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1st FACULTY OF MEDICINE

Department of Pathophysiology

PROTEIN SEPARATION AND IMMOBILIZATION ON CHELATED COBALT IONS

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VYUŽITÍ CHELATOVANÝCH IONTŮ KOBALTU PRO IMOBILIZACI A SEPARACI PROTEINŮ

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● Zatloukalová E., Kučerová Z.: Originální příprava imunoafinitních nosičů pomocí afinitní sorpce na imobilizovaných kovových iontech, *5. studentská vědecká konference 1. LF UK, Praha 24. května 2004.*

● Zatloukalová E., Kučerová Z.: New Immunoaffinity Resin Prepared Via Affinity Sorption of Antibodies to Immobilized Cobaltic Ions, *12th International Symposium, Advances and Applications of Chromatography in Industry, Bratislava, Slovak Republic June 29 – July 1, 2004 (sborník na CD).*

● Zatloukalová E., Kučerová Z.: Afinitní sorpce protilátek na imobilizované ionty kobaltu, *Pokroky v chromatografii a elektroforéze, Chiranal 2005, Olomouc, Czech Republic, February 7 – 10, 2005 (sbornik na CD).*

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CONTENT

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● Zatloukalová E.: Afinitní chromatografie na imobilizovaných kobaltnatých iontech a její využití, *Chem. Listy 98 (2004) 254 – 259.*

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● Zatloukalová E., Kučerová Z.: Cobalt Binding Proteins from Mouse Liver, *Pokroky v chromatografii a elektroforéze, Chiranal 2002, Olomouc 24. – 27. 6. 2002 (str. 90).*

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1. INTRODUCTION

Metal ions have an important role in every living organism. If we want to understand the role of metal ions in the human body, we need to comprehend the interactions between metal ions and proteins. The affinity of metal ions to proteins can be studied, for example by immobilized metal ion affinity chromatography.

Immobilized metal ion affinity chromatography (IMAC) is a method useful for the detection, separation or immobilization of molecules. IMAC can be used for various purposes, including a study of surface topography [1], removal of contaminating proteins or the enzymic modification of biomolecules [2]. The efficient purification of recombinant proteins with engineered histidine affinity tags (attached to the N- or C- terminus) is another important application of this method [3-8].

IMAC is a pseudobioaffinity (or chemoselective or group selective) type of chromatography [9-12]. IMAC is based on the specific interactions between biomolecules in solution and metal ions fixed to a solid support [13-15]. Metal ions are immobilized by means of chelating ligands. Since metal ions are electron acceptors, they interact with the electron-donor groups of biomolecules. In biomolecules, electrons can be donated by the surface-exposed atoms of nitrogen, sulphur and oxygen, and potentially phosphorus [9, 16].

IMAC-Co^{2+} relies on the formation of weak coordinate bonds between cobalt ions and the basic functional groups of proteins, primarily histidyl residues. As biomolecules (e.g. proteins) are bound to a cobalt chelate complex, weakly bound molecules of ligands (e.g. water) are displaced from the complex. Elution of the target proteins is mainly achieved by

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protonation (at lower pH) or by the addition of a competing agent (e.g. imidazole) [17].

There are clear benefits to IMAC-Co^{2+}: stability of the separated biomolecules, high loading, mild elution conditions, simple regeneration and low cost.

The mild elution conditions of IMAC-Co^{2+} are mainly advantageous for protein separation. For protein immobilization, it is a good idea to raise the stability of the bonds between the chelated cobalt ions and immobilized proteins. It is possible to modify IMAC-Co^{2+} so that the bonds between the chelated cobalt ions and immobilized proteins become more kinetically stable [18]. Kinetically stable complexes can be formed by changing the oxidation state of the chelated cobalt ions from Co^{2+} to Co^{3+} with hydrogen peroxide [18]. This enables the formation of an "irreversible" complex between the chelated cobalt ion and the bound protein. However, in contrast with J.E. Hale [18], we used a different approach that does not involve direct contact of the protein ligand with the oxidising agent (hydrogen peroxide).

The preparation of a stable immunoaffinity carrier can be achieved by antibody immobilization via chelated Co^{3+} ions. Binding antibodies to chelated cobalt ions is an example of oriented immobilization that avoids random coupling of the antibody. It was proved that a metal-binding site was localised to the Fc fragment of the antibody [19]. Another advantage of protein immobilization via chelated Co^{3+} ions is that it enables some antibody subclasses to bind that do not otherwise react with proteins A or G.

Immobilized antibodies are a powerful tool not only for the isolation of specific proteins but also for the stabilization of labile proteins and for the

preparation of immunoaffinity reactors [20, 21]. Figure 1 shows some examples of immunoaffinity carrier applications.

4. CONCLUSION

Two chromatographic systems with chelated cobalt ions were used for protein immobilization and separation.

III. IMMOBILIZED COBALT ION AFFINITY CHROMATOGRAPHY (IMAC-Co^{2+})

1. Mouse liver proteins induced by the treatment of mice with cobalt (II) chloride were separated by IMAC-Co^{2+}.
2. The separated proteins were made visible with SDS-PAGE and 2D electrophoresis. Electrophoretic bands were densitometrically evaluated.
3. Samples from the Co^{2+}-treated mice showed a higher number of electrophoretic spots in the areas of Mr about 37 000, 32 000 and 26 000 and pH 6.5–7.5.

IV. IMMUNOAFFINITY CHROMATOGRAPHY WITH ANTIBODIES IMMOBILIZED THROUGH CHELATED COBALT IONS

1. A technique for the immobilization of antibodies through chelated cobalt ions was developed. We worked towards the immobilization of various antibody izotypes (rabbit IgG, chicken IgY and human IgE).
2. The antigen-binding ability of immobilized rabbit IgG was verified.
3. The capacity of the immunosorbent and the recovery of OVA were determined.
4. The immunosorbent was stable and could be reused.
5. It was deduced that the interactions between antibodies and chelated cobalt ions were of a coordinate nature
6. The vital contribution of unchelated Co^{2+} ions in the interaction between IgG and Co^{3+}-IDA-Agarose was confirmed.

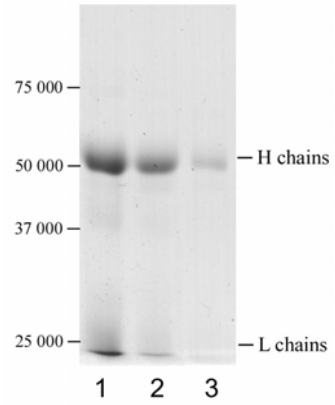

Fig. 16: SDS-PAGE of rabbit IgG immobilized to Co^{3+}-IDA-Agarose in the presence of unchelated Co^{2+} ions.

1) IgG used for the immobilization, 2) IgG fraction not immobilized to the carrier in the presence of unchelated Co^{2+} ions, 3) IgG immobilized to Co^{3+}-IDA-Agarose in the presence of unchelated Co^{2+} ions and extracted from the carrier. Electrophoresis conditions : 12.8% running gel; 4x concentrated reducing sample buffer; 180V; 40 - 50 min; Mini PROTEAN® 3 Cell; Coomassie Brilliant Blue R-250.

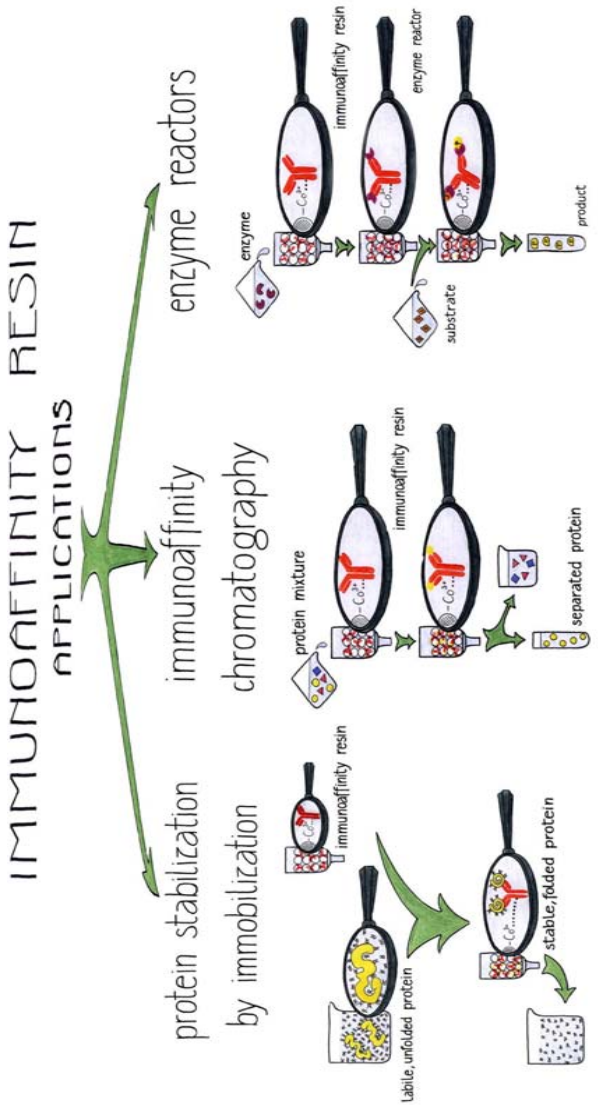

Figure 1: Examples of applications of immunoaffinity carriers with the antibodies bound via chelated cobalt ions

2. AIM OF THE STUDY

The main goal of this study was protein immobilization and separation using chelated cobalt ions. The following chromatographic systems with chelated cobalt ions were used: immobilized metal (cobalt) ion affinity chromatography (IMAC-Co^{2+}) and immunoaffinity chromatography with antibodies immobilized through chelated cobalt ions.

I. IMMOBILIZED COBALT ION AFFINITY CHROMATOGRAPHY (IMAC-Co^{2+})

- Separation of mouse liver proteins induced by the treatment of mice with cobalt (II) chloride. Evaluation of the separated proteins by electrophoretic methods.

II. IMMUNOAFFINITY CHROMATOGRAPHY WITH ANTIBODIES IMMOBILIZED THROUGH CHELATED COBALT IONS

- Development of a technique that is suitable for the immobilization of antibodies through chelated cobalt ions. Working towards the immobilization of various antibody izotypes.

- Verification of the antigen-binding ability of immobilized antibodies.

- Study of the binding interactions between the antibodies and chelated cobalt ions.

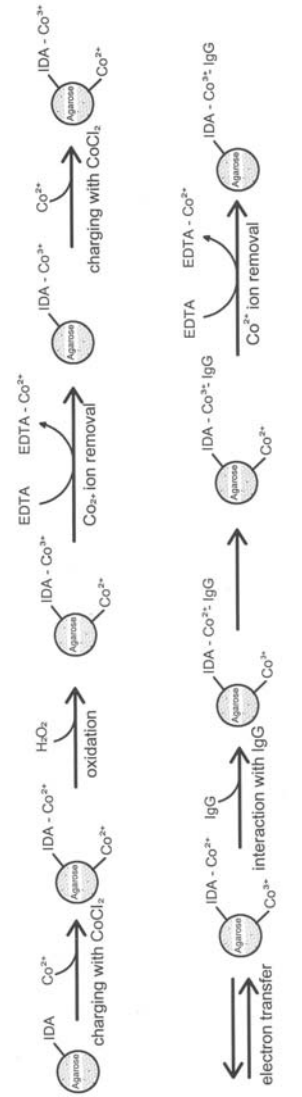

Fig. 15: Scheme of the experiment confirming the vital role of Co^{2+} ions in the interaction between IgG and Co^{3+}-IDA-Agarose

A similar mechanism of electron transfers $M^{3+} + e^- \rightarrow M^{2+}$ is known from the literature [38, 39]. These authors referred to a mechanism of dissolution of the coordination polymer $CrCl_3$ due to the action of Cr^{2+} ions. We expect that Co^{2+} ions could act similarly, undergoing a transfer of the electron from the electron shell to Co^{3+}-IDA-Agarose. This electron transfer resulted in the formation of Co^{3+} ions and Co^{2+}-IDA-Agarose. This Co^{2+}-IDA-Agarose was kinetically fast and was able to bind the antibody in reasonable time period. When IgG was bound to Co^{2+}-IDA-Agarose, the whole complex of IgG-Co^{2+}-IDA-Agarose changed to IgG-Co^{3+}-IDA-Agarose because of repeated electron transfer. This repeated electron transfer also enabled the change of Co^{3+} ions to Co^{2+}, that were able to catalyze additional antibody binding. Electrons were probably transferred via a so-called bridge mechanism.

The vital contribution of unchelated Co^{2+} ions in the interaction between IgG and Co^{3+}-IDA-Agarose was confirmed by the results of the following experiment (see figure 15): Co^{2+} ions unchelated by IDA were removed by EDTA solution. In the next step, Co^{2+} ions that were thought to be essential to the interaction with IgG were resupplied by incubating the carrier with a solution of $CoCl_2$. The Co^{2+} ions supplied in this step catalyzed the interaction between IgG and Co^{3+}-IDA-Agarose. As we expected, IgG was bound to Co^{3+}-IDA-Agarose in the presence of catalytic Co^{2+} ions. The amount of bound antibody was lower (see the SDS-PAGE in Fig. 16) than with Co^{3+}-IDA-Agarose that was not first treated with EDTA and then with $CoCl_2$ solutions. The lower effectiveness of the interaction could be explained by the rather slow reaction rate and equilibrium mechanism of the reaction between IDA-chelated and unchelated cobalt ions [40].

3. RESULTS AND DISCUSSION

3.1 SEPARATION OF MOUSE LIVER PROTEINS USING IMMOBILIZED COBALT ION AFFINITY CHROMATOGRAPHY (IMAC-Co^{2+})

3.1.1 Sample prepurification

Liver tissue was homogenised in a phosphate buffer and the homogenate was centrifuged. The supernatant was then prepurified by gel permeation chromatography on Sephadex G-25 to remove low-molecular mass compounds. After gel permeation chromatography, the sample was inserted into a column of IDA (iminodiacetic acid)-Sepharose. IDA-Sepharose removed any potential metal (Co^{2+}) ions present, particularly in the samples from the Co^{2+}-treated mice. Metal ions were removed so as not to inhibit subsequent binding interactions between the liver proteins and immobilized cobalt ions (Co^{2+}-IDA-Sepharose). IDA-Sepharose was also used as a matrix for affinity chromatography on immobilized cobalt ions. The use of IDA-Sepharose for sample prepurification reduced the subsequent nonspecific interactions between proteins and Co^{2+}-IDA-Sepharose.

3.1.2 Affinity chromatography on immobilized cobalt ions (IMAC-Co^{2+})

Prepurified samples of mouse liver proteins were separated in a column of Co^{2+}-IDA-Sepharose. The chromatogram is shown in Fig. 2. It is conceivable that proteins were bound to Co^{2+}-IDA-Sepharose according to the level of histidine residues in the protein [22]. E. Sulkowski [23] stated in his paper that chromatographic carriers with immobilized cobalt ions

required at least two vicinal histidine residues at the surface of the protein for protein binding.

The proteins adsorbed to Co^{2+}-IDA-Sepharose were only a small minority of all the proteins applied to the carrier. This fact could be explained by the low average amount (approx. 2 %) of histidine residues in the protein molecules [24].

Proteins adsorbed to Co^{2+}-IDA-Sepharose were released by a buffer containing a competing agent (imidazole). Protein molecules bound to the carrier were replaced by molecules of imidazole [4, 5, 12, 13, 25]. The concentration of imidazole in the elution buffer was optimised (gradient 0.05-1 M). In the end, 0.06 M imidazole was used for elution.

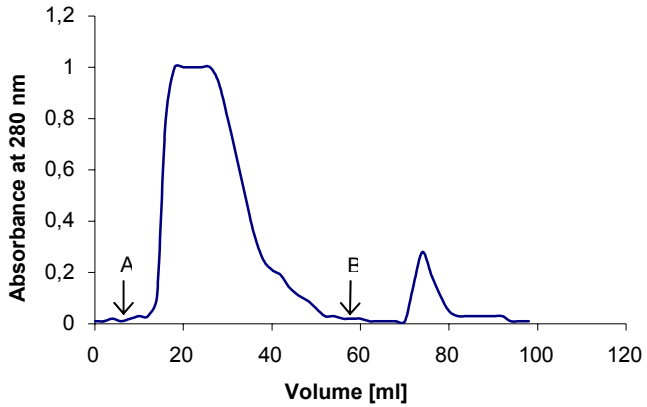

Fig. 2: Chromatograph of liver proteins separated by IMAC-Co^{2+}.
*Chromatography conditions: A prepurified sample was applied to a column of Co^{2+}-IDA-Sepharose (65 x 10 mm I. D.) in an equilibration buffer **(A)**. Adsorbed proteins were eluted by 60 mM imidazole in the equilibration buffer **(B)**. Protein content was determined by BCA assay [26] and by measurement of $A_{280\,nm}$. Fraction volume: 1 ml; flow rate: 0.25 ml/min.*

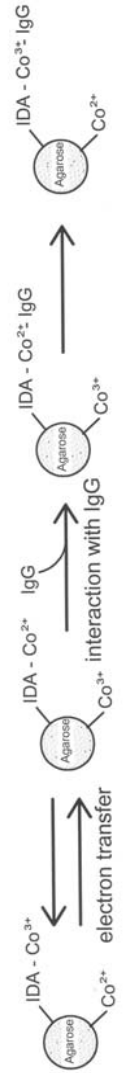

Fig.14: Schematic representation of the putative interactions between the antibodies (IgG), Co^{2+}ions and IDA-Agarose

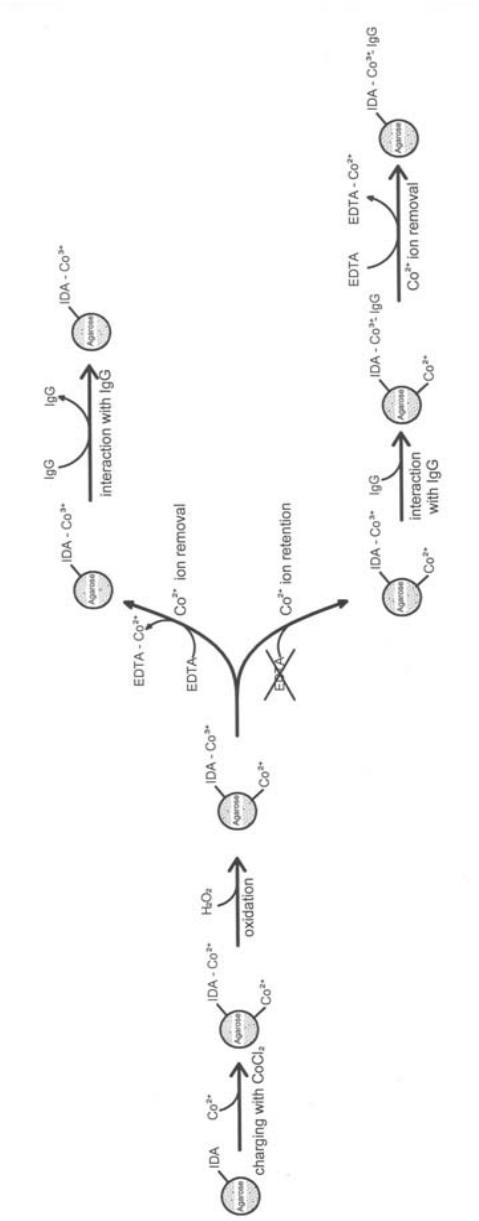

Fig. 13: Schematic representation of putative interactions on IDA-Agarose

3.1.3 Characterization of separated proteins by SDS-electrophoresis and two-dimensional electrophoresis

The protein fractions obtained after sample prepurification and after affinity chromatography on immobilized cobalt ions were evaluated by SDS-PAGE [27]. Samples from the Co^{2+}-treated and control mice were compared. Additional bands of proteins were found in the sample from the Co^{2+}-treated mice in comparison to the untreated ones. The relative molecular mass of these additional protein bands was determined to be between 25 000 and 37 000.

Electrophoretic bands were then densitometrically evaluated using the program ElfoMan2.5. The relative mobility of proteins from Co^{2+}-treated mice was determined to be 43, 49, 57, 66, 72, 81 and 91 units. Values of relative molecular mass (61 000, 53 000, 45 000, 37 000, 32 000, 26 000 and 21 000) were calculated using a relative molecular mass standard.

The adsorption of proteins from the Co^{2+}-treated and control mice to Co^{2+}-IDA-Sepharose was compared. Protein bands with relative mobility 66, 72 and 81 units only appeared in the sample from the Co^{2+}-treated mice. The relative molecular mass of mouse liver proteins that were induced by cobalt (II) chloride and separated on a column with chelated Co^{2+} ions was approximately 37 000, 32 000 and 26 000.

The differences between the samples were also evaluated by two-dimensional electrophoresis (Fig. 3). Samples from the Co^{2+}-treated mice showed a higher number of electrophoretic spots in the area of Mr ~ 30,000 and pH 6.5 – 7.5.

The differing intensity of electrophoretic spots in the area of Mr ~15 000 and pH 7.5 – 8.0 was caused by an unequal amount of haemoglobin subunits in the samples from the Co^{2+}-treated and control mice.

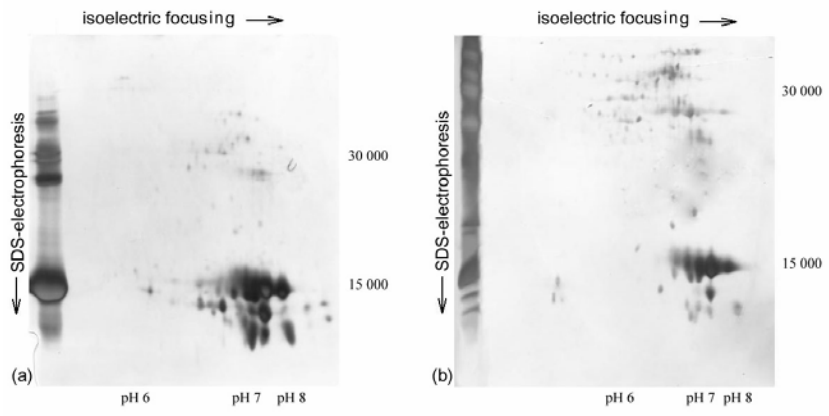

Fig. 3: Two-dimensional electrophoresis.

Comparison of the ***(a)*** *control and* ***(b)*** *Co^{2+}-treated mouse liver proteins. Proteins eluted from Co^{2+}-IDA-Sepharose by 60 mM imidazole in the equilibration buffer. First dimension: IEF in an immobilized pH gradient (pH 3 – 10 in an 18 cm long gel strip). Second dimension: SDS – PAGE in a 12.8% T, 2.6%C gel (a sample not treated with IEF, was applied alongside the gel). Silver stained.*

3.2 IMMUNOAFFINITY CHROMATOGRAPHY WITH ANTIBODIES IMMOBILIZED THROUGH CHELATED COBALT IONS

3.2.1 Immobilization of antibodies to Co^{3+}-IDA–Agarose

Co^{2+}-IDA-Agarose was oxidised prior to antibody binding using hydrogen peroxide to obtain Co^{3+}-IDA-Agarose. Polyclonal rabbit anti-ovalbumin (OVA) IgG, egg yolk (IgY) chicken antibodies and human IgE were bound to Co^{3+}-IDA-Agarose columns (see table 1). The individual steps of antibody immobilization were made visible with SDS – PAGE (Fig. 4).

c) cobalt ions were removed from the carrier with EDTA much more easily if they were presented as unchelated Co^{2+} ions than if they were present as IDA-chelated Co^{3+} ions [37]

$Co^{2+} + EDTA >> Co^{3+}\text{-}IDA\text{-}Agarose + EDTA$

d) IDA-Agarose that was used for cobalt ions and antibody immobilization was a porous carrier with high saccharide moiety content. If $CoCl_2$ solution was applied to IDA-Agarose, most of the Co^{2+} ions were chelated by IDA. A small amount of Co^{2+} ions were probably not chelated by IDA and Co^{2+} ions entered into the pores of agarose or were bound to hydroxylic groups of saccharide moieties. Some Co^{2+} ions may also have been caught in the netting formed by the molecules of Co^{3+}-IDA-Agarose and the solvent.

3.3.6 Effect of unchelated Co^{2+} ions on the interactions between antibodies and chelated cobalt ions

The above-mentioned facts were also summarized in figure 13, where putative interactions on Co^{3+}-IDA-Agarose are shown. It is evident that Co^{2+} ions presented e.g. in the pores of Agarose or on its saccharide moieties had a key role in the interaction between IgG and Co^{3+}-IDA-Agarose. These Co^{2+} ions are thought to catalyze the interaction between IgG and Co^{3+}-IDA-Agarose. Co^{3+}-IDA-Agarose without Co^{2+} ions is probably too kinetically slow to allow the interaction to occur within a reasonable time period. Mutual interactions of the antibody with Co^{2+} ions and Co^{3+}-IDA-Agarose are shown in figure 14.

Firstly, EDTA solution was applied to half of the Co^{3+}-IDA-Agarose before antibody application. Antibody immobilization was compared between carriers treated and untreated with EDTA solution. The results (see Fig. 12) showed that interaction of IgG with Co^{3+}-IDA-Agarose was prevented if the carrier was treated with EDTA solution before antibody application. In the other experiment, 78 % of the applied IgG was bound to the carrier untreated with EDTA (estimated from the amount of unbound antibody using a BCA assay [26]).

Next, the carrier untreated with EDTA was washed with EDTA solution after antibody immobilization. Changes in antibody content were monitored. The results (obtained by SDS-electrophoresis and shown in Fig. 12) showed that antibody content was maintained in this case. An explanation was sought why washing with EDTA solution prevented antibody immobilization but did not remove antibody that was previously immobilized.

The characteristics of Co^{3+}-IDA-Agarose are summarized below:

a) oxidation of Co^{2+}-IDA-Agarose to Co^{3+}-IDA-Agarose with hydrogen peroxide was practically complete (according to ^{1}H-NMR results, data not shown)

b) the value of redox potential for the reaction $Co^{3+} + e^{-} \rightarrow Co^{2+}$ was much lower for cobalt ions chelated to IDA than for cobalt ions unchelated to IDA [36]
Co^{2+}-IDA-Agarose - e^{-} → Co^{3+}-IDA-Agarose >> Co^{2+} - e^{-} → Co^{3+}

Antibody subclass	IgG	IgY	IgE
Source	rabbit	chicken (egg yolk)	human
Antibody applied to Co^{3+}-IDA-Agarose	1,2 mg	1,4 mg	1,3 mg
Antibody bound to Co^{3+}-IDA-Agarose	90 %	76 %	59 %

Table 1: Immobilization of antibody subclasses to Co^{3+}-IDA-Agarose
The amount of immobilized antibody was estimated from the difference in protein content prior to / after antibody application using a BCA assay.

No loss in antibody content was noticed during immunosorbent preparation. Solutions of 50 mM EDTA, 6 M urea, 3 M NaCl, 20 % v/v dioxane, 0.1 M imidazole and buffers of pH 2.5 and pH 11.0 did not release the bound antibodies. On the other hand, if needed, antibodies could be released from the carrier by reducing the Co^{3+} ions of the metal-chelate carrier, for example with DTT or 2-mercaptoethanol.

Antibodies immobilized to chelated cobalt ions are thought to be oriented with the antigen binding sites towards the mobile phase. The antibodies interact with cobalt ions via the histidine residues in the Fc fragment of the antibody [28]. Hale and Beidler determined an endogenous metal-binding site near the C-terminus of human, rabbit, mouse and guinea pig IgG [19]. This metal-binding site was shown to be highly conserved in various antibody subclasses from different species. The immobilization of antibodies to Co^{3+}-IDA–Agarose also allowed the oriented immobilization

of other antibody subclasses. According to our results, it was also possible to bind chicken immunoglobulins (IgY) and human IgE.

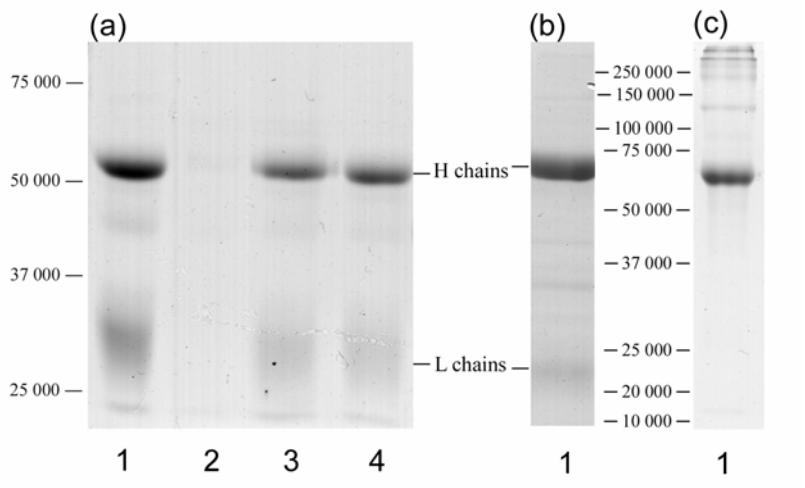

Fig. 4: SDS – PAGE. Immobilization of (a) anti-OVA IgG, (b) egg yolk chicken IgY and (c) human IgE to Co^{3+}-IDA-Agarose.

a: *1) IgG used for the immobilization, 2) IgG fraction not immobilized to Co^{3+}-IDA-Agarose, 3) IgG ligand extracted from Co^{3+}-IDA-Agarose, 4) IgG ligand extracted from Co^{3+}-IDA-Agarose after washing with 0.12 M imidazole, 0.05 M EDTA and 6M urea solutions;* ***b:*** *1) IgY ligand extracted from Co^{3+}-IDA-Agarose after washing with 0.12 M imidazole, 0.05 M EDTA and 6M urea solutions;* ***c:*** *IgE ligand extracted from Co^{3+}-IDA-Agarose after washing with 0.12 M imidazole, 0.05 M EDTA and 6M urea solutions. Electrophoresis conditions: 12.8% running gel; 4x concentrated reducing sample buffer; 180V; 40 - 50 min; Mini PROTEAN® 3 Cell; Coomassie Brilliant Blue R-250.*

were interested how Co^{3+}-IDA-Agarose participated in the interaction and how the interaction was influenced by EDTA solution.

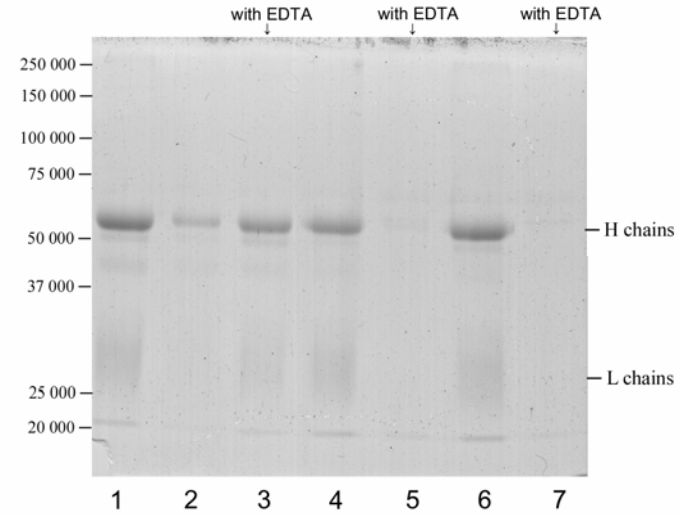

Fig. 12: SDS-PAGE of rabbit IgG immobilized to Co^{3+}-IDA-Agarose in the presence of EDTA.

1) IgG used for the immobilization, 2) IgG fraction not immobilized to the carrier in the presence of EDTA, 3) IgG immobilized to Co^{3+}-IDA-Agarose and extracted from the carrier, 4) IgG immobilized to Co^{3+}-IDA-Agarose in the presence of EDTA and extracted from the carrier, 5) IgG immobilized to Co^{3+}-IDA-Agarose, after washing the immunosorbent with solutions of EDTA and urea and extraction from the carrier, 6) IgG immobilized to Co^{3+}-IDA-Agarose in the presence of EDTA, after washing the immunosorbent with solutions of EDTA and urea and extraction from the carrier. Electrophoresis conditions: 12.8% running gel; 4x concentrated reducing sample buffer; 180V; 40 - 50 min; Mini PROTEAN® 3 Cell; Coomassie Brilliant Blue R-250.

IgY and Co^{3+}-IDA-Agarose proceeded in the presence of imidazole. The results obtained by SDS-electrophoresis are shown in Fig. 11. The interaction between IgY and Co^{3+}-IDA-Agarose in the presence of imidazole was appreciably reduced in comparison to the results obtained with the antibody in the absence of imidazole. This result also supported our hypothesis of the coordinative nature of the interaction.

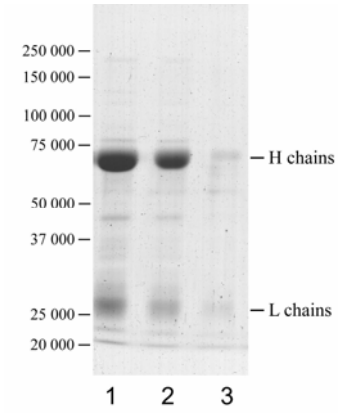

Fig. 11: SDS-PAGE of chicken IgY immobilized to Co^{3+}-IDA-Agarose in the presence of imidazole.

1) IgY used for the immobilization, 2) IgY not bound to the carrier in the presence of imidazole, 3) IgY immobilized in the presence of imidazole and extracted from the carrier. PAGE conditions: 12.8% running gel; 4x concentrated reducing sample buffer; 180V; 40-50 min; Coomassie Brilliant Blue.

3.3.5 Effect of EDTA on the interactions between antibodies and chelated cobalt ions

Another interesting point concerns the fact that Co^{3+}-IDA-Agarose was expected to be highly thermodynamically stable and kinetically slow. So, we

Both chicken IgY and human IgE are important biomolecules in current research. Chicken antibodies (IgY) have several advantages over IgG [17]. A major advantage of chicken IgY results from the evolutionary distance between chicken and mammals, which allows for greater immunogenicity against conserved mammalian proteins such as albumin [1]. IgY also offers the production and maintenance of high levels of specific antibodies during its separation, lower cost and compatibility with animal protection regulations because of the lower stress on the animal [3, 4]. IgE molecules (together with mast cells, basophils and eosinophils) constitute the essential elements in allergic inflamation [29, 30, 31]. This is why IgE molecules are frequently investigated today.

The rabbit anti-OVA IgG, egg yolk chicken antibodies (IgY) and human IgE used for the immunosorbent preparations were model antibodies and the technique described here is thought to be also usable for other antigen-antibody systems.

3.2.2 Immunoaffinity chromatography of egg white proteins on immobilized anti-OVA IgG

Affinity chromatography of egg white proteins (as a complex mixture) or of pure ovalbumin (OVA) (as a model antigen) was carried out using anti-OVA IgG immobilized to Co^{3+}-IDA-Agarose. Antibody-antigen recognition, antigen binding and subsequent elution from the carrier were examined with SDS-PAGE (Fig. 5). OVA adsorbed to the immunoaffinity carrier was eluted using elution buffer A (0.2 M NaCl, pH adjusted to 2.8 with 0.5 M HCl). OVA eluted with elution buffer A was not contaminated with other egg white proteins (Fig. 5, line 4). The antigen was completely released from the carrier, because no OVA was eluted using 6 M urea in the next step (Fig. 5, line 6). Urea (4 - 6 M) was also used to regenerate the

carrier. The carrier could be used several times; repeated experiments (4 – 5 times) showed no decrease in antibody binding ability. No adsorption of OVA was noticed after incubating egg white proteins with Co^{3+}-IDA-Agarose without the immobilized antibody and with Co^{3+}-IDA-Agarose containing an immobilized non-specific control antibody (data not shown).

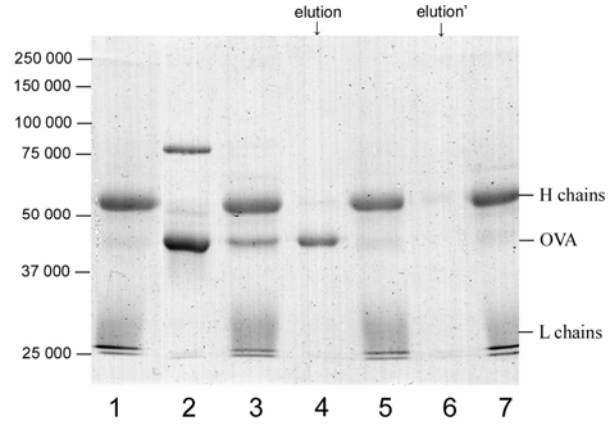

Fig. 5: SDS –PAGE. Immunoaffinity chromatography of egg white proteins on immobilized anti-OVA IgG.

1) anti-OVA IgG, 2) complex mixture of egg white proteins, 3) IgG ligand and OVA extracted from Co^{3+}-IDA-Agarose, 4) OVA eluted from the immunoaffinity carrier with elution buffer A (pH 2.8), 5) IgG ligand extracted from the carrier after elution of OVA with elution buffer A, 6) fraction eluted from the immunoaffinity carrier with 6 M urea, 7) IgG ligand extracted from the carrier after urea regeneration. Electrophoresis conditions: 12.8% running gel; 4x concentrated reducing sample buffer; 180V; 40 - 50 min; Coomassie Brilliant Blue R-250.

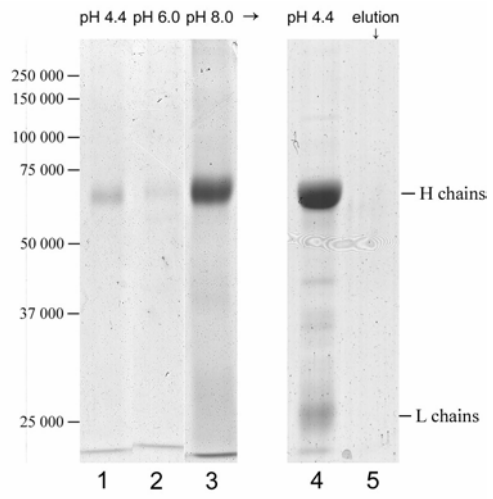

Fig. 10: SDS-PAGE of chicken IgY immobilized to Co^{3+}-IDA-Agarose at pH 4.4, 6.0 and 8.0.

1) IgY immobilized to Co^{3+}-IDA-Agarose at pH 4.4 and extracted from the carrier, 2) IgY immobilized to Co^{3+}-IDA-Agarose at pH 6.0 and extracted from the carrier, 3) IgY immobilized to Co^{3+}-IDA-Agarose at pH 8.0 and extracted from the carrier, 4) IgY immobilized at pH 8.0 and extracted from the carrier after washing the carrier with pH 4.4 buffer, 5) fraction eluted from the immunosorbent with pH 4.4 buffer. Electrophoresis conditions: 12.8% running gel; 4x concentrated reducing sample buffer; 180V; 40 - 50 min; Mini PROTEAN® 3 Cell; Coomassie Brilliant Blue R-250.

3.3.4 Effect of a competing agent on the interactions between antibodies and chelated cobalt ions

The participation of imidazol groups in the antibody binding to Co^{3+}-IDA-Agarose [12, 13, 16, 17, 19, 22, 23, 28] was confirmed by an experiment with a competing agent (imidazole). Interaction between chicken

3.3.3 Effect of pH on the interactions between antibodies and chelated cobalt ions

It is thought that antibodies are bound with chelated Co^{2+} ions via coordination bonds [16]. However, it is not well known what types of bonds prevail between antibodies and chelated Co^{3+} ions.

The effect of pH on the interaction between chicken antibody (IgY) and chelated Co^{3+} ions was examined. Antibody interaction with Co^{3+}-IDA-Agarose was compared using buffers of pH 4.4, 6.0 and 8.0. The results, obtained by SDS-electrophoresis (Fig. 10), showed a much higher amount of IgG immobilized to Co^{3+}-IDA-Agarose with pH 8.0 buffer than with those ofpH 4.4 or 6.0.

This fact can be explained by the protonization of the antibody's imidazol groups at pH 4.4 or 6.0. Values of pK are (depending on the environment) about 6-7 for the imidazole groups of His [35] and about 4 for the carboxylic groups of Glu and Asp. The participation of the imidazol groups in the interaction at pH 8.0 indicates coordinate bonding while the carboxylic groups participating at pH 4.4 or 6.0 would indicate more of an ionic interaction.

A substantial contribution by ionic interactions in the interaction of IgY with Co^{3+}-IDA-Agarose at pH 8.0 was excluded by the following experiment: IgY was immobilized to Co^{3+}-IDA-Agarose at pH 8.0 and the carrier was washed with pH 4.4 buffer after IgY immobilization. As no antibody was washed off from the carrier after the wash with the pH 4.4 buffer, it was deduced that the interaction was of a coordinate nature.

3.2.3. Immunosorbent capacity determination.

Immunosorbents were prepared with varying levels of immobilized anti-OVA IgG. The capacities of the immunosorbents were determined from the amount of OVA released from the carrier. The results are shown in table 2.

Recovery of the OVA was calculated from the difference between the amount of the protein applied / released during immunoaffinity chromatography. Recovery of OVA was 97 % and elution could be considered complete.

ANTIBODY IMMOBILIZATION			
Antibody applied to Co^{3+}-IDA-Agarose	4 mg	8 mg	16 mg
Antibody bound to Co^{3+}-IDA-Agarose	89 %	92 %	79 %
CAPACITY DETERMINATION			
OVA applied to the immunosorbent	1 mg	1 mg	1 mg
OVA bound to the immunosorbent	0.06 mg	0.15 mg	0.25 mg

Table 2: Immunosorbent Capacity determination

The amount of immobilized antibody was calculated from the amount of unbound protein. Protein concentration was determined using a BCA assay. Elution: 0.2 M NaCl, pH adjusted to 2.8 with 0.5 M HCl.

The amount of OVA eluted during capacity determination is in accordance with the amount of immobilized antibody. Fig. 6 shows a chromatograph of capacity determination and Fig. 7 shows the SDS-PAGE of the individual protein fractions.

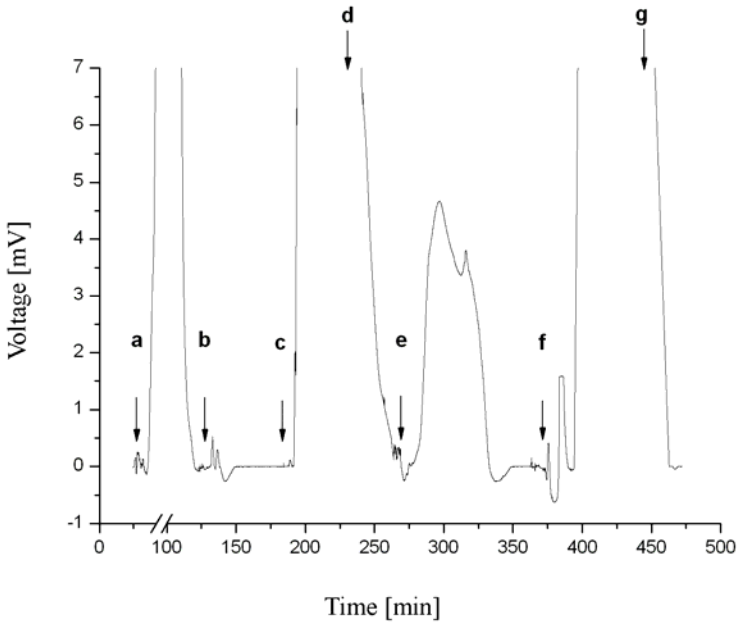

Fig. 6: Immunosorbent Capacity determination.

Affinity chromatography of OVA on anti-OVA IgG immobilized to Co^{3+}-IDA-Agarose. Chromatography conditions: 1 mg of OVA was applied to the affinity carrier (25 x 10 mm I.D.; 13 mg of IgG) in buffer ***a*** *(PBS). Elution with buffers:* ***b*** *(1 M NaCl in PBS),* ***c*** *(120 mM imidazole in PBS),* ***d*** *(PBS),* ***e*** *(0.2 M NaCl, pH adjusted to 2.8 with 0.5 M HCl),* ***f*** *(6 M urea in water) and* ***g*** *(PBS). Solutions* ***c*** *and* ***f*** *absorb at 280 nm.*

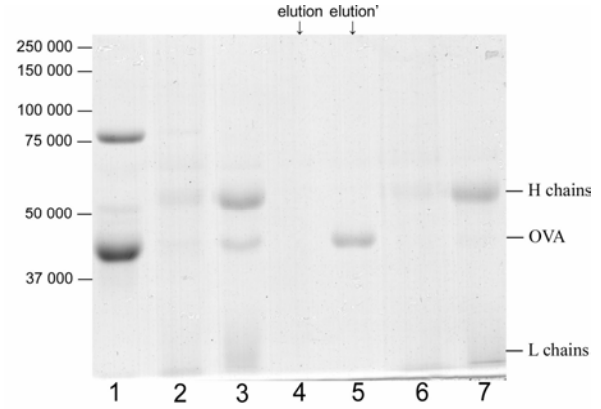

Fig. 9: SDS-PAGE of fractions obtained after immunoaffinity chromatography of egg white proteins.

Comparison of immunosorbents prepared by immobilization of anti-OVA IgG prior to or after oxidation of the carrier. 1) egg white proteins, 2) IgG ligand (affected by oxidation) after OVA binding and extraction from the carrier, 3) IgG ligand (not affected by oxidation) after OVA binding and extraction from the carrier, 4) OVA released with elution buffer A (pH 2.8) from the immunosorbent with the antibody affected by oxidation, 5) OVA released with elution buffer A from the immunosorbent with the antibody not affected by oxidation, 6) IgG ligand (affected by oxidation) after elution of OVA (buffer A) and extraction from the carrier, 7) IgG ligand (not affected by oxidation) after elution of OVA (buffer A) and extraction from the carrier. Electrophoresis conditions: 12.8% running gel; 4x concentrated reducing sample buffer; 180V; 40 - 50 min; Mini PROTEAN® 3 Cell; Coomassie Brilliant Blue R-250.

According to the above results, it was obvious that the immunosorbent prepared using the new technique recognised, bound and subsequently released the antigen.

In the first method, polyclonal rabbit anti-OVA IgG was immobilized to the Co^{3+}-IDA-Agarose. According to the SDS-PAGE (Fig. 8), no decrease in antibody content was detected after washing the immunosorbent with solutions of imidazole, EDTA and urea (Fig. 8, line 4). The Immunosorbent was suitable for immunoaffinity chromatography and the antibody was not subsequently treated with hydrogen peroxide.

In the other method, polyclonal rabbit anti-OVA IgG was applied to Co^{2+}-IDA-Agarose prior to immunosorbent oxidation and 86 % of the applied IgG [26] was immobilized to the carrier (Fig. 8, line 2). After washing, the carrier was oxidised with hydrogen peroxide. The amount of immobilized antibody decreased (Fig. 8, line 3) after oxidation of the immunosorbent with hydrogen peroxide.

The ability of both immunosorbents to bind the specific antigen was tested by affinity chromatography. The affinity chromatography of egg white proteins was followed by SDS-PAGE and the results are shown in Fig. 9. Adsorbed egg white proteins were eluted with elution buffer A (0.2 M NaCl, pH adjusted to 2.8 with 0.5 M HCl).

In the first case, OVA was successfully eluted from the immunosorbent (Fig. 9, line 5). Elution buffer A completely released the OVA from the carrier, because no OVA was eluted using 6 M urea (data not shown). We did not observe contamination of the eluate with other egg white proteins. The immunosorbent with antibody bound to Co^{3+}-IDA-Agarose was suitable for immunoaffinity chromatography.

In the second case, the fractions eluted from the carrier with the antibody treated with the hydrogen peroxide showed no traces of OVA (SDS–PAGE, Fig. 9, line 4). Neither did 6 M urea release any OVA from this carrier (data not shown).

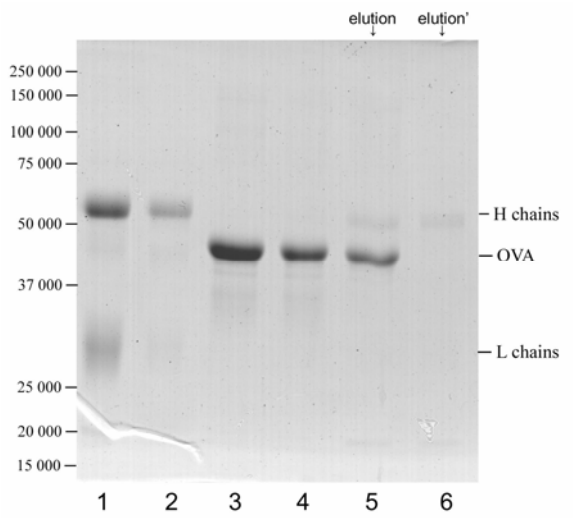

Fig. 7: SDS-PAGE. Immunosorbent capacity determination.

1) IgG used for the immobilization, 2) IgG fraction not immobilized to Co^{3+}-IDA-Agarose, 3) OVA applied to the immunosorbent, 4) OVA not bound to the immunosorbent, 5) OVA eluted from the immunosorbent with elution buffer A (pH 2.8), 6) fraction eluted from the immunosorbent with 6 M urea. Electrophoresis conditions: 12.8% running gel; 4x concentrated reducing sample buffer; 180V; 40-50 min; Mini PROTEAN® 3 Cell; Coomassie Brilliant Blue R-250.

3.3 STUDY OF INTERACTIONS BETWEEN ANTIBODIES AND CHELATED COBALT IONS

3.3.1 Effect of hydrogen peroxide on immobilized antibody

A study of the interactions between the antibodies and chelated cobalt ions preceded the preparation of the immunoaffinity carriers. Preliminary

experiments were performed with immunosorbent preparation according to the literature [Hale]. The technique described in the literature [18, 32-34] consisted of the immobilization of antibodies to the Co^{2+}-chelated support, followed by oxidation of the support with 0.03 % H_2O_2. According to our results, the antibody was released from the carrier after the oxidation step with hydrogen peroxide. This is why the effect of hydrogen peroxide on the immobilized antibody was studied.

Chicken antibody (IgY) was immobilized to Co^{2+}-IDA-Agarose and 88 % of the applied IgY was bound to the carrier (estimated from the difference in protein content prior to / after antibody application using a BCA assay [26]). The antibody-Co^{2+}-IDA-Agarose complex was exposed to 0.01 %, 0.03 % and 0.05 % hydrogen peroxide. According to the electrophoretic evaluation (data not shown), antibodies were released from the immunosorbents with increasing hydrogen peroxide concentration. The decrease in antibody content was so extensive, that even a hydrogen peroxide concentration of 0.01 % could not be used for immunosorbent oxidation.

Therefore, preparation of the immunoaffinity carrier was modified in our laboratory. The new technique consisted of oxidising the Co^{2+}-IDA-Agarose to Co^{3+}-IDA-Agarose prior to antibody binding. Using this technique suppressed oxidation-mediated effects on the antibody.

A Hydrogen peroxide concentration of 0.03-0.05 % resulted in complete oxidation of the Co^{2+}-IDA carrier. Incomplete oxidation of the Co^{2+}-IDA-Agarose could result in the binding of unwanted proteins to Co^{2+}ions. We preferred complete oxidation of the support together with no need to expose the antibody to the oxidising agent. Therefore, 0.05 % hydrogen peroxide concentration was used for the preparation of Co^{3+}-IDA-Agarose.

3.3.2 Comparison of two approaches used for antibody immobilization

We compared the approach for antibody immobilization presented in this paper with that described by J.E. Hale [18]. The difference was in the oxidising step. The approach described in [18] involved oxidising the antibody-Co^{2+}-IDA-Agarose complex. The approach used in our laboratory instead oxidised Co^{2+}-IDA-Agarose to Co^{3+}-IDA-Agarose prior to antibody application.

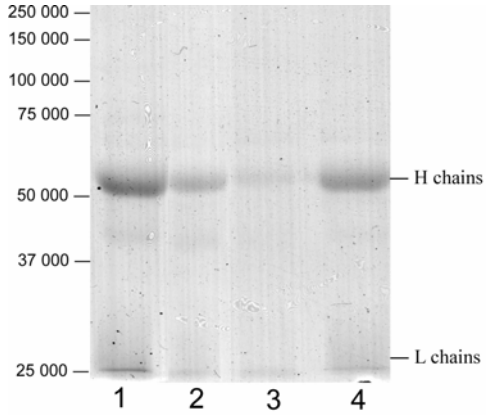

Fig. 8: SDS-PAGE of IgG immobilized to the carrier before or after carrier oxidation.

1) IgG used in the immobilization, 2) IgG ligand extracted from Co^{2+}-IDA-Agarose, 3) IgG ligand affected by oxidation during its immobilization and extracted from the carrier, 4) IgG ligand not affected by oxidation during its immobilization and extracted from the carrier. Amount of immobilized antibody was determined from the amount of unbound antibody using a BCA assay. Electrophoresis conditions: 12.8% running gel; 4x concentrated reducing sample buffer; 180V; 40 - 50 min; Mini PROTEAN® 3 Cell; Coomassie Brilliant Blue R-250.