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PHYSICO-CHEMICAL AND CHROMATOGRAPHIC PROPERTIES OF NEW ION EXCHANGERS

I. CM-TRISACRYL M

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SUMMARY

A new synthetic chromatographic cation exchanger, with carboxyl groups particularly well adapted for separating biological macromolecules, is described. It is characterized by a high hydrophilicity and optimal degree of ionic substitution, leading to good protein sorption. Its physico-chemical properties and its chemical and mechanical stability, and the effects of the main chromatographic parameters (gradient slope, column length, flow-rate and pH) on the resolving power and elution molarities of several proteins, are reported.

INTRODUCTION

Ion exchangers for the chromatography of biological macromolecules, especially proteins, are generally ionic derivatives obtained by chemical modification of materials originally conceived for other purposes. Cellulose-based ion exchangers, described for the first time in 1956[1], are a good example. More recently developed ion exchangers, such as those based on agarose, dextran or hydroxyalkyl methacrylate polymers[2,3], are also derivatives of gels used for gel filtration purposes.

This paper describes a new synthetic, hydrophilic carboxylic ion exchanger obtained by copolymerization of three acrylic monomers: a principal monomer which forms the matrix, a monomer with a functional carboxyl group and a bifunctional cross-linking monomer. The principal monomer, which represents the main novelty, is N-[tris(hydroxymethyl)methyl]acrylamide, obtained as described recently[4], using tris(hydroxymethyl)aminomethane as the amine.

Physico-chemical studies of very hydrophilic monomers of the same type were published in 1966 and 1967[5,6]. This monomer is characterized by an easily polymerizable moiety (acrylic double bond) and by a high hydrophilic neutral moiety. Preliminary communications have appeared concerning the polymers obtained from this monomer, especially regarding their applications in affinity chromatography[7,8]. These reports have shown that it is suitable for the chromatography of biological materials and is promising for other applications in various fields of chromatography.

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EXPERIMENTAL

Materials

N-[Tris(hydroxymethyl)methyl]acrylamide was prepared according to Brown *et al.*[4]. Acrylic acid and N,N-diallyltartradiamide were purchased from Aldrich-Europe (Beerse, Belgium) and N,N,N′,N′-tetramethylethylenediamine, ammonium persulphate and Triton X-100 from Fluka (Buchs, Switzerland). Pure proteins (egg white lysozyme, cytochrome *c*, bovine serum albumin, β-lactoglobulin, human immunoglubin G and human haemoglobin), were obtained from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical-reagent grade.

Preparation of the ion exchanger CM-Trisacryl M

CM-Trisacryl M was prepared on the laboratory scale according to the method described in French Patent No. 7,702,391. A 1-l volume of an aqueous solution of monomers was prepared by dissolving N-[tris(hydroxymethyl)methyl]acrylamide at a concentration of 330 mg/ml, acrylic acid at a concentration of 30 mg/ml and N,N′-diallyltartradiamide as a cross-linking agent at a concentration of 40 mg/ml. The pH of the solution was adjusted to 6.5 by addition of 2 *M* sodium hydroxide solution. The temperature of the solution was adjusted to 55°C in a water-bath, and when it was perfectly equilibrated, 120 mg of ammonium persulphate and 1.6 ml of N,N,N′,N′-tetramethylethylenediamine were added. This mixture was immediately emulsified in stirred paraffin oil (2 l). Under these conditions, polymerization occurs to form spherical solid particles with an increase in temperature. The particles were then recovered by decantation, washed with a 1 % aqueous solution of Triton X-100, sieved to obtain the fraction between 40 and 80 μm and finally washed with 1 *M* sodium chloride solution.

This ion exchanger thus obtained is now commercially available from Réactifs IBF, France.

Determination of the pressure–flow-rate relationship

This parameter was studied using pressure-resistant glass columns (I.D. 1.26 cm) filled with CM-Trisacryl M. The chromatographic system consisted of a volumetric pump at the column entrance and a precision manometer mounted in the by-pass between the pump and the column. The flow-rate was progressively increased and the resulting pressure at the column entrance was recorded.

Determination of the practical specific capacity, Q_A (number of carboxylic groups per millilitre)

This determination was performed by the method of frontal analysis[9]. About 10 ml of CM-Trisacryl M were loaded into a 1.6 cm I.D. column and the column was washed with 0.2 *M* hydrochloric acid and then with deionized water until the acid was totally eliminated. Titration was performed by using 0.1 *M* sodium hydroxide solution at a flow-rate of 30 ml/h. The column output was monitored and the curve was recorded on a strip-chart recorder. This capacity was calculated according to the equation

$$Q_A = \frac{(V - V_m - V_0) \cdot [\mathrm{NaOH}]}{V_t} \qquad (1)$$

where

V = volume (ml) of the titration solution used for the assay from the beginning until the inflection point of the curve;
V_m = volume (ml) of the mobile phase or retention volume of a compound that does not interact with the ion exchanger;
V_0 = dead volume (ml);
V_t = total volume (ml) of the ion exchanger;
[NaOH] = concentration (μmole/ml) of the sodium hydroxide solution.

Determination of pK_a of the anionic groups

pK_a values were determined on an experimental curve obtained by determining the breakthrough capacity (Q_B) at different pH values. These determinations were performed by frontal analysis over the pH range 2.5–6.5.

Determination of the protein sorption of the ion exchanger

Experiments were carried out according to the frontal analysis method described above, using protein-buffered solutions instead of sodium hydroxide solution. A column containing 5 ml of ion exchanger was equilibrated in a 16 mM citrate buffer covering the pH range 3–6.5. Experiments were performed with 5 mg/ml protein solutions. All determinations were made at 25°C. The proteins used for these determinations were cytochrome c, bovine serum albumin, β-lactoglobulin, human haemoglobin and human immunoglobulin G.

Chromatographic methods

Columns of ion exchanger were prepared by loading an aqueous suspension of CM-Trisacryl M into glass tubes, then washing the ion exchanger with the buffer used for the experiment. The dimensions of the columns and the composition of the buffer are given in the legends of the figures.

RESULTS AND DISCUSSION

Physico-chemical properties

The formula of the cross-linked CM-Trisacryl M copolymer is shown in Fig. 1. The main characteristic of this anionic macromolecule is that it bears three hydroxymethyl groups, a carboxyl group and one alkylamide group for each principal repeating unit. Because of these chemical functions, the polymer is very hydrophilic and suitable for the separation of biological macromolecules, especially proteins. The amino-2-hydroxymethyl-2-propanediol residues create a micro-environment that includes numerous water molecules, which thus favour the approach of hydrophilic solutes (proteins) towards the polymer and the resulting ion exchange with the carboxyls. This matrix has an obvious advantage over polyacrylamide- or hydroxymethylmethacrylate-based supports, which have pronounced hydrophobic character. In addition, the molecular weight of N-[tris(hydroxymethyl)methyl]acrylamide is much higher than that of acrylamide. Thus, for an identical molar concentration (comparable porosity), the matrices obtained contain more dry material and are mechanically more resistant.

Mechanical resistance was measured under real working conditions. Fig. 2

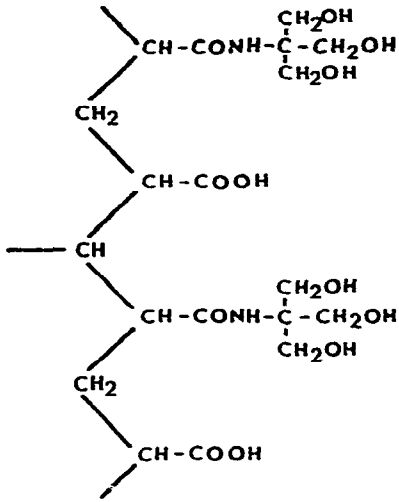

Fig. 1. Structure of the ion exchanger.

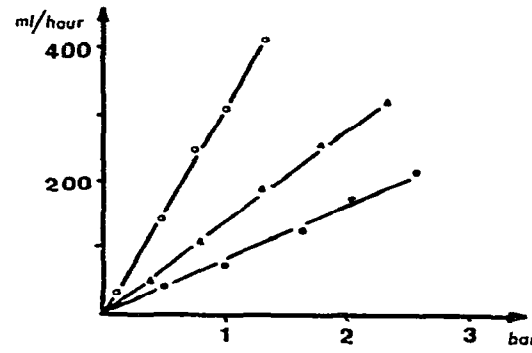

Fig. 2. Effect of pressure on flow-rate of a CH-Trisacryl column. Column I.D.: 1.26 cm. Column length: (○) 7 cm; (▲) 12.8 cm; (●) 24.5 cm.

shows that the pressure–flow-rate relationship at pressures up to 3 bar follows the law of d'Arcy[10], demonstrating the rigidity of the support in this range. This interesting characteristic of the cation exchanger permits the flow-rate that will be obtained when the chromatographic separations are performed at a pressure below 3 bar to be easily calculated.

The copolymerization method used for the preparation of the ion exchanger in the presence of ammonium persulphate and N,N,N′,N′-tetramethylethylenediamine as catalysts leads to very reproducible results, as the practical specific capacity determined for eight different batches was 210 ± 5 μequiv./ml. The results agree with those previously published[4].

The pK_a value of the carboxyl groups was 4.7 (Fig. 3). The curve shows that at pH 4 about 90% of the anionic groups were protonated and that at pH 6 at least 90% of these groups were in the COO^- form. The pH range in which the ion exchanger exhibits a useful exchange capacity for proteins is thus 4–6.

The protein sorption of the ion exchanger is dependent on the chemical composition of the medium and on the nature of the protein. However, it is interesting to examine the variation of this property with pH. The complete curves in Fig. 4b particularly show that they have a maximum which differs from protein to protein. These results are in accordance with theoretical curves calculated for protein models with a given isoelectric point (p*I*) and shown in Fig. 4a. The calculations were made using eqn. 2 and on the basis of two simple hypotheses: (i) the protein sorption is proportional to the concentration of the ionized carboxyl group; (ii) the net charge of the protein is a linear function of pH in the range studied.

$$S = KQ_A\left(1 - \frac{1}{10^{pH-pK_a} + 1}\right)\left(1 - \frac{pH}{pI}\right) \qquad (2)$$

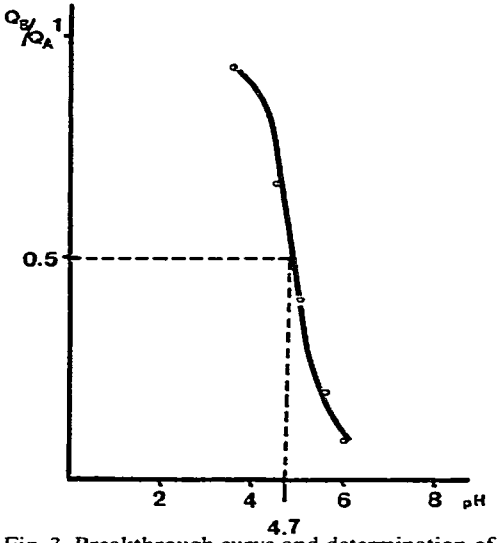

Fig. 3. Breakthrough curve and determination of the pK_a of the ionic group (4.7). Q_A = practical specific capacity; Q_B = breakthrough capacity. Experimental points were determined using 0.1 *M* sodium hydroxide solution as titration solution after equilibration of the column at given pH (sodium citrate buffer) and extensive washing with demineralized water.

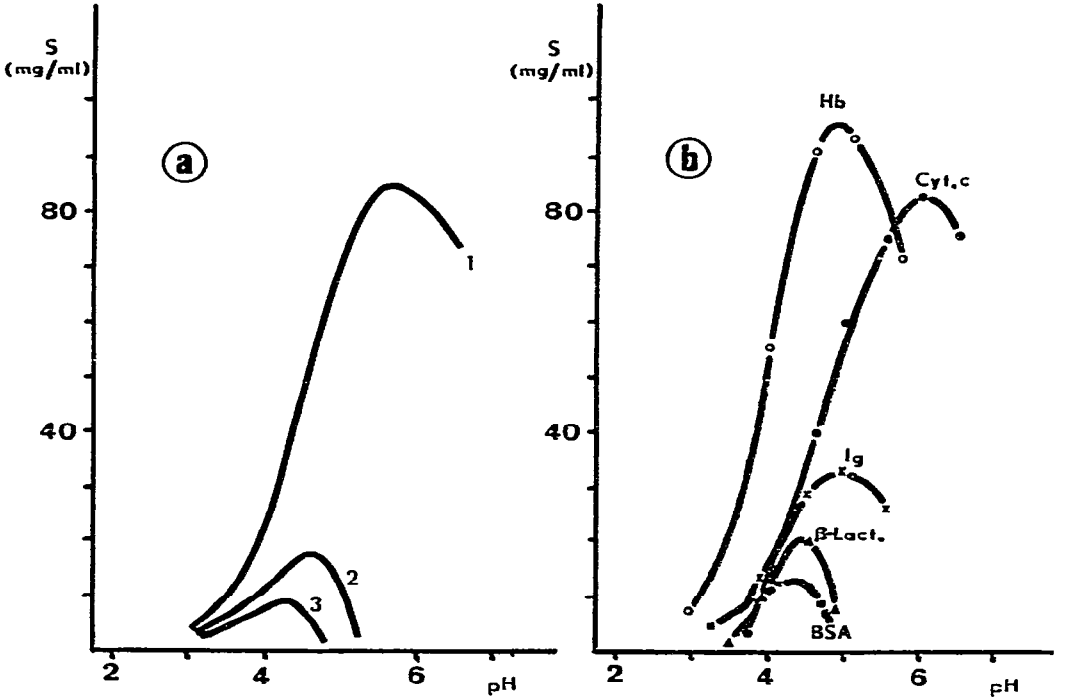

Fig. 4. Protein sorption (*S*) of CM-Trisacryl M as a function of pH. (a) Theoretical curves determined by eqn. 2 for isoelectric points of 9.5 (curve 1), 5.2 (curve 2) and 4.8 (curve 3). (b) Experimental curves determined in 16 m*M* citrate buffer and protein solutions at a concentration of 5 mg/ml. Cyt. c = cytochrome *c*; Hb = human haemoglobin; Ig = human immunoglobulin; β-lact = β-lactoglobulin; BSA = bovine serum albumin.

where S represents the protein sorption of the ion-exchanger and K is a coefficient that depends on the nature of the protein and on the counter-ion concentration.

It is interesting that the maxima of the curves in Fig. 4a are progessively shifted toward a more basic pH as the isoelectric point of the protein increases. The relationship between the isoelectric point and the pH corresponding to maximum protein sorption is represented in Fig. 5. The curve is a theoretical one calculated from eqn. 2 and the points represent experimental results. This curve is of a practical interest because it may be used to choose the pH of the buffer that permits an optimal interaction between a protein with a given isoelectric point and the ion exchanger.

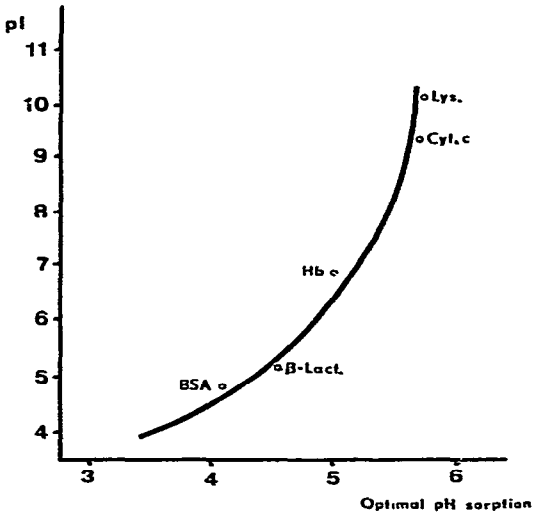

Fig. 5. Relationship between protein isoelectric point and optimal pH for protein sorption of the ion exchanger. The curve is a theoretical model; the points are experimental results.

Chromatographic properties

The major chromatographic properties of CM-Trisacryl M were determined by separating artificial mixtures of proteins (β-lactoglobulin, cytochrome c and lysozyme). We limited the study to easily accessible parameters such as elution gradient slope, column length, flow-rate and buffer pH. Particular attention was paid to the influence of these parameters on a retention value [sodium chloride elution molarity (m_{elu})] and on resolution. We chose elution molarity as a retention parameter because the classical parameters (retention volume[11], capacity factor[12], and K_{av}[13]) are significant only under isocratic conditions, whereas in ion-exchange chromatography elution is more often carried out with an elution gradient of known slope.

The results indicate that the elution molarity of proteins increases considerably with an increase in the slope of the elution gradient (Fig. 6), decreases linearly with the reciprocal of the column length and remains constant with varying column flow-rate. Also, the elution molarity varies with pH to the extent that the ionization of proteins may also vary with pH.

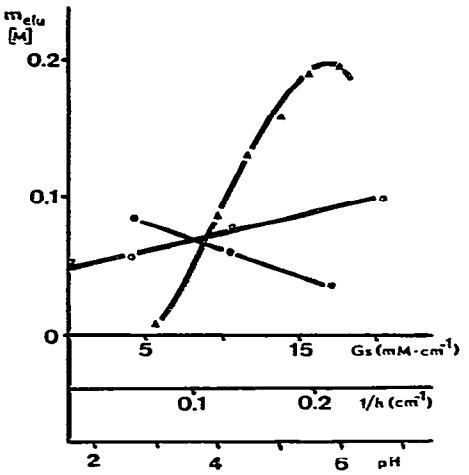

Fig. 6. Influence of elution gradient slope (Gs) (○), column length (h) (●) and pH (▲) on the elution molarity (m_{elu}) of cytochrome c. Experiments were carried out in 16 mM citrate buffer.

Variations of the elution molarity can be summarized by the following empirical equation:

$$m_{elu} = aG_s - \left(\frac{b}{h} - d\right) \tag{3}$$

where G_s (mM/cm) is the sodium chloride gradient slope and h (cm) is the length of the column. The terms a, b and d depend on the support on the protein and on the buffer; d corresponds to the elution molarity of a given protein when the slope is zero and the column is infinitely long. As an example and for the lysozyme under the conditions described, $a = 4.75 \cdot 10^{-3}$, $b = 0.365$ and $d = 0.288$.

The resolution increases with increasing column length but decreases with increasing gradient slope and with increasing flow-rate, as expected on the basis of theoretical considerations[14].

As a practical application, we studied the separation of proteins from human serum. We initially performed separations under relatively empirical conditions, but using a buffer whose pH and molarity conformed to the recommendation of the supplier of the chromatographic support (Fig. 7a). We then decreased the sodium chloride gradient slope (Fig. 7b) and then with the same gradient slope we increased the column length (Fig. 7c). As flow-rate also plays an important role in a separation process, this parameter was decreased and a separation under optimal conditions was thus obtained (Fig. 7d).

This procedure can be followed in the opposite direction when the separation of a few components mixture is easy to achieve, occasionally leading to savings of time. For the complete optimization of a separation, it should be recalled that ionic strength elution gradients can have forms other than linear and can be combined with pH changes.

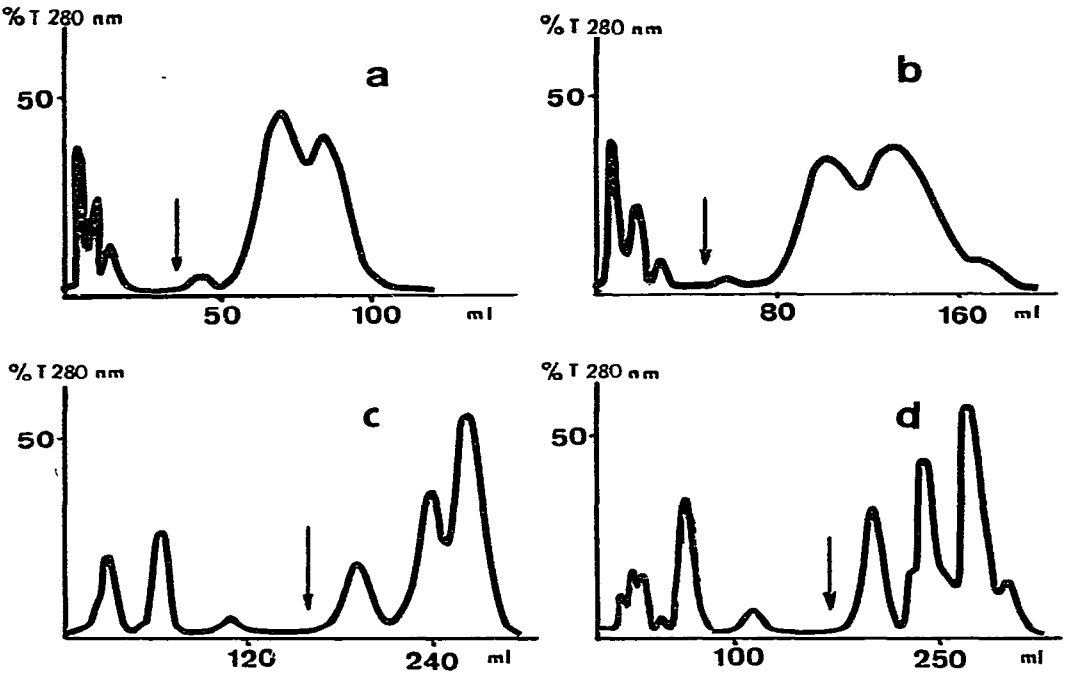

Fig. 7. Experimental optimization of the separation of human serum proteins on CM-Trisacryl M. The variables were elution gradient slope (b), column length (c) and flow-rate (d). Columns used: 5.4 × 1.6 cm I.D. (a and b) and 27 × 1.6 cm I.D. (c and d). Sample: 1 ml of human serum. Buffer: 0.05 *M* acetate (pH 4.5). Sodium chloride gradient slope in sodium acetate buffer (0.05 *M*, pH 3) from 0 to 0.1 *M*: 2.44 m*M*/cm (a) or 1.22 m*M*/cm (b–d). Flow-rate: 58.5 cm/h (a–c) or 6.3 cm/h (d). The arrows indicate the starting points of the elution gradient.

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