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Characterization and application of new macroporous membrane ion exchangers

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Abstract

A new ready-to-use unit for high-performance membrane chromatography has been characterized. Its dynamic capacity, resolving power and protein recovery were measured at different flow-rates. The binding capacity was 0.5–2 mg/cm^2 with a 95% recovery at 10 ml/min irrespective of the protein concentration up to 10 mg/ml. For very-high flow-rates (50 and 100 ml/min) the recovery was 90% and 70%. At these flow-rates, the maximum back-pressure was about 0.1 MPa and was independent of the filtration area. By increasing the filtration area, a proportional capacity increase was obtained, indicating an easy scale-up. High flow-rates had only a slight effect on resolution. This new adsorber was able to purify IgM from supernatant of cell culture of a human hybridoma in less than 8 min with a high degree of purity (95%). © 1998 Elsevier Science B.V.

Keywords: Macroporous membrane ion exchangers; Immunoglobulin M

1. Introduction

Since the late 1950s, the liquid chromatography of biomolecules has progressed with the successive use of new supports, such as polysaccharide [1–3], silica [4–7] and organic polymers [8–10]. However, porous beads have deep pores which require long mass transfer times, resulting in band broadening and loss of resolution.

In the late 1980s Unger and coworkers [11,12], Maa et al. [13,14] and Duncan et al. [15] developed non-porous particles for reducing resistance to stagnant mobile phase mass transfer; however, these very small particles produced high back-pressure. A few years later, Afeyan and coworkers [16–19] proposed an alternative to the mass transfer problem with the Perseptive chromatography technique. After modification of their porosity and permeability, the supports exhibited low back-pressure at high flow-rate by combination of convective and diffusive transport modes. This adsorber was followed by hyperdiffusion chromatography using a composite support

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intended to reduce the mass transfer problem [20–23]. Simultaneously, membrane chromatography (membrane adsorber, MA) using hollow fibres and thick discs was introduced and developed [24–39]. This chromatography allows the mass transfer of biological molecules to the membrane surface mainly by convection [39–44]. The advantage of this method compared with high-performance liquid chromatography (HPLC) consists in a lower back-pressure and higher capacity. Tennikova et al. [38] called this technique ''high-performance membrane chromatography'' (HPMC).

Indeed, HPMC seemed to combine the advantages of membrane technology (easy scale-up, low back-pressure) and column chromatography (selectivity, efficiency, capacity). Accordingly, membrane adsorbers have been used in ion-exchange, hydrophobic interaction and reversed-phase modes, and membrane chromatography is now used more in affinity chromatography [28,30,32–34,36,45–51] than the other techniques. Despite promising results and the availability of several membrane adsorbers on the market [43,52–54], membrane technology has lagged behind column chromatography both in analytical and preparative biochromatography. Recently, bioprocessors have begun to introduce membrane adsorbers into bioprocessing [55].

In this paper a new ready-to-use ion-exchange MA is characterized and used for fast and efficient protein separation. Two types of results have been obtained: (i) high flow-rates with only a slight effect on capacity and resolution; (ii) no high back-pressure.

In addition, the adsorption capacity is proportional to the membrane surface and scale-up is easy. This adsorber has been used for fast purification of immunoglobulin M (IgM) from supernatant of cell culture of a human hybridoma, and the good elution conditions allowed the IgM to be kept intact for radiolabelling.

2. Experimental

2.1. Membrane adsorbers

The ready-to-use units S_5, S_{15}, S_{100}, are strongly acidic cation exchangers with the sulfonic group and Q_5, Q_{15}, Q_{100} are strongly basic anion exchangers with quaternary ammonium group. These units were kindly supplied by Sartorius (Palaiseau, France).

The characteristics of the units are described in Table 1.

2.2. Instruments

The chromatographic systems used throughout this study were the FPLC and Biopilot workstations from Pharmacia (Saclay, France). The data were collected and evaluated using the FPLC director and Unicorn 1-12 Data system.

For recovery studies, we used a Uvikon 930 spectrophotometer (Kontron, Montigny Lebretonneux, France) to measure absorbance at 280 nm.

Table 1
Technical specification of MA units

Parameter	MA5	MA15	MA100
Membrane			
pore size: 3–5 μm	Stabilized modified cellulose	Stabilized modified cellulose	Stabilized modified cellulose
Housing	Cyrolite	Polysulfone	Polysulfone
Filtration area[a]	5 cm^2	15 cm^2	100 cm^2
Flow-rate	>100 ml/min×unit	>50 ml/min×unit	>75 ml/min×unit
Max. pressure	0.6 MPa	0.7 MPa	0.6 MPa
Thickness (μm)	250–300	750–900	1250–1500
Layer	1	3	5
Diameter (mm)	25	25	50

[a] 50 cm^2 filtration area=1 ml membrane volume.
Membrane adsorbers used were sulfonic acid group (S) and quaternary ammonium group (Q).

2.3. Chemicals

Lysozyme, ovalbumin, β-lactoglobulin and bovine serum albumin were purchased from Sigma (l'Isle d'Abeau Chesnes, France) and prefiltered using a 0.22-μm membrane filter. Human monoclonal antibody (IgM) was obtained from EBV-transform B cell as described in [56].

Cell cultures were produced with Technomouse bioreactors (hollow fibre technology) according to Integra Biosciences instructions.

All salts were HPLC grade, and the buffers were filtered through a 0.22-μm membrane filter.

2.4. Determination of basic behaviour of Sartobind units

Anion-exchange units (quaternary ammonium functional group) were equilibrated with 50 m*M* Tris–HCl buffer (pH 8.0 for low flow-rate and pH 8.6 for high flow-rate), and proteins were eluted with a step gradient up to 1 *M* NaCl.

Capacity and recovery studies were performed with bovine serum albumin.

Cation-exchange units (sulfonic acid functional groups) were equilibrated with 50 m*M* sodium acetate buffer (pH 5.6 for low flow-rate and pH 4.0 for high flow-rate), and proteins eluted with a step gradient to 1 *M* NaCl.

Lysozyme was chosen for capacity and recovery studies.

Measurements of the sorption capacity of the ready-to-use units for standard proteins were based on breakthrough curves and calculations made at 10% and 50% breakthrough and by the peak collection method.

2.5. Separation properties

Under similar conditions, resolution was determined by using mixtures of pure compounds, ovotransferrin and β-lactoglobulin for anion-exchange chromatography, lysozyme and ovotransferrin for low flow-rate cation-exchange chromatography, and lysozyme and ovalbumin for high flow-rate cation-exchange chromatography. The low flow-rates were 0.13 ml/min to 10 ml/min and high flow-rates were 1.0 ml/min to 100 ml/min. The elution was performed by linear salt gradient.

2.6. Regeneration of units

Between each study, the units were washed with 1 *M* sodium hydroxide without loss of performance.

2.7. Purification of human monoclonal antibody (IgM)

The membrane adsorber used was Q_5 unit, which was equilibrated with 25 m*M* MES pH 7.0, and proteins eluted with a multiple-step gradient to 2 *M* NaCl. Human monoclonal antibody was produced by cell culture in complex media (RPMI 1640, SVF 1%, OPI 1%, Ultroser 1%). After centrifugation, the supernatant was diluted with the equilibration buffer and injected onto the MA.

IgM purification was studied by specific enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot.

3. Results and discussion

3.1. Capacity and recovery

3.1.1. Capacity

To measure the dynamic capacities of the three sizes of membrane adsorber units, the frontal analysis method was used (bovine serum albumin for anion-exchange and lysozyme for cation-exchange, 4 to 5 mg/ml according to the experiments — Fig. 1).

Fifty and 10% breakthrough points were taken to calculate the capacity. Moreover, we also used a peak collection method after step gradient to 1 *M* NaCl. Breakthrough curves were measured at two flow-rates (Fig. 2), and we noticed only a slight fluctuation (7%) for 50% and 10% breakthrough points. However, no fluctuation for peak collection was observed. The capacities of the different units are summarized in Table 2. Under these experimental conditions, the capacities of the units were 2–10 mg for 5 cm^2 filtration area, 10–30 mg for 15 cm^2 filtration area and 70–200 mg for 100 cm^2 filtration area. As 50 cm^2 filtration area corresponds to 1 ml

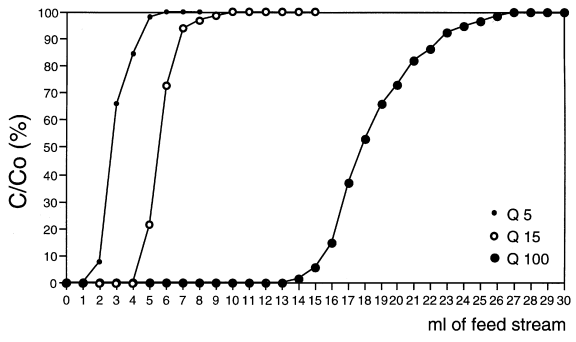

Fig. 1. Solute breakthrough curves with Q_5, Q_{15}, Q_{100} units; 5 mg/ml bovine serum albumin dissolved in 50 m*M* Tris–HCl pH 8; detection at 280 nm; flow-rate 10 ml/min.

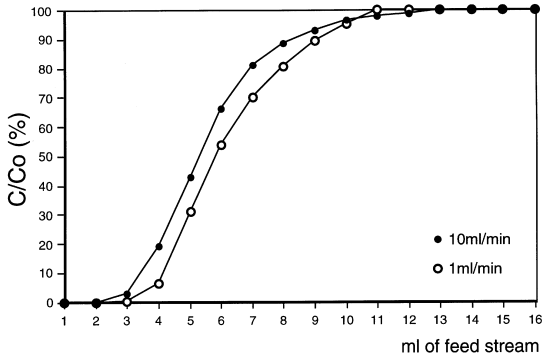

Fig. 2. Solute breakthrough curves with S_{15} unit at two flow-rates (1 and 10 ml/min); 5 mg/ml lysozyme dissolved in 50 m*M* sodium acetate pH 5.6; detection at 280 nm.

membrane volume, we could estimate the capacity of the 1 ml classical gel as being equivalent to 35–100 mg. By increasing the filtration area, a proportional increase in capacity was obtained. This indicated an easy scale-up for preparative chromatography.

3.1.2. Capacity and recovery

In a MA, ionic groups are directly accessible via convective flow for binding biomolecules since they are on the inner surfaces of the membrane pores. In the following experiments we also studied the capacity of MAs and recovery of proteins at high concentration and high flow-rate (10 ml/min).

Increasing volumes of sample at high concentration were injected into the units Q_{15} and S_{15}: bovine albumin (15 mg/ml) for Q_{15} unit (Fig. 3A) and lysozyme (17 mg/ml) for S_{15} unit (Fig. 3B).

The capacity of the Q_{15} unit for bovine serum albumin increased almost linearly up to 10 mg protein and reached a plateau. The recovery remained above 95% up to 10 mg protein and decreased for higher protein amounts.

In the study with S_{15} unit, similar results were observed with a capacity increasing to reach a plateau at 13 mg. Until that point, the recovery was about 95%, and beyond the recovery decreased.

At these concentrations in the feed stream, the capacity was limited to 10 mg. This result was not expected from the breakthrough curve experiment, and may indicate that a flow of protein at high concentration limited the dynamic capacity. We also noticed a high recovery of proteins (95%) up to 10 mg/ml in the feed stream.

3.1.3. Recovery

In these experiments, the S_{15} and Q_{15} ready-to-use units were tested for the same quantity of protein (lysozyme for S_{15} and bovine albumin for Q_{15}) at several concentrations (5 mg/ml; 2.5 mg/ml; 1.25 mg/ml; 0.625 mg/ml). The recovery was obtained by step gradient to 1 *M* NaCl at 10 ml/min. The results are summarized in Table 3. We obtained more

Table 2
Dynamic capacities (mg) of ready-to-use Sartobind units at 50% and 10% breakthrough point and peak collection

Unit	S_5	S_{15}		S_{100}	Q_5	Q_{15}	Q_{100}
Filtration area (cm^2)	5	15	15	100	5	15	100
Flow-rate (ml/min)	10	1	10	10	10	10	10
50% Breakthrough	15	28	26	164	15	30	98
10% Breakthrough	12	20	18	138	11	25	82
Peak collection	5	22	22	95	5	14	78

S_5, S_{15}, S_{100} experiments were performed with 50 m*M* sodium acetate pH 5.6 with step gradient to 1 *M* NaCl for lysozyme.
Q_5, Q_{15}, Q_{100} experiments were performed with 50 m*M* Tris–HCl pH 8.0 with step gradient to 1 *M* NaCl for bovine serum albumin.

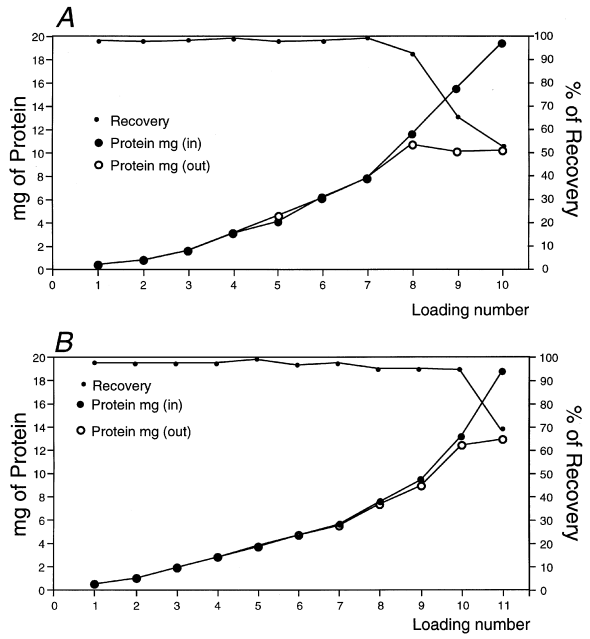

Fig. 3. (A) Capacity and recovery at high protein concentration with Q_{15} unit; 15 mg/ml bovine serum albumin dissolved in 50 m*M* Tris–HCl pH 8; detection at 280 nm; sample volume (25 μl to 1.25 ml). (B) Capacity and recovery at high protein concentration with S_{15} unit; 17 mg/ml lysozyme dissolved in 50 m*M* sodium acetate pH 5.6; detection at 280 nm; sample volume (25 μl to 1.25 ml).

than 95% of recovery for each unit and concentration. This demonstrates that at such ranges of concentration, the recovery did not depend on the protein concentration in the sample.

Using the Q_{15} unit we also studied the effect of high flow-rate on the recovery of proteins during step gradient (0–1 *M* NaCl) elution. One milliliter of 5 mg/ml bovine serum albumin dissolved in 50 m*M* Tris–HCl pH 8.6 was used at various flow-rates (10, 25, 50, 100 ml/min). The results are shown in Fig. 4. The recovery decreased from 99% to 93%, 87% and 70%, respectively at the flow-rates of 10, 25, 50 and 100 ml/min. Such recoveries at high flow-rates demonstrate that the Sartobind unit can be used for fast preparative chromatography.

Table 3
Protein recovery (%) for S_{15} and Q_{15} units

Sample	5 mg/ml	2.5 mg/ml	1.25 mg/ml	0.625 mg/ml
S_{15}	98	97	98	98
Q_{15}	96	98	97	96

Sample: lysozyme for S_{15}; bovine serum albumin for Q_{15}.
Buffer S_{15}: 50 m*M* sodium acetate pH 5.6 with step gradient to 1 *M* NaCl.
Buffer Q_{15}: 50 m*M* Tris–HCl pH 8.0 with step gradient to 1 *M* NaCl.

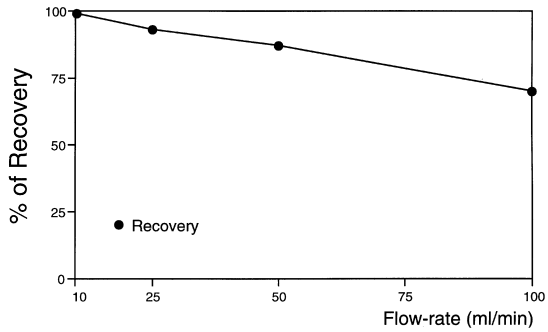

Fig. 4. Effect of the high flow-rate with Q_{15} unit on recovery in step gradient elution; 5 mg/ml bovine serum albumin dissolved in 50 m*M* Tris–HCl pH 8.6; sample volume: 1 ml; buffer A: 50 m*M* Tris–HCl pH 8.6; Buffer B: 1 *M* NaCl in buffer A, detection at 280 nm.

3.2. Resolution

These studies were performed with S_{15} (sulfonic acid group) and Q_{15} (quaternary ammonium group) units with the FPLC workstation for low flow-rate and Biopilot Workstation for high flow-rate.

3.2.1. Resolution studies at low flow-rate (0.13 to 10 ml/min)

Anion-exchange chromatography was performed with a Q_{15} unit with two protein mixtures, β-lactoglobulin and ovotransferrin (ratio: 2.2) (see Fig. 5A). We observed that when the flow-rate increased 80-fold, the resolution decreased by 37%. The resolution decrease was high (26%) for the very low flow-rate (up to 1 ml/min), particularly between (0.13 to 0.25 ml/min) (15%), but between 1 to 10 ml/min the resolution loss did not exceed an additional 16%.

Cation-exchange chromatography was performed with an S_{15} unit with two protein mixtures, ovotransferrin and lysozyme (ratio: 2.3) (see Fig. 5B). In this experiment, the resolution decreased by 15%. The resolution decrease for a very low flow-rate at 0.13 to 1 ml/min was 4% and reached 11% for 1 to 10

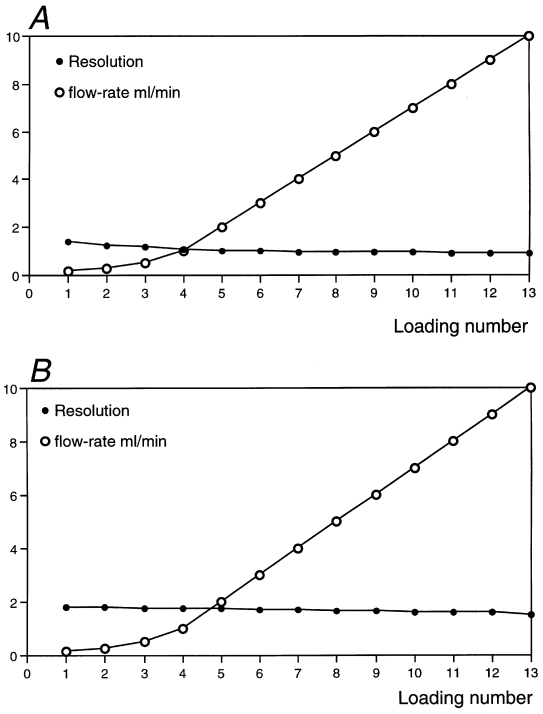

Fig. 5. (A) Effect of separation speed (low flow-rate) with Q_{15} unit on resolution in linear gradient elution; 200 μg of β-lactoglobulin and ovotransferrin were separated using linear salt gradient (40 ml). Buffer A: 50 m*M* Tris–HCl pH 8; buffer B: 0.3 *M* NaCl in buffer A; detection at 280 nm. (B) Effect of separation speed (low flow-rate) with S_{15} unit on resolution in linear gradient elution; 200 μg of ovotransferrin and lysozyme were separated using linear salt gradient (length 32 ml). Buffer A: 50 m*M* sodium acetate pH 5.6; buffer B: 1 *M* NaCl in buffer A; detection at 280 nm.

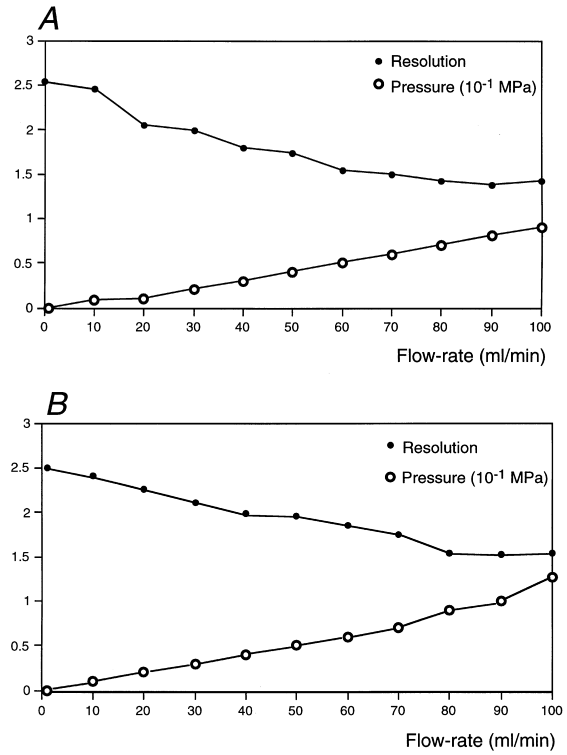

Fig. 6. (A) Effect of separation speed (high flow-rate) with Q_{15} unit on resolution in linear gradient elution and on back-pressure; 200 μg of β-lactoglobulin and ovotransferrin were separated using linear salt gradient (length: 80 ml). Buffer A: 50 m*M* Tris–HCl pH 8.6; buffer B: 0.3 *M* NaCl in buffer A; detection at 280 nm. (B) Effect of separation speed (high flow-rate) with S_{15} unit on resolution in linear gradient elution and on back-pressure; 200 μg of ovalbumin and lysozyme were separated using linear salt gradient (length: 42 ml). Buffer A: 50 m*M* sodium acetate pH 4; buffer B: 1 *M* NaCl in buffer A; detection at 280 nm.

ml/min. In summary, the resolution decreased only by a half unit of the resolution scale, even when the flow-rate increased 80-fold. The effect on resolution decrease was not significant compared with the high increase in the flow-rate.

3.2.2. Resolution study at high flow-rate (1 to 100 ml/min)

Anion-exchange chromatography was carried out with Q_{15} (quaternary ammonium group) unit with two protein mixtures, β-lactoglobulin and ovotransferrin (ratio: 2.2) (Fig. 6A). We observed that when the flow-rate increased 100-fold, the resolution decreased by 40%. For the 1 to 10 ml/min range, the decrease was limited to 4%. For the 10 to 50 ml/min range, the decrease was 25%, and for the range 50 to 100 ml/min, the resolution decreased by 11% with stabilization at 80 ml/min.

Cation-exchange chromatography was performed with an S_{15} (sulfonic acid group) unit with two protein mixtures, ovalbumin and lysozyme (ratio: 1.5) (Fig. 6B). The resolution factor decreased in the same way with identical stabilization at 80 ml/min. In summary, the resolution decreased only by one unit of the resolution scale when the flow-rate

increased 100-fold, with a stabilization at 80 ml/min (1000 cm/h).

For some experiments (data not shown) we found a slight increase in the resolution factor, between 60 and 100 ml/min. This stabilization or slight increase beyond 1000 cm/h has been observed for macroporous beads [18] and for membranes by Tennikova and Svec [44]. They demonstrated an increase in resolution at increased flow-rate (1 to 10 cm/min), ending in stabilization. Our results are slightly different because our experiments were performed up to 20 cm/min using different experimental conditions. This stabilization or increase can be explained by the protein diffusivity which is essentially enhanced by convective flow through the membrane [44]. This concept has already been used in perfusion chromatography [18,19].

When the resolution is higher than unity, the flow-rate can be increased and fast separations achieved (Fig. 7B). Simultaneously, when back-pressure was measured (Fig. 6A,B), the maximum occurred at around 0.1 MPa. This back-pressure was independent of the filtration area since similar results were found with 5 cm^2 and 100 cm^2 filtration areas. The effects of the high flow-rates on resolution and recovery were estimated. Results are illustrated in the chromatograms shown in Fig. 7.

Two extreme flow-rates of 1.0 ml/min and 100 ml/min were used. During these two chromatograms, no modification of the protein behaviour was observed. However, there was a shift to a high salt concentration for elution at 100 ml/min. This effect can be explained by the fact that ions at very high speed have less time for competitive interactions with adsorbed proteins, thus they are released from the membrane at higher NaCl concentrations. We noticed a 30% decrease in the height of the peaks corresponding to the value already described in Section 3.1.

3.2.3. Purification of human monoclonal antibody (IgM)

The chromatogram (Fig. 8) showed seven peaks, one at each step. IgM was detected by specific ELISA in the fourth peak at the 20% step (0.4 *M* NaCl). This result was confirmed by Western blot. SDS–PAGE showed a high degree of purity (95%), and the purification was performed at 10 ml/min for 8 min. This time can be shortened by one step at 10% before and one step at 100% after the step at 20%. Thus, purification could be done in less than 5 min. The good elution conditions allowed the IgM to be kept intact for radiolabelling.

4. Conclusion

The performance of a new membrane ion exchanger was studied. No packing was necessary and drying by bubble did not occur. Reversed-flow was used and sanitization with sodium hydroxide was possible. Analytical and preparative chromatography could be performed with the same unit. In addition, the new membrane ion exchanger which may be installed on any workstation with appropriate connectors has good capacity and offers excellent resolution at a high flow-rate.

The absence of inter-particular voids in the membrane allows the mobile phase to flow through the pores; consequently, this increases diffusivity by convective flow. High flow-rates are obtained with pressure around 0.1 MPa. Scale-up is easy and is obtained merely by enlarging the membrane area.

In comparison with the polymethacrylate membrane developed by Tennikova and Svec [44], the membrane adsorber studied in this paper (for an equivalent surface and diameter) has a higher pore size (3–5 μm instead of $<$0.4 μm). Combined with a lower thickness (250–300 μm instead of 1 mm), this large pore size allows higher flow-rates (100 ml/min) with a lower back-pressure (0.1 MPa). At the flow-rate in the experiments of Tennikova and Svec (10 ml/min), the back-pressure was only 0.01 MPa instead of a value higher than 1 MPa.

This new adsorber provides fast and easy purification of IgM.

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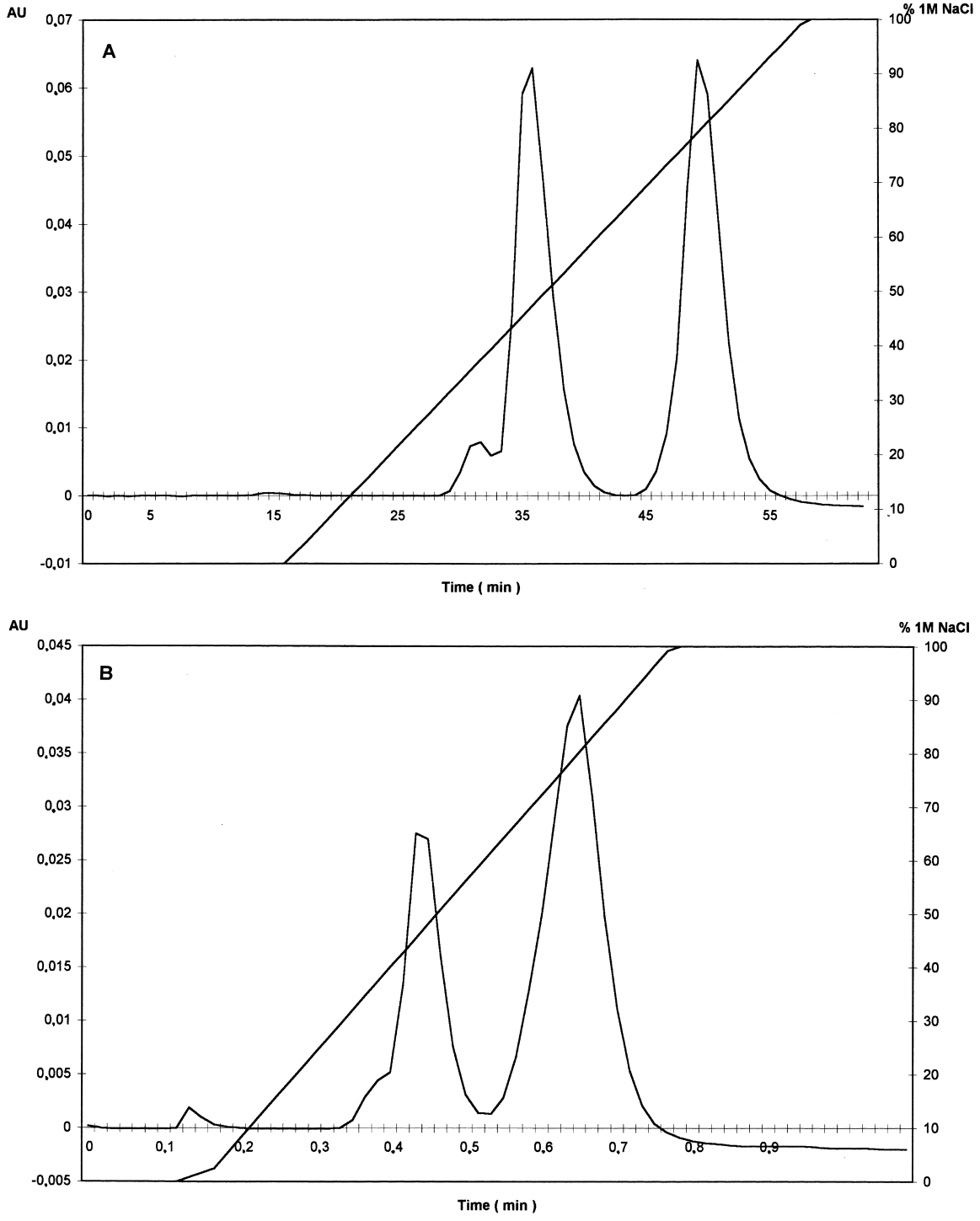

Fig. 7. (A) Chromatographic purification with S_{15} unit of a 200 μg mixture of ovalbumin and lysozyme: sample volume: 200 μl; buffer A: 50 m*M* sodium acetate pH 4; buffer B: 1 *M* NaCl in buffer A (gradient length=80 ml); detection at 280 nm; flow-rate: 1.0 ml/min. (B) Chromatographic purification with S_{15} unit of a 200 μg mixture of ovalbumin and lysozyme; sample volume: 200 μl; buffer A: 50 m*M* sodium acetate pH 4; buffer B: 1 *M* NaCl in buffer A (gradient length=80 ml); detection at 280 nm; flow-rate 100 ml/min.

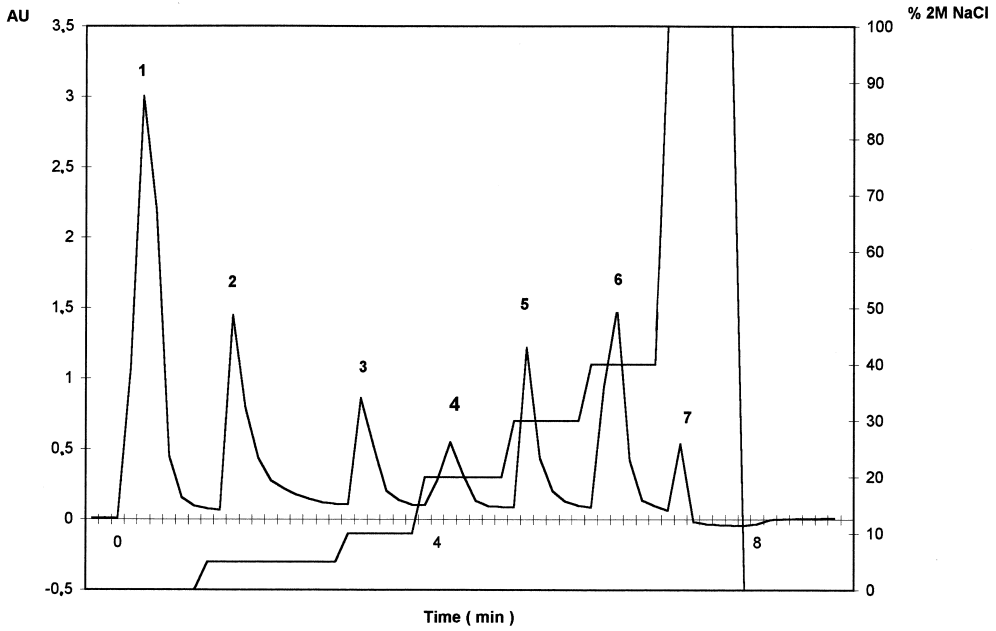

Fig. 8. Chromatographic purification with Q_5 unit of diluted supernatant of cellular culture in buffer A, sample volume: 9 ml; buffer A: 25 m*M* MES pH 7.0; buffer B: 2 *M* NaCl in a buffer A multiple-step gradient; detection at 280 nm; flow-rate 10 ml/min.

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