Journal of Chromatography, 172 (1979) 351–356

CHROM. 11,652

SUCCESSFUL ISOELECTRIC FOCUSING OF WOOL LOW-SULPHUR PROTEINS

ROBERT C. MARSHALL and R. J. BLAGROVE
Division of Protein Chemistry, CSIRO, Parkville (Melbourne), Victoria 3052 (Australia)
(Received October 20th, 1978)

SUMMARY

Isoelectric focusing in polyacrylamide gel containing 8 *M* urea has been used to estimate the isoelectric points of S-carboxymethylated low-sulphur proteins from wool and to demonstrate charge heterogeneity within the families of proteins present. Two-dimensional electrophoresis has been employed to relate stained bands on the isoelectric gels to wool low-sulphur components resolved by alkaline–urea or sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Similar two-dimensional patterns were obtained when the protein was loaded at either the acidic or basic end of the first dimension. Furthermore, a purified protein, component 7c, gave a single band when loaded at either end of the gradient. These results conflict with the report by Frater (*J. Chromatogr.*, 50 (1970) 469) of artifacts in isoelectric focusing attributed to the formation of complexes between Ampholines and a low-sulphur protein from wool.

INTRODUCTION

Isoelectric focusing has become established as a standard method for the separation and analysis of complex protein mixtures as well as a criterion for homogeneity of isolated components. Important reasons for its widespread use, especially in polyacrylamide gels, are high resolution and convenience for determining isoelectric points. With the development of two-dimensional electrophoretic methods, isoelectric focusing has gained added importance as an analytical tool; for example, in combination with sodium dodecyl sulphate (SDS)–polyacrylamide gel slabs it may be possible[1] to resolve several thousand proteins on a single gel.

The S-carboxymethylated low-sulphur protein fraction (SCMK-A) from wool contains a closely related group of proteins which are difficult to separate because of similarity in molecular size and amino acid composition. There have been eight components described[2] following alkaline urea– and SDS–polyacrylamide gel electrophoretic studies. Isoelectric focusing should be suited to investigate heterogeneity within this class of proteins, but Frater[3] has reported artifacts during focusing experiments in 8 *M* urea with component 8, a major acidic fraction extracted from reduced and S-carboxymethylated wool. The methods used to demonstrate the presence of

artifacts included application of the sample at different positions on the gel and refocusing of a discrete band to give multiple zones. On the other hand, Righetti and co-workers[4–6] have demonstrated that the binding of carrier ampholytes to strongly acidic polyelectrolytes such as heparin and polyglutamic acid is abolished in 8 *M* urea. We have studied isoelectric focusing of SCMK-A from wool with wide-range Ampholines and narrow-range Servalyts.

MATERIALS AND METHODS

Materials

Isoelectric focusing was carried out using Ampholines (range pH 3–10) from LKB (Bromma, Sweden) and Servalyts (ranges pH 2–4, 4–6, 5–7) from Serva (Heidelberg, G.F.R.). Acrylamide and N,N′-methylenebisacrylamide were from Eastman-Kodak (Rochester, N.Y. U.S.A.), SDS was from BDH (Poole, Great Britain) specially pure grade and the urea (p.a.) was from Merck (Darmstadt, G.F.R.). All solutions were freshly prepared in glass-distilled water.

Using the procedure of Maclaren and Kilpatrick[7], wool was extracted with tributylphosphine in 5 *M* sodium iodide–25% 1-propanol and the solubilized proteins were alkylated with iodoacetate at pH 8.5. After dialysis against water, SCMK-A was precipitated by adjusting the pH to 4.4. A purified protein, component 7c, was isolated from SCMK-A by chromatography over DEAE-cellulose and SP-Sephadex[8].

Isoelectric focusing

Gels for isoelectric focusing contained 5% acrylamide, 0.15% N,N′-methylenebisacrylamide, 8 *M* urea (to ensure solubility of the proteins near their isoelectric point), and 1.4% pH 2–4, 0.7% pH 4–6 and 0.7% pH 5–7 Servalyts. Polymerisation was initiated with 0.25% riboflavin. The anolyte solution was 8 *M* urea–0.03 *M* orthophosphoric acid and the catholyte solution was 8 *M* urea–0.05 *M* sodium hydroxide. Samples were dissolved in 8 *M* urea–0.03 *M* phosphoric acid–20% glycerol (pH 4.0) or 8 *M* urea–0.05 *M* Tris–20% glycerol (pH 8.0) for loading at the acidic or basic end of the gel, respectively. All experiments were performed at 20° in a Model 155 Bio-Rad apparatus in which the cylindrical gel tubes (130 × 3.5 mm) were cooled by immersion in the lower electrode solution. The gels were prefosused for 2 h at 400 V, the samples were applied and focusing was continued for 17 h at 400 V. The gels were stained directly[9] for 3 h with 0.01% Coomassie brilliant blue R 250 (Ciba-Geigy, Basel, Switzerland) in 25% ethanol–10% acetic acid–0.1% copper sulphate and destained in 10% ethanol–10% acetic acid. The staining solution was discarded after use.

The pH gradient was determined at 20° on an unstained gel using a surface electrode (type Lot 403-30 from Ingold, Zurich, Switzerland). Pieces of wire were inserted into several gels at about 1-cm intervals, the pH was measured at these positions and the gels were subsequently stained and photographed to locate the protein bands accurately. The results for at least four gels were averaged to obtain final apparent p*I* values.

Two-dimensional analysis

The cylindrical gel rods described above, or sections cut from them containing

the protein bands (determined by staining another gel loaded with 25 μg of protein for 30 min), were used as the first dimension. In the second dimension, a 1.5-mm slab gel (10% acrylamide) measuring 14 × 8 cm (Bio-Rad apparatus, Model 220) was used following the procedure of Laemmli[10] for SDS–polyacrylamide gel electrophoresis. Following isoelectric focusing, the gel rods were equilibrated in SDS–stacking-gel buffer for 30 min and were set into the top of the 2-cm stacking gel (4.5% acrylamide). Electrophoresis was performed at 100 V for 1 h, then 200 V for a further 3.5 h. The slab gels were stained with Coomassie brilliant blue G 250 (0.2% in 50% methanol–10% acetic acid) and stored in 10% acetic acid solution.

RESULTS AND DISCUSSION

Initially, SCMK-A from wool was subjected to isoelectric focusing in polyacrylamide gel rods using pH 3–10 Ampholines and 8 *M* urea. Closely similar patterns were observed no matter whether the protein was loaded at the acidic or basic end of the gradient. The apparent isoelectric points of all the components were in the range 4.7 to 5.5. In subsequent experiments, a mixture of narrow range Servalyts was used to give a more suitable gradient. The well documented effects of urea[11,12] on the pK_a of the ionizable groups of ampholytes and the buffering action of urea at low pH presumably account for the need to incorporate significant amounts of the pH 2–4 range, although the final gradient was from pH 4.5 to 6.0.

The isoelectric focusing patterns for SCMK-A and a purified protein (component 7c) derived from it are shown in Fig. 1. It is our impression that the pattern for SCMK-A is more reproducible with slightly better resolution when the sample is loaded at the acidic end of the gradient. Nevertheless, a single band for component 7c was obtained with gels loaded at either end of the gradient. There are other reports in the literature[13,14] suggesting that the site of sample application is important in electrofocusing, but no reasons for this have been proposed.

Two-dimensional electrophoresis has been used to demonstrate that the bands shown in Fig. 1 represent separate single components of SCMK–A. The results shown in Fig. 2A and 2B were obtained using gels loaded at either the acidic or basic end of the first dimension and an SDS–polyacrylamide slab gel as the second dimension. The patterns are very similar which shows that under the conditions of our experiments there are no artifacts produced by formation of complexes between the SCMK-A proteins and the ampholytes. The components marked in Fig. 2A were assigned by two-dimensional electrophoresis[15], alkaline urea followed by SDS–polyacrylamide slab gel. There are discrete spots for components 8a, 8b, 8c-1 and 8c-2. In contrast,

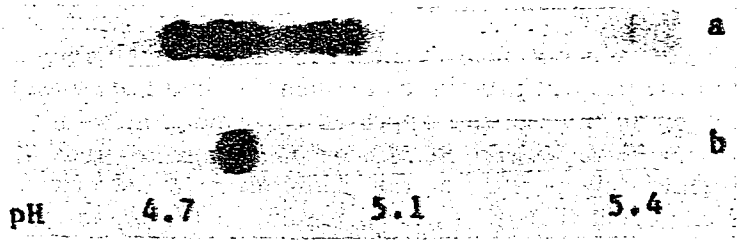

Fig. 1. Isoelectric focusing of (a) 10 μg SCMK-A; (b) 2 μg component 7c. Samples loaded at the acidic end of the gradient.

components 7a, 7b and 7c give rise to smeared regions of apparently constant molecular weight encompassing a range of p*I* values. When a limited amount of protein is loaded, a number of discrete spots can be seen within each component 7 region. Likewise, component 5 gives one major spot but a smeared region, with other spots, extends to either side as the amount of protein loaded is increased.

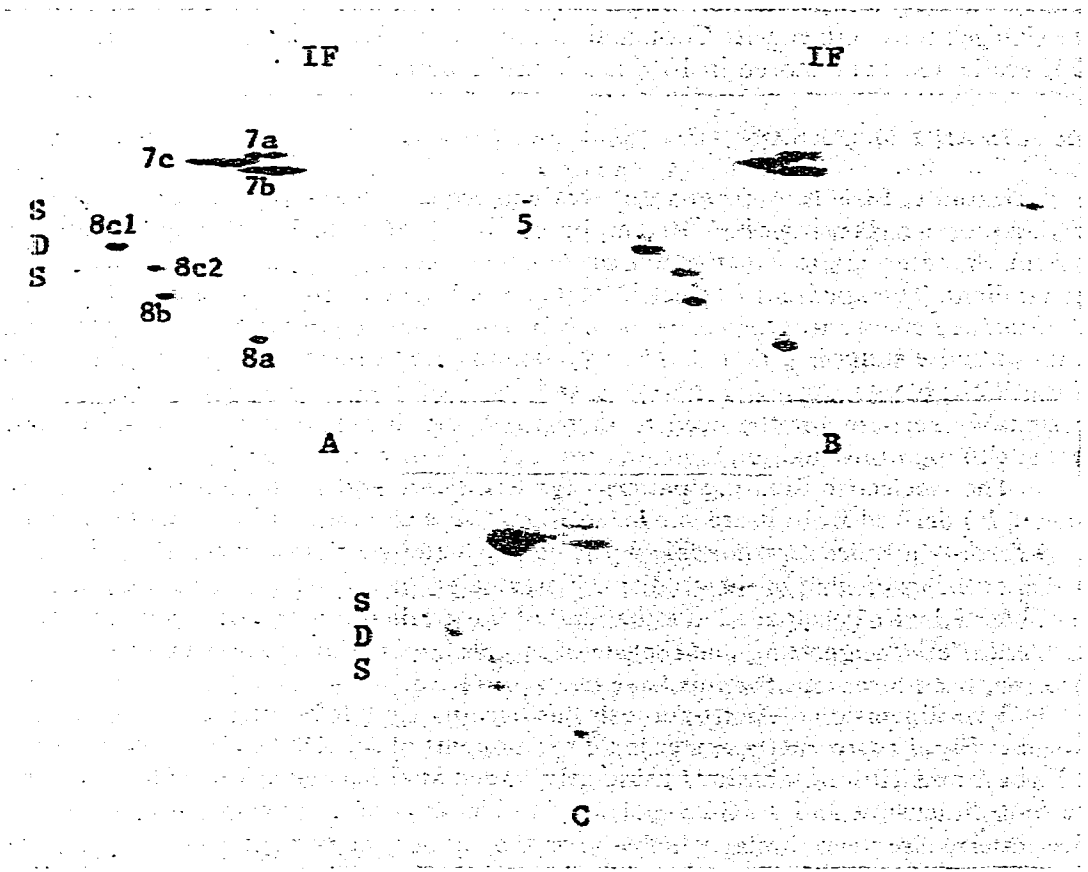

Fig. 2. Two-dimensional electrophoresis of wool low-sulphur proteins. First dimension (horizontal): isoelectric focusing (IF) in urea. (A) SCMK-A (10 μg) loaded at acidic end of gradient, left-hand side; (B) SCMK-A (10-μg) loaded at basic end of gradient, right-hand side; (C) mixture of SCMK-A (6 μg) and component 7c (2 μg) loaded at acidic end of gradient. left-hand side. Second dimension (vertical): SDS–polyacrylamide gel electrophoresis.

The result shown in Fig. 2C was obtained by electrofocusing a small amount of SCMK-A to which some of the purified protein, component 7c, had been added. In the second dimension slab gel an intense spot appeared corresponding with the lower p*I* part of the component 7c region for SCMK-A. Thus it seems that the purified component 7c represents only portion of this component in the original SCMK-A and this confirms our belief that the smeared regions in Fig. 2 represent charge heterogeneity and not artifacts.

The apparent isoelectric points of those components of SCMK-A identified in

this study are given in Table I. It is emphasized that these p*I* values refer to the particular protein component in 8 *M* urea solution at 20°. While the values for components 8a, 8b, 8c-1 and 8c-2 are for discrete spots, those for components 7a, 7b, 7c and 5 encompass a small section of the gradient. The measured p*I* values decrease in the same order that these components elute[2] from DEAE-cellulose in 8 *M* urea at pH 7.4 and are consistent with the report[3] of a broad, poorly staining smear extending from pH 4.5 to 5.2 when component 8 was focused using narrow-range (pH 3–6) Ampholines.

TABLE I

ISOELECTRIC POINTS OF THE PROTEIN COMPONENTS OF SCMK-A DERIVED FROM WOOL

Component	*pI**
5	5.3–5.4
7a	5.0–5.1
7b	5.0–5.1
7c	4.8–5.0
8a	5.0
8b	4.8
8c-1	4.7
8c-2	4.7_5

* Estimated error in p*I* values is $\pm$ 0.05.

It is not apparent why artifacts were found[3] during previous attempts to electrofocus a low-sulphur protein from wool. We have used flat-bed gels polymerized with persulphate in the manner described by Frater[3]. The banding patterns for both SCMK-A and a purified component 8c-1, at a high sample load of 200 μg[3], were independent of the position of application. This suggests that the artifacts observed were related to differences in the protein preparation or batch of Ampholines used rather than to the conditions of isoelectric focusing. The results presented here show that these proteins can be successfully focused in polyacrylamide gel, enabling this method to be exploited in the isolation and characterization of low-sulphur proteins from keratin.

ACKNOWLEDGEMENTS

We thank L. M. Dowling for preparation of SCMK-A and component 8c-1, Dr. L. G. Sparrow for preparation of component 7c and J. E. Burns for technical assistance.

REFERENCES

1 P. H. O'Farrell, *J. Biol. Chem.*, 250 (1975) 4007.
2 W. G. Crewther, L. M. Dowling, K. H. Gough, A. S. Inglis, N. M. McKern, L. G. Sparrow and E. F. Woods, in K. Ziegler (Editor), *Proc. Int. Wool Text. Res. Conf. 5th, Aachen, Sept. 2–11, 1975*, Vol. II, Deutches Wollforschungsinstitut, Aachen, 1976, p. 233.
3 R. Frater, *J. Chromatogr.*, 50 (1970) 469.
4 P. G. Righetti and E. Gianazza, *Biochim. Biophys. Acta*, 532 (1978) 137.

5 E. Gianazza and P. G. Righetti, *Biochim. Biophys. Acta*, 540 (1978) 357.
6 P. G. Righetti, R. P. Brown and A. L. Stone, *Biochim. Biophys. Acta*, 542 (1978) 232.
7 J. A. Maclaren and D. J. Kilpatrick, *Aust. J. Biol. Sci.*, 22 (1969) 1081.
8 L. G. Sparrow, N. M. McKern, W. G. Crewther and L. M. Dowling, *Aust. J. Biol. Sci.*, submitted for publication.
9 P. G. Righetti and J. W. Drysdale, *J. Chromatogr.*, 98 (1974) 271.
10 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
11 H. B. Bull, K. Breese, G. L. Ferguson and C. A. Swenson, *Arch. Biochem. Biophys.*, 104 (1964) 297.
12 N. Ui, *Biochim. Biophys. Acta*, 229 ((1971) 567.
13 D. Graesslin, A. Trautwein and G. Bettendorf, *J. Chromatogr.*, 63 (1971) 475.
14 A. Hamann and R. Drzeniek, *J. Chromatogr.*, 147 (1978) 243.
15 L. M. Dowling, personal communication.