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Polymer versus monomer as displacer in immobilized metal affinity chromatography

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Abstract

Successful immobilized metal affinity chromatography (IMAC) of proteins on Cu^{2+}-iminodiacetic acid Sepharose has been carried out in a displacement mode using a synthetic copolymer of vinyl imidazole and vinyl caprolactam [poly(VI-VCL)] as a displacer. Vinyl caprolactam renders the co-polymer with the thermosensitivity, e.g., property of the co-polymer to precipitate nearly quantitatively from aqueous solution on increase of the temperature to 48°C. A thermostable lactate dehydrogenase from the thermophilic bacterium *Bacillus stearothermophilus* modified with a $(His)_6$-tag [$(His)_6$-LDH] has been purified using an IMAC column. For the first time it was clearly demonstrated that a polymeric displacer [poly(VI-VCL)] was more efficient compared to a monomeric displacer (imidazole) of the same chemical nature, probably due to the multipoint interaction of imidazole groups within the same macromolecule with one Cu^{2+} ion. Complete elution of bound $(His)_6$-LDH has been achieved at 3.7 m*M* concentration of imidazole units of the co-polymer (5 mg/ml), while this concentration of free imidazole was sufficient to elute only weakly bound proteins. Complete elution of $(His)_6$-LDH by the free imidazole was achieved only at concentrations as high as 160 m*M*. Thus, it was clearly demonstrated, that the efficiency of low-molecular-mass displacer could be improved significantly by converting it into a polymeric displacer having interacting groups of the same chemical nature.

Keywords: Immobilized metal affinity chromatography; Polymers; Monomers

1. Introduction

Successful chromatographic purification of proteins requires specific binding of the target protein to the chromatographic matrix. Although, this is not the whole story. The bound protein should be eluted preferably in a specific way, completely in a small volume and preserving its intact conformation and hence biological activity. Elution of proteins is usually realized by creating conditions (changing pH, salt concentration or addition of some specific chemicals) where the binding force between the target matrix and the protein is decreased. Literally the protein is ''pulled out'' from the matrix. There is another option – to ''push out'' the bound protein by applying a substance with higher affinity towards the matrix compared to the bound protein. This approach is used in displacement chromatography.

Displacement chromatography has recently at-

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tracted a lot of attention due to its obvious advantages of high loading capacities, resolving power, final product purity and concentration [2–6,12,13,15,17]. In fact, elution in traditional immobilized metal affinity chromatography (IMAC) is often carried out in a displacement mode when high concentrations of imidazole are used [18]. Imidazole molecules compete with protein molecules bound to metal ions via histidine residues and finally displace bound protein. Usually, imidazole is used as displacer in concentrations higher than 0.1 *M* [16]. At this high concentration, some leaching of metal ions from the matrix could take place contributing to the elution of protein bound to metal ion ligands.

Imidazole is a monodentate ligand. Four imidazoles are capable of binding one copper ion with a dissociation constant, *K*, for each imidazole ligand decreasing from $K_1 = 0.17$ m*M* for binding of the first imidazole ligand to $K_4 = 2.2$ m*M* for binding of the fourth imidazole ligand [14]. One could expect binding of polydentate imidazole-containing ligands (e.g., polymer-containing imidazole groups) to be more efficient, especially taking into consideration the fact that *N*-substituted imidazole derivates have proven to be as efficient displacers as the free imidazole itself [18]. Hence such an imidazole-containing polymer could be a better displacer for protein from IMAC than plain imidazole. Experimentally it was demonstrated by using ribonuclease A or myoglobin as ''imidazole-containing polymers'' [10–12]. But the idea to purify one protein by using large amounts (concentrations 10–30 mg/ml) of another protein as a displacer does not look that attractive.

Synthetic metal chelating polymers like polyacrylic and polyglutamic acids have been used successfully for the displacement of proteins bound to hydroxy- and fluoroapatite [2,9,15]. As the mechanism of protein binding to hydroxy- and fluoroapatite remains still obscure, the mechanism of displacement is unclear as well. One could simply imagine partial ''dissolution'' of the matrix caused by leaching Ca^{2+}-ions bound to chelating polymer.

The objective of the present paper was to evaluate a synthetic copolymer of vinyl imidazole and vinyl caprolactam [poly(VI-VCL)] as a displacer for IMAC of proteins on Cu^{2+}-iminodiacetic acid Sepharose (Cu^{2+}-IDA-Sepharose). Vinyl caprolactam has been chosen as a co-monomer as it renders the co-polymer with the thermosensitivity, e.g., property of the co-polymer to precipitate nearly quantitatively from aqueous solution on increasing temperature to 48°C [7]. Short (about 1–2 min) heating of the eluate at this temperature followed by centrifugation allows separation of the polymer from the protein solution and regeneration of the former after dissolution at room temperature. The relatively high temperature required for the polymer separation could be damaging for most proteins, but fully acceptable for thermostable proteins isolated from thermophilic microorganisms, like lactate dehydrogenase (LDH) from the thermophilic bacterium *Bacillus stearothermophilus* [1]. This enzyme was genetically modified with a $(His)_6$-tag [$(His)_6$-LDH] to introduce affinity for IMAC columns and used in our experiments.

2. Materials and methods

2.1. Materials

Sodium pyruvic acid (pyruvate), bicinchoninic acid solution (BCA), iminodiacetate (IDA), EDTA-tetrasodium salt, 3-(*N*-morpholino)propanesulfonic acid (MOPS), ethanolamine, ampicillin, isopropyl β-D-thiogalactopyranoside (IPTG), sodium borohydride, 1,4-butanediol digycidyl ether, imidazole and β-NADH were purchased from Sigma (St. Louis, MO, USA). Albumin was purchased from ICN Biomedicals (Aurora, OH, USA). Tryptone and yeast extract were bought from Difco (Detroit, MI, USA). Recombinant strain of *Escherichia coli* TG1 with pUC $(His)_6$-LDH was a gift from Professor Leif Bülow (Department of Pure and Applied Biochemistry, Center for Chemistry and Chemical Engineering, Lund University, Lund, Sweden). Sepharose CL-6B was a product of Pharmacia BioProcess Technology (Uppsala, Sweden). Copper sulfate, potassium carbonate and sodium chloride were supplied by Merck (Darmstadt, Germany). Tris was purchased from USB (Cleveland, OH, USA). 1,4-Dioxane was dried over potassium hydroxide and distilled (101–102°C). *N*-Vinylcaprolactam (was a gift from ISP, Guildford, UK) and was recrystallized from hexane, m.p. 38–

39°C. *N*-Vinylimidazole (Aldrich, Steinheim, Germany) was distilled under vacuum (74–76°C, 10 mmHg; 1 mmHg=133.322 Pa). 2,2′-Azobis (2-methylenpropionitrile) was purchased from Acros (Malmö, Sweden). The synthesis of poly(VI-VCL) is described in detail elsewhere [7]. The synthesis of homopolymer, poly(vinyl caprolactam) has been carried out in the same way omitting vinyl imidazole from the reaction mixture.

2.2. Epoxy activation of Sepharose

A 20-ml volume of Sepharose CL-6B was thoroughly washed with distilled water and then suspended in 20 ml 0.6 *M* NaOH containing 38 mg sodium borohydride. Under agitation, 20 ml 1,4-butanediol digycidyl ether was slowly added, then the suspension was incubated overnight on a shaking table, 120 rpm at room temperature. Excess reagent was removed by extensive washing with distilled water.

2.3. Preparation of Cu^{2+}-iminodiacetate Sepharose

A 20-ml volume of epoxy-activated Sepharose CL-6B was washed with 200 ml of distilled water in a sintered glass funnel, suction dried and moved to a 250-ml shaking flask containing 2.5 g IDA in 20 ml 2 *M* potassium carbonate. The flask was placed in a 60°C shaking incubator over night. The gel suspension was then filtered and washed with 1 l 1 *M* NaCl followed by 1 l distilled water. The excessive reactive groups were blocked by suspending the gel in 15 ml 1 *M* ethanolamine solution, pH 9.0 and incubating the gel suspension on shaking table at room temperature for 4 h followed by filtering and washing with 1 l 1 *M* NaCl and 1 l distilled water. The resulting IDA-Sepharose gel was degassed and 1.3 ml was packed in a column (inner diameter 1.0 cm). In all chromatographic experiments 20 m*M* Tris–HCl, 150 m*M* NaCl, pH 7.3 was used as running buffer at a flow-rate of 1 ml/min and for chemical preparation and dilutions unless stated differently. Finally Cu^{2+} was bound to the IDA-Sepharose by passing 20 ml 5 m*M* $CuSO_4$ (dissolved in distilled water) through the packed gel.

2.4. Cultivation and recovery of recombinant *E. coli* containing thermostable $(His)_6$-LDH

A recombinant strain of *E. coli* TG1 with pUC $(His)_6$-LDH was cultivated in LB medium (tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l, ampicillin 50 mg/l) at 37°C in a shaking bath at 160 rpm for 2 h. When the absorbance at 550 nm (A_{550}) reached 1.0, IPTG was added to a final concentration of 48 μg/ml to induce $(His)_6$-LDH gene expression. After 24 h cells were harvested by centrifugation at 16 200 *g* for 10 min. Pellets were washed with 50 m*M* Tris–HCl, pH 7.0 and recentrifuged at 16 200 *g* for 10 min. After resuspension in the same buffer, the cells were disrupted by sonication four times for 1 min followed by 1 min of interruption. The cells were kept in ice bath and the sonicator was set to 70% amplitude and 0.5 cycles/s, maximum sonic intensity 105 W/cm^2 with the chosen probe. After disruption the cell debris was removed by centrifugation at 11 900 *g* and the supernatant was divided into fractions and kept at −20°C.

2.5. Estimation of maximum binding capacity of Cu^{2+} ions to poly(VI-VCL)

Samples of 12 mg poly(VI-VCL) in 2.5 ml 5 m*M* MOPS, 0.1 *M* NaCl, pH 7.0 containing 0.5 to 10 m*M* $CuSO_4$ were prepared. Precipitate of Cu^{2+}–copolymer complex appeared directly after addition of $CuSO_4$, but to assure complete precipitation the sample was then heated in a water bath for 2 min at 50°C. The precipitate and supernatant were separated by centrifugation at 3000 *g* for 5 min. Cu^{2+} ions were analyzed in the supernatant as described below. Control samples of 0.5 to 10 m*M* $CuSO_4$ in 2.5 ml 5 m*M* MOPS, 0.1 *M* NaCl, pH 7.0 but without polymer were exposed for the same treatment. The amount of Cu^{2+} ions bound to poly(VI-VCL) was calculated from the difference in Cu^{2+} concentration between the supernatants and the control samples.

2.6. Estimation of Cu^{2+} ions in solution

A 0.2-ml volume of 10% aqueous ammonia was added to 0.8 ml of $CuSO_4$-containing samples. Absorbance of the copper–ammonia complex (λ_{max}=610 nm) was measured directly and after 30

min which gave the same result. Linear calibration of the control samples gave $\epsilon_{610}=40\ M^{-1}\ cm^{-1}$.

2.7. Elution chromatography with increasing concentration of imidazole

A 0.55-ml volume of crude cell supernatant from cultivation of recombinant *E. coli* containing $(His)_6$-LDH was thawed from −20°C, diluted 10 times in running buffer and loaded to the 1.3 ml Cu^{2+}-IDA-Sepharose column. The unbound fraction (13.2 ml) was collected for control and elution of bound protein with 3.7, 10, 20, 40, 80, 160 and 320 m*M* imidazole was performed. Of each concentration six fractions of 3.3 ml (2.54 column volumes) was collected. The column was regenerated with EDTA and Cu^{2+}. The chromatography processes were monitored using LKB UVI-cord equipped with a 276 nm filter.

2.8. Relative poly(VI-VCL) content established by A_{450}-dispersion

Samples of 100 μl of fractions from a poly(VI-VCL) elution were diluted 10 times and heated for 10 min in a 48°C water bath. The samples were then transferred to a preheated cuvette (48°C) and moved to a spectrophotometer where the absorbance at 450 nm was read immediately.

2.9. Displacement chromatography with poly(VI-VCL)

A 0.55-ml volume of crude cell supernatant of recombinant *E. coli* containing $(His)_6$-LDH was thawed from −20°C, diluted 10 times in running buffer and loaded to the 1.3 ml Cu^{2+}-IDA-Sepharose column. The unbound fraction (15.8 ml) was collected for control. Then fractions of two column volumes (2.6 ml) were collected. First other proteins than $(His)_6$-LDH were eluted with 10 column volumes of 3.7 m*M* imidazole followed by washing with running buffer. Secondly, displacement of $(His)_6$-LDH was performed by loading 24 column volumes of 5 mg/ml poly(VI-VCL) followed by washing with running buffer. Finally 10 column volumes of 250 m*M* imidazole were applied to control whether further elution of protein occurred. The column was regenerated with EDTA and Cu^{2+}.

2.10. $(His)_6$-LDH activity

The method used is based upon determining the decrease in absorbance at 340 nm from oxidation of β-NADH. Sample solutions were diluted to give a rate of 0.01–0.05 ΔA_{340}/min. In a 1.0-ml cuvette 0.967 ml of a reaction mixture containing 0.225 m*M* β-NADH and 1.0 m*M* pyruvate in 0.2 *M* Tris–HCl, pH 7.3 was mixed with 33 μl of appropriately diluted sample. The decrease of A_{340}/min was recorded for 5 min.

2.11. Protein content estimation with BCA

A standard solution of 0, 100, 200, 400 and 800 μg/ml of albumin was prepared. A 950-μl volume of a reaction mixture containing one part 4% (w/v) $CuSO_4$ and 50 parts BCA solution was mixed with 50 μl of appropriately diluted sample or standard. The preparations were incubated for 60 min in darkness at room temperature and then the absorbance at 562 nm was read. The fractions of the 250 m*M* imidazole elution in the displacement experiment had to be dialyzed five times for 30 min against 2 l 20 m*M* Tris–HCl, pH 7.3 in order to be analyzed successfully.

2.12. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Samples were mixed with sample buffer and applied on a 15% SDS–PAGE gel under reducing conditions according to the instructions by the manufacturer (Bio-Rad Labs., Hercules, CA, USA).

3. Results and discussion

The step-wise imidazole elution of $(His)_6$-LDH bound to Cu^{2+}-IDA-Sepharose after application of crude homogenate on the column is presented in Fig. 1. Pronounced elution of $(His)_6$-LDH started at 40 m*M* and to achieve complete elution imidazole concentration as high as 160 m*M* was required.

The elution of $(His)_6$-LDH with 5 mg/ml poly-

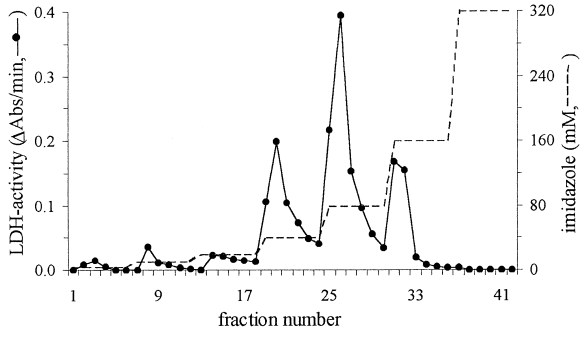

Fig. 1. Elution curve of $(His)_6$-LDH bound to Cu^{2+}-IDA-Sepharose after application of crude homogenate. Elution was performed by increasing the imidazole concentration in seven steps, from 3.7 m*M* up to 320 m*M*. Experimental conditions: 20 m*M* Tris–HCl, 150 m*M* NaCl, pH 7.3 was used as running buffer at a flow-rate of 1 ml/min. Column volume was 1.3 ml and fraction size 3.3 ml.

(VI-VCL) as well as a breakthrough profile of the polymer are presented in Fig. 2. The breakthrough profile of the poly(VI-VCL) is sharp with $(His)_6$-LDH eluting mainly before the polymer. This indicates the high rate of displacer binding to the Cu^{2+}-IDA-Sepharose. Tailing of $(His)_6$-LDH elution profile and overlapping with the elution profile of the polymer indicates a slow rate of protein desorption proceeding even when the sorbent is already saturated with the displacer. The same effect has been observed for the poly(VI-VCL) displacement of ovalbumin and lysozyme from egg white on the Cu^{2+}-IDA-Sepharose column [8]. No $(His)_6$-LDH was eluted with homopolymer, poly(*N*-vinylcaprolactam), containing no imidazole groups indicating that the polymer backbone by itself had no effect on specific protein interaction with Cu^{2+} ligands.

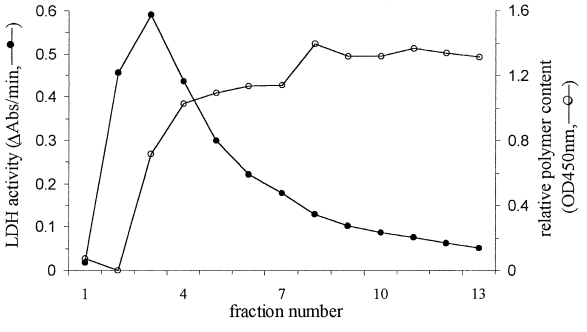

Fig. 2. Displacement of $(His)_6$-LDH (filled circles) bound to Cu^{2+}-IDA-Sepharose after application of crude homogenate by 5 mg/ml poly(VI-VCL) and breakthrough profile of the polymer (open circles). The relative polymer content was established by heating samples of the fractions for 10 min in 48°C water bath and measure A_{450}. Experimental conditions as in Fig. 1.

The poly(VI-VCL) is a co-polymer of *N*-vinylcaprolactam (VCL) and *N*-vinylimidazole (VI) with a molar ratio of 9:1 corresponding to 7.00% (w/w) VI. In the displacement experiments a concentration of 5 mg/ml poly(VI-VCL) has been used since lower co-polymer concentrations resulted in incomplete elution of bound $(His)_6$-LDH (data not shown). Consequently the VI part of the polymer is 0.35 mg/ml which is equal to 3.7 m*M*. Thus, the complete elution of $(His)_6$-LDH with the polymeric displacer is achieved at about 50-fold less concentrations of imidazole group when bound to the polymer backbone as compared to free imidazole molecules present in solution.

The maximum binding capacity of 8.3 μmol Cu^{2+} ions per μmol poly(VI-VCL) (Fig. 3) corresponds to the binding of a Cu^{2+} ion to on average 1.1 imidazole group [M_r=12 000 of poly(VI-VCL)]. The dissociation constant could be estimated (as Cu^{2+} concentration corresponding to half-saturation) as 1.5 m*M*. Hence, imidazole groups of the polymer are quite similar to that of free imidazole with regards to

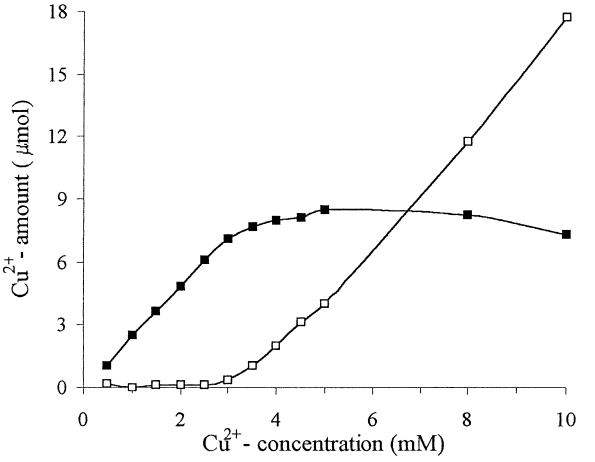

Fig. 3. Amount of bound Cu^{2+} (filled squares) and non bound Cu^{2+} (open squares) by 1 μmol poly(VI-VCL) as a function of Cu^{2+} concentration in a total volume of 2.5 ml 5 m*M* MOPS, 0.1 *M* NaCl, pH 7.0. After separation of Cu^{2+}–poly(VI-VCL) complex the amount of Cu^{2+} ions bound to poly(VI-VCL) was calculated from the difference in Cu^{2+} concentration between the supernatants and control samples without polymer.

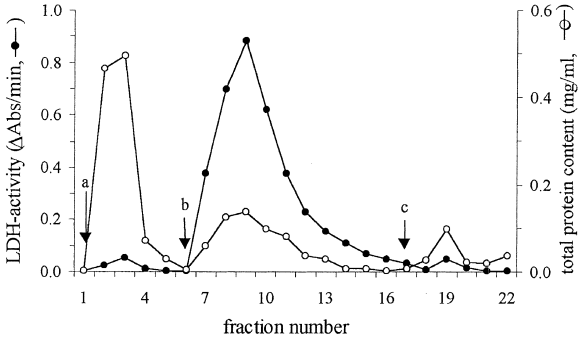

Fig. 4. Elution in three steps of $(His)_6$-LDH bound to Cu^{2+}-IDA-Sepharose after application of crude homogenate. Arrow (a) indicates start of elution with 3.7 m*M* imidazole, arrow (b) – displacement with 5 mg/ml poly(VI-VCL) and arrow (c) – elution with 250 m*M* imidazole. Filled circles represent $(His)_6$-LDH activity and open circles the total protein content of each fraction estimated with BCA. Experimental conditions as in Fig. 1 except that fraction size was 2.6 ml.

the strength and stoichiometry of Cu^{2+} binding. Enhanced elution with imidazole-containing polymer at low-imidazole-group concentration in solution could be explained by the high local concentration of imidazole groups created in the vicinity of the Cu^{2+} ligand when poly(VI-VCL) binds to the Cu^{2+} ligand at least via one imidazole group. High local concentrations of imidazole groups could also promote multi-site interactions between one polymer molecule and the Cu^{2+} ligand. One could also not exclude the effect of the polymer backbone, which could change the microenvironment of Cu^{2+} ligand, when poly(VI-VCL) binds to it.

Depending on the nature of the displacer, monomeric (free imidazole) or polymeric [poly(VI-VCL)] with the same concentration of the active imidazole unit affects proteins bound from the crude extract in a completely different way (Figs. 4 and 5, Table 1). Elution with 3.7 m*M* imidazole displaces weakly bound contaminating proteins (fractions 1–5; lane 4 for peak fraction 3) and accompanied with only minor elution of $(His)_6$-LDH. The subsequent elution with 5 mg/ml poly(VI-VCL) (giving in solution exactly the same concentration of imidazole groups, 3.7 m*M*) results in essentially complete displacement of strongly bound $(His)_6$-LDH (fractions 6–17; lane 5 for peak fraction 9) purified about eightfold (Table 1). Subsequent elution with 250 m*M* imidazole (fractions 18–22) results in minor amounts of both enzymatic activity and protein proving that displacement of $(His)_6$-LDH with poly(VI-VCL) was essentially quantitative.

To the best of our knowledge this is the first reported protein purification by displacement IMAC using synthetic polymer as a displacer. It was clearly demonstrated, that the efficiency of low-molecular-mass displacer could be improved significantly by converting it into a polymeric displacer having interacting groups of the same chemical nature. Copolymers containing vinyl imidazole units were

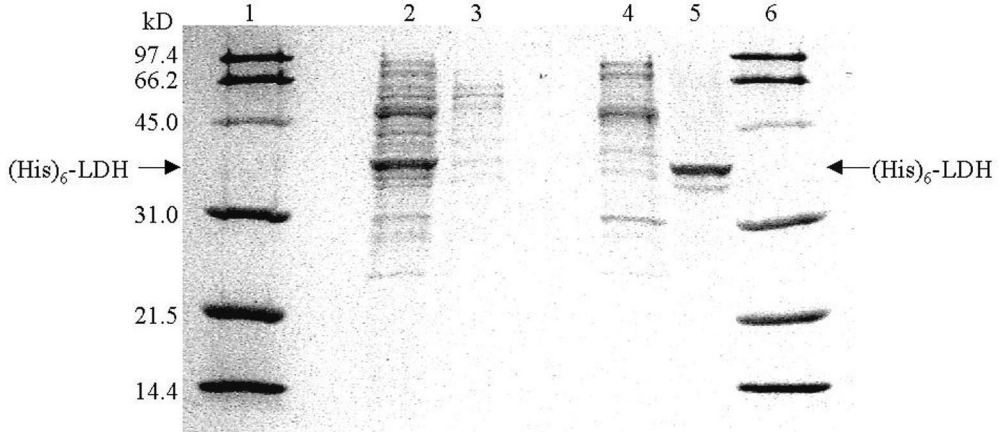

Fig. 5. SDS–PAGE assay of the fractions obtained in the three-step elution of $(His)_6$-LDH from crude homogenate. Lanes: 1 and 6=molecular mass standards, 2=crude homogenate, 3=unbound fraction, 4=peak fraction of protein with 3.7 m*M* imidazole displacement, 5=peak fraction of $(His)_6$-LDH with 5 mg/ml poly(VI-VCL) displacement.

Table 1
Elution of $(His)_6$-LDH bound to Cu^{2+}-IDA-Sepharose with imidazole and poly(VI-VCL)

	Cell supernatant	Unbound fraction	3.7 m*M* imidazole peak fraction	Poly(VI-VCL) peak fraction	Poly(VI-VCL) combined fractions 7–11	250 m*M* imidazole peak fraction
Volume (ml)	0.55	16	2.6	2.6	13	2.6
Protein content (mg/ml)	18.6	0.17	0.50	0.14	0.51	0.10
Total protein content (mg)	10.2	2.7	1.3	0.36	6.6	0.26
Total $(His)_6$-LDH activity (ΔA/min)	8.4	0.32	0.14	2.3	7.7	0.13
Total $(His)_6$-LDH activity (U)	1.4	0.05	0.02	0.4	1.2	0.02
Recovery (%)		3.8	1.6	27	92	1.5
Specific activity (U/mg)	0.13	0.02	0.02	1.02	0.19	0.08
Purification factor		0.14	0.13	7.7	1.4	0.60

demonstrated to be efficient displacers in IMAC of proteins. Moreover, proper choice of the polymeric backbone provides polymeric displacers with new useful properties, e.g., easy separation from the eluted protein and regeneration.

Acknowledgements

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