A method for the preparation of low-pH dodecyl sulphate/polyacrylamide-gradient gels

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1. A low-pH lithium dodecyl sulphate/polyacrylamide-gradient slab-gel system, suitable for electrophoresis, is described, and the migration properties of standard proteins are compared on this and conventional high-pH gels. 2. Cytochrome oxidase may be partially resolved into its component polypeptides. The order of migration of these is, however, dependent on the pH of the gel system.

There are occasions when low-pH dodecyl sulphate/polyacrylamide gels are essential to the study of pH-labile biological molecules (Bader *et al.*, 1966). A method for preparing constant-polyacrylamide-composition gels has been described by Lichtner & Wolf (1979), and relies on the substitution of Li^+ for Na^+ ions to increase the solubility of dodecyl sulphate at low pH and temperatures.

It is of particular interest to us to perform low-pH electrophoresis of bovine cytochrome *c* oxidase (EC 1.9.3.1), since we wish to identify those polypeptide subunits of the enzyme that specifically bind the Cr^{2+} ion. Protein- Cr^{2+} complexes are, however, labile at high pH and cannot therefore be separated on conventional gel systems.

To improve the resolution of the low-pH gels described by Lichtner & Wolf (1979), we have modified their method so that it incorporates a polyacrylamide gradient and discontinuous buffer system. High-pH polyacrylamide-gradient gels offer a much improved resolution over constant-composition gels. The results reported below show a similar improvement for low-pH gels, and also show that the pH of the gel system can alter the order of migration of the polypeptides in some cases.

Methods and materials

Low-pH LDS/polyacrylamide-gradient-gel electrophoresis

The electrophoretic method used was a simplified version of that reported by Lambin (1978). The gradient of acrylamide was delivered between two glass plates 20 cm long $\times$ 15 cm wide, held apart at

the edges by two 1 mm-thick Perspex [poly(methyl methacrylate)] strips. The bottom of the glass plates was sealed with an $\approx 16\%$ -acrylamide plug prepared from a 15 ml solution of the following components: 8 ml of acrylamide [stock solution: 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide], 1.2 ml of 1 M-citric acid/0.125 M-phosphoric acid/Tris buffer, pH 4.0, 0.15 ml of LDS (10%, w/v), 2.82 ml of sucrose (saturated solution), 2.5 ml of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution (0.025%), 0.15 ml of ascorbic acid (8.33%, w/v) and 0.18 ml of H_2O_2 (2.5%, w/v). The last three components are the catalyst system, and were freshly prepared before use. At room temperature the gel forms within 5 min on addition of the H_2O_2 .

A linear gradient was established on top of this 'plug' so that the highest concentration of acrylamide was at the bottom of the plate. Solutions of 18% and 10% acrylamide solutions were mixed to form the gradient. Both of these solutions contained 2.0 ml of 1 M-citric acid/0.125 M-phosphoric acid/Tris buffer, pH 4.0, 0.25 ml of LDS (10%, w/v), 0.9 ml of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.025%), 0.25 ml of ascorbic acid (8.33%, w/v) and 0.3 ml of H_2O_2 (2.5%, w/v). The 18% acrylamide solution also contained 15.6 ml of acrylamide stock solution and 4.6 ml of saturated sucrose solution. The 10% acrylamide solution contained 8.4 ml of stock acrylamide solution. Both of the solutions for the gradient were made up to a total volume of 25 ml with distilled water. The gel forms within 15 min at room temperature.

A stacking gel of 6.7% acrylamide, also used to form the protein-sample wells, was placed on top of the gradient gel, and contained the following (20 ml prepared): 4.5 ml of acrylamide stock solution, 0.5 ml of 1 M-citric acid/0.125 M-phosphoric acid/Tris buffer, pH 3.0, 0.2 ml of LDS, 1.29 ml of water, 1.5 ml of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution (0.025%), 0.2 ml of

Abbreviations used: LDS, lithium dodecyl sulphate; SDS, sodium dodecyl sulphate.

ascorbic acid (8.33%, w/v) and 0.24 ml of H_2O_2 (2.5%, w/v).

This type of slab gel produces satisfactory resolution of polypeptides of molecular weights between 90 000 and 4000.

Protein samples were prepared for electrophoresis by incubating them at room temperature in 0.25 M-citric acid/0.031 M-phosphoric acid/Tris buffer, pH 4.0, containing 8 M-urea, 5% (w/v) LDS and 4% (v/v) 2-mercaptoethanol for 1½–2 h.

The electrophoresis buffer was 0.51 M-L-glutamic acid/Tris buffer, pH 4.0, containing 0.1% LDS. This was circulated slowly between the anode and cathode reservoirs in order to prevent pH changes, which occur as a consequence of electrophoresis. At room temperature a constant voltage of 150 V moved the tracker dye, basic Fuchsin, to the bottom of the gel in 20 h. The gel was then fixed and stained as described previously (Wilson *et al.*, 1980).

Two-dimensional gel electrophoresis

Electrophoresis in the first dimension was performed as described above, but the leading edge was allowed to migrate only two-thirds of the length of the gel. A 1 cm-wide section of the unstained low-pH gel was removed, and placed on top of the stacking gel of a high-pH (8.6) SDS/polyacrylamide-gradient gel and subjected to electrophoresis in the second dimension. The procedure for running the polypeptides in a second dimension on a high-pH gel was essentially identical with that described above, but the materials and conditions were as described by Wilson *et al.* (1980).

Materials

Chemicals for SDS/ and LDS/polyacrylamide-gel electrophoresis were purchased from BDH Chemicals (Poole, Dorset, U.K.) and were of the highest purity available. Proteins used for molecular-weight determinations were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Results and discussion

SDS/polyacrylamide-gradient-gel electrophoresis, which is normally performed at alkaline pH, offers excellent resolution of the polypeptide subunits of protein molecules (Lambin *et al.*, 1976). The low-pH constant-polyacrylamide-composition gels described by Lichtner & Wolf (1979) were unable to achieve comparable resolution. We sought, therefore, to alter the well-tested high-pH system in order to satisfy the conditions outlined in the introduction.

We draw attention, below, to a number of changes that we have made and procedures that should be employed to ensure good resolution.

Fig. 1. Plots of $\log(\text{apparent molecular weight})$ versus $\log(\text{relative mobility})$ for standard proteins

Proteins used were: bovine serum albumin, catalase, ovalbumin, lactate dehydrogenase, D-amino acid oxidase, myoglobin, avidin, haemoglobin, lysozyme, chymotrypsinogen A, chymotrypsin and cytochrome c. The pH values of the gels were 4.0 (O) and 8.6 (●). The mobility is relative to that of the tracking dye (=10): Bromophenol Blue at high pH and basic Fuchsin at low pH.

(a) The catalyst system of Avruch & Fairbanks (1972) was used. We found, however, that ascorbic acid at concentrations of approx. 8% enhanced the rate of polymerization, but at higher concentrations (approx. 10%) formation of rigid gels was prevented. To ensure rapid polymerization we have increased the FeSO_4 concentration.

(b) As emphasized by Lichtner & Wolf (1979), it was important to exclude Na^+ and K^+ ions from the system in order to prevent precipitation of the dodecyl sulphate.

(c) Protein bands were poorly resolved if a stacking gel was not used.

(d) Glutamic acid was used in the running buffer. Aspartic acid or malic acid could be used instead, but glutamic acid was found to be the most convenient since it has the highest solubility of the three.

(e) In comparison with the high-pH gel system, the stacking gel was less efficient at concentrating the polypeptide chains when employed at low pH values. Nevertheless, good resolution of proteins was achieved.

High-pH SDS/polyacrylamide-gradient gels, when calibrated with standard polypeptides, show a linear relationship between $\log(\text{molecular weight})$ and $\log(\text{migration distance})$ (Lambin *et al.*, 1976; Lambin, 1978), but, as with constant-composition gels (Fish *et al.*, 1970), a deviation below apparent molecular weight approx. 17 000 occurs. Fig. 1 shows such a plot for some marker proteins on

Fig. 2. Two-dimensional dodecyl sulphate/polyacrylamide-gradient-gel electrophoresis of cytochrome *c* oxidase

The arrows indicate the direction of migration and the pH values of the gels. The inset shows a densitometric scan at 600nm of the low-pH gel stained with Kenacid Blue. An unstained gel, positioned identically with that in the inset, was subjected to electrophoresis in the second dimension (pH 8.6). A second sample of cytochrome *c* oxidase, incubated at pH 4.0, was included in the pH 8.6 gel for comparison and is shown at the extreme left.

the same in the low-pH system as at high pH, we undertook two-dimensional electrophoretic experiments (Fig. 2). Cytochrome *c* oxidase samples were subjected to electrophoresis on a low-pH gel in the first dimension and on a high-pH gel in the second dimension, at right-angles to the first. These two-dimensional gels revealed that the distance migrated by a particular polypeptide, measured relative to its neighbours, was affected by the nature of the gel system. However, in only one instance did this lead to a change in the order of migration of the polypeptides. One of the low-molecular-weight chains, contaminant *c*, migrates relatively more slowly on the low-pH gels. The behaviour of this latter polypeptide shows that caution must be exercised in the use of low-pH gel systems. Where a multi-peptide complex is to be studied, e.g. cytochrome oxidase, it cannot be assumed that the migratory behaviour of the individual components is identical in both high-pH and low-pH gel systems. Changes in migration order may also arise from proteolysis occurring during incubation. However, this seems not to be the case here, as identically treated samples (i.e. incubated at pH 4.0) show differences in order of migration when subjected to electrophoresis at low pH and high pH (see Fig. 2).

We have described an LDS/polyacrylamide-gradient-gel electrophoresis system that seeks to provide both good resolution of polypeptides and conditions that allow separation of polypeptide-ligand complexes that may require acid conditions for their stability. Such slab gels are suitable for radioautography.

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References

- Avruch, J. & Fairbanks, G. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1216–1220
- Bader, H., Sen, A. K. & Post, R. L. (1966) *Biochim. Biophys. Acta* **118**, 106–115
- Fish, W. W., Reynolds, J. A. & Tanford, C. (1970) *J. Biol. Chem.* **245**, 5166–5168
- Lambin, P. (1978) *Anal. Biochem.* **85**, 114–125
- Lambin, P., Rochu, D. & Fine, J. M. (1976) *Anal. Biochem.* **74**, 567–575
- Lichtner, R. & Wolf, H. U. (1979) *Biochem. J.* **181**, 759–761
- Ludwig, B., Downer, N. W. & Capaldi, R. A. (1979) *Biochemistry* **18**, 1401–1407
- Wilson, M. T., Lalla-Maharajh, W., Darley-Usmar, V., Bonaventura, J., Bonaventura, C. & Brunori, M. (1980) *J. Biol. Chem.* **255**, 2722–2728

low-pH and high-pH gels. It can be seen that these plots are very similar in form, indicating that the migration properties of the standard proteins are not greatly affected by the change in gel system (e.g. pH, high Fe^{2+} ion concentrations etc.)

As an example of the resolution of a molecular complex into component polypeptide subunits, we have studied bovine cytochrome *c* oxidase. This enzyme is membrane-bound and is composed of at least ten to twelve polypeptides (Ludwig *et al.*, 1979; Wilson *et al.*, 1980). The inset to Fig. 2 depicts a densitometric scan of the polypeptides of the enzyme resolved by the low-pH gel. In order to determine whether the order of migration of the subunits was