Journal of Chromatography, 606 (1992) 291–296
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 276

Short Communication

Improvements in the method developed for performing isoelectric focusing in uncoated capillaries

Jeff R. Mazzeo and Ira S. Krull

Department of Chemistry, Barnett Institute, 341 MU, Northeastern University, Boston, MA 02115 (USA)

(First received January 7th, 1992; revised manuscript received April 6th, 1992)

ABSTRACT

Capillary isoelectric focusing run times were reduced from 30 to 5 minutes through reversing the polarity and shortening the separation distance. Complete resolution was only obtained by increasing the concentration of tetramethylethylenediamine in the sample solution. Problems previously seen with acidic proteins were shown to be due to anodic drift in the ampholyte gradient, and were minimized by increasing the concentration of phosphoric acid in the anode buffer. The inability of the method to tolerate protein samples with salt is also discussed.

INTRODUCTION

In a previous report [1], we described the development of a method for performing isoelectric focusing (IEF) in uncoated capillaries without the need for performing salt mobilization, as opposed to the traditional method of performing IEF in coated capillaries with salt mobilization [2–8]. This procedure relied upon the maintenance of some electroendosmotic flow (EOF), such that proteins spent sufficient time in the capillary to focus but were mobilized past a stationary detection point by residual EOF. Control of EOF was achieved by adding methyl cellulose to the sample–ampholyte mixture. Furthermore, it was necessary to supplement the pH 3–10 ampholytes with tetramethylethylenediamine (TEMED) so that basic proteins would focus in the region prior to the detection point, with the TEMED acting to block the region after the detection point [9,10]. A similar method has been reported by Thormann *et al.* [11], with the difference being that only a small plug of the sample–ampholyte mixture is introduced into the capillary filled with catholyte, as opposed to filling the whole capillary with the sample–ampholyte mixture, as in our case. In their method, it is not necessary to add TEMED to the ampholytes.

In this note, we wish to describe two improvements we have made to our capillary IEF (cIEF) method. Firstly, we will describe instrumental changes allowing run times to be decreased to less than 15 min with no sacrifice in resolution, and to about 5 min with some loss in resolution, compared to run times of about 30 min previously [1]. Furthermore, we will show that the problems with poor peak shape of acidic proteins are due to pH gradient decay, and can be improved by increasing the anode buffer concentration. We will also report the maximum concentration of salt in the sample which can be tolerated before serious loss in resolution is seen.

Correspondence to: Professor I. S. Krull, Department of Chemistry, Barnett Institute, 341 MU, Northeastern University, Boston, MA 02115, USA.

0021-9673/92/$05.00 © 1992 Elsevier Science Publishers B.V. All rights reserved

EXPERIMENTAL

Chemicals

All chemicals, capillaries and instrumentation were as previously reported [1]. β-Lactoglobulin was from Sigma (St. Louis, MO, USA).

Capillary isoelectric focusing

In all cases, the sample was 0.5 mg/ml cytochrome *c*, chymotrypsinogen A, β-lactoglobulin A and 0.25 mg/ml myoglobin, 5% Pharmalyte 3–10 and 0.1% methyl cellulose TEMED concentration was in the range 0.5–1.6%. The catholyte was always 20 m*M* NaOH, while the anolyte varied from 10–100 m*M* H_3PO_4. In the salt concentration study, the sample was made 5, 10, 25 and 50 m*M* NaCl. The instrument, ISCO Model 3850 (Lincoln, NE, USA), was operated in the reverse polarity mode, such that the position of the cathode was 40 cm away from the detection point, and the anode was 20 cm further away. In all cases, the applied voltage was 24 kV, generating a field of 400 V/cm. Detection was at 280 nm.

RESULTS AND DISCUSSION

Decreasing migration time

The migration time of proteins in this method depends on which point in the capillary they are detected. Due to EOF, the migration direction is towards the cathode; the closer to the cathode they are detected, the longer the migration time. Thus, an apparently easy way to decrease migration time is to detect the proteins closer to the anode. However, there are several things which must be taken into account. First, wherever the detection point is in the capillary, it is required that all proteins focus on the anodic side of the window for them to be detected. The place where proteins focus in the capillary can be controlled by changing the concentration of TEMED. Higher concentrations of TEMED block more of the cathodic end of the capillary, causing proteins to focus closer to the anode. Secondly, it is important to detect the proteins at a point when they are completely focused. Otherwise, they will not be completely resolved. For any set of experimental conditions, there is a certain amount of time which is required to achieve complete focusing and resolution. In cases where the highest resolution is required, the total run time is limited to some time greater than this minimum amount of time. Of course, one can throw away some resolution in order to achieve faster run times.

In the previously reported separations [1], the total separation distance, that is, the distance from anode to detection point, was 40 cm, with a total capillary length of 60 cm. The instrument we are using requires a minimum capillary length of about 40 cm from the "hot" electrode (+ or −) to the detection point, and a minimum capillary length of 20 cm from the detection point to the ground electrode. Thus, shortening the separation distance and the run time could only be achieved by reversing the polarity and making the ground electrode the anode and the "hot" electrode the cathode. This leads to a separation distance of 20 cm, half of that previously used.

When the optimal conditions from the previous set-up are used in the reverse polarity set-up, the separation in Fig. 1 is obtained. Note that the migration time of the peak corresponding to p*I* 6.8, peak 4, is about 6 min, compared to 21 min in the previous set-up [1]. However, the peaks are broader than in the previous case and resolution is incomplete between the peaks corresponding to p*I* 7.2 and 6.8, peaks 3 and 4. Furthermore, chymotrypsinogen A, peak 2, shows up as only one peak, whereas before there were several minor peaks of lower p*I* just after the main peak. The broader peaks and lower resolution can be explained by assuming that the proteins have not yet fully focused. This suggests that the concentration of TEMED should be increased, with all other conditions the same.

Fig. 2. shows the same separation as in Fig. 1, with the exception that the TEMED concentration has been increased from 1.0 to 1.2%. Note that the migration time of the peak corresponding to p*I* 6.8, peak 4, has increased from 6 to 8.5 min, and that the peaks are much sharper and better resolved, as was expected. Theoretical plates for the peaks in Fig. 2 range from 200 to 400 000. However, since IEF is not an equilibrium-based separation technique, it is really inappropriate to report plates for the peaks.

When even higher concentrations of TEMED were used, better resolution and longer run times were achieved. At higher concentrations of TEMED, the proteins are focusing closer to the anode, further and further away from the detection

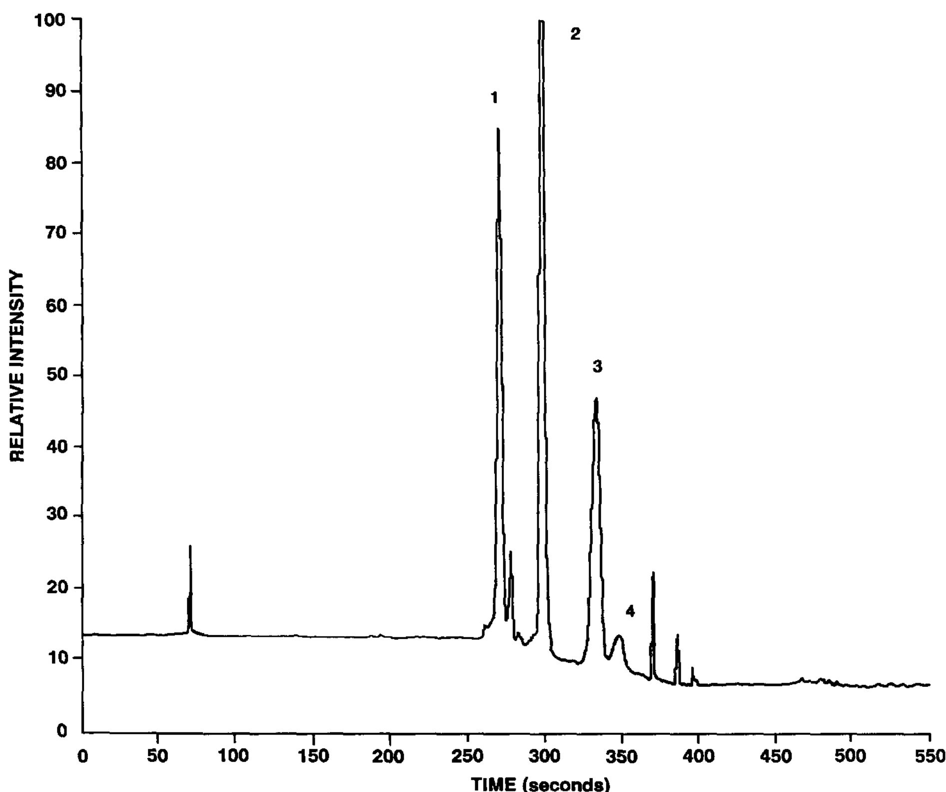

Fig. 1. cIEF of protein mixture using reverse polarity set-up. Capillary: 75 μm I.D., uncoated, 60 cm total length, 20 cm anode to detection. IEF: anolyte 10 m*M* H_3PO_4, catholyte 20 m*M* NaOH, voltage 24 kV. Detection: UV, 280 nm. Sample: 0.5 mg/ml cytochrome *c*, chymotrypsinogen A and β-lactoglobulin A, 0.25 mg/ml myoglobin, 5% Pharmalyte 3–10, 1.0% TEMED, 0.1% methyl cellulose. Peaks: 1 = cytochrome *c*, p*I* 9.6; 2 = chymotrypsinogen A, p*I* 9.1; 3 = myoglobin, p*I* 7.2; 4 = myoglobin, p*I* 6.8.

window. Thus, changing the TEMED concentration is a convenient way of changing run time and resolution. Faster run times can be achieved by lowering the concentration of TEMED, with a sacrifice in resolution. Better resolution can be achieved with higher concentrations of TEMED, at the expense of longer run times. However, there is a limited window of TEMED concentration which can be used. If the TEMED concentration is too low, some basic proteins may focus past the detection point. If the concentration is too high, excessive current will be generated, leading to problems with joule heating. We have found that TEMED concentrations of 0.5–1.6% can be employed with the 20-cm separation distance.

Improved separation of acidic proteins

In both Figs. 1 and 2, it is very difficult to determine which peak, if any, corresponds to β-lactoglobulin A, p*I* 5.1. One could argue that within the time frame monitored, β-lactoglobulin A did not migrate past the detection point. In Fig. 2, a plot of migration time *vs.* p*I* was linear with r^2 = 0.997. Based on the equation for this line, the expected migration time for β-lactoglobulin was 11 min. This assumes that all proteins are moving with the same velocity, which would be true if EOF was the sole source of mobilization in the capillary. However, as has been discussed and studied extensively [12–14], ampholyte gradients are known to suffer from pH gradient decay, whereby the basic end of the gradient migrates towards the cathode (cathodic drift)

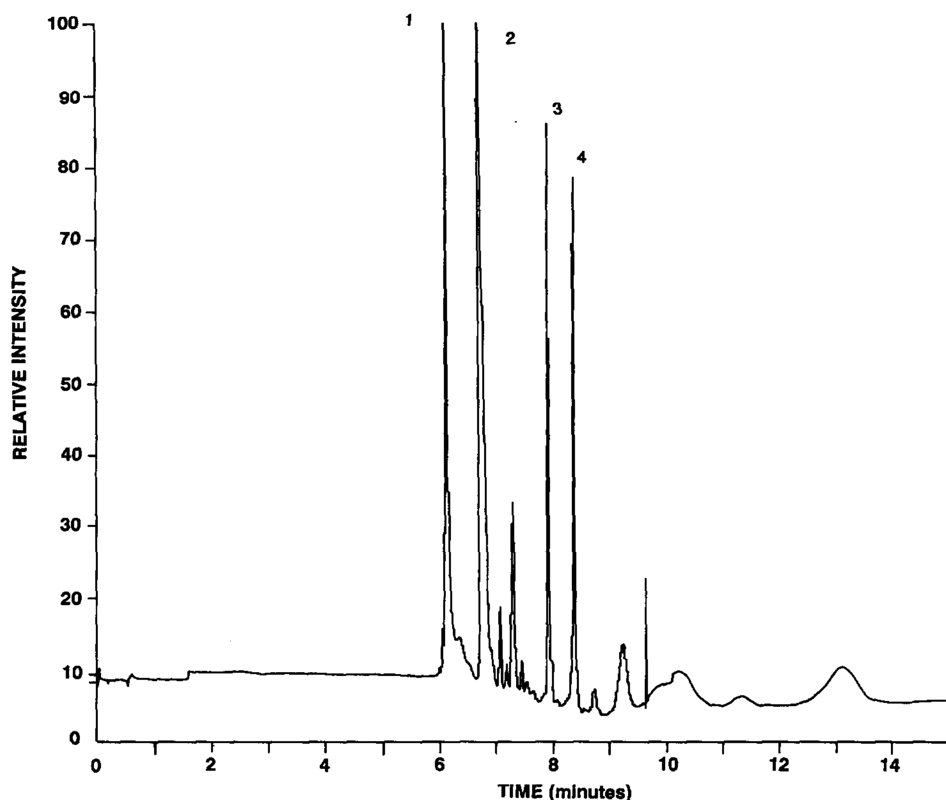

Fig. 2. cIEF of protein mixture using reverse polarity set-up, 1.2% TEMED. Conditions as in Fig. 1, except 1.2% TEMED. Peaks as in Fig. 1.

and the acidic end migrates toward the anode (anodic drift). We must assume that pH gradient decay is also occurring in our system [15]. Thus, the assumption that all proteins are migrating with the same velocity is not correct. Basic proteins will migrate with the additive sum of EOF and any cathodic drift, since they are in the same direction. Neutral proteins will migrate with EOF predominantly. Acidic proteins experience EOF toward the cathode and, in opposition to that, anodic drift toward the anode.

The clear solution to the problems with acidic proteins is to minimize any anodic drift. In a recent paper by Mosher and Thormann [12], it was pointed out that the extent of drift in ampholyte systems strongly depends on the phosphoric acid and sodium hydroxide concentrations. In all of our work, we have used 10 m*M* phosphoric acid and 20 m*M* sodium hydroxide, as was most commonly used in other cIEF separations [2–8]. According to the work of Mosher and Thormann, this ratio of concentrations leads to both cathodic and anodic drift, with the anodic drift being much worse. This would explain the poor peak shapes and inability to detect acidic proteins in our system. The solution, again according to the work of Mosher and Thormann, is to use higher concentrations of phosphoric acid, which will minimize anodic drift while increasing cathodic drift.

It is clear that the concentration of phosphoric acid must be optimized to successfully separate acidic proteins in our system. The concentration must be high enough to minimize anodic drift, but not so high that it increases cathodic drift to the point that basic proteins are detected when they have not yet fully focused and resolved.

Holding all other conditions the same as in Fig. 2, we have investigated phosphoric acid concentra-

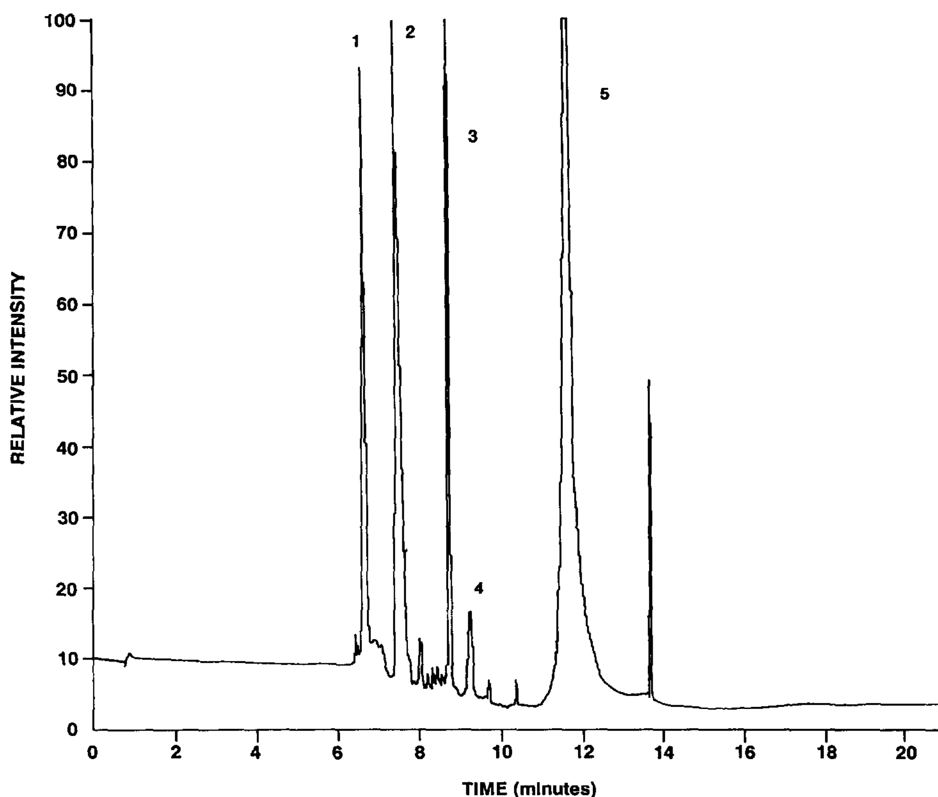

Fig. 3. cIEF of protein mixture using reverse polarity set-up, 25 m*M* H_3PO_4. Conditions as in Fig. 1, except 1.2% TEMED and 25 m*M* H_3PO_4. Peaks: 1 = cytochrome *c*, p*I* 9.6; 2 = chymotrypsinogen A, p*I* 9.1; 3 = myoglobin, p*I* 7.2; 4 = myoglobin, p*I* 6.8; 5 = β-lactoglobulin A, p*I* 5.1.

tions of 10, 15, 20, 25, 30, 50 and 100 m*M*. Fig. 3 shows the same separation in Fig. 2, with the exception that the concentration of phosphoric acid was 25 m*M*. A full scale peak at 12 min is now seen, peak 5, which corresponds to β-lactoglobulin A. We have found that concentrations of phosphoric acid ranging from 20 to 30 m*M* give about the same results, with lower concentrations giving no peak and higher concentrations leading to poor resolution in the basic region of the gradient.

Notice that the peak corresponding to β-lactoglobulin A is much broader than the basic and neutral protein peaks. This is most likely due to the fact that it is not moving with the same velocity as the other peaks, since the width of a peak in this system is a function of the speed with which a protein migrates past the detection point. At best, it would be expected that the fastest an acidic protein like β-lactoglobulin A could move is the speed of EOF. It is unlikely that a protein as acidic as β-lactoglobulin A would experience any cathodic drift, as the basic and neutral proteins do. From the data in Fig. 3, a plot of migration time *vs.* p*I* gave $r^2 = 0.951$ when the migration time of β-lactoglobulin A was included, and $r^2 = 0.997$ when not included. This also suggests that β-lactoglobulin is not migrating at the same speed as the other proteins.

This less than ideal peak shape and separation of acidic proteins is a problem which may be overcome by using capillaries which show EOF in the direction toward the anode, *i.e.*, capillaries which have been modified with a cationic sufactant. In this case, EOF and anodic drift will be in the same direction. However, then there will be a problem with basic proteins. Thus, using EOF-driven cIEF, it may never be possible to achieve optimal resolution throughout the entire pH gradient. Nevertheless, it is possible to do all proteins, acidic, neutral and

basic, using one set of experimental conditions. It is clear that cIEF is a unique and informative way to study the anodic and cathodic drift associated with ampholyte gradients.

Effect of salt concentration

In applying the method to samples of recombinant proteins for characterization, we have found that salt in the protein sample can greatly affect the separation, usually leading to higher initial focusing current and poor separation. It is well known that IEF is intolerant to salt. We wished to determine the maximum salt concentration which could be tolerated by our method. Using the same conditions in Fig. 3, we found that when the concentration of NaCl in the sample exceeded 10 m*M*, the separation was totally lost. Thus, it is imperative that samples be desalted prior to separation using this method. The same has also been said when using other cIEF methods [9].

CONCLUSIONS

We have described a simple way to decrease run times in the cIEF method from 30 min to as low as 5 min, with some sacrifice in resolution. Previous problems with the poor separation of acidic proteins have been shown to be due to anodic drift present in the ampholyte gradient, and were minimized by increasing the concentration of phosphoric acid. Finally, it has been shown that a maximum of 10 m*M* salt can be tolerated by this method.

ACKNOWLEDGEMENT

This work was supported, in part, by a grant from ISCO, Inc., as well as via donation of an ISCO HPCE instrument to Northeastern University. This is contribution number 538 from the Barnett Institute at Northeastern University.

REFERENCES

1 J. R. Mazzeo and I. S. Krull, *Anal. Chem.*, 63 (1991) 2852.
2 S. Hjertén and M. D. Zhu, *J. Chromatogr.*, 346 (1985) 265.
3 S. Hjertén, *J. Chromatogr.*, 347 (1985) 191.
4 S. Hjertén, J. L. Liao and K. Ya, *J. Chromatogr.*, 387 (1987) 127.
5 S. Hjertén, K. Elenbring, F. Kilar, J. L. Liao, J. C. Chen, C. Siebert and M. D. Zhu, *J. Chromatogr.*, 403 (1987) 47.
6 F. Kilar and S. Hjertén, *J. Chromatogr.*, 480 (1989) 351.
7 F. Kilar and S. Hjertén, *Electrophoresis*, 10 (1989) 23.
8 M. D. Zhu, D. L. Hansen, S. Burd and F. Gannon, *J. Chromatogr.*, 480 (1989) 311.
9 M. D. Zhu, R. Rodriguez and T. Wehr, *J. Chromatogr.*, 559 (1991) 479.
10 G. Yao-Jun and R. Bishop, *J. Chromatogr.*, 234 (1982) 459.
11 W. Thormann, J. Caslavska, S. Molteni and J. Chmelik, *J. Chromatogr.*, 589 (1992) 321.
12 R. A. Mosher and W. Thormann, *Electrophoresis*, 11 (1990) 717.
13 R. A. Mosher, W. Thormann and M. Bier, *J. Chromatogr.*, 351 (1986) 31.
14 R. Hagedorn and G. Fuhr, *Electrophoresis*, 11 (1990) 281.
15 J. R. Mazzeo and I. S. Krull, *J. Microcolumn Sep.*, 4 (1992) 29.