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Effect of porous structure of macroporous polymer supports on resolution in high-performance membrane chromatography of proteins

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Abstract

The effect of porous structures of 2-mm thick diethylamine functionalized monolithic polymethacrylate discs on their chromatographic behavior in ion-exchange mode has been studied. Discs with small pores did not perform well because they exhibited high back pressure and substantial peak broadening. Discs characterized with pores larger than 1 000 nm did not provide good separations either because the time required for some protein molecules to traverse the length across the pore to reach the wall for adsorption/desorption process that is essential for the separation may be longer than their residence time within the matrix. Optimum pore size is centered at about 700 nm. Excellent separations have been achieved with these discs even at very steep gradients and high flow-rates which allow to shorten the separation times substantially. © 1998 Elsevier Science B.V.

Keywords: Polymer supports; Membrane chromatography; Poly(glycidyl methacrylate–ethylene dimethacrylate) discs; Stationary phases, LC; Proteins

1. Introduction

The fast growth of biotechnology and the pharmaceutical industry requires development of new isolation and purification methods for biopolymers. Besides high throughput, these methods have to be gentle to preserve the native structure of the separated molecules that are important for their activity. This means that it is necessary to avoid work at extreme values of pH and temperature, the undesirable contacts with aggressive, denaturing surfaces and long operation times. Membrane chromatography can be considered one such method [1–12].

Membrane chromatography is a separation technique that combines advantages of both high-performance liquid chromatography (HPLC) and membrane technology [13]. The concept of membrane chromatography is similar to that of shallow packed columns in which the traditional packing is replaced by one or more porous discs (membranes) provided with active ligands. The separation is achieved using interactions well known from ion-exchange, hydrophobic interaction, reversed-phase, and affinity HPLC modes. The current market offers several designs of separation systems that involve chromatographic membranes in various shapes such as hollow fibers, stacked sheets, and individual discs [11–17].

Obviously, the understanding of the fundamental chromatographic processes is extremely important for the optimization of separation methods that involve the membrane devices. For example, the effects of well-known parameters, such as mass

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transport (convection, dispersion, and diffusion), adsorption, and back mixing (extra column band spreading) on the separation were discussed by Roper and Lightfoot [11]. However, less attention has been paid to the porous structure of the solid matrix itself, although its contribution to the separation processes has been recognized for HPLC in packed columns despite their controversial role in these chromatographic separations [18–20]. The pores seem to be required to increase the area of surface on which the adsorption–desorption processes occur. On the other hand, they are also the source of the resistance to mass transport by diffusion. Therefore, Unger and co-workers suggested to eliminate the pores from separation media completely and to use very small non-porous particles [21,22]. Indeed, his approach provides highly efficient columns for chromatographic separations of biopolymers. However, the very small loading capacity makes these columns less attractive for preparative scale separations.

The recognition of the 'on–off' mechanism that affects the protein separations and is typical of the interactive HPLC modes such as ion-exchange, hydrophobic interaction, and reversed phase, that are sometimes called selective desorption [23,24], has led to the development of an entirely new class of stationary phases. It can be assumed that separations based on this mechanism would benefit from design of porous media which provide a free path for the desorbed molecules from the solid surface into the stream of the mobile phase. The perfusive beads characterized by a bidisperse porous structure with both flow-through and diffusive pores are a good approximation of an ideal chromatographic sorbent [25,26]. The role of pores in the mass transport of separated macromolecules has been discussed in detail in numerous publications [27–29]. The large convective transport pores divide the beads into smaller entities, thus decreasing the length of the diffusional paths. However, if the mass transport within the separation medium is really important, the diffusive pores must create an obstacle for a rapid return of desorbed protein molecules from the beads to the stream of the mobile phase and deteriorate the separation. Hence, separation media that do not contain these small 'diffusive' pores should perform better in the separation of large molecules.

We have developed separation media in the shape of porous disks with a well-controlled porous structure [1,30–32] that have later been used in various separations [33–37]. In contrast to perfusion chromatography, our approach is characterized by flow of *all* the mobile phase through the disk. Although a few excellent papers have recently concerned different aspects of the theory of membrane chromatography using cartridges with stacked modified cellulose sheets [10,11,38–41] and porous discs [42–45], the effect of porous properties of the separation media on their chromatographic behavior has never been studied in detail.

We have demonstrated in our previous work [32] that an increase in the pore size of the disc does not affect the loading capacity while the resolution improves. As a result, excellent separations are achieved even at very high flow-rates that could be easily tolerated by the matrix due to the low back pressure. This suggests that the major part of the chromatographic process occurs in large transport channels, and indicates the possible negative effect of small 'diffusive' pores. Significant contribution of smaller pores to the separation would result in band spreading of the protein peaks at higher flow-rates. However, this is in contrast to the narrowing of the chromatographic zone observed experimentally.

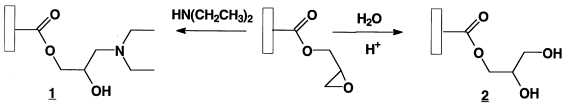
This report describes the effect of the pore size distribution of the porous matrix on the high-performance membrane chromatography (HPMC) of proteins in the anion-exchange separation mode. Our aim is to demonstrate that the absence of small pores does not influence the separation properties of the disc because only large pores of an optimal size are involved in the separation.

2. Experimental

2.1. Materials

The macroporous polymer monoliths were prepared by free radical polymerization of a mixture of glycidyl methacrylate, ethylene dimethacrylate, 2,2′-azobisisobutyronitrile, and porogenic solvents (cyclohexanol and dodecanol) in barrels of polypropylene syringes using a procedure described elsewhere [46]. The monolith was cut to 2-mm thick

discs using a lathe. The discs were washed with methanol, a methanol–water mixture (50:50), water, and methanol, and dried. Dry discs were immersed in diethylamine and kept at the room temperature for 24 h to afford functionalities (**1**) well suited for anion-exchange chromatography of proteins [47]. The content of diethylamine ligands was determined by elemental analysis. Discs with diol functionalities (**2**) were prepared by hydrolysis of epoxide groups of polymerized glycidyl methacrylate units using 0.5 mol/l sulfuric acid for 3 h at a temperature of 50°C [48].



Standard protein mixture for anion-exchange HPLC (Bio-Rad) consisting of horse heart myoglobin, conalbumin, chicken egg albumin and soy bean trypsin inhibitor was used in the chromatographic experiments.

2.2. Methods

The discs were installed in a cartridge that was designed specifically for HPMC by Knauer (Berlin, Germany). A detailed description of this cartridge has been published elsewhere [34]. Each experiment was performed with only a single 2-mm thick membrane placed in the cartridge.

The chromatographic experiments with a gradient of the mobile phase were performed using a Gilson liquid chromatograph (Middleton, WI, USA) consisting of two piston pumps with a maximum flow-rate of 5 ml/min, a UV detector, and a Rheodyne C7125 20-μl loop injector. The data were processed with the Chromatography Station for Windows software program (Data Apex, Prague, Czech Republic).

The gradient elutions were carried out with the mobile phase consisting of 0.05 mol/l Tris–HCl, pH 7.0 (buffer A) and 1 mol/l sodium chloride solution in buffer A (buffer B). Linear gradients from 100% A to 100% B were used in all of the experiments. Individual gradient times and flow-rates are shown in the figures and tables.

Isocratic elution of selected markers (acetone, hydrogen peroxide, chicken egg albumin) was used for the estimation of porosity under operating conditions. Distilled water and 1 mol/l NaCl in 0.05 mol/l Tris–HCl buffer (pH 7.0) were used as the mobile phases, and acetone, hydrogen peroxide, and ovalbumin as the markers. The pore size distribution in the dry state was determined by mercury porosimetry using an automated mercury porosimeter for Porous Materials (Ithaca, NY, USA).

3. Results and discussion

3.1. Properties of porous discs

The porous properties of the discs are controlled in a broad range by both composition of polymerization mixture and polymerization temperature. Based on our extensive study of these effects that has been published recently [48,50], we have chosen polymerization conditions that afford discs with large differences in their pore size distributions. Fig. 1

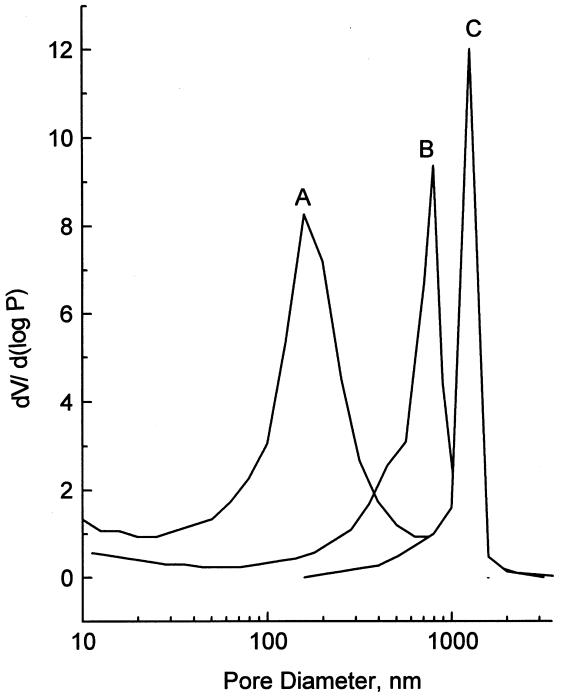


Fig. 1. Differential pore size distribution profiles for the poly-(glycidyl methacrylate–*co*-ethylene dimethacrylate) discs measured by mercury intrusion porosimetry.

Table 1
Preparation conditions and properties of modified poly(glycidyl methacrylate–*co*-ethylene dimethacrylate) discs

Disc	GMA (%)[a]	T (°C)[b]	DEA (mmol/g)[c]	V_p (ml/g)[d]	D_p (nm)[e]
A	50	70	1.2	1.22	166
B	50	60	1.0	1.27	700
C	60	60	1.7	1.52	1130

[a]Percentage of glycidyl methacrylate in the polymerization mixture.
[b]Polymerization temperature.
[c]Content of diethylamine functionalities in modified discs.
[d]Total pore volume.
[e]Pore diameter at the highest peak in the pore size distribution profile (mode).

shows the pore size distribution profiles for the discs prepared under conditions summarized in Table 1. The modal pore size of 700 and 1130 nm found for discs B and C, respectively, is sufficiently large to ensure even high flow-rate at modest back pressure. In contrast, the flow resistance of disc A is much higher and the back pressure increases very steeply as the flow-rate increases. However, the back pressure versus flow-rate dependency remains linear even at high flow-rates [48] and clearly documents the rigidity of the matrix.

Table 2
Porosities of discs calculated from elution volumes of unretained compounds[a]

Analyte	Pore volume (ml/ml)[b]		
	Disc A	Disc B	Disc C
H_2O_2	0.45	0.60	0.60
Acetone	0.45	0.60	0.60
Ovalbumin	0.29	0.48	0.50

[a]Disc volume, 1 ml; flow-rate, 0.5 ml/min. Data are corrected with respect to extra column volumes that have been determined experimentally.
[b]Pore volume is calculated from retention volume of the analyte V_R divided by the volume of the disc ($V_D = 1$ ml).

The porosity of the discs can also be assessed from the retention time of an unretained compound. This technique is very valuable because it shows the porosity under conditions in which the discs are typically used. Markers such as acetone, hydrogen peroxide, and chicken egg albumin allow calculation of porosities that reflect only those pores that are accessible for these markers during the chromatographic process. The porosity data of Table 2 obtained with low-molecular-weight markers for large pore discs reported in ml/ml units can be recalculated to the specific pore volume (ml/g) using the apparent density of 3 ml/g that is typical of this type of material, and compared with data obtained from mercury porosimetry. For example, the pore volume of 0.6 ml/ml calculated for disc C from chromatographic measurement is equal to a specific pore volume of 1.8 ml/g which relates well to the pore volume of 1.52 ml/g determined by mercury intrusion in the dry state. In contrast, a significantly lower pore volume is found with large molecules of albumin because the protein is excluded from small

Table 3
Chromatographic conditions used for the separation of protein mixture

Run no.	Disc	Flow-rate (ml/min)	Gradient time (min)	X_o (mm)[a]	X_o/L[b]
1	B	0.5	240	50	25
2	A	0.5	240	50	25
3	B	5.0	24	50	25
4	C	5.0	24	50	25
5	B	2.5	48	50	25
6	B	2.5	24	25	12.5
7	B	2.5	10	10	5

[a]Length of the separation layer.
[b]Thickness of the disc, $L = 2$ mm.

pores and, therefore, elutes in a volume that is smaller than that obtained with a small marker. Obviously, a large number of pores smaller than the hydrodynamic size of albumin is typical of disc A with a pore size distribution shifted toward smaller pores.

The epoxide groups of the original discs were converted to diethylamine functionalities by a simple reaction with diethylamine at room temperature for 24 h. The contents of ligands in the discs that are in the range of 1.0 to 1.7 mmol/g are shown in Table 1. Obviously, the degree of substitution depends on the percentage of glycidyl methacrylate in the porous polymer and on its morphology.

3.2. Chromatographic evaluation

The chromatographic experiments were carried out using the conditions listed in Table 3. While the thickness of discs, L, has been kept constant at 2 mm in all of the experiments, flow-rate and gradient time were varied. Using the specific separation conditions, the length of the separation layer X_o is calculated from Eq. (1) [45]:

$$X_o = \lambda U C_c / ZB \quad (1)$$

where λ is the auxiliary parameter, C_c is the critical concentration of the displacing salt, Z is the effective charge on the solute ion divided by the charge of the mobile phase ion, and B is the steepness of gradient of the mobile phase defined as the ratio of composition change and gradient volume which, in turn, is a product of flow-rate and gradient time. It should be emphasized that the length of the separation layer X_o is a theoretical value showing the thickness of the layer of the separation medium within which the chromatographic separation process occurs and may substantially differ from the actual thickness of the membrane (2 mm).

Table 3 shows the chromatographic conditions used for the separations. Constant gradient volume which determines the length of the separation layer is used in runs 1–5, while gradient time and flow-rate are changed. These changes define the rate of change in composition and, therefore, the X_o value [45]. Clearly, the gradient time does not determine time of analysis and the peaks may elute well before the

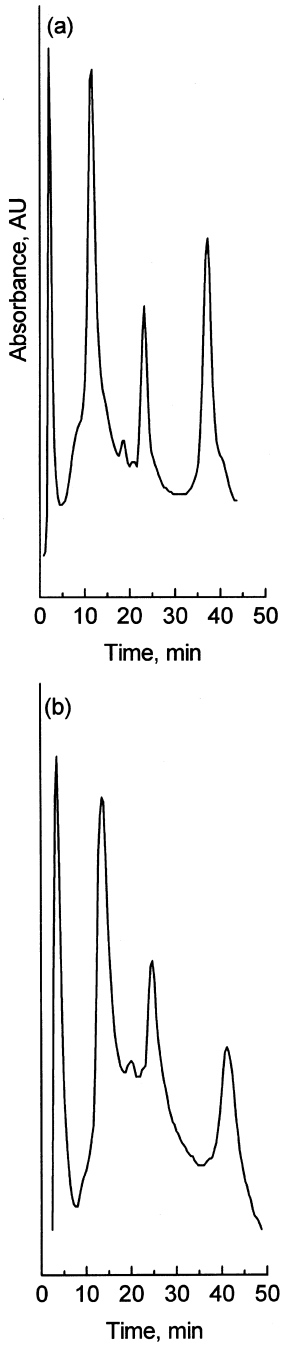


Fig. 2. Separation of standard protein mixture by ion-exchange chromatography on discs B (a) and A (b). Conditions: disc, 25×2 mm; proteins, myoglobin, conalbumin, chicken egg albumin, soya bean trypsin inhibitor (order of elution); protein concentration, 4.5 mg/ml in buffer A; buffer A, 0.05 mol/l Tris–HCl, pH 7.0; buffer B, 1 mol/l NaCl in buffer A; flow-rate, 0.5 ml/min; gradient time, 240 min.

overall gradient time elapses. It is impossible to use a flow-rate of 5 ml/min with disc A to characterize its chromatographic properties at this high flow-rate because the back pressure is very high. Therefore, all comparative measurements are related to disc B.

Fig. 2 compares the separations of protein mixture using discs A and B at a flow-rate of 0.5 ml/min. The separation with disc B is much better than that with A because of the presence of a number of pores with hindered flow that leads to substantial peak broadening. The peak width of conalbumin at the half height is 2.10 ml for A, while it is only 1.40 ml for C (Table 4). Disc A also exhibits the highest back pressure which, together with the poor separation properties, makes it unsuitable for chromatography of proteins.

The overall linear flow velocity U of the mobile phase through the disc in a cartridge is

$$U = FA^{-1}\epsilon^{-1} \quad (2)$$

where F is the volumetric flow-rate, A is the cross-section of the disc, and ϵ is the porosity. This equation does not reflect the fact that the flow velocities in the individual pores are not necessarily equal to the overall velocity U and depend on the size of the pores. According to the Hagen–Poiseuille law:

$$U = \Delta P r^2/8\eta L_t \quad (3)$$

where ΔP is the pressure drop along the liquid path and η is the viscosity of the liquid, the flow through a tube is proportional to the square of its diameter. Although Eq. (3) is exactly valid for the flow through a straight cylindrical tube of radius r and length L_t, it has been demonstrated recently, that the flow through a macroporous monolith approximately obeys this rule [48]. After rearranging to:

$$\Delta P/U = 8\eta L/r^2 \quad (4)$$

this equation shows that the pressure drop per unit of linear flow velocity increases exponentially with decreasing tube diameter. In other words, the larger the pore, the lower the flow resistance, and the more liquid flows through this pore at given pressure. As a result, the flow through the small pores is very slow and can even approach zero because the overall back pressure within the cartridge, that has to be used to drive the liquid through the porous matrix, is lower than that required for the flow through some small pores. The limited flow through some parts of the disc also leads to the decrease in the surface area that is effective in the adsorption/desorption process. Therefore, discs with a broad pore size distribution are not desirable because large volume of pores may not be active in the transport of the mobile phase. This is in contrast to the situation in monolithic columns that have an opposite aspect ratio compared to the thin discs. The path through the medium is longer and, due to their tortuosity, the individual, pore size-dependent flow-rates do not vary too much from the average in different parts of the column.

Another negative effect of small pores can result from size-exclusion phenomena. In particular, large molecules such as proteins cannot pass pores that are smaller than their size and follow only the flow through the larger pores. Discs with very small pores

Table 4
Spreading of the chromatographic zones and peak resolutions for the component of the protein mixture separated in discs with different porous structure using ion-exchange chromatographic mode[a]

Run no.	$w_{1/2,\text{conalb}}$ (ml)[b]	$w_{1/2,\text{ovalb}}$ (ml)	$w_{1/2,\text{STI}}$ (ml)	$R^{c}_{S,2-3}$	$R_{S,3-4}$
1	1.40	1.40	1.80	3.0	3.2
2	2.10	—	2.20	—	—
3	1.25	1.30	1.55	3.5	3.6
5	1.40	1.30	1.50	3.2	3.4
6	1.00	0.80	1.00	2.3	2.7
7	0.60	0.45	0.55	1.9	2.2

[a]Chromatographic conditions: proteins, myoglobin, conalbumin, chicken egg albumin, soya bean trypsin inhibitor (order of elution); protein concentration, 4.5 mg/ml in buffer A; buffer A, 0.05 mol/l Tris–HCl, pH 7.0; buffer B, 1 mol/l NaCl in buffer A.
[b]Peak width at the half height.
[c]Resolution of specified peaks.

might behave as efficient 'filters' for large molecules provided the flow through would be possible at reasonable back pressure. This is in contrast to packed columns in which any small and large molecule can always find their way through the interparticular voids regardless the pore size of the separation medium.

This discussion may lead to a conclusion that an ideal matrix will be characterized by large pores with a narrow distribution. Sarfert and Etzel [41] have recently described the effect of pores on the chromatographic separation of proteins using typical membranes in terms of the residence time of the mobile phase within the membrane t_{res} and the time scale t_{film} representing the mass transfer through the film of liquid to the pore wall and diffusion into the wall. While the former is a simple function of the thickness of the separation layer L and the flow velocity U

$$t_{res} \approx L/U \tag{5}$$

the latter increases exponentially as the pore diameter d_p increases:

$$t_{film} \approx d_p^2/4D \tag{6}$$

where D is the diffusion coefficient of the protein which is a characteristic of each protein and generally is small for large molecules. To achieve good separation, t_{res} must be larger than t_{film}. The residence time can be controlled through the flow-rate and thickness of the membranes, while the transport time is controlled by the pore size. They found that a very thin layer of separation medium with very large pores is not well suited for the separation of proteins.

Although the diffusion coefficient D is a constant, diffusivity of the large molecules within pores of a slab can be accelerated by convection resulting from flow of the liquid through the pore [49]. The diffusivity can be visualized as a vector the length and direction of which is a sum of axial (flow of the liquid) and spatial (diffusion) motions. An increase in one of these parameters, the axial flow, increases to some extent the rate of the transport of protein to the wall. Indeed, excellent separations have been achieved with the chromatographic discs at high flow-rates of the mobile phase [37].

The above discussion leads to the conclusion that

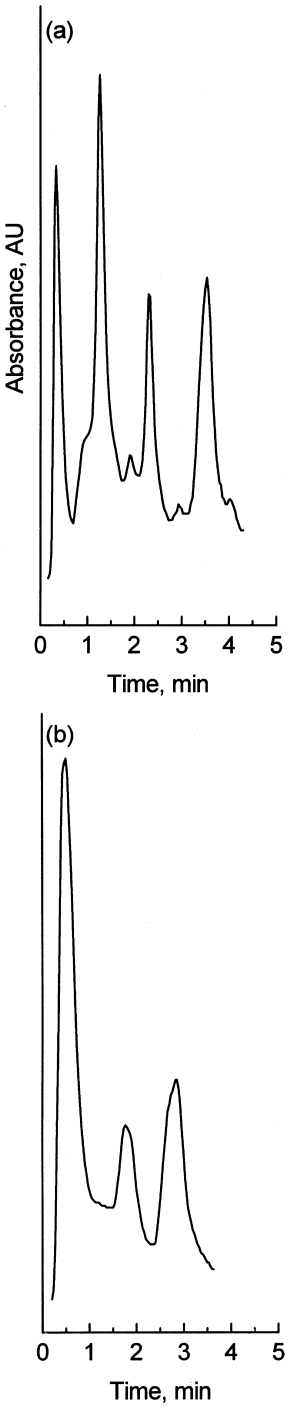


Fig. 3. Separation of standard protein mixture by ion-exchange chromatography on discs B (a) and C (b). Conditions: flow-rate, 5 ml/min; gradient time, 24 min; for other conditions, see Fig. 2.

the best separation can be achieved with discs provided with *optimal* size pores that are sufficiently small to allow the protein molecules to reach the wall, while they are also sufficiently large for fast flow at a reasonable back pressure. The separation may further benefit from a narrow distribution of the pore sizes.

In order to substantiate this conclusion we compared chromatographic properties of discs B and C that contain very large pores at a flow-rate of 5 ml/min. Fig. 3 documents the excellent separation achieved with disc B while the separation with disc C is poor under identical conditions, and only three peaks are obtained. This is not surprising in view of the conclusions of the discussion presented above. Some of the pores in C are too large to allow the protein molecules to reach the pore wall prior to their elution from the disc, the number of separative absorption/desorption steps decreases, and the separation deteriorates. An alternative interpretation that assumes a decrease in surface area resulting from the increase in the pore size is not very likely. The overall surface areas of both membrane materials are lower than 5 m^2/g [48] and the difference, if any, can hardly explain the dramatic effect of pore size on the chromatographic properties.

Our experiments document that the properties of disc B are best suited for chromatography of proteins. Therefore, this disc was used for the determination of the effect of gradient volume on the separation at a constant flow-rate of 2.5 ml/min. The chromatograms shown in Fig. 4 demonstrate the expected decrease in the separation time resulting from the increase in the steepness of the gradient. While about 8 min are required for the separation of four proteins at a gradient volume of 120 ml, only about 4 min are required at 60 ml, and the separation is finished in only less than 3 min with a 25 ml gradient volume. The decrease in the separation time is not the only benefit of the shorter gradient time. The other advantage of this is the narrowing of the peaks reflected in the peak width. As expected, the

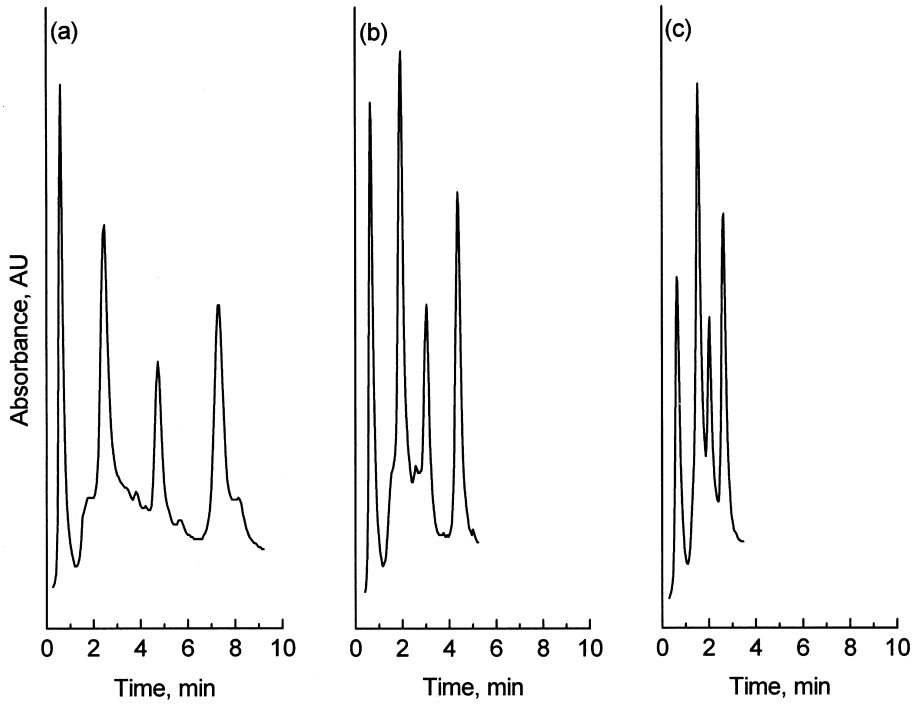


Fig. 4. Separation of standard protein mixture by ion-exchange chromatography on disc B using different gradient volumes. Conditions: flow-rate, 2.5 ml/min; gradient time, 48 (a), 24 (b), and 10 min (c); for other conditions, see Fig. 2.

resolution decreases as the peaks cumulate within a shorter period of time (Table 4).

4. Conclusion

Although much remains to be done in the study of thin monolithic separation layers for high-performance chromatography of proteins, this work confirms that the optimization of the porous structure may help to achieve outstanding separations in very short period of time.

Acknowledgements

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