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Surfactant-mediated hydrophobic interaction chromatography of proteins: gradient elution

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ABSTRACT

Addition of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) to mobile phases in gradient elution hydrophobic interaction chromatography (HIC) on SynChropak Propyl causes changes in observed elution times for nine globular proteins. The nine proteins showed different percentage reductions in capacity factor, k', demonstrating the ability of CHAPS to change the selectivity of the separations. Three basic types of gradient experiments have been explored for surfactant-mediated gradient elution HIC. Type I gradients are conducted with constant salt and variable surfactant concentration. Type II gradients with variable salt and constant surfactant concentration, and Type III gradients with variable salt and surfactant concentrations. By the criterion of a linear relationship between gradient time and retention time the linear solvent strength condition applies to Type II and Type III gradients. Type III gradients, with the fastest re-equilibration time, are preferable for repetitive analyses. Type I gradients are relatively ineffective in making use of the solvent strength of CHAPS, and Types I and II gradients require long equilibration times due to large changes in surface concentration of CHAPS which occur during elution. The presence of CHAPS had a negligible effect on peak shapes of the proteins examined, except for bovine serum albumin which yielded a narrower, less distorted peak in the presence of CHAPS.

INTRODUCTION

High-performance hydrophobic interaction chromatography (HIC) is growing in prominence for separation of proteins [1–5]. In this technique proteins are eluted from weakly hydrophobic stationary phases using decreasing salt gradients. These relatively mild conditions favor the recovery of proteins in their native condition (*i.e.* with retention of biological activity) [6]. This contrasts with reversed-phase (RP) HPLC separations of proteins where the use of acidic conditions, organic solvents and more hydrophobic stationary phases commonly induces protein denaturation [8-11].

Surfactants, due to fact that they may interact both with proteins [12] and stationary phase surfaces [13-18], are expected to have potential as eluting agents in HIC. Recent studies from this laboratory have shown some of the potential of surfactants in HIC as eluting agents [19,20]. It is suggested that non-ionic or zero net charge surfactants will have the highest potential for practical non-denaturing protein separations. This is due to the fact that these materials are known to be gentler in their action towards proteins, with less tendency to cause denaturation [21].

Recent isocratic HIC elution studies in this laboratory [20] with the zero net charge surfactant 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate

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(CHAPS), have shown that this surfactant can act as an eluting agent for proteins and can also provide increased selectivity in protein separations.

However, most practical protein separations employ gradient elution to obtain maximum peak capacity and resolution. Since most proteins exhibit steep elution isotherms on RP-HPLC (and to a lesser degree on HIC stationary phases), they generally elute over a narrow range of solvent strength, making development of suitable conditions for isocratic elution of a protein mixture more difficult. For these and other well-known advantages of gradient elution, it was necessary to examine surfactant-mediated HIC in the gradient mode.

Recent studies of protein retention characteristics in both gradient elution HIC and RP-HPLC have shown them to be predictable in terms of the linear solvent-strength formalism [22–26]. Linear relationships have been observed between log k' (where k' is the capacity factor) and salt concentration for proteins in isocratic HIC [27–29]. As a consequence, protein retention in gradient elution HIC can also be modeled in terms of the linear solvent-strength formalism [24]. Thus standard HPLC optimization techniques can be applied to gradient elution protein separations by HIC.

Results from this laboratory [20] have shown that the linear relationships between the log k' and salt concentration in HIC are also observed in the presence of the surfactant CHAPS. Thus addition of a surfactant to HIC mobile phases does not affect the chromatographic predictability of the system, although changes in the eluting power and selectivity of the mobile phase are introduced. We therefore decided to determine whether chromatographically useful modifications of protein retention and selectivity can be obtained with CHAPS under various gradient conditions.

EXPERIMENTAL

Carbonic anhydrase B (human erythrocyte), ribonuclease A (bovine pancreatic), bovine pancreatic trypsin inhibitor (BPTI), enolase (Yeast Type III), α-amylase (from Bacillus, Type IIA), α-chymotrypsinogen A, aldolase (rabbit muscle, Type III) and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, U.S.A.). Lysozyme (hen eggwhite) was obtained from Miles Labs. (Elkhart, IN, U.S.A.). The proteins were used without further purification. Ammonium sulfate (ultra pure) was obtained from Schwarz Mann (Cambridge, MA, U.S.A.) and potassium phosphate (A.C.S. reagent) was obtained from Fisher (Fair Lawn, NJ, U.S.A.). HPLC-grade water was produced with a Milli-Q purification system. CHAPS was synthesized according to the procedure of Hjelmeland [26] and twice recrystallized from methanol.

The chromatographic system consisted of a Perkin-Elmer Model Series 3B pumping system (Norwalk, CT, U.S.A.), Perkin-Elmer Model LC75 variable-wavelength UV detector and a Waters WISP automatic sample injector (Milford, MA, U.S.A.). The chromatograms were integrated with a Spectra-Physics Model 4270 computing integrator and stored on a Spectra-Physics Chromstation AT. The chromatographic stationary phase used was 6.5-μm SynChropak Propyl HIC packing obtained from SynChrom (Linden, IN, U.S.A.). This material was packed into a 10 cm × 4.6 mm I.D. HPLC column. The column was thermostatted with a circulating water bath at 30.0 ± 0.1°C.

TABLE I

CLASSIFICATION OF GRADIENTS

All these gradients were linear, both in salt and in CHAPS.

Initial concentration		Final concentration		Gradient type
Ammonium sulfate (*M*)	CHAPS (m*M*)	Ammonium sulfate (*M*)	CHAPS (m*M*)	
1.7	0.0	1.7	0.6	I
1.7	0.6	0.0	0.6	II
1.7	0.1	0.0	3.0	III
1.7	0.6	0.0	3.0	III

Chromatographic mobile phases were prepared according to standard methods and filtered through 0.5-μm filters prior to use. All mobile phases contained 0.02 *M* potassium phosphate buffer at pH 6.1. All gradients contained an initial ammonium sulfate concentration of 1.7 *M*, with CHAPS concentrations ranging from 0 to 0.6 m*M*. The final buffer was in all cases 0.02 *M* potassium phosphate buffer with CHAPS concentrations ranging from 0 to 3 m*M*. CHAPS concentrations were chosen to be below the critical micelle concentrations [20]. All gradients were linear, with gradient times ranging from 10 to 60 min. The elution of proteins was routinely accomplished at a flow-rate of 1 ml/min, and monitored at a detector wavelength of 280 nm. Column equilibration experiments were conducted with a detector wavelength of 215 nm for the amide chromophore of CHAPS. Protein solutions were prepared at a concentration of *ca.* 1 mg/ml in 0.02 *M* phosphate buffer, pH 6.1 without CHAPS, and filtered through a 0.5-μm filter prior to injection. The injection volume was 25 μl for all the proteins.

Three basic combinations of surfactant and salt gradient are possible and were investigated in this study. They are as follows:

Type I: hold salt concentration constant while varying surfactant concentration.

Type II: vary salt concentration while holding surfactant concentration constant.

Type III: vary salt and surfactant concentrations simultaneously.

Description of the specific gradients used in this study is summarized in Table I; all gradients employed were linear. In the plots of retention time *vs.* gradient time (Figs. 1, 2, 4 and 5), lines were fit to the data with a linear least-squares program.

RESULTS AND DISCUSSION

Linear solvent strength theory

Since a linear relationship between log k' and salt concentration is observed in HIC, the linearly decreasing salt concentration gradients commonly employed for elution will yield a linear relationship between log k' and time [24]. Therefore, under these conditions the linear solvent strength (LSS) theory may be employed. This theory has been successfully employed in describing small-molecule gradient sep-

arations [22,25], and for a general description of large-molecule separations [23]. We also find an example of its application to HIC gradient separations of proteins [24]. The basic retention relation for linear solvent-strength conditions is as follows:

$$\log k_i = \log k_0 - b(t/t_0) \tag{1}$$

Where k_i is the value of k' at the column inlet during the gradient at time t, t_0 is the column dead time and k_0 is the value of k' at $t = 0$. The implication of eqn. 1 is that a linear relationship between the log of the instantaneous k' at the column head and time must apply during the gradient. The gradient steepness parameter may be described as:

$$b = \Delta\varphi S V_m / t_G F \tag{2}$$

Here $\Delta\varphi$ is the change in volume fraction of strong solvent in the gradient (*i.e.* for a 0–100% gradient $\Delta\varphi$ is 1.0), S is the slope of $\log k'$ *vs.* φ, V_m is the column dead volume, F the flow-rate, and t_G the gradient time. It can be shown that retention in gradient elution then may be expressed as:

$$t_g = (t_0/b)\log(2.3k_0 b + 1) + t_0 + t_D \tag{3}$$

Here, t_D is the correction for instrumental dwell time. In order to simplify this relationship we make the approximation $2.3bk_0 \gg 1$. Thus eqn. 3 simplifies to the following relationship:

$$t_g = t_G/\Delta\varphi S \log k_0 + t_0 + t_D \tag{4}$$

According to eqn. 4, we expect that retention time, t_g, will be linearly related to the gradient time, t_G. Since we previously observed that linear relationships are observed for $\log k'$ *vs.* salt concentration in the presence of the surfactant CHAPS in HIC [20], we would like to know if gradients containing CHAPS can be run under LSS conditions. To test this hypothesis t_g was plotted *vs.* t_G for the nine proteins in this study under Type III gradient conditions. Linear relationships were observed for all nine

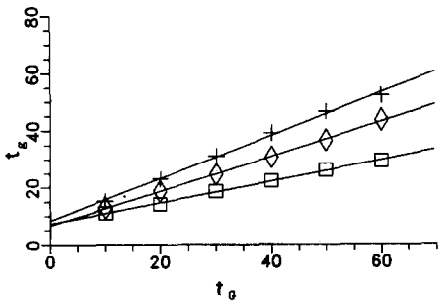

Fig. 1. Dependence of retention time (min) on gradient time (min) for the proteins carbonic anhydrase B (□), α-chymotrypsinogen A (+) and aldolase (◇). Linear gradient without CHAPS, 100% 1.7 *M* ammonium sulfate + 0.02 *M* potassium phosphate, pH 6.1 to 100% 0.02 *M* potassium phosphate, pH 6.1.

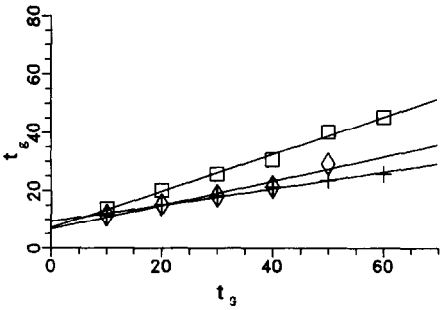

Fig. 2. Dependence of retention time (min) on gradient time (min) for the proteins α-amylase (□), BPTI (+) and enolase (◇). LSS gradient analysis, Type III bilinear gradient elution with CHAPS, 100% 1.7 *M* ammonium sulfate + 0.1 m*M* CHAPS + 0.02 *M* potassium phosphate, pH 6.1 to 100% 3.0 m*M* CHAPS + 0.02 *M* potassium phosphate, pH 6.1.

proteins. In Fig. 1, t_g *vs.* t_G is plotted for three representative proteins under gradient elution conditions without CHAPS in the mobile phase. In Fig. 2, t_g *vs.* t_G is plotted for representative data collected under Type III gradient conditions (with CHAPS present). As can easily be seen in Fig. 2, linear relationships between t_g and t_G are also observed for CHAPS-containing mobile phases in gradient elution HIC of proteins. Similarly, linear plots are obtained for Type II gradients and for the other six proteins under Type III conditions. Therefore we find that HIC separations with CHAPS can be modeled in terms of the LSS theory.

Selectivity

In order to examine the effects of CHAPS on selectivity, t_g was determined as $f(t_G)$ for the nine proteins with CHAPS (Type III gradients) and without CHAPS. Linear relationships for t_g *vs.* t_G were found in all cases, consistent with the LSS model. As shown in Table II, the effect of CHAPS varied greatly, depending on the

TABLE II

CHANGES IN t_g DUE TO CHAPS

Gradients of 60 min. Type III gradients were employed, with salt decreasing linearly from 1.70 *M* to 0 *M* ammonium sulfate, and [CHAPS] increasing linearly from 0.6 m*M* to 3.0 m*M*. With values of t_G ranging from 10 to 60 min, plots of t_g *vs.* t_G were fit with a linear least-squares program. Least-squares values of t_g with and without CHAPS were used to obtain $\Delta t_g = t_g$ (with CHAPS) $- t_g$ (without CHAPS).

Protein	t_g (without CHAPS)	Δt_g (min)
α-Amylase	45.9	− 3.7
Aldolase	43.1	− 0.8
BPTI	31.1	− 6.9
BSA	60.6	−17.5
Carbonic anhydrase	29.8	− 9.5
α-Chymotrypsinogen	53.3	− 7.9
Enolase	28.0	−13.7
Lysozyme	29.9	− 7.1
Ribonuclease A	16.7	− 4.9

protein. With t_g = 60 min the decrease in t_g due to CHAPS ranged from 0.8 min (aldolase) to 17.5 min (BSA). These reductions in t_g correspond to 1.8% and 30% respectively. It is therefore evident that the CHAPS shows strong selectivity in the gradient mode, consistent with what we have already shown for isocratic HIC [20].

Operational characteristics

Selectivity effects in Type I gradient elution were not investigated, as this mode of chromatography is more difficult to carry out. This is because of the difficulty of finding a suitable retention window of salt and surfactant concentrations such that proteins are retained strongly without CHAPS and weakly retained with CHAPS in the mobile phase. However, one such window was found for the protein carbonic anhydrase B. Fig. 3 shows the elution of this protein under Type I gradient conditions. The Type I gradient experiment was carried out using an initial CHAPS step gradient of 0–0.6 m*M* at a constant ammonium sulfate concentration of 1.7 *M*. The large peak observed in the chromatogram at about 5–10 min is caused by the breakthrough of a small amount of solvent impurity in the CHAPS (identified by gas chromatography as dimethylformamide). The detector was re-zeroed at about 10 min to keep the entire chromatogram on scale. Breakthrough of CHAPS ocurred at approximately 60 min, followed by the carbonic anhydrase B peak at approximately 70 min, as [CHAPS] in the mobile phase continued ro rise, approaching equilibrium.

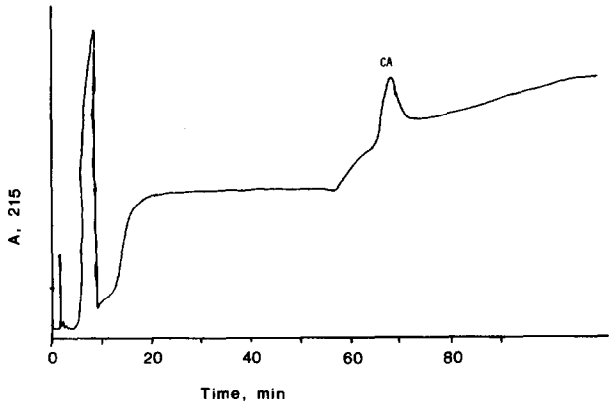

Fig. 3. Type I gradient elution of carbonic anhydrase B (CA). At t_0 a step gradient of 0 to 0.6 m*M* CHAPS in 1.70 *M* ammonium sulfate + 0.02 *M* potassium phosphate was introduced. The apparent peak at 5–10 min is an artifact caused by the breakthrough of a small amount of solvent impurity, followed by re-zeroing the detector at a time corresponding to the apparent peak maximum. Re-zeroing was necessary to keep the entire chromatogram on scale.

The extent of change of retention time for Type III gradient elution is affected by the initial CHAPS concentration. Fig. 4 shows the effect of increasing the initial surfactant concentration from 0.1 to 0.6 m*M* on the retention time of BPTI. As this example shows, the magnitude of the change in retention time increases with the initial CHAPS concentration. The protein BPTI shows a small decrease in retention with an initial mobile phase concentration of 0.1 m*M* CHAPS in Type III gradient elution. When the initial CHAPS concentration is increased to 0.6 m*M*, a significantly

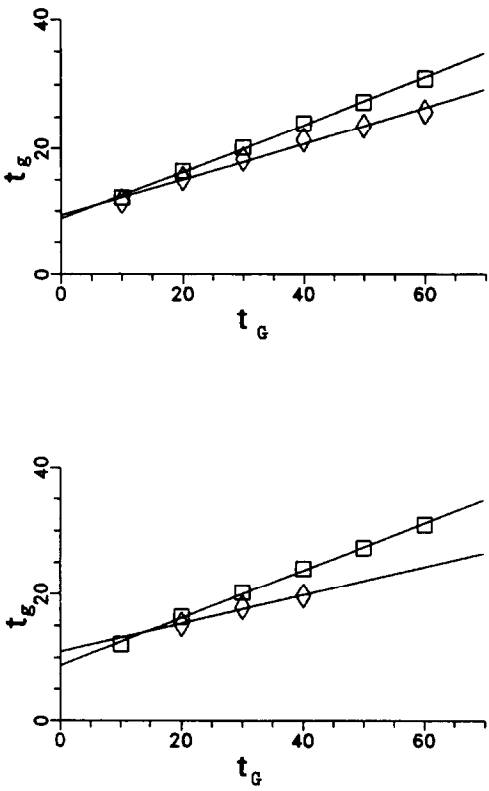

Fig. 4. Effect of initial CHAPS concentration on the retention of the protein BPTI in Type III gradient elution. Type III bilinear gradient elution with CHAPS (◇), 100% 1.7 *M* ammonium sulfate + 0.1 m*M* CHAPS (top) or 0.6 m*M* CHAPS (bottom) to 100% 3 m*M* CHAPS + 0.02 *M* potassium phosphate, pH 6.1. Gradient elution without CHAPS (□), 100% 1.7 *M* ammonium sulfate + 0.02 *M* potassium phosphate, pH 6.1 to 100% 0.02 *M* potassium phosphate, pH 6.1. Retention and gradient times in min.

larger decrease in retention time is seen for BPTI. A similar pattern was observed for lysozyme.

Protein retentions in Type II and Type III gradient elution modes were also compared. Fig. 5 shows the effect of the two gradient modes on α-chymotrypsinogen A. Contrary to our expectation of shorter retention times with Type III gradients (compared to Type II), the data show no significant differences. We believe that this is the result of the appreciable lag time between t_0 and the time required to load the stationary phase with surfactant. Substantial lag times are evident in both Fig. 3 (0–60 min) and Fig. 6 (50–85 min). This is discussed further in the following section.

Equilibration

In order to study equilibration requirements of the HIC column after execution of a surfactant gradient, blank elution profiles were recorded, using a detector wavelength where CHAPS exhibits significant UV absorbance, *i.e.* 215 nm. The resulting blank elution profiles are shown in Fig. 6 (Type II) and Fig. 7 (Type III). For Type II

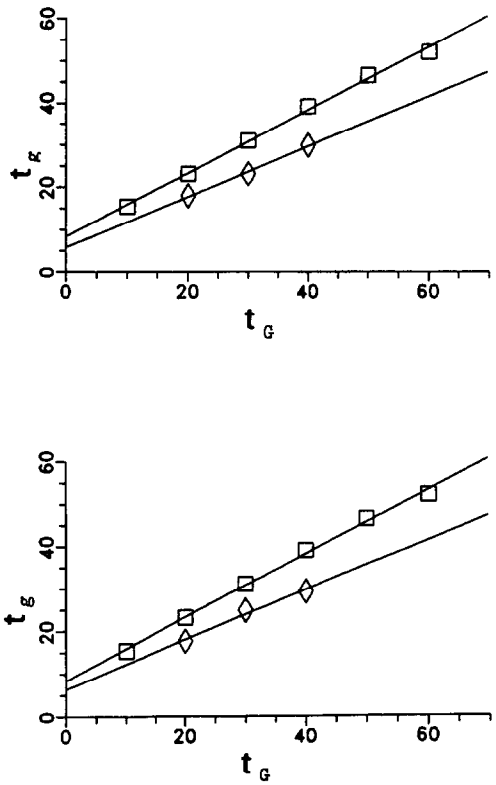

Fig. 5. Type II *vs.* Type III gradient elution for α-chymotrypsinogen A. (Top) Type II linear gradient elution with CHAPS (◇): 100% 1.7 *M* ammonium sulfate + 0.6 m*M* CHAPS + 0.02 *M* potassium phosphate, pH 6.1 to 100% 0.6 m*M* CHAPS + 0.02 *M* potassium phosphate, pH 6.1. (Bottom) Type III bilinear gradient elution with CHAPS (◇): 100% 1.7 *M* ammonium sulfate + 0.6 m*M* CHAPS + 0.02 *M* potassium phosphate, pH 6.1 to 100% 3 m*M* CHAPS + 0.,02 *M* potassium phosphate, pH 6.1. Gradient elution without CHAPS, 100% 1.7 *M* ammonium sulfate + 0.02 *M* potassium phosphate, pH 6.1 to 100% 0.02 *M* potassium phosphate, pH 6.1. □ = Retention times without CHAPS. Retention and gradient times in min.

gradients a number of features are observable. First, at a point near the end of the gradient (approximately 22 min), a large peak is observed. This is believed to be due to the elution of CHAPS from the stationary phase. Shortly after returning to initial conditions at the end of the gradient, a large negative deflection is observed at approximately 48 min. This corresponds to removal of CHAPS from the mobile phase by adsorption onto the stationary phase for reequilibration of the stationary phase surface. This is expected, since with a decrease in mobile phase salt concentration, the equilibrium amount of CHAPS adsorbed on the SynChropak propyl stationary phase surface decreases [20]. Thus, CHAPS adsorbed on the stationary phase at the initially high salt concentration at the start of the gradient would be expected to be eluted from the column as the salt concentration decreases in the gradient. After reequilibration of the stationary phase surface, breakthrough of the CHAPS-containing mobile phase is seen at 80–90 min followed by a return to the original baseline at

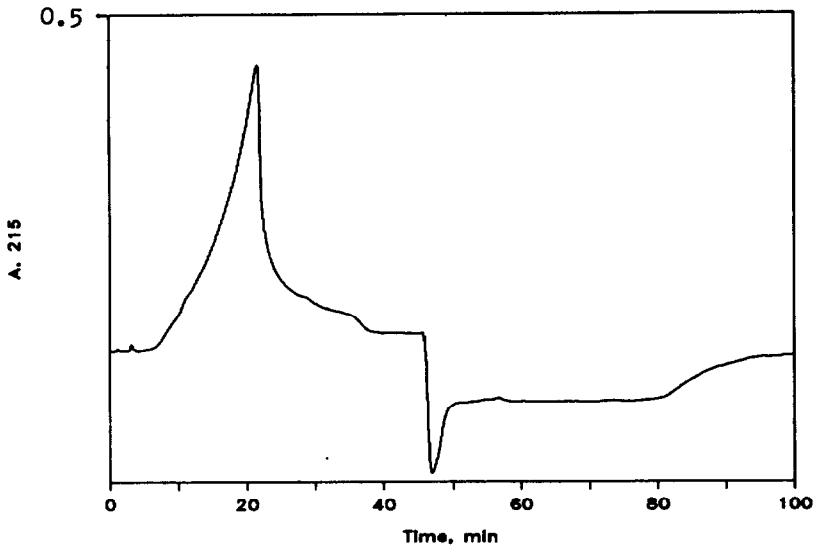

Fig. 6. Type II gradient equilibration with 0.2 m*M* CHAPS. Blank gradient on SynChropak propyl, 10 cm × 4.6 mm I.D., 100% 1.7 *M* ammonium sulfate + 0.2 m*M* CHAPS + 0.02 *M* potassium phosphate, pH 6.1 to 100% 0.2 m*M* CHAPS + 0.02 *M* potassium phosphate, pH 6.1. A.u.f.s. = 0.5.

100 min. In these studies reequilibration times of 60–90 min were employed, depending on column volume and surfactant concentration. Increase in the mobile phase concentrations of CHAPS or ammonium sulfate leads to increased adsorption of CHAPS on the stationary phase and a longer time for equilibration. Longer equili-

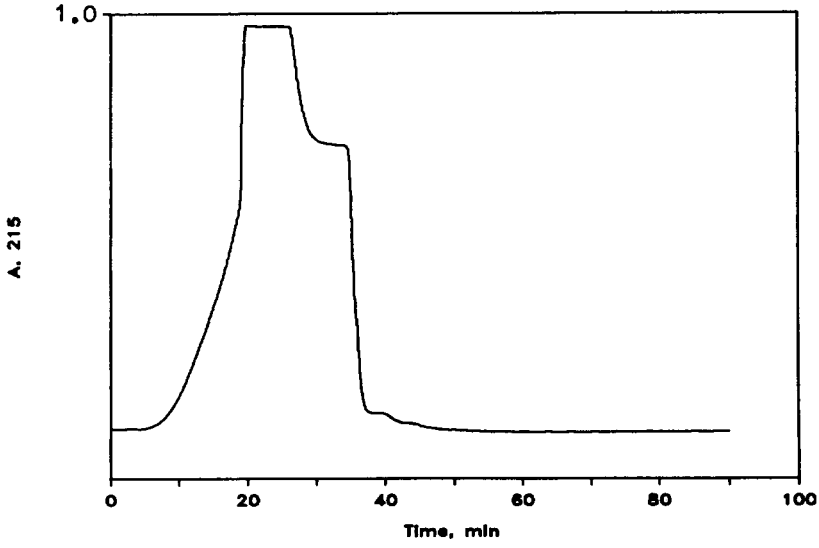

Fig. 7. Type III gradient equilibration. Blank bilinear gradient on SynChropak propyl, 10 cm × 4.6 mm I.D., 100% 1.7 *M* ammonium sulfate + 0.6 m*M* CHAPS + 0.02 *M* potassium phosphate, pH 6.1 to 100% 3 m*M* CHAPS + 0.02 *M* potassium phosphate, pH 6.1. A.u.f.s. = 1.0.

bration times result from atypical adsorption isotherms and the marked depletion of surfactant from the mobile phase [20,31,32] while the stationary phase is being loaded. The effect is seen clearly in the determination of adsorption isotherms by frontal analysis [20,31,32].

Type III gradients (Fig. 7), exhibit some significant differences from Types I and II. (Note: comparison of Figs. 6 and 7 should allow for the difference in absorbance scales.) First, the magnitude of the peak observed near the end of the gradient (*i.e.* the "CHAPS peak") is larger than for Type II. This is probably due to the combined effects of increasing [CHAPS] and decreasing $[(NH_4)_2SO_4]$ in the Type III gradient as compared with only decreasing $[(NH_4)_2SO_4]$ in the Type III gradient. Furthermore, the negative deflection observed in Type II gradient elution is not present in Type III. The breakthrough of CHAPS-containing mobile phase after reequilibration of the column is also missing. No negative peak is observed at the return to initial conditions at 40 min, and the detector signal returns immediately to the original baseline value. Also no breakthrough of CHAPS was noted at the end of the equilibration period. Therefore the required equilibrations for our Type III gradients are much shorter than for Type II gradients. Typically, equilibration times of 15–20 min were used in this study. These equilibration requirements are comparable to those required in conventional high-performance HIC separations without surfactant. Since we have not carried out an extensive survey of different Type III gradients, we cannot be sure that rapid equilibration will always be obtained. Nonetheless it appears likely that Type III gradient will be preferred for repetitive analyses.

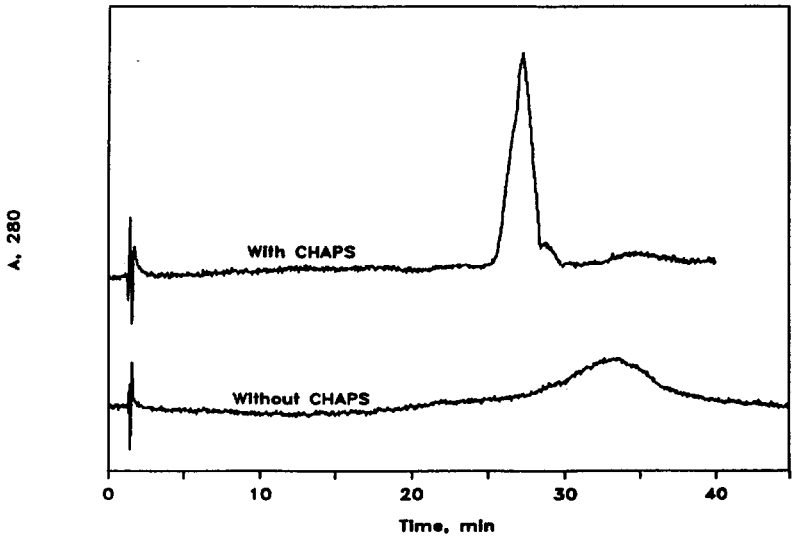

Fig. 8. The effect of CHAPS on the peak shape of bovine serum albumin in gradient elution. Lower curve: 100% 1.7 *M* ammonium sulfate + 0.02 *M* potassium phosphate, pH 6.1 to 100% 0.02 *M* potassium phosphate, pH 6.1. Linear gradient of 30 min with a 15-min hold at final conditions. Gradient elution with CHAPS (upper curve) is Type III: 0.6 m*M* CHAPS linearly increasing to 3.0 m*M* CHAPS at 30 min, superimposed on the salt gradient for the lower curve.

Peak shapes

Almost all the proteins examined in this study produced chromatographic peaks of normal shape with or without the presence of CHAPS. However, the protein bovine serum albumin (BSA) has been reported to yield distorted peak shapes in HIC [4]. We therefore decided to examine the effect of CHAPS on the peak shape of this protein in gradient elution HIC. When BSA is eluted from a SynChropak propyl HIC column it elutes as a broad distorted peak with a much larger bandwidth than the other proteins in this study (Fig. 8). When CHAPS is added to the mobile phase, the retention time is considerably reduced and a narrowed, less distorted peak results. The presence of CHAPS had negligible effects on peak shapes of the eight other proteins in this study. We assume that distortion of the BSA peak in the absence of CHAPS is the result of sluggish conformational equilibria in the protein [27]. On this assumption it appears that CHAPS has the effect of either shifting the equilibrium or of speeding rate-limiting conformation interconversions. Another possibility is binding of BSA to the stationary phase surface followed by a conformational change induced by the stationary phase. This mechanism has been reported for the reversed-phase chromatography of proteins [9]. In the presence of CHAPS we know that BSA is bound less tightly to the stationary phase surface, possibly reducing conformational instability induced by contact with the stationary phase.

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