ELSEVIER

Journal of Chromatography A, 865 (1999) 27–34

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Polycations as displacer in high-performance bioseparation

Beata Schmidt[a], Christine Wandrey[a], Sabine Vogt[a], Ruth Freitag[a,*], Helmut Holzapfel[b]

[a]*Institute of Chemical Engineering, Swiss Federal Institute of Technology Lausanne, 1015 Ecublens, Switzerland*

[b]*ACIMA, 9471 Buchs, Switzerland*

Abstract

Displacement chromatography is an interesting but up to now rarely used type of preparative biochromatography. The lack of well-engineered and accessible displacer contributes to this phenomenon. In this paper a novel type of displacer is introduced for cation-exchange displacement chromatography, which will soon become commercially available. The molecule is a well-defined PolyDADMAC [poly(diallyldimethylammonium chloride)] with a molar mass of less than 35 000 g/mol, an exclusively linear structure and a molar mass polydispersity of less than 1.5. A method for synthesizing such a polymer at high yields is described. The PolyDADMAC is shown to be an efficient displacer of basic proteins from strong cation-exchange columns.

Keywords: Displacement chromatography; Displacers; Proteins

1. Introduction

Displacement chromatography is an independent mode of chromatography, which allows a very efficient separation of multi-component mixtures in the non-linear region of the adsorption isotherm [1]. Contrary to the more commonly known elution mode of chromatography, in displacement chromatography the components are resolved into consecutive zones of the pure substances rather than into ''peaks''. The separation is based on a competition between the sample components for the surface binding places. This competition is enforced during the operation by an advancing displacer front.

Generally, displacement chromatography is considered to have some advantages in comparison to conventional separation techniques [2,3]. In spite of these advantages however, the method has not been widely accepted as a preparative technique at the present. A major reason for this stems from the current lack of well-engineered and commercially available displacers. Although the synthesis and application of various displacers has been reported very few if any of these were actually designed for the purpose of displacement chromatography. A real breakthrough has not occurred.

In order to function as such, a putative displacer should bind more strongly than the substances of interest to the stationary phase. In addition the substance should be non-toxic, stable, cheap and easily detectable. Good solubility in the mobile phase and the possibility of easy column regeneration are additional requirements. In case of biopolymer and especially protein displacement chromatography, it is in addition often assumed that a suitable displacer needs to be a large molecule itself. Thus the combination between a polyionic displacer and an ion-exchange material has become a classic in protein displacement chromatography [4]. Lately small molecules have also been discussed, since it has been shown that stationary phase affinity is much more important in a protein displacer than size [5–

*Corresponding author.

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00809-2

8]. However, such extremely small molecules tend to be very sensitive to the experimental conditions and the switch from displacer to elution agent is abrupt [9].

More recently, a number of ''intelligent'' displacer molecules have been proposed. Freitag et al. have presented a set of thermoprecipitable displacers for ion-exchange and hydroxyapatite chromatography [10]. A somewhat similar approach has been taken by Patrickios et al. [11]. They used group transfer polymerization to produce tri-block polymethacrylates of a dual-charge nature. These polymers show isoelectric points (p*I* values) much like proteins and can be recovered by precipitation at their isoelectric point. While such substances offer intriguing features, their synthesis is involved and they are not likely to become commercially available in the near future.

Considering the requirements for an ideal displacer such as (i) high solubility in aqueous solution even if they contain a variety of different ions, (ii) chemical stability, (iii) defined chemical structure, (iv) easily available on a large scale, and (v) high level of purity and homogeneity, polymers on the basis of the pyrrolidinium structure should be good candidates for cationic displacement chromatography. Such polymers were first prepared in the 1950s from the monomer diallyldimethylammonium chloride (DADMAC) [12]. The kinetics and mechanism of the polymerization process [13–23] as well as the structure of the resulting polyelectrolytes were elucidated in the 1980s [24–26]. In spite of this long-standing investigation, interest in research on poly(diallyldimethylammonium chloride) (PolyDADMAC) polymers has not diminished. On one hand, this is based on its unique chemical structure and on the other hand on its versatile applicability [22,27–37]. PolyDADMAC was, for example, the first polymer to be approved by the US Food and Drug Administration for the use in potable water treatment [38].

The molecule investigated in this paper as putative displacer for cation-exchange displacement chromatography is a novel type of PolyDADMAC (patent pending [39]). Contrary to the commercially available substances of that chemistry this PolyDADMAC is characterized by a homogeneous structure, narrow distribution of the molar mass, and defined affinity to the stationary phase.

2. Materials and methods

2.1. Materials

Proteins, fine chemicals, as well as bulk chemicals for buffer and eluent preparation were from Sigma, Deisenhofen, Germany. Bio-Rad, Munich, Germany, supplied the strong cation-exchange column, UNO S1 (35×7 mm, 1.35 ml, continuous-bed). For reversed-phase liquid chromatography (RPLC) analysis of the displacement fractions, a Hytach C_{18} column (30×4.6 mm) (Glycotech, Hamden, CT, USA) filled with non-porous particles was used.

2.2. Polymer analysis, purification and characterization

The conversion of the polymerization was directly detected by the peak high and/or peak area via gel permeation chromatography (GPC) analysis in 0.2 *M* NaCl aqueous solution using a Shodex OHpak SB-803 HQ column (Showa-Denko, Tokyo, Japan) at a flow-rate of 0.5 ml/min. For the calibration pure monomer was used. With the same equipment and conditions the purity and the molecular parameters were investigated. Additionally, the molar mass was determined by dilution viscometry with a Vicologic TI1 (SEMATech, Nice, France) [37]. Dependent on the quantity of the polymer two ultrafiltration systems were employed for the purification, the Hollow-Fiber Concentrator CH 2A (Amicon, Beverly, MA, USA) and the Pelicon System (Millipore, Lausanne, Switzerland). Following the purification, the polymers were freeze–dried with a Beta 1-16 (Christ, Osterode, Germany).

2.3. Displacement chromatography

The chromatographic system for displacement chromatography was assembled from an ERC HPLC pump 64 (ERC, Alteglofsheim, Germany) and a Valco 10-port valve (Valco, Houston, TX, USA). A 1-ml loop was used for sample injection; the displacer was introduced from a 5-ml preparative sample loop (Knauer, Berlin, Germany). A flow-rate of 0.2 ml/min and a displacer concentration of 20 mg/ml were used. The carrier was a 0.005 *M* phosphate buffer, pH 7.2. Displacement separations were monitored by collecting fractions twice per

minute. Fractions were analyzed by analytical high-performance liquid chromatography (HPLC) as described previously [5]

2.4. Isotherm measurements

Isotherms were measured as described previously [41] using the displacement chromatography system. The buffer was 0.005 *M* phosphate, pH 7.2. The flow-rate was 0.5 ml/min.

2.5. Fraction analysis

The analytical HPLC system was assembled from a degasser ERC-3112 (Ercatech, Bern, Switzerland) and a binary gradient pump (Techlab, Braunschweig, Germany) controlled by a Chromatography Station for Windows (Techlab). Detection (214 nm) was by an SPD-10 UV detector (Shimadzu, Kyoto, Japan) equipped with an 8-μl micro-flow cell (0.1 s filter time). Sample injection (10 μl) was done by an autosampler (Spark Holland, Emmen, The Netherlands). Data collection and interpretation were carried out with the APEX Andromeda chromatography software (Techlab). Buffer B was deionized water with 0.1% (v/v) trifluoroacetic acid (TFA), buffer A was acetonitrile with 0.08% (v/v) TFA added. The gradient was run from 20% B to 60% B (3 min, 1.0 ml/min, 60°C). All components of interest including the displacer were quantified by this method.

3. Results and discussion

3.1. Synthesis of PolyDADMAC protein displacer molecules

As a result of the chosen form of polymerization (cyclopolymerization of DADMAC), all PolyDADMAC molecules possess a backbone of cyclic units [27,40]. Additionally, the highly hydrophilic permanently charged quaternary ammonium group found in each chain unit provides the polymer with a high water solubility and solution properties corresponding to those of strong polyelectrolytes [36,43]. The ideal cyclopolymerization of DADMAC is shown in Fig. 1.

Based on this general scheme the chemical structure and the macromolecular parameters of PolyDADMAC are determined by (i) the ring size of the cyclic units, (ii) portions of cyclic and linear structure units resulting from non-ideal cyclopolymerization or impurities, (iii) extent of branching or crosslinking resulting from the reaction of the pendent double bonds which remain from non-ideal cyclopolymerization and impurities, (iv) the number of chain units (degree of polymerization), and (v) polydispersity of the degree of polymerization.

With the exception of the ring size and the 6:1 ratio of *cis*- and *trans*-substitution all other parameters and characteristics can be influenced and regulated by the synthesis process. As will be demonstrated below, effective polycationic protein displacers have a limited molar mass and should be uniform in structure (low degree of branching) and mass (low polydispersity). However, most of the developed technologies and production processes for PolyDADMAC are oriented towards high-molar-mass polymers. Up to now, the commonly used method to obtain linear (non-branched) polymers with the desired narrow molar mass distribution, was to terminate the radically initiated polymerization reaction at conversions of less then 10%. None of the existing preparations offer a method to obtain, without special fractionation, a PolyDADMAC-type polymer having a low-molar-mass and a narrow molar mass distribution at economical conversions of more than 40%. Here a polymerization procedure is described, which achieves the dual goal of high conversion but low average molar masses and uniform structure. This is possible by maintaining a constant low monomer concentration up to high conversions thereby avoiding side reactions and high polydispersity.

Fig. 2 demonstrates the monomer, polymer and total concentration profiles of the applied polymerization procedure starting with a monomer concentration of 2 mol/l. This monomer concentration is kept constant while the polymer is formed by continuous addition of a highly concentrated (4 mol/l) monomer solution at a rate sufficient to replace exactly the monomer portion inserted into polymers. If low-molar-mass polymers are produced, as it is here the case for the displacer production, the polymerization kinetics is not remarkably changed by this procedure, although both the polymer concentration and the total concentration of monomeric units increase with the conversion. Therefore, uni-

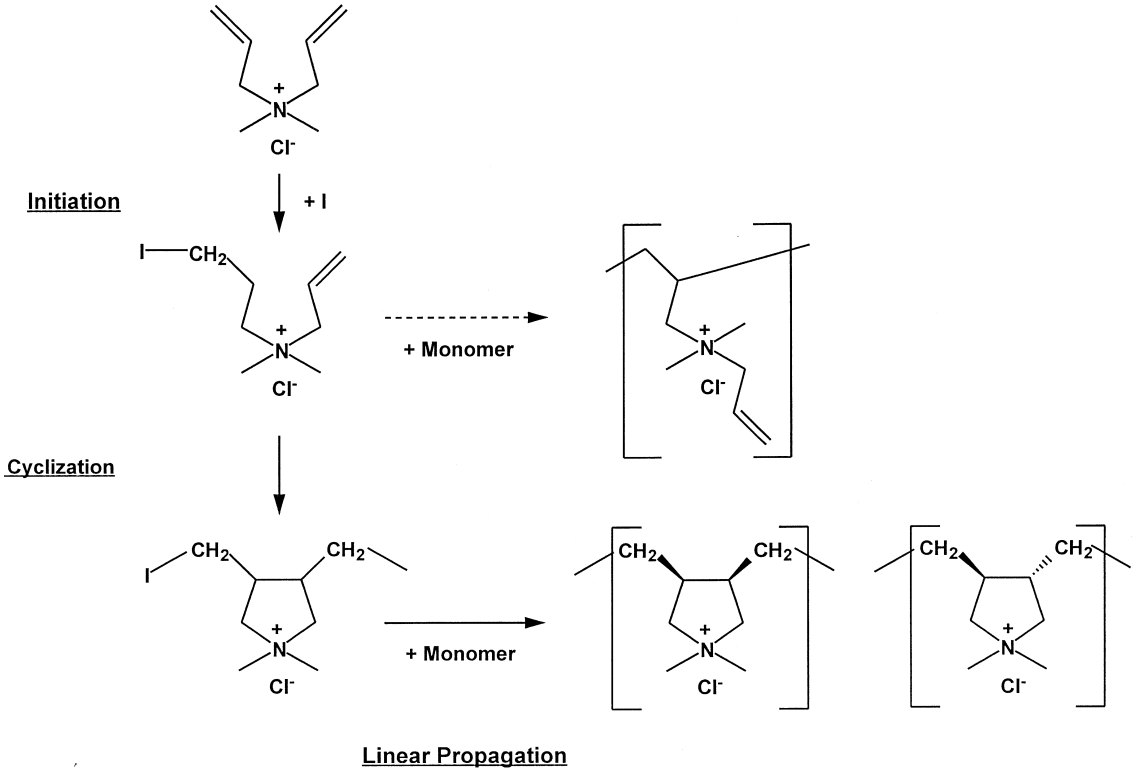

Fig. 1. Ideal cyclopolymerization of the diallyldimethylammonium chloride (DADMAC).

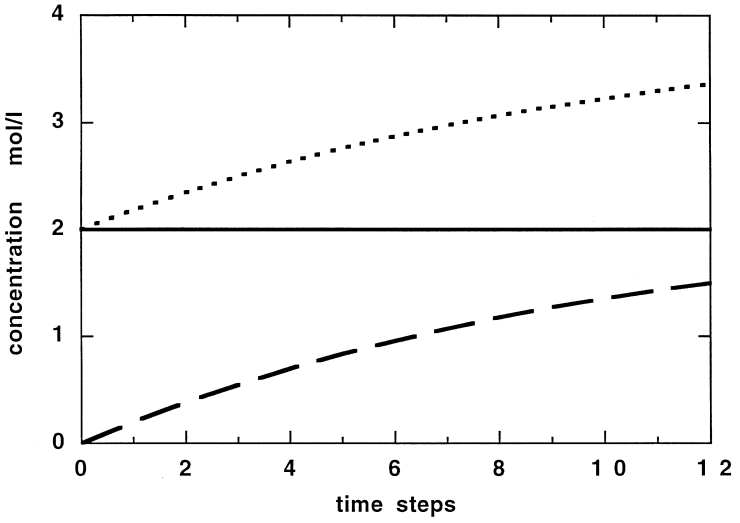

Fig. 2. Principle of the polymerization process. Change of concentrations during the polymerization. — Monomer concentration, - - - polymer concentration, · · · total concentration. The polymer concentration is related to the concentration of monomer units (monomol/l).

form and narrow distributed polymers can be formed up to conversions between 40 and 50%.

Several PolyDADMACs with different but well-defined molar masses between 10 000 g/mol and 40 000 g/mol were prepared according to the principle of Fig. 2. The different samples could be polymerized by variation of the monomer concentration (2 to 3 mol/l), initiator concentration ($1 \cdot 10^{-3}$–$50 \cdot 10^{-3}$ mol/l), time step (0.5–2.0 h) and temperature (35–50°C). A PolyDADMAC with a molecular mass of 200 000 g/mol was also prepared and used for comparison. The process solutions were purified by ultrafiltration (cut-off 3000 g/mol) in order to remove the residual monomer and initiator. The purified solutions were either directly prepared for the use in displacement chromatography (buffer exchange) or the solid polymer was obtained first by freeze–drying. Polymers of defined different molar masses were employed to show the separation performance

3.2. Characterization of the novel displacer molecules

The characterization of the polymers by GPC shows the expected narrower molar mass distribution and high purity, which is not found in the commercially available products. Fig. 3a–c demonstrate that the purification of the polymers by ultrafiltration was successful and that the synthesized polymers have the desired molecular characteristics. The chromatogram depicted in Fig. 3a (reaction mixture before ultrafiltration) shows three main peaks, which were ascribed as follows: (1) PolyDADMAC, (2) NaCl, (3) DADMAC (residual monomer). A fourth, very small peak, can be seen in addition, which is a methodical salt peak. After the purification by ultrafiltration with a cut-off of 3000 g/mol the monomer peak is no longer seen, Fig. 3b. Fig. 3c, by comparison, represents a chromatogram obtained under identical conditions for a low-molar-mass PolyDADMAC produced by a conventional polymerization process. Clearly, the molar mass polydispersity of this preparation is much higher in this case.

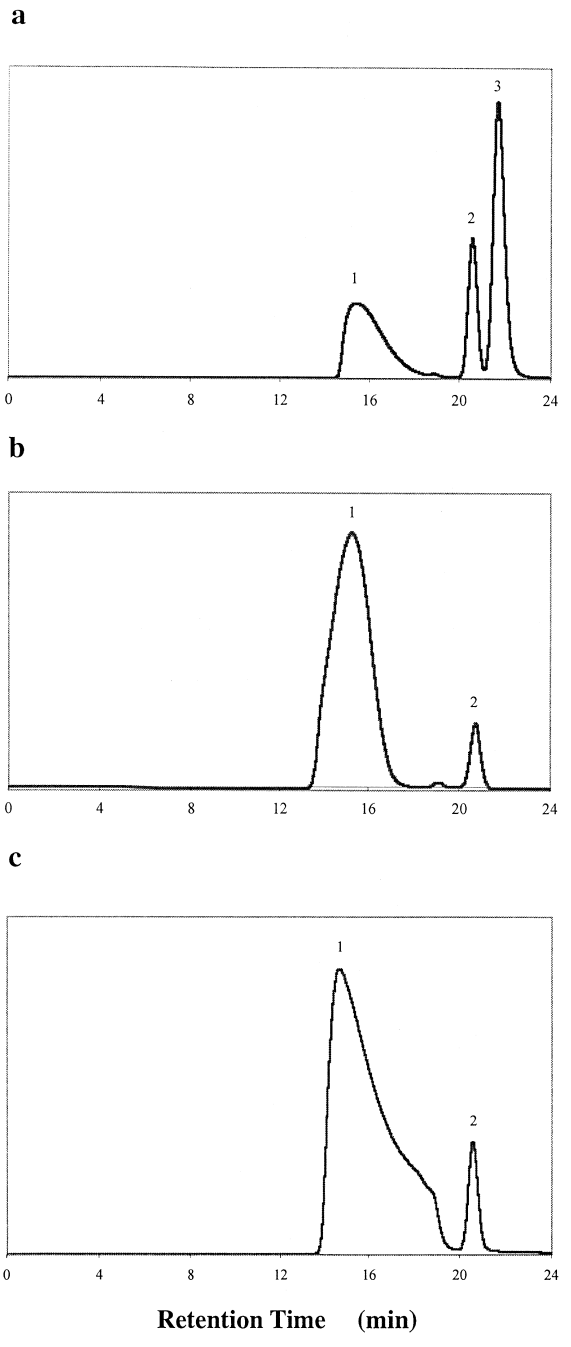

Fig. 3. GPC chromatograms. Peaks: 1=PolyDADMAC, 2=NaCl, 3=DADMAC. (a) PolyDADMAC solution, 40% conversion, before purification. (b) Purified PolyDADMAC (ultrafiltration, cut-off 3000 g/mol). (c) Purified conventional PolyDADMAC (same purification as b).

3.3. Evaluation of the chromatographic properties of PolyDADMAC

PolyDADMAC is a polycation. Each of its chain units carries one charge. The charge density of the molecule is therefore a constant. As a consequence, the range of the ionic interaction is mainly influenced by the ionic strength of the solution, which in turn is determined by the total ion concentration in the system. This offers a unique possibility to investigate the influence of the molecule's size on a displacer's performance. For a first evaluation, PolyDADMAC isotherms were recorded in correlation to those of two proteins, namely lysozyme and cytochrome *c*. Since any suitable displacer should bind more strongly to the stationary phase than the sample components, the displacer isotherm should be above that of either protein. Fig. 4 shows that this is only the case for the isotherm of the PolyDADMAC with an average molar mass of 12 000 g/mol. The isotherm of the PolyDADMAC having a molar mass of 200 000 g/mol, by comparison, is flatter than that of either protein. It can already be deduced from this experiment and related ones that only PolyDADMAC with a molar mass of less than 35 000 g/mol is a possible displacer for the two proteins lysozyme and cytochrome *c*.

This assumption was corroborated for factual chromatographic separations. In order to reduce the possible influence of steric and/or diffusional mass transfer effects on the outcome of these experiments, a novel type of continuous-bed cation-exchange column, the UNO S1 column, was chosen. This stationary phase contains no intraporous space. The entire adsorptive surface should in principle be equally accessible to molecules of different sizes.

However, as demonstrated in Fig. 5 and in accordance with the isotherm measurements, only the PolyDADMAC with the average molar mass of 12 000 g/mol was capable of displacing cytochrome *c* from the continuous-bed cation-exchange column, Fig. 5a. Concomitantly, the protein was concentrated by a factor of 5. With more than 90% the recovery was also satisfactory. During a similar experiment with the larger displacer (PolyDADMAC, 200 000 g/mol), only a small amount of the protein (<10%) did elute at low concentration in the displacer zone. No actual displacement was observed, Fig. 5b.

Since the charge densities of the two PolyDADMACs must be identical, the reason for the different

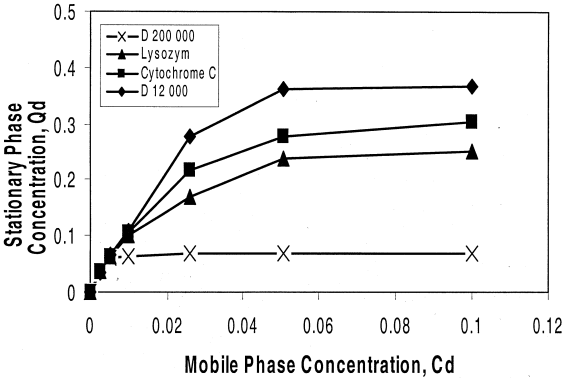

Fig. 4. Single component isotherms recorded for PolyDADMAC (molar masses 12 000 and 200 000 g/mol, respectively) and two basic proteins (lysozyme and cytochrome *c*).

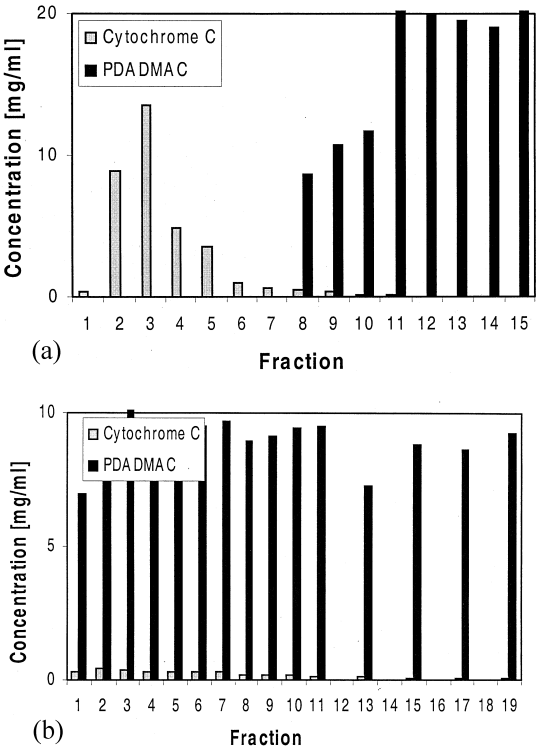

Fig. 5. Displacement of cytochrome *c* using PolyDADMAC as displacer. (a) Molar mass 12 000 g/mol, (b) molar mass 200 000 g/mol. Column: UNO S1, buffer: 0.005 *M* phosphate, pH 7.2, flow-rate: 0.2 ml/min.

capabilities can only be speculated upon. Accessibility of the adsorptive surface cannot be the major cause, since a continuous-bed column was used in the experiments. It is however possible, that the difference is caused by steric hindrance. Under conditions of strong competition a given space recently liberated by a protein molecule (molecular mass cytochrome *c*: 12 500 g/mol), may allow a small PolyDADMAC to interact with all monomer units. The larger molecule on the other hand could interact only with a certain percentage of its units, thus leaving the others to interact with mobile ions from the buffer. It is also known that shorter polyelectrolyte chains posses a more extended chain conformation than longer ones. Therefore, they tend to a flat adsorption on charged surfaces whereas the long polyelectrolyte chains create ''loops'' and ''tails''. As a result such a polyelectrolyte interacts only with a fraction of its charges.

3.4. Application of the displacer for protein separation

In order to investigate whether a small (<35 000 g/mol) PolyDADMAC is indeed capable to separate two basic proteins by cation-exchange displacement chromatography, a separation of cytochrome *c* (p*I* 11.2) and lysozyme (p*I* 10.2) was attempted. Fig. 6 demonstrates that this is indeed the case. Cytochrome *c* is found in pure and concentrated form (by a factor of 5) at the beginning of the displacement train. The substance continues to appear in the following zones, which contain mainly the lysozyme. As a consequence, lysozyme can not be recovered in pure form. It becomes, however, also concentrated. The recovery is again satisfactory (>85%) for both proteins. It must be added that up to this point neither the displacer concentration nor the column length has been optimized. Both are known to influence the success of a displacement separation to a large extent [42].

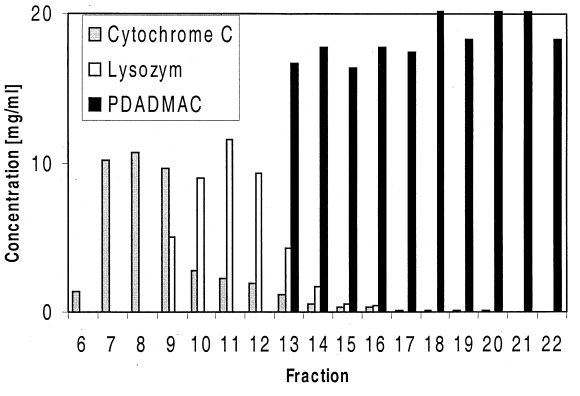

Fig. 6. Separation of cytochrome *c* and lysozyme by cation-exchange displacement chromatography using PolyDADMAC (12 000 g/mol) as displacer. Column: UNO S1, buffer: 0.005 *M* phosphate, pH 7.2, flow-rate: 0.2 ml/min.

4. Conclusions

In view of the results presented in this paper, a better utilization of the possibilities offered by modern polymer chemistry seems to be desirable for the advancement of displacement chromatography. The know-how for the preparation of well suited substances for biopolymer and more specifically protein displacement is in principle available.

Acknowledgements

The development is a cooperation between the Laboratory of Polymers and BioMaterials and the Center of Biotechnology of the Swiss Federal Institute of Technology in Lausanne, Switzerland, and ACIMA, a producer of fine chemicals, also Switzerland. The research is supported by the Commission for Technology and Innovation, CTI (grant No: 3843.1). A. Bartkowiak is acknowledged for the support in GPC.

References

[1] R. Freitag, Nature Biotechnol. 17 (1999) 300.
[2] J.A. Gerstner, BioPharm 9 (1996) 30.
[3] R. Freitag, in: G. Subramanian (Ed.), Bioseparation and Bioprocessing, Biochromatography, Vol. 1, VCH, Weinheim, New York, 1998, p. 89.
[4] S.C.D. Jen, N.G. Pinto, React. Polym. 19 (1993) 145.
[5] R. Freitag, J. Breier, J. Chromatogr. A 691 (1995) 101.
[6] S.D. Gadam, S.M. Cramer, Chromatographia 39 (1994) 409.
[7] G. Jayaraman, Y.-F. Li, J.A. Moore, S.M. Cramer, J. Chromatogr. A 702 (1995) 143.

[8] C. Kasper, S. Vogt, J. Breier, R. Freitag, Bioseparation 6 (1996) 247.
[9] A. Kundu, K. Barnthouse, S.M. Cramer, Biotech. Bioeng. 56 (1997) 119.
[10] R. Freitag, S. Vogt, M. Mödler, Biotechnol. Progr., (1999) in press.
[11] C.S. Patrickios, S.D. Gadam, S.M. Cramer, W.R. Hertler, T.A. Hatton, Biotechnol. Progr. 11 (1995) 33.
[12] G.B. Butler, F.L. Ingley, J. Am. Chem. Soc. 72 (1951) 894.
[13] C. Wandrey, W. Jaeger, G. Reinisch, Acta Polym. 32 (1981) 197.
[14] C. Wandrey, W. Jaeger, G. Reinisch, Acta Polym. 32 (1981) 257.
[15] D.A. Topchiev, G.T. Nashmetdinova, Vysokomol. Soed. A25 (1983) 636.
[16] D.A. Topchiev, G.T. Nashmetdinova, A.I. Kartashewskij, A.V. Netshaeva, V.A. Kabanov, Isv. Akad. Nauk SSR, Ser. Khim. 10 (1983) 2232.
[17] M. Hahn, W. Jaeger, C. Wandrey, G. Reinisch, Acta Polym. 35 (1984) 350.
[18] W. Jaeger, M. Hahn, C. Wandrey, F. Seehaus, G. Reinisch, J. Macromol. Sci. Chem. A21 (1984) 593.
[19] V.A. Kabanov, D.A. Topciev, Vysokomol. Soed. A30 (1988) 675.
[20] P.C. Huang, K.H. Reichert, Angew. Makromol. Chem. 162 (1988) 19.
[21] P.C. Huang, K.H. Reichert, Angew. Makromol. Chem. 165 (1989) 1.
[22] W. Jaeger, U. Gohlke, M. Hahn, C. Wandrey, K. Dietrich, Acta Polym. 40 (1989) 161.
[23] W. Jaeger, M. Hahn, C. Wandrey, in: K.H. Reichert, W. Geiseler (Eds.), Polymer Reaction Engineering, VCH, Weinheim, 1989, p. 239.
[24] J.E. Lancaster, L. Bacchei, H.P. Panzer, J. Polym. Sci., Polym. Lett. 14 (1976) 549.
[25] R.M. Ottenbrite, D.D. Shillady, in: E. Goethals (Ed.), Polymeric Amines and Ammonium Salts, Pergamon Press, Oxford, 1980, p. 143.
[26] C. Wandrey, W. Jarger, G. Reinisch, M. Hahn, G. Engelhard, H. Jancke, D. Ballschuh, Acta Polym. 32 (1981) 179.
[27] G.B. Butler, Cyclopolymerization and Cyclocopolymerization, Marcel Dekker, New York, 1992.
[28] G.B. Butler, N.Z. Zhang, in: S.W. Shalaby, C.L. McCormick, G.B. Butler (Eds.), Water Soluble Polymers, ACS Symposium Series, No. 467, American Chemical Society, Washington, DC, 1991, p. 25.
[29] M.F. Hoover, J. Macromol. Sci.-Chem. A4 (1970) 1327.
[30] R.M. Ottenbrite, W.S. Ryan, Ind. Eng. Chem. Prod. Res. Dev. 19 (1980) 529.
[31] G.B. Butler, in: E. Goethals (Ed.), Polymeric Amines and Ammonium Salts, Pergamon Press, Oxford, 1980, p. 125.
[32] F.M. Hoover, H.E. Carr, Tappi 51 (1968) 552.
[33] R. Nicke, Zellstoff Papier 31 (1982) 19.
[34] W. Jaeger, L.T. Hong, B. Philipp, G. Reinisch, C. Wandrey, Zellstoff Papier 28 (1979) 268.
[35] C. Wandrey, W. Jaeger, W. Starke, J. Wotzka, Wasserwirtschaft Wassertechnik 34 (1984) 1.
[36] H. Dautzenberg, W. Jaeger, J. Kötz, B. Philipp, C. Seidel, D. Stscherbina, Polyelectrolytes – Formation, Characterization, Application, Carl Hanser Verlag, Munich, 1994.
[37] C. Wandrey, J. Hernàandez-Barajas, D. Hunkeler, Adv. Polym. Sci. 144 (1999) 123.
[38] Anonymous, Chem. Eng. News, (1968) 46.
[39] R. Freitag, Ch. Wandrey, Quarternary Ammonium Polymer, its Preparation, its Use for Separating Biomolecules (Polycationic Protein Displacers), European Pat. Appl. No: 98810231.5 (date of deposit: 18 March 1998), Int. Pat. Appl. No: PCT/IB 99/00455.
[40] G.B. Butler, R.J. Angelo, J. Am. Chem. Soc. 79 (1957) 3128.
[41] S. Vogt, R. Freitag, Biotechnol. Progr. 14 (1998) 742.
[42] R. Freitag, S. Vogt, J. Biotechnol., (1999) submitted for publication.
[43] Anonymous, Chem. Eng. News, (1968) 46.