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ISOELECTRIC FOCUSING IN AGAROSE UNDER DENATURATING CONDITIONS

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SUMMARY

A method for running isoelectric focusing under denaturing conditions in agarose is described. The unfolding of protein is obtained with urea, reduction of disulphides with mercaptoethanol and an increased solubility of hydrophobic proteins with a non-ionic detergent. This method minimizes the risk of carbamylation of proteins when using urea in combination with agarose. The difficulty in making agarose gels in 8 *M* urea has also been overcome.

INTRODUCTION

Isoelectric focusing (IEF) in combination with denaturating and/or reducing conditions is a convenient method for the study of protein structure, subunit composition or single peptide chains of proteins[1]. It is also suitable for the study of poorly soluble proteins such as hydrophobic proteins[2] or proteins that have been fully or partially denatured.

The concentration of urea necessary for unfolding different proteins varies between 4 and 9 *M*[3]. In the denaturing process urea acts on both hydrogen bonds and hydrophobic interactions. By unfolding the protein with urea the Stokes radius is increased[3], which puts an increasing demand on the porosity of the IEF matrix.

To obtain a higher porosity in a polyacrylamide gel, the most commonly used matrix, the total concentration of acrylamide can be reduced or the concentration of the cross-linking agent N,N′-methylenebisacrylamide (BIS) can be increased. Both methods lead to soft gels, and in the latter instance also to more hydrophobic gels. Another possibility is to use alternative cross-linkers; diallyltartardiamide (DATD) has been used for this purpose. However, these gels have been shown to contain high concentrations of unpolymerized DATD, which is capable of reacting with proteins[4].

Thus agarose, with a macroporous structure that minimizes molecular sieving effects, appears to be the matrix of choice, especially when studying proteins of high molecular weight. The inertness and the non-toxic nature of agarose are also attractive qualities.

Agarose of standard quality cannot be used in IEF because of its high content of charged groups, which give rise to severe electroendosmosis and concomitant gradient drift followed by disruption of the gel. Agarose-IEF, however, is a specially

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treated agarose that is suitable for IEF. It has an extremely low content of negatively charged groups and the effect of these are counter-balanced by positively charged groups that are covalently bound to the matrix. This gives a gel with practically no electroendosmosis and gradient drift in the central pH region.

Isoelectric focusing in Agarose-IEF under denaturing conditions offers a number of potential advantages. However, there are also some inherent difficulties, mainly:

(1) Problems connected with the effects of urea on the agarose gel matrix itself. At higher concentrations of urea, the well organized structure of the agarose matrix is disturbed. This is shown by a much lower gelling temperature, a longer time for gelling and a gel of lower mechanical strength than normal.

(2) Urea and ammonium cyanate form an equilibrium pair (see Fig. 1). Thus, there is always the risk of carbamylation when using urea, not so much for the Pharmalyte (very few primary amino groups) as for the proteins[6]. The risk is fairly low with fresh urea solution at room temperature, but increases as the temperature rises. An 8 *M* solution of urea, at equilibrium at 20°C (pH $\geqslant$ 6), contains 0.02 *M* cyanate ions[5] and at 100°C urea reaches its equilibrium with cyanate within less than 30 min[6].

Fig. 1. Equilibrium between urea and ammonium cyanate.

This paper describes a reliable method for focusing in agarose with urea that has been developed to overcome the above problems.

EXPERIMENTAL

The IEF runs were carried out on an FBE 3000 flat-bed electrophoresis apparatus (Pharmacia, Uppsala, Sweden), cooled with water thermostated at 15°C using a MGW Lauda Thermo-temp. An ECPS 3000/150 electrophoresis constant-power supply was used in conjunction with a VH-1 volt-hour integrator, both from Pharmacia. The pH gradients were measured with a Beckman Phasar I pH meter with an Ingold surface electrode. The field strength was measured with a Simpson 270 voltmeter.

Gel casting

The agarose gel was cast on plastic film (Gel Bond) applied on a levelling table with a film of water in between and two plastic frames on it, as illustrated in Fig. 2.

To prepare 1-mm thick 2% Agarose-IEF gel with 8 *M* urea and 2% Nonidet NP-40, the following procedure was used; the first group of chemicals were weighed into a 50-ml E-flask:

(I) 0.6 g of Agarose-IEF
3.6 g of Sorbitol
15 g of water

(II) 14.4 g of urea
1.9 ml of Pharmalyte 3-10
0.6 ml of Nonidet NP-40
(0.3 ml of Mercaptoethanol)

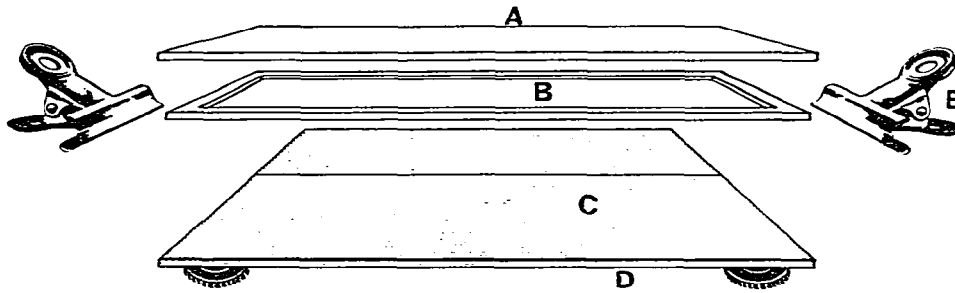

Fig. 2. Mould for casting agarose gels (from Pharmacia). A = glass plate; B = two frames (5 mm thick); C = Gel Bond; D = levelling table; E = clamps.

Agarose and sorbitol were dissolved by boiling in water, then the temperature was decreased to 65°C and Pharmalyte and urea were added and dissolved without further heating. This decreased the temperature to about 30°C. If a non-ionic detergent and/or mercaptoethanol were used they were added last and dissolved carefully without foaming. The solution was then poured into the casting frames and the glass plate was clamped on top as a lid, preventing the gel from drying out. The solution was then left for 15–20 h (overnight) at 21°C to achieve the necessary mechanical strength.

Running conditions

The cooling plate was thermostated at 15°C. The electrode solutions were 1 *M* sodium hydroxide as catholyte and 0.05 *M* sulphuric acid as anolyte. The following settings on the ECPS 3000/150 were found to give good results when running over a short distance: 10 W, 50 mA and 1500 V. Prefocusing was carried out for 500 V h before the samples were applied. The gel was then run for a further 1300 V h to 1800 V h in all. The sample should be applied in the neutral region of the gel.

Fixing and staining

Different procedures were followed, depending on whether or not the gel contained a non-ionic detergent:

(a) Gel without non-ionic detergent; the procedure described in the Agarose-IEF instruction sheet was used:

(1) immediately after the run was finished the proteins were fixed in 10% trichloroacetic acid (TCA) solution in water for 30 min;

(2) the gel was washed for 2 × 15 min in destaining solution (acetic acid–ethanol–water, 10:30:60);

(3) the gel was pressed for 15 min under a 3-mm thick layer of filter-paper, a glass plate and a weight of 1 kg;

(4) the paper was removed and the gel dried with a hair dryer;

(5) the film was placed in staining solution: 0.1% Coomassie R250 dissolved in destaining solution (see a2) for 15 min;

(6) the gel was destained in destaining solution until a clear background was obtained;

(7) finally, the film was dried with a hair dryer.

(b) Gel containing non-ionic detergent;

(1) the proteins were fixed in TCA–ethanol–water (10:33:57) for 30 min;
(2) the gel was washed in TCA–ethanol–water (5:33:62) for 2 × 45 min or until the precipitate was dissolved;
(3) the procedure was continued from a2 in the previous method.

RESULTS AND DISCUSSION

Gel casting

The optimal concentration of Agarose-IEF in 8 *M* urea was found to be 2%. Lower concentrations decreased the band sharpness and gave unacceptably soft gels, and at higher concentrations two very pronounced ditches appeared in the middle of the gel, giving an extremely high field strength in that area and eventually causing the gel to burn off.

It is extremely important that the temperature be as low as possible during the whole casting procedure. An advantage is that agarose solidifies very slowly in higher concentrations of urea. This makes it possible to cast the gel at a very low temperature, *ca.* 25°C, which is far below its normal gelling temperature. Artefacts that could be ascribed to ammonia and cyanate were observed when the casting solution was exposed to excessive heating (100°C for 3 min): the pH gradient extended 1 pH unit higher than normal towards the cathode and in this region there was also a very low field strength, ascribed to the accumulation of ammonium ions. Samples applied in this area did not migrate or showed a very smeary protein pattern.

An additional precaution that could be taken against cyanate is to add 1% mercaptoethanol to the gel. The released cyanate will then react with the thiol groups in preference to amino groups[6].

Running conditions

It is important not to run the gel at a temperature lower than 15°C because urea then precipitates. Pre-focusing is carried out to remove the cyanate and ammonia from the gel. Applying the samples in the middle of the gel creates a minimum of disturbances and gives a short distance for the protein to migrate, thus minimizing the focusing time.

Fixing and staining

The use of a special washing procedure when non-ionic detergents are incorporated into the gel is necessary on account of the precipitate formed between ampholytes and non-ionic detergents at low pH[1]. If the precipitate is not removed before staining, a very strong complex with Coomassie Blue R250 stain is produced.

Protein patterns

A typical example of the separating power and results obtained with this technique is shown in Fig. 3. Protein mixtures of different complexity were run in 8 *M* urea. Cheese and soybean meal are examples of foodstuffs containing proteins denatured to various extents, which, under standard conditions, will give smeary and ill-defined protein patterns. However, after reduction and complete unfolding in urea both gave distinct protein bands.

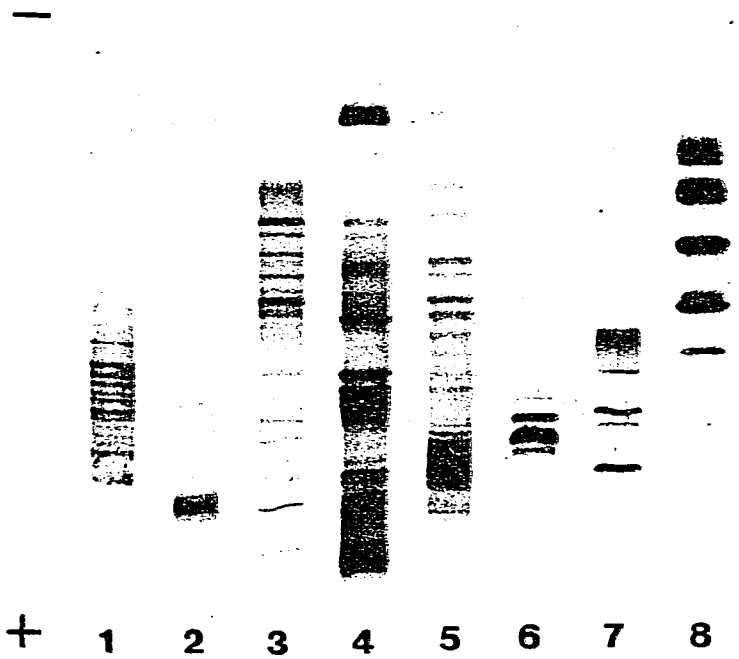

Fig. 3. Agarose-IEF gel run in 8 *M* urea for 1800 V h, 1500 V, 50 mA, 10 W. Samples: 1 = *E. coli* lysate; 2 = cellulase from *Trichoderma reesei* QM 9414; 3 = mackerel extract; 4 = cheese; 5 = soybean meal; 6 = soybean lectin; 7 = lentil lectin; 8 = Helix pomatia lectin (all lectins from Pharmacia). The samples were dissolved in final concentrations of 8 *M* urea and 2% mercaptoethanol. *E. coli*, mackerel and cheese were first mechanically disrupted in solution at low temperature. The samples were then frozen and thawed twice, and applied without further treatment.

pH measurements in urea

Fig. 4 shows the apparent pH gradient after IEF in 8 *M* urea. The values were measured with an Ingold surface electrode calibrated with ordinary standard buffers and reported without any corrections. The interpretation of pH readings in concentrated urea solution is difficult. However, it has been shown that the p*I* of ampholytes are increased by 0.4 pH unit when run in 6 *M* urea[7]. In principle, ampholytes

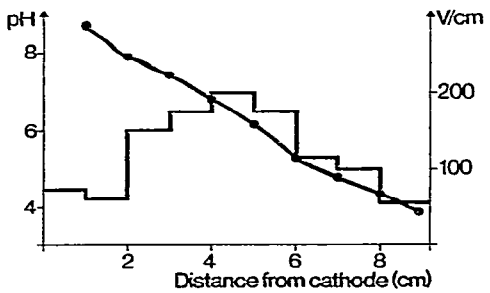

Fig. 4. Apparent pH gradient and field strength in an Agarose-IEF gel with 8 *M* urea after focusing for 1800 V h.

and protein contain the same kind of ionizable groups. However, the correlation of p*I* in urea and in water is much more complicated for proteins as some of the ionizable groups have anomalous p*K* values in the native state, depending on their environment[7]. Without urea the p*I*s of the two isomers of lentil lectin have been determined to 8.15 and 8.65[8], and in 8 *M* urea these are shifted to apparent pH values of about 5.0 and 5.5 (see Fig. 3). Insulin and haemoglobin, however, show very little change in their p*I* points[7].

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