Determination of isoelectric points of acidic and basic proteins by capillary electrophoresis

Y.J. Yao[a], K.S. Khoo[b], M.C.M. Chung[b], S.F.Y. Li[*,a]

[a]Department of Chemistry, National University of Singapore, Kent Ridge Crescent, Singapore 0511, Singapore
[b]Department of Biochemistry, National University of Singapore, Kent Ridge Crescent, Singapore 0511, Singapore

Abstract

The isoelectric point (pI) of a protein is of practical importance in many separation procedures, both analytical and preparative. The pI is defined as the pH where the net charge of the protein is zero. Therefore, by plotting the mobilities of the proteins against pH, the intercept at zero mobility should yield the pI value. Isoelectric points have traditionally been determined by isoelectric focusing. In this paper, the potential of capillary electrophoresis as an alternative technique for the determination of pI values of both acidic and basic proteins was investigated. The problem commonly encountered with adsorption of the positively charged proteins with the unprotonated silanol groups of the fused-silica wall is solved by applying a dynamic coating of a polycationic reagent to the wall. The advantages of this technique of determining the pI values are simplicity, speed and minimal sample requirement.

1. Introduction

The isoelectric point (pI) of a protein may be defined as the pH of a buffer of specified composition at which no net migration of the protein is produced by application of an electric field. Knowledge of the pI values of proteins may be of interest in the purification of proteins, characterization of small peptides or active areas of enzymes, prediction of ionic interactions between different proteins or between proteins and other macromolecules, design of purification procedures for peptides synthesized from DNA of known sequence, etc. The experimentally determined pI value can be taken as a rough indication of the proportion of diamino *versus* dicarboxylic amino acids, plus the contribution of both, the charges of other amino acids with

potentially ionizable acid–base groups (tyrosine, cysteine, histidine, and arginine), and the post-translational addition of charged residues (P_i, AMP, ADP-Rib, etc.) on the protein [1].

The pI values of proteins have been conventionally determined by experimental methods involving electrofocusing [2] and chromatofocusing [3]. Both methods use a pH gradient to establish the pH at which the proteins have no net charge. In order to assign the pI value, either the pH gradient in the gel is measured at several points, or suitable markers of known pI are included in the same run. The ability of capillary zone electrophoresis (CZE) to analyze and separate charged species rapidly and with minimum sample requirement offers an attractive alternative to the determination of the pI values of proteins. However, analysis of proteins at pH lower than their pI values in untreated fused-silica capillaries is generally difficult, due to non-

* Corresponding author.

specific adsorption between the proteins and the silanol groups on the capillary surface. To circumvent this problem, several approaches have been attempted, including chemical modification of the silica surface [4,5], manipulation of buffer pH [6,7], and dynamic modification of the capillary surface by additives in the buffer solution [8–10]. ·

Our approach to counteract this adsorption problem is to coat the capillary wall with a polycationic reagent so that repulsion between the positive capillary wall and the cationic proteins could minimize protein adsorption. Polybrene, a polymeric quaternary ammonium salt, was originally introduced by Tarr et al. [11] in order to avoid excessive extraction of proteins and peptides in the course of the liquid-phase protein sequencing procedure. It has the capacity to form a thin film on glass surfaces, which cannot be removed by washing with water. This property of polybrene renders it a possible candidate to serve the purpose of analyzing cationic proteins.

2. Experimental

2.1. Chemicals and reagents

The proteins investigated were purchased as lyophilized powders from Sigma (St. Louis, MO, USA) and were either isoelectric focusing (IEF) standards (proteins a–c, e–h in Table 1) or were of the highest purity available (proteins d, k–m). Two basic cytolysins, magnificalysins I and II, purified from a tropical sea anemone, *Heteractis magnifica* [12] were also analyzed. Protein samples were prepared in water at concentrations of ca. 0.1 mg/ml, with 0.004% of mesityl oxide as the neutral marker.

The buffers used for the p*I* determinations were shown in Table 1. MOPS [3-(N-morpholino)propanesulfonic acid], MES [2-(N-morpholino)ethanesulfonic acid], and polybrene were obtained from Sigma; bicine [N,N-bis(2-hydroxyethyl)glycine] from Aldrich (Milwaukee, WI, USA); sodium tetraborate and sodium acetate from Fluka (Buchs, Switzerland); glycine

Table 1
Buffer systems used for the p*I* determination

	Protein	Buffer system
a	Amyloglucosidase	Glycine–HCl
b	Trypsin inhibitor	Na acetate–acetic acid
c	β-Lactoglobulin A	Na acetate–acetic acid
d	β-Lactoglobulin B	Na acetate–acetic acid
e	Carbonic anhydrase II	MES–MOPS–NaOH
f	Carbonic anhydrase I	MES–MOPS–NaOH
g	Myoglobin	MOPS–NaOH
h	L-Lactic dehydrogenase	MOPS–bicine–NaOH
i	Magnificalysin I	Na borate–boric acid
j	Magnificalysin II	Na borate–boric acid
k	Ribonuclease	Na borate–boric acid
l	Cytochrome c	Na borate–boric acid
m	Lysozyme	Na borate–boric acid

and boric acid from Merck (Darmstadt, Germany). Other chemicals used were of analytical reagent grade or better. Water was purified from an Alpha-Q system (Millipore, Bedford, MA, USA). The pH of the buffer solutions was measured at the same temperature as the electrophoretic runs, i.e., 30°C. All solutions were filtered through a 0.45-μm filter membrane (Whatman, Ann Arbor, MI, USA) before use.

2.2. Capillary electrophoresis

Capillary electrophoresis was performed on an Applied Biosystems Model 270A System (ABI, Foster City, CA, USA). Fused-silica capillaries of 50 μm I.D. obtained from Polymicro Technologies (Phoenix, AZ, USA) were treated with 1 M NaOH (10 min), followed by another 10-min flush with 0.1 M NaOH. After rinsing, the capillary was coated with polybrene by flushing the capillary with 0.05% polybrene solution, prepared in the electrophoretic buffer, followed by flushing with the electrophoretic buffer. The rinsing and coating procedures were repeated whenever a new pH was used. Prior to each sample analysis, the capillary was rinsed with the polybrene-containing buffer for 1 min, followed by a 2-min rinse with the electrophoretic buffer. Samples were introduced by application of vacuum (127 mmHg) for 0.5 s. Electrophoretic runs were carried out at −15 kV, with UV detection at

214 nm. The temperature of the column was maintained at 30°C. Electropherograms were recorded with a HP 3390A integrator (Hewlett Packard, Palo Alto, CA, USA).

3. Results and discussion

The principle underlying the method for determining the pI values of proteins using capillary zone electrophoresis is rather straightforward and the measurements are simple to perform. In general, a protein is cationic in solutions at pH values below its pI value. On increasing the pH, the net positive charge decreases in magnitude, accompanied by a decreasing mobility, which diminishes to zero at the isoelectric pH value. Hence from the plot of mobility *versus* pH, the intercept at zero mobility yields the pI value of the protein.

Polybrene is a hydrophobic polyquaternary amine polymer having the repeated unit:

$$[-N^+(CH_3)_2-(CH_2)_6-N^+(CH_3)_2-(CH_2)_3-]_n \cdot$$

$$2nBr^-$$
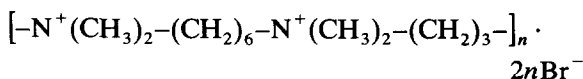
Through multi-site electrostatic interaction, polybrene binds strongly onto the silica surface, neutralizing the negative capillary surface charge. More polybrene can be attached to the surface-bound polybrene via hydrophobic interaction, resulting in the capillary surface acquiring a net positive charge. With just 0.002% (w/v) of polybrene present in the buffer, charge reversal is achieved and reversed polarity (*i.e.*, injection at the cathode end) is required to move the bulk solution through the capillary.

It was found that the capillary retained the positive charge characteristic in the absence of polybrene in the electrophoretic buffer, even after prolonged washing with aqueous NaOH and HCl. This strong binding property of polybrene has allowed it to be excluded from the electrophoretic buffer, yet achieving charge reversal of the capillary. The capillary, however, is flushed with the coating solution before each sample analysis, in order to maintain reasonable reproducibility in migration times. Advantages
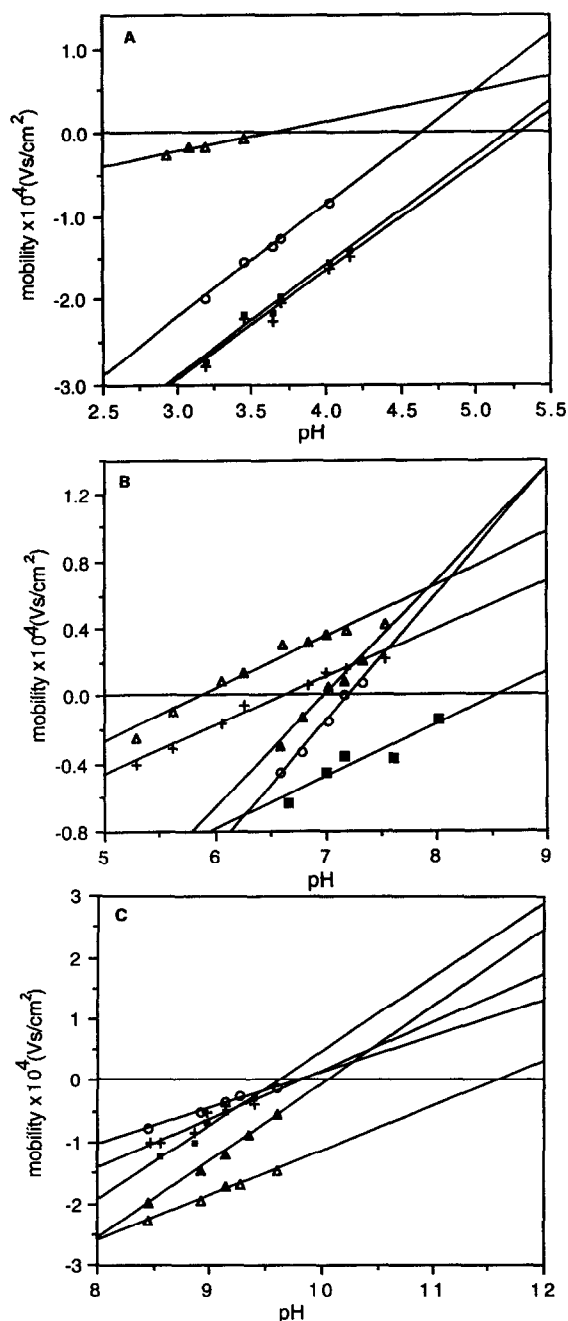

Fig. 1. Plots of electrophoretic mobility of proteins as a function of buffer pH. (A) △ = amyloglucosidase; ○ = trypsin inhibitor; ■ = β-lactoglobulin A; + = β-lactoglobulin B. (B) △ = carbonic anhydrase II; + = carbonic anhydrase I; ▲ = myoglobin (minor component); ○ = myoglobin (major component); ■ = L-lactic dehydrogenase. (C) ○ = ribonuclease; + = magnificalysin II; ■ = magnificalysin I; ▲ = cytochrome *c*; △ = lysozyme.

that can be derived by excluding polybrene from the electrophoretic buffer include better sensitivity of the system, a consequence from the lowering of the background absorbance, and minimization of possible interaction between proteins and polybrene.

Fig. 1 shows plots of electrophoretic mobilities of the proteins as a function of the buffer pH. The pI values obtained are presented in Table 2. The buffer systems used for the determination of the pI values are shown in Table 1. Each protein was determined in a pH range with the same set of buffering ions in order to simplify data interpretation. In general there is good agreement between the pI values obtained by the CZE method and literature values, most of which were obtained by IEF technique. However, a difference between the pI determined by different methods is not unexpected. It is well known that the pI determined by electrophoresis is dependent on the kind of buffer and the ionic strength used [13,14]. In our study, we have also observed that the choice of buffer can have a significant effect on the "observed" pI value. For example, when succinate buffer was used in the determination of pI of carbonic anhydrase I and II, the values obtained were 0.4 pH units lower than the reported values obtained by IEF meth-

od (*i.e.*, 6.6 and 5.9, respectively). However, with MES–MOPS buffer, the reported pI values for the proteins can be obtained. This discrepancy possibly arises from the binding of the divalent succinate anions to the proteins, partially neutralizing the positive charges on the proteins and thus resulting in the lowering of the observed pI values. Usually, higher pI values are obtained in buffers of lower ionic strength. This has also been explained by binding of anions to proteins at electrophoresis [14]. The ionic strength of the buffers employed in our determination was *ca.* 0.02 M. This limits the operating current to less than 15 μA, minimizing Joule heating effects which might result in temperature variations in the capillary. Temperature is an important parameter, as it affects protein stability and the dissociation constants of the ionizable groups in the proteins. This in turn influences the pI value obtained experimentally for the same protein.

4. Conclusions

This paper has demonstrated a simple and reliable method for the determination of pI values of proteins. The use of the polybrene-

Table 2
Comparison of pI values determined by the CZE method with literature values

	Protein	Isoelectric point	
		CZE	Literature[a]
a	Amyloglucosidase (*Aspergillus niger*)	3.6	3.6
b	Trypsin inhibitor (soybean)	4.6	4.6
c	β-Lactoglobulin A (bovine milk)	5.2	5.1
d	β-Lactoglobulin B (bovine milk)	5.3	5.3 [15]
e	Carbonic anhydrase II (bovine erythrocytes)	5.9	5.9
f	Carbonic anhydrase I (human erythrocytes)	6.6	6.6
g	Myoglobin (horse heart)	7.0 and 7.2	6.8 and 7.2
h	L-Lactic dehydrogenase (rabbit muscle)	8.6	8.6
i	Magnificalysin I (sea anemone)	9.6	9.4 [12]
j	Magnificalysin II (sea anemone)	9.8	10.0 [12]
k	Ribonuclease (bovine pancreas)	9.7	9.5 [16]
l	Cytochrome *c* (horse heart)	10.1	10.2 [17]
m	Lysozyme (hen egg white)	11.6	11.3 [18]

[a] Isoelectric points for proteins a–c, e–h (IEF markers) are provided by the manufacturer.

coated capillary provided several advantages. In contrast to chemical modification of the capillary surface, which generally resulted in drastic reduction or elimination of the electroosmotic flow, the electroosmotic flow is preserved in a polybrene-coated capillary, although it is reversed in direction. This allows both cationic and anionic species to be investigated in the same run. In addition, the pH of the electrophoretic buffer remains a freely adjustable parameter, which greatly increase the versatility and usefulness of the technique.

Acknowledgements

References

[1] P.H. O'Farrell, *J. Biol. Chem.*, 250 (1975) 4007.
[2] H. Rilbe, *Ann. N.Y. Acad. Sci.*, 209 (1973) 11.
[3] L.A.Æ. Sluyterman and O. Elgersma, *J. Chromatogr.*, 150 (1978) 17.
[4] A. Malik, Z. Zhao and M.L. Lee, *J. Microcol. Sep.*, 5 (1993) 119.
[5] J.T. Smith and Z.E. Rassi, *J. High Resolut. Chromatogr.*, 15 (1992) 573.
[6] M. Zhu, R. Rodriguez, D. Hansen and T. Wehr, *J. Chromatogr.*, 516 (1990) 123.
[7] P.D. Grossman, K.J. Wilson, G. Petrie and H.H. Lauer, *Anal. Biochem.*, 173 (1988) 265.
[8] Å. Emmer, M. Jansson and J. Roeraade, *J. Chromatogr.*, 547 (1991) 544.
[9] J.A. Bullock and L. Yuan, *J. Microcol. Sep.*, 3 (1991) 241.
[10] M.M. Bushey and J.W. Jorgenson, *J. Chromatogr.*, 480 (1989) 301.
[11] G.E. Tarr, J.F. Beeches, M. Bell and D.J. McKean, *Anal. Biochem.*, 84 (1978) 622.
[12] K.S. Khoo, W.K. Kam, H.E. Khoo, P. Gopalakrishnakone and M.C.M. Chung, *Toxicon*, 31 (1993) 1567.
[13] A. Tiselius and H. Svensson, *Trans. Faraday Soc.*, 36 (1940) 16.
[14] J. Steinhardt and S. Beychok, in H. Neurath (Editor), *The Proteins: Composition, Structure, and Function*, Academic Press, New York, 1964, Ch. 8, p. 140.
[15] B.D. Polis, H.W. Schmukler, J.H. Custer and T.L. McMeekin, *J. Am. Chem. Soc.*, 72 (1950) 4965.
[16] E.A. Anderson and R.A. Alberty, *J. Phys. Colloid Chem.*, 52 (1948) 1345.
[17] H.H. Lauer and D. McManigill, *Anal. Chem.*, 58 (1986) 166.
[18] T. Imoto, L.N. Johnson, A.C.T. North, D.C. Phillips and J.A. Rupley in P.D. Boyer (Editor), *The Enzymes*, Academic Press, New York, 1972, Vol VII, p. 666.