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Cation exchangers for selective sorption of large proteins☆

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Abstract

The sorption of bovine serum albumin, cytochrom *c* and fibrinogen on the series of carboxylic cation-exchangers with various concentrations of ionogenic groups has been investigated. The dependence of sorption selectivity on protein size and on concentration of ionogenic groups was demonstrated.

Keywords: Adsorption; Ion exchangers; Proteins; Competition

1. Introduction

In recent years, several studies [1–3] were centered around the influence of ionogenic group concentration in the sorbent on separation of protein mixtures. It should be noticed that such influence was described only in terms of making protein desorption easier when using sorbents with low content of ionogenic groups. However, this is not the only advantage of low-capacity sorbents. The key-point to improve the efficiency of chromatographic protein separation is the experimental study of multicomponent processes. An examination of multicomponent protein sorption is highly complicated, so usually model systems, including sorbent and two dissolved proteins are used.

The study of multicomponent protein sorption on swelling sorbents that are used in preparative low-pressure ion-exchange chromatography shows that under experimental conditions close to the requirements of maximal protein binding during sequential and simultaneous sorption on ion-exchangers with high capacity values, the synergetic effects are observed. Synergetic effects appear as the increase of protein interaction with ion-exchanger in the presence of another protein [4–8]. Protein binds so strongly to the sorbent that no displacement by another protein is possible, and if all sorption centers are occupied the further process goes on through interprotein interaction. In this case the dynamic experiments show that the sequential protein addition to the column results in no displacement of the first protein by the second one. With weakening of the protein–sorbent interaction, that can be achieved by decreasing either the ionization degree [8] or the concentration of ionogenic groups [8], the transfer from synergism to competition (displacement of one protein from the sorption centers by another one) takes place.

To preserve structural features of polyelectrolyte networks ensuring enough permeability for protein macromolecules and high-rate mass-transfer at lower ion-exchange group concentrations, the series of polyelectrolyte networks with various contents of carboxylic groups was synthesized. The variety of the ionogenic-group concentration was achieved by introducing a non-ionogenic monomer *N*-(2-hydroxypropyl)methacrylamide (HPMA) into the polymer with the constant maintenance of the cross agent *N*,*N*′-ethylenedimethacrylamide (EDMA) [7]. At the same time it is essential to avoid the formation of ionogenic blocks [8]. An even distribution of ionogenic groups in the polymeric network was achieved due to the use of compounds with similar structure: β-methacryloylalanine (MAA) as a carboxylic monomer and HPMA as a non-ionic one. These sorbents demonstrated the transfer from synergetic to competitive sorption of protein pairs with the decrease of MAA content [7].

However, until recently these phenomena were investigated on proteins with similar sizes and molecular masses hence there was no effect of macromolecular size on sorption selectivity. The binding of sorbent with proteins during competitive sorption has been realized presumably only via one-pointed ion-ion interac-

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Table 1
Model proteins

Protein	Isoelectric point	Molecular mass (Da)	Molecular size (nm)
Insulin [13]	5.4	11500	4.0 × 2.0 × 2.0
Cytochrom *c* [13]	10.5	12300	5.9 × 5.9 × 4.2
BSA [14]	4.7	66400	14.1 × 4.1 × 4.2
Fibrinogen [15]	5.6	370000	10.0 × 15.8 × 11.0

tions. The increase of protein size should ultimately result in the increasing of number of ion-ion interactions between protein and polymer.

In this paper we focus on results of sequentional and simultaneous sorption of model protein pairs, in which proteins significantly differ in their molecular masses and sizes, in the series of carboxylic cation-exchangers with various contents of ionogenic groups.

2. Materials and methods

Pig insulin (main component content 93%), bovine serum albumin (BSA) (Calbiochem-Behring, purity 98%), cytochrom *c* (St-Petersburg Plant of Medical Preparations) and fibrinogen (MP Biomedicals, approx.75% protein) were used as model proteins (Table 1).

Carboxylic network polyelectrolytes synthesized at the Institute of Macromolecular compounds, RAS are represented by a series of MAA, HPMA and EDMA copolymers with various contents of carboxylic groups and constant cross-agent concentration (20% EDMA). Various ionogenic group concentrations were achieved by varying MAA/HPMA ratio in the reaction mass. The copolymerization of MAA with HPMA and EDMA was performed under argon at room temperature in an aqueous 10% acetic acid solution. The concentration of the monomer mixture in the solution was 20%. The process was initiated by an ammonium persulfate–sodium bisulfite redox system. The block copolymer formed was aged at 80 °C for 1 h. After cooling, the block was ground, washed with distilled water to pH 6–7 and dried in air. A fraction with particle size of 100–200 μm in air-dry state was taken for future experiments. Some properties of these sorbents are shown in Table 2.

The potentiometric titration of sorbents was performed using 0.1 M NaOH in 0.1 M NaCl. Portions of air-dry sorbent (100 mg) were added to different volumes of alkaline and the total volume in each flask was brought up to 10 ml with 0.1 M NaCl. The pH values were measured periodically until they became constant.

Table 2
The characteristics of MAA-HPMA-EDMA copolymers

Sorbent	Molar ratio MAA:HPMA	Total exchange capacity (mg/equiv./g)	Specific volume V_s (ml/g)
KMA-E20	1:0	4.62	7.46
KMAH-1-1-E20	1:1	2.40	7.87
KMAH-1-3-E20	1:3	1.35	9.66
KMAH-1-4-E20	1:4	1.02	9.1
KMAH-1-5-E20	1:5	0.92	7.37

To estimate the specific volume of swollen sorbents (V_s), 1 g of each air-dry pattern had been put in a preliminary weighted flask, dried at 50 °C, cooled to the room temperature and weighed. Then sorbents were transferred into calibrated cylinders and put into distilled water. After 24 h exposure sorbents volume in a swollen state was measured. The value of specific volume was calculated as a ratio of sorbents volume in a swollen state to its dry weight.

Sorption process for individual and binary protein solutions was studied in 0.1 M acetate buffer with pH 3.8 (buffer A), i.e. under conditions close to maximal binding for BSA and fibrinogen. The same buffer solution was used for washing of the chromatographic columns.

To obtain sorption isotherms for individual proteins the batch experiments were conducted. Solutions (10 ml) with various protein concentrations were poured into glass vessels containing weighed portions of the sorbent. After stirring for 48 h sorbent was saturated with the protein. Then the protein concentration in individual solution was measured and the protein quantities adsorbed were calculated.

Sequential and simultaneous sorption processes were studied under dynamic conditions using steel "Milichrom" columns (2 mm × 62 mm). The flow rate of protein solution (with concentration 1 mg/ml) was 15 μl/min. When the sorbent was saturated, the column was washed with buffer A and the solution of the second protein was added. Then the column was washed with buffer A and the proteins were desorbed by 0.3% orthophosphoric acid (flow rate 15 μl/min). It was shown previously that desorption of proteins under these conditions is complete. To study protein sorption from mixtures, the solution containing equal concentrations of both proteins (with a total concentration 2 mg/ml) was passed through the column and after desorption the composition of eluates had been estimated.

Protein concentrations in individual solutions were evaluated from optical density at a wavelength of 280 and 410 nm using spectrophotometer SPECORD M40 (Germany). Protein concentrations in mixed solutions (if one of the components was cytochrom *c*) were calculated due to the additivity of optical density value [9,10]. The accuracy of the technique is ±5%.

Results of simultaneous sorption of a model pair fibrinogen-BSA have been analyzed by SDS-PAAG electrophoresis (Laemmli) with 4% concentrating and 9% separating gels. Proteins were colored with Coomassie brilliant blue [11]. Quantitative analysis of protein zones in the gel was carried out on densitometer Epson 1600; data obtained were processed with software "LabWork 4.0" (dot-blotting). To make a calibration plot, samples of BSA and fibrinogen with concentrations 0.2, 0.4, 0.5, 0.75 and 1.0 mg/ml were inflicted into a gel.

3. Results and discussion

3.1. Synthesis of carboxylic networks

Cation-exchangers are polymer networks with densely crosslinked regions comprising microglobules and with regions

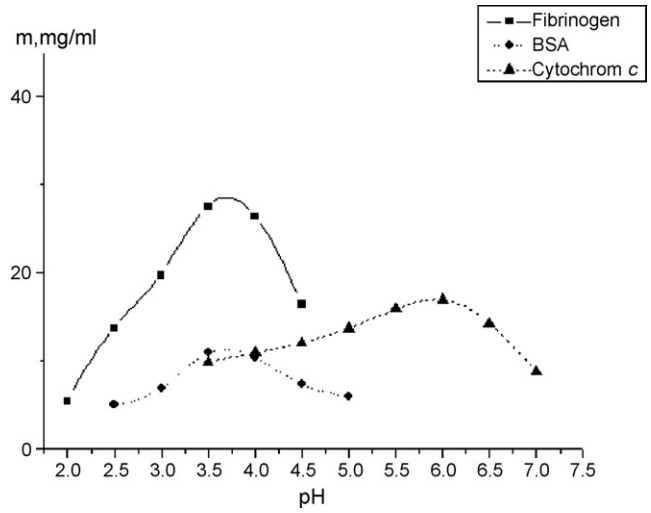

Fig. 1. Model protein sorption depending on PH.

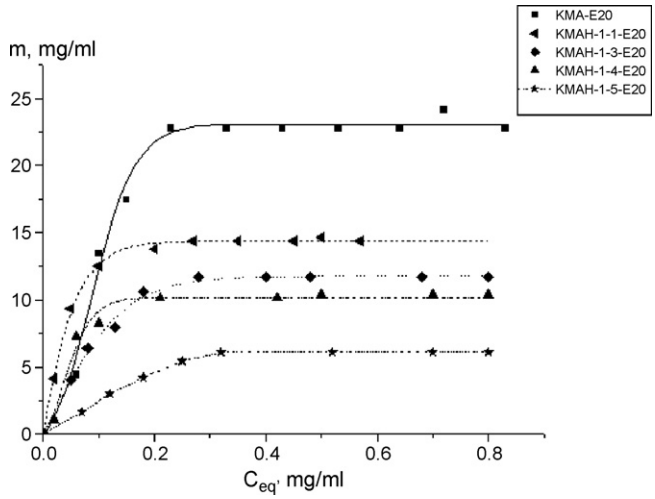

Fig. 3. Sorption isotherms of BSA on the series of carboxylic cation exchangers.

of widely spaced polymer chains. This structure contains a considerable amount of a solvent and permits transport of macromolecules to the sorption centers.

For more details see [7].

3.2. Sorption isotherms and dependence on the pH value

Fig. 1 shows the dependence of KMAH-1-3-E20 cation-exchanger sorption capacity for proteins under study on the pH value. For our experiments on protein sorption, we selected the pH value close to pH optimal for BSA and fibrinogen. Cytochrom's pH optimum is in neutral range, however, its adsorption at given pH 3.8 still remains significant.

Figs. 2–4 present sorption isotherms for individual protein solutions on cation-exchangers with various densities of ionogenic groups. Obviously, the lowering of the ionogenic group content in the sorbent reduces its capacity for proteins due to the decreased number of interactions between amino groups of the protein and carboxyl groups of the cation-exchanger. At the same time, the dependence of cytochromes sorption capacity on MAA content is linear, but the decrease of the capacity for BSA and fibrinogen is not so evident (Fig. 5). One could expect that this phenomenon is related to the size difference of model proteins: when the binding for cytochrom *c* is hindered because of a low ionogenic group density, the binding for BSA and fibrinogen (having a larger molecular size and hence higher ability to contact with sorbents groups) is still possible.

Fig. 5 distinctly shows that in case of KMA-E20, i.e. sorbents with high density of ionogenic groups (80% ionogenic monomer) sorption capacity for cytochrom *c* is about 2 times higher than that for BSA, but there is a decrease in protein sorption capacity differences when the carboxylic groups quantity is reduced. Curves for BSA and cytochrom *c* cross at the point on the graph corresponding to KMAH-1-3-E20 (20% of ionogenic monomer), which means equal capacities for both proteins. With further decrease of carboxylic group content the reversion of sorbent-protein affinity occurs, and the capacity of KMAH-

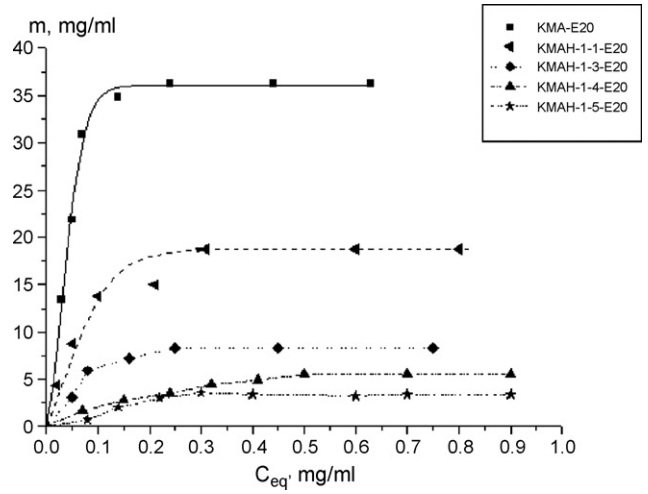

Fig. 2. Sorption isotherms of cytochrom *c* on the series of carboxylic cation exchangers.

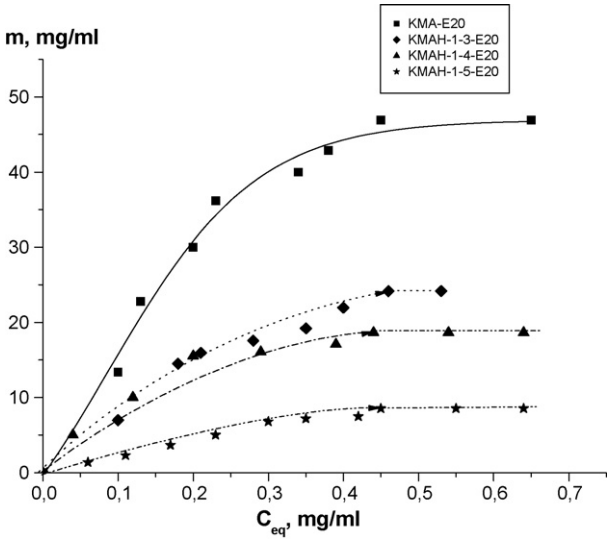

Fig. 4. Sorption isotherms of fibrinogen on the series of carboxylic cation exchangers.

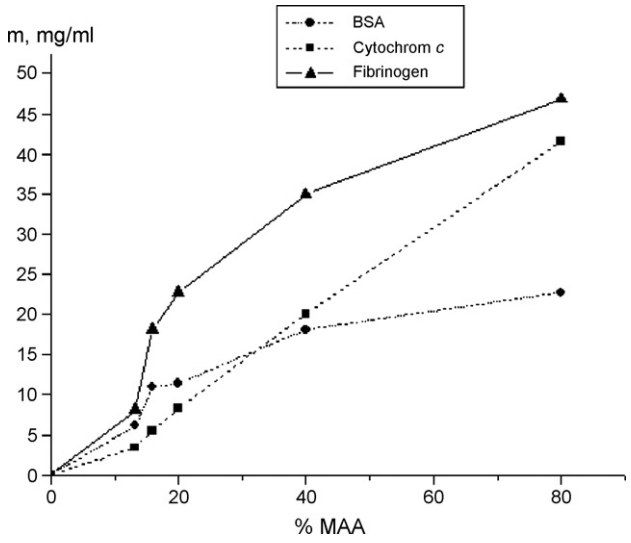

Fig. 5. Dependence of sorption capacity on MAA content.

1-4-E20 and KMAH-1-5-E20 (16% and 13.3% of ionogenic monomer, respectively) for BSA is higher than for cytochrom. Fibrinogen having much larger size than BSA molecule is adsorbed with a higher capacity on all cation-exchangers used. It might be seen that the bigger the difference between sorption capacities is the higher the sorption selectivity should be during the multicomponent process. However, as [12] shows, the speculation about multicomponent sorption based on data obtained for individual proteins is possible only in case of competition for a limited number of sorption centers. Without the replacement of a protein by another one the final protein composition in the sorbent phase is determined by kinetics of sorption process. Utilization of high-capacity ion-exchangers inevitably results in primary sorbent binding (saturation) with more mobile proteins. Less mobile proteins are adsorbed due to interprotein interactions. We have not conducted kinetic studies, nevertheless, it is clear that the smaller protein molecule is the faster it passes through polymer network cells. The decrease of the number of protein–sorbent bonds should cause the replacement of a protein by another one, i.e. the competition. As it was mentioned above, the easiest way to determine the presence of such replacement is to study sequentional protein sorption in dynamic experiments.

Table 3
Sequential sorption of model proteins

Sorbent	BSA/Cyt *c*	Fibr/Cyt *c*	Cyt *c*/BSA	Cyt *c*/Fibr
KMA-E20	–	–	–	–
KMAH-1-1-E20	–	–	–	–
K KMAH-1-3-E20	–	–	–	+
K KMAH-1-4-E20	–	–	+	+
K KMAH-1-5-E20	–	–	+	+

A previously sorbed protein is written in the first place and potential replacement agent – in the second. Replacement is signed by "+", absence of replacement – by "–".

3.3. Sequential sorption of model proteins

Fig. 6 demonstrates the data on sequential sorption of BSA and cytochrom *c* on two sorbents.

Thus, for the sorbent with maximal concentration of carboxylic groups (Fig. 6a), addition of the second protein does not lead to the displacement of the first one that is strongly bound with the surface (irrespective of the molecular size). In another case (Fig. 6b), if the first protein is BSA there is no replacement by cytochrom, but on the other hand, when BSA is added to the equilibrated system including sorbent and cytochrom *c*, BSA does replace significant amounts of cytochrom from the sorbent and the quantity of cytochrom adsorbed is decreased by more than two times.

Results of sequential protein sorption are presented in Table 3. We can see that cytochrom *c* cannot replace the larger protein at any ionogenic group concentration. At the same time if preliminarily sorbed, it is displaced from the sorbents with low content of ionogenic groups by BSA or fibrinogen.

3.4. Simultaneous sorption of model proteins

Now let us consider how the presence or absence of replacement affects sorption from mixed solutions. Data on composition of eluates are shown in Table 4. Composition of the eluat was cal-

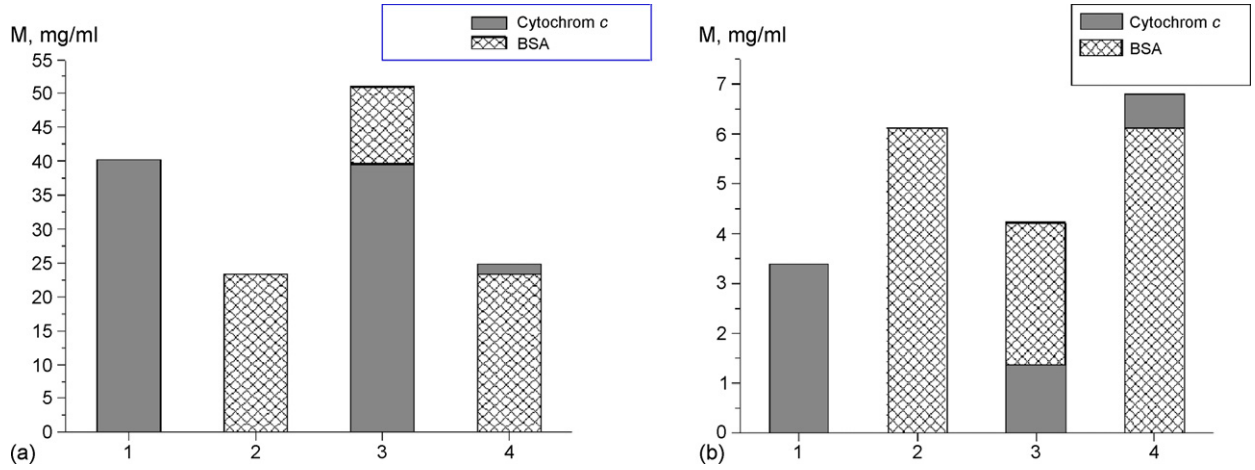

Fig. 6. Sorption of cytochrom *c* and BSA on KMA-E20 (a) and KMAH-1-5 (b).

Table 4
Composition of eluates after sorption from binary protein solutions

Sorbent	C_{cyt}/C_{BSA}	$C_{cyt}/C_{fibrinogen}$	$C_{cyt}/C_{insulin}$	$C_{BSA}/C_{fibrinogen}$
KMA-E20	2.8		1	0.9
KMAH-1-1	1.8	1.05	–	–
KMAH-1-3	1.0	0.18	0.4	0.52
KMAH-1-4	0.12	0.17	0.5	0.45
KMAH-1-5	0.12	0.12	0.5	0.09

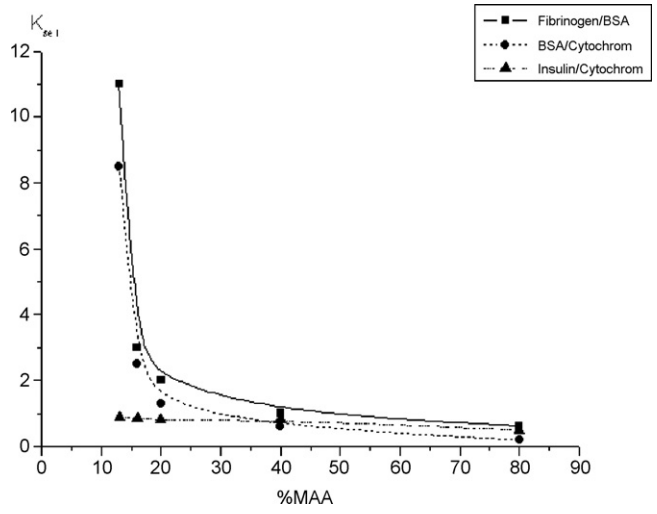

Fig. 7. The dependence of selectivity coefficients on ionogenic group content.

culated as a mass ratio of protein quantities adsorbed (estimated after desorption).

Table 4 shows that the ratio of quantities of proteins adsorbed (cytochrom *c*: BSA) changes from 2.8 (sorbent with 80% MAA content) to 0.12 (16 and 13.3 % MAA). It means that sorption selectivity for BSA on the sorbent with the lowest density of ionogenic groups has been increased by 25 times when compared with highly activated sorbents. As mentioned above, a higher selectivity of cytochroms sorption (in contrast to BSA and fibrinogen) on high-capacity sorbents could be explained in terms of its higher mobility, thus cytochrom *c* can occupy more advantageous sorption centers, and BSA cannot replace it from the network. In the case of sorbents with ionogenic group concentration reduced by introducing the non-ionogenic monomer HPMA, under the same conditions BSA (capable to larger number of ion-ion interactions) displaces cytochrom *c* from the sorbent phase.

Fibrinogen has larger size than BSA and diminishes sorption of cytochrom *c* already when using KMAH-1-3-E20. Data on simultaneous sorption of cytochrom *c* and insulin on the same sorbents are to support the idea of the essential role of protein size for sorption selectivity. These proteins have a similar size and the decrease of ionogenic group content does not result in a significant change of selectivity.

To illustrate the above-said, Fig. 7 shows the dependence of selectivity coefficients on ionogenic group content. Selective coefficients were calculated for protein concentrations used in experiments on simultaneous sorption.

Selectivity coefficient was calculated according to the equation:

$$K_{sel} = \frac{m_1 \times C_2}{m_2 \times C_1}$$

where C_1 and C_2 – protein concentrations in solution, m_1 and m_2 – protein concentrations in the sorbent phase, respectively.

The solution on the outlet of the column has equal concentrations of both proteins, so the selectivity coefficient was found as a ratio of protein amounts in the sorbent phase estimated from the composition of the eluate (obtained after desorption).

4. Summary

The sorption of model proteins, which significantly differ in their sizes has been studied. The possibility of transfer from synergetic to competitive sorption on ion-exchangers was demonstrated. Using of carboxylic cation-exchangers with reduced ionogenic group concentration results in selective sorption of larger proteins. Presumably this kind of sorbents could be used for selective separation of large proteins from complex mixtures.

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