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Nickel and copper complexes of a chelating methacrylate sorbent in the purification of chitinases and specific immunoglobulin G_1 by immobilized metal ion affinity chromatography

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Abstract

The isolation of the isoforms of endo- and exochitinases of *Clostridium aminovalericum* T1 and of the horseradish peroxidase (HRP)-specific immunoglobulin G_1 from natural sources by immobilized metal ion affinity chromatography was studied. The effect of Cu^{2+} and Ni^{2+} complexes of iminodiacetic acid incorporated in porous glycidyl methacrylate–*co*-ethylene dimethacrylate and in agarose (Sepharose Fast Flow) beads on separation of the target polypeptides was analyzed. It was found that the Cu^{2+} complexes bound both the HRP-specific IgG_1 and some isoforms of chitinases more strongly than the Ni^{2+} complexes. From the former complexes, both target polypeptides were eluted by a stepwise imidazole concentration gradient of 5–100 m*M*. The lower strength of Ni^{2+} complex binding with the HRP-specific IgG_1 resulted in its easy elution with a pH gradient of 5.5–5 while some isoforms of chitinases required imidazole for their elution. The ''fraction elution degree'' of a target polypeptide (i.e., the ratio of its amounts in each eluate fraction and in the combined fractions) was used for the evaluation of the sorption selectivity and binding affinity of the separating components to the studied metal complexes.

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1. Introduction

Immobilized metal ion affinity chromatography (IMAC) is an effective method for isolation of various proteins [1–3], monoclonal antibodies [4–6] and enzymes [7,8] from raw biological media. It is known as a method with medium to high affinity [9], which mainly depends on the type of interacting partners, polypeptides and coordinated metal ions. Being coordinated with a chelating ligand, the metal ions (Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+} or Fe^{3+}, Al^{3+}, Ca^{2+}), mostly used in IMAC, exhibited different selectivity and binding affinity to proteins because of

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the difference in their reactivity towards amino acid residues in proteins [10]. According to Pearson [11], the hard metal ions (e.g., Fe^{3+}, Ca^{2+}, Al^{3+}) prefer oxygen; the soft ones (Cu^{+}, Ag^{+}, Hg^{2+}) sulfur while the transition metal ions (Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}) coordinate to nitrogen, oxygen and sulfur. Inasmuch the most amino acid residues contain the above mentioned atoms, their interactions with coordinated metal ions are probable.

Due to a key role of these interactions in metal–protein binding affinity, their study is of a special interest. At present, they are the subject of intensive investigations in the field of bioinorganic chemistry. In a recent theoretical study [12], the affinity of metal ions (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+}) to the side chains of amino acids such as asparagine (Asn), glutamine (Gln), methionine (Met), serine (Ser), aspartic acid (Asp), cysteine (Cys), histidine (His), threonine (Thr), glutamic acid (Glu), lysine (Lys) and tyrosine (Tyr) was analyzed. The following order of amino acid residues according to their affinity to the selected metal ions was found: His (N_{ϵ})>His (N_{δ})>Asn≅Gln>Lys>Tyr>Thr≅Met>Ser[1]. These findings are in good agreement with the experimental observations that His is the most common binding amino acid in proteins when separated by IMAC [15]. Under defined conditions, the ionized amino acid residues can also contribute to interactions with metal ions. Their affinity order was determined as follows: Cys^{-}> Asp^{-}, Glu^{-}>Tyr^{-}>Ser^{-}>Thr^{-}. The computed interaction energies of the studied amino acids with metal ions correlated with the Irving–Williams series of stability constants [16], which decrease with increasing metal ion radius in the range Cu^{2+}> Ni^{2+}>Co^{2+}≅Zn^{2+}. This order of the metal binding affinity to proteins is very often observed in IMAC and is in accord with Sulkowski definition [17] of the minimal requirements for protein binding with coordinated transition metal ions. In accordance with this prediction, the coordinated Cu^{2+} ions would bind any protein with a single exposed histidine (His), while coordinated Ni^{2+} ions would bind only to a protein with two exposed vicinal His residues rather strongly and with two non-vicinal His residues less strongly. A protein with two exposed vicinal His would be able to bind the coordinated Zn^{2+} ions while two adjacent His are required for protein binding with coordinated Co^{2+} ions. However, exceptions from the rule indicated that additional factors exist affecting the protein binding [18]. Nevertheless, the knowledge of the key role of His in protein binding affinity helped in increase of the recombinant protein purification efficiency by IMAC after attaching to them of poly-His tags [19–21]. It could be concluded that the further investigations of metal–protein interactions by computer modeling and their experimental verification in IMAC separations seem to be a way of improving the process efficiency.

In the present study, the effect of Cu^{2+} and Ni^{2+} complexes on the isolation of the isoforms of endo- and exochitinases from culture filtrate of *Clostridium aminovalericum* T1 and of the horseradish peroxidase (HRP)-specific immunoglobulin G_1 (IgG_1) from mouse ascitic fluid by IMAC was studied. An attempt was made to find a correlation between both the sorption selectivity and binding affinity of the target polypeptides and the type of coordinated metal ions. The separation efficiency of a porous chelating glycidyl methacrylate–*co*-ethylene dimethacrylate sorbent and the chelating Sepharose Fast Flow differing in polymer structure and concentration of iminodiacetic acid (IDA) groups was compared.

2. Experimental

2.1. Chelating sorbents

The chelating porous glycidyl methacrylate–*co*-ethylene dimethacrylate (GMA-*co*-EDMA beads, 7–13 μm) were prepared by suspension polymerization and modified with IDA as described in Ref. [22]. The sorbent had a porous structure with the pore surface of 68 m^2/g and with concentration of IDA groups, 128±2 μmol/ml. The IDA-GMA sorbent and the chelating Sepharose Fast Flow (IDA-SFF,

[1] The occurrence (%) of the selected amino acid in proteins was found as follows: Asn, 5.22; Gln, 4. 11; Met, 2.27; Ser, 7.34; Asp, 5.22; Cys, 1.82, His, 2.23; Thr, 5.96; Glu, 6.26; Lys, 5.82; Tyr, 3.25 [13]. However, only part of them (e.g., ca. 50% for His) is on the surface [14].

Amersham Pharmacia Biotech, Uppsala, Sweden) with coordinated Cu^{2+} and Ni^{2+} ions were used in the study.

2.2. Preparation and analysis of the chitinases of Cl. aminovalericum T1

The chitinolytic bacterium *Cl. aminovalericum* strain T1 was isolated from the feces of takin. A 1-l volume of the modified medium M10 [23] was supplemented with 4 g of the colloidal chitin, which was prepared from the crab shell chitin (Sigma, St. Louis, MO, USA) by a known method [24]. The medium was inoculated with 50 ml of the overnight culture and allowed to grow at 39 °C for 120 h. The cells were harvested by centrifugation at 10 000 *g* at 4 °C for 20 min. The supernatant was concentrated to 1/20 of its original volume by ultrafiltration using PM-10 filters with an M_r 10 000 cut-off (Amicon, Millipore, USA) and kept at −20 °C for the further analyses.

The endochitinase activity was determined using 0.5% (w/v) carboxymethylchitin as a substrate. The carboxymethylchitin was prepared from the chitin (Sigma) by the published method [25]. The enzymes were incubated with the substrate and with 100 m*M* phosphate buffer, pH 6 at 40 °C for 60 min. The enzymatic reaction was stopped by adding equal volumes of 0.3 *M* $ZnSO_4$ and of 0.3 *M* $Ba(OH)_2$ and the reduced groups were detected with 4-hydroxybenzohydrazide [26]. The absorbance of the yellow-colored product was measured at 410 nm using a 8451A spectrophotometer (Hewlett-Packard, CA, USA).

The exochitinase activity was measured with *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitobiose (Sigma) according to Roberts and Selitrennikoff's method [27]. The enzyme was incubated with 0.2% substrate solution in 100 m*M* phosphate buffer, pH 6 at 40 °C for 30 min. The reaction was stopped with a double volume of 2% Na_2CO_3. The absorbance of the solution with released *p*-nitrophenol was measured at 410 nm. In the same way *N*-acetyl-β-glucosaminidase was assayed with *p*-nitrophenyl-β-D-*N*-acetylglucosaminide (Sigma) according to Ref. [28]. The enzyme activities were measured at least in triplicate if not otherwise stated.

2.3. Mouse ascitic fluid containing the horseradish peroxidase-specific IgG_1

The crude mouse ascitic fluid containing HRP-specific IgG_1 (Seva-Imuno, Prague, Czech Republic) was clarified by filtration through Whatman paper (twice) followed by dialysis through a Cuprophane membrane against distilled water to remove sodium azide. Dialysis proceeded for 16 h at 5 °C. The clarified fluid contained proteins (19.95 mg/ml) and specific IgG_1 (2.05 mg/ml). Conductivity of the dialyzed fluid was 2.686 mS/cm, pH 7.77. After dialysis, the fluid was 1.25 times diluted with a buffer A (0.25 *M* potassium phosphate solution, pH 8.5, containing 2.5 *M* NaCl), filtered through a Millex-GP (0.22 μm) filter (Millipore, Bedford, MA, USA) and stored in a refrigerator.

2.4. Column IMAC

The IMAC procedure was carried out using a HR-5 column (Pharmacia Biotech, Sweden) packed with 1.5 or 2 ml of a chelating sorbent in the Ni^{+2} or Cu^{2+} form. The coordination of metal ions with a sorbent was carried out under static conditions at room temperature. The sorbent previously equilibrated with 0.1 *M* sodium acetate solution containing 0.5 *M* NaCl, pH 4.5 was shaken for 16 h with 5–10 ml of 0.1 *M* $NiCl_2$ or $CuSO_4$ dissolved in the same buffer. The equilibration solution was separated, the sorbent was washed with the same buffer and 25 ml of filtrate was collected. The sorbent transferred into a column was equilibrated with the loading buffer (0.05 *M* potassium phosphate solution containing 0.5 *M* NaCl, pH 8, 10 bed volumes, BVs). The clarified HRP-IgG_1 solution (2 ml) or the chitinase filtrate (0.9–2.5 ml) was passed through a sorbent at a flow-rate of 0.25 ml/min. Unsorbed proteins were displaced by washing the sorbent with the loading buffer (15 BVs). The sorbed target polypeptides were eluted at decreasing pH (6, 5.5, 5, 4.5) followed by an imidazole concentration gradient (0.005, 0.01, 0.1, 0.5 *M*) and finally with 0.05 *M* Tris buffer containing 0.5 *M* NaCl and 0.05 *M* EDTA, pH 8. Concentration of the polypeptides in the eluate fractions was determined by the method of Warburg and Christian [29]. Eluate fractions with maximum protein and HRP-IgG_1 concentrations were analyzed

by the Bradford method [30], enzyme-linked immunosorbent assay (ELISA) and sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE). Enzyme activity in the eluate fractions was analyzed by the above-mentioned methods.

2.5. SDS–PAGE

SDS–PAGE was carried out in a vertical Mini-Hoefer SE 200 apparatus by the discontinuous buffer method under the nonreducing conditions described by Laemlli [31]. Gels[2] were stained, fixed and dried in accordance with the manufacturer instructions. A standard protein mixture (Pharmacia Biotech) was used as a high-molecular-mass protein marker.

3. Results and discussion

The studied target polypeptides (chitinases and the HRP-IgG_1) are the metabolic products of *Cl. aminovalericum* T1 bacterium and mice, respectively. They are the minor components in the studied biological media. The mouse ascitic fluid contained a number of enzymes and proteins of various molecular masses but transferrin and serum albumin were the main proteins accompanying the HRP-IgG_1. The culture filtrate of *Cl. aminovalericum* T1 prepurified by ultrafiltration was free of the components with a molecular mass lower than 10 000. It contained an exo- and endochitinase mixture consisting of minimum six isoforms with molecular masses of 43 500–65 000 [33].

For evaluation of the sorption selectivity and binding affinity of Cu^{2+} and Ni^{2+} complexes to the target polypeptides, a portion of a component in eluate fractions (‘‘the fraction elution degree’’) was used. This is a useful parameter, which shows the distribution of a component in eluate fractions and reflects the difference in its sorption selectivity and binding affinity to metal complexes. It was defined as the ratio (%) of the amounts of a component in each eluate fraction and in the combined eluate fractions. In IMAC of the mouse ascitic fluid and of the culture filtrate of *Cl. aminovalericum* T1, the fraction elution degree of the components was calculated from the HRP-IgG_1 and protein concentrations and the endo- and exochitinase activities, respectively. The HRP-IgG_1 specific concentration and the chitinase specific activity were also calculated for evaluation of the target polypeptide purity in the eluate fractions. These dependences were used for analysis of the separation efficiency of Ni^{2+} and Cu^{2+} complexes of the IDA-GMA and IDA-Sepharose Fast Flow sorbents in IMAC of the target polypeptides.

It is clearly seen (Fig. 1A–C) that ca. 50% of the loaded mouse ascitic proteins was displaced from the studied metal complexes with the loading buffer[3], pH 8 due to their low binding affinity to these proteins. In contrast, the binding affinity of HRP-IgG_1 to both types of metal complexes of both the studied chelating sorbents was high. The HRP-IgG_1 was completely adsorbed on both types of metal complexes. At pH decreasing to 6, the elution of contaminating proteins was higher when eluted from the Ni^{2+} complexes (Fig. 1A) than from the Cu^{2+} ones (Fig. 1C), indicating a lower protein binding affinity of the former complexes. As can be seen from Table 1, the Cu^{2+}–IDA–GMA sorbent exhibited only somewhat higher sorption capacity for the contaminating proteins compared with the Ni^{2+}–IDA–GMA one. This difference can be explained by the above-mentioned Sulkowski’s definition. Inasmuch as the difference in protein sorption capacities of Cu^{2+} and Ni^{2+} complexes is not so big, it is possible to assume that not only His residues but others were responsible for protein binding.

The main amount of the HRP-IgG_1 was eluted from Ni^{2+} complexes at pH 5.5–5 while a competitor (imidazole) was needed to desorb it from

[2]The stacking gel (T 4%, C 2.5%) and the separating gradient gel (T 7.5–10%, C 2.5%) of a spacer thickness of 1 mm were used; T=[g acrylamide/g *N,N′*-methylenebisacrylamide (Bis)]/100 ml solution, C=g Bis/% T.. A photocopy of SDS–PAGE of fractions eluted from the Ni^{2+} and Cu^{2+} complexes of IDA-GMA sorbents in separation of HRP-IgG_1 is available on request.

[3]It should be noted that a volume of the loading buffer twice as high was needed for removing the contaminating proteins from Ni^{2+} complexes of IDA-Sepharose Fast Flow in comparison with IDA-GMA sorbent.

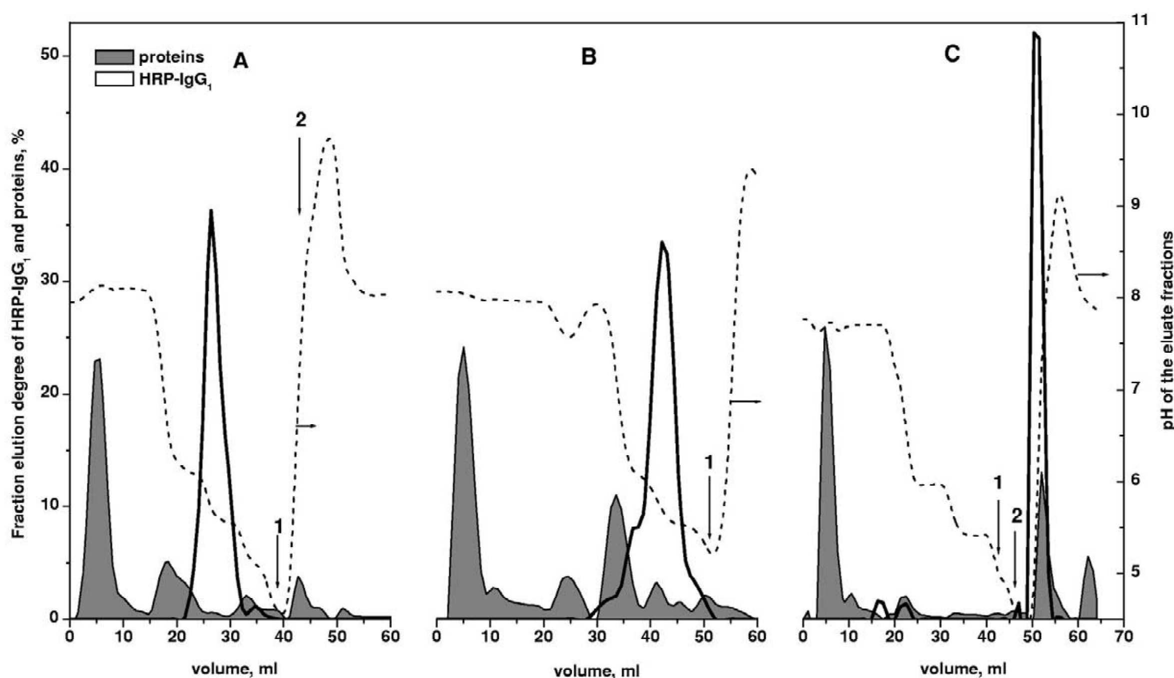

Fig. 1. Dependence of the fraction elution degree of HRP-IgG_1 and accompanying proteins on the eluate volume in IMAC of the raw mouse ascitic fluid. Loaded ascitic fluid, 2 ml. Flow-rate, 0.25 ml/min. Elution was carried out under pH gradient in 0.1 *M* acetate buffer containing 0.5 *M* NaCl, pH 6, 5.5, 5; followed by imidazole concentration gradient: 1–10 m*M*, 2–100 m*M* and with 0.05 *M* Tris buffer containing 0.05 *M* EDTA and 0.5 *M* NaCl. (A, C) IDA-GMA sorbent (1.5 ml), (B) IDA-Sepharose Fast Flow (2 ml); (A, B) Ni^{2+} complexes, (C) Cu^{2+} complexes.

Cu^{2+} complexes. In the latter case (Fig. 1C), the peak of HRP-IgG_1 appeared a little earlier than the peak of contaminating proteins (transferrin and serum albumin) but the HRP-IgG_1 specific concentration in the first imidazole fraction was rather high. One can see (Table 1) that the total HRP-IgG_1 recovery with both metal complexes of IDA-GMA sorbent is comparable but its purity and yield in the eluate fractions with the highest protein concentration (peak yield) was remarkably higher when eluted from Ni^{2+} complexes. The Ni^{2+}–IDA–GMA sorbent retained the HRP-IgG_1 somewhat more strongly than Ni^{2+}–IDA–SFF (Fig. 1B and C). Very likely, the observed difference is caused by the difference in their polymer structures and in concentration of IDA groups, which was approximately three times higher in the IDA-GMA sorbent [32] thus ensuring higher probability of the ligand–protein interactions. The HRP-IgG_1 specific concentration was the highest (84%) when eluted from the Ni^{2+}–IDA–GMA sorbent than from the Ni^{2+}–IDA–SFF (61.4%) (Table 1).

The difference in sorption selectivity and binding affinity of Ni^{2+} and Cu^{2+} complexes of the studied chelating sorbents for endo- and exochitinases was also observed in their isolation from the culture filtrate of *Cl. aminovalericum* by IMAC. As can be seen from Table 1, the sorption capacity of both metal complexes of the IDA-GMA sorbent for the total protein mixture was twice as low as that of the Cu^{2+}–IDA–SFF sorbent. However, lower values of the protein and exochitinase recovery with the Ni^{2+}–IDA–GMA sorbent reflected its higher binding affinity to exochitinases. A great difference in sorption capacity of Cu^{2+} complexes of the IDA-GMA and IDA-SFF sorbents for endochitinases was observed. The considerably higher activity of endochitinases eluted from the Cu^{2+}–IDA–SFF sorbent can be

Table 1
Separation of HRP-IgG_1 from the raw mouse ascitic fluid and of endo- and exochitinases from the culture filtrate of *Cl. aminovalericum* T1 by IMAC on Cu^{2+} and Ni^{2+} complexes of the IDA-GMA sorbent and IDA-Sepharose Fast Flow

		IDA-GMA		IDA-SFF	
		Ni^{2+}	Cu^{2+}	Ni^{2+}	Cu^{2+}
Raw mouse ascitic fluid containing HRP-IgG_1					
Total proteins	Sorbed (mg/ml sorbent)	13.36	14.19	12.74	–
	Eluted (mg/ml sorbent)	10.49	12.64	10.7	–
	Yield (%)	79	89	84	–
HRP-IgG_1	Sorbed (mg/ml sorbent)	2.73	2.73	2.05	–
	Eluted (mg/ml sorbent)	2.42	2.36	2.0	–
	Yield (%)	88.7	86.5	97.0	–
Peak of HRP-IgG_1	Purity (%)	84.0	30.0	61.4	–
	Yield (%)	79.4	50.2	80.7	–
Culture filtrate of *Cl. aminovalericum* T1 containing endo- and exochitinases					
Total proteins	Sorbed (mg/ml sorbent)	2.63	2.57	–	4.90
	Eluted (mg/ml sorbent)	1.73	2.25	–	4.26
	Yield (%)	68.0	85.0	–	78.0
Endochitinase	Sorbed (EU/ml sorbent)	455	212	–	4071
	Eluted (EU/ml sorbent)	374	174	–	3257
	Yield (%)	82.0	83.0	–	80.0
Exochitinase	Sorbed (EU/ml sorbent)	24.5	27.5	–	27.6
	Eluted (EU/ml sorbent)	14.2	23.1	–	22.1
	Yield (%)	58.0	84.0	–	80.0

associated with its higher sorption selectivity for these enzymes than for contaminating proteins. Cu^{2+} complexes of both the studied sorbents exhibited practically the same sorption capacity for exochitinases.

As can be seen from the dependences of the fraction elution degree (Fig. 2A and B), the highest and the lowest amounts of both endo- and exochitinases were removed from Cu^{2+} complexes of IDA-GMA and IDA-SFF sorbents, respectively, by washing with the loading buffer, pH 8. As before, the observed difference could be explained by the extremely high sensitivity of metal ions to the steric arrangement of the interacting ligands depending on morphology and chemical structure of the polymer support, to which they have been attached. This assumption is supported by the conclusion [12] that the preference of metal ions to the definite coordination geometry and the optimum metal ion–ligand distance help in their selective binding with amino acids.

The endo- and exochitinases were partly separated under washing the sorbents with the loading buffer. The sorbed enzymes were further separated at decreasing pH (6, 5.5, 5) and imidazole concentrations (5, 10, 100 m*M*) by gradient elution. They were retained more strongly with Cu^{2+} complexes of IDA-GMA sorbent (Fig. 2A) than with Ni^{2+} ones (Fig. 2C) and eluted from the former in overlapped peaks with the imidazole gradient. The endochitinases eluted somewhat earlier than the exochitinases. The last eluate fractions of the imidazole peak contained an isoform of exochitinase with very high specific activity. If the Ni^{2+}–IDA–GMA sorbent was used, an isoform of highly purified exochitinase was desorbed within the pH interval of 7–6. The rest of sorbed enzymes eluted from Ni^{2+} complexes at a lower imidazole concentration (5 m*M*) than from Cu^{2+} complexes of IDA-GMA sorbent. Some isoforms of chitinases were retained weaker with Cu^{2+}–IDA–SFF and eluted at pH 6–5. Their peaks partly overlapped. Nevertheless, the sorption capacity of

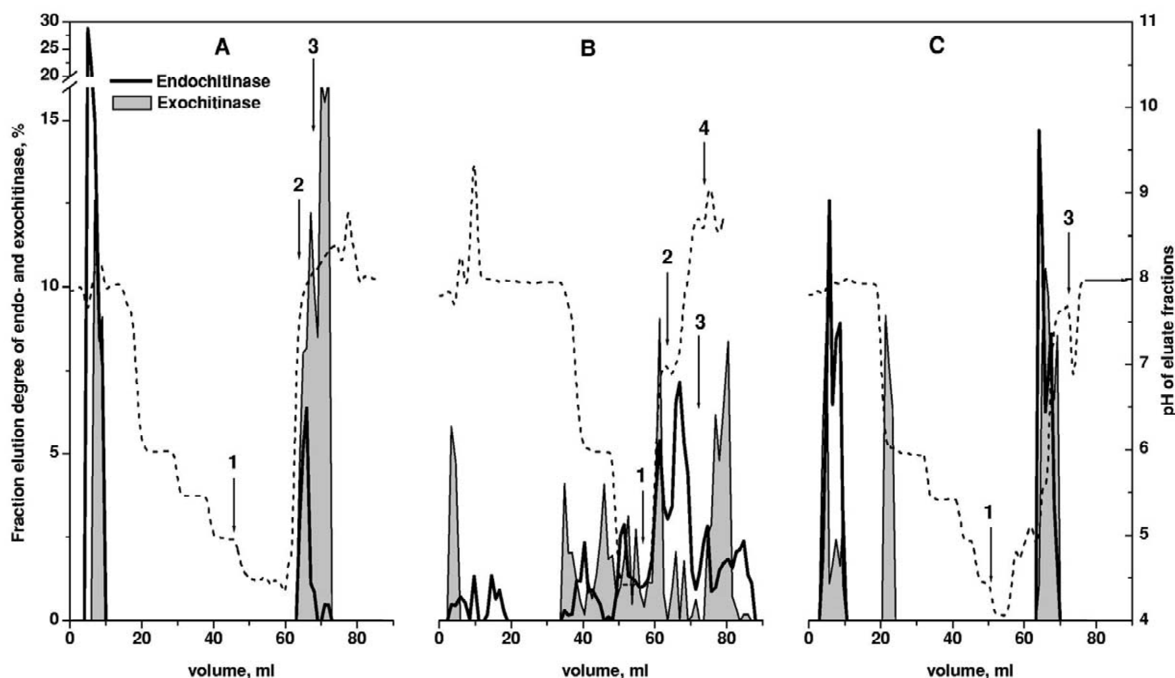

Fig. 2. Dependence of the fraction elution degree of chitinases and accompanying proteins in IMAC of the culture filtrate of *Cl. aminovalericum* T1 on the eluate volume. Flow-rate, 0.25 ml/min. Elution was carried out under the pH gradient in 0.1 *M* acetate buffer containing 0.5 *M* NaCl, pH 6, 5.5, 5; followed by imidazole concentration gradient: 1–5 m*M*, 2–10 m*M*, 3–100 m*M*, 4–500 m*M* and with 0.05 *M* Tris buffer containing 0.05 *M* EDTA and 0.5 *M* NaCl. (A, C) IDA-GMA sorbent (2 ml) and the loaded filtrate, 0.9 ml; B: IDA-Sepharose Fast Flow (2 ml) and the loaded filtrate, 2.5 ml; (A, B) Cu^{2+} complexes; (C) Ni^{2+} complexes.

the Cu^{2+}–IDA–SFF sorbent for endo- and exochitinases and its separation was indisputably better (Fig. 2B) compared with the studied metal complexes of IDA-GMA sorbent.

4. Conclusions

Analysis of sorption selectivity and binding affinity of Cu^{2+} and Ni^{2+} complexes to the target polypeptides (HRP-IgG_1, endo- and exochitinases) in their separation from biological fluids by IMAC on the IDA-GMA and IDA-Sepharose Fast Flow sorbents has shown that:

(1) Ni^{2+} complexes of the IDA-GMA sorbent exhibited somewhat lower binding affinity to the HRP-IgG_1 than Cu^{2+} complexes, thus ensuring its better separation from the contaminating proteins under pH gradient elution at pH 5.5–5. It was found that HRP-IgG_1 with 84% purity and 79% yield could be isolated from the raw mouse ascetic fluid on the Ni^{2+}–IDA–GMA sorbent by IMAC.

(2) The isoforms of highly purified exo- and endochitinases of *Cl. aminovalericum* T1 could be isolated on the Ni^{2+}–IDA–GMA and Cu^{2+}–IDA–GMA sorbents, respectively. An isoform of exochitinase was eluted from the Ni^{2+} complexes in the pH range of 7–6 owing to its low binding affinity to the coordinated metal ions. The isoforms of endochitinases having very low affinity to Cu^{2+} complexes was displaced from the sorbent with the loading buffer, pH 8 while the others with a very high binding affinity was eluted with imidazole concentration gradient of 10–100 m*M*.

(3) The chromatographic behavior of the Ni^{2+}–IDA–SFF sorbent was very similar to that of the Ni^{2+}–IDA–GMA sorbent with the exception of somewhat lower binding affinity of the former to

HRP-IgG_1. In IMAC of the culture filtrate of *Cl. amnovalericum* T1, the Cu^{2+}–IDA–SFF sorbent exhibited a considerably higher sorption selectivity to exo- and endochitinases than the Cu^{2+}–IDA–GMA sorbent and better separation efficiency.

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