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Synergistic effects in the processes of protein multicomponent sorption

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Abstract

Investigations of sequential and simultaneous sorption of protein pairs on cation exchangers demonstrated a number of synergistic effects. The synergism of protein sorption is determined by multipoint binding of protein macromolecules with the sorbent surface and by the possibility of multilayer packing of proteins in the sorbent phase. It was shown that selectivity of multicomponent protein sorption changes during the process. This effect influences protein desorption dynamics. The band front of the elution peak is enriched with the protein component which was selectively sorbed in the final stage of the sorption process.

Keywords: Sorption; Proteins; Ribonuclease; Lysozyme

1. Introduction

A stoichiometric displacement model is generally used in the theoretical description of phenomena occurring during ion-exchange sorption of proteins [1–3]. According to this model the bonding of the protein molecule carrying a certain number of charged groups to one or several fixed groups is accompanied by the displacement of an equivalent number of counter-ions. After fixed groups are no longer available the process can occur only as a result of the displacement of one sorbed molecule by another. Hence, in this case the sorption process is considered exclusively as competition for a limited number of sorption centres. Helfferich and Klein [4] have pointed out that synergistic phenomena are possible during multicomponent sorption. These phenomena can appear when two types of molecules have a tendency to form aggregates or complexes in the stationary phase. In these cases the addition of molecules of one type to the solution phase increases the concentration of the other component in the sorbent.

The transition to industrial methods of protein isolation and purification requires high capacity sorbents to be used. Several factors (large inner surface attaining tens of square meters per sorbent gram, high density of ionogenic groups, and the introduction into the sorbent matrix of groups favouring the interaction of nonionic nature) lead to increasing sorption capacities for proteins [5]. Protein concentrations in pores of these sorbents attains tens and even hundreds of mg per ml. It is impossible to understand sorption processes correctly without taking into account protein–protein interactions in the sorbent phase. In our opinion, protein sorption on these sorbents is usually accompanied by synergistic phenomena [6–8].

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2. Materials and methods

Sorbents were synthesized in the laboratory of physical chemistry of polyelectrolytes (Institute of Macromolecular Compounds RAS, St. Petersburg, Russia). Their properties are given in Table 1. CMT is a copolymer of methacrylic acid and hexahydro-1,3,5-triacryloyltriazine [9]. Composite sorbents (CS) were synthesized on the basis of CMT and a macroporous sulfocation exchanger CU-23. They are cation exchanger dispersions immobilized in grains of a high permeable cellulose matrix [10].

As model proteins we used bovine insulin (Plant of Medical Preparations, St. Petersburg, Russia), ribonuclease from bovine pancreas (Biolar, Olaine, Latvia), lysozyme from chicken egg white (Ferein, Moscow, Russia) and protamine sulfate from salmon (Sigma, St. Louis, MO, USA). Some properties of the proteins used are given in the Table 2.

Sorption processes were studied in glass vessels in which various concentrations of protein solutions were added to sorbent feeds of certain humidity. After stirring for 48 h sorbent was saturated by protein. Protein concentrations in individual solutions were determined from optical density at a wavelength of 278 nm and protein quantities sorbed by 1 ml of the sorbent in the swollen state were calculated. In order to determine protein concentrations in mixed solutions, Reversed-phase high-performance liquid chromatography methods were used. Chromatography was carried out on a micro-column liquid chromatograph Milichrom (Nauchpribor, Orel, Russia). A stainless steel column (65×2 mm) was packed by LiChrosorb C_{18} (Merck, Darmstadt, Germany). Eluent consumption was 0.1 ml/min, and the wavelength of spectrometric detector was 210 nm. Effective protein separation was obtained at stepped gradient elution by a mixture of acetonitrile (Kriochrom, St. Petersburg, Russia) and 0.3% aqueous phosphoric acid (Sigma) (acetonitrile content was 0, 10, 20, 30, 40, 50%). Protein concentrations were determined from calibration plots of concentration dependences of peak areas. These plots were constructed for standard protein solutions at concentrations from 0.1 to 1.5 mg/ml. The error of concentration determining by this method was about 4%.

Table 2
Some properties of the proteins used

Protein	Molecular mass	Isoelectric point	Dimensions (nm)
Insulin dimer	11 500	5.4	4.0×2.0×2.0
Ribonuclease	13 680	9.8	3.8×2.8×2.0
Lysozyme	14 600	11.1	4.5×3.0×3.0
Protamine sulfate	6 000–8000	10.4	-

Sequential and simultaneous sorption of protein pairs was investigated at such pH values at which protein bonding with the sorbent is at a maximum: for sorption on CS-CU-23 at pH 2.5, for sorption on CMT and CS-CMT at pH 5.5. Since insulin is insoluble at pH values close to its isoelectric point, the sorption of the insulin–ribonuclease pair was studied at pH 4.0. The ionic strength of the solution was 0.1 *M* in all experiments.

3. Results and discussion

The synergistic character of protein multicomponent sorption is clearly shown in experiments on sequential sorption of insulin and ribonuclease. Properties of these proteins are well known and their interactions with CS-CU-23 and CMT were studied earlier [10,11]. If the CMT is brought into equilibrium with the ribonuclease solution and subsequently

Table 1
Some properties of the sorbents

Cation exchanger	Group type	Partical size (μm)	TEC for Na^+ (mg-equiv./g)	Humidity in the swollen state (%)
CS-CU-23	SO_3^-	315–500	1.3	90
CMT	COO^-	315–500	9.8	94
CS-CMT	COO^-	315–500	2.4	90

TEC = total exchange capacity of the dry sorbent.

different amounts of insulin are added to the solution, in such a way that its initial concentrations in the solution are equal to initial concentrations of ribonuclease, ribonuclease concentration in the sorbent increases (Fig. 1). The synergistic effect may be caused by either protein sorption in the form of a polyelectrolytes complex or by protein–protein interactions in a sorbent phase. At the chosen experimental conditions the probability of formation of an insulin–ribonuclease complex is small because at pH 4.0 both proteins are polycations. Moreover, there is no proportionality between the amounts of additionally sorbed ribonuclease and sorbed insulin. The affinity of insulin for sorbent depends on the amount of previously sorbed ribonuclease (Fig. 2). It may be assumed that at high ribonuclease concentration in the sorbent insulin molecules are bonded not to ionogenic groups of sorbent but to ribonuclease molecules. It should be noted, that insulin sorption on a cation exchanger saturated by ribonuclease proceeds even in the case when ion–ion interaction with fixed exchanger groups is excluded. At pH 8.1 insulin is polyanion and is not sorbed on the CS-CU-23, but it is sorbed in considerable amounts on the same cation exchanger with previously sorbed ribonuclease [6].

The insulin–ribonuclease cation exchanger system is not unique. Fig. 3 shows data on sequential and simultaneous sorption of protein pairs. In one series of experiments the sorbent was brought into contact with 10 ml of protein solution at a concentration of 1 mg/ml, and after 48 h 10 mg of the other protein was added. In the other series sorbent was brought into contact with the binary protein solution at equal volume concentrations of proteins (total concentrations 2 mg/ml). The results are shown in the form of

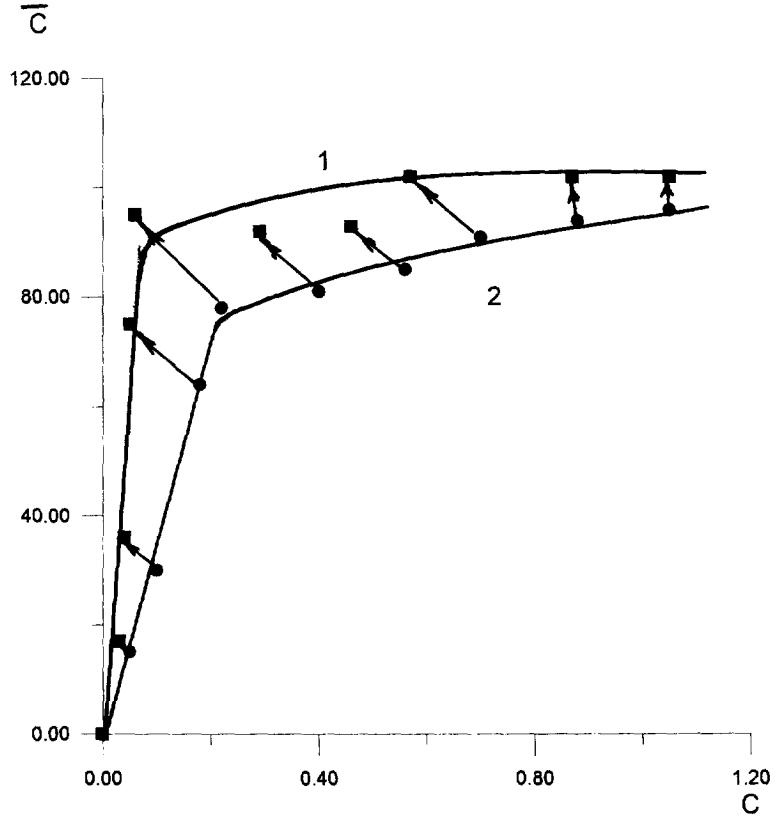

Fig. 1. Curves of ribonuclease distribution between the solution and CMT at pH 4.0: (1) from individual solution, (2) after insulin addition; C = protein concentration in solution (mg/ml), $\bar{C}$ = protein concentration in sorbent (mg/ml). Arrows show the increasing of ribonuclease concentration after insulin addition.

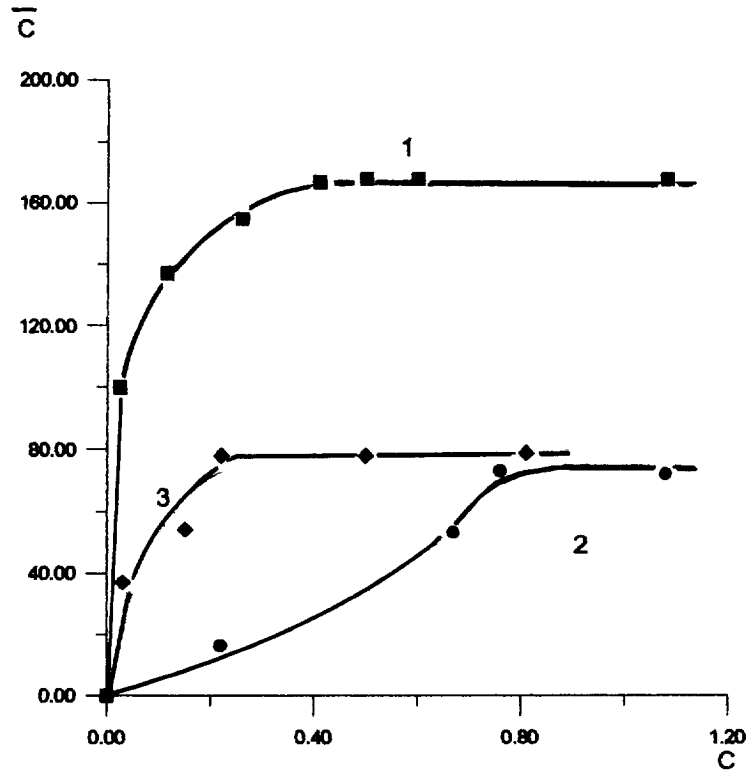

Fig. 2. Curves of insulin distribution between the solution and CMT at pH 4.0: (1) sorption on CMT, (2) sorption on CMT saturated with ribonuclease, (3) sorption on CMT modified by ribonuclease at concentrations equal to initial ribonuclease concentrations. C and $\bar{C}$ as in Fig. 1.

histograms. For each sorbent six histograms are given, the two on the left refer to individual protein sorption, the next two to sequential sorption and the two on the right to simultaneous sorption.

Even a limited set of data permits the following conclusions:

(1) synergistic effects taking place in the insulin–ribonuclease pair are independent of (a) the type of charged sorbent groups (Fig. 3a,c), (b) sorbent type: composite sorbent or not (Fig. 3b,c), (c) hydrophobicity of sorbent matrix: CU-23 having the polystyrene matrix is more hydrophobic than carboxylic cation exchanger CMT (Fig. 3a,b),

(2) the difference in protein isoelectric points does not influence the character of the observed effects; for the lysozyme–ribonuclease pair the addition of the second protein also leads to an increasing of the first protein concentration in the sorbent (Fig. 3d),

(3) the systems in which one of the components is protamine sulfate are evidently an exception (Fig. 3e,d).

The main difference between protamine sulfate and the other proteins is its conformation. Insulin, ribonuclease and lysozyme are globular proteins, whereas protamine sulfate in solution is a random coil [12]. Fig. 4 shows sorption isotherms of individual proteins on CMT. Proceeding from the concept of competitive sorption it may be assumed that the ability to displace previously sorbed protein should increase with selectivity in the ribonuclease–protamine sulfate–insulin line. However, as shown above, insulin does not displace ribonuclease under these conditions but even increases its concentration in the sorbent. Passing into the phase of the sorbent the protein macromolecule tends to occupy the state with minimum free energy. In order that the displacing protein could take the place of its competitor, it should approach the sorbent surface. Sorption

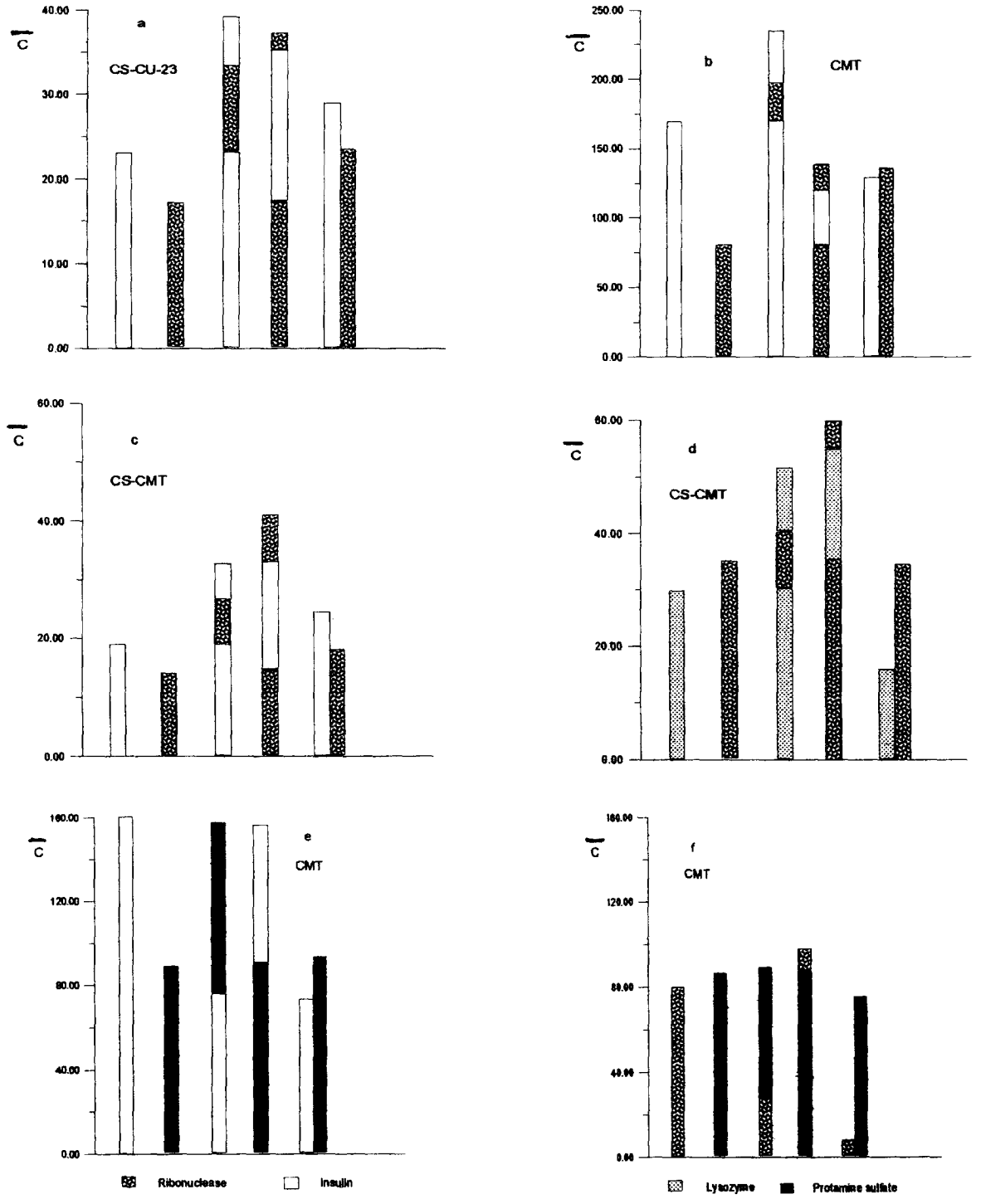

Fig. 3. Sequential and simultaneous sorption of protein pairs. For each case six histograms are given, the two on the left refer to individual protein sorption, the next two to sequential sorption (bottom: protein contacted with sorbent in the first stage, top: protein sorbed on the sorbent saturated with first protein) and the two on the right to simultaneous sorption. $\bar{C}$ as in Fig. 1.

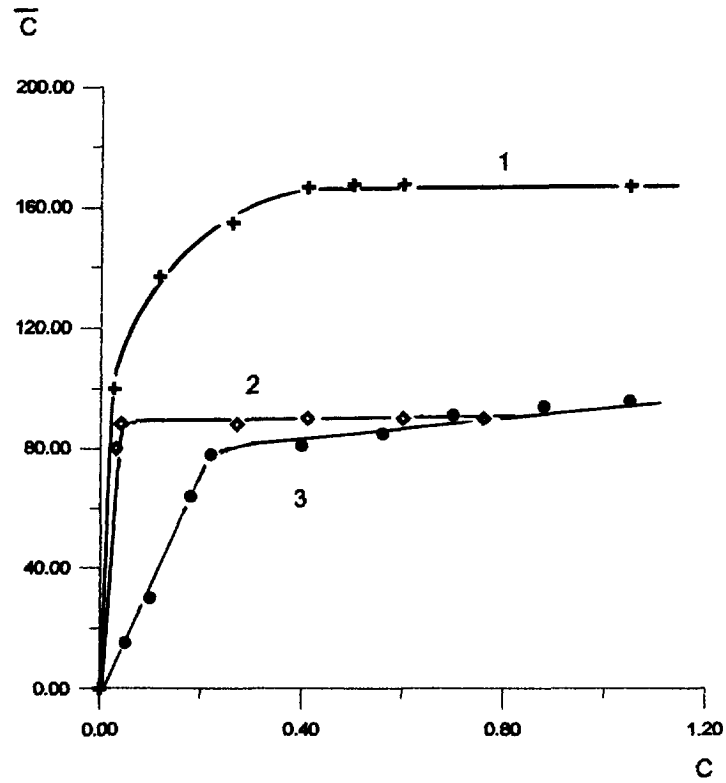

Fig. 4. Sorption isotherms for individual proteins on CMT. C and $\bar{C}$ as in Fig. 1. (1) insulin, (2) protamine sulfate, (3) ribonuclease.

capacity of sorbent for protein can exceed that of the monolayer. For globular proteins this fact makes the sorbent surface sterically inaccessible. The sorption of the second component can proceed only as a result of protein–protein contacts in the sorbent phase. However, it can be seen from the scheme proposed in [2] that even when the packing of globular proteins has maximum density, a considerable number of fixed groups which do not participate in the exchange process remain on the sorbent surface. For a linear protamine sulfate molecule which easily changes its conformation, these groups are accessible. Once the protamine sulfate molecule comes into contact with the sorbent surface, the process will continue cooperatively and protamine sulfate will displace globular proteins. It is for this reason that other displacing agents besides protamine sulfate, which are used in displacement chromatography, are also linear polyelectrolytes (e.g. diethlyaminoethyldextrane and sulfodextrane).

Our work is similar to the investigation of Arai and Norde [13]. The lysozyme–ribonuclease pair, which has been employed by these authors among other protein pairs, was used by us especially for comparison of results. Arai and Norde [13] did not observe synergistic effects in the sorption of two proteins on the surface of polystyrene latices with charged sulfo and amino groups. It should be pointed out that although they used the concept of competitive sorption, some of their data do not conform this model. For instance, sequential sorption was not always accompanied by the displacement of the protein previously sorbed. It may be suggested that the first protein interacts with the surface of the latice by the ion-exchange mechanism, whereas the second protein is sorbed by interacting with hydrophobic polystyrene parts. A certain basis of this suggestion is the fact that the sorption capacity of latices for the two proteins did not exceed the monolayer capacity. Synergistic effects are probably pronounced only when the sorption capacity for proteins exceed that of the monolayer.

In order to determine the monolayer capacity, data on the size of the surface on which proteins are sorbed should be available. The area of swelling carboxylic cation exchangers can be evaluated only approximately by using the method of small-angle X-ray scattering. It attains several tens of square meters per cm^3. The pore surface area can be determined more precisely for strong macroporous ion exchangers. The surface area of a sample of CU-23 was determined by mercury porosimetry and was 70 m^2/g. Proceeding from the proportionality of total exchange capacity for Na^+ ions (for CU-23 this value is equal 2.5 mg-equiv./g) to area of cation-exchange surface, we obtained the value of the sorbing surface of 1 g of wet cellosorbent as equal to 3.6 m^2. The maximum monolayer capacity (m_0) calculated, e.g. for ribonuclease, is 14 mg/g.

$$m_0 = \frac{S \cdot M}{N \cdot A_0}$$

where S is the surface area of 1 g of the sorbent, M the molecular mass, N the Avogadro number, and A_0 the area occupied by one molecule. At pH 2.5 1 g of CS-CU-23 sorbs 17 mg of ribonuclease. For insulin the calculated value is 17 mg/g and the experimental value is 23 mg/g. It should be emphasized that the calculation is based on the assumptions of maximum packing density and complete accessibility of pore surface, which is hardly possible.

The possibility of multilayer protein packing was considered theoretically in Fraaije and Lyklema's works, see e.g. [14]. If multilayer packing is possible for macromolecules of one type, the interaction of a previously sorbed protein with macromolecules of another protein differing in charge distribution and hydrophobic–hydrophilic balance is also possible. The sorbed protein completely covers the accessible sorbent surface and thus decreases the surface charge density. It can even reverse the sign of the electrostatic potential. This reversion can explain the fact that insulin is sorbed on the CS-CU-23 modified by ribonuclease at a pH higher than the isoelectric point for insulin [6].

As a result of multilayer packing in the sorbent phase, the final state of the system depends on its history. For competitive sorption from a limited volume, the final content of protein components in the solid-phase can be determined by the ratio of coefficients of individual protein distribution between the phases. In competitive sorption from solution at a constant concentration, the solid-phase is gradually enriched with selectively sorbed component. In synergistic sorption selectivity changes during the filling of sorbent by proteins. Moreover the percentage of components in different layers can differ. This fact should influence the desorption process.

Fig. 5 shows the curves of ribonuclease (1,3) and lysozyme (2,4) distribution between the phases. The selectivity depends on the sorbent saturation by the other protein. The sorption process proceeds without the displacement of previously sorbed protein. It may be assumed that during the sorption from a binary solution in the first stage proteins are bonded as a result of sorbate–sorbent interactions (inner layer) and in the second stage their bonding is due to the interactions between sorbate molecules (outer layer). Then if sorption from a binary solution with equal ribonuclease and lysozyme concentrations is considered, the composition of the outer layer must depend on protein concentrations in solution. At high concentrations this layer should be enriched with lysozyme and at low concentrations with ribonuclease. This phenomenon must be manifested during protein desorption from the sorbent.

In order to evaluate the effect of the type of sorbent filling on protein desorption, a procedure for studying protein sorption and desorption by passing the solutions through a thin sorbent layer was developed. A column (2 mm in diameter) was packed with a CS-CMT feed of such a volume that approximately 1 mg of protein could be sorbed at saturation. In this work effective separations of two proteins have not been investigated. Column experiments were undertaken only to confirm the hypothesis of protein selectivity variation during the filling of the cation exchanger with proteins. Hence, the desorption of a lysozyme and ribonuclease mixture was carried out under the conditions when both proteins are completely desorbed. Let us assume that protein bonding in the initial stage of sorption process proceeds on the most advantageous sorption centers and becomes weaker during their consumption. In this case during desorption protein–protein contacts should be broken first, and the band front of

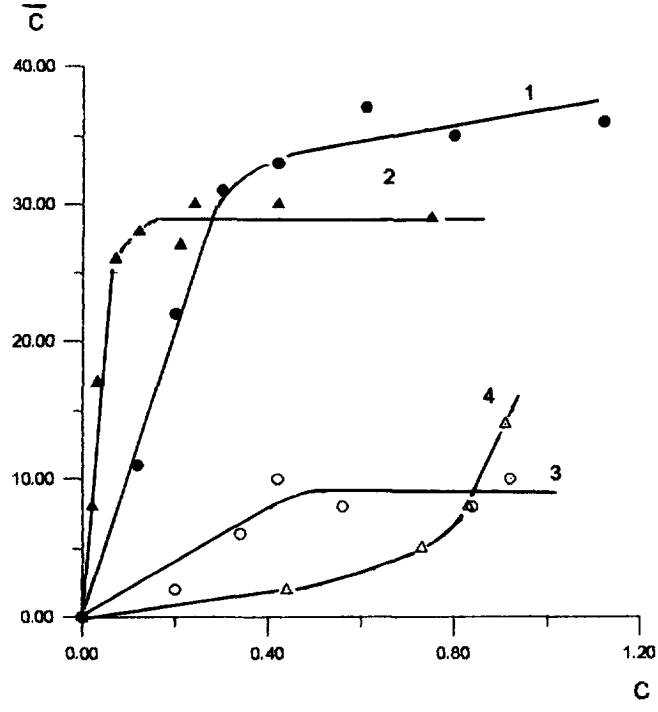

Fig. 5. Curves of protein distribution between phases. C and $\bar{C}$ as in Fig. 1. (1) sorption of ribonuclease on CS-CMT, (2) sorption of lysozyme on CS-CMT, (3) sorption of ribonuclease on CS-CMT saturated with lysozyme, (4) sorption of lysozyme on CS-CMT saturated with ribonuclease.

the elution peak will be enriched with the protein sorbed in the final stages of the sorption process. To check this assumption, lysozyme and ribonuclease solutions at a concentration of 1 mg/ml were passed through the column in different sequences. In this case the displacement of the protein sorbed in the first stage did not occur. The column was washed with distilled water and then desorption was carried out by dividing the elution peak into fractions. It can be seen in Fig. 6. that the band front of the peak is actually enriched with (a) lysozyme and (b) ribonuclease sorbed in the second stage.

Simultaneous sorption was carried out from solutions with a total concentration of (a) 2 mg/ml and (b) 0.8 mg/ml in a ratio of volume concentrations of lysozyme and ribonuclease 1:1. The total amount of proteins brought into contact with the sorbent and the experimental time remained invariable. As it may be seen in Fig. 5 in the case of a more concentrated solution in the final stage lysozyme should be sorbed more selectively, whereas in the opposite case ribonuclease should be preferred. Fig. 7 shows that the band front is enriched by lysozyme in the experiment of sorption from a more concentrated solution. Consequently, it was demonstrated that at the same composition of the protein solution it is possible to control the selectivity of the sorption process and, thus, the solid-phase composition by varying its concentrations.

4. Conclusions

The investigations of sequential and simultaneous sorption of protein pairs on cation exchangers showed that the choice between competition and synergism depends on the conformation of protein molecules. The competition model is valid when one of the components in solution is protamine sulfate which adopts random coil conformation in solution. When globular proteins (insulin, ribonuclease, lysozyme) are sorbed competition is replaced by syner-

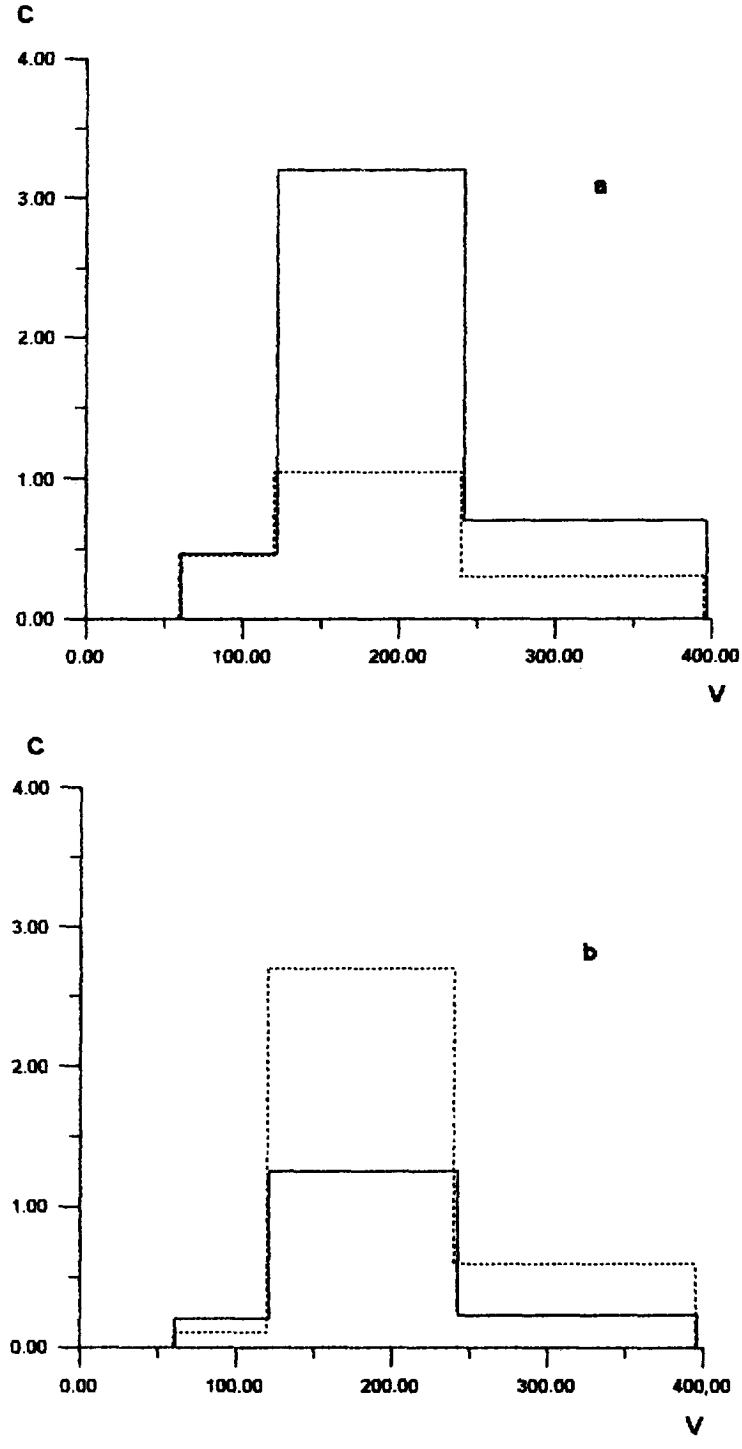

Fig. 6. Protein elution from CS-CMT after sequential sorption of (a) ribonuclease–lysozyme, (b) lysozyme–ribonuclease. C = protein concentration on the exit of column (mg/ml), V = elution volume (μl). Solid line = ribonuclease, broken line = lysozyme.

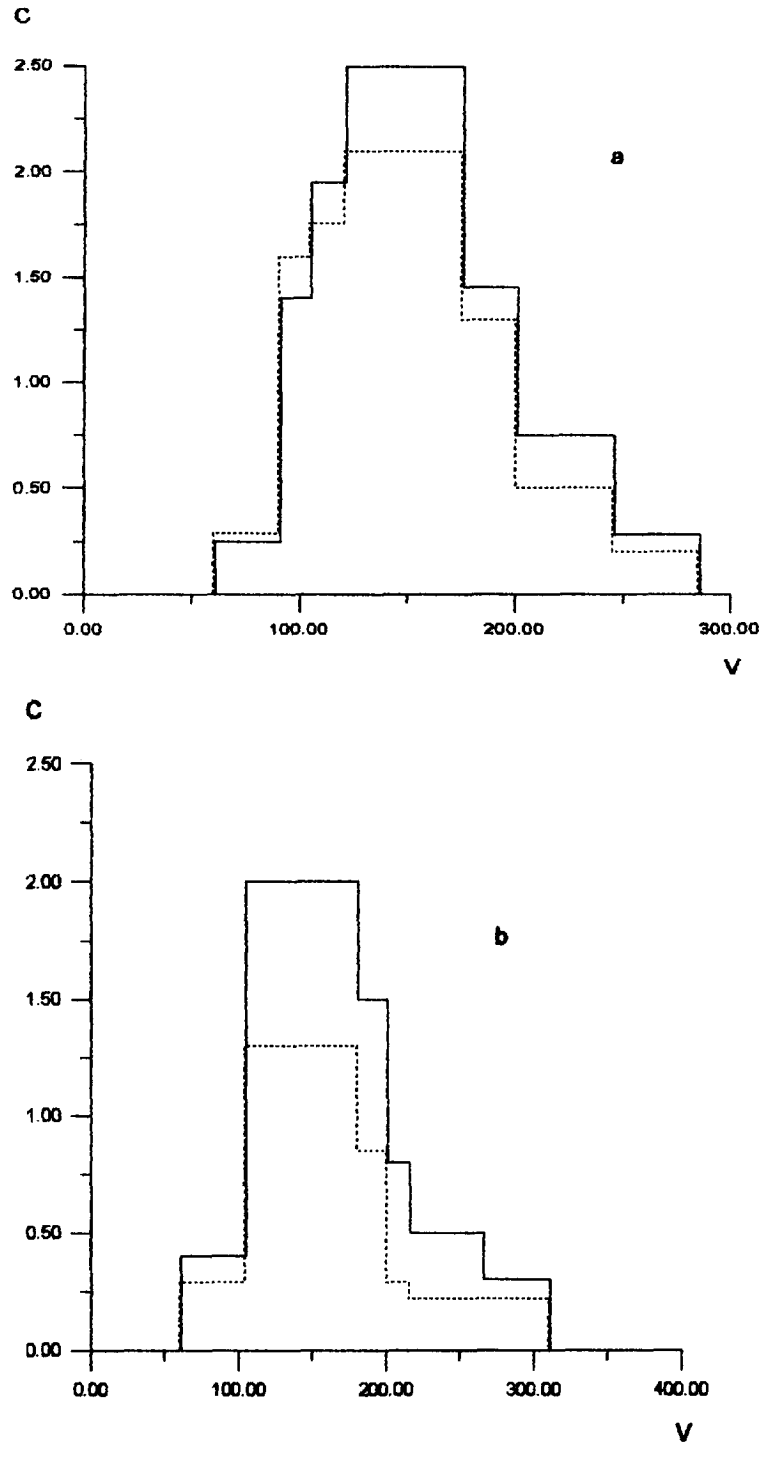

Fig. 7. Protein elution from CS-CMT after simultaneous sorption of lysozyme and ribonuclease solution with a total concentration of (a) 2 mg/ml and (b) 0.8 mg/ml. Solid line = ribonuclease, broken line = lysozyme. C and V as in Fig. 6.

gism. The synergism of the protein sorption is caused by multipoint bonding to the sorbent surface and the possibility of multilayer packing of protein globules in sorbent pores. Sorption selectivity can change with sorbent filling. This phenomena may be interpreted as a result of the transition from protein contacts with sorbent to protein–protein contacts. The changes in selectivity leads to the difference in layer composition and influences the dynamics of desorption processes.

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