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Use of cholate derivatives with submicellar concentration for controlling selectivity of proteins in hydrophobic interaction chromatography

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Abstract

Hydrophobic interaction chromatography (HIC) of proteins using a phenyl column has been performed in the presence of various surfactants with micellar and submicellar concentration ranges. Most surfactants were effective for a decrease in the retention of proteins in both concentration ranges. However, the use of anionic cholate derivatives increased the retention of the proteins with high isoelectric point, such as lysozyme, cytochrome *c*, and trypsin, in submicellar concentration range, and then decreased it above the critical micellar concentration, while the retention of the other proteins was monotonously decreased. The results of frontal chromatographic analysis of the surfactant and capillary electrophoresis for the proteins in the presence of surfactant show that in the submicellar concentration range, cholate derivatives allowed to be adsorbed on the stationary phase, while they exhibited no interactions with the proteins. Thus, it appeared that the increase in the retention of basic proteins was due to the electrostatic attraction between the proteins and cholate-modified stationary phase. We have applied the unique property of cholate to the separation of ovalbumin and lysozyme in egg white sample using hydrophobic chromatography.
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Keywords: Hydrophobic interaction chromatography; Micelles; Retention behavior; Proteins; Surfactants; Cholates

1. Introduction

In protein separation, high-performance liquid chromatography (HPLC) is to be essential in view of the high resolution and of simple operation [1]. Most commonly, hydrophobic silica supports with C4, C8, C18, and longer alkyl modification as available have been used in reversed-phase HPLC (RP-HPLC). However, the interaction of proteins with the absorbents tends to be so strong that drastic conditions such as high concentration of non-aqueous solvent are required for protein elution, which will result in protein denaturation in RP-HPLC [2–4]. Recently, the use of hydrophobic interaction chromatography (HIC), in which protein elution is conducted by a decrease in salt concentration, has increased due to its capability of non-denaturing protein separation [5], and thus the number of commercially available HPLC support of this mode has

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also increased (HI-HPLC). In RP- and HI-HPLC, surfactants are also effective for reducing retention of proteins [6–15]. As expected, they can interact with the stationary phase supports and the proteins to inhibit direct interaction of the supports and the proteins. The use of surfactant micelles in the mobile phase has been widely accepted as micellar liquid chromatography [16–18], which provides unique and selective properties in the separation of hydrophobic solutes as well as micellar electrokinetic chromatography, in which micelles are used as a pseudo-stationary phase [19–21].

In HIC with surfactant mobile phase, adsorption of the surfactant molecules onto the hydrophobic supports and the proteins depending on the surfactant concentration should alter their surface properties [8,10,14]. Thus, consideration of the interaction between surfactant-modified stationary phase supports and proteins should be required for assessing retention of the proteins in HIC. As a few systematic studies on the use of a variety of surfactant mobile phases have been reported in protein separations [7,8,10,14], we have tested a phenyl–ligand support which is in frequent use for protein separations, with submicellar and micellar mobile phases. In this study, employing a set of six proteins as retention probes, the role of surfactants in HI-HPLC with a phenyl column was investigated in a broad concentration range below and above critical micelle concentration (CMC). We have demonstrated that anionic cholate and its derivatives have shown the unique property for retention of proteins in comparison with other surfactants tested, and that it has be mainly due to the weak hydrophobic interaction between cholates and proteins.

2. Experimental

2.1. Reagents

Sodium dodecyl sulfate (SDS), sodium cholate (SC), sodium deoxycholate (SDC), and sodium taurocholate (STC) were obtained from Wako Pure Chemical (Osaka, Japan). Cetyltrimethylammonium bromide (CTAB) was obtained from Kishida Chemical (Tokyo, Japan). 3-[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid (CHAPS) and disodium salt of bicinchoninic acid (BCA) were from Dojindo Laboratories (Kumamoto, Japan), and Triton X-100 (TX100) for a grade of liquid scintillation was purchased from Nacalai Tesque (Kyoto, Japan). All surfactants were used as received.

Lysozyme (chicken egg white grade I), albumin (bovine serum), trypsin (bovine pancreas), myoglobin (horse heart), hemoglobin (horse erythrocyte), concanavalin A (*Canavalia ensiformis*) were supplied from Sigma (St. Louis, MO). Cytochrome *c* (horse heart) was from Wako Pure Chemical (Osaka, Japan).

All other reagents used were of analytical grade.

2.2. Instruments

All high-performance liquid chromatographic measurements were made with a hydrophobic phenyl column (TOSOH TSKgel phenyl-5PW, 7.5 cm × 7.5 mm i.d., pore size 100 nm), which was incorporated with a pump (Shimadzu LC-6A), a 100 μl loop injector (Rheodyne model 7125), and a UV-Vis detector (Hitachi L-4200). A Hitachi D-2500 Chromato-Integrator was utilized for the peak area measurement in the chromatogram. The HPLC system was temperature-controlled. For frontal chromatographic analysis of a surfactant, the same instrument as above was employed. Capillary electrophoresis was conducted with a fused-silica capillary column (GL Sciences, 70 cm × 50 μm i.d.) in combination with a power supply (Matsusada Precision Devices HCZE-30P NO.25), a data processor (Hitachi D-2500), and a UV-Vis detector (JASCO CV40).

2.3. Procedures

In HPLC, mobile phases were prepared by dissolving a certain surfactant with various concentrations in 0.1 M phosphate buffer solution (pH 7.0). After equilibration of the column with the mobile phase at a flow rate of 0.5 ml min^{-1}, a 100 μl portion of aqueous protein–surfactant solution was injected, in which the surfactant concentration was the same as that in the mobile phase. The elution of all the proteins was made with the same solution at 0.5 ml min^{-1} as was used in the column equilibration, and monitored by measurement of absorbance at 280 nm. For analysis

of lysozyme and ovalbumin in egg white samples, a mobile phase was a Tris–buffer solution (50 mM, pH 9.0) containing sodium chloride (0.2 M) and a surfactant. After equilibration of the column with the mobile phase, a 100 μl portion of diluted samples of egg white was injected. The retention of the two proteins was monitored by measuring absorbance at 282 nm. Egg white samples were prepared according to the methods of Awade et al. [22]. To a 5 ml aliquot of egg white, 10 ml of a 50 mM Tris–buffer solution containing 0.4 M sodium chloride and 10 mM 2-mercaptoethanol were added. The egg white solution thus obtained was kept overnight while being stirred gently, and was used after dilution.

In the frontal chromatographic analysis, the column was first equilibrated with an aqueous phosphate buffer (0.1 M, pH 7.0) and without addition of any surfactants at a flow rate of 0.5 ml min^{-1}. Then, another phosphate buffer solution (0.1 M, pH 7.0) containing a fixed amount of a surfactant was continuously loaded at the same flow rate, and the frontal elution of a surfactant was monitored by measurement of absorbance at 210 nm.

The interstitial volume (V_0) of the chromatographic system was determined by injecting different solutions such as methanol, methanol–water, or water into the column. This volume, approximately 1.0 ml, was used for calculations for retention factors $\{k' = (V_e - V_0)/V_0\}$, where V_e is the retention volume for a certain protein. The k' values determined in this study were averages of at least triplicate determinations. Deviations in individual retention factor values were never greater than 5%.

In the capillary electrophoresis, a capillary was purged with a CHAPS buffer solution (10 mM, pH 12.0) involving a definite concentration of a surfactant. Setting such high pH condition can prevent proteins from adsorption onto the inner wall of the capillary. Protein solutions were introduced into the capillary by applying 10 kV for 15 s to both ends of the capillary. Then, the positive end of the capillary was placed into the buffer solution. Electrophoresis was made by applying 15 kV and was monitored by measurement of the absorbance of a protein at 280 nm.

Total proteins in the egg white samples were determined by the modified BCA method of Hill and Straka [23] with bovine serum albumin as a standard.

3. Results and discussion

3.1. Effect of surfactants on retention of proteins

For comparing the trends of retention with surfactant concentrations, we have exploited the normalized retention parameter K of the proteins [8] defined as:

$$K = \frac{k'}{k'_0} \quad (1)$$

where the denominator (k'_0) and the numerator (k') are the retention factors found in the absence and the presence of surfactants, respectively.

Fig. 1 shows the K values of the proteins as a function of the concentrations of various surfactants. Except for SC in Fig. 1D, all surfactants induced decreases in the K values for all the proteins with an increase in surfactant concentrations. Most surfactants are accepted to be effective for only decreasing retention of many proteins in the concentration range below and above the CMC by inhibiting the hydrophobic interaction between the protein and the stationary phase. Thus, the effect of surfactant could be independent upon the net charges of proteins and those of surfactants. In the absence of surfactants, lysozyme showed the longest retention time among the proteins tested, indicating strong hydrophobicity of lysozyme, so that the retention of lysozyme was greatly decreased in the use of CTAB, CHAPS, and TX100. In contrast, the effect of SDS on the retention seems likely not to be selective for the proteins. This could be due to strong interaction between the proteins and SDS monomer as described later.

As is evident in Fig. 1D, the effect of SC was different from others in view of longer retention of lysozyme, trypsin, and cytochrome *c* in the concentration range below CMC, which is reported to be 15 mM. The K values of these proteins were increased in an SC concentration range below the CMC, but were decreased above the CMC. Especially, retention factor of lysozyme at the CMC was almost 10 times greater than that in the absence of SC. The retention of myoglobin and hemoglobin were almost unchanged, and that of concanavalin A was slightly decreased. Additionally, these three proteins were almost unaffected by an increase in SC concentration even above the CMC.

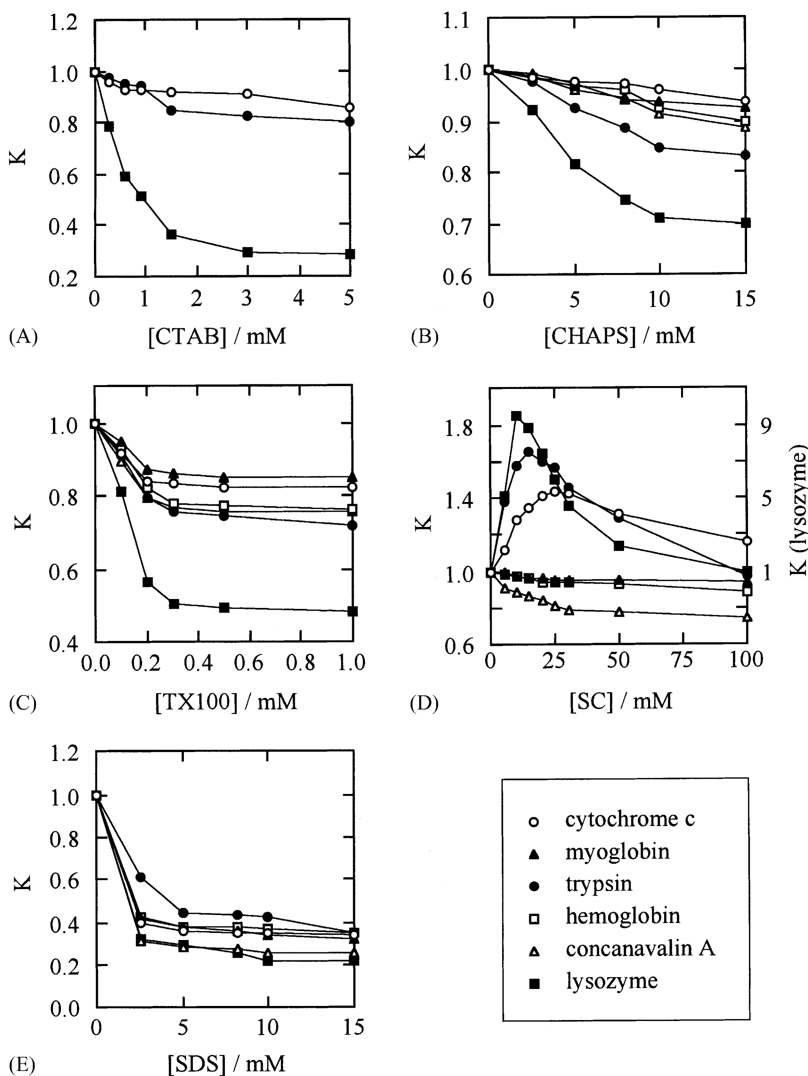

Fig. 1. Effect of surfactant concentration in the mobile phase on normalized retention parameter, K, of six proteins. Hydrophobic interaction chromatography was performed on a TSKgel phenyl-5PW (7.5 mm i.d., 7.5 cm length) column with 0.1 M phosphate buffer (pH 7.0) containing surfactant as a mobile phase at flow rate of 0.5 ml/min.

The marked effect of SC is probably dependent on a net charge of proteins. Table 1 lists the isoelectric points (pI) of the proteins and their K values at CMC of SC. This clearly indicates that the greater the pI, the greater the K value. Fig. 2 shows the influence of pH on the retention of six proteins, where the ordinate was expressed as a ratio of a retention factor (k') at an appropriate pH to that at pH 7.0 (k'(pH = 7.0)). The ratio was decreased with an increase in pH, namely with a decrease in the net charge of a protein. It is clear from the results in Table 1 and Fig. 2 that the charge of proteins plays a dominant role in the retention behavior, when SC is present in a submicellar concentration. This situation may be seen as formally analogous to the proposals for ion-pairing mechanisms in hydrophobic interaction chromatography of small molecules, in which surfactant molecules distribute onto the stationary phase to alter its surface

Table 1
Isoelectric point and K values of proteins

Protein	pI[a]	K[b]
Lysozyme	11.0	8.84
Trypsin	8.7	1.66
Cytochrome c	9.0	1.35
Myoglobin	7.3	0.97
Hemoglobin	7.4	0.96
Concanavalin A	4.4	0.88

[a] pI values were obtained from [31–33].
[b] K values were calculated from the retention factors without surfactant and with SC at its critical micellar concentration, 15 mM.

properties [24,25]. An electrostatic attraction between the proteins with high pI and the SC adsorbed on the stationary phase seems to play a direct role in increasing the retention of proteins. However, the situation can be applicable to the use of SDS and other ionic surfactants. As mentioned above, SDS as well as other surfactants only reduces the retention of proteins even in a submicellar concentration range. Thus, this is a part of the answer as to why the retention of the proteins with high pI was increased by using SC, but was decreased by using SDS in a submicellar concentration range. Meanwhile, the decrease in the retention of proteins at micellar concentration levels of SC and SDS could be ascribable to the predominant interactions of proteins and micelles in the mobile phase, as is likely to be in micellar chromatography.

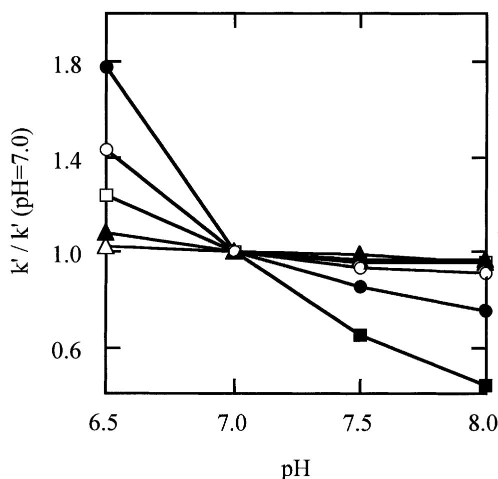

Fig. 2. Effect of pH on normalized retention factor of proteins. Each symbol indicates the same protein as in Fig. 1.

The surfactants can interact with proteins in the mobile as well as the stationary phase. Thus, two factors, the modification of the stationary phase by adsorbed surfactant and the interaction of the proteins with surfactants, could have profound implications with regard to the retention. As there have been great differences in the effects of SC and other surfactants on the retention of proteins, in the subsequent studies we have chosen SC and SDS for clarifying their distribution on the stationary phase based on frontal chromatography, and their interaction with proteins based on capillary electrophoresis.

3.2. Frontal chromatography of SC and SDS

Distribution isotherms of SC and SDS between the stationary and mobile phases in HIC were obtained by their frontal chromatographic analysis. Frontal analysis involves changing the concentration of a surfactant at the column inlet in a stepwise fashion. When the concentration is increased, a step develops into a sharp front at the column outlet. Fig. 3 depicts a typical elution profile of SC upon changing its concentration from C_a to C_b, and an integral mass balance shows that the stationary phase concentration of SC, $q(C_b)$, in equilibrium with C_b in a mobile phase is given by:

$$q(C_b) = q(C_a) + \frac{(C_b - C_a)(V_e - V_0)}{V_{sp}} \quad (2)$$

where $q(C_a)$ is the concentration of adsorbed surfactant in equilibrium with C_a in a mobile phase [26,27]. V_0, V_e, and V_{sp} are the void volume, the retention

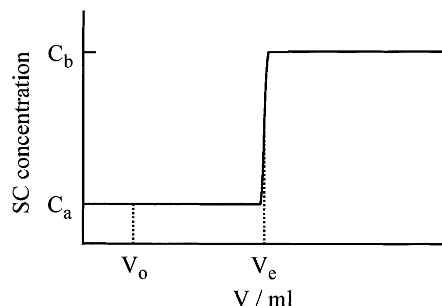

Fig. 3. Chromatogram of cholate in frontal analysis. V_0, void volume in the column; V_e frontal elution volume of cholate; C_a and C_b, cholate concentrations in the mobile phase at the beginning and the end of the analysis, respectively.

volume of a front, and the volume of the stationary phase, respectively. Repeating this operation with successively higher concentrated solutions of a surfactant yields additional discrete points on the distribution isotherm.

In the frontal chromatograms of SC and SDS, where total concentration of the respective surfactants was changed from 0 to 5 mM, the retention time of surfactant front was longer than that of the solvent front, thus indicating distribution of the two surfactants on the stationary phase as expected above.

In Fig. 4, the amounts of both surfactants, SC and SDS, adsorbed onto the stationary phase, $q(C_b)$, were increased with an increase in their equilibrium concentrations in the mobile phase even below the CMC. Additionally, SDS was found to distribute onto the stationary phase more readily than SC. A large amount of SDS distribution would give rise to a modified stationary phase, which is hydrophilic in nature. The shorter retention times of proteins on the SDS-modified stationary phase are partly due to an increase in the polarity of the stationary phase resulting from SDS distribution. However, the amounts of the surfactant adsorbed cannot explain the differences in the retention behavior in SC and SDS. Thus, for clarifying the factors which define the retention of proteins, we also have to take into account the interaction of proteins with the monomeric surfactant. For this purpose, we have examined the behavior of proteins in capillary electrophoresis in the presence of a surfactant with various concentrations below and above CMC.

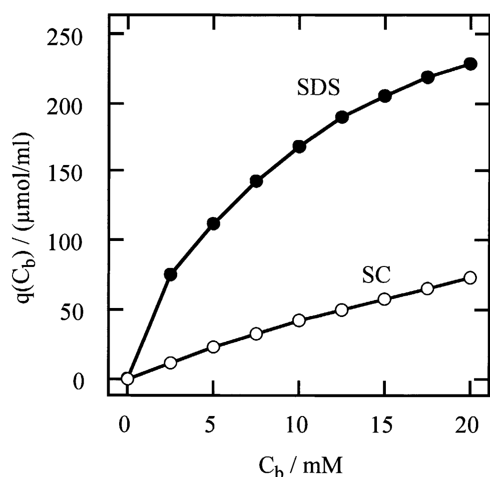

Fig. 4. Adsorption isotherms of SDS and SC on a TSKgel phenyl-5PW measured by frontal chromatography.

3.3. Capillary electrophoresis of proteins

In spite of the observation that both SC and SDS were adsorbed onto the stationary phase below the CMC, SC enhances the retention of proteins in a submicellar concentration range, while SDS only reduces the retention. The two surfactants are likely to show different interactions of monomeric surfactant with proteins in a mobile phase. In view of this, we examined the concentration effects of two surfactants below and above CMC on the capillary electrophoresis of proteins. We have chosen lysozyme and cytochrome *c* as a probe protein because of their distinctively different behavior in their retention.

Fig. 5 shows the capillary electropherograms for cytochrome *c* at pH 12.0 and at two concentration regimes of the respective surfactants. It appeared that SDS tends to decrease considerably the migration time of cytochrome *c* in comparison with SC. Furthermore, the migration time in the presence of SC below CMC was almost unaffected in comparison with that in the absence of SC. On the other hand, the migration time even in the presence of SDS below CMC was longer

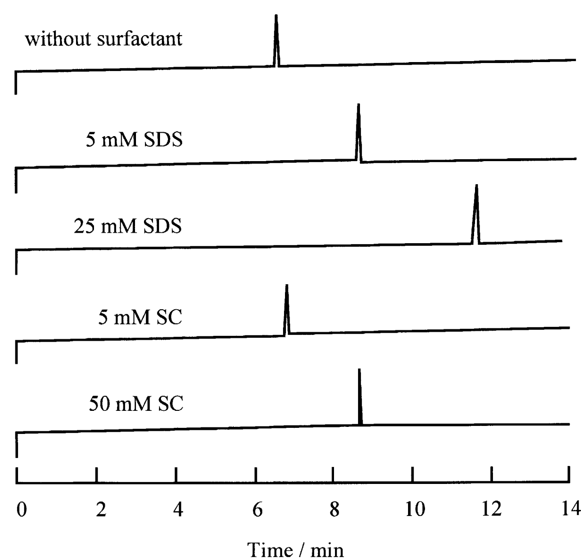

Fig. 5. Capillary electropherograms of cytochrome *c* in the presence of SDS or SC below and above respective critical micellar concentrations.

than that in the absence of SDS. The same results were obtained when lysozyme was used as a retention probe.

The effect of surfactant on the elution of the proteins can be estimated by using the retention factor k'_{ce} [20,21] given by:

$$k'_{ce} = \frac{U_{eo} + U_{ep} - U_{p}}{U_{p} - U_{mc}(\text{or } U_{mo})} \quad (3)$$

where U_{eo} is the electro-osmotic velocity of solution, U_{ep} the electrophoretic velocity of the protein, U_{p} the apparent velocity of the protein, U_{mc} the electrophoretic velocity of the micelle, and U_{mo} is the electrophoretic velocity of the surfactant monomer. U_{mc} and U_{mo} were used above and below CMC, respectively. Fig. 6 shows the effect of the surfactant concentrations on the k'_{ce} values of cytochrome *c* and lysozyme. The retention factors of both proteins increased with an increase in the concentration of SDS even below CMC, while in the case of SC, they increase only above CMC of SC. The increase of retention factor means the increase in the apparent negative charge on the protein, which should be due to the hydrophobic interaction between protein and surfactant molecules or surfactant micelles, because both of the proteins and surfactants have negative charge at the experimental condition, pH 12. In comparing the two proteins, k'_{ce} of lysozyme was more affected by the surfactants than that of cytochrome *c*, indicating a strong hydrophobicity of lysozyme as was observed in HIC.

These facts suggest the strong interaction of proteins with SDS even in the monomer state. Thus, independent upon the state of SDS, namely above or below CMC, SDS–protein interactions could define the retention behavior of proteins in capillary electrophoresis. It has already been reported that SDS in aqueous solutions strongly associates with proteins below the CMC [28,29]. The present result is consistent with those reported by other workers, as evidenced by the greater k'_{ce} value of proteins with increasing SDS concentration in a range below CMC.

The SDS–protein complex as a result of the strong hydrophobic interaction would have a negative charge in spite of a net charge of the protein even at pH 7, the condition in the chromatography. The formation of the complex could give rise to the electrostatic repulsion with SDS-modified stationary phase. Meanwhile, the increase in surface polarity of the stationary phase in HIC as a result of SDS distribution would also lead to a decrease in hydrophobic interactions between proteins and the stationary phase. Any or all of these factors can contribute to a great decrease in retention of proteins in a broad range of SDS concentrations compared to other surfactants.

On the other hand, as a comparison of the results in Fig. 6 indicates, SC has little effect on the migration time of the proteins, thus suggesting the very weaker interaction of proteins with SC than with SDS. Furthermore, in Fig. 6B, SC below CMC has insignificant effect on k'_{ce} values, while SC above CMC increases k'_{ce} values. From this result, it is

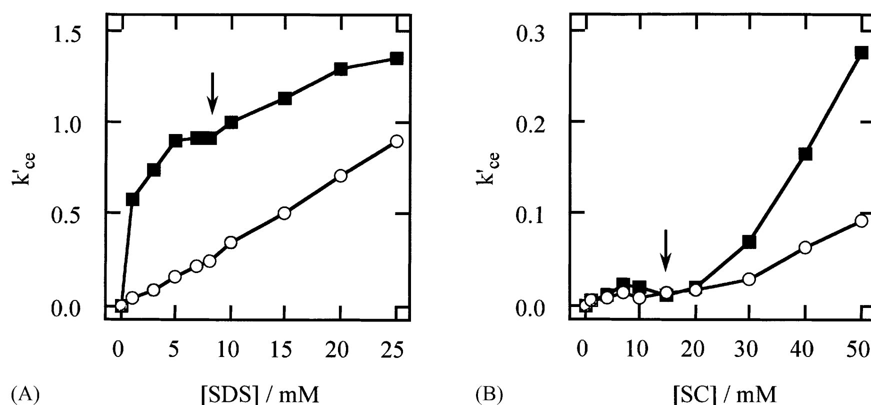

Fig. 6. Relationships between k'_{ce} and surfactant concentration in capillary electrophoresis of lysozyme and cytochrome *c*. The arrows indicate the critical micellar concentrations of the respective surfactants.

reasonably concluded that the hydrophobic interaction of monomeric SC with proteins is weaker than that of SC micelles with proteins. That is, SC can adsorb onto the stationary phase below CMC, but it can interact with proteins only above CMC. Thus, the electrostatic attraction between the proteins with a positive charge and SC-modified stationary phase could give rise to an increase in the retention below CMC of SC, while above CMC the retention decreases as the same manner as in the case of SDS. The specific interaction of SC with proteins, which is highly dependent on the solution state of SC, is probably due to its structural nature, i.e. the hydrophobic moiety of SC is in the single side of its steroid ring [30]. This structure could provide the strong constraint on the hydrophobic interaction with proteins, but not with phenyl groups on the stationary phase.

3.4. Use of various bile acids on the retention of proteins in hydrophobic chromatography

In Fig. 1, SC was characteristic in view of enhancing retention of proteins having a high isoelectric point in the SC concentration range below CMC, while the retention of other proteins with a low isoelectric point was reduced. This property was seen only for SC among surfactants tested. Even in the use of CHAPS, zwitterionic derivative of cholate, an increase in the retention was not observed. Buckley and Wetlaufer [8] also reported that below CMC, CHAPS decreased the retention of protein, where a propyl HIC packing was used as a stationary phase. From these results, CHAPS would act only as an inhibitor for the interaction between the stationary phase and proteins. Thus, the specific property of SC in the retention of proteins seems to be dependent upon its structure and charge. For further elucidation of the nature of SC, we tested sodium deoxycholate and sodium taurocholate, having the same basic structure and charge as those of SC, in the hydrophobic chromatography of proteins.

In Fig. 7, the relative retention factors (K value) of five proteins were shown as a function of surfactant concentration. The retention behavior of the five proteins in the use of SDC and STC was almost the same as that in the use of SC (Fig. 1D). The K values for trypsin and cytochrome c with a high pI increased, then reached to a maximum, and decreased with increasing concentrations above the respective CMC of SDC and STC. The K values of other three proteins, myoglobin, hemoglobin, and concanavalin A, having a low pI decreased monotonously with an increase in surfactant concentrations. These properties were in common to cholates having the same basic structure as SC.

It is apparent in Fig. 7 that SDC gives the K values higher than SC or STC, for proteins having a high pI, and gives the K values smaller than SC or STC for proteins with a low pI. It is also certain that there is no distinct deference between SC and STC. For further elucidation of functions of three cholates, we conducted their frontal chromatographic analysis. Data

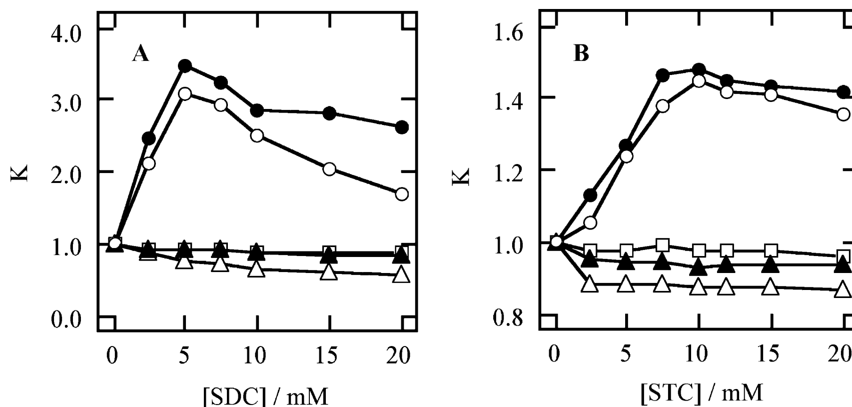

Fig. 7. Effect of the concentration of cholate derivatives on normalized retention parameter, K, of six proteins in hydrophobic interaction chromatography. Each symbol indicates the same protein as in Fig. 1.

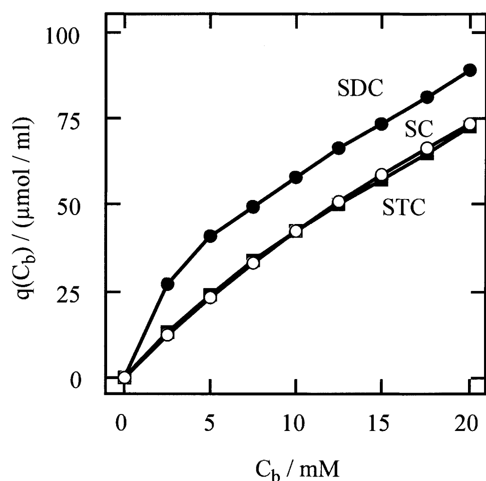

Fig. 8. Adsorption isotherms of cholate derivatives on a TSKgel phenyl-5PW measured by frontal chromatography.

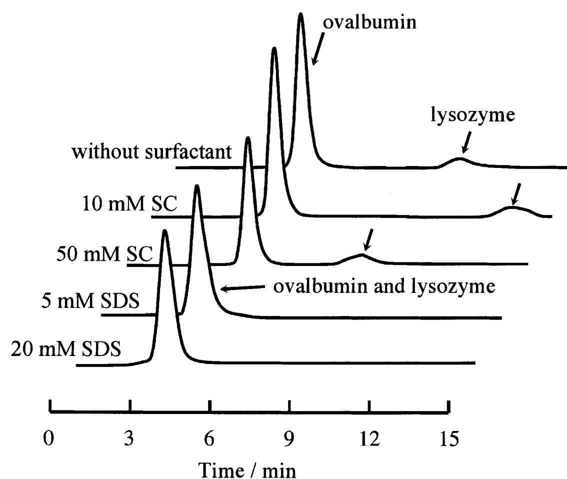

Fig. 9. Chromatograms of ovalbumin and lysozyme in egg white sample in the presence of SDS or cholate below and above respective critical micellar concentrations.

obtained was summarized in distribution isotherms for respective surfactants. As is seen in Fig. 8, the amount of a surfactant adsorbed onto the stationary support ($q(C_b)$) is increased with the increase in the equilibrium concentration of the surfactant in the mobile phase (C_b). The extent of the adsorbed amount is the highest for SDC, and is comparable to SC and STC. The large distribution of SDC is ascribed to the hydrophobicity of SDC stronger than that of SC and STC, due to a lesser number of hydroxyl groups of SDC than those of the others. The carboxyl group of SDC and SC, and the sulfonic group of STC dissociate completely at pH 7.0 and thus the surface net charge of the stationary support is highly dependent upon the amount of a surfactant adsorbed onto the stationary support. Accordingly, the presence of SDC in large amounts on the stationary support can be favorable for the strong electrostatic interaction with proteins, which gives the largest K values below CMC among the three surfactants. On the other hand, no distinct differences were found between the functions of SC and STC as well as their amounts on the stationary support. Thus, the strength of their interaction with proteins is similar to each other. As a result, cholate derivatives would show particular promise in alteration of selectivity in hydrophobic chromatography of proteins. Finally, we have tested the effectiveness of SC for controlling retention of lysozyme in egg white samples.

3.5. Effect of surfactants on the hydrophobic chromatography of lysozyme and ovalbumin in egg white sample

As discussed above, SC appears to be a good means for controlling retention of proteins that have a high pI. Therefore, we tested SC in separation of proteins in egg white samples, in which basic lysozyme and acidic ovalbumin are major components. For comparison with SC, SDS was also tested.

Fig. 9 indicates the effects of SC and SDS on the retention of egg white samples at two concentration regimes (10 and 50 mM for SC, or 5 and 20 mM for SDS) and pH 9.0. In the presence and absence of SC, two peaks were observed. The first peak was confirmed to be ovalbumin and the second was lysozyme. The elution of lysozyme was delayed by using SC below CMC, while that of ovalbumin was remained unchanged. On the other hand, early elution of lysozyme was observed with SC above CMC. In contrast to SC, in the presence of SDS below and above CMC only one overlapped peak was observed, which was attributable to lysozyme and ovalbumin. Thus, SC is effective for separation of lysozyme from ovalbumin in egg white samples.

These results using egg white samples show a good agreement with those using aqueous samples. That is, it is apparent that SC is effective for increasing or

decreasing retention of lysozyme even in egg white samples without giving any effect on the retention of ovalbumin, and that SDS only decreased the retention of the two proteins in egg white samples as well as in aqueous samples containing only a specified protein.

4. Conclusion

In micellar chromatographic separations of proteins, most attention is focused to facilitate protein elution without denaturation. In the present study, we realized that cholate-assisted elutions were effective for changing the selectivity and thus for discrimination of proteins with similar hydrophobicity. If preservation of the native protein is an objective, a substantial amount of experimentation is usually required to solubilize and purify a particular protein without denaturation. In these respects, cholate derivatives are superior to other surfactants, there being likelihood of strong and denaturing interactions of cholates with proteins.

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