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EFFECT OF SALT CONCENTRATION ON THE ELUTION PROPERTIES OF COMPLEXES FORMED BETWEEN SODIUM DODECYLSULPHATE AND PROTEIN POLYPEPTIDES IN HIGH-PERFORMANCE SILICA GEL CHROMATOGRAPHY

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SUMMARY

Complexes formed between sodium dodecylsulphate and protein polypeptides were applied to high-performance silica gel (TSK-GEL G3000SW) chromatography at various concentrations of sodium phosphate buffer of pH 7. The retention time was markedly dependent on the buffer concentration, and the resolution of protein polypeptides was satisfactory only at buffer concentrations between 0.05 and 0.15 *M*. The effect of buffer concentration could be ascribed only partially to the change in the effective size of the complexes, having a polyelectrolyte-like nature, with salt concentration. It is suggested that the ionic exclusion effect due to the negatively charged matrix of the silica gel must be taken into consideration when interpreting the phenomenon.

INTRODUCTION

Gel chromatography in the presence of sodium dodecylsulphate (SDS) has been used to estimate molecular weights of the polypeptides constituting proteins and to establish the protein composition of biomembranes.[1] As the resolution is relatively low and the method is time consuming, it has not frequently been used as SDS–polyacrylamide gel electrophoresis. The recent introduction of high-performance liquid chromatography in protein analysis is changing the situation.

Imamura *et al.*[2] showed the efficiency of the method for the analysis of protein polypeptides in the presence of SDS using a TSK-GEL G3000SW pre-packed column (Toyo Soda, Tokyo, Japan). The dependence of the observed retention times on the buffer concentration described in their paper attracted our interest, because it was just as expected from the flexible polyelectrolytic nature proposed by Takagi and co-workers[3,4] for the complexes formed between SDS and protein polypeptides. This

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study was carried out primarily to examine whether or not the above-mentioned dependence of the elution property of the complexes could be interpreted by the effect of salt concentration on the hydrodynamic effective sizes of the complexes.

EXPERIMENTAL

Sodium dodecylsulphate (SDS) designated PSP-4 was obtained from Nakarai Chemicals (Kyoto, Japan) as a specially prepared reagent. It was 97% pure by gas chromatography, the major contaminants being decyl- and tetradecylsulphates. All proteins used were best commercially available products. The most frequently used proteins, bovine serum albumin, hen ovalbumin and bovine β-lactoglobulin, were reduced and carboxyamidomethylated according to the method described previously[3], except that SDS was used instead of urea as the denaturant and the three proteins were treated in a single batch. Because the modified proteins had been dialysed against 8 m*M* SDS and then lyophilized, the preparation could be instantaneously solubilized in aqueous solvents.

Six kinds of proteins contained in the Pharmacia (Uppsala, Sweden) kit of standard proteins for gel electrophoresis (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactoalbumin), rabbit immunoglobulin G and bovine pancreatic ribonuclease A were reduced in a batch (when using the kit) or separately by dithiothreitol in about a ten times greater amount on a weight basis in sodium phosphate buffer (pH 7.0) containing 30 times the amount of SDS on a weight basis. The reaction was carried out so as to make the total protein concentration about 2 mg/ml. No sign of re-oxidation of sulphydryl groups of the reduced proteins was observed as far as their elution property was concerned. The reduced proteins were found to be eluted with retention times identical with those of the corresponding reduced–carboxyamidomethylated proteins.

The preparation of reduced–carboxyamidomethylated bovine serum albumin used in the viscosity and binding measurements was identical with that used in a previous study[4]. The concentration of the modified bovine serum albumin was determined assuming the abosrbance of a 1% solution at 280 nm for a 1 cm path length to be 6.78[5].

Sodium phosphate buffers (pH 7.0) of various concentrations, were prepared by mixing aqueous solutions of Na_2HPO_4 and NaH_2PO_4 of appropriate concentration. Sodium azide (0.01%) was added to all of the buffers.

Gel chromatography was carried out at room temperature (25 $\pm$ 2°C) and a flow-rate of 0.50 ml/min using a high-speed liquid chromatograph (Model HLC-803) and a TSK-GEL G3000SW column (60 cm $\times$ 0.75, Lot No. C36556) from Toyo Soda.

The binding of SDS was measured by the equilibrium dialysis technique according to the method described previously[4]. Dialysis was continued for 15–30 days with shaking in a thermostated water-bath at 25 $\pm$ 0.2°C. The protein concentration was 0.5–1.0 mg/ml.

Viscosity was measured with an Ubbelohde-type viscometer with a flow time for water of 260 sec. Reduced–carboxyamidomethylated bovine serum albumin was dissolved in sodium phosphate buffer (pH 7.0) of the desired concentration containing 1.5 times the amount of SDS by weight to give a final concentration of *ca.* 3

mg/ml, and dialyzed in a thermostated water-bath at 25 ± 0.2°C against 1 l of each of the above buffers containing SDS in concentrations just sufficient to attain the maximal amounts of SDS binding under each set of conditions. The outer solution was changed every day for the first 3 days. For dilution of a sample solution in the viscometer, the outer solution was used as the solvent.

RESULTS

The major part of these experiments was designed as follows. A pre-packed silica gel column (TSK-GEL G3000SW) was equilibrated with sodium phosphate buffer (pH 7.0) of various concentrations between zero and 0.25 *M*, in the presence of 0.01 % of sodium azide. The results of preliminary experiments suggested that the concentration of micellar SDS is another factor that affects the mode of separation. The presence of excess of SDS therefore, seemed unfavourable when examining purely the effect of buffer concentration. The SDS concentration in the elution buffer was therefore always kept at the critical micellar concentration (CMC) in the particular buffer. The CMC (m*M*) was found to change with the concentration of the sodium phosphate buffer (*c*, *M*) according to the equation $\ln \mathrm{CMC} = -0.67 \ln c - 1.85$. CMC in the absence of the phosphate buffer was assumed to be 8 m*M*, which is the value in pure water and, therefore, slightly higher than the estimate in the presence of 0.01 % of sodium azide.

Three kinds of protein polypeptides derived from bovine serum albumin, hen ovalbumin and β-lactoglobulin were selected as models. They were prepared as a mixture containing the three kinds of protein polypeptides in a ratio to give peaks of roughly equal areas when the elution was monitored at 280 nm. The sample solution was prepared by the addition of sodium phosphate buffer of the desired concentration, and applied to the high-pressure silica gel column equilibrated with the buffer. A 100-μl volume of a sample solution containing 0.2–0.5 mg of the total protein polypeptides were applied. This applied correspondingly to other experiments using samples other than the polypeptide mixture.

Typical examples of the elution patterns obtained are shown in Fig. 1. In the

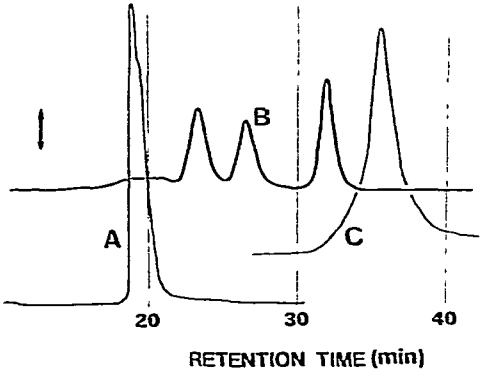
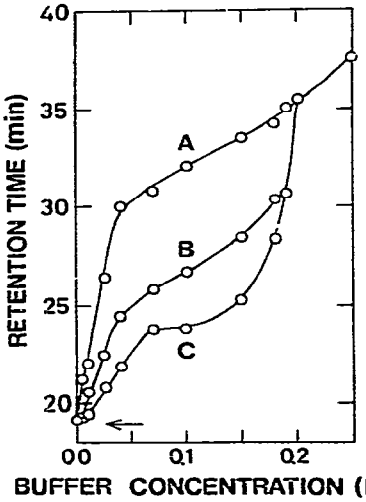


Fig. 1. Typical examples of the elution patterns of the three protein polypeptides A, 8 m*M* SDS–0.01 % NaN_3 (pH 7); B, 0.74 m*M* SDS–0.10 *M* sodium phosphate buffer (pH 7.0)–0.01 % NaN_3, C, 0.46 m*M* SDS–0.20 *M* sodium phosphate buffer (pH 7.0)–0.01 % NaN_3. Absorbance scale indicated by the arrow is 0.016, 0.008 and 0.004 for A, B and C, respectively.

presence of moderate concentrations of the sodium phosphate buffer (0.05–0.15 *M*) the three components could be definitely separated, as shown by pattern B. With decrease in the buffer concentration, the polypeptides were eluted earlier with decreased resolution and finally as a single peak at the void volume, as shown by pattern A. The increase in the buffer concentration increased the retention time of the three peaks, accompanying a decrease in the resolution to give a single peak above 0.2 *M*. The change in retention time as a function of the buffer concentration is illustrated in Fig. 2.



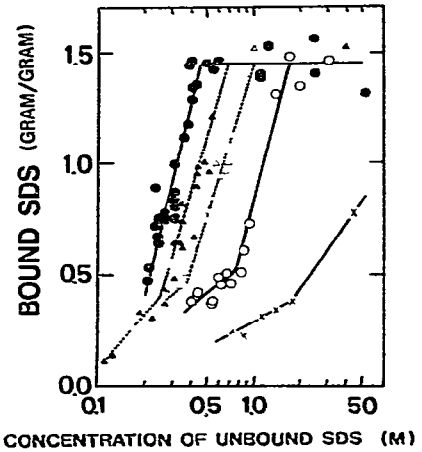


Fig. 2. Retention times of the three protein polypeptides *versus* concentration of the buffers A = β-Lactoglobulin; B = ovalbumin; C = bovine serum albumin (all in the reduced–carboxyamidomethylated states). The arrow indicates the void volume.

Fig. 3 Binding isotherms of SDS to reduced–carboxyamidomethylated bovine serum albumin at various concentrations of sodium phosphate buffer (pH 6.4) containing 0.01 % of NaN_3 at 25°C. ●, 0.4 *M*; ▲, 0.3 *M*; △, 0.2 *M*; ○, 0.1 *M*; ×, 0.01 *M*

Possible changes in the amount of SDS bound might affect the hydrodynamically effective size of a complex between the protein polypeptide and SDS and consequently the retention time for the polypeptides. To examine this point, the binding of SDS to reduced–carboxyamidomethylated bovine serum albumin was measured at various concentrations of the sodium phosphate buffer*. Fig. 3 shows the binding isotherms obtained. Although the points are scattered, the results clearly indicate that the amount of SDS bound is insensitive to the concentration of the buffer within the range between 0.1 and 0.4 *M*. The measurements could not be extended to lower buffer concentration owing to Donnan equilibrium of SDS. The change in the reten-

* The pH of the sodium phosphate buffer was adjusted to 6.4 instead of 7.0 in other experiments described in this paper, because the measurements had been initiated under a different project. No ionizable group having a p*K* value in the above pH region plays a major role in the formation of the complexes under study. It may be safely assumed that the isotherms obtained at pH 6.4 are not significantly different from those at pH 7.0

tion time at least above 0.1 *M* in Fig. 2 therefore cannot be ascribed to the change in the amount of bound SDS.

The complexes formed between protein polypeptides and SDS are polyelectrolytic owing to the acquisition of a high net charge introduced by the bound dodecylsulphate ions. The hydrodynamic effective size of such a complex is expected to decrease with increase in salt concentration in the same manner as is observed with intrinsic polyelectrolytes[6]. To examine this possibility and also to examine the change in the hydrodynamic effective size directly, the viscosity of the solution of the complex between reduced–carboxyamidomethylated bovine serum albumin and SDS was measured at various concentrations of the complex and of the buffer salt. The concentration of unbound SDS was kept less than three times the CMC of SDS in order to avoid possible interference by excess SDS micelles. Fig. 4 shows the results obtained.

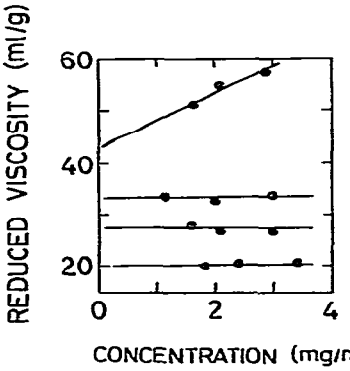


Fig. 4. Relative viscosity of complexes formed between SDS and reduced–carboxyamidomethylated bovine serum albumin at various concentrations of the buffer salts at 25°C. Concentrations of the sodium phosphate buffers were 0, 0.050, 0.20 and 0.40 *M*, from top to bottom. Concentrations of SDS in the buffers were 8.0, 2.0, 1.5 and 0.5 m*M*, respectively.

The intrinsic viscosity, $[\eta]$, of the complex can be related to the effective hydrodynamic radius, R_e, according to the equation[7]

$$[\eta] = 2.5 \cdot \frac{N}{M} \cdot \frac{4\pi}{3} \cdot R_e^3 \tag{1}$$

where N and M are Avogadro's number and the molecular weight of the polypeptide, respectively. The radii estimated from the intrinsic viscosity obtained in Fig. 4 using eqn. 1 were 7.6, 7.0, 6.6 and 5.9 nm in 0, 0.1, 0.2 and 0.4 *M* sodium phosphate buffer, respectively. It should be noted that the radius decreased slowly and monotonously with increasing buffer concentration.

The radius of the complex was expected to be estimated also from the data for the high-performance silica gel chromatography shown in Fig. 2. According to Ackers[8], the effective radius of a polymer applied to a molecular sieve column is correlated with the partition coefficient, K_d, according to the equation

$$R_e = A + B \operatorname{erf}^{-1}(1 - K_d) \tag{2}$$

where A and B are purely empirical constants for a given chromatographic system. Values of R_e for more than twenty kinds of protein polypeptides have been tabulated by Fish[1]. The values of R_e were estimated in 0.04 M sodium phosphate buffer (pH 7) in the presence of 0.1% of SDS and 0.02% of sodium azide. If the silica gel matrix is acting purely as a rigid molecular sieve, the constants A and B may be approximated as universal constants which are insensitive to buffer concentration. Assuming as above, the values of R_e given by Fish[1] were plotted *versus* corresponding values of $\text{erf}^{-1}(1 - K_d)$. Values of K_d were estimated for bovine serum albumin, rabbit immunoglobulin G heavy and light chains, hen ovalbumin, carbonic anhydrase and bovine β-lactoglobulin, all in reduced states. A good linear plot was obtained, giving the following equation:

$$R_e = -24.2 + 77.8\ \text{erf}^{-1}(1 - K_d) \quad (3)$$

In the calculation of K_d, the retention times corresponding to the void volume and the total volume were assumed to be 19.0 and 53.0 min, respectively. These values were determined using Blue Dextran 2000 and D_2O, respectively. Their elution was monitored by measurements of absorbances at 280 nm for the former and at 2500 cm^{-1} for the latter. Fig. 5 shows the values of the apparent effective radii determined for the three protein polypeptides by the application of the data shown in Fig 2 to eqn. 3.

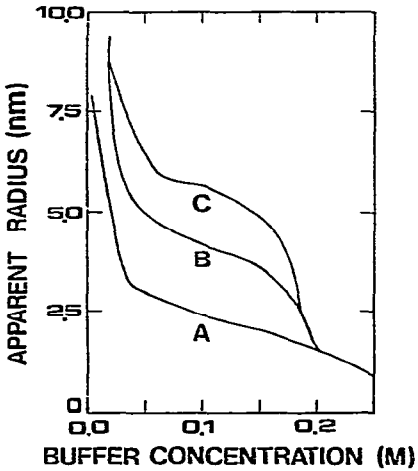


Fig 5. Apparent radii of the complexes *versus* concentration of the buffers The radii were calculated from the data shown in Fig 2 using the equation of Ackers[8] (eqn. 3) A, B and C as in Fig. 2

DISCUSSION

High-performance silica gel chromatography in the presence of SDS gives high resolution and very good reproducibility. For example, the use of two TSK-GEL G3000SW columns in series (each 60 × 0.75 cm) gave a resolution comparable to that of SDS–polyacrylamide gel electrophoresis with higher reproducibility and using only 5–20 μg of each protein (Takagi, unpublished results). In gel electrophoresis, the

presence of a moderate concentration of buffer salts is a prerequisite for maintaining an electrical conductivity sufficient for carrying out electrophoresis. In high-performance silica gel chromatography, the solvent composition seemed to be very variable. Binding of SDS to proteins was expected to protect them from unfavourable adsorption to the matrix.

We therefore expected that high-performance silica gel chromatography in the presence of SDS could be carried out in the presence of various salt concentrations and even in the absence of added salt, to make various modes of separation possible. The results obtained, however, indicate that the separation of protein polypeptides in the presence of unbound SDS with a concentration equal to the CMC is satisfactory only in the presence of sodium phosphate buffer with concentrations between 0.05 and 0.15 *M*. As the complexes formed between protein polypeptides and SDS are flexible and polyelectrolytic in nature[3,4], their effective size was expected to change as a function of the buffer concentration. The intrinsic viscosity of reduced–carboxyamidomethylated bovine serum albumin was found to change in a way that suggested that the complex did behave as expected. On the other hand, an unrealistically large change in the hydrodynamic effective size was suggested from the retention times in high-performance silica gel chromatography as shown in Fig. 5.

At low salt concentrations, the complexes were excluded from the silica gel matrix irrespective of their sizes. It should be noted that even SDS micelles with an effective radius of 2.5 nm were eluted at the void volume at zero buffer concentration in the presence of 0.01 % of sodium azide (data not included)[9]. It is known that the repulsive interaction between the gel matrix and solutes both with charges of the same sign, called "ionic exclusion"[10], causes such an effect. The complexes between SDS and protein polypeptides are undoubtedly highly charged. Silica gel is well known to have silanol groups that are ionizable, giving negative charges to its matrix. Although the silanol groups of the silica gel of the TSK-GEL column are reportedly modified to minimize their interaction with proteins, part of the silanol groups are probably left unreacted, giving the gel negative charges. Preliminary experiments suggested that the silica gel binds a small amount of SDS to give further additional charges to the matrix.

The ionic exclusion effect suggested above seems to be the major factor causing the effect of buffer salt concentration on the performance of the silica gel column in high-performance liquid chromatography in the presence of SDS. Even in the presence of moderate concentrations of the buffer salts, the apparent radius estimated from the gel chromatographic experiments changed more markedly than expected from the viscosity data. The results obtained in this study should be taken into consideration when designing experiments using high-performance silica gel chromatography in the presence of SDS and interpreting the results obtained.

As shown in Fig. 2, the resolution of the three kinds of protein polypeptides decreased markedly at buffer concentrations above 0.15 *M*, finally giving a single peak above 0.2 *M*. Apparently the effective size of each of the complexes is reduced to a level where the column cannot separate the components. Further studies, including viscosity measurements of the complexes formed between SDS and polypeptides derived from proteins with different molecular weights, are needed in order to clarify this point.

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