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Thermosensitive copolymers of *N*-vinylimidazole as displacers of proteins in immobilised metal affinity chromatography ☆

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Abstract

Synthetic copolymers of *N*-vinylcaprolactam (VCL) and *N*-vinylimidazole (VI) were studied as thermosensitive, reusable displacers for immobilised metal affinity chromatography (IMAC) of proteins. The copolymer with weight-average molecular mass of 11 700 g/mol prepared by free radical polymerisation at a 9:1 monomer molar ratio was separated into several fractions by IMAC and thermal precipitation. The fraction with an average VI content of 8.5% was most efficient as a reusable displacer for IMAC of ovalbumin, lysozyme and other proteins of egg white on Cu^{2+}-IDA-Sepharose. The displacer exhibited a sharp breakthrough curve and binding capacity of 16–20 mg/ml gel, depending on the flow-rate. The recovery of egg white proteins in the course of displacement chromatography was >95%. The displacer could be removed quantitatively from the protein fractions by thermal precipitation at 48°C. Co-precipitation of lysozyme with the displacer was minimal in the presence of 3% (v/v) acetonitrile, while the lysozyme enzymatic activity in the supernatant was completely retained. Addition of free imidazole to the mobile phase increased the rate of protein desorption and allowed better separation of egg white proteins and the displacer in the course of chromatography. The displacement profile of the egg white extract consisted of three zones with different distributions of individual proteins characterised by SDS–PAGE. Regeneration of the column was easily performed with 0.02 *M* EDTA in 0.15 *M* sodium chloride, pH 8.0, followed by washing with distilled water and reloading with Cu^{2+}. The displacer could also be regenerated by thermal precipitation at 48°C and subsequent dialysis against dilute hydrochloric acid (pH 2.5).

Keywords: Immobilized metal affinity chromatography; *N*-Vinylimidazole; Proteins

1. Introduction

Displacement chromatography is a potentially powerful technique for preparative separation of proteins. Theoretically, the displacement mode of elution offers many advantages over conventional bio-chromatographic techniques based on gradient elution, i.e. the gradual weakening of the interactions between the sorbent and the adsorbed solutes. Being carried out under conditions of constant and high affinity of the above components, the displacement elution mode ensures the separation of individual solutes as a sequence of highly concentrated contacting zones exiting in the order of increasing binding

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strength (a ''displacement train'') [1–3]. The potential of displacement chromatography in terms of high loading capacities, resolving power, final product purity and concentration has been recognised by many authors [4–7].

Although the fundamentals of displacement chromatography are well established and the predictions made for improved bioseparations are highly promising, the actual implementation of the method is still rather poor. Separations of model protein mixtures have mostly been studied, while the protein purification techniques assisted by the displacement mode of elution are rarely described. The limitations of the method were comprehensively summarised by Gerstner et al. [8]; the most important among them are the slow flow-rates, difficult on-line control of the running separations and column regeneration. The former is a consequence of the slow mass-transfer and macromolecular displacement interaction rates. While the new, perfusive sorbents with flow-through properties enable improved mass-transfer in various modes of bio-chromatography [8,9], the rates of adsorption, desorption and displacement of proteins are much more difficult to control. In our opinion, the problem may be partially solved by the chemical design of appropriate polymeric displacers capable of a fast interaction with the affinity sites of the sorbent and efficient displacement of the adsorbed proteins.

Among the polymers used as displacers in biochromatography one can refer to carboxymethyldextrans [6,10] and dextran sulphate [8], chondroitin sulfate, a carbohydrate polymer [11], polyacrylic and polyglutaminic acids [7], polyethyleneimine [7,12] and end-group-modified polyethylene glycols [7]. Proteins with high affinities for the sorbents are also often used as displacers [5,7,13]. The use of low-molecular-mass displacers such as imidazole in immobilised metal affinity chromatography [14] or antibiotics [15] and protected amino acids [16] in ion-exchange chromatography was recently advocated by Cramer and colleagues. Although no definite rules have been formulated for the correct choice and/or directed design of the displacers, it seems that flexible-chain, hydrophilic polymers with multiple interactive functions are most promising for this purpose.

Recently, 1-vinylimidazole (VI) copolymers with *N*-isopropylacrylamide or *N*-vinylcaprolactam (VCL) were studied as carriers for immobilised metal affinity precipitation [17–20]. Besides the effective chelation of transition metal ions and subsequent binding to the histidine residues of the target proteins, the inherent feature of the above copolymers is their thermosensitivity. When the temperature of an aqueous solution of a thermosensitive polymer rises above a critical point, designated *the cloud point*, phase separation into a concentrated polymer phase and an aqueous phase takes place. The lower temperature where this transition takes place when varying the polymer concentration is known as the Lower Critical Solution Temperature (LCST).

It seems attractive to study the function of these copolymers as displacers in immobilised metal affinity chromatography (IMAC): the thermosensitivity may facilitate, first, their repeated use after regeneration of the column and, secondly, their removal from the fractions of late-eluting proteins.

The use of the thermosensitive polymer, poly(*N*-vinyl caprolactam), as displacer in dye-affinity chromatography has been reported [21]. A similar approach to the synthesis of thermoresponsive displacers for anion-exchange and hydroxyapatite chromatography was recently taken by radical copolymerization of *N*-dimethylacrylamide and a vinyl derivative of hydroxyindanone [22]. Owing to the calcium-chelating properties of the latter monomer, on one hand, and its hydrophobicity, on the other, the resulting copolymers displayed both affinity for hydroxyapatite and thermosensitivity in aqueous solution. Two major whey proteins, α-lactalbumin and β-lactoglobulin, were separated by displacement chromatography on hydroxyapatite, although some admixtures of the displacer were noticeable in the zone of the eluted β-lactoglobulin. The removal of the displacer from the protein zone was possible, however, by its quantitative precipitation on heating the samples to 10°C above the cloud point

Owing to the importance of IMAC among modern methods of bioseparation [23], the chemical design and detailed investigation of reusable metal-chelating polymeric displacers, including their structure, molecular dimensions and regeneration techniques, appears to be very much warranted.

2. Materials and methods

2.1. Materials

1,4-Dioxane was dried over potassium hydroxide and distilled (101–102°C). *N*-Vinylcaprolactam was a generous 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 mm Hg). 2,2′-Azobis(2-methylpropionitrile) was purchased from ACROS (Malmö, Sweden). Lysozyme from egg white was a product of Sigma (St. Louis, MO, USA). Fresh eggs were available from a local supermarket. Copper sulfate, EDTA disodium salt, sodium chloride, sodium dihydrogen phosphate and potassium dihydrogen phosphate were of analytical grade and supplied by Merck (Darmstadt, Germany). Deuterium oxide 99.9 atomic % D was from Eurisо-Top (Gif-sur-Yvette, France). Iminodiacetate Sepharose CL-6B (IDA-Sepharose) was prepared according to Porath and Olin [24].

2.2. Copolymerization of N-vinylimidazole with N-vinylcaprolactam

VCL (3.75 g, 0.02 mol), VI (0.273 ml, $2\cdot10^{-3}$ mol) and 180 mg AIBN were dissolved in 60 ml 1,4-dioxane. The prepared solution was placed in a two-necked 100 ml round-bottom flask equipped with a reflux condenser and a gas-supplement tube for nitrogen bubbling. Free radical polymerisation was started by heating the reaction mixture to 80°C under nitrogen bubbling in a water bath and carried out for 20 h (10 h+10 h, with an overnight break). Two additional portions of AIBN (80+80 mg, each in 2 ml dioxane) were added as the reaction proceeded after 5 h and at the beginning of the second heating cycle. The volume of the reaction mixture was then reduced to 6 ml by evaporation of the solvents under reduced pressure; the solution of the copolymer became viscous. Two portions of the solution (3 ml) were each dropped into 60 ml diethyl ether with vigorous magnetic stirring, separated from the solvent by filtration through filter paper, washed with ca. 50 ml ether on the filter and dried in air. The yield of the copolymer was 75%.

2.3. Thermal precipitation of (VCL–VI) copolymer

(VCL–VI) copolymer (10 mg/ml) solution in 10 m*M* sodium phosphate, containing 0.3 *M* NaCl, pH 8.5 (10 ml), was heated to 48°C in a water bath with vigorous shaking until the copolymer precipitated on the walls of the test-tube, while the supernatant became almost clear (8–10 min). The precipitate was dissolved in 5 ml distilled water and the UV absorbance of a sample of the copolymer solution diluted 50-fold was detected at 220 nm. With the addition of salts, distilled water and sodium hydroxide, the solution was then adjusted to the conditions of repeated precipitation or chromatographic displacement.

2.4. Estimation of Cu^{2+} in solution

Ten percent aqueous ammonia (0.4 ml) was added to 1.6 ml of a solution of copper sulphate ($2.5\cdot10^{-4}$–$4\cdot10^{-3}$ *M*) in distilled water. The absorbance of the copper–ammonia complex develops to a maximum ($\lambda_{max} = 610$ nm) in 20–30 min under these conditions. Linear calibration gave $\varepsilon_{610} = 40\ M^{-1}\ cm^{-1}$.

2.5. Estimation of Cu^{2+}-binding capacity of IDA-Sepharose

Copper sulphate aqueous solution (10^{-3} *M*) was applied in a frontal mode with a flow-rate of ca. 0.5 ml/min to a ''Varian Bond Elut LPC Reservoir'' column packed with the tested sorbent (0.85×2.7 cm, 1.6 ml). Fractions of 1.6 ml eluate were collected and assayed for Cu^{2+} as described above. After saturation of the sorbent (the concentrations of Cu^{2+} in the feed and the fractions become equal), the column was washed with 8 ml distilled water (a volume sufficient to attain zero concentration of Cu^{2+} in the fractions) and the bound Cu^{2+} was desorbed by 0.2 *M* imidazole, pH 7.0. The Cu^{2+} concentration was quantified in the imidazole eluate as described above. The amount of desorbed Cu^{2+} divided by the volume of the packing gave the Cu^{2+}-binding capacity of the sorbent.

2.6. Adsorption of (VCL–VI) copolymers onto Cu^{2+}-IDA-Sepharose

Cu^{2+}-IDA-Sepharose placed in a standard ''Varian Bond Elut LRC Reservoir'' column (1.6 ml) was washed with 15 ml distilled water. A solution of (VCL–VI) copolymer in distilled water (1 mg/ml) was applied in a frontal mode to the column at a flow-rate of 0.5 ml/min. Fractions of 1.6 ml were collected, diluted two-fold with distilled water and monitored at 220 nm (amide group absorption; $\varepsilon_{220} = 7.2$ ml/mg·cm). The non-bound fractions (from the 7th to the 18th, see Fig. 1) were combined, reduced in volume to ca. 10 ml by vacuum evaporation, dialysed against distilled water and freeze-dried.

2.7. Displacement chromatography of lysozyme from egg white

2.7.1. Low protein loading

Lysozyme solution (0.5 ml, 2 mg/ml) in 20 m*M* sodium phosphate, containing 0.2 *M* NaCl, pH 7.0, was applied to a ''Varian Bond Elut LPC Reservoir'' column (1.6 ml) with Cu^{2+}-IDA-Sepharose followed by the same buffer (10 ml), then a 5 mg/ml solution of (VCL–VI) copolymer was applied in a frontal mode to the column. The flow-rate was 0.5 ml/min in all cases. The absorbance at 220 and 280 nm was estimated in the collected 1.6 ml fractions. The copolymer obtained as described in Section 2.2 was used as a displacer without purification.

2.7.2. High protein loading

Lysozyme solution (2 mg/ml) in 10 m*M* sodium phosphate, containing 0.15 *M* NaCl, pH 7.0, was applied to the column described in Section 2.7.1 in a frontal mode with a flow-rate of 0.5 ml/min. After saturation of the column by lysozyme, the column was washed with the starting buffer until the absorbance of the washings (280 nm) decreased to 3% of the breakthrough curve maximum. The solution of previously precipitated (48°C) (VCL–VI) copolymer (5 mg/ml, see Section 2.3) was applied to the column in a frontal mode. The fractions were collected and assayed as described in Section 2.7.1.

2.8. Displacement elution of ovalbumin and lysozyme at various imidazole concentrations

Ovalbumin (2 ml, 3 mg/ml) or its mixture with lysozyme (3 mg) was applied to a 1×5.5 cm column with Cu^{2+}-IDA-Sepharose equilibrated with 10 m*M* sodium phosphate containing 0.15 *M* NaCl, pH 7.0,

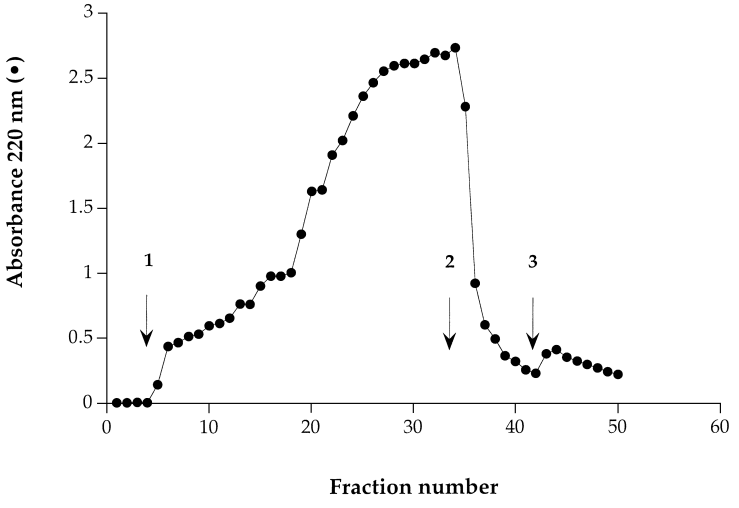

Fig. 1. Breakthrough curve of (VCL–VI) copolymer (1 mg/ml solution in distilled water) on a Cu^{2+}-IDA-Sepharose column (1.6 ml) at a flow-rate of 0.5 ml/min. Fractions of 1.6 ml were collected, diluted two-fold with distilled water and monitored at 220 nm (amide group absorption). Arrow 1, application of the copolymer solution; arrow 2, washing with distilled water; arrow 3, washing with 0.2 *M* NaCl in 10 m*M* sodium phosphate, pH 7.0.

or the same buffer containing 0.1 or 0.5 m*M* imidazole. Elution was carried out with 5 mg/ml (VCL–VI) copolymer, previously purified by IMAC and thermal precipitation, at a flow-rate of 0.25 or 1 ml/min. Fractions of 1 ml were collected. The UV absorbance of the eluate at 280 nm as well as the lysozyme activity were estimated in the collected fractions. For detection of the displacer, the fractions were diluted 10-fold, heated to 48°C and their turbidities (apparent absorbances) were registered at 450 nm to detect the presence of thermosensitive polymer.

2.9. Displacement chromatography of egg white extract

Egg white (3 ml) was extracted with 27 ml of 50 m*M* sodium acetate, containing 0.15 *M* sodium chloride (pH 4.5). The extraction mixture was shaken slowly in a shaker at 20 rpm and 4°C for 3 h, then filtered through Munktell No. 3 filter paper, adjusted to pH 7.0 and again filtered to remove insoluble matter. The extract (2 ml, 11.7 mg protein/ml) was applied to a 1×5.5 cm column with Cu^{2+}-IDA-Sepharose equilibrated with 10 m*M* sodium phosphate containing 0.15 *M* NaCl and 0.1 m*M* imidazole, pH 7.0, and eluted with the same buffer at a flow-rate of 0.25 ml/min. Fractions of 1 ml were collected. After the non-adsorbing components of the egg white were eluted, a 5 mg/ml solution of (VCL–VI) copolymer previously purified by IMAC and thermal precipitation was applied to the column in a frontal mode. The UV absorbance of the eluate at 280 nm as well as the lysozyme enzymatic activity and turbidity at 48°C were estimated in the collected fractions (see Section 2.8).

2.10. Estimation of lysozyme activity

The lysozyme activity was estimated by the turbidimetric assay procedure [25]. A suspension of 0.15 mg/ml *Micrococcus lysodeikticus* cells was prepared in 66 m*M* potassium phosphate buffer (pH 6.24) containing 15 m*M* NaCl at room temperature. To 2.5 ml of the suspension, a 5–50 μl sample was added, followed by immediate mixing. The absorbance at 450 nm (A_{450}) was recorded every 15 s for 2 or 3 min. The initial rate of decrease of the absorbance was calculated by linear regression for both the test and the blank. One unit of lysozyme activity (U) was defined as the activity that can cause a decrease of A_{450} by 1 unit per minute.

2.11. Gel-electrophoresis

SDS–PAGE was carried out in a BioRad mini protein II system according to the instructions of the manufacturer, using 15% polyacrylamide gel. The samples were obtained by dialysis of the chromatographic fractions, followed by concentration to ca. 1 mg protein/ml by evaporation under vacuum. The proteins and copolymers were stained with Coomassie Blue R-250.

2.12. Light scattering and NMR measurements

Light scattering measurements were performed with a Malvern 4700c System (UK). An argon ion laser (Uniphase 2213-75 SL, 30 mW) operating at 488 nm wavelength was used as a light source. The spectrometer was calibrated with distilled water and toluene to ensure that the scattering intensity from water and toluene had no angular dependence in the range 10–150°. The weight-average molecular mass (M_w) of the copolymer was determined by the static light scattering technique using the concentration and angular independence of the scattering intensity [26] registered under the above conditions. M_w/M_n was calculated from dynamic light scattering measurements. The intensity autocorrelation function was registered at 90° and the data were processed by cumulant analysis using the PCS software program (version 1.35) supplied by Malvern Instruments.

The fractions of (VCL–VI) copolymer separated either by thermal precipitation or by IMAC were dialysed against distilled water and freeze-dried. The samples for light scattering measurements were prepared by dissolution of freeze-dried copolymer fractions in 10 m*M* sodium phosphate containing 0.3 *M* NaCl (pH 8.5) followed by filtration through a 0.45 μm Sartorius Minisart filter into a round-wall sample cuvette (10 mm diameter). The temperature dependencies were studied at a copolymer concentration of 0.15 mg/ml.

NMR spectra were registered on a Bruker DRX500 spectrometer operating at 500.2 MHz for

^{1}H at 27°C. The samples for NMR spectrometry measurements were prepared by dissolving the freeze-dried copolymer fractions in deuterium oxide at 2 mg/ml. Chemical shifts are reported as ppm downfield from tetramethylsilane.

3. Results and discussion

3.1. Adsorption and separation of (VCL–VI) copolymers on Cu^{2+}-IDA-Sepharose columns. Regeneration of the displacer

Depending on the ratio of the monomer units in copolymers of *N*-vinylcaprolactam (VCL) and 1-vinylimidazole (VI) as well as the pH and ionic strength of their aqueous solutions, the relevant cloud point values vary significantly [17,20]. The cloud point increases with increasing content of VI monomer units, decreasing pH and decreasing ionic strength of the aqueous solution. Owing to the different reactivity ratios of the monomers, the (VCL–VI) copolymers prepared by free radical polymerisation with a fixed initial ratio of the monomers and high polymerisation yields [20] necessarily contain chains with different molar percentages of monomers. In order to prepare a chromatographic displacer with a narrow temperature range of the phase transition and defined affinity for the metal-chelating sorbent, we attempted to separate a sample of (VCL–VI) copolymer into several fractions by means of thermal precipitation and immobilised metal affinity chromatography (IMAC). Co-polymerisation of VCL and VI in the molar proportion 9:1 leads to a product with a well-expressed affinity for Cu^{2+} and a reasonable cloud point range (from 30°C in 1 *M* NaCl to 50°C in distilled water) [20]. This sample was chosen for further separation in the present study.

The ultimate property of a polymeric displacer is the capability of strong binding to the relevant affinity sorbent. Thus adsorption of the (VCL–VI) copolymer sample to the metal-chelating carrier was studied first. Fig. 1 illustrates the breakthrough curve of the copolymer obtained by frontal chromatography on a Cu^{2+}-IDA-Sepharose column. Clearly, the synthesised copolymer contained some adsorbing as well as some non-adsorbing fractions. Judging by the absorbance ($\lambda = 220$ nm) of the combined non-adsorbing fractions (see Section 2.5) one could presume a content of ca. 5 mg or 11% of the applied copolymer therein. After these fractions were dialysed and freeze-dried, their actual mass turned out to be only 2 mg. Apparently, some lower-molecular-mass chains of the copolymer did not interact with the sorbent and were removed by dialysis. The higher-molecular-mass fraction of the non-interacting copolymer was isolated and studied as described below. The specific binding capacity of the affinity sorbent was estimated as 15 mg copolymer/ml gel. The Cu^{2+}-binding capacity of the parent IDA-Sepharose was 8 μmol/ml.

After the weakly bound portions of the copolymer were removed by washing with distilled water and 0.2 *M* sodium chloride in 10 m*M* sodium phosphate, pH 7.0, the specifically adsorbed copolymer was eluted from the column with 0.02 *M* EDTA in 0.15 *M* NaCl, pH 8.0. This stage leads to regeneration of both the affinity sorbent and the polymeric displacer and sometimes appears to be most laborious during displacement chromatography separations [4]. Although Cu^{2+} chelated by IDA-Sepharose may be readily desorbed by 0.2 *M* aqueous imidazole, pH 7.0 (see Section 2.5), the specifically adsorbed (VCL–VI) copolymer could not be eluted under these conditions. Instead, it was found that 0.02 *M* EDTA in 0.15 *M* NaCl, pH 8.0, quantitatively desorbs both Cu^{2+} and the copolymer, so that two column volumes of the above eluent followed by two column volumes of 0.15 *M* NaCl appear to be sufficient for the regeneration of IDA-Sepharose. After subsequent washing with distilled water (ca. five column volumes) the carrier may again be loaded with Cu^{2+}.

Separation of the desorbed copolymer from Cu^{2+}, EDTA and the salts may be performed by thermal precipitation of the copolymer. The success of copolymer isolation strongly depends, however, on its concentration and pH. At a copolymer concentration of 10 mg/ml and pH 8.5 (most imidazoles in the monomer units are deprotonated, $pK_b \approx 7$) preparative isolation of the copolymer is feasible at a sodium chloride concentration of ca. 0.3 *M* at 48°C. At lower ionic strengths the collectable precipitates do not form, although the fine suspension of copolymer makes the solution cloudy. At much higher

ionic strengths, the co-precipitation of proteins, which may also be present in the EDTA eluate, is highly probable, as discussed in detail below (Section 3.3).

The copolymer concentration in the eluate is usually in the range 3–5 mg/ml, whereas the concentration of sodium chloride is slightly below 0.15 *M*. Thus the two- to three-fold reduction of the volume of the eluate by evaporation under vacuum leads to conditions optimal for thermal precipitation of the copolymer. On heating to 48°C and vigorous shaking, the copolymer precipitates in the form of bulky aggregates sticking to the walls of the test-tube. The almost clear supernatant, containing Cu^{2+} salts and EDTA, is decanted, while the precipitated copolymer is then dissolved in distilled water. Independent experiments have shown that, under similar conditions (10 mg copolymer per ml of 0.3 *M* NaCl in 10 m*M* sodium phosphate, pH 8.5), ca. 80% copolymer may be recovered after the first thermal precipitation and 70–75% after repeated precipitation. Some highly soluble fractions of the copolymer were, therefore, separated in this way.

According to the above chromatographic technique, the fraction of the high affinity (VCL–VI) copolymer was obtained in a yield of about 60% (w/w) from the parent copolymer sample. Some traces of EDTA are, however, still present in the thus-purified copolymer. They cause leakage of Cu^{2+} from Cu^{2+}-IDA-Sepharose when attempting to reload it with regenerated copolymer. In order to accomplish purification of the displacer, dialysis against dilute hydrochloric acid (pH 2.5) was necessary to remove all detectable low-molecular-mass compounds.

3.2. Characterization of (VCL–VI) copolymers

The weight-average molecular mass (M_w) of the (VCL–VI) copolymer prepared according to Section 2.2 and dialysed against distilled water was 11 700 g/mol and M_w/M_n was 1.1, as determined by a combination of static and dynamic light scattering [27].

NMR spectra of isolated fractions of (VCL–VI) copolymer are presented in Fig. 2. The spectra of the parent (Fig. 2A) and purified copolymer (Fig. 2B) are rather similar, which is not surprising because only a minor portion of the copolymer was removed in the course of IMAC and further precipitation. In contrast, the non-adsorbed fraction of the copolymer (Fig. 2C) contains much fewer imidazole monomer units (5%) compared to the purified copolymer (8.5%) and the parent copolymer (11%). Moreover, the relevant resonance band of the imidazole ring protons (chemical shift 6.95 ppm) is poorly resolved and somewhat wider than that of the purified copolymer. This testifies to the longer relaxation times and restricted mobility of these protons. Although it is hard to presume the structural features underlying this phenomenon, it is likely that a certain portion of the vinyl imidazole units in the copolymer suffers from constrained reactivity, so that the relevant chains having reduced affinity for Cu^{2+} could be separated from the rest of the copolymer by means of IMAC.

Thermal precipitation of the studied polymeric displacers is the key property allowing their purification and regeneration as discussed above. Although many studies have addressed the accurate physicochemical characterisation of the phase transitions in thermoresponsive polymers [28,29], the relevant behaviour of affinity polymeric reagents has not yet been studied in detail. Usually, the cloud points of these polymers are registered by the decrease in optical transmittance of their aqueous solutions observed on heating [18,21]. In our opinion, the method of dynamic light scattering is more informative. It is well known that the scattered intensity is proportional to the spatial fluctuations of the polymer concentration $I \sim \langle \Delta c^2 \rangle$ [30]. This value is extremely sensitive to temperature changes when the system approaches the cloud point. As increasing fluctuations precede phase separation, the light scattering technique enables one to follow quantitatively a thermoprecipitation process over a wide range of temperatures.

Fig. 3 illustrates the light scattering intensity registered with different fractions of (VCL–VI) copolymer as a function of temperature. The fraction of copolymer remaining in the supernatant (3) after thermal precipitation at 48°C undergoes a phase transition at much higher temperatures than the parent copolymer (1) and its thermally precipitated fraction (2). One should note that only a minor portion of the readily soluble copolymer (up to 25%)

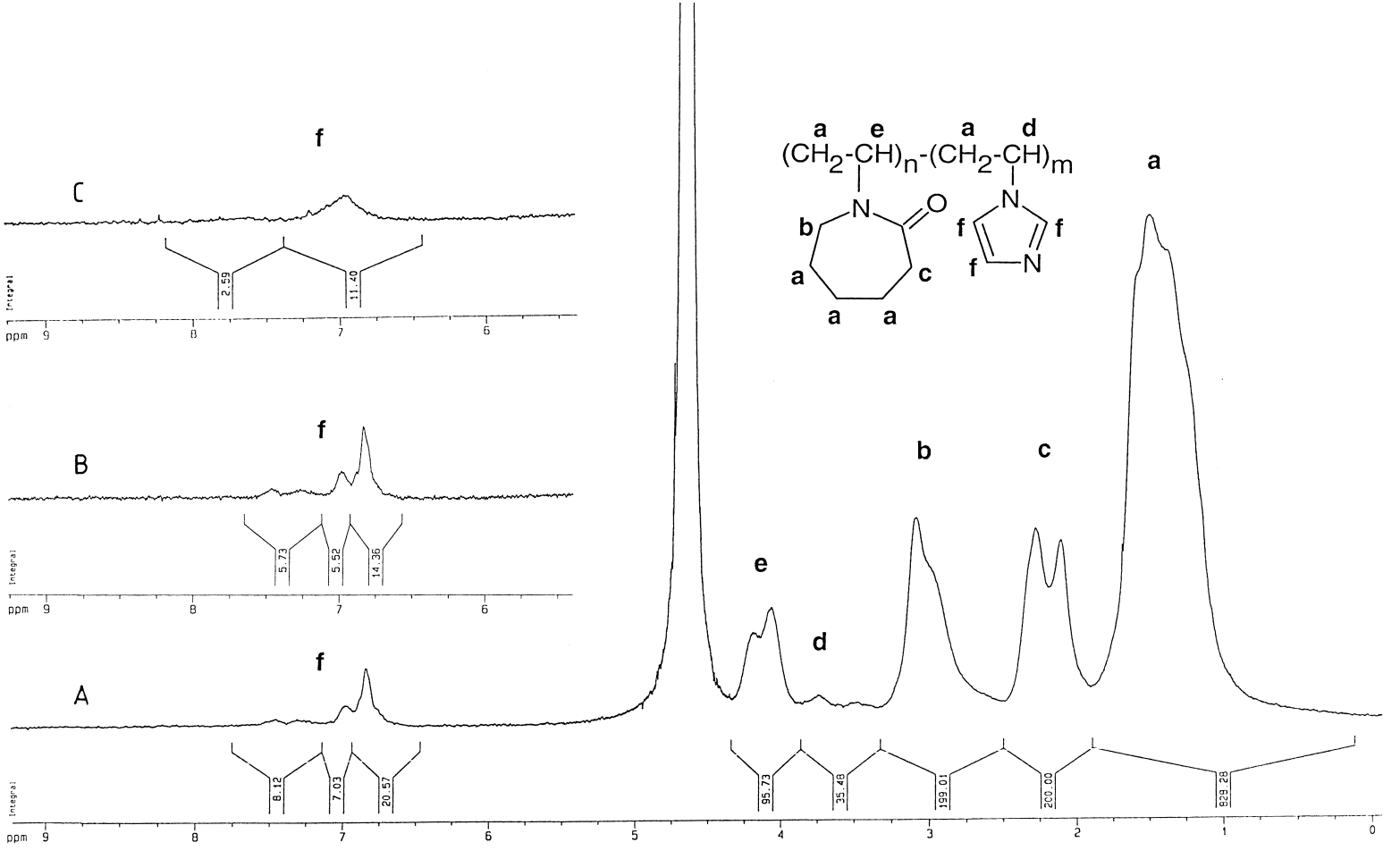

Fig. 2. ^{1}H-NMR spectra (500 MHz) of the parent (VCL–VI) copolymer (A), the copolymer purified by IMAC (B) and the non-adsorbing copolymer fraction (C). The samples were dialysed against distilled water and freeze-dried. Solvent: D_2O.

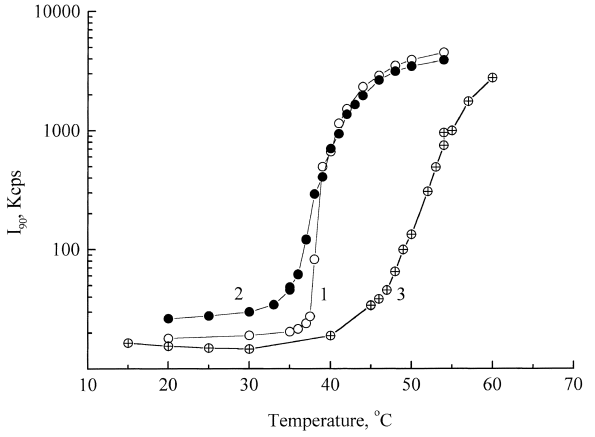

Fig. 3. Intensity of light scattering from 0.15 mg/ml copolymer solutions as a function of temperature: (1) the parent (VCL–VI) copolymer; (2) the copolymer purified by IMAC and thermal precipitation; (3) the copolymer fraction remaining soluble in the course of thermal precipitation at 48°C. All samples were dissolved in 10 m*M* sodium phosphate containing 0.3 *M* NaCl, pH 8.5.

remains in solution after thermal precipitation in 10 m*M* sodium phosphate containing 0.3 *M* NaCl, pH 8.5 (see Section 3.1), so the cloud point of the precipitated fraction (2) shifted only slightly to lower temperatures. The better solubility of fraction 3 can be explained either by its lower molecular mass (ca. 30% of the fraction was removed by dialysis) or, more likely, by the higher molar percentage of VI in the copolymer.

3.3. Displacement chromatography of lysozyme as a model protein. Removal of the displacer from the protein solution

Lysozyme from egg white is a well studied protein whose binding to metal-chelating affinity sorbents has been described many times in the literature [31,32]. Among the other model proteins used for characterisation of displacement chromatographic systems (α-chymotrypsinogen, cytochrome *c*, lactalbumin, lactoferrin, etc.), lysozyme binds to the immobilised metal most strongly, so that its elution zone is sometimes observed within the zone of the displacer [5]. The displacement of lysozyme by a novel displacer most probably means that many other proteins can also be displaced. It seemed reasonable, therefore, to choose this protein as a model for studying the efficiency of the novel displacer and the completeness of displacer removal from the obtained protein eluates.

Lysozyme hardly interacted with Cu^{2+}-IDA-Sepharose loaded with (VCL–VI) copolymer as described in Section 1: the protein was chromatographed without retardation with 94% recovery. Lysozyme was quantitively adsorbed, however, when applied to uncoated Cu^{2+}-IDA-Sepharose. Further frontal application of 5 mg/ml (VCL–VI) copolymer displaced the protein with a recovery of 75%, as shown in Fig. 4. Although the elution of lysozyme took place slightly before the zone of the displacer, the eluted protein definitely contained (VCL–VI) copolymer at a rather high concentration. The conditions of the given chromatographic run were apparently not optimal for the formation of a narrow zone of the protein: the column was too short and the flow-rate too high, which caused peak tailing. Nevertheless, the displacement of lysozyme was registered and the problem of displacer removal from the protein eluate has to be solved.

The concentration of displacer is another parameter to be optimised. The binding capacity of the parent copolymer to Cu^{2+}-IDA-Sepharose did not depend strongly on the concentration, at least in the range from 1 to 10 mg/ml, and remained ca. 15 mg/ml gel as mentioned in Section 1. Displacement concentrations of 1 and 2 mg/ml, however, provided very broad zones of eluted lysozyme (14 and 10 column volumes, respectively), while a concentration of 10 mg/ml made the volume available for the ''displacement train'' too small. In all cases the displaced lysozyme contained some copolymer.

In order to quantify the contamination of the protein with the displacer one may estimate the ratio of absorbances $R = D_{220}/D_{280}$ in the protein fractions (see Table 1). As the extinction coefficient (ε_{280}) of the copolymer is low, the above ratio is very sensitive to even slight contamination of the protein: $R_{\text{lysozyme}} = 6.5$, $R_{\text{displacer}} = 480$. Provided both D_{220} and D_{280} are known, the concentrations of (VCL–VI) copolymer can easily be calculated. Thermal precipitation of the displacer from the protein fractions may be followed quantitatively, therefore, by UV spectrophotometry.

To obtain high precipitation yields of the co-

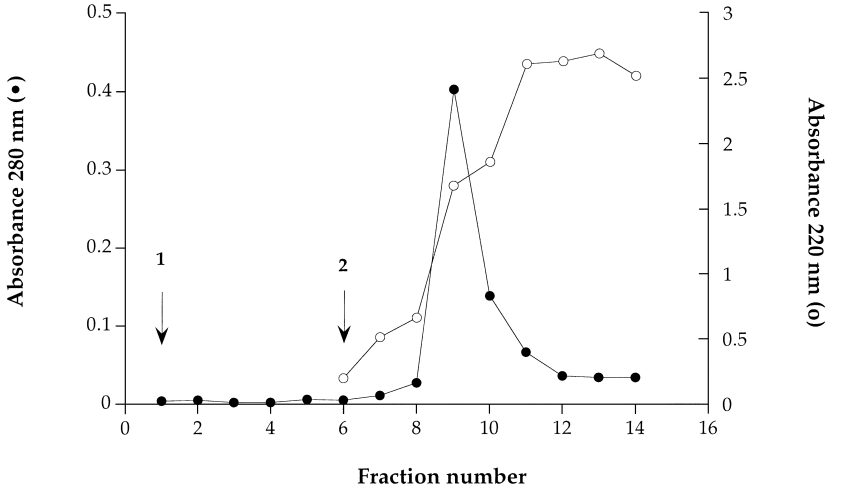

Fig. 4. Displacement chromatography of lysozyme (0.5 ml, 2 mg/ml) applied to a Cu^{2+}-IDA-Sepharose column equilibrated with 20 m*M* sodium phosphate, 0.2 *M* NaCl, pH 7.0 (0.85×2.7 cm, 1.6 ml), followed by 5 mg/ml (VCL–VI) copolymer solution in the same buffer. Flow-rate 0.5 ml/min. Absorbance at 280 nm was detected in fractions diluted two-fold. The concentration profile of (VCL–VI) copolymer was monitored by the eluate absorbance at 220 nm in collected fractions (1.6 ml) diluted 10-fold. Arrow 1, application of lysozyme; arrow 2, application of the copolymer. For details, see Section 2.7.1.

polymer it is convenient to use a high concentration of sodium chloride added to the chromatographic fractions. This leads, however, to a significant non-specific co-precipitation of lysozyme with the co-polymer pellet, presumably due to enhanced hydro-phobic interactions (see Table 2).

The concentrations of lysozyme and the copoly-mer taken for this study (see footnote, Table 2) correspond approximately to those in the concen-trated chromatographic fractions (Fig. 4), two- or three-fold reduced in volume by vacuum evapora-tion. It should be noted, however, that in real separated mixtures the protein loading capacities, as well as the protein concentration in the eluates, may be much higher so that the actual uptake of proteins during displacer precipitation is assumed to be lower than that given in Table 2. It follows from Table 2 that the lower ionic strengths in the copolymer solution leads to a reduced uptake of lysozyme, which becomes minimal in the presence of small amounts of acetonitrile.

On the other hand, after the removal of the larger share of the displacer by thermal precipitation, a

Table 1
Extinction coefficients of (VCL–VI) copolymer and lysozyme at different wavelengths (ml/mg·cm)

	Wavelength	
	$\varepsilon_{220\ nm}$	$\varepsilon_{280\ nm}$
(VCL–VI) copolymer	7.2	0.015
Lysozyme	14.3	2.2

Table 2
Co-precipitation of lysozyme with (VCL–VI) copolymer from aqueous solutions at 48°C[a]

Solution composition	Co-precipitation of lysozyme (%)
1. 0.8 *M* NaCl	64
2. 0.3 *M* NaCl	22
3. 0.3 *M* NaCl + 1% EtOH	20
4. 0.3 *M* NaCl + 3% EtOH	18
5. 0.3 *M* NaCl + 8% EtOH	20
6. 0.3 *M* NaCl + 1% MeCN	12
7. 0.3 *M* NaCl + 3% MeCN	11
8. 0.2 *M* NaCl	No collectable precipitate

[a] 10 mg/ml copolymer in 20 m*M* sodium phosphate, pH 8.5, 1 mg/ml lysozyme.

small amount still contaminates the protein fractions. In order to reduce this amount, twice repeated precipitation of the copolymer from 0.3 *M* NaCl containing 10 m*M* sodium phosphate (pH 8.5) was performed prior to the experiments as illustrated by Table 2. Under optimal conditions (Table 2, line 7), the copolymer concentration in lysozyme solution was reduced 17.5-fold. Although purified by IMAC and thermal precipitation, the displacer contained some minor fractions undergoing a phase transition above 48°C (see Fig. 3, curve 2). For complete removal of the displacer from the protein fractions, repeated thermal precipitation at higher temperatures may be helpful if acceptable from the viewpoint of protein stability. To verify this proposal, displacement chromatography of lysozyme was carried out under conditions of high loading, which is closer to real separations.

The relevant elution profile is illustrated in Fig. 5. Unlike in the chromatogram recorded at low lysozyme loading (Fig. 4), the elution zone at the higher loading acquired a form more typical of displacement chromatograms. The lysozyme fractions were collected, two-fold reduced in volume by evaporation under vacuum and heated to 48°C in order to precipitate the displacer under optimal conditions (Table 2, line 7). The protein solution was separated from the precipitated displacer by decantation, dialysed against distilled water and further three-fold reduced in volume by evaporation. The ionic strength of the solution was adjusted to 0.3 *M* by the addition of 1.5 *M* sodium chloride. After incubation in a water bath at 60°C for 2 min the obtained lysozyme solution became somewhat cloudy because of precipitation of residual displacer. This fine suspension was quickly filtered through a Sartorius 0.45 μm cartridge filter preheated at 60°C. The yield of lysozyme found in the filtrate was 70% from that contained in the collected fractions, while the absorbance ratio of $R = 6.3$ indicated the recovery of pure protein. The enzymatic activity of lysozyme was completely retained (55 U/mg versus 50 U/mg in the commercial sample), and SDS–PAGE analysis of the sample showed a single intensive band of lysozyme (data not shown). Thus the possibility of preparative separation of the displacer from protein solution has been demonstrated, at least for a relatively thermostable protein such as lysozyme.

3.4. Kinetics of protein desorption. Influence of free imidazole as a mobile-phase modifier

Strictly speaking, the presence of a polymeric displacer in fractions of eluted proteins testifies to the deviation of the real displacement process from

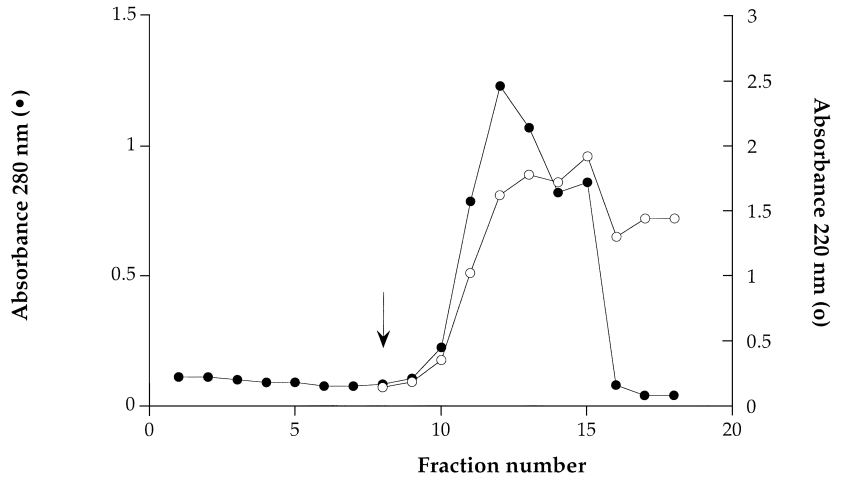

Fig. 5. Displacement elution of excessively loaded lysozyme by 5 mg/ml (VCL–VI) copolymer in 10 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.0. Column and flow-rate are as given in Fig. 4. Absorbance at 280 nm was detected in fractions diluted two-fold. The (VCL–VI) copolymer fraction purified by IMAC and double thermal precipitation was used as a displacer and detected by its absorbance at 220 nm in the collected 1.6 ml fractions diluted 20-fold. Arrow: application of the copolymer. For details, see Section 2.7.2.

theoretical models [1,3,8] predicting the elution of contacting, non-overlapping zones. Such a deviation may result from non-homogeneity of the displacer or from the slow rates of adsorption and desorption displayed both by the separated proteins and the displacer. In the mode of gradient chromatographic elution, the desorption of proteins usually proceeds in a narrow range of concentrations of a mobile-phase modifier, which almost excludes repeated adsorption of an immediately desorbed solute [33]. In contrast, frequent adsorption and desorption of protein molecules is presumed to underlie the mechanism of their separation in displacement chromatography. This is the reason why the rates of sorbent–solute interaction are, in the latter case, of much greater importance.

In order to evaluate the role of the above kinetic phenomena, displacement chromatography of ovalbumin was carried out with (VCL–VI) copolymer previously purified by IMAC and thermal precipi-

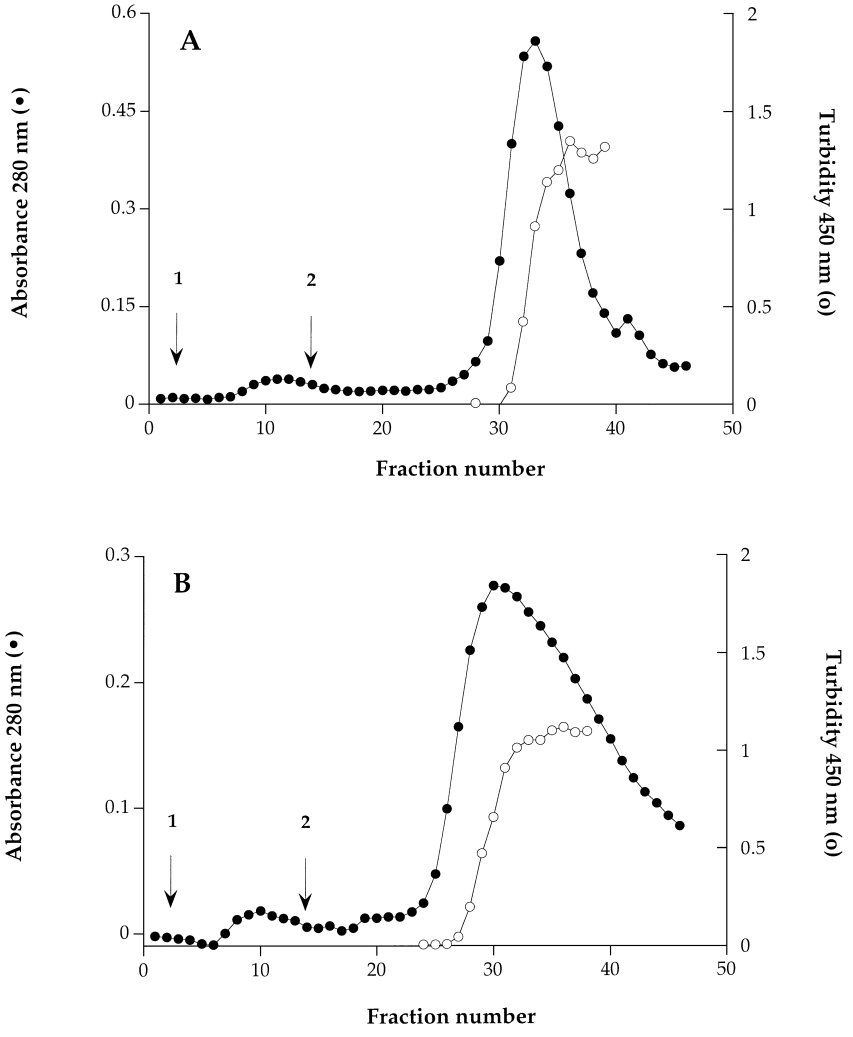

Fig. 6. Displacement elution of ovalbumin (6 mg) from Cu^{2+}-IDA-Sepharose by 5 mg/ml purified (VCL–VI) copolymer in 10 m*M* phosphate, 0.15 *M* NaCl, pH 7.0. Column, 1×5.5 cm; flow-rate, 0.25 ml/min (A) and 1 ml/min (B). One millilitre fractions were diluted 10-fold to detect the displacer profile at 450 nm and 48°C. Arrow 1, application of the protein; arrow 2, application of the displacer. For details, see Section 2.8.

tation as discussed in Section 3.1. Besides its narrow molecular mass distribution ($M_w/M_n = 1.1$), the displacer exhibits high chemical homogeneity (see Section 3.2): as estimated by ^{1}H-NMR, each chain of the displacer contained about eight vinylimidazole units capable of complex formation with immobilised Cu^{2+}.

The displacement elution of ovalbumin registered at two different flow-rates is shown in Fig. 6. The breakthrough curves of the displacer are sharp and rather similar: copolymer adsorption capacities of 16 and 19 mg/ml gel were registered at flow-rates of 1 and 0.25 ml/min, respectively. This indicates the presence of a high rate of displacer mass-transfer in the pores of Sepharose as well as its fast interaction with immobilised Cu^{2+}. On the other hand, the elution profiles of ovalbumin obtained under these conditions are very different. The broader peak of the protein registered at the higher flow-rate testifies that ovalbumin desorption is slow and takes place by the time the sorbent is already saturated by the displacer.

To minimise the effect of the slow desorption rates in displacement from the IMAC column, the mobile phase was modified with free imidazole as previously proposed [5]. Indeed, the presence of imidazole strongly changed the elution profile of ovalbumin (see Fig. 7). The protein appeared in the eluate much earlier than the front of the displacer. Obviously, the rate of desorption is increased significantly so that protein tailing vanished. On the other hand, ovalbumin was eluted as an asymmetrical peak due to its slower adsorption and partial breakthrough in the presence of 0.5 m*M* imidazole. Apparently, imidazole competed with the protein for binding to the immobilised Cu^{2+}. Displacement of ovalbumin in the mixture with lysozyme exhibited a similar chromatographic pattern (see Fig. 8A), the lysozyme zone being eluted after that of ovalbumin, although minor lysozyme activity was also found in the ovalbumin zone.

The lower concentration of imidazole in the mobile phase (0.1 m*M*, Fig. 8B) prevented both the early breakthrough of ovalbumin and its tailing, while the distribution of lysozyme activity within the eluted fractions remained the same. Judging from the data obtained, the concentration of free imidazole in the mobile phase is a powerful tool for optimisation of the displacement process in IMAC. Although the front of the displacer became more diffuse in the presence of imidazole (see Figs. 6 and 7), the separation of proteins from the polymeric displacer within the course of chromatography was better due to the absence of protein tailing. In order to evaluate the applicability of the polymeric displacer to the separation of real-world protein mixtures, IMAC of egg white protein extract was carried out in the presence of 0.1 m*M* imidazole.

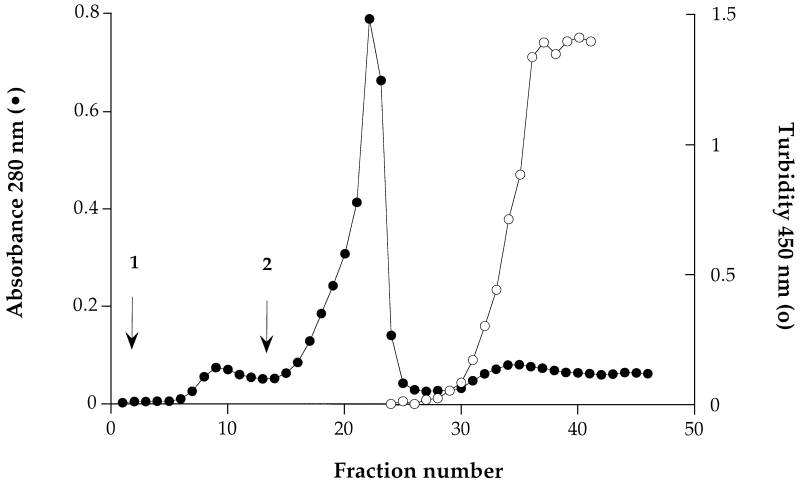

Fig. 7. Displacement elution of ovalbumin (6 mg) from Cu^{2+}-IDA-Sepharose in the presence of 0.5 m*M* imidazole by 5 mg/ml purified (VCL–VI) copolymer. Column and eluent are as given in Fig. 6. Flow-rate 0.25 ml/min.

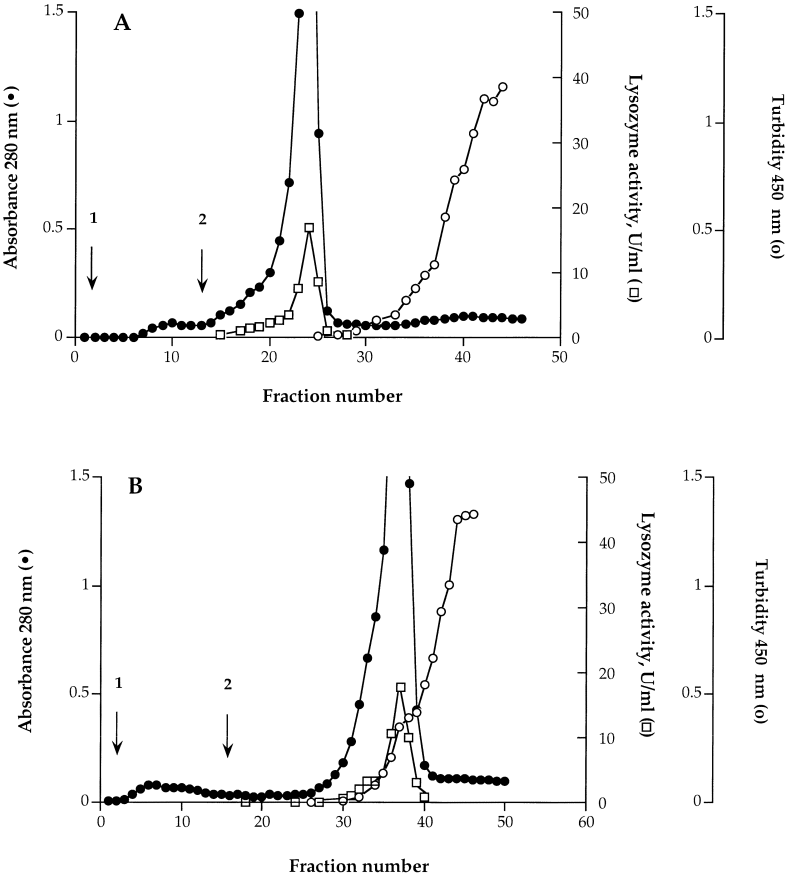

Fig. 8. Displacement elution of ovalbumin (6 mg) and lysozyme (3 mg) by 5 mg/ml purified (VCL–VI) copolymer in the presence of 0.5 m*M* (A) or 0.1 m*M* imidazole (B). Column and eluent are as given in Fig. 6. Flow rate 0.25 ml/min.

3.5. Displacement chromatography of the proteins of egg white

Fig. 9 illustrates the displacement IMA chromatogram of egg white extract. Cu^{2+}-IDA-Sepharose bound more than 90% of the proteins contained therein. As found by SDS–PAGE, the non-bound fraction of the extract contained bands of ovomucoid (30–35 kDa) and a slight trace of ovalbumin (45 kDa). The other main components of egg white, conalbumin (77 kDa) and lysozyme (14.4 kDa), were not found in this fraction. After washing the column with phosphate buffer (see Fig. 9), introduction of the displacer caused the appearance of the first protein zone (fractions 23–30), where the major component was ovalbumin (Fig. 10, lane 3). The second zone (fractions 31–34) contained most of the proteins of egg white and the larger share of lysozyme enzymatic activity (Fig. 10, lane 4). The third zone (fractions 35–41) appeared with the front of the displacer and contained conalbumin (77 kDa) and traces of ovalbumin and lysozyme. The displacer was separated from the latter fractions by thermal precipitation at 48°C, as described in Section 3. Its breakthrough curve (Fig. 9) registered by turbidity at 450 nm and 48°C (see Materials and methods) was rather sharp and corresponded to a binding capacity of 20 mg/ml, which agrees well with that registered in experiments with pure ovalbumin. Judging by the UV absorbance of the protein eluates (280 nm), the overall recovery of proteins in the course of displacement was above 95%.

The front of the displacer follows the zone with maximal protein content, so that the separation as a

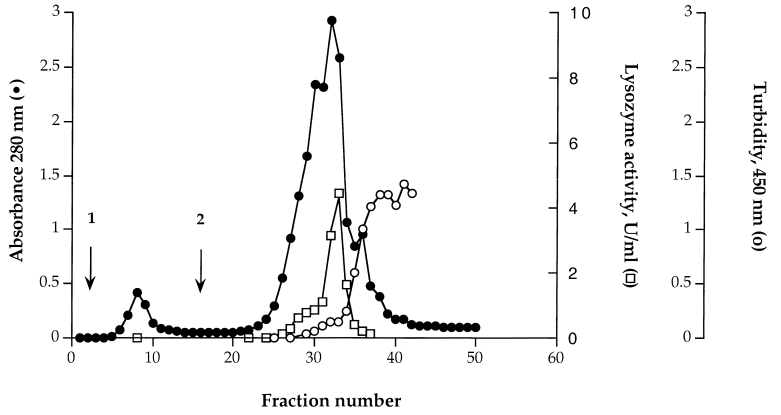

Fig. 9. Separation of egg white extract by displacement chromatography on Cu^{2+}-IDA-Sepharose in 10 m*M* sodium phosphate, 0.15 *M* NaCl, 0.1 m*M* imidazole, pH 7.0. Column, 1×5.5 cm; flow-rate, 0.25 ml/min. Arrow 1, application of egg white extract (2 ml, 11.7 mg protein/ml); arrow 2, application of the displacer, 5 mg/ml (VCL–VI) copolymer purified by IMAC and repeated thermal precipitation, in the above buffer. The 1 ml fractions were diluted 10-fold to detect the displacer profile at 450 nm and 48°C.

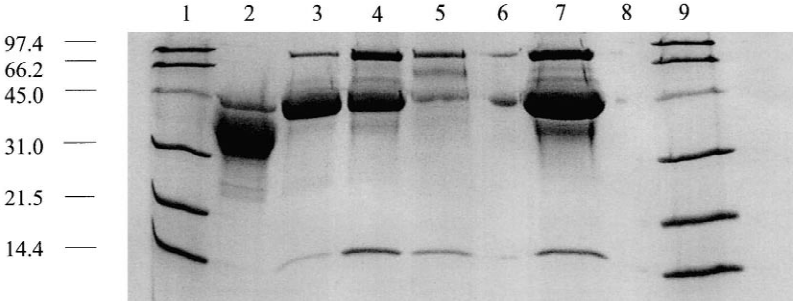

Fig. 10. SDS–PAGE assay of the fractions obtained in the course of displacement chromatography of egg white extract. Lane 1, molecular mass standards; lane 2, non-bound proteins of egg white (fractions 6–11, Fig. 9); lane 3, fractions 23–30, Fig. 9; lane 4, fractions 31–34, Fig. 9; lane 5, fractions 35–41, Fig. 9; lane 6, fractions 42–50, Fig. 9; lane 7, egg-white extract; lane 8, empty; lane 9, molecular mass standards.

whole looks like a displacement process. On the other hand, the selectivity of the obtained separation was not high. At the present stage of research it seemed more important, however, to demonstrate the general feasibility of (VCL–VI) copolymers as displacers in IMAC and to determine the basic routes to optimisation of the relevant chromatographic processes. In our view, quantitative desorption of proteins bound to Cu^{2+}-IDA-Sepharose, combined with facile regeneration of both the affinity column and the displacer, make the studied thermosensitive copolymers quite promising for various modifications of IMAC such as displacement chromatography or polymer-shielded affinity chromatography [34].

4. Nomenclature

Cu^{2+}-IDA-Sepharose	iminodiacetate-Sepharose loaded with Cu^{2+}
EDTA	ethylenediaminetetraacetic acid, disodium salt
IDA	iminodiacetic acid
IDA-Sepharose	iminodiacetic acid-Sepharose
IMAC	immobilised metal affinity chromatography
LCST	lower critical solution temperature
SDS–PAGE	polyacrylamide gel electro-

	phoresis in the presence of sodium dodecylsulfate
VCL	*N*-vinylcaprolactam
VI	1-vinylimidazole
(VCL–VI) copolymer	co-polymer of *N*-vinylcaprolactam and 1-vinylimidazole

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