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MINIMIZING ADSORPTION OF PROTEINS ON FUSED SILICA IN CAPILLARY ZONE ELECTROPHORESIS BY THE ADDITION OF ALKALI METAL SALTS TO THE BUFFERS

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SUMMARY

A method for minimizing the adsorption of proteins on fused-silica capillaries in capillary zone electrophoresis has been devised, involving the use of K^+ concentrations of 0.3 *M* and above in the operating buffer. The increased ionic strength results in a competition between K^+ and proteins for cation-exchange sites on the silica surface. The resulting increase in conductivity requires the use of lower voltages and capillaries of smaller diameter to allow adequate heat dissipation. With a voltage of 5 kV applied to a 50-cm capillary filled with a buffer of pH 9 containing 0.25 *M* potassium sulfate, a separation of five proteins was obtained. Two of these protein bands adsorbed irreversibly without added salt, but showed no apparent adsorption in the presence of 0.25 *M* potassium sulfate.

INTRODUCTION

Silica has been known for many years to possess cation-exchange properties[1]. These properties arise from the acidic nature of the silanol (Si–OH) groups, which make up a large proportion of the exposed surfaces of the silica. The groups are weakly acidic, leaving a negatively charged surface capable of ion exchange.

Proteins consist of numerous amino acids, many of which contain acidic or basic side-chains capable of giving substantial charge to a protein. Proteins can therefore be thought of as large "polyelectrolytes" which can ion exchange either as cations or as anions. Kopaciewicz *et al.*[2] showed that even at the isoelectric point (p*I*) of a protein it can still have regions of localized positive or negative charge. The proteins can orient themselves such that these regions of charge can make a close approach to an ion exchanger. It is not surprising, therefore, to find that proteins adsorb strongly on siliceous materials.

As early as 1954, Holt and Bowcott[3], studying the reactions of soluble silicic acid with proteins and heptadecylamine, suggested the formation of an ionic band between the basic amine functionality on the proteins and the silanoate ($Si–O^-$) on the silicic

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acid. In 1967, Weldes[4] atributed the interactions between proteins and alkali metal silicates with hydrogen bonding. Hydrogen bonding alone, however, cannot adequately explain the adsorption, as proteins fail to desorb from the silicates in the presence of high concentrations of urea[5], a reagent that generally disrupts hydrogen bonds. Messing[5] proposed a combination of the two mechanisms, on the basis that desorption of the protein with urea alone does not work, but that a mixture of urea and either dilute or concentrated acid results in nearly quantitative desorption. Messing[5] suggested that the urea breaks hydrogen bonds while the acid protonates the silanoates. Morrisey and Stromberg[6] suggested a hydrogen bonding interaction between the carbonyls in the proteins and the silica surface, although they failed to explain how this takes place at higher pH where the silica is highly ionized. Hiatt *et al.*[7] also proposed a hydrogen bonding interaction when they found a rabies virus with a net negative charge adsorbed on silica. Voegel *et al.*[8], and many others, simply refer to adsorption in a generic way without offering any proposed mechanism. Regardless of the exact mechanism(s) involved, much effort has been spent on attempts to deactivate silica to make it non-adsorptive toward proteins.

Initially, the need for surface deactivation in the liquid chromatography (LC) of biological macromolecules was circumvented by the use of carbohydrate-based supports and stationary phases. The principal advantage of these is their substantial hydrophilicity and the resulting lack of denaturation of the proteins. However, carbohydrate matrices are not well matched with current high-pressure LC systems, as they have poor mechanical stability and compress or collapse under typical operating pressures. Silica provides an abundant, easy to manufacture alternative as a support, but suffers from high adsorptivity toward many solutes.

Regnier and Noel[9] were among the first to formulate an accetable alternative in the form of a "carbohydrate" [3-(glycidoxypropyl)trimethoxysilane; GPTS] bonded to a controlled porosity glass (CPG) support. Chang *et al.*[10] used the GPTS as an intermediate coupling agent to which they bonded various ion-exchange, hydrophobic and hydrophilic groups, making a wide range of CPG-based packings. Since that time, many researchers have continued to pursue improvements in CPG- and silica-based packing materials for protein analyses[11], striving for biocompatability (no denaturation) and high recovery.

Our research has centred on the capillary zone electrophoresis (CZE) of proteins and peptides in glass and fused-silica capillaries. Short peptides, because of their limited number of charged groups, tend to migrate electrophoretically without significant adsorption. Proteins, however, possess so many ionic sites that adsorption to the capillary wall becomes significant. Walbroehl[12] predicted that capacity factors (k') as small as 0.05 are sufficient to reduce plate numbers for proteins 20-fold, making it a necessity that steps be taken to eliminate adsorption. The "glycophase" used by Regnier and Noel[9] has worked to some extent[13], but the efficiencies are still lower than predicted by theory, suggesting that some adsorptive sites might remain. Additional problems with the glycophase are the time required for column deactivation (several hours) and the limited lifetime of the deactivated surface (several days). An ideal solution would be one that required no pretreatment at all.

In traditional forms of ion-exchange chromatography, retained compounds can be eluted by increasing the ionic strength of the mobile phase. Following this line of reasoning, and assuming that Holt and Bowcott[3] were correct in their assertion of

ionic interaction-based adsorption, it should be possible to prevent adsorption in CZE by operating in buffers with high ionic strength. It is difficult to look at the effects of ionic strength in an electrophoretic system because of the number of possible complications and interferences which can confound data interpretation. During CZE, zone broadening and peak asymmetry can be caused by such things as sample concentration overloading[14] and the thermal effects arising from the joule heating that occurs in electrophoretic systems. This paper examines the effects of alkali metal chlorides on protein adsorption in fused-silica capillaries, using a "chromatographic" approach. In this way, the effectiveness of the salts can be examined in a straightforward manner without complications from electrophoretic effects.

EXPERIMENTAL

Columns

Bare fused silica (50 cm × 75 μm I.D.) (Polymicro Technologies, Phoenix, AZ, U.S.A.) was used for all chromatographic runs, and bare fused silica (50 cm × 25 μm I.D.) (Scientific Glass Engineering, Austin, TX, U.S.A.) was used for electrophoresis. All columns were pretreated with 1 *M* KOH for 20 min, followed by 45-min rinses with each of 0.1 *M* KOH and water, using the method described by Lauer and McManigill[15]. In addition, the column was rinsed for 10 min with 0.1 *M* KOH, followed by 10 min with water and then 10 min with buffer after each series of injections. If protein adsorbed during a run, the column was rinsed for 5 min with 0.1 *M* KOH, followed by 5 min with water and then 5 min with buffer. This procedure will remove any adsorbed protein and provide a fresh surface for each subsequent injection.

Detection

A variable-wavelength ultraviolet absorption detector constructed in this laboratory was used. It includes a 30-W deuterium lamp (Hamamatsu, Middlesex, NJ, U.S.A.) as a source and an Instruments SA (Metuchen, NJ, U.S.A.) concave holographic grating monochromator to select the wavelength of interest. The wavelength for this work was 193 nm, corresponding to the peak UV absorption of proteins. Light was detected in the signal and reference paths by two end-on photomultiplier tubes (R759, Hamamatsu), connected to current amplifiers and to an IBM-PC computer for data acquisition and manipulation. Further details of the detector design will be published elsewhere.

Electrophoretic and chromatographic apparatus

The basic electrophoretic apparatus has been described in detail elsewhere[16]. The high-voltage power supply (RHR30PN30/RVC10, Spellman High-Voltage Electronics, Plainview, NY, U.S.A.) is capable of delivering up to ±30 kV. Electrophoretic injections were carried out by the electromigration technique described previously[16]. Electrophoresis was done at +5 kV. The chromatographic system was set up in a gravity-driven flow mode by placing the inlet buffer reservoir 23 cm higher than the outlet. Chromatographic injections were made by lowering the inlet buffer reservoir to the level of the outlet reservoir, replacing it with a sample vial, and raising the vial 23 cm for 5 s. The vial was then lowered to the height of the outlet

reservoir and replaced with the buffer reservoir, then raised back up 23 cm. Data acquisition was begun at this point.

Reagents

The buffer for chromatography and electrophoresis was 0.1 *M* 2-[N-cyclohexylamino]ethanesulfonic acid (CHES) (Sigma, St. Louis, MO, U.S.A.) of pH 9.0, containing varying amounts of KCl (J. T. Baker, Phillipsburg, PA, U.S.A.), NaCl (EM Science, Cherry Hill, NJ, U.S.A.), LiCl (Baker and Adamson, Morristown, NJ, U.S.A.) and CsCl (Aldrich, Milwaukee, WI, U.S.A.). All proteins were purchased from Sigma and were used as received. Additional salts used for comparison in absorbance studies were K_2SO_4 (Baker and Adamson), KNO_3 (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and KBr (Harshaw Chemical, Solon, OH, U.S.A.).

RESULTS AND DISCUSSION

In order to determine the extent of adsorption and describe it in quantitative terms, k' (column capacity factor) was chosen as the best means of expressing the data:

$$k' = (t_R - t_m)/t_m \quad (1)$$

where t_R is the "retention time" of the protein (lysozyme) and t_m is the dead time as defined by elution of a neutral marker (acetone). The choices of a probe protein and a buffer system are important if one is to gain useful information from this study. It is necessary to select a protein that will adsorb under typical operating conditions, but should not adsorb so badly that drastic measures must be taken to reduce its adsorption. Lauer and McManigill[15] have shown that adsorption can be reduced by selecting a buffer pH that is above the isoelectric points (p*I*) of all proteins in solution. In such a system, the proteins have a net negative charge and are repelled from the negatively charged silica surface. If their idea is kept in mind, one can select a buffer pH that is far enough removed from the p*I* of lysozyme that it does not have an overall negative charge, but close enough to the p*I* that the number of adsorptive sites on the protein is reduced. CHES buffer, with a pH of 9.0, is only 2 pH units below the p*I* of lysozyme (11.0), so it satisfies the above requirements. Hence, the system selected for this study was lysozyme in 0.1 *M* CHES buffer.

As the chromatographic peaks in this system are broad, determination of minute changes in k' values would be impossible if the dead-time marker and protein were co-injected. It is important to measure minute changes in k', as Walbroehl[12] has predicted that k' values as small as 0.05 can reduce the efficiency of the electrophoresis of proteins 20-fold. Therefore, the data were collected by first injecting acetone dissolved in the buffer of interest, calculating the first statistical moment of the peak and calling that the "dead time", then injecting lysozyme and measuring its "retention time". This was repeated three times for each salt concentration. In an attempt to randomize any effects of the order in which salts were examined or the order of concentrations of salt, three sets of triplicate data were collected, alternately changing the order of salts and order of concentrations. The result was a total of nine pieces of data for each concentration of each salt. Following the nine sets of injections, a k' value was calculated using a statistical treatment outlined by Skoog and West[17]. Table

TABLE I

CAPACITY FACTORS AND ABSOLUTE STANDARD DEVIATIONS FOR 0.2% LYSOZYME RUN CHROMATOGRAPHICALLY IN 0.1 *M* CHES BUFFER OF pH 9.0

Each data value is the mean of nine trials.

Salt concentration (M)	*Capacity factor (k')*			
	LiCl	*NaCl*	*KCl*	*CsCl*
0.1	—[a]	0.35 ± 0.13	0.49 ± 0.06	0.39 ± 0.11
0.3	0.10 ± 0.02	0.04 ± 0.03	0.02 ± 0.02	0.03 ± 0.02
1.0	0.00 ± 0.02	0.00 ± 0.03	−0.003 ± 0.007	0.00 ± 0.04

[a] k' unmeasurable owing to irreversible adsorption.

I shows the k' values and their standard deviations for 0.2% lysozyme run in the four salts at three different concentrations. The negative k' value is an artifact of the lack of co-injection; occasionally, the dead-time marker had a slightly longer "retention time" than the solute when the two were injected separately. However, the negative value was smaller than its associated uncertainty.

There are considerable uncertainties associated with most of the data in Table I. Much of this is likely to arise from the dynamic nature of the capillary surface; between two sets of data, new silanols have formed as siloxane bonds have been hydrolysed. As stated earlier, the order in which data were collected was randomized, so that any effects of the progressive surface changes could be minimized.

In spite of the uncertainties in the data, there are some trends that are worthy of note. All four of the salts examined performed an equivalent job of preventing adsorption at a concentration of 1.0 *M*. At a concentration of 0.3 *M*, some differences begin to emerge. LiCl was the worst at preventing adsorption, giving a k' for lysozyme approximately three times higher than those given by the other salts. All of the other salts yield k' values that are significant but are approximately equal to each other. At a concentration of 0.1 *M*, K^+, Na^+ and Cs^+ all had high levels of adsorption, but it was not so severe as to be irreversible. Li^+, however, at 0.1 *M* concentration was so ineffective at minimizing adsorption that a solute peak was never observed. Li^+ is the most highly hydrated of these ions and, with its sphere of hydration included, it is effectively the largest in the alkali metal series. As the largest alkali metal ion, it is the most weakly bound. The expected order of effectiveness of the four salts is therefore $Cs^+ > K^+ > Na^+ > Li^+$. As Li^+ is clearly inferior and Cs^+ suffers from unacceptably high optical absorbance at short wavelengths, these two ions were eliminated. K^+ and Na^+ were essentially equal in their effectiveness, and K^+ was arbitrarily chosen for further studies.

Gooding and Schmuck[18] have shown that the choice of buffer anion in cation-exchange chromatography plays some role in solute retention. Hence it would seem logical to expect some type of effect in this study. A series of potassium salts were selected as buffer additives to investigate the effects of the anion chosen on solute retention. The four salts, KCl, KNO_3, KBr and K_2SO_4, all yielded equivalent k' values at potassium concentrations of 1.0, 0.3 and 0.1 *M* (0.5, 0.15 and 0.05 *M* in K_2SO_4), suggesting that in this system the anion does not have a measurable effect. The four salts, however, had widely varying optical absorbances (see Table II). KBr and KNO_3

TABLE II
ABSORBANCE OF 0.1 *M* POTASSIUM SALTS AT 193 nm IN WATER VS. WATER DRAWN THROUGH A 75-μm I.D. COLUMN

Salt	*Absorbance*	*Salt*	*Absorbance*
K_2SO_4	0.0178	KBr	6.36
KCl	0.1343	KNO_3	4.76

had absorbances more than two orders of magnitude higher than K_2SO_4. With such high absorbances, it was actually necessary to dilute these two salt solutions 100-fold and then to extrapolate back to the values reported in Table II. The extremely high absorbances result in stray light becoming a significant proportion of the remaining photocurrent, thereby pushing the detector into a region of poor linearity. Clearly, K_2SO_4 is far superior in transparency to the other salts which were examined. Given this fact, and the fact that there is no apparent difference in the abilities of the various potassium salts to prevent adsorption, it appears that K_2SO_4, at a concentration between 0.15 and 0.5 *M* (0.3–1.0 *M* in K^+), is the salt of choice for further work.

Capillary zone electrophoresis requires the use of high voltages, passing current through the buffer medium which fills the capillary tube, and generating joule heat which must be dissipated at the capillary walls. Using a typical capillary of I.D. 75 μm and a typical operating voltage of 20 kV, a buffer containing 0.3 *M* K^+ would pass current and generate heat well in excess of that which the system can tolerate. Three approaches can be taken to minimize the effects of the heat. The applied voltage can be lowered, but simple theory predicts that separation efficiency is directly proportional to applied voltage and analysis times are inversely proportional to applied voltage[19]. Consequently, lowering the voltage will probably lower the peak efficiencies and increase the analysis times. The column length can be increased, providing greater electrical resistance and thus lower joule heat, in addition to providing a greater surface area for heat dissipation. However, analysis times are proportional to the square of the column length, so this approach would result in significantly longer analysis times. Another approach is to reduce the inner diameter of the capillary, thereby increasing the surface area-to-volume ratio and improving the heat dissipating ability of the capillary. The problem with this approach is that it significantly shortens the path length of the on-column UV absorption detector, making detection of the zones more difficult. The only way to compensate for this is to increase the sample concentration or improve detection limits. In capillary electrophoresis it is necessary to keep the sample concentration approximately 100 times lower than the buffer and salt concentrations in order to prevent sample overloading[14], so care must be taken to avoid increasing the sample concentration too much. Fortunately, the increased ionic strength allows the use of increased sample concentrations.

Fig. 1 shows an electropherogram of a mixture of five proteins [1% (w/v) each] in 0.1 *M* CHES buffer (pH 9.0) containing 0.25 *M* K_2SO_4 and 0.001 *M* EDTA. The first two components are lysozyme and trypsinogen, both of which ordinarily show significant adsorption in a pH 9.0 buffer which does not contain any additional salt. As their isoelectric points are 11 and 9.3, respectively, both of these proteins still contain significant regions of positive charge at pH 9.0, making them likely to adsorb strongly

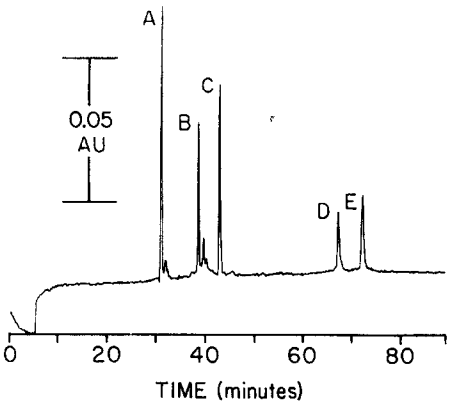

Fig. 1. Zone electrophoretic separation of five proteins on a 100 cm × 25 μm I.D. bare fused-silica column. (A) Hen-egg lysozyme, N = 68 000; (B) bovine pancreatic trypsinogen, N = 140 000; (C) horse heart myoglobin, N = 78 000; (D) bovine milk β-lactoglobulin B, N = 95 000; (E) bovine milk β-lactoglobulin A, N = 95 000. The concentration of each protein is 1% (w/v) in 0.1 M CHES buffer containing 0.25 M K_2SO_4 and 1 mM EDTA at pH 9.0. Injection was for 3 s at 5 kV; electrophoresis at 5 kV. UV detection at 193 nm. The electropherogram was subjected to a nine-point Savitzky–Golay smoothing three consecutive times. (Reprinted with permission from ref. 20.)

on the capillary wall. Neither of them shows any evidence of serious adsorption in the presence of K_2SO_4. One advantage of this approach to the prevention of adsorption lies in the fact that no initial surface treatment was required, and no conditions that might be unfavourable for the column or proteins were required. The obvious drawbacks are the relatively long analysis time and the inordinately high protein concentrations in the original sample. Both problems could be solved with more sensitive detection, as a better detector would allow the use of even smaller diameter capillaries, allowing the application of higher voltages, and/or lower concentrations of protein, minimizing the likelihood of sample concentration overloading.

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REFERENCES

1 K. K. Unger, *Porous Silica. Its Properties and Use as Support in Column Liquid Chromatography*, Elsevier, Amsterdam, 1977, p. 130.
2 W. Kopaciewicz, M. A. Rounds, J. Fausnaugh and F. E. Regnier, *J. Chromatogr.*, 266 (1983) 3.
3 P. Holt and J. Bowcott, *Biochem. J.*, 57 (1954) 471.
4 H. Weldes, *Adhes. Age*, 10 (1967) 32.
5 R. A. Messing, *J. Am. Chem. Soc.*, 91:9 (1969) 2370.
6 B. W. Morrissey and R. R. Stromberg, *J. Colloid Interface Sci.*, 46 (1974) 152.
7 C. W. Hiatt, A. Shelokov, E. J. Rosenthal and J. M. Galimore, *J. Chromatogr.*, 56 (1971) 362.
8 J. C. Voegel, N. De Baillou, J. Sturm and A. Schmitt, *Colloids Surf.*, 10 (1984) 9.
9 F. E. Regnier and R. Noel, *J. Chromatogr. Sci.*, 14 (1976) 316.
10 S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 120 (1976) 321.

11 K. K. Unger, B. Anspach and H. Giesche, *J. Pharm. Biomed. Anal.*, 2 (1984) 139.
12 Y. Walbroehl, *Ph. D. Dissertation*, University of North Carolina, 1986.
13 Y. Walbroehl and J. W. Jorgenson, *J. Chromatogr.*, 315 (1984) 135.
14 J. W. Jorgenson and K. D. Lukacs, *Clin. Chem.*, 27 (1981) 1551.
15 H. H. Lauer and D. McManigill, *Anal. Chem.*, 58 (1986) 166.
16 J. W. Jorgenson and K. D. Lukacs, *Science (Washington, D.C.)*, 222 (1983) 266.
17 D. A. Skoog and D. M. West, *Fundamentals of Analytical Chemistry*, Holt, Rinehart & Winston, New York, 3rd ed., 1976, p. 74.
18 K. M. Gooding and M. N. Schmuck, *J. Chromatogr.*, 266 (1983) 633.
19 J. W. Jorgenson and K. D. Lukacs, *Anal. Chem.*, 53 (1981) 1298.
20 H. H. Lauer and D. McManigill, *Trends Anal. Chem.*, 5 (1986) 11.