10 m*M* β-mercaptoethanol; (C) linear gradient to 5 m*M* β-mercaptoethanol (fractions 9–19), further, elution with 5 and 10 m*M* β-mercaptoethanol (arrows mark the position of the eluent changes); ■, protein concentration, mg/ml; ●, Hg^{2+}concentration, μmol/ml.

demonstrate that the eluate fractions contained the target protein in its prepurified state. The same was determined, if the elution of adsorbed rhG-CSF is performed by eluents containing β-mercaptoethanol. RP-HPLC analysis of the protein samples from eluate fractions revealed 66 and 72% purity of rhG-CSF recovered from the column by a linear gradients to 10 m*M* of β-mercaptoethanol (Fig. 5) or imidazole gradient, respectively. Bioassay of the protein sample eluted from Sepharose–IDA–Hg(II) column under imidazole gradient had showed the biological function of rhG-CSF (data not shown here). This enabled us to assume that maybe the protein is recovered from Hg(II)-loaded column in a conformational state close to that of the correctly folded protein. This may not be unexpected, because we have already exploited the technique of IMAC to refold recombinant IL-3 and G-CSF by the chromatography of the respective inclusion body solution in GdmCl on Sepharose–IDA column charged with Zn^{2+} or Ni^{2+} ions [42]. Data in Table 3 and Figs. 2–3 support our assumption that chelated mercury has a remaining site for coordination of available electron-donor groups in proteins. Among such, functional groups bearing sulfur and/or nitrogen atoms might be assumed since respective agents such as β-mercaptoethanol and imidazole are able to cause the recovery of rhG-CSF extracted from its

Table 3
Retention behaviour of bromelain (1), and rhG-CSF (2) on Sepharose–IDA–Hg(II) gel[a]

Exp. no.	Amount of adsorbed protein (mg)	Eluent	Protein recovery (%)	Released Hg^{2+} ions (%)
1	6.7	L.g[b] to 5 m*M* β-mercaptoethanol	20	–
		10 m*M* β-mercaptoethanol	78	67
		Starting buffer	1.5	24
		1.0 *M* NaCl	–	9
2	3.5	L.g. to 5 m*M* β mercaptoethanol	–	–
		5 m*M* β-mercaptoethanol	86	24
		10 m*M* β-mercaptoethanol	2	42
		Starting buffer	–	
		1.0 *M* NaCl	–	

[a] Procedures of chromatography as in Fig. 2B and C.
[b] L.g., linear gradient.

inclusion bodies by the Hg(II)–IDA–Sepharose column.

3.2. Evaluation of chromatographic behaviour of chemically-modified rhG-CSF on Hg(II)- and Ni(II)-charged Sepharose–IDA gels

Recombinant human G-CSF from *E. coli* is a 175-amino acid polypeptide chain containing an extra Met at its N-terminus. The molecule contains a free cysteine at the position 17, and two intramolecular disulfide bonds important for the maintenance of its three-dimensional structure, and consequently its biological function. The protein molecule also contains five histidine residues [43,44]. To discriminate whether the free SH group of cysteine-17 and/or the histidine residues may be involved in interaction with chelated mercury, the SH group was modified with iodoacetamide and histidine residues were modified with DEP and chromatography of such protein derivatives was performed on both Hg(II) and Ni(II) ions-charged Sepharose–IDA gels at pH values of 5.0 and 7.0. As can be seen from Table 4, non-modified rhG-CSF was retained at pH 5.0 by metal-free Sepharose–IDA gel via ion–ion interaction that can be suppressed by Na_2SO_4. The inclusion of 0.13 *M* of salt into the buffer of pH 5.0 was sufficient to eliminate such interactions. In the presence of this salt the protein interaction with the Ni(II)-loaded column was also practically suppressed and indicated that the function of His residues at pH 5.0 is abolished. In contrast to Ni(II) ions, mercury chelated by IDA gel maintained the retention ability towards the protein molecule under similar conditions of chromatography. Table 4 shows that rhG-CSF adsorbed at pH 5.0 in the presence of 0.13 *M* Na_2SO_4 was quantitatively recovered with eluents containing β-mercaptoethanol. This may indicate that free SH group might be considered as the group interacting with the chelated mercury.

At pH 7.0, non-modified rhG-CSF interacted with Ni(II)-loaded column (Table 4). Its elution from the column occurred in the presence of imidazole (0.15 *M*), while eluents containing β-mercaptoethanol (5–10 m*M*) or NaCl (1 *M*) were inefficient. This indicated that the protein interaction with chelated Ni(II) occurred mainly via histidine residues and the behaviour of iodoacetamide-modified protein on Ni(II)-loaded column remained analogous, i.e. its elution was also caused by 0.15 *M* imidazole. In addition to this, the DEP-modified protein completely lost its ability to be retained on Ni(II)-loaded column and was found in the breakthrough fractions (Table 4). The chromatographic behaviour of iodoacetamide-modified rhG-CSF on Hg(II)-loaded column at pH 7.0 was not evaluated having in mind a possible function of His residues in such an interaction. Non-modified rhG-CSF can be released at pH 7.0 from Hg(II)-charged column with eluents containing 5 m*M* β-mercaptoethanol (Table 3, Fig. 2C). The same type of eluent caused the elution of DEP-modified protein (Table 4). This fact favours the assumption that histidine residues might not be responsible for interaction with chelated Hg(II) ions

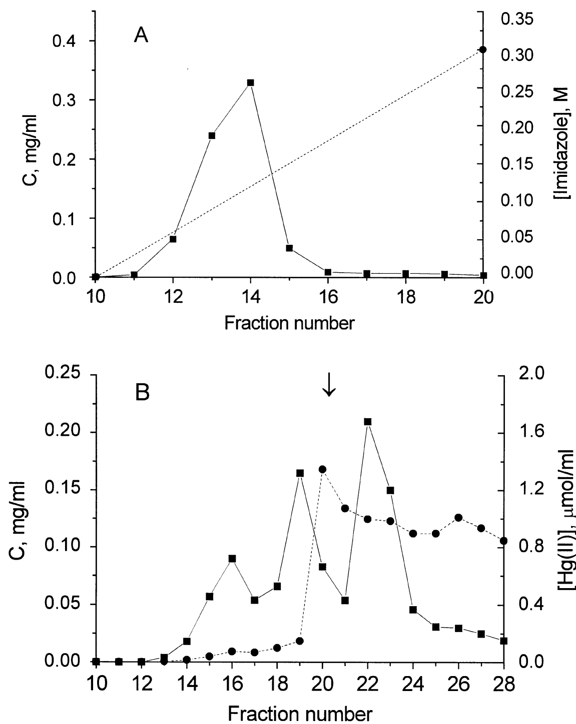

Fig. 3. RhG-CSF inclusion bodies chromatography onto Sepharose–IDA–Hg(II) columns. Profiles of protein recovery. 3 ml (bed volume) of Sepharose–IDA–Hg(II) in 10 m*M* HEPES buffer, pH 7.0; flow-rate 30 ml/h, fraction volume 3.5 ml. (A) An 8.8-ml volume of inclusion body solution (2.85 mg/ml) was loaded onto the column; 17.6 mg of unadsorbed protein was washed out by buffer, pH 7.0; adsorbed protein was recovered under a linear imidazole gradient to 300 m*M*. ■, protein concentration, mg/ml; ●, imidazole concentration, *M*. (B) A 10.6-ml volume of inclusion bodies solution (2.1 mg/ml) was loaded onto column; 16.6 mg of unadsorbed protein was washed out by buffer, pH 7.0; adsorbed protein was eluted under a linear gradient of β-mercaptoethanol to 10 m*M*, further, 10 m*M* solution of β-mercaptoethanol (arrow indicates the position of eluent changes). ■, protein concentration, mg/ml; ●, Hg^{2+}concentration, μmol/ml.

at pH 7.0. Thus, the involvement of Cys-17 in the interaction with chelated Hg(II) ions at pH 7.0 might be possible. The elusive support for this may be drawn from the chromatographic behaviour of iodoacetamide-modified and non-modified rhG-CSF on Hg(II)-charged column at pH 5.0. Despite that iodoacetamide-modified protein was retained by Hg(II)-charged column at pH 5.0 (Table 4), the recovery of its major portion (94%) occurred under linear gradient elution to 5 m*M* of β-mercaptoethanol. In the case of non-modified rhG-CSF,

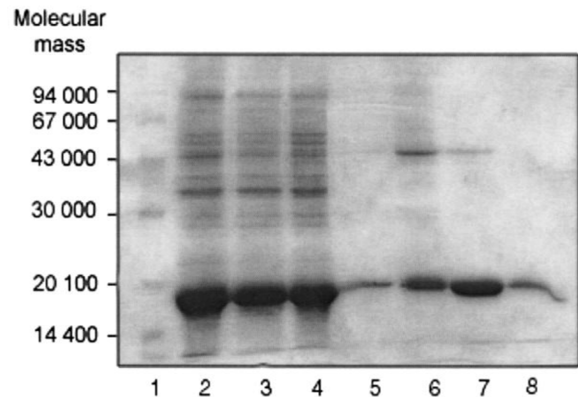

Fig. 4. SDS–PAGE under non-reducing conditions of fractions eluted from Sepharose–IDA–Hg(II) column performing chromatography of inclusion bodies solution, containing rhG-CSF. Lanes: 1 = molecular mass markers, 2 = samples of loads; 3,4 = samples of breakthrough fractions; 5–8 = samples of imidazole eluate fractions (Fig. 3A).

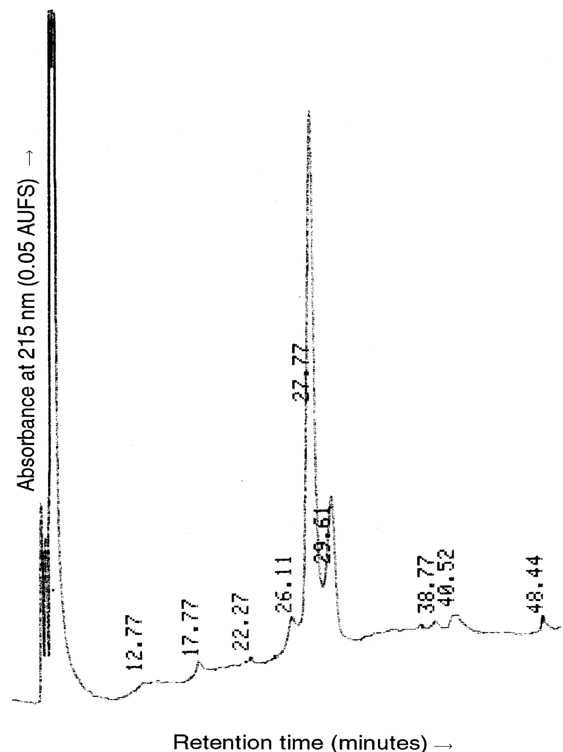

Fig. 5. RP-HPLC profiles of rhG-CSF recovered from Sepharose–IDA–Hg(II) column. Approximately 250 μl sample from eluate fraction (No 16, Fig. 3B) eluted under gradient of β-mercaptoethanol was analysed.

Table 4
Chromatographic behaviour of purified rhG-CSF (1–4), its iodoacetamide (5,6) and diethyl pyrocarbonate (7,8) -modified derivatives on metal ion-loaded Sepharose-IDA gels[a]

Exp. no.	Metal ion	pH of chromatography	Amount of loaded protein (mg)	Amount of adsorbed protein (mg)	Eluent	Protein recovery (%)
1	None	5.0	3.5	3.5	L.g[a] to 0.25 M Na_2SO_4	91
2	Hg^{2+}	5.0+0.13 M Na_2SO_4[b]	3.6	3.6	L.g to 5 mM β-mercaptoethanol	10
					5 mM β-mercaptoethanol	90
3	Ni^{2+}	5.0+0.13 M Na_2SO_4[b]	3.1	0.4	0.15 M imidazole	20
4	Ni^{2+}	7.0[c]	3.0	3.0	L.g to 5 mM β-mercaptoethanol	–
					5 mM β-mercaptoethanol	–
					10 mM β-mercaptoethanol	–
					Starting buffer	–
					1.0 M NaCl	–
					Starting buffer	–
					0.15 M imidazole	78
5	Hg^{2+}	5.0+0.13 M Na_2SO_4[b]	2.3	2.3	L.g to 5 mM β-mercaptoethanol	94
					5 mM β-mercaptoethanol	6
6	Ni^{2+}	7.0[c]	3.4	3.4	L.g to 5 mM β-mercaptoethanol	–
					5 mM β-mercaptoethanol	–
					10 mM β-mercaptoethanol	–
					Starting buffer	–
					1.0 M NaCl	–
					Starting buffer	–
					0.15 M imidazole	86
7	Ni^{2+}	7.0[c]	2.2	–		96
			2.5	–		97
8	Hg^{2+}	7.0[c]	2.3	1.5	L.g to 10 mM β-mercaptoethanol	99
			2.5	2.5	L.g to 10 mM β-mercaptoethanol	82
					10 mM β-mercaptoethanol	16

[a] Chromatography performed onto 3 ml (bed volume) Sepharose–IDA gel columns. Columns were operated at a flow-rate of 30 ml/h. L.g, linear gradient.
[b] Chromatography of rhG-CSF performed in 10 mM MES buffer, pH 5.0 containing 0.13 M Na_2SO_4.
[c] 10 mM solution of HEPES was used for buffer of pH 7.0.

gradient to 5 mM of β-mercaptoethanol was insufficient, so further elution with 5 mM of β-mercaptoethanol was necessary to cause the quantitative recovery of the protein (Table 4). This indicated a weaker interaction of iodoacetamide-modified rhG-CSF compared to non-modified protein at pH 5.0. On the other hand, data in Table 4 allows us to consider that donor groups other than free SH might also be involved in the interaction with Sepharose–IDA–Hg(II) column at pH 5.0. Thus, based on the results of Table 4, it is not strictly possible to conclude that the free SH group of rhG-CSF is directly involved in the interaction with chelated Hg(II) ions; further studies are necessary to answer this. But the fact that such a column may extract protein from its inclusion bodies may strengthen the support for the assumption that such an interaction might be possible.

4. Comments and conclusion

Despite the fact that mercury is one of the most

toxic heavy metals in the environment [45], its soluble compounds may be met in biochemistry in regard to purification [46] or kinetic and structural studies of cysteine containing enzymes [47–50]. Ultimately, mercuric ions might be found in coordinated state in metalloproteins [51]. We are pursuing the idea for the possible application of chelated mercury to probe the availability and functional activity of free-SH-group present onto protein surface. Such interest might also be related to probe the available free-SH group in the part of inclusion body proteins produced by recombinant DNA technology, that often might be found in their solubilized state as reduced polypeptide chains or folding intermediates rich with free-reduced SH groups. It is evident, that for this purpose heterogeneous systems containing tightly chelated mercury would be most appropriate. Owing to this, it was of interest to evaluate how mercuric ions might behave on chelation by IDA gel, one of most widely used chelating adsorbent in IMAC [22].

Mercuric ions can bind to some part of functional groups (amino, carboxyl, phosphate, hydroxyl, thiol, as noted in [49]), hence, these group-containing compounds may be selected as eluents for chromatography on Sepharose–IDA gel charged with Hg^{2+} ions. Their type and the concentration range may vary with regard to the extent of chelated-Hg(II) ions leaching, as it is shown in Table 1.

Preliminary evaluation of Sepharose–IDA–Hg(II) column towards bromelain in a buffer containing no additives of salts at pH 7.0 (Table 2) did not show any function of metal ion. But, it exhibited protein retention ability when the appropriate salt, such as Na_2SO_4 was introduced into the buffer. The presence of this salt at a concentration sufficient to suppress the charge function of the column seems likely dependent on the isoelectric point of chromatographed protein and the chosen pH value for chromatography. The two tested proteins, bromelain and rhG-CSF, interacted with the chelated mercuric ions if chromatography was performed in buffers of pH value below the p*I* of the proteins in the presence of 0.13 *M* Na_2SO_4 (Fig. 2B, Table 3 and Table 4). In case of rhG-CSF, chromatography at pH 7.0 occurred without the addition of salt (Fig. 2C, Fig. 3 and Tables 3–4). In this respect the Sepharose–IDA–Hg(II) column operates similarly to the column charged with commonly used metal ions like Cu(II), Ni(II) or Zn(II), except that the use of salts like as sodium chloride or compounds containing chloride anions, like guanidine hydrochloride should be excluded.

The comparative retention studies of rhG-CSF and its chemically modified derivatives on Hg(II)- and Ni(II)-loaded Sepharose–IDA columns (Table 4) did not reveal the direct interaction of free SH group of the protein with chelated mercury, but showed that histidine residues were not essential for this interaction. It is possible that the involvement of one or other donor groups of a protein into interaction is dependent on their microenvironment and accessibility. Depending on this one can suppose, that the free SH group, histidine or other amino acid side group might bind to coordinated mercury. For example, it was considered that the interaction of Hg^{2+} ions with proteinase K having a cysteinyl residue near its active site might occur via the involvement of the latter with the His from the active-site [47]. In metalloproteins that contained coordinated mercury(II) ion, functional groups such as Cys, His, Glu, Tyr and others, were found as the binding residues [51]. We assume that the direct interaction of free-reduced SH groups with Sepharose–IDA–Hg(II) column might be possible when the solution of rhG-CSF inclusion bodies is chromatographed. In the oxidative folding pathway of this protein, the longer existing folding intermediate I_2 that has a single correctly formed disulfide bond and three free cysteines was detected [31]. In the solution of inclusion bodies that were chromatographed on the Hg(II) loaded column at pH 7.0, the presence of such a folding intermediate and its interaction with chelated mercury might be possible. In contrast to native G-CSF, whose desorption from Sepharose–IDA–Hg (II) column occurred at pH 7.0 with 5 m*M* β-mercaptoethanol (Fig. 2C, Table 3), the retention of the target protein from its inclusion body was stronger since its elution requires a gradient elution to 10 m*M* solution of β-mercaptoethanol (Fig. 3B). This is consistent with our recent studies, that enabled us to show the presence of ≈3 mol of titrable by Ellman reagent cysteines in the $(His)_6$–rhG-CSF inclusion body solution at pH 7.0, and data on the extraction of the target protein from its inclusion body solution into top phase of aqueous

two-phase systems containing PEG–dye–Hg(II), which occurs more efficiently then in case of Ni(II) ions (data not shown here).

Based on data presented here, it is possible to conclude, that mercury(II) in its chelated state might find application in protein chromatography following the methodology of IMAC. However, mercury leaching should be kept in mind when elution systems are selected. Metal ion leaching still remains the common problem of IMAC and limits its application for biotechnological and analytical purposes. To overcome these limitations a range of new chelating adsorbents has been recently proposed for IMAC of amino acids, peptides and proteins [52–55]. One could believe that the evaluation of new chelators will be also in favour of mercury loaded columns.

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