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DISC ELECTROPHORESIS: AVOIDING ARTIFACTS CAUSED BY PERSULFATE

E. E. KING*
Crops Research Division, Agricultural Research Service, United States Department of Agriculture, State College, Miss. 39762 (U.S.A.)
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SUMMARY

Artifacts of separation and inactivation of enzymes in polyacrylamide disc electrophoresis may be avoided by substituting riboflavin for persulfate, or by incorporating an antioxidant into stacking gels and samples. Polymerization with riboflavin results in gels having poor resolution and reproducibility. Addition of an antioxidant to stacking gels and samples is the more reliable alternative for general use.

INTRODUCTION

Polyacrylamide gels for electrophoresis are generally prepared with ammonium persulfate as the polymerization catalyst. It has been shown that the use of persulfate leads to artifacts in separation patterns and inactivation of enzymes[1-3]. Pre-runs of separating gels without sample have been proposed to remove residual persulfate[3], but this technique affords no more protection against artifact and enzyme inactivation than does a riboflavin-polymerized stacking gel as employed in the standard disc electrophoresis procedure. The mobility of persulfate in alkaline gels is greater than that of the Bromphenol Blue tracking dye[4]. Therefore if a riboflavin-polymerized stacking gel is interposed between sample and separating gel, protein should never encounter persulfate. By the time protein has migrated into the separating gel, persulfate will have migrated ahead of it and will remain ahead throughout the run. Nevertheless, inactivation of enolase and multiple bands from a single protein species have been detected with these conditions[1]. These effects are therefore likely caused by nonionic by-products of the polymerization reaction in the separating gel instead of by persulfate *per se*.

Polymerization of separating gels with riboflavin instead of persulfate has been suggested to avoid the difficulties caused by persulfate[1,2], as has incorporation of an antioxidant into the sample, stacking gel, or both[1]. Either of these methods should prevent separation artifacts and enzyme inactivation due to residual persulfate or nonionic oxidizers. A comparison of these two methods is the subject of this report.

* Address correspondence to: E. E. King, P. O. Box 5367, State College, Miss. 39762, U.S.A.

EXPERIMENTAL

Materials and methods

Formulations and techniques of disc electrophoresis were those of DAVIS[5] except that no sample gel was used. Samples were prepared in 5 % sucrose and layered beneath the tank buffer onto the stacking gels. Gels were prepared in glass tubes 75 × 5 mm I.D. Photolysis of riboflavin was accomplished with two 15 W cool white fluorescent lamps. Twelve gels at a time were run at room temperature; electrode buffers were used only once. Constant current of 1 mA per tube was applied until the Bromphenol Blue tracking dye entered the separating gel; current was then increased to 2 mA per tube for the remainder of the run. Low current was used to test the resolving capabilities of experimental gel formulations by maximizing any lack of resolution caused by diffusion of protein within gels.

At the completion of each electrophoretic run, gels were removed from the glass tubes, cut off at the tracking dye front, fixed for 30 min in cold 15 % trichloroacetic acid (TCA) and stained overnight with Coomassie Blue in 10 % TCA as described by CHRAMBACH *et al.*[6]. Excess dye was removed by rinsing with 10 % TCA.

Migration of protein bands was measured on a fluorescent light box by using a metric rule and hand lens; accuracy of measurement was ± 0.25 mm. Results are expressed as relative mobilites (R_M), the ratio of protein migration to tracking dye migration. Mean R_M values shown in Fig. 1 are based on values obtained from six to eight gels representing two separate electrophoretic runs. Diagrammatic representations of gels are used to present mean R_M values and to overcome the limitations of photography which are particularly noticeable with faint bands.

Sample material was a partially purified, salt-free lyophylized preparation from bovine pancreas marketed by Worthington Biochemical Corp., Freehold, N. J. under the designation protease*. 300 μg of sample in 25 μl of 5 % sucrose was applied to each gel. Electrophoresis in 7 % acrylamide persulfate-polymerized gels in the DAVIS[5] system yields thirteen bands (Fig. 1a) which are diverse in size, staining intensity, and R_M. This pattern is the standard with which all others were compared; two such gels were included in each electrophoretic run as controls.

Experimental variables

Preliminary experiments showed that riboflavin concentration has a marked effect on gel strength and porosity. High concentrations of riboflavin produce soft, porous gels which are seldom removed from the glass tubes without damage. R_M values of proteins are greater in riboflavin-polymerized gels than in persulfate-polymerized gels of the same acrylamide concentration. One of the problems then became that of producing a riboflavin-polymerized separating gel with physical strength and sieving characteristics similar to the standard persulfate-polymerized gel. To attempt this, the concentrations of riboflavin, N,N'-methylenebisacrylamide (bis) and acrylamide were varied.

BRACKENRIDGE AND BACHELARD[7] show that porosity of riboflavin-polymerized gels is reduced by increasing polymerization time to 1 h or more. DAVIS[5], however, warns that extended polymerization times are likely to produce gel inhomogeneities,

* Mention of a specific trade name is made for purposes of identification only, and does not imply endorsement by the United States Department of Agriculture.

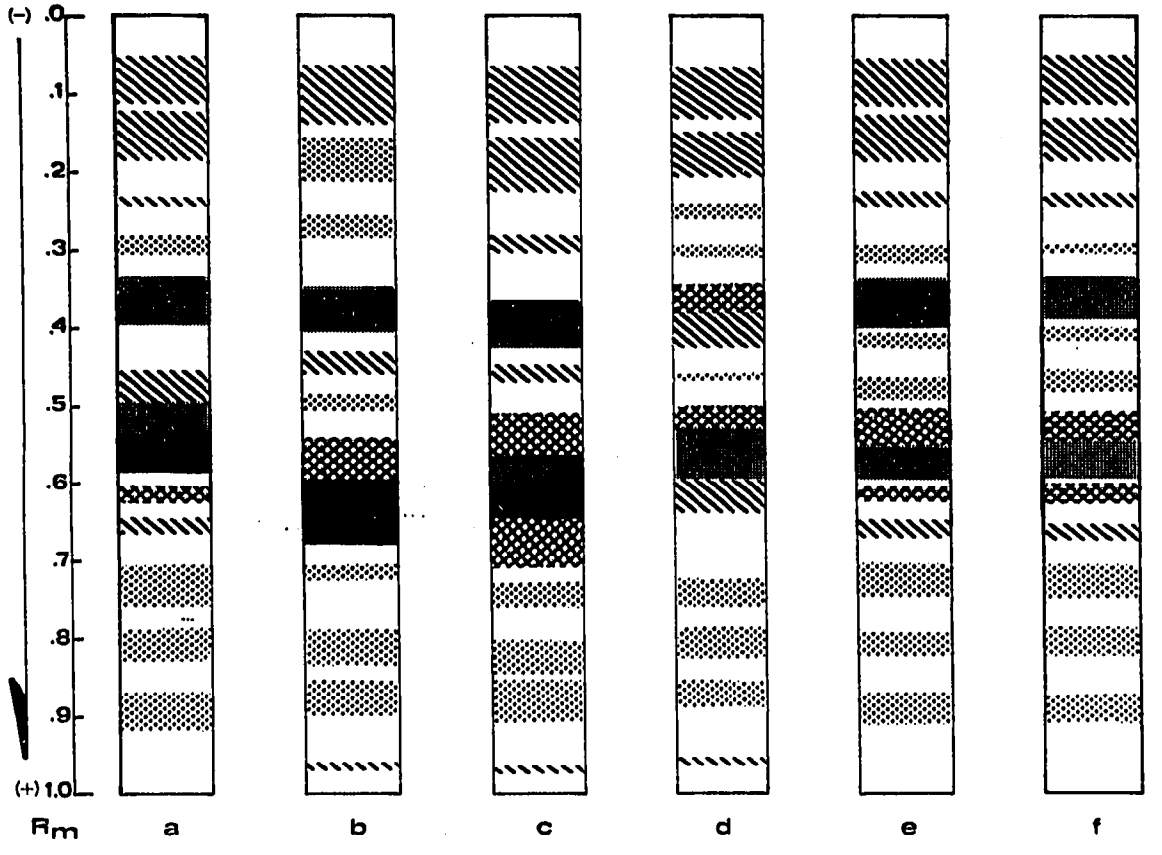

Fig. 1. Protein separation patterns in standard and experimental gel formulations. (a) 7% acrylamide, 0.184% bis, polymerized with persulfate; (b) 7% acrylamide, 0.184% bis, polymerized with riboflavin; (c) 7% acrylamide, 0.5% bis, polymerized with riboflavin; (d) 8.25% acrylamide, 0.184% bis, polymerized with riboflavin; (e) as (a) with 2.5 m*M* 2-mercaptoethanol in stacking gel and sample. (f) as (a) with 2.5 m*M* dithiothreitol in stacking gel and sample. Arrow indicates direction of current flow.

giving rise to localized zones of decreased resolution. Therefore, the effect of varying riboflavin concentration was studied using the standard 30 min polymerization time. To find the lowest concentration that would accomplish polymerization, riboflavin concentration was varied from 2×10^{-3} to 2.5×10^{-5} % using a separating gel of 7 % acrylamide and 0.184 % bis as employed in the DAVIS[5] disc technique.

There is conflict in the literature concerning the influence that bis concentration has on the porosity or sieving characteristics of a gel[8,9]. To determine the effect of varying bis concentrations on gel strength and the separation pattern of the heterogeneous sample, bis concentration was varied from 0.184 % to 0.875 % (2.6 to 12.5 % expressed as percent of monomer concentration). Acrylamide and riboflavin concentrations were constant at 7 % and 5×10^{-5} % respectively.

Acrylamide concentration, the parameter most influential on pore size, was varied from 7 to 9.75 %. Bis and riboflavin concentrations were constant at 0.184 % and 5×10^{-5} % respectively.

Dithiothreitol and 2-mercaptoethanol were incorporated into sample solutions and stacking gels used with 7 % acrylamide persulfate-polymerized separating gels. Both antioxidants were employed at concentrations of 2.5 m*M* and 5.0 m*M*.

RESULTS

Gels polymerized with riboflavin

Of the riboflavin concentrations tested, 5×10^{-5} % was most suitable. Higher concentrations produced soft, weak gels; lower concentrations resulted in incomplete polymerization during the thirty min exposure to light. Although 5×10^{-5} % riboflavin produced gels with physical strength approaching that of persulfate-polymerized gels of equivalent acrylamide concentration, protein separation patterns were dissimilar (Fig. 1a,b). Fewer bands were apparent, R_M values were greater, and bands were wider and less distinct in the riboflavin-polymerized gels.

Increasing the bis concentration from the usual 0.184 % produced gels with good physical strength, but also with decreased transparency. Gels with bis concentrations greater than 0.5 % were white and opaque; only the most intense bands were visible. Bis concentration of 0.5 % yielded the same number of bands as the standard persulfate-polymerized gel, but in a different pattern (Fig. 1a,c). R_M values were generally greater, and it is difficult to identify many bands with those in the standard gel.

Riboflavin-polymerized gels were prepared in which the bis-concentration was constant at 0.184 % and acrylamide concentration was increased from 7 to 9.75 %. In terms of general pattern similarity and R_M comparability, 8.25 % gels are closest to the standard 7 % persulfate-polymerized gel, although differences are present (Fig. 1a,d). Though many bands have comparable R_M values, some bands cannot be positively identified with any in the standard gel, and one band present in the standard gel (R_M 0.65–0.67) is not visible in the riboflavin-polymerized gel.

Reproducibility of R_M values between riboflavin-polymerized gels of similar acrylamide concentration in the same electrophoretic run was poor, and even poorer between gels in separate runs. Bands in these gels were often badly distorted and indistinct (Fig. 2), whereas this was seldom the case with gels polymerized with persulfate.

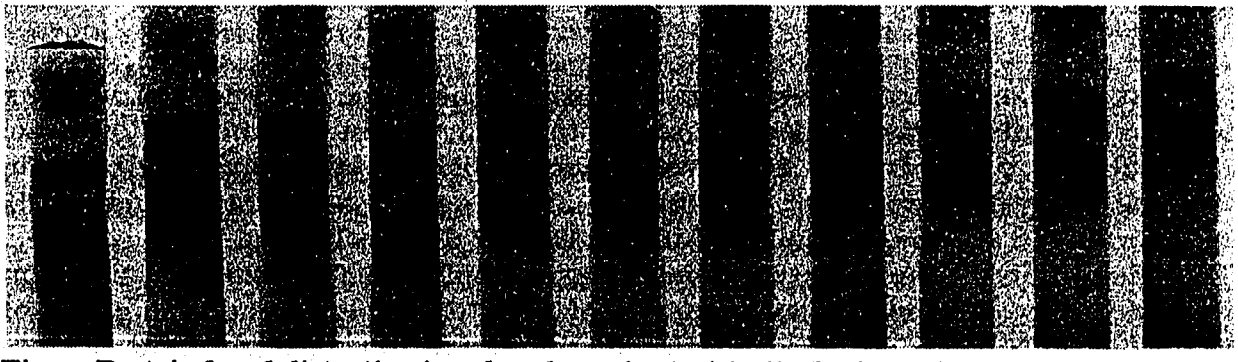

Fig. 2. Protein band distortion in gels polymerized with riboflavin. Gel on extreme left polymerized with persulfate, others contain various concentrations of acrylamide polymerized with riboflavin. All gels were from the same electrophoretic run.

Antioxidants in stacking gel and sample

Addition of dithiothreitol or 2-mercaptoethanol to stacking gels and sample solutions in conjunction with persulfate polymerization of separating gels produced separation patterns nearly identical with those in the standard gel (Fig. 1a,e,f). The poor resolution and distortion of bands characteristic of riboflavin-polymerized gels was missing, and reproducibility was good. There were no apparent differences in

separation patterns between 2.5 mM and 5 mM concentrations of either antioxidant.

The only detectable differences in protein separation patterns between the standard gel (Fig. 1a) and gels containing antioxidants (Fig. 1e,f) were in the regions of R_M 0.33–0.43 and 0.45–0.50. In the first region two bands exist in the gels with antioxidant *versus* one in the standard gel; in the second region, resolution of a band was markedly improved in the gels containing antioxidant over that in the standard gel.

DISCUSSION

Results of this investigation show that it is possible to produce a riboflavin-polymerized separating gel which approximates the standard persulfate-polymerized gel in terms of gross mechanical properties and sieving characteristics. There are differences between the two types of gels in terms of separation patterns, quality of resolution and reproducibility.

Differences in separation patterns are a logical result, since the major objection to the use of ammonium persulfate[1–3,7] is that separation artifacts are produced by the adverse action of the persulfate ion or its reaction products upon sensitive proteins. BREWER[1] and MITCHELL[3] have shown that such oxidation may produce multiple bands from a single protein species; FANTES and FURMINGER[2] report decreased staining intensity with Amido Black and band absence. The differences between separation patterns of persulfate- and riboflavin-polymerized gels in this study may represent similar circumstances, the pattern in the riboflavin-polymerized gel being the more realistic.

A comparison of the pattern in the 8.25 % acrylamide riboflavin-polymerized gel (Fig. 1d) with those in the gels containing antioxidants (Fig. 1e,f) tends to substantiate this view. Aside from generally increased resolution in the gels containing antioxidant, differences in the separation patterns are few. The areas of marked difference are the regions of R_M 0.60–0.70 and 0.95–0.97, where patterns in the gels containing antioxidant resemble the standard gel rather than the riboflavin-polymerized gel.

CONCLUSION

The band distortion, poor resolution, and inadequate reproducibility associated with riboflavin polymerization preclude its recommendation as a routine substitute for persulfate polymerization. Instead, the use of persulfate-polymerized separating gels with the addition of a suitable antioxidant such as dithiothreitol or 2-mercaptoethanol to stacking gels and sample material is recommended. Resolution and reproducibility are at least equal to that of the standard persulfate procedure, and the risk of artifacts due to oxidation is minimized.

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