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ANALYTICAL SEPARATION OF HORDEIN AND ITS FRACTIONS BY DISC ELECTROPHORESIS ON ACRYLAMIDE GEL

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SUMMARY

An analytical method requiring 200 to 500 μg of protein sample for the investigation of hordein and its fractions is described, in which disc electrophoresis on 7.5 to 20% acrylamide gels, in the presence of 6 and 2 M urea, is used. Among the currently used methods the proposed procedure is characterized by its very high resolution. Hordein is fractionated, at pH 4.6, into 6 clear and well-resolved fractions moving towards the cathode. In addition 3 to 4 other fractions, in very small quantities, can also be observed. The influence of different factors is studied with the view of obtaining optimum resolution. The protein discs were stained with Coomassie Brillant Blue R 250. The method was used in the investigation of the total hordein from "Winter barley 1337", crops 1963, and its fractions isolated by gel chromatography.

INTRODUCTION

The known analytical methods used for the characterization of hordein (prolamine of barley) require either a large amount of material, much time or special apparatus; furthermore, the resolving power of these methods is not satisfactory. They have been used for lack of better ones. The ultracentrifuge method, used by QVENSEL AND SVEDBERG[1], showed hordein preparations to be homogeneous. Tiselius electrophoresis applied to total hordein by BISERTE AND SCRIBAN[2] showed the presence of five fractions, two of which run together and are defined with difficulty as individual fractions. The quantity necessary for a separate determination in a modern apparatus for Tiselius-type electrophoresis is about 9–12 mg of hordein. The resolving power of paper electrophoresis was found to be much poorer when applied to hordein by KLAUSHOFER et al.[3]. The quantity of the sample necessary for analysis in this case was 1 mg, only three diffuse hordein fractions were found and these were hardly distinguished. The continuous carrier-free electrophoresis by HANNIG used as an analytical method by IVANOV et al.[4] had the same resolution and required the same quantity of the sample as the Tiselius electrophoresis. However, it showed better reproducibility. Fractionation of hordein in the presence of all the other barley proteins was attempted by electrophoresis on starch gel[5] and exhibited a very bad resolution. The method of separation of hordein fractions according to their different

molecular volumes, introduced by MESROB et al.[6] for preparative work, may also be used as an analytical method; however, the method was relatively slow and required more than 10 mg of protein sample.

The method of disc electrophoresis on acrylamide gel, suggested by ORNSTEIN AND DAVIS[7], was mainly developed for water-soluble proteins. An attempt has been made to use this method for the separation of barley proteins[8,9]. The results obtained so far were completely satisfactory, but they mainly cover the water-soluble basic proteins of barley[8] and the mixtures extracted by pyrophosphate buffer and 0.05 M acetic acid[9]. However, both solutions extract many other water-soluble proteins of barley and only some of the lowest molecular hordein components.

The aim of the present work was to develop a rapid analytical method with a high resolving power which can characterize the alcohol-soluble barley protein, defined by OSBORNE[10] as hordein. The disc electrophoresis method of ORNSTEIN AND DAVIS[7], with some modifications owing to the properties of hordein, was chosen. Hordein is only soluble in the presence of urea and in acetic acid at concentrations greater than 0.5 M. Hordein is not readily soluble in acetic acid at lower concentrations; in such cases turbid suspensions are obtained, from which a part of the protein precipitates very quickly. According to our preliminary investigations the solubility of hordein in the more dilute acetic acid solutions is selective, some lower molecular weight fractions being more soluble than the rest of the protein. Disc electrophoresis permits a separation of the hordein fractions, based on the electric charges and molecular volumes (and respectively on molecular weight).

MATERIALS AND METHODS

Acrylamide—pure, (Koch-Light, London).
Bisacrylamide (Fluka, Switzerland).
Tetramethylenediamine (TEMED), synthesized in our laboratory.
Dimethylamino-propionitril (DMAPN) (American Cyanamid Co., U.S.A.).
Urea—reagent grade, crystallized from methanol–water.
Total hordein and its fractions, separated according to molecular weight differences, were obtained by gel chromatography according to MESROB et al.[6].
Coomassie Brillant Blue R 250 was kindly provided by ICI, London.
The other reagents used were of "reagent grade" quality.
The disc electrophoresis procedure, described by ORNSTEIN AND DAVIS[7], was modified as follows.

The apparatus was prepared for use according to the detailed description given by DAVIS[11]. It consists of 6 tubes of 5 mm I.D. and 65 mm in length arranged in a circle around the cylindrical graphite electrodes. Both electrode vessels were placed in such a manner, that the anode vessel was above that of the cathode. It was supplied with direct current by a stabilized electronic amplifier. In accordance with ORNSTEIN's theoretical basis of disc electrophoresis[12], K$^+$ was chosen as a fast cation, while the glycine and the acetate anion were used as the slow cation and counterion respectively. With this ion system and the hordein cation a narrow triple boundary can be achieved. Urea was incorporated in all the gels; the urea concentration for 10% acrylamide gel was 6 M and for an acrylamide concentration of 20%—2 M. The pH values of the solutions was determined directly with a pH-meter, the computation of these values

for solutions containing so much urea being very difficult. The actual pH value for the large-pore gel was 6.3 and for the small-pore gel 4.6. Because of the considerable amount of urea in the gel solutions, DMAPN was added also to the large-pore upper gel, which led to complete polymerization of that gel even in the presence of the protein solution.

The following stock solutions were necessary for the preparation of the different gels (for 7.5% acrylamide in the small-pore gel).

(A)   1 N Potassium hydroxide   48.0 ml
      Glacial acetic acid   17.2 ml
      TEMED   4.0 ml
      Water added to make   100.0 ml
(B)   1 N potassium hydroxide   48.0 ml
      Glacial acetic acid   2.87 ml
      TEMED   0.46 ml
      Water added to make   100.0 ml
(B1) (2 times higher concentration than B)
      2 N potassium hydroxide   24.0 ml
      Glacial acetic acid   2.87 ml
      TEMED   0.46 ml
      Water added to make   50.0 ml
(C)   Acrylamide   30.0 g
      Bis-acrylamide   0.8 g
      Water added to make   100.0 ml
(D)   Acrylamide   10.0 g
      Bis-acrylamide   2.5 g
      Water added to make   100.0 ml
(D1) (2 times higher concentration than D)
      Acrylamide   10.0 g
      Bis-acrylamide   2.5 g
      Water added to make   50.0 ml
(E)   Riboflavin, 4 mg in 100 ml of water. To each 5 ml of this solution 100 μl of DMAPN are added.
(E1) (2 times higher concentration than E)
      4 mg of riboflavin in 50.0 ml of water. To each 2.5 ml of this solution 100 μl DMAPN are added.
Note: The solutions containing DMAPN only have a lifetime of 2–3 days.
      (AP) 28 mg of ammonium persulfate was dissolved in 5 ml of water (this was mixed fresh daily).

The following solution (C1) was also used for the preparation of 20% acrylamide in the small-pore gel:

(C1) Acrylamide   3.4 g
      Bis-acrylamide   7.8 mg
      Water added to make   10.0 ml

Those solutions not already mentioned regarding shelf-life can be stored in brown glass bottles in a refrigerator for more than a month. On account of its low stability it is recommended that the addition of urea to the solutions is done daily.

Preparation of the small-pore gel (7.5%)

2.5 ml of solution A, 5.0 ml of solution C, 7.2 g of urea and 5.0 ml of solution AP were taken and water was added to make 20.0 ml.

0.8 ml of the above mixture was poured into each tube, and a layer of *ca.* 2 mm water was formed over the gel and the solution then polymerized in UV light.

Preparation of the large-pore middle gel (3.5%)

0.50 ml of solution B, 1.0 ml of solution D, 0.5 ml of solution E and 1.44 g urea were mixed with water to make 4.0 ml. 0.2 ml of this mixture was poured into each tube which was then layered with *ca.* 2 mm of water and polymerized in UV light.

Preparation of the large-pore upper gel (3.5%)

0.5 ml of solution B1, 1.0 ml of solution D1, 0.5 ml of solution E1 and 1.44 g of urea mixed with water to make 4.0 ml. 0.1 ml of this solution was poured into each tube and 0.1 ml of 6 M aqueous urea solution containing 0.5 mg of hordein preparation was added; the mixture was then polymerized in UV light.

Preparation of the small-pore gel (20%)

3.5 ml of solution C1, 1.0 ml of solution A and 0.72 g of urea were made up to 6 ml with AP solution. The polymerization was carried out as before and the large-pore middle and upper gels were prepared according to the above description.

Buffer for the electrode vessels consisted of 26.3 g glycine, 8.0 ml glacial acetic acid and water was added to 1000 ml.

The introduction of gel solutions into the apparatus and the polymerization was carried out according to DAVIS[11]. Electrophoresis was carried out at 140 V. At the start the potential was increased till the current density reached 5 mA per tube, but after 10 min a current density of 3 mA per tube was used. The apparatus was kept at room temperature. The considerable thermostability of hordein made additional cooling of the apparatus unnecessary.

The current was passed for 100 min. The gels were removed from the tubes and colored according to CHRAMBACH *et al.*[13]. The gels were placed in test tubes with about 10 ml 12.5% solution of trichloroacetic acid as fixing agent for the protein. After 30 min of fixing, the gels were transferred to a 0.05% solution of Coomassie Brillant Blue R 250 (a 1% aqueous solution of the dye diluted 20 times with a 10% trichloroacetic acid solution) where the gel remains for 60 min. Washing the colored gels with a 10% trichloroacetic acid solution led to well-outlined discs on a clean background. The intensity of the coloring increased in the following 2 days.

The photometric measurements were performed on a Jouan Model E.S.A. C.-5-57 photometer for paper electropherograms, adapted by making an aperture on the carrier table and reducing the slit to 0.4 mm.

RESULTS AND DISCUSSIONS

The possibility of carrying out the disc electrophoresis in 0.5 M and 1 M acetic acid media as in the case of known electrophoretic methods for hordein separation was first examined. This resulted in only two diffuse discs being obtained, a large amount of protein remaining in the upper gel as stationary material. In order to
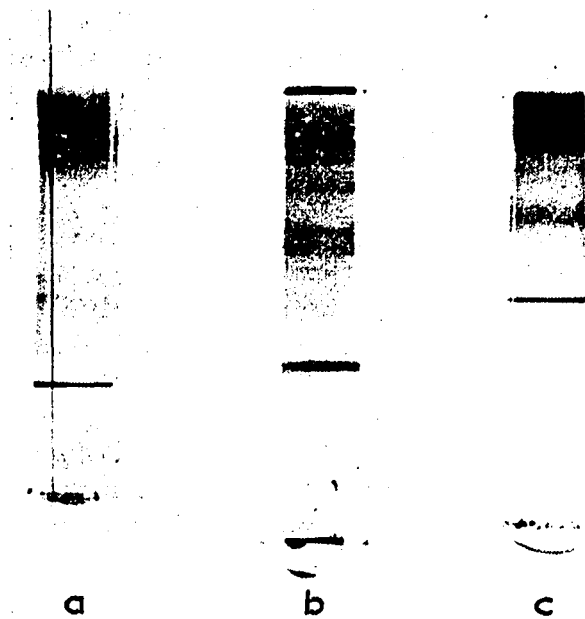
Fig. 1. Total hordein resolved in a 7.5% acrylamide gel in the presence of urea. All the other data are as given for the standard method of electrophoresis. The samples were separated at the following concentrations of urea: a = 8 M; b = 6 M; c = 4 M.

achieve a good separation of hordein into its fractions, electrophoresis in the presence of urea was attempted. The effect of different concentrations of urea in the gel was studied; 6 and 8 M concentrations of urea (Fig. 1) were found to be most suitable.

Since the density of the gel influenced the fractionation of the protein into
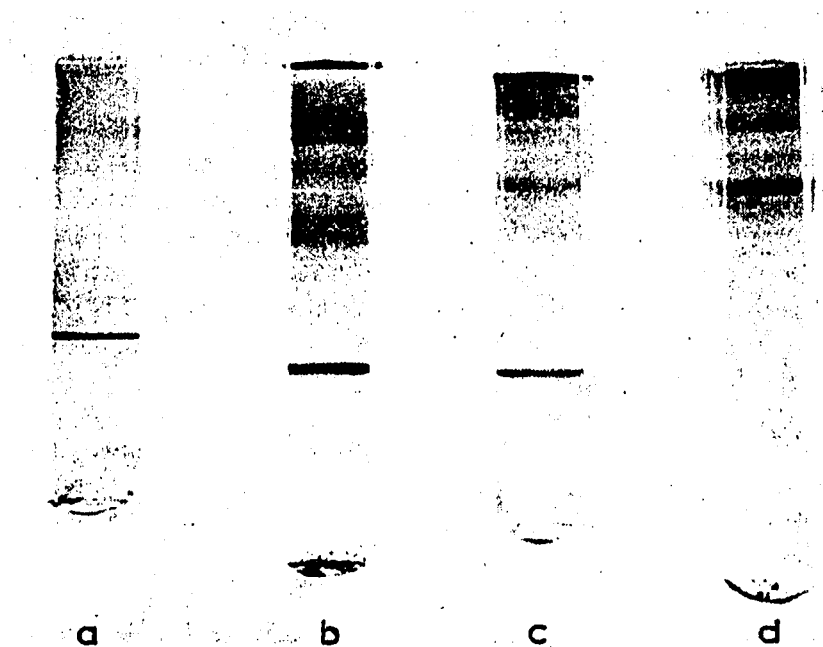

Fig. 2. Total hordein resolved by the described standard disc electrophoresis with 6 M urea, the concentration of the acrylamide in the small-pore gel being: a = 5%; b = 7.5%; c = 10%; d = 20% acrylamide with 2 M of urea.

components differing in molecular volume, the disc electrophoresis was investigated at different concentrations of acrylamide in the lower gel. The choice of different concentrations was limited, because the polymerization of acrylamide became worse in the presence of 6 M urea in the gel solution. With a concentration of more than 10% acrylamide, the gels were soft and unstable. 20% acrylamide in the lower gel was achieved by use of 2 M of urea. Disc electropherograms of hordein at different percentages of acrylamide in the small-pore gel are shown in Fig. 2.

The effect of current density and duration was studied maintaining the other conditions standard.

It was shown, that the optimal time for electrophoresis was 100 min. During this time the fastest fractions moved up to 35–40 mm. In the first 20 min the protein hardly reached the boundary of the lower gel.

At a higher current density (5 mA per tube) distorted discs were obtained. At 3 mA per tube the electrophoresis proceeds without increasing the temperature and yields good discs. The influence of the pH values of the different gels on the running and the separation of the hordein fractions was also investigated. At pH values of 8.4 for the lower gel and 10.3 for the upper gel, the mobility of the hordein fractions was negligible. Only two strong diffuse discs moved 5 mm towards the anode in 200 min. At a pH of 6.6 in the lower gel (8.3 in the upper gel) the movement was only 7–8 mm towards the cathode in 100 min. In this case the discs were also very diffuse.

The internal resistance of the small-pore gel exerts a sharp separation influence on the components of the hordein mixture, because of the large intervals of their molecular weights (and respectively their molecular volumes) from 15,200 to 350,000[14]. As can be seen from Fig. 2 the slow fractions were separated better in the gel with 5% acrylamide than in the gel with 10%. On the other hand, the fastest fractions were separated by a higher density in the small pore gel. Thus a gel with 7.5% acrylamide was chosen as the standard resolution method for total hordein. Study, however, of the purity of the different hordein fractions gives us grounds for recommending the use of 5% and 7.5% gels for the slow components of the hordein and 10% and 20% for the fast ones; especially in the case of fraction 4, where the most suitable gel was 20% acrylamide, but the buffer system should contain only 2 moles of urea (see Fig. 3). This is possible, because of the comparatively high solubility of this fraction in a 2 M urea solution.

It can be seen that under these experimental conditions the hordein fractions were, in general, separated more slowly. An attempt was made to accelerate the electrophoresis by increasing the current density. This, however, led to distortion of the discs as a result of increased temperature. An optimum value for the current density was established at 3 mA per tube for the buffer system employed and the standard conditions of electrophoresis.

In order to obtain good separation, the duration of electrophoresis should be 100 min. With some hordein preparations, especially when taken in larger quantities, complications were noted in the normal polymerization of the upper large-pore gel. In order to avoid this difficulty the use of Sephadex G-25 instead of acrylamide gel as an anticonvectional medium in the upper space of the tube was investigated. This was accomplished by introducing dry Sephadex G-25 into the protein solution being studied. The dextran swelled instantly and filled the liquid volume with a gel of sufficient stability. The results from this method of introducing the protein sample
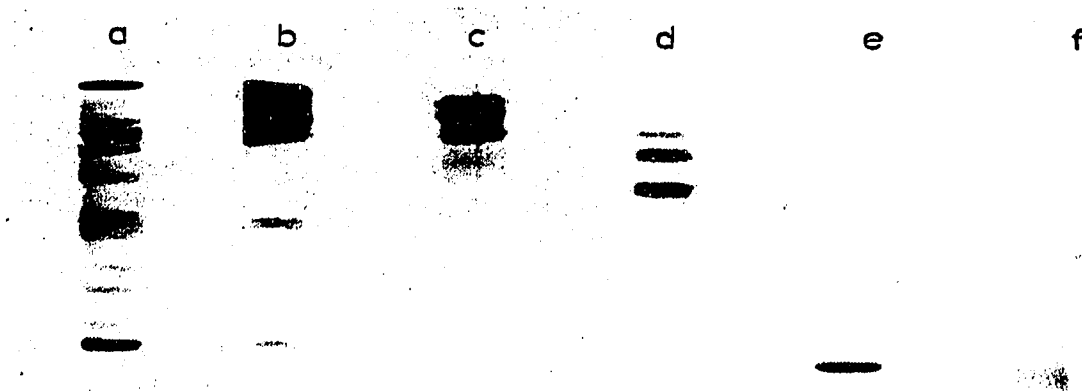
Fig. 3. Disc electrophoretic pattern according to the standard conditions: a = total hordein from "Winter barley 1337" crops 1963. Patterns due to fractions of the same hordein obtained by gel chromatography: b = fraction 1; c = fraction 2; d = fraction 3; e = fraction 4; f = fraction 4, resolved on 20% gel with 2 M of urea, for 220 min electrophoresis.

were similar to those obtained by the standard procedure. This method, however, allowed larger volumes of protein solutions to be employed.

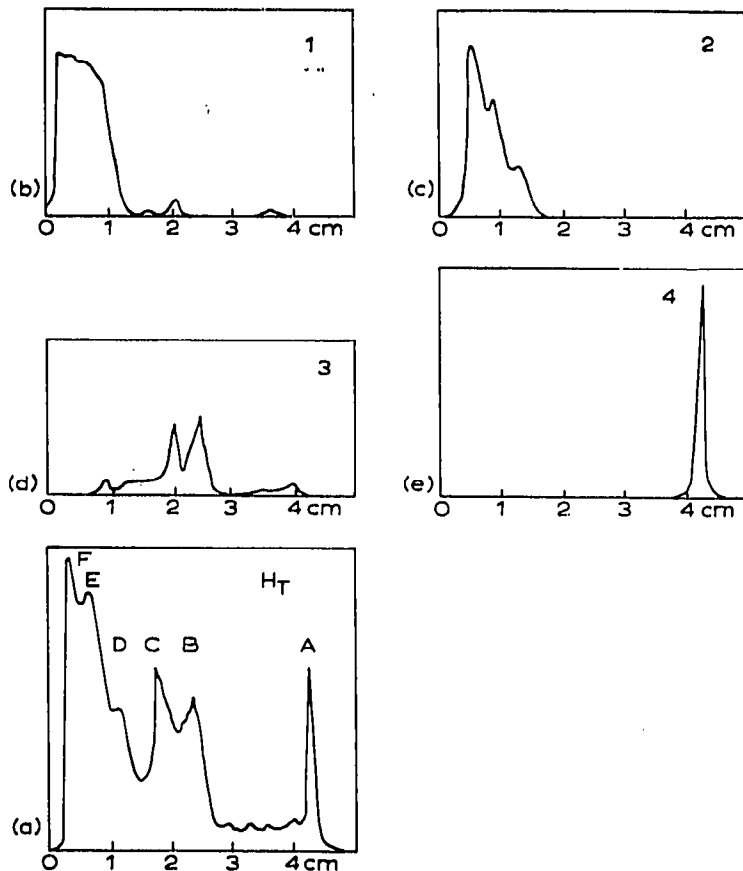The optimum quantity of protein for resolution per tube was found to be 500 μg

Fig. 4. Photometric scans of the disc electrophoretic separations given in Fig. 3. a–e as in Fig. 3; A–F cf. Table I.

of the total hordein; for the different hordein fractions an amount of 200–250 μg was quite sufficient. Satisfactory fractionation of the total hordein was established with even 300 μg to 1 mg.

The composition of total hordein isolated from "Winter barley 1337", crops 1963, by IVANOV et al.[4] was subjected to the described electrophoretic method of characterization. It was found to consist of 10 electrophoretically different fractions (Fig. 3).

It can be seen that hordein was resolved in 6 main fractions (A to F) and 4 secondary ones. Fraction C differed from the others in its strong red-violet color. Fractions E and F were grey-blue, and all others were greenish-blue. Fractions (1–4) also belonging to the same hordein, obtained by gel chromatography on Sephadex G-200[6], were separated by the same method. It can be seen from Fig. 3, that the fractions 1, 2 and 3 were not homogeneous but mixtures of the hordein fractions A–F in different ratios. These fractions were separated only to a certain degree. Thus, fraction 1 represented a protein zone with very little electrophoretic mobility and contained traces of A and B. Fraction 2 represented a mixture of D, E and F electrophoretic fractions. The mobility of these three fractions was very similar. Fraction 3 contained mainly B and C, and fraction 4 contained only the fraction A. The presence of the lower molecular weight fractions in the higher molecular weight ones is quite probably due to contamination in the gel chromatographic separation.

With a view to determining the amounts of individual fractions present in the total hordein, the discs obtained were photoscanned (Fig. 4). The disadvantage of this method of determination is the uncertainity of whether the different fractions were equally colored. The amounts of the different hordein fractions based on photometric determination are given in Table I.

TABLE I

PERCENTAGE OF THE DIFFERENT DISC ELECTROPHORETIC FRACTIONS IN TOTAL HORDEIN AND IN INDIVIDUAL HORDEIN FRACTIONS

Isolated on Sephadex G-200; amounts based on planimetry of curves given in Fig. 4.

Hordein preparation	Electrophoretic fraction					
	A	B	C	D	E	F
Total hordein	9.6	22.4	18.3	16.0	14.2	19.4
Fraction 1	1.2	1.8	slow moving material 97.0			
Fraction 2			12	13	66	9
Fraction 3	2	49	38	11		
Fraction 4	100					

The results obtained indicate that this method permits an electrophoretic analysis of hordein which gives better resolution than other known analytical methods. This procedure gives well-differentiated fractions combined with good reproducibility. The least amount of material necessary for the electrophoretic examination is 500 μg, the individual fractions requiring even less. The method does not require an expensive apparatus. The use of the Coomassie Blue dyeing procedure decreased the duration of the determination and increased the sensitivity of the method.

The Amidoschwarz 10B coloring technique leads to the dissolution of a considerable part of the protein discs because of the good solubility of hordein in acetic acid solutions. In trichloroacetic acid, however, hordein precipitates and the discs are clearly outlined.

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