ELSEVIER

Journal of Chromatography A, 664 (1994) 13–25

JOURNAL OF CHROMATOGRAPHY A

Immobilized metal affinity membrane adsorbers as stationary phases for metal interaction protein separation

Oscar-Werner Reif[a], Volker Nier[a], Ute Bahr[b], Ruth Freitag*,[a]

[a]*Institut für Technische Chemie, Universität Hannover, Callinstrasse 3, 30167 Hannover, Germany*

[b]*Institut für medizinische Physik und Biophysik, Universität Münster, Robert-Koch Strasse 31, 48149 Münster, Germany*

(First received August 25th, 1993; revised manuscript received November 18th, 1993)

Abstract

Novel immobilized metal affinity membrane adsorbers (IMA-MA) were studied for potential use as stationary phases for protein separation. Protein adsorption on IMA-MA loaded with Cu(II), Ni(II), Zn(II) and Co(II) ions was compared as a function of the flow-rate and the ionic strength of the elution buffer. To exclude the possibility of mixed-mode interaction in the experiments, the binding of proteins similar in terms of hydrophobicity, isoelectric point, size and mass-to-charge ratio but differing in their number of surface histidine residues was investigated. Matrix-assisted laser desorption/ionization mass spectrometry was used to distinguish between these proteins in the eluted fractions. Salt concentration of at least 0.5 *M* NaCl and flow-rates below 2 ml min^{-1} were found suitable to ensure an adsorption mechanism based on affinity interaction between the proteins and the chelated metal ions. In an application study, the IMA-MA and conventional chelating Sepharose fast flow columns were compared for the isolation of a recombinant fusion protein (EcoR V), which carried a polyhistidine sequence (HIS_6-tag) at the N-terminus.

1. Introduction

Immobilized metal affinity chromatography (IMAC) is a sensitive method for protein separation, which allows one to distinguish between proteins differing only by a single histidine residue on their respective surfaces [1]. Starting in 1975, when Porath *et al.* [2] first extended the concept of ligand-exchange chromatography, introduced more than a decade before by Helfferich [3], to the separation of biomolecules, IMAC has been used to separate complex protein mixtures [4–7] and to investigate the surface topography of their histidine residues [8,9]. Moreover, recent advances in genetic engineering may prove to be a great incentive towards the development of efficient IMAC techniques [10–13]. It has become possible to fuse an affinity tag to either terminus of a recombinant protein, and this has been shown to facilitate protein purification considerably. While larger tags such as protein A or glutathione-S-transferase have to be cleaved off to obtain the active and stable protein, a small polyhistidine sequence at the N-terminus of a recombinant protein serves to enhance the affinity of the fusion protein to IMAC phases to a high degree without influencing the protein's integrity and activity overmuch [14,15].

IMAC is based on the interaction of a Lewis acid (electron pair donor), *i.e.*, a chelated metal ion, with an electron acceptor group on the

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(93)E1261-W

surface of the protein. Save for some exceptions, such as the well documented affinity of certain phosphoproteins for Fe(III) ions [16], proteins are assumed to interact mainly through their histidine and, to a lesser extent, their tryptophan residues with the immobilized metal ions. Nitrogen as a Lewis base of intermediate hardness should interact best with Lewis acids of similar polarizability, such as Cu(II), Zn(II), Ni(II) or Co(II). Such ions can be fixed to a chromatographic support through chelating agents such as the terdentate ligand iminodiacetic acid (IDA). The nature of the chelating agent is of great importance. If a large number of the coordination sites of the metal ion are involved, the ion is stably immobilized and bleeding becomes less likely. The affinity to proteins is, however, decreased. Elution in IMAC involves most commonly the lowering of the pH of the mobile phase below 6, which causes protonation of the side-chains of most histidine residues. Competitive elution in a gradient of increasing histidine, glycine or imidazole concentration, elution in a salt or organic modifier gradient and elution of the protein–metal ion complexes with EDTA have also been described.

Compared with other chromatographic methods based on affinity interactions, IMAC is taken to be superior in terms of ligand stability, capacity, recovery of active protein and cost. Columns can be repeatedly stripped and reloaded with metal ions. Compared with other liquid chromatographic (LC) methods, however, examples of practical applications have been few in IMAC. One reason may be the limited theoretical basis currently available for the design of the chromatographic experiment, and another the paucity of available stationary phases for IMAC, compared with, *e.g.*, ion-exchange or reversed-phase chromatography. Traditionally, soft and semi-rigid polysaccharide-based stationary phases carrying IDA chelating groups are used in IMAC. In addition, a few silica- and polymer-based carriers suitable for HPLC applications are available [6,17–20]. The aim of the work described in this paper was to investigate the potential of a new type of stationary phase, namely membrane adsorbers (MA) carrying IDA groups, for protein separation.

2. Experimental

2.1. Chemicals

Proteins were of analytical-reagent grade or better and were purchased from Sigma. Bulk chemicals were obtained from Fluka. Buffer and sample solutions were prepared with deionized water and prefiltered through a 0.2-μm membrane (Sartorius, Göttingen, Germany).

2.2. Stationary phases

Immobilized metal affinity membrane adsorbers (IMA-MA) were obtained from Sartorius. The membranes themselves consisted of a hydrophilic copolymer carrying chelating IDA groups. They are between 170 and 190 μm thick, have an average pore size of 0.45 μm and are stable in the pH range 2–13. Two types of ready-to-use IMA-MA units were available. In the first option, a single membrane with a cross-sectional area of 5.4 cm^2 was encased in a suitable filter holder; in the second option, a stack of five membranes each with a cross-sectional area of 20 cm^2, *i.e.*, a total filtration area of 100 cm^2, was placed in a larger filter holder. The maximum operating pressures of the units were 400 and 700 kPa, respectively. Both filter holders were equipped with male and female Luer lock connectors for easy insertion into the chromatographic systems. New IMA-MA were stored dry at room temperature. Between use the IMA-MA were kept in the appropriate equilibration buffer with 0.001 *M* NaN_3 added as a bacteriostatic agent. For the preparation of the active IMA-MA, 20 ml of a 0.5 *M* solution of the sulphate salt of the metal ion to be immobilized were passed through the unit at a flow-rate of 1 ml min^{-1}. The respective equilibration buffers were used to prepare the loading solution. Prior to the loading step the IMA-MA were washed with pure equilibration buffer. If a decrease in capacity was observed during the experiments, the IMA-MA were stripped of all metal ions by washing with 10 ml of 0.5 *M* EDTA solution at a flow-rate of 1 ml min^{-1}. Reloading was carried out after excessive washing with equilibration buffer as described above.

Chelating Sepharose fast flow (Pharmacia, Uppsala, Sweden) was used to slurry pack IMAC columns (1 cm I.D., various lengths). Three column volumes of water were pumped through followed by 30 ml of 0.1 *M* $NiSO_4$ or $CuSO_4$ solution at a flow-rate of 1 ml min^{-1}. Subsequently the column was flushed with 120 ml of water. The column was equilibrated with three column volumes of the initial buffer before sample injection and washed with methanol–water (1:5, v/v) after use.

2.3. Instrumentation

For the experiments concerning the purification of recombinant EcoR V, the IMA-MA and the chelating Sepharose fast flow columns were integrated into a fast protein liquid chromatographic (FPLC) system (Pharmacia), which consisted of two P 500 pumps, a Model 2141 UV detector, a Superrac fraction collector and an MV7 injection valve. All components were controlled by an LCC 500 unit. Data analysis was carried out using conventional FPLC software. All other experiments were carried out using an HPLC system assembled from a Model 64 pump (Knauer, Berlin, Germany) which allowed flow-rates up to 40 ml min^{-1}, an injection valve (Valco, Houston, TX, USA) and a Model 7215 UV–Vis detector (ERMA, Tokyo, Japan). The gradient was controlled by an Autochrom System 300 and an Autochrom valve box (obtained from ERC, Regensburg, Germany). An SP 4290 integrator (Spectra-Physics, San Jose, CA, USA) and an R4 integrator (Shimadzu, Kyoto, Japan) were used for collection and analysis of the chromatographic data. Protein fractions were collected using a Retriever 500 (ISCO, Lincoln, NE, USA).

2.4. Methods

Protein concentrations were established according to Bradford [21] or Lowry *et al.* [22] using bovine serum albumin (BSA) as a standard protein. Concentrations of metal ions in solution were established by titration with 0.1 *M* EDTA against murexide as indicator [23]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [24] using a laboratory-made apparatus. The activity of EcoR V was determined as follows. EcoR V splits λ-DNA into 22 fragments [25], which can be separated on agarose slab gels and quantified after staining with ethidium bromide [26]; 1 U of the restriction enzyme will split 1 μg of λ DNA within 1 h at 37°C.

To determine the protein-binding capacity of the IMA-MA, human serum albumin (HSA, 0.5 mg ml^{-1}) was loaded on to the metal ion-saturated IMA-MA at a flow-rate of 1 ml min^{-1} until saturation was reached, *i.e.*, until the protein concentration in the eluate was constant again. After washing, the retained protein was eluted and quantified. A 0.025 *M* phosphate buffer (pH 8.0) containing 0.5 *M* NaCl was used for loading and washing. For elution a 0.025 *M* acetate buffer (pH 3.5) containing 0.5 *M* NaCl was used. All chromatographic experiments were repeated three times. The capacity of a given stationary phase for metal ions was established by pumping 0.1 *M* $NiSO_4$ or $CuSO_4$ solution through the unit (flow-rate 1 ml min^{-1}) until saturation. By monitoring the metal ion concentration in the eluate using the methods described above, the amount of metal ions retained on the chromatographic support could be calculated. The larger IMA-MA were used in the capacity measurements. Capacities were calculated as μmol cm^{-2} and mg cm^{-2} for metal ions and proteins, respectively, by dividing the total amount retained on the IMA-MA by the cross-sectional area (20 cm^2).

For matrix-assisted laser ionization/desorption mass spectrometry (MALDI-MS) measurements, a Vision 2000 laser time-of-flight mass spectrometer (Finnigan MAT, Bremen, Germany) was used. Samples were dissolved in acetonitrile–water containing 0.1% trifluoroacetic acid (1:2 v/v) at a concentration of *ca.* 1 mg ml^{-1}. A 10 g l^{-1} solution of 2,5-dihydroxybenzoic acid in the same solvent was used as a matrix. For measurements, 1 μl of sample and 1 μl of matrix were mixed on a metal target and allowed to dry.

2.5. Bacterial culture

Escherichia coli LK 111 λ/pLBM/pUHE/His Th RV (2) was used as the expression system. The strain produces the restriction endonuclease EcoR V with the chelating peptide (CP) His_6 at the N-terminus. Expression is controlled by the lac-promotor. The strain is resistant to the antibiotics ampicillin and chloramphenicol. Strain maintenance was done on agar plates using LB medium (10 g l^{-1} casein peptone, 10 g l^{-1} NaCl, 5 g l^{-1} yeast extract) with an ampicillin concentration of 75 μg ml^{-1} and a chloramphenicol concentration of 30 μg ml^{-1}. *E. coli* cultures were performed in shaking flasks filled with 200 ml of LB medium with similar antibiotic concentrations added. After inoculation, the culture was left to grow overnight at 30°C. Production of EcoR V was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) at concentrations between 0.2 and 2 m*M*. After 2–3 h the bacteria were centrifuged off at 3000 *g* (4°C) and stored at −20°C. Cell lysis was achieved by sonication (5 min, 100 W, 0°C). Subsequently the cell debris was removed by centrifugation at 35 000 *g* (4°C). The supernatant was filtered through a 0.2-μm membrane (Sartorius).

3. Results and discussion

3.1. Characterization of immobilized metal affinity membrane adsorbers

For assessment of the general suitability of the IMA-MA as stationary phases for protein IMAC, the capacities of the IMA-MA for metal ions and proteins were determined. In Table 1 the capacities for Cu(II) and Ni(II) ions, *i.e.*, the two most commonly used ions in IMAC, are compiled, together with the protein capacities established for IMA-MA saturated with the respective metal ions, taking HSA as a probe. The metal ion capacity of the IMA-MA is higher for Cu(II) than for Ni(II). At the same time, more HSA is bound per μmole of immobilized Cu(II) than per μmole of immobilized Ni(II). In all instances capacities found for the 20 cm^2 IMA-MA were sufficiently high for the semi-preparative tasks required in our laboratory. When HSA was repeatedly adsorbed on the IMA-MA and eluted in a pH gradient (pH 8.0–3.5) at a flow-rate of 1 ml min^{-1}, a decrease in capacity was observed after five cycles. After 25 cycles, the capacity had decreased by *ca.* 10%. The effect was seen for both the Cu(II)–IMA-MA and the Ni(II)–IMA-MA systems. In order to prevent the capacity decrease from biasing the experiments, all metal ions were removed from the IMA-MA with an EDTA wash after five chromatographic experiments and the MA reloaded with fresh metal ions during the investigations described below. Under these circumstances, an IMA-MA could be used for several hundred experiments without a decline in performance. The protein recovery was well over 90% and no evidence of unspecific or irreversible protein binding was found.

3.2. Influence of the chelated metal on protein separation

Cu(II), Ni(II), Zn(II) and Co(II) are the most commonly used metal ions in protein IMAC. In order to investigate the influence of the metal ion species on the protein separation power of the IMA-MA, a mixture of proteins that differ in

Table 1
Capacities of the IMA-MA for Cu(II) and Ni(II) ions and capacities of the saturated supports for human serum albumin (HSA)

Species	IMA-MA	Cu(II)–IMA-MA	Ni(II)–IMA-MA
Ni(II)	59.25 μmol cm^{-2}		
Cu(II)	65.75 μmol cm^{-2}		
HSA		1.13 mg cm^{-2}	0.81 mg cm^{-2}

the number of their surface histidine residues (HIS) had to be designed. Cytochrome *c* from tuna heart (t-Cyt *c*, no HIS), cytochrome *c* from horse heart (e-Cyt *c*, 1 HIS) and dog myoglobin (d-Myo, 2 HIS) were chosen. The 20 cm^2 IMA-MA units were used for these investigations. Proteins are most commonly eluted from IMAC phases by protonation of the histidine residues, *i.e.*, by lowering the pH of the eluent to <6. As metal ions are substantially washed out from the chelating phase if the pH is lowered too much, pH gradients were only applied down to a value of 4 in our experiments. Proteins that did not elute under these conditions were eluted in an imidazole gradient, *i.e.*, through the introduction of a competing electron donor. A 5-ml volume of a sample containing a total of 3.5 mg of each cytochrome species and 1.5 mg of d-Myo was loaded on to the IMA-MA at pH 7 and eluted at a flow-rate of 0.5 ml min^{-1} in a two-step gradient of decreasing pH (first step) and increasing imidazole concentration (second step). Fractions of 1 ml were collected and the protein content was determined. On the Cu(II)–IMA-MA only t-Cyt *c*, *i.e.*, the protein without surface histidine residues, appears in the breakthrough, whereas e-Cyt *c* and d-Myo are retained and well separated (Fig. 1a). This corresponds to results published in the literature, where one HIS residue is usually reported as sufficient for the retention on a Cu(II)–IMAC column, and proteins varying by only one HIS residue can be

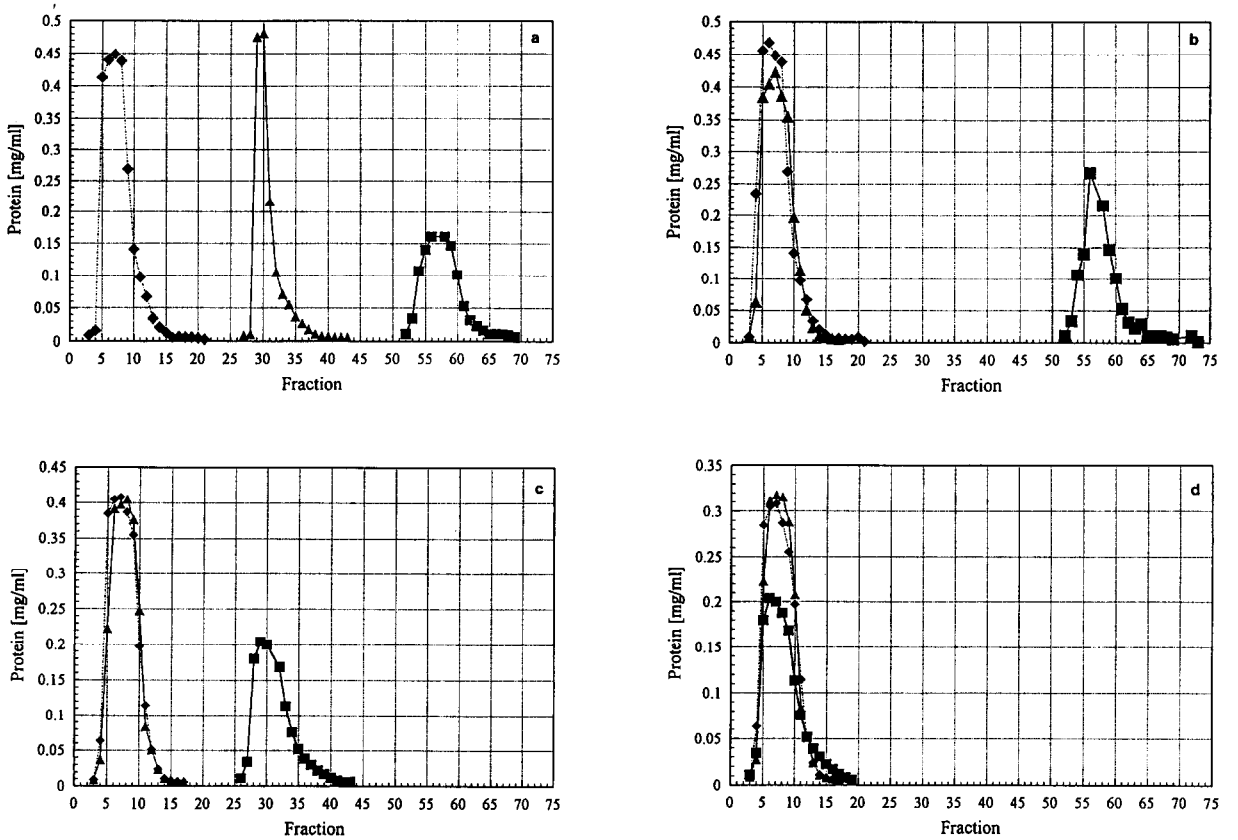

Fig. 1. Separation of proteins on IMA-MA with different chelated metal ions: (a) Cu(II); (b) Ni(II); (c) Zn(II); (d) Co(II). IMA-MA size: 20 cm^2. Buffer A = 0.02 *M* phosphate–0.75 *M* NaCl (pH 7.0); buffer B = 0.05 *M* acetate–0.75 *M* NaCl (pH 4.0); buffer C = 0.05 *M* acetate–0.75 *M* NaCl–0.1 *M* imidazole (pH 4.2). Sample: e-Cyt *c* 3.5 mg, t-Cyt *c* 3.5 mg, d-Myo 1.5 mg. Gradient: fractions 0–24 buffer A, fractions 24–49 buffer B, fractions 49–75 buffer C. Flow-rate: 0.5 ml min^{-1}. Sample volume: 5 ml. ◆ = t-Cyt *c*; ▲ = e-Cyt *c*; ■ = d-Myo.

separated [9,27,28]. Whereas e-Cyt *c* is eluted in the pH-step, d-Myo is desorbed only by introducing the competing electron donor imidazole. On the Ni(II)– (Fig. 1b) and Zn(II)–IMA-MA (Fig. 1c) the two cytochromes appear together in the breakthrough, whereas d-Myo is retained in both instances. However, whereas the elution of d-Myo from the Ni(II)–IMA-MA requires conditions similar to those for its elution from Cu(II) phases, *i.e.*, an imidazole gradient, a simple pH decrease will elute the protein from the Zn(II) phase. A higher affinity of the d-Myo to the Ni(II)– than to the Zn(II)–IMA-MA can thus be assumed. None of the test proteins were retained on the Co(II)–IMA-MA (Fig. 1d). On the whole, the retention behaviour of the test proteins on the IMA-MA mirrors that established for conventional IMAC phases, where the affinity of most proteins towards the immobilized metals follows the order Cu(II) > Ni(II) > Zn(II) > Co(II). Only the fact that not even d-Myo was retained on the Co(II)–IMA-MA is surprising, as proteins with two HIS residues are usually reported to show some affinity to Co(II)-saturated IMAC phases [8].

Some workers, *e.g.*, Sulkowski [8], recommend the addition of 1 m*M* imidazole to the equilibration buffer, especially if an imidazole gradient is to be used for elution in IMAC, as a beneficial influence on the separation efficiency is often observed. In order to establish how such an addition influences the performance of the IMA-MA, t-Cyt *c* and e-Cyt *c* were separated in a pH gradient on a Cu(II)–IMA-MA in the presence of 1 m*M* imidazole in both the equilibration and the elution buffer (Fig. 2). Under these conditions neither t-Cyt *c* nor e-Cyt *c* is retained on the IMA-MA, even though the latter protein had been retained well on the same stationary phase in the absence of imidazole. Apparently, the affinity of e-Cyt *c* for the Cu(II)–IMA-MA is reduced sufficiently to prevent retention in the presence of imidazole.

3.3. *Influence of flow-rate of elution buffer on protein separation*

MA phases have no interparticular void volume. Concomitantly, mass transport through the pores is convective. As a consequence, adsorption equilibria are generally reached quickly and high volumetric flow-rates can be used without impairing the separation efficiency [29]. In addition, MA can be used at higher volumetric flow-rates than conventional LC and HPLC columns, owing to their negligible flow resistance. Fast separations therefore become possible, often cited as a major advantage of MA phases over conventional chromatographic supports. The effect of the volumetric flow-rate on the sample distribution over an MA has already been investigated using a strong ion exchanger MA of a geometric design similar to that of the IMA-MA used in these investigations [29]. The feasibility of using flow-rates of 60 ml min^{-1} and more has been established for protein separations on these ion exchanger MA. The general possibility of fast protein separation as a result of an interaction with immobilized metal ions, on the other hand, has already been suggested by Bonn *et al.* [30], who demonstrated the superiority of micropellicular, *i.e.*, non-porous, supports over conventional porous stationary phases in this respect. The fastest volumetric flow-rates applied in that case were 2 ml min^{-1}. El Rassi and Horvath [6] found the efficiency of chelated metal phases to be lower than expected, which

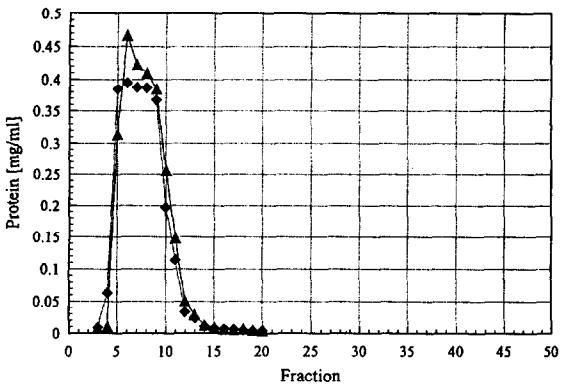

Fig. 2. Separation of proteins on Cu(II)–IMA-MA in the presence of imidazole. IMA-MA size: 20 cm^2. Buffer A = 0.02 *M* phosphate–0.75 *M* NaCl (pH 7.0)–1 m*M* imidazole; buffer B = 0.05 *M* acetate–0.75 *M* NaCl (pH 3.9)–1 m*M* imidazole. Sample: t-Cyt *c* 3.5 mg, e-Cyt *c* 3.5 mg. Gradient: fractions 0–24 buffer A, fractions 24–50 buffer B. Flow-rate: 0.5 ml min^{-1}. Sample volume: 5 ml. ◆ = t-Cyt *c*; ▲ = e-Cyt *c*.

they ascribed to the slow interaction kinetics of the affinity-based adsorptive reaction. Such slow interaction kinetics, however, should limit the maximum applicable flow-rate. A study of the influence of the elution buffer flow-rate on protein separation on the IMA-MA was therefore considered necessary.

A mixture of 0.1 mg each of α-chymotrypsinogen, β-lactoglobulin and lysozyme in 0.02 *M* phosphate buffer (pH 6) was separated in a two-step NaCl gradient (0–0.5 *M* NaCl) on a Ni(II)–IMA-MA at various flow-rates; the 5.4-cm^2 units were used. Protein fractions were collected and analysed by SDS-PAGE. β-Lactoglobulin was not retained under these conditions, α-chymotrypsinogen was eluted in the first salt step (*ca.* 20% B) and lysozyme in the second step, *i.e.*, at 100% B. Fig. 3 shows that an efficient separation could be achieved even at flow-rates of 35 ml min^{-1}, thus decreasing the time required for complete separation to less than 2 min. According to these results, the flow-rate of the elution buffer does not present a limiting factor on the efficiency of protein separations on the IMA-MA. This finding was surprising and considered to require further investigation. Mixed-mode retentions, where the metal–chelate affinity interaction is, *e.g.*, overlaid with a hydrophobic or ion-exchange interaction, are possible with the supports used in protein IMAC. The low salt concentration of the loading buffer used in the experiment above may conceivably have furthered such an ion-exchange interaction. Taking the isoelectric points of the proteins into account, a chromatogram similar to that depicted in Fig. 3 would also ensue if the separation had been carried out on a cation exchanger MA. Moreover, such a separation should indeed be independent of the mobile phase flow-rate in the range examined, as we have recently been able to demonstrate [29].

To investigate this further, e-Cyt *c* and d-Myo were separated at various flow-rates in the presence of at least 0.75 *M* NaCl in the equilibration and the elution buffer, on both Cu(II)– and Ni(II)–IMA-MA. As before, a two-step pH and imidazole gradient was used for elution. At a flow-rate of 1 ml min^{-1} a chromatogram similar

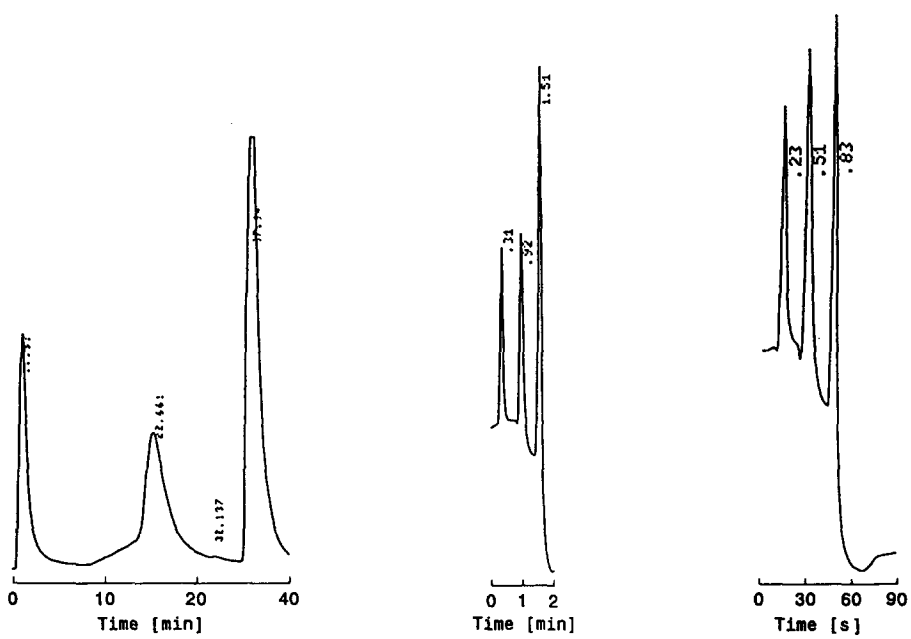

Fig. 3. Separation of β-lactoglobulin, α-chymotrypsinogen and lysozyme on a Cu(II)–IMA-MA as a function of flow-rate. Flow-rate: (a) 1, (b) 15 and (c) 35 ml min^{-1}. IMA-MA size: 5.4 cm^2. Buffer A = 0.02 *M* phosphate (pH 6.0); buffer B = 0.02 *M* phosphate–0.5 *M* NaCl (pH 6.0). Sample: 0.1 mg β-lactoglobulin, 0.1 mg α-chymotrypsinogen, 0.1 mg lysozyme; sample volume, 1 ml. Gradient: step 1, 0% B; step 2, 20% B, step 3, 100% B.

to that shown in Fig. 1a was obtained for the Cu(II)–IMA-MA (Fig. 4a). Whereas a small amount of both proteins appears in the breakthrough, the major part of the e-Cyt *c* is eluted by the pH shift and the major part of the d-Myo by the imidazole step. At 2.5 ml min^{-1} the protein concentration in the breakthrough is much higher than at 1 ml min^{-1} (Fig. 4b). Analysis of the peak fractions by SDS-PAGE revealed that fractions 25–40 contain both e-Cyt *c* and d-Myo. Apparently some d-Myo is already eluted by the pH shift under these conditions. At a flow-rate of 5 ml min^{-1} only d-Myo is retained at all on the IMA-MA (Fig. 4c). However, this protein is now split into two equally sized portions, one of which is eluted by the pH shift (fractions 25–40) and the other by the imidazole step (fractions 52–70). Similar results were obtained when e-Cyt *c* and d-Myo were separated on a Ni(II)–IMA-MA, using a step gradient from 0.02 *M* phosphate buffer (pH 7.0) containing 0.75 *M* NaCl to 0.04 *M* acetate buffer (pH 4.0) containing 0.8 *M* NaCl and 0.1 *M* imidazole. The two proteins were separated well at a flow-rate of 1 ml min^{-1}. At a flow-rate of 2.5 ml min^{-1} only a fraction of the d-Myo was retained, while most of the d-Myo broke through with the e-Cyt *c*.

From the data presented, it can be deduced that the efficiency of some protein separations on the IMA-MA decreases rapidly with increasing flow-rate of the elution buffer, whereas other protein mixtures separate almost independently of this parameter. So far the reason for this difference can only be speculated upon. The salt concentration used in the separations of e-Cyt *c* and d-Myo may have been sufficiently high to suppress an ion-exchange interaction, whereas such an effect may have been operative in the separation of β-lactoglobulin, α-chymotrypsinogen and lysozyme. A definite statement concerning the flow-rate dependence of metal–chelate affinity interaction chromatography would require the study of the separation of two proteins that vary in the number of surface histidine residues, but only insignificantly in their isoelectric point, their hydrophobicity, their ternary structure or their size, as a function of flow-rate.

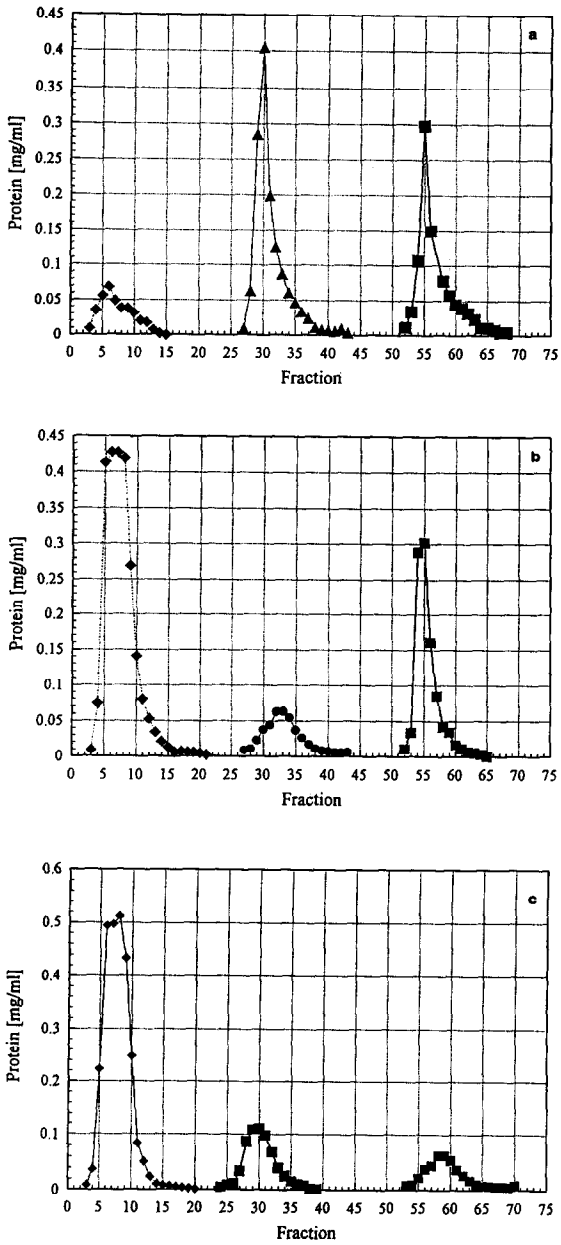

Fig. 4. Separation of e-Cyt *c* and d-Myo on a Cu(II)–IMA-MA as a function of flow-rate. Flow-rate: (a) 1, (b) 2.5 and (c) 5 ml min^{-1}. IMA-MA size: 20 cm^2. Buffer A = 0.02 *M* phosphate–0.75 *M* NaCl (pH 7.0); buffer B = 0.04 *M* acetate–0.8 *M* NaCl (pH 4.5); buffer C = 0.04 *M* acetate–0.8 *M* NaCl–0.1 *M* imidazole (pH 4.0). Gradient: fractions 0–24 buffer A, fractions 24–49 buffer B, fractions 49–75 buffer C. Sample: e-Cyt *c* 3 mg, d-Myo 2.5 mg; sample volume 5 ml. ◆ = Breakthrough; ▲ = e-Cyt *c*; ■ = d-Myo; ● = e-Cyt *c* and d-Myo.

t-Cyt *c* and e-Cyt *c* are two proteins that resemble each other strongly but differ in the number of their surface histidine residues, as pointed out before. Owing to their similarity, however, the determination of the individual concentrations of these two proteins in the collected protein fractions by conventional, *i.e.*, chromatographic or electrophoretic, methods would be difficult. Here recent progress in mass spectrometry becomes significant. New soft-ionization methods allow the ionization and measurement of sensitive proteins *in toto*. As the molecular masses of t-Cyt *c* and e-Cyt *c* differ by about 100 u, the analysis of the fraction composition by MALDI-MS is possible. Thus, the separation of t-Cyt *c* and e-Cyt *c* on a Cu(II)–IMA-MA with a pH step gradient as a function of flow-rate was investigated and MALDI-MS was used to analyse each peak composition.

At a flow-rate of 1 ml min^{-1} two protein peaks were collected, one in the breakthrough and the other during the pH gradient. According to the MALDI mass spectra, only t-Cyt *c* is found in the breakthrough fractions (Fig. 5a), e-Cyt *c* being retained on the IMA-MA (Fig. 5b). If the flow-rate is raised to 2.5 ml min^{-1}, again two protein peaks are observed in the chromatograms. However, a mixture of t-Cyt *c* and e-Cyt *c* is now found in the breakthrough, according to the MALDI mass spectrum (Fig. 5c). Still, significant amounts of e-Cyt-*c* are retained on the IMA-MA and eluted by the pH step. The trend becomes more pronounced if the flow-rate is raised to 5 ml min^{-1}. Under these circumstances, only a small portion of the e-Cyt *c* is retained on the IMA-MA at all, while most of this protein co-elutes with the t-Cyt *c* in the breakthrough. It must therefore be assumed that the flow-rate limit for protein separation on IMA-MA phases is indeed considerably lower than that determined for MA used as stationary phases in other, less specific, chromatographic techniques.

3.4. *Influence of ionic strength of elution buffer*

Ion exchange-based interactions seem to be of consequence in protein separations on IMA-MA

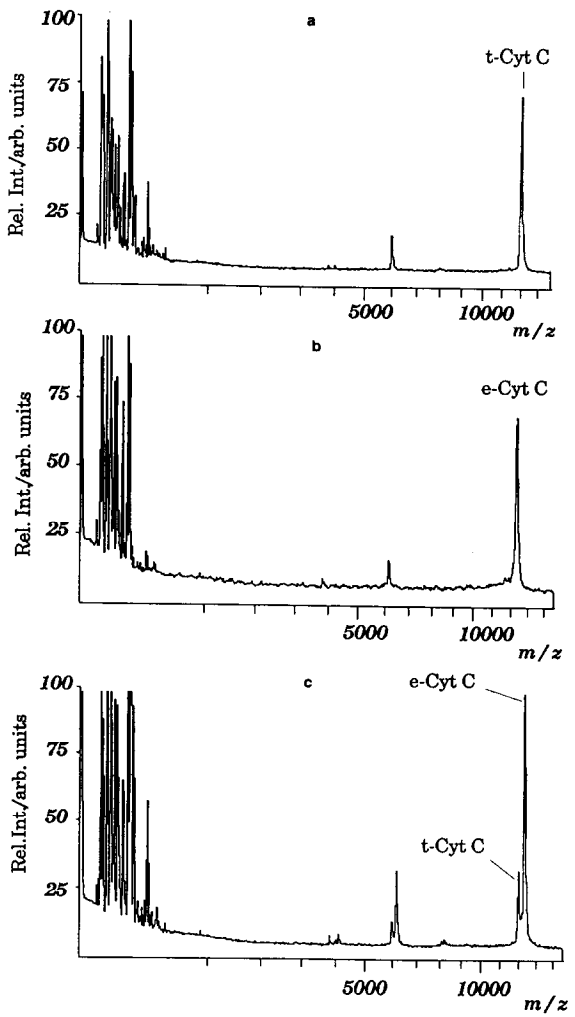

Fig. 5. (a) Mass spectrum of the protein-containing fractions of the breakthrough collected during the separation of t-Cyt *c* and e-Cyt *c* on a Cu(II)–IMA-MA at a flow rate of 1 ml min^{-1}. The singly and doubly charged molecules of t-Cyt *c* are detected. IMA-MA size: 20 cm^2. Buffer A = 0.02 *M* phosphate–0.75 *M* NaCl (pH 7.0); buffer B = 0.05 *M* acetate–0.75 *M* NaCl (pH 4.2). Gradient: fractions 0–24 buffer A, fractions 24–50 buffer B. Sample: 3-Cyt *c* 2.5 mg, t-Cyt *c* 3.5 mg; sample volume, 5 ml. (b) Mass spectrum of the protein-containing fractions eluted by the pH gradient during the separation of t-Cyt *c* and e-Cyt *c* on a Cu(II)–IMA-MA at a flow-rate of 1 ml min^{-1}. The singly, doubly and triply charged molecules of e-Cyt *c* are detected. Separation conditions as in (a). (c) Mass spectrum of the protein-containing fractions of the breakthrough collected during the separation of t-Cyt *c* and e-Cyt *c* on a Cu(II)–IMA-MA at a flow-rate of 2.5 ml min^{-1}. The singly, doubly and triply charged molecules of t-Cyt *c* and e-Cyt *c* are detected. Separation conditions as in (a).

phases. The influence of the ionic strength of the elution buffer therefore had to be investigated. A high salt concentration should suppress the ion exchange mechanism by shielding charges present on either the proteins or the support. However, at too high a salt concentration, hydrophobic interactions between the proteins and the support may occur [6,7]. For the purpose of these investigations t-Cyt *c* and e-Cyt *c* were separated on Cu(II)–IMA-MA in a pH step gradient. Various amounts of NaCl were added to the buffers. The composition of each protein peak was again analysed using MALDI-MS. If a minimum of 0.5 *M* NaCl was added, t-Cyt *c* and e-Cyt *c* were well separated (Fig. 6a). As their average hydrophobicities, isoelectric points and surface charge distributions are nearly identical, the separation can safely be attributed to differences in their affinity to the chelated Cu(II) ions. When the NaCl concentration of the buffers was lowered to 0.1 *M* the chromatogram depicted in Fig. 6b ensued. Again, two separate peaks are present. However, according to the MALDI-MS measurements, neither peak consists of a pure protein. In fact, there seems to be no difference in the retention behaviour of the two cytochrome *c* species under these conditions, which strongly argues for an ionic interaction between the proteins and the stationary phase, rather than an interaction based on the affinity for the chelated metal ions. A similar picture emerged when no NaCl was added to the buffer (Fig. 6c). The major portion of both the t-Cyt *c* and the e-Cyt *c* was retained on the stationary phase, presumably owing to an ion-exchange mechanism, and eluted in the pH gradient. According to the MALDI-MS measurements, no separation takes place between the proteins and their elution profiles are identical.

3.5. Application of IMA-MA to the purification of $(HIS)_6$-tagged EcoR V

The isolation of recombinant proteins that carry a polyhistidine tag is currently perhaps the most promising application of IMAC. Even trace amounts of tagged proteins can be removed from complex cell lysates in a single-step operation. As an example of a successful application of the

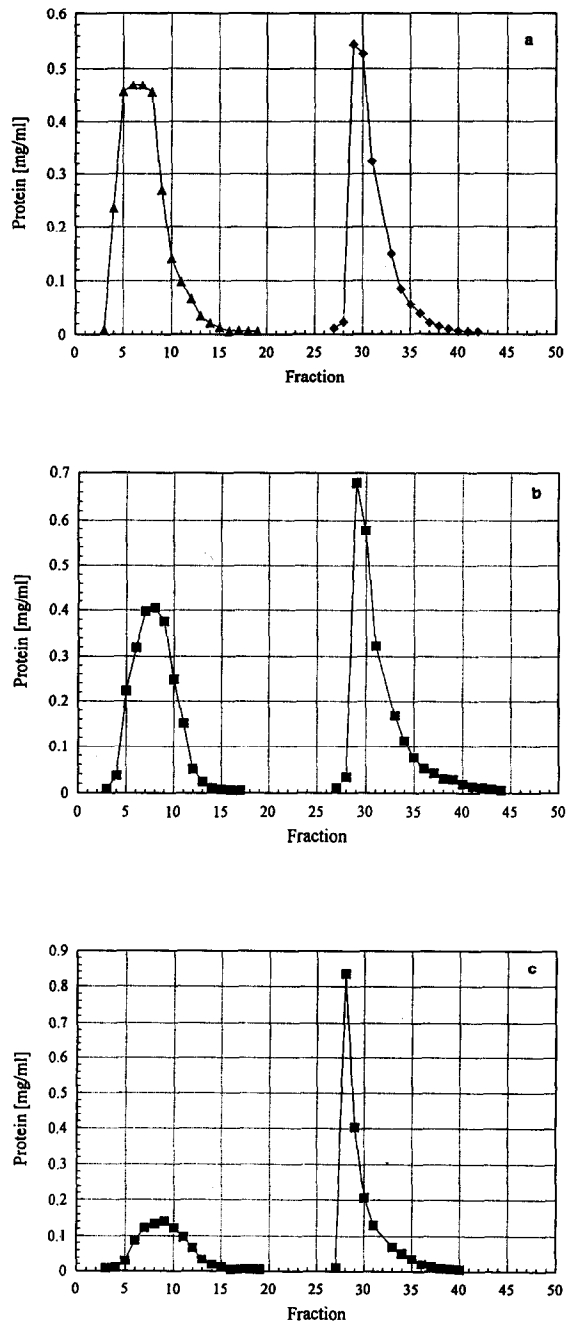

Fig. 6. Separation of e-Cyt *c* and t-Cyt *c* on a Cu(II)–IMA-MA with different NaCl concentrations in the buffer: (a) 0.5, (b) 0.1 and (c) 0 *M* NaCl in buffers A and B. IMA-MA size: 20 cm^2. Buffer A = 0.025 *M* phosphate (pH 7.0); buffer B = 0.05 *M* acetate (pH 4). Gradient: fractions 0–24 buffer A, fractions 24–50 buffer B. Sample: e-Cyt *c* 2.6 mg, t-Cyt *c* 3.2 mg; sample volume, 5 ml. Flow-rate: 1 ml min^{-1}. ▲ = t-Cyt *c*; ◆ = e-Cyt *c*; ■ = t-Cyt *c* and e-Cyt *c*.

IMA-MA, the 5.4-cm^2 Ni(II)–IMA-MA units were used to isolate $(HIS)_6$-tagged EcoR V, an *E. coli* restriction endonuclease, from cell lysates of *E. coli*. The results obtained with the IMA-MA were compared with those achieved with conventional Ni(II)–IMAC columns, made by slurry packing of chelating Sepharose fast flow. The protein capacity of one 5.4-cm^2 Ni(II)–IMA-MA unit was found to be roughly the same as that of 1 ml of swollen gel. Stacks of up to ten MA could be used with a concomittant linear increase in the capacity before the pressure limit of the individual units was surpassed.

For the isolation of EcoR V the filtered cell lysate was loaded on to the stationary phase which had been equilibrated with the initial buffer. Subsequently the support was washed with this buffer until all UV-active, non-adsorbed components had been flushed out. Elution could be achieved in a pH gradient (7.0–5.8), a salt gradient (0.5–1.5 *M* NaCl) or an imidazole gradient (0–1 *M*). Fractions of 1 ml were collected and analysed using SDS-PAGE. The enzyme activity was determined as described above. Fractions collected during the pH and salt gradients contained a number of proteins. EcoR V was not isolated by these operations. This is not surprising, as both of these agents obviate the histidine–metal interaction in an all-or-nothing fashion. The number of histidine residues present is of little consequence under these circumstances. When imidazole is added to the eluting buffer, however, adsorption equilibria are readjusted instead. In this case the number of histidine residues is of great significance. Consequently, EcoR V could be separated from the other *E. coli* proteins in a gradient of increasing imidazole concentration.

When the fractions were checked for co-eluting metal ions by adding dimethylglyoxime, traces of Ni(II) ions were found. By decreasing the final imidazole concentration of the gradient to 0.25 *M*, the washing out could be prevented, while EcoR V was still isolated. Fig. 7a shows the separation on the Ni(II)-loaded Sepharose fast flow column under optimized conditions. According to the analysis by SDS-PAGE, the first peak contains a number of proteins, while the second peak contains one major protein together with one impurity. EcoR V activity is found only in the second peak. As according to the SDS-PAGE analysis the molecular mass of the main protein found in the second peak is *ca.* 30 000, we assumed this protein to be EcoR V. The impurity, which amounts to 13% of the total protein content of the second peak, has a molecular mass of *ca.* 89 000. The total protein content of the *E. coli* cell lysate after centrifugation and filtration was 10.2 mg ml^{-1} and that of the pooled EcoR V containing fractions 0.22 mg ml^{-1}. As a result of the chromatographic purification process, the specific activity was increased by a factor of 33 from $1.36 \cdot 10^4$ to $4.55 \cdot 10^5$ U mg^{-1}.

When a similar linear imidazole gradient was used to isolate EcoR V on a Ni(II)–IMA-MA, the chromatogram shown in Fig. 7b ensued. No distinct protein peaks could be collected. Analysis of the fractions by SDS-PAGE showed, however, that a certain amount of separation did take place, *i.e.*, fractions collected after *ca.* 40 min contained large amounts of EcoR V. Better results in terms of resolution were obtained when the final buffer concentration was reached in a step gradient (Fig. 7c). Whereas a linear gradient has a sharpening effect on the analyte zones in column gradient elution, in MA gradient elution a step gradient is more suitable, or else radial differences in the volumetric flow-rate across the MA will lead to zone broadening. The purities and specific activities of the EcoR V isolated on the IMA-MA were only insignificantly higher than those found for EcoR V purified on the Sepharose column. The IMA-MA were stable for at least 4 months of use. In daily laboratory routine they proved to be less labour intensive than the Sepharose columns, less time being required for washing and equilibration. The capacity of the system was easily adapted to the product concentration determined in the cell lysate by choosing the optimum number of MA units, rather than packing a new column.

4. Conclusions

IMAC is a sensitive and selective method for protein separation. Proteins similar in their

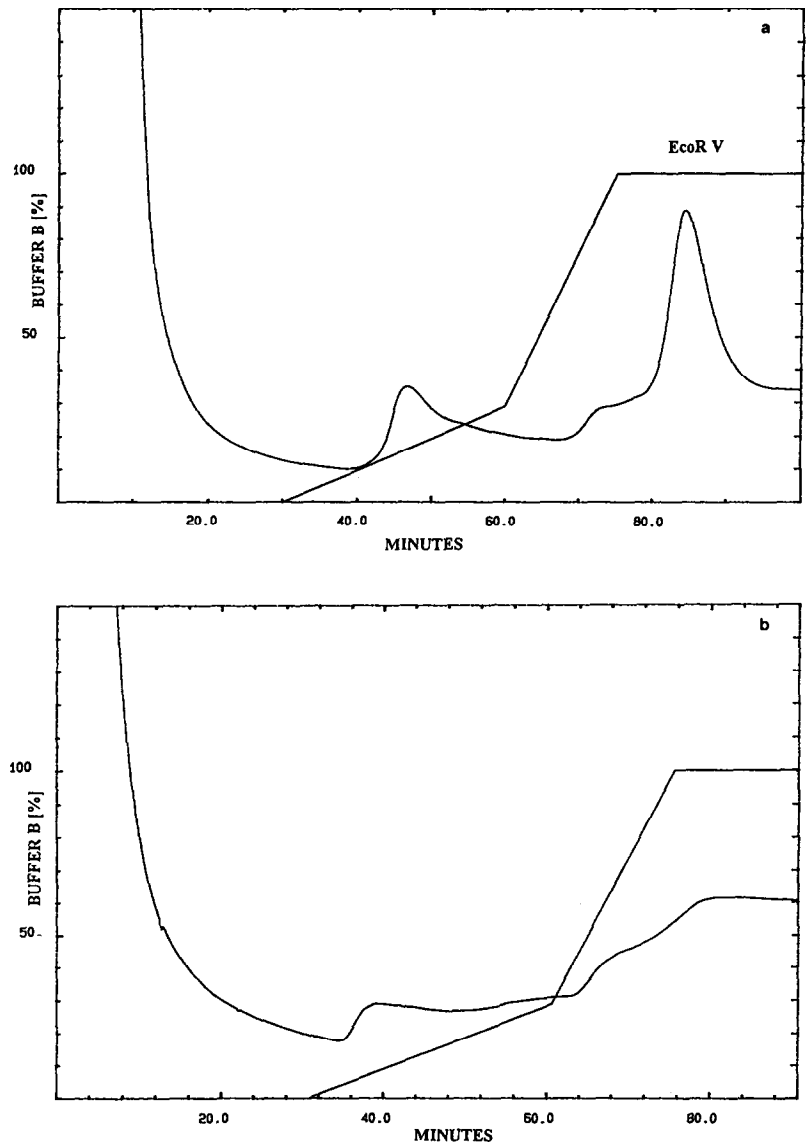

Fig. 7. Purification of $(HIS)_6$-tagged EcoR V by metal–chelate affinity chromatography. (a) Separation on a Ni(II) Sepharose fast flow column; (b) separation on a Ni(II)–IMA-MA using a linear gradient; (c) separation on a Ni(II)–IMA-MA using a step gradient. IMA-MA size: 5.4 cm^2. Buffer A = 0.3 *M* phosphate–1 m*M* dithioerythritol–0.5 *M* NaCl–10 m*M* imidazole (pH 7.0); buffer B = 0.3 *M* phosphate–1 m*M* dithioerythritol–0.5 *M* NaCl–0.25 *M* imidazole. Flow-rate: 1 ml min^{-1}.

physico-chemical parameters can be separated. However, the chromatographic conditions have to be controlled carefully, otherwise mixed-mode retention may become a problem. The membrane absorber units introduced here are interesting alternatives to conventional LC columns in protein IMAC. The IMA-MA are commercially available, comparatively inexpensive units that can be used instantaneously and are easy to handle. Scale-up is extremely simple, as up to ten units can be used in a stack, with a concomitant linear increase in the overall capacity. Conditions can be found where the IMA-MA are similar to standard IMAC columns in terms of

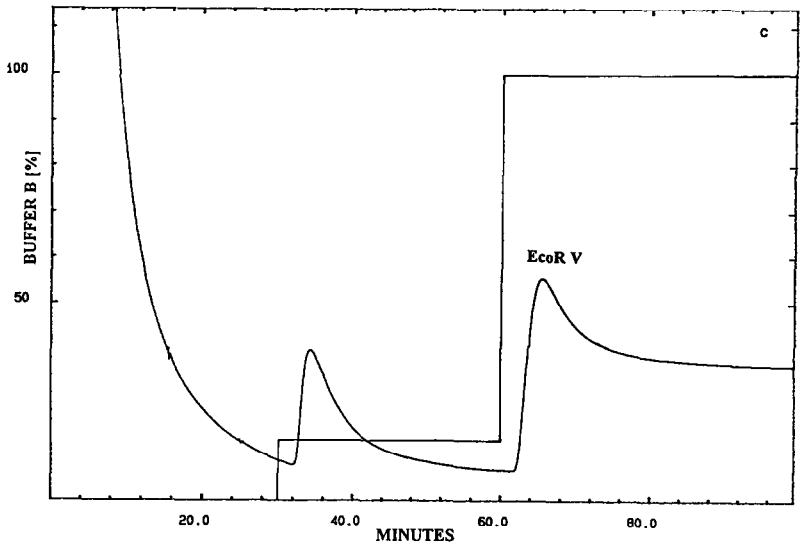

Fig. 7 *(continued).*

resolution and capacity. First attempts to use the IMA-MA for the routine isolation of a biotechnological product have yielded promising results.

5. References

[1] J.W. Wong, R.L. Albright and N.H.L. Wang, *Sep. Purif. Methods*, 20 (1991) 49.

[2] J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature*, 258 (1975) 598.

[3] F. Helfferich, *Nature*, 189 (1961) 1001.

[4] B. Lönnerdal and C.L. Keen, *J. Appl. Biochem.*, 4 (1982) 203.

[5] L. Fanou-Ayi and M. Vijayalakshmi, *Ann. N.Y. Acad. Sci.*, 413 (1983) 300.

[6] Z. El Rassi and Cs. Horváth, *J. Chromatogr.*, 359 (1986) 241.

[7] Z. El Rassi and Cs. Horváth, in K.M. Gooding and F.E. Regnier (Editors), *HPLC of Biological Macromolecules, Methods and Applications*, Marcel Dekker, New York, 1990, pp. 179–212.

[8] E. Sulkowski, *Trends Biotechnol.*, 3 (1985) 1.

[9] E.S. Hemdan, Y.-J. Zhao, E. Sulkowski and J. Porath, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 1811.

[10] R. Gentz, C.-H. Chen and C.A. Rosen, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 821.

[11] T. Moks, L. Abrahmsen, B. Oesterlöf, S. Josephson, M. Oestling, S.O. Enfors, I. Persson, B. Nilsson and M. Uhlen, *Bio/Technology*, 5 (1987) 379.

[12] J. Germio and D. Bastia, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 4692.

[13] H.M. Sassenfeld and S.J. Brewer, *Bio/Technology*, 2 (1984) 76.

[14] E. Hochuli, W. Bannwarth, H. Döbeli, R. Gentz and D. Stüber, *Bio/Technology*, 6 (1988) 1321.

[15] R. Janknecht, G. de Martynoff, J. Lou, R.A. Hipskind, A. Nordheim and H.G. Stunnenberg, *Proc. Natl. Acad. Sci. U.S.A.*, 88 (1991) 8972.

[16] L. Andersson and J. Porath, *Anal. Biochem.*, 154 (1986) 250.

[17] T. Seshadi, U. Kampschulze and A. Kettrup, *Fresenius' Z. Anal. Chem.*, 300 (1980) 124.

[18] T. Seshadi and A. Kettrup, *Fresenius' Z. Anal. Chem.*, 310 (1982) 1.

[19] M. Gimpel and K. Unger, *Chromatographia*, 17 (1983) 200.

[20] H. Takayanagi, D. Hatano, K. Fujimura and T. Audo, *Anal. Chem.*, 57 (1985) 1840.

[21] M. Bradford, *Anal. Biochem.*, 72 (1976) 248.

[22] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, 193 (1951) 25.

[23] G. Jander and E. Blasius, *Einführung in das Anorganisch-Chemische Praktikum*, S. Hirzel Verlag, Stuttgart, 12th ed., 1984, p. 326.

[24] U.K. Laemmli, *Nature*, 227 (1970) 680.

[25] G.V. Kholmina, B.A. Rebentish, Y.S. Skoblov, A.A. Mironov, Y. Yankovskii, Y.I. Kozlov, L.I. Glatman, A.E. Moroz and V.G. Debabov, *Dokl. Akad. Nauk SSSR*, 253 (1980) 495.

[26] K. Hebenbrock, *Ph.D. Thesis*, University of Hannover, Hannover, 1993.

[27] M. Belew, T.T. Yip, L. Andersson and R. Ehrnström, *Anal. Biochem.*, 164 (1987) 457.

[28] T.T. Yip, Y. Nakagawa and J. Porath, *Anal. Biochem.*, 183 (1989) 159.

[29] O.-W. Reif and R. Freitag, *J. Chromatogr. A*, 654 (1993) 29.

[30] G.K. Bonn, K. Kalghatgi, W.C. Horne and Cs. Horváth, *Chromatographia*, 30 (1990) 484.