STARCH-GEL ELECTROPHORESIS OF WHEAT FLOUR PROTEINS

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[*Manuscript received November* 22, 1962]

Summary

An improved apparatus and procedures for starch-gel electrophoresis of proteins of wheat flour are described; highly reproducible separation of the protein components was achieved. By starch-gel electrophoresis it was shown that similar protein components occur in the extracts of wheat flour obtained with a variety of solvents; however, there were marked differences in the proportions of these components in various extracts. Several protein components were present in the fractions separated by ion-exchange chromatography of the proteins soluble in sodium pyrophosphate and of those soluble in acetic acid; some fractions contained a number of similar protein components.

With standardized procedures it was found that there are considerable differences in the protein components of different wheat flours. Varieties of similar genotype had similar protein components; the most marked differences between flours of different genotype occur among the slow-moving protein components.

I. INTRODUCTION

The classification of wheat proteins proposed by Osborne (1907) has remained without any major modification as the basis for their extraction and subsequent separation. Pence and Mecham (1962) have summarized the results of many recent investigations on wheat proteins with ion-exchange chromatography and electrophoresis. Starch-gel electrophoresis has already proved to be effective for the separation of wheat proteins (Elton and Ewart 1960, 1962; Coulson and Sim 1961; Woychik, Boundy, and Dimler 1961). The classes of wheat proteins as defined by Osborne (1907) and their fractionation by ion-exchange chromatography (Coates and Simmonds 1961; Simmonds and Winzor 1961; Simmonds 1962) have now been examined by starch-gel electrophoresis.

II. MATERIALS AND METHODS

(*a*) *Flour Samples*

Samples of Australian wheats were milled to approximately 70% extraction on a Buhler laboratory mill. Flour obtained from *Triticum vulgare* cv. Gabo, grown at the Waite Institute in 1959, was used for the extraction and fractionation studies.

(*b*) *Extraction Procedure*

In general the extraction procedure was that described by Coates and Simmonds (1961), except that 10-g samples of flour were extracted with 20 ml solvent. For the initial extraction in water-saturated n-butanol, the flour was homogenized with 20 ml n-butanol for 2 min at 14,000 r.p.m. in an M.S.E. top-drive homogenizer.

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The mixture was then transferred to a 50-ml polypropylene centrifuge tube. In all other extraction steps the flour residue was resuspended and stirred by hand for 5 min, then the mixture in the centrifuge tube was agitated for 10 min on a "Microid" flask-shaker (Griffin and George, Ltd.). After centrifuging at 6000 *g* on a Lourdes "Versa-fuge" for 5 min the supernatant was decanted. All extractions were carried out at 2°C.

The standard procedure was a single extraction in water-saturated n-butanol followed by four extractions in 0·01M sodium pyrophosphate (pH 7·4) and four extractions in 0·05N acetic acid (pH 3·5). To examine the extraction of proteins by other solvents, samples were also extracted with water prior to pyrophosphate extraction, and with a 70% (v/v) ethanol–water mixture before acetic acid extractions. In these experiments, to obtain complete extraction of the proteins soluble in each solvent, the residue was extracted six times with each solvent except that a single extraction with n-butanol was used. Nitrogen determinations by a microKjeldahl procedure were made on the supernatant after each individual extraction and also on the bulked extracts. The ethanol extract was exhaustively dialysed against distilled water, and all the extracts were freeze-dried. The dried samples were resuspended in 2M urea to give a final concentration of 2·5 mg protein nitrogen per millilitre.

(*c*) *Fractionation by Ion-exchange Chromatography*

The proteins soluble in pyrophosphate were separated by chromatography on diethylaminoethyl (DEAE)-cellulose (Coates and Simmonds 1961; Simmonds 1962) and those soluble in acetic acid by chromatography on carboxymethyl (CM)-cellulose (Simmonds and Winzor 1961). For each fractionation three columns (1·8 by 15 cm) loaded with 100 ml of protein extract (40–80 mg N), were eluted simultaneously with buffer pumped from a common reservoir by a bank of micro-pumps. The contents of the tubes comprising each of the eight fractions obtained from the DEAE-columns (Simmonds 1962) and each of the 10 fractions separated on the CM-columns (Simmonds and Winzor 1961) were combined and freeze-dried.

Since the dried material eluted from the CM-cellulose column could only be suspended satisfactorily in 2·0M urea, this solvent was used for resuspending each of the fractions from the DEAE- and CM-cellulose columns. The material of each fraction was then separately dialysed for 48 hr against water, which was changed frequently, thus removing salts and non-protein nitrogen. After dialysis, the volume of the solution was measured, a sample was removed for determination of the nitrogen content, and the solution was freeze-dried. The material was then finally suspended in 2·0M urea to give a concentration of 2·5 mg protein nitrogen per millilitre.

(*d*) *Starch-gel Electrophoresis*

(i) *Apparatus.*—The starch-gel trays were constructed according to Smithies' (1955) specifications but with certain modifications.* To provide efficient cooling, the "Perspex" base was replaced by a hollow brass plate (2 cm thick) through which water from an ice-water bath could be circulated. To provide complete electrical

* Units of this design are made by Paton Industries, Ltd., Glen Osmond, S. Aust.

insulation, the brass plate was covered by "Melinex" (I.C.I.A.N.Z. Ltd.) polyester film (0·003 in. thick) which offered a negligible barrier to heat transfer yet proved to be very durable. Adhesion of the "Melinex" film to the brass plate was achieved with "Bostik 1768" adhesive (B.B. Chemical Co. Aust. Pty. Ltd.). The units were also provided with a second brass plate, similarly insulated, which could be placed over the top surface of the gel if further cooling was required.

Platinum electrodes, soldered to small brass rods set in removable "Perspex" plugs, were inserted at each end of the tray. Thus the voltage could be conveniently measured throughout electrophoresis. Plate 1, Figure 1, is a photograph of the apparatus.

Electrophoresis was carried out with the gel in the vertical position. Electrical connections between the gel and the bridge solution were made with "Wettex" (cellulose sponge); two pieces of "Wettex" were clamped to each end of the gel by the removable end sections of the tray and the free ends of the "Wettex" dipped into the bridge solution (aluminium lactate buffer). The apparatus in use is shown in Plate 1, Figure 2.

Direct current was supplied from a stabilized power unit (Paton Industries, Ltd.) capable of delivering 300–2000 V and 200 mA. As the current output for each gel was only 40 mA, by using a potential divider it was possible to apply the required voltage to two gels simultaneously from one power supply unit.

(ii) *Preparation of Gel.*—A stock solution of 0·017M aluminium lactate buffer of ionic strength 0·1 (Jones, Taylor, and Senti 1959) was prepared by activating 5·47 g aluminium foil as described by Elton and Ewart (1962), and placing this in a 2-l. beaker containing 38·5 ml water. This was allowed to dissolve overnight and then filtered through "Hyflo Supercel" (Johns-Mansville Corp., California). The filtrate was made up to 10 l. and adjusted to pH 3·2 with lactic acid. The conductivity of the buffer was checked on a Wheatstone bridge.

A starch gel was prepared by mixing in a 3-l. round-bottom "Pyrex" flask 84 g hydrolysed starch (Connaught Medical Research Laboratories, Toronto, Canada) with 700 ml of aluminium lactate buffer at pH 3·2 containing 2M urea. The gel was heated according to Smithies (1955); the mixture was vigorously swirled over a Meker bunsen flame and then boiled under vacuum for 30 sec. After pouring, any small air bubbles were rapidly removed by suction through a Pasteur pipette.

After cooling, the lid was removed and a small drop of 2% $HgCl_2$ solution was added to each slot to inhibit β-amylase activity (Elton and Ewart 1962). Electrophoresis was carried out either at 10 V/cm for $5\frac{1}{4}$ hr or at 12 V/cm for 10 hr. Gels were sliced horizontally with a skin grafting knife blade and were stained by continuous shaking for about 12 hr. Stock solutions of stains were prepared by dissolving either 250 mg of nigrosine (B.D.H. Ltd.) or 250 mg each of nigrosine and naphthalene black 10 B (Gurr Ltd.) in 2 l. of solution consisting of 966 ml methanol, 172 ml glacial acetic acid, and 862 ml water. The stained gels were washed and photographed as described by Elton and Ewart (1962). When stained with naphthalene black 10 B as described by Smithies (1955), slow-moving protein components of wheat faded rapidly, whereas when stained with nigrosine as above, a permanent pattern was obtained.

III. Results

To compare those proteins which are extracted by different solvents, samples of flour were extracted with water, with dilute salt solution, with 70% ethanol, and with dilute acid. The amount of nitrogen extracted into each solvent is given in Table 1. Samples of the extracts IC, IIC, IIIB, IIIC, IVD, IIID, IIIE, and IE (Table 1) were separated by starch-gel electrophoresis and the results are shown in Plate 2, Figure 1.

Table 1

EXTRACTION OF WHEAT FLOUR PROTEINS BY DIFFERENT SOLVENTS

10 g flour (*Triticum vulgare* cv. Gabo) containing 190·2 mg nitrogen was extracted with 20 ml of each of the following solvents: A, water-saturated n-butanol (once); B, distilled water (six times); C, 0·01M sodium pyrophosphate, pH 7·4 (six times); D, 70% ethanol (v/v) (six times); E, 0·05N acetic acid, pH 3·5 (six times). The results give the total nitrogen extracted by each solvent as a percentage of the total nitrogen of the flour; values are means of four determinations. The variation between determinations was within the range ± 5%

Expt. No.	Percentage of Total Nitrogen of Flour Extracted by the Successive Solvents Indicated:				
	A	B	C	D	E
I			12·6		49·3
II	0·3		9·1		46·7
III		10·7	4·9	8·7	39·3
IV		10·7		22·8	28·2

As shown in Table 1, extraction with water-saturated n-butanol results in a decrease in the amount of protein subsequently extracted by pyrophosphate and acetic acid. However, electrophoretic patterns (Plate 2, Fig. 1) of the pyrophosphate extracts show that the initial butanol treatment has no effect on the number of components extracted into pyrophosphate. As compared with the pyrophosphate extract, the water extract contains a larger proportion of the slower-moving components and the fastest-moving components are absent. Treatment of the water-extracted residue with pyrophosphate results in the extraction of all of the components present in the original pyrophosphate extract (Plate 2, Fig. 1, column 1); there is an increased concentration of the fastest-moving components. Electrophoretic patterns of the ethanol- and acetic acid-soluble proteins are similar, and differ from those of the water- and pyrophosphate-soluble proteins in the absence of certain of the faster-moving components and the presence of a greater proportion of the slow-moving components. It is evident that no new components are present to distinguish these proteins which are soluble in ethanol and acetic acid from those which are extracted into either water or dilute salt solution.

The proteins soluble in pyrophosphate and acetic acid were fractionated on DEAE-cellulose (Simmonds 1962) and on CM-cellulose (Simmonds and Winzor 1961) respectively. Each of the fractions separated in this way was examined by starch-gel electrophoresis and the results are shown in Plate 2, Figure 2, and Plate 3, Figure 1. Fraction K (Simmonds 1962) and Fraction 10 (Simmonds and Winzor 1961) could not be examined by starch-gel electrophoresis, as these are eluted by 0·1N NaOH, and therefore these fractions were omitted (see Section IV). The results show that the fractions separated by ion-exchange chromatography are not homogeneous and that similar electrophoretic components are present in several of the chromatographic fractions. The pyrophosphate fractions A, D, and E were examined by electrophoresis at 12 V/cm for 10 hr and the results are shown in Plate 3, Figure 2. The presence of each of the slow-moving components of the acetic acid extract is shown in the pyrophosphate fractions A, D, and E. Electrophoresis of the pyrophosphate extract before chromatographic fractionation does not show all of these components as they are present in low concentration in comparison to the fast-moving components. In addition, it is seen that the distribution of the slow-moving components in the DEAE-cellulose fractions is different from that in the fractions obtained from chromatography of the acetic acid-soluble proteins on CM-cellulose (Plate 3, Fig. 1).

The application of starch-gel electrophoresis to the separation of proteins of different wheat varieties was also examined. Plate 4, Figures 1 and 2, shows the patterns of the pyrophosphate- and acetic acid-soluble proteins from flours of *Triticum vulgare* cv. Beacon, Olympic, Gabo, Koda, Mengavi, Spica, and Free Gallipoli, and of *T. durum* cv. Dural. The most marked differences occur in the slow-moving components of the acetic acid-soluble proteins. As shown in Plate 4, Figure 2, there is similarity in the patterns obtained for varities of similar genotype such as Beacon and Olympic (columns 1 and 2), and Gabo, Koda, and Mengavi (columns 3, 4, and 5).

IV. Discussion

No attempt has been made to number the protein components separated by starch-gel electrophoresis as it has been found that the number of components increases with an increase in concentration of either urea or lactate buffer. Furthermore electrophoresis of the acetic acid-soluble proteins at 12 V/cm for 10 hr results in an increase in the number of components than can be obtained under the standard electrophoresis conditions of 10 V/cm for $5\frac{1}{4}$ hr. The conditions described in the present investigation were adopted as a standard procedure because they enabled good separations for comparative studies and also provided a gel which was easily prepared and of a good texture to handle. The addition of urea to aluminium lactate buffer improved the resolution of the components; contrary to Coulson and Sim (1961), reduction of the starch content of the gel to 10% resulted in decreased resolution of the components. Initially, considerable difficulty was encountered in separating the acetic acid-soluble proteins; this was due to the high concentration of protein nitrogen in the samples which resulted in streaking of the slow-moving components, and this defect was overcome by loading smaller quantities of protein.

Coulson and Sim (1961) have also reported the presence of pyrophosphate-soluble components in the acetic acid extract and they attributed this to incomplete extraction. In the results reported here (Plate 2, Fig. 1) precautions were taken to carry out an exhaustive extraction with each solvent; nitrogen determinations on the individual extracts showed that negligible protein was extracted after the fourth extraction in either pyrophosphate or water, and that in each case the first extract of the subsequent solvent contained a large proportion of the total nitrogen extracted by that solvent.

The presence of both slow- and fast-moving components in all extracts examined cannot be attributed to any particular treatment; similar patterns were obtained when the extracts were concentrated by filtration through dialysis tubing under vacuum instead of by freeze-drying. Moreover, reproducible patterns were obtained with samples which had been stored for over 12 months at 2°C in 2M urea containing a small quantity of thymol to prevent bacterial contamination. As the acetic acid extracts after freeze-drying could not be completely redispersed in either water or aluminium lactate buffer, 2M urea was used to resuspend all the freeze-dried samples. Identical components were obtained with both pyrophosphate and acetic acid extracts when these were redispersed in either water or aluminium lactate buffer, and hence the use of 2M urea to resuspend the freeze-dried protein did not modify the electrophoretic patterns.

The results with fractions obtained by ion-exchange chromatography of the pyrophosphate and acetic acid extracts provide good evidence that the electrophoretic components are not produced by modification of the proteins during electrophoresis. The electrophoretic patterns of these fractions show a separation of slow- and fast-moving components, and the number of components present varies according to the type of protein applied. This is evident from the patterns obtained not only with protein fractions separated by ion-exchange chromatography (Plate 3, Fig. 2) but also with different wheat varieties (Plate 4, Figures 1 and 2). Individual protein components which were isolated after separation by starch-gel electrophoresis re-ran as single components with the same mobilities (Graham, unpublished data). This provides additional evidence against the existence of an equilibrium of slow- and fast-moving components due to protein dissociation during electrophoresis.

Osborne's classification (1907), based on relative solubility of proteins in different solvents, has been the basis of much of the subsequent chemical and physical studies on wheat proteins. As the classes of proteins described by Osborne (1907) differ so markedly in their solubility characteristics, this has led to the belief that they are constituted of very different proteins. However, the results described here show that similar protein components occur in extracts obtained with a wide variety of solvents. Nevertheless, there is a much greater proportion of slow-moving protein components in the acetic acid-soluble as compared with the salt-soluble proteins. As yet, the proteins soluble in 0·1N NaOH have not been examined by starch-gel electrophoresis as no resolution into components is obtained with these extracts, even after exhaustive dialysis to remove all sodium hydroxide. It appears that

certain conditions can modify some of the proteins which are normally soluble in pyrophosphate and acetic acid, thereby rendering them soluble only in 0·1N NaOH (Graham, Morton, and Simmonds 1963).

Electrophoresis of those fractions, separated from the pyrophosphate- and acetic acid-soluble proteins by ion-exchange chromatography, show that these fractions are not homogeneous. It would appear that each chromatographic fraction is made up of a complex of protein components. Differences in the chromatographic elution patterns have been reported for flours obtained from different wheat varieties (Simmonds and Winzor 1961; Simmonds 1962). It is now suggested that these differences can be much more rapidly and clearly defined by starch-gel electrophoresis.

Plate 4, Figures 1 and 2, represents a survey of the protein compositions of different wheat varieties. It is clear that there are similar protein components in varieties of similar genotype. Between different genotypes the most marked differences occur in the slow-moving components. A single treatment of flour in 0·05N acetic acid gives an extract which gives a representative electrophoretic pattern showing all the protein components. This is useful for comparative studies. Further development will involve procedures for complete extraction of all protein components and their quantitative estimation after separation by electrophoresis.

V. Acknowledgments

Milling of the flour samples was carried out by the Bread Research Institute of Australia, Sydney.

The author wishes to thank Professor R. K. Morton for his valuable suggestions and interest in this work, and also Miss M. Ryder and Mr. D. Morton for their technical assistance.

Grateful acknowledgment is made of financial support from the Wheat Industry Research Fund.

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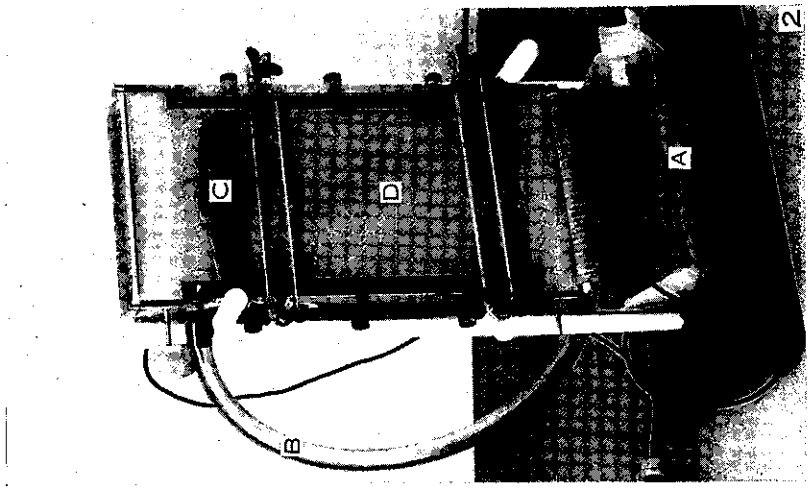

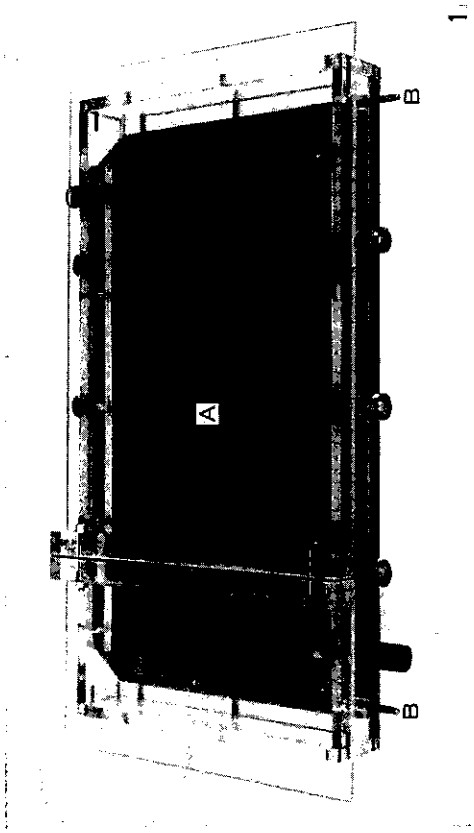

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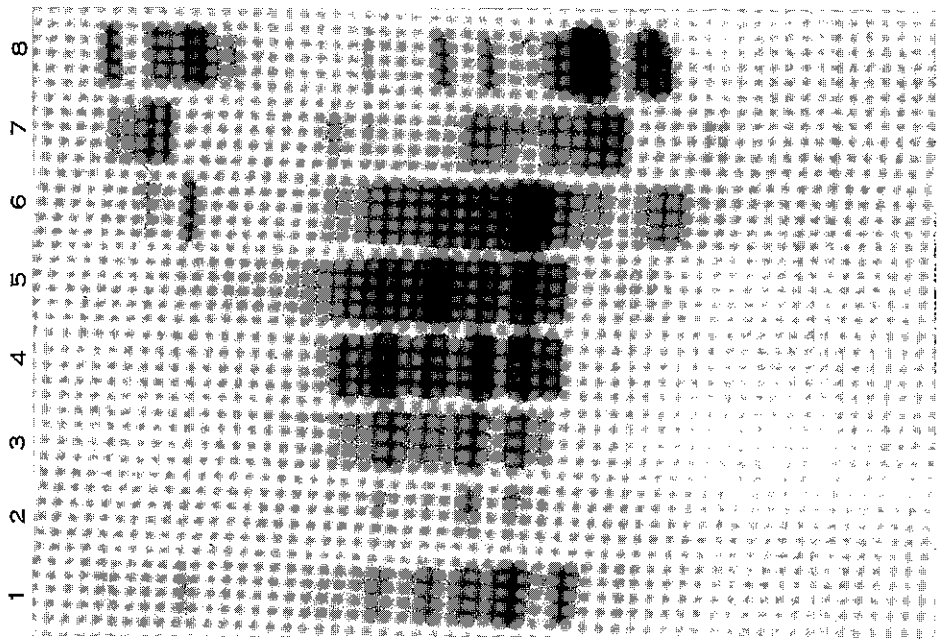

Fig. 2

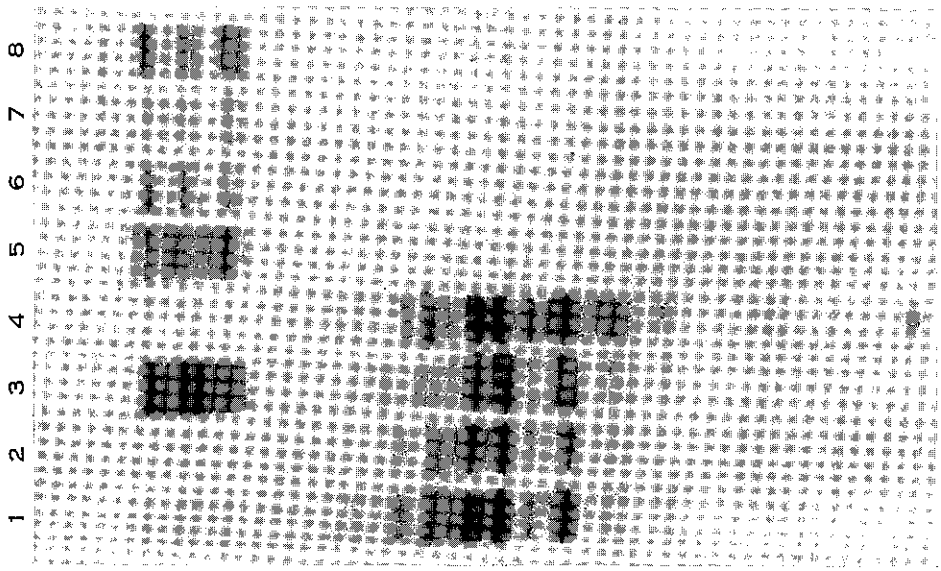

Fig. 1

STARCH-GEL ELECTROPHORESIS OF WHEAT FLOUR PROTEINS

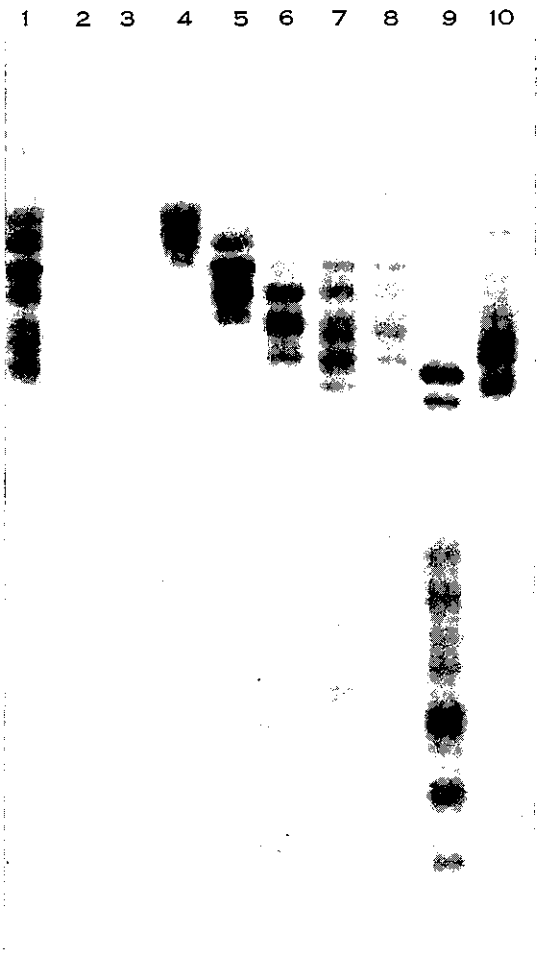

Fig. 1

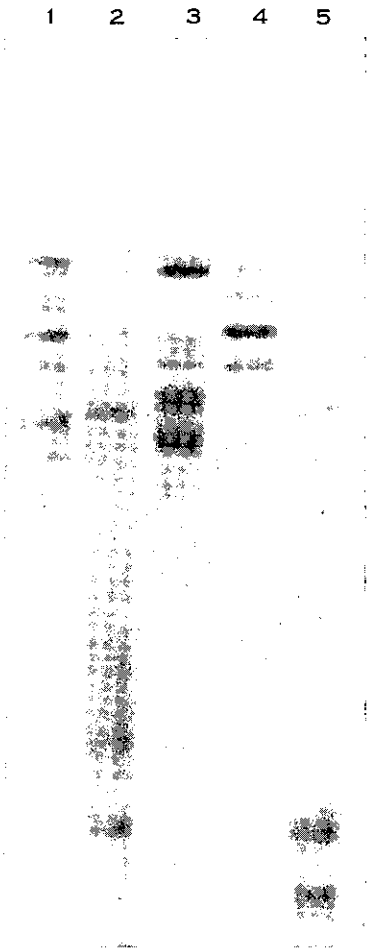

Fig. 2

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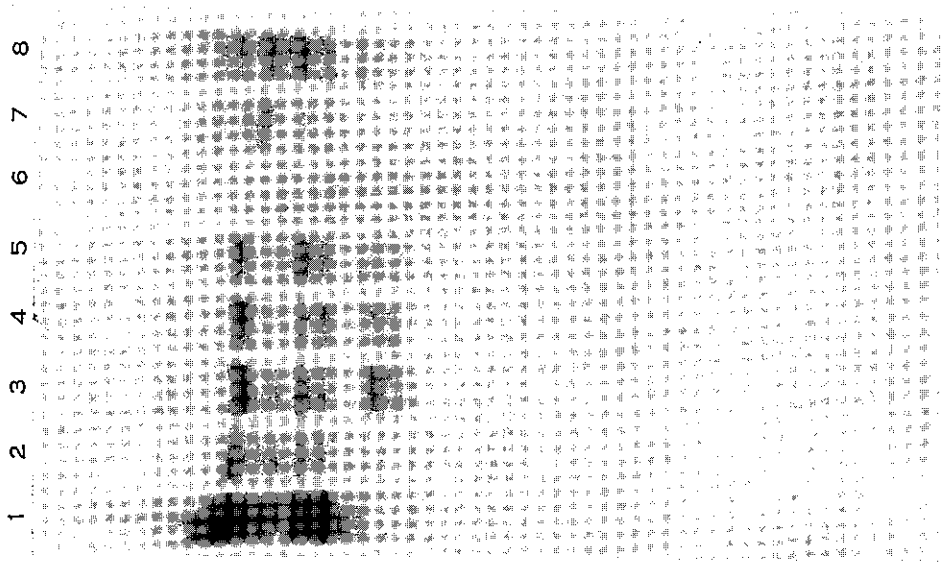

Fig. 2

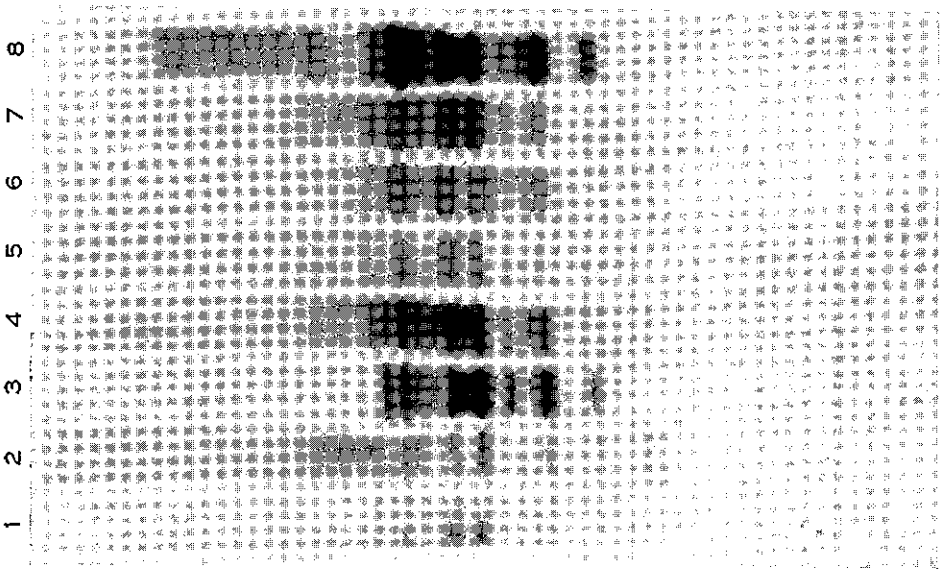

Fig. 1

Explanation of Plates 1–4

Plate 1

Fig. 1.—Apparatus for starch-gel electrophoresis: *A*, brass cooling plate; *B*, platinum electrode.

Fig. 2.—Apparatus for vertical starch-gel electrophoresis in use: *A*, container for bridge solution; *B*, outlet for coolant; *C*, "Vaseline" covering slots containing samples; *D*, polythene sheet covering gel.

Plate 2

Fig. 1.—Protein components of extracts from flour of *T. vulgare* cv. Gabo as separated by vertical starch-gel electrophoresis. The gel containing 12% starch was prepared in 0·017M aluminium lactate buffer (ionic strength 0·1) and 2M urea. Samples (0·1 ml) containing 2·5 mg protein nitrogen were resuspended in 1 ml 2M urea. Electrophoresis was at 10 V/cm for 5¼ hr. Gel was stained with 0·0125% nigrosine for 12 hr. Columns *1–8* are the flour proteins extracted as shown below (see also Table 1). *1*, 0·01M sodium pyrophosphate, pH 7·4 (IC); *2*, 0·01M sodium pyrophosphate, pH 7·4, after previous extraction in n-butanol (IIC); *3*, water (IIIB); *4*, 0·01M sodium pyrophosphate, pH 7·4, after previous extraction in water (IIIC); *5*, 70% ethanol (v/v) after previous extraction in water (IVD); *6*, 70% ethanol (v/v) after previous extraction in (i) water and (ii) 0·01M sodium pyrophosphate, pH 7·4 (IIID); *7*, 0·05N acetic acid, pH 3·5, after previous extraction in (i) water, (ii) 0·01M sodium pyrophosphate, pH 7·4, and (iii) 70% ethanol (v/v) (IIIE); *8*, 0·05N acetic acid, pH 3·5, after previous extraction in 0·01M sodium pyrophosphate, pH 7·4 (IE).

Fig. 2.—Protein components of chromatographic fractions of proteins from flour of *T. vulgare* cv. Gabo which are soluble in 0·01M sodium pyrophosphate (pH 7·4). Conditions for starch-gel electrophoresis were as for Plate 2, Figure 1. Pyrophosphate-soluble proteins were separated into eight fractions by ion-exchange column chromatography on DEAE-cellulose (Simmonds 1962). Columns *2–8* are the protein components present in seven of the chromatographic fractions as follows: *1*, 0·01M sodium pyrophosphate, pH 7·4, before chromatographic fractionation; *2*, fraction J; *3*, fraction F; *4*, fraction H; *5*, fraction G; *6*, fraction E; *7*, fraction D; *8*, fraction A.

Plate 3

Fig. 1.—Protein components of chromatographic fractions of proteins from flour of *T. vulgare* cv. Gabo which are soluble in 0·05N acetic acid (pH 3·5). Conditions for starch-gel electrophoresis were as for Plate 2, Figure 1, except that electrophoresis was at 12 V/cm for 10 hr. Acetic acid-soluble proteins were separated into 10 fractions by ion-exchange chromatography on CM-cellulose (Simmonds and Winzor 1961). Columns *2–10* are the protein components present in nine of the chromatographic fractions as follows: *1*, 0·05N acetic acid, pH 3·5, before chromatographic fractionation; *2*, fraction 1; *3*, fraction 2; *4*, fraction 3; *5*, fraction 4; *6*, fraction 5; *7*, fraction 6; *8*, fraction 7; *9*, fraction 8; *10*, fraction 9.

Fig. 2.—Protein components extracted from flour of *T. vulgare* cv. Gabo. Conditions for starch-gel electrophoresis were as for Plate 3, Figure 1. Sodium pyrophosphate-soluble proteins were fractionated by ion-exchange chromatography on DEAE-cellulose (Simmonds 1962). Columns *1–5* are the protein components present in the extracts or chromatographic fractions as follows: *1*, 0·05N acetic acid extract, pH 3·5; *2*, 0·01M sodium pyrophosphate extract, pH 7·4; *3*, fraction A; *4*, fraction D; *5*, fraction E.

Plate 4

Fig. 1.—Protein components of sodium pyrophosphate extracts of flours of several wheat varieties. Conditions for starch-gel electrophoresis were as for Plate 2, Figure 1. Columns *1–7*, *T. vulgare* cv. Beacon (*1*), Olympic (*2*), Gabo (*3*), Koda (*4*), Mengavi (*5*), Spica (*6*), Free Gallipoli (*7*). Column *8*, *T. durum* cv. Dural.

Fig. 2.—Protein components of acetic acid extracts of flours of different wheat varieties. Conditions for starch-gel electrophoresis were as for Plate 3, Figure 1. Columns *1–8* as for Plate 4, Figure 1.