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New ion exchangers for the chromatography of biopolymers

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ABSTRACT

A group of new ion exchangers for the separation of biopolymers is described in which the ionic groups are exclusively located on linear polymer chains grafted on the support surface. This arrangement markedly reduces the contact between the analyte and the matrix, thus suppressing unspecific side activities of the support. Further, this "tentacle-like" arrangement of the ionic groups allows ionic interactions between the ion exchanger and the analyte which are impossible with standard-type exchangers for steric reasons. These changes in the interaction mode manifest themselves in appreciable differences in the selectivities of the two types of ion exchangers. In addition, the new type of arrangement of the binding groups avoids distorsions of the analyte which may occur on interacting with the conventional type.

INTRODUCTION

Ion exchangers suitable for the chromatography of functionally active biopolymers consist in general of natural or synthetic polymers or inorganic materials such as silica, which carry defined ionic groups on their surface. Such materials play the most important role in the isolation and purification of biopolymers, especially proteins with and without catalytic properties. Their basic function may be described as an electrostatic binding of counter-ionic analytes and their release at distinct concentrations of a competing salt. Despite this simple concept, a comprehensive formal treatment, *e.g.*, relating the eluting salt concentration to molecular parameters, is not easy.

The importance of ion-exchange matrices in downstream processing and special fractionations of proteins and nucleic acids is uncontested and they are widely accepted as very potent tools in research and production. However, in order to exert their full potential, they should possess the following properties: (i) the basic support material should be highly inert, *i.e.*, essentially free from non-specific side affinities; (ii) the support should be highly porous with pore sizes large enough to allow larger analytes to freely penetrate the support particles; the internal volume and the surface area accessible to the analyte should be large enough to provide an acceptable binding capacity; and (iii) the support should be rigid enough to allow its application at

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flow-rates high enough to avoid substantial degradations by proteases and nucleases. Although these demands seem reasonable, there are very few materials available that rigorously fulfil all three requirements.

In this paper it is shown that, apart from the basic properties listed above, the arrangement of the ionic groups on or in the ion exchanger seems to be more important than generally expected. In a standard ion exchanger the ionic groups are fixed via short arms on the support surface, thus forming a rigid array of binding sites for the poly-counter-ionic analyte. This implies that the analyte may be distorted during the process of maximizing the number of ion pairs formed between the ion exchanger and the analyte. It is obvious that this process may be of special relevance at low ionic strength at which the electrostatic effects are sufficiently large, *i.e.*, under conditions under which the analyte is bound. The extent to which such a distortion is reversible or able to induce denaturation or irreversible binding by turning hydrophobic areas to the outside is difficult to establish. One can avoid this effect, however, by fixing the ionic groups on linear polyelectrolytes bound to the support surface. This is partially realized in ion exchanger consisting of cross-linked polysaccharide strands to which the ionic groups are bound, or in silica particles that are coated with ionic polymers and cross-linked in an additional step[1,2].

In both instances the true mobility of the groups as provided by the flexibility of the cross-linked polymer chains can hardly be estimated. A maximum motional freedom of the groups is guaranteed for the ion exchangers described in this paper because the polymer chains carrying the charges are grafted on the support surface in the absence of cross-linkers. Preliminary notes on these materials have been presented recently[3,4].

EXPERIMENTAL

All buffer components were analytical-reagent grade materials obtained from E. Merck (Darmstadt, F.R.G.). The water used was deionized with a Milli-Q system equipped with an additional UF filter (Millipore).

The test proteins used were as follows: chymotrypsinogen A (E. Merck; Cat. No. 2306), cytochrome *c* (E. Merck; Cat. No. 24804), lysozyme (E. Merck; Cat. No. 5281), conalbumin (ovotransferrin) (Serva, Heidelberg, F.R.G.; Cat. No. 17466), ovalbumin (Sigma, St. Louis, MO, U.S.A.; Grade VI, Cat. No. A-2512) and human serum albumin (Behring-Werke, Marburg, F.R.G.; ORHA 20/21).

The plasmid digest pBR 322 × HpaII was obtained from E. Merck (Cat. No. 6117); the mixture of the 2.99 kb (kilobase pairs) and 7.8 kb DNA fragments was a gift from Dr. Reiffen (E. Merck).

A fast protein liquid chromatographic (FPLC) system (Pharmacia, Uppsala, Sweden) and a high-performance liquid chromatographic (HPLC) system (Merck–Hitachi, Inert Version, E. Merck, Darmstadt, F.R.G.) were used.

"Conventional" ion exchangers of the Fractogel TSK type were obtained from E. Merck. The "tentacle"-type ion exchangers were prepared as described elsewhere[5]. All these tentacle types [based on Fractogel, EMD types or on silica (LiChrosphers)] are available from E. Merck.

RESULTS AND DISCUSSION

Grafting charged monomers on polymers or modified silica to provide a family of ion exchangers

When a porous chromatographic support consists of a basic material on which vinyl polymer chains may be grafted in sufficient lateral density, ion exchangers of the following types may be formed from the monomers listed:

$CH_2=CHCONH(CH_2)_2N(CH_3)_2$ → "DMAE" type, weak anion exchanger

$CH_2=CHCONH(CH_2)_2N(C_2H_5)_2$ → "DEAE" type, weak anion exchanger

$CH_2=CHCONH(CH_2)_2N^+(CH_3)_3$ → "TMAE" type, strong anion exchanger

$CH_2=CHCOO^-$ → "COO^-" type, weak cation exchanger

$CH_2=CHCONHC(CH_3)_2CH_2SO_3^-$ → "SO_3^-" type, strong cation exchanger

The grafting reaction requires primary or secondary aliphatic hydroxyl functions as initiation sites and uses Ce^{IV} ions as the catalyst; it was developed by Mino and Kaizerman[6] for grafting polymers in a homogeneous phase. It is obvious that the density of the hydroxyl groups on the support surface and the amount of catalyst used determine the density of the grafting. Similarly, the amount of monomer present governs the mean length of the grafted chains. Indirect figures and microscopic studies indicate that chains consisting of a few monomers only are equally accessible as long chains, forming brush-like layers up to 50 μm in thickness. The chain length optimally suited for the functioning of an ion-exchange process will definitely depend on the type of analyte used; according to present experience, chains of 5–50 monomers seem to function properly for proteins and nucleic acids. The fact that 50 monomers correspond to a length of about 10 nm in the fully extended state emphasizes the importance of using supports with sufficiently wide pores; materials with 100–500 nm diameter pores have been found to be adequate.

One might expect the following features for materials prepared properly by the procedure described above: (i) the capacity of the ion exchanger no longer depends exclusively on the surface area of the support; (ii) the analyte barely contacts the support surface and is thus prevented from non-specific interactions; (iii) owing to the high flexibility of the uncross-linked polyelectrolyte chains, the charges can easily adopt a configuration that is optimum for their electrostatic interaction with the analyte, exhibiting a tentacle-like function.

Comparative tests of conventional and tentacle-type ion exchangers and special applications

The experimental verification of the statements made above was possible owing to the availability of ion exchangers prepared from a vinyl polymer (Fractogel) in the conventional way and the polymer-grafting process described above.

Comparative data for the capacities of the two types of ion exchangers are given in Table I. The data were obtained by passing solutions of the test proteins through

TABLE I

BINDING CAPACITIES OF CONVENTIONAL AND "TENTACLE-TYPE" ION EXCHANGERS FOR CERTAIN STANDARD PROTEINS

Ion exchanger	*Protein (buffer)*	*Capacity (mg/ml)*
Fractogel TSK 650(s) DEAE (conventional)	Bovine serum albumin (50 m*M* Tris–HCl, pH 8.0)	30
Fractogel EMD 650(s) DEAE ("tentacle")	As above	70–140
Fractogel TSK 650(s) SP (conventional)	Lysozyme (20 m*M* phosphate, pH 7.0)	55
Fractogel EMD 650(s) SO_3^- ("tentacle")	As above	70–140
Fractogel TSK 650(s) CM (conventional)	Haemoglobin (10 m*M* acetate, pH 5.0)	50
Fractogel EMD 650(s) COO^- ("tentacle")	As above	70–140

small column packings until their saturation became detectable by the breakthrough of the protein front (an increase in the absorbance at 280 nm of more than 0.2). The data indicate that a substantial increase in the capacities may be achieved by the tentacle-type modification.

The reduced non-specific interaction between an analyte and the support resulting from grafting polymer chains on the surface could be observed for two smaller proteins known to stick to gel permeation media owing to their hydrophobicity and basicity.

These proteins, lysozyme and chymotrypsinogen A, elute from a diol-modified support of porous glass (pore size 90 nm) at 1.5 and 0.89 column volumes, respectively, in a medium consisting of 100 m*M* sodium chloride and 10 m*M* phosphate (pH 7). These values exceed the expected values by 95% in the first and by 15% in the second case owing to hydrophobic and ionic interactions of the proteins with the support. After grafting neutral polyacrylamide on the support surface, the elution volumes decreased to 1.2 and 0.78 bed volumes, respectively, which are the correct value for the chymotrypsinogen A and a much improved value for the lysozyme.

The third statement is confirmed by some comparative separations on conventional and tentacle-type ion exchangers. All these separations were performed on ion exchangers prepared from the same support material, the vinyl polymer Fractogel TSK HW 65(s). The conventional (TSK) types carry their ionic groups directly on the surface whereas the tentacle (EMD) types carry them on the grafted polymers only. In all instances columns of 150 × 10 mm I.D. were used.

In Figs. 1 and 2, separations of chymotrypsinogen A, cytochrome *c* and lysozyme (1 mg each) by gradient elution are shown. Fig. 1 shows the separation on strong cation exchangers carrying SO_3 groups. Compared with the conventional type, the tentacle type exhibits a marked increase in selectivity coupled with a clear band sharpening for the lysozyme. When the corresponding pair of weak cation exchangers

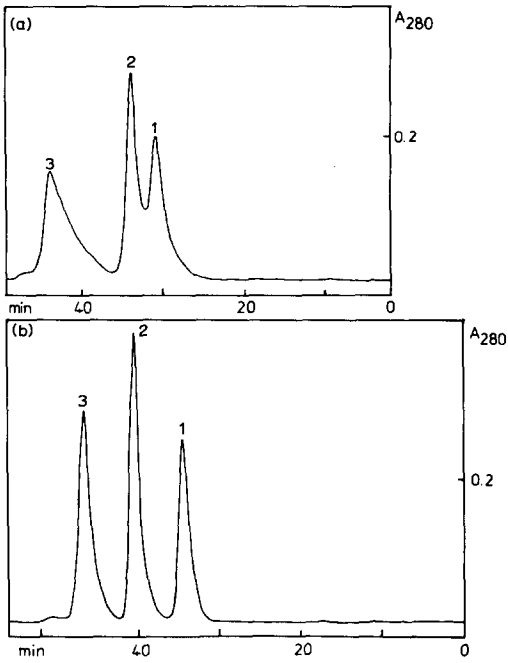

Fig. 1. (a) Fractionation of (1) chymotrypsinogen A, (2) cytochrome *c* and (3) lysozyme on strong cation exchangers. Support, Fractogel TSK 650(s) SP (conventional type); sample, 1 mg each; flow-rate, 1 ml/min; column size, 150 × 10 mm I.D. Solvent A = 20 m*M* phosphate, pH 6.0; solvent B = A + 1 *M* NaCl; gradient, 0–10 min, 0% B; 10–70 min, 0–100% B. Monitor, UV2 (Pharmacia). (b) Fractionation of (1) chymotrypsinogen A, (2) cytochrome *c* and (3) lysozyme on strong cation exchangers. Support, Fractogel EMD 650(s) SO_3^- (tentacle type); conditions as in (a).

with carboxyl groups is used, a very similar effect is observed (Fig. 2); a substantial increase in selectivity is accompanied by a slight band sharpening.

The effects seen with the basic enzymes on cation exchangers are also observed when acidic proteins such as albumins are separated on DEAE-type anion exchangers

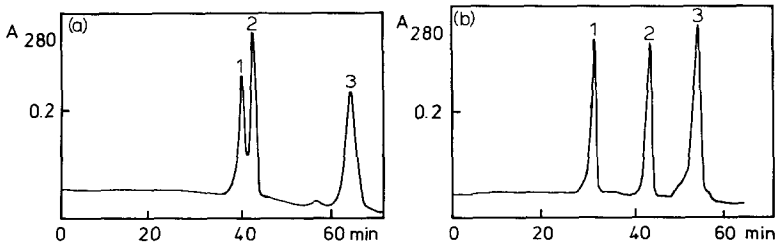

Fig. 2. (a) Fractionation of (1) chymotrypsinogen A, (2) cytochrome *c* and (3) lysozyme on weak cation exchangers. Support, Fractogel TSK 650(s) CM (conventional type); sample, flow-rate and column size as in Fig. 1a. Solvent A = 20 m*M* acetate, pH 5.0; solvent B = A + 1 *M* NaCl; gradient, 0–100% B in 100 min. Monitor, L4200 (Merck–Hitachi). (b) Fractionation of (1) chymotrypsinogen A, (2) cytochrome *c* and (3) lysozyme on weak cation exchangers. Support, Fractogel EMD 650(s) COO^- (tentacle type); conditions as in (a).

by gradient elution. In Fig. 3 the separation of conalbumin, ovalbumin and human serum albumin on a conventional and a tentacle-type anion exchanger is shown. In this instance the selectivity increase due to the tentacle arrangement of the ionic groups is less pronounced; the band sharpening is larger, however.

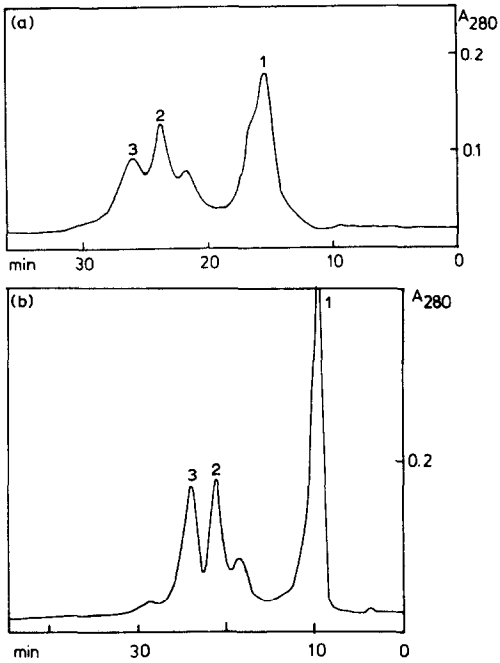

Fig. 3. (a) Fractionation of (1) conalbumin, (2) ovalbumin and (3) human serum albumin on weak anion exchangers. Support, Fractogel TSK 650(s) DEAE (conventional type); flow-rate and column size as in Fig. 1a; sample, 1 mg each. Solvent A = 20 m*M* Tris–HCl, pH 8.0; solvent B = A + 1 *M* NaCl; gradient, 0–100% B in 100 min. Monitor, UV2 (Pharmacia). (b) Fractionation of (1) conalbumin, (2) ovalbumin and (3) human serum albumin on weak anion exchangers. Support, Fractogel EMD 650(s) DEAE (tentacle type); conditions as in (a).

Both effects, sharpening of elution bands and change in selectivity, are most easily explained in terms of the tentacle arrangement of the charges. The band sharpening may be attributed to the reduction of non-specific interactions between the analyte and the support, which eliminates unnecessary kinetic barriers; the change of the selectivity results directly from the fact that tentacles may reach counter-ionic charges in or on the macromolecular analyte, which are not accessible to the surface-fixed charges present in the conventional type of ion exchanger.

The promising results observed for preparative ion exchangers of the tentacle type prompted us to test this structural arrangement of the binding groups also for anaytical materials based on porous silica. In Figs. 4 and 5 two elution profiles with standard test proteins are shown. Comparison of Figs. 1b and 4 reveals that the tentacle-specific selectivity is fully preserved when the matrix is changed.

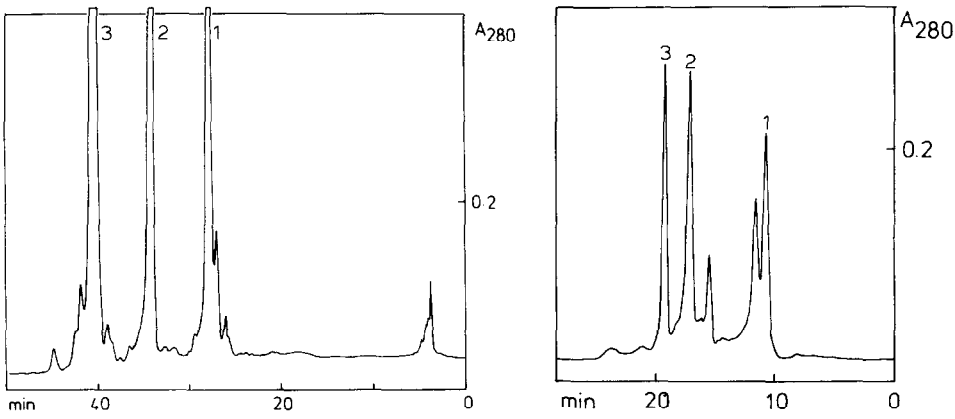

Fig. 4. Fractionation of (1) chymotrypsinogen A, (2) cytochrome *c* and (3) lysozyme (0.5 mg each) on LiChrospher SO_3^- (5 μm) (tentacle type). Column size, 40 × 10 mm I.D.; flow-rate, 1 ml/min; buffers and gradient as in Fig. 1a; monitor, UV1 (Pharmacia).

Fig. 5. Fractionation of (1) conalbumin, (2) ovalbumin and (3) human serum albumin (0.5 mg each) on LiChrospher TMAE (5 μm) (tentacle type). Column size, 40 × 10 mm I.D.; flow-rate, 1 ml/min; buffers and gradient as in Fig. 3a; monitor, UV1 (Pharmacia).

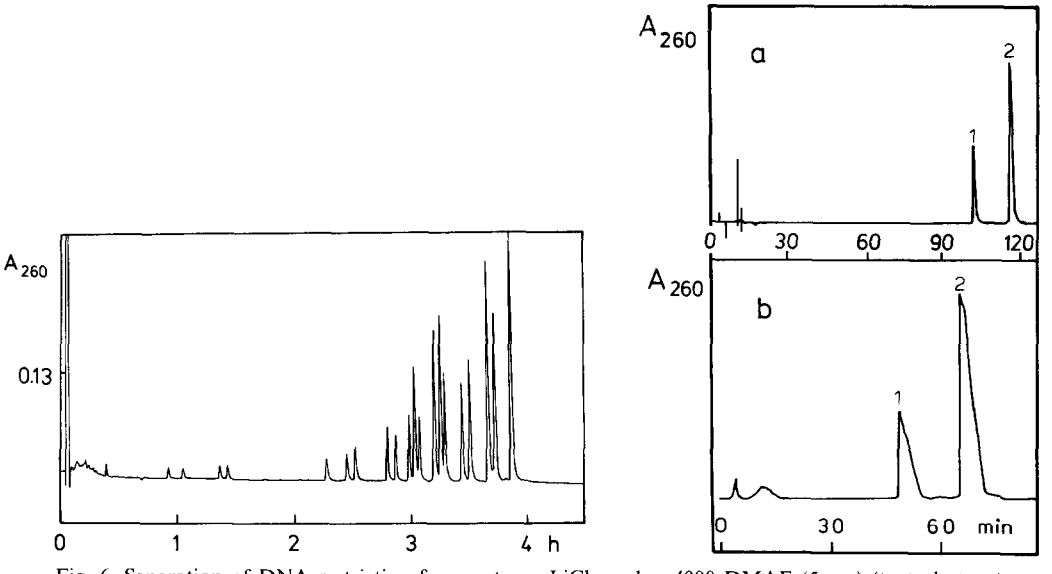

Fig. 6. Separation of DNA restriction fragments on LiChrospher 4000 DMAE (5 μm) (tentacle type). Column size, 40 × 10 mm I.D.; sample, pBR 322 × HpaII (50 μg) containing fragment lengths of 2 × 9, 15, 2 × 26, 2 × 34, 67, 76, 90, 110, 122, 2 × 147, 2 × 160, 180, 190, 201, 217, 238, 242, 309, 404, 527 and 622 base pairs; flow-rate, 1 ml/min; temperature, 23°C. Solvent A = 20 m*M* Tris–HCl + 0.6 *M* NaCl (pH 6.8); solvent B = 20 m*M* Tris–HCl + 1.1 *M* NaCl (pH 6.8); gradient, 400 min, 0–100% B. Monitor, L4200 (Merck–Hitachi).

Fig. 7. Separation of larger DNA fragments on LiChrospher 4000 DMAE (5 μm) (tentacle type). Fragment sizes, (1) 2.99 and (2) 7.8 kb. Solvent: A = 20 m*M* Tris–HCl + 0.4 *M* NaCl (pH 6.8); solvent B = 20 m*M* Tris–HCl + 1.1 *M* NaCl (pH 6.8). Column size, 40 × 10 mm I.D.; flow-rate, 1 ml/min. (a) Analytical run (50-μg sample). Gradient, 0–10 min, 0–57% B; 10–300 min, 57–100% B. (b) Preparative run (450 μg). Gradient, 0–10 min, 0–64% B; 10–250 min, 64–100% B. Monitor, L4200 (Merck–Hitachi).

The most convincing effects were observed, however, when DNA restriction digests were fractionated on a tentacle-type DMAE-LiChrospher (pore size 400 nm, particle size 5 μm). The unexpected degree of resolution shown in Figs. 6 and 7 is not restricted to microgram amounts but also holds for milligram amounts.

CONCLUSIONS

The most interesting properties observed for the tentacle-type ion exchangers are the increased mass transfer rates and the marked changes in selectivity in comparison with the conventional types. The first effect is easy to understand as a result of the reduced non-specific interaction between the analyte and the support matrix, thus providing "cleaner" dissociation kinetics.

The change in selectivity is in perfect agreement with the ion-exchange mechanism for proteins proposed by Kopaciewicz *et al.*[7], according to which an analyte will interact with a conventional ion-exchange matrix only with a certain, distinct area of its surface. In tentacle-type exchangers the flexibility of the charge arrangement allows additional or other electrostatic interactions that should account for the changed selectivity. In this respect, it seems justified to conclude that the action of the tentacle-type ion exchanger involves a new separation parameter, the overall steric distribution of the charges on the analyte.

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