PROTEINS OF WHEAT AND FLOUR. THE SEPARATION AND PURIFICATION OF THE PYROPHOSPHATE-SOLUBLE PROTEINS OF WHEAT FLOUR BY CHROMATOGRAPHY ON DEAE-CELLULOSE

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ABSTRACT

Extraction of mature wheat flour with 0.01M sodium pyrophosphate at pH 7 yields a solution containing the albumin and globulin protein components, together with pentosan-protein and nucleoprotein fractions. Chromatography of this solution on diethylaminoethyl-cellulose (DEAE-cellulose) has now been improved to yield eight subfractions. Three of these peaks, D, E, and F, eluted by a gradient of increasing sodium chloride concentration, have been rechromatographed on a large scale on DEAE-cellulose to yield symmetrical elution patterns. When purified, these components also gave symmetrical electrophoretic patterns showing only small amounts of cross-contamination. Determination of the amino acid residues occupying the N-terminal position by Sanger's DNP-technique showed that peaks D and E contained serine only and peak F contained serine and threonine as N-terminal amino acid residues. A comparison of the elution patterns from DEAE-cellulose of the pyrophosphate extracts of ten different flours showed a marked difference in the relative amounts of the various components present.

A previous paper (4) described methods which had been developed for the extraction from mature wheat flour of the sodium pyrophosphate-soluble proteins and their separation by chromatography on DEAE-cellulose. The present paper describes improvements in the separations obtained by introducing slight modifications to these methods. Three of the components, peaks D, E, and F, have been prepared on a large scale and rechromatographed to a state of chromatographic and electrophoretic homogeneity. The homogeneity of these preparations has also been examined by determination of their N-terminal amino acid residue. Comparative separations have been conducted on the pyrophosphate extracts of ten flour samples of different types.

Materials

The flours used in this study were selected from those investigated by Bell and Simmonds (2). Flours 1–4 correspond to samples C4266,
TABLE IV

Properties of Products Obtained by "Percolation" and "Dry" Reactions

<table>
<thead>
<tr>
<th>Reaction Conditions</th>
<th>Intrinsic Viscosity</th>
<th>Clarity of 1% Paste</th>
<th>Alkali Number</th>
<th>Brookfield Viscometer Data *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dt./g.</td>
<td>%</td>
<td></td>
<td>Viscosity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-Hour</td>
</tr>
<tr>
<td>Percolation reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry reaction,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dry starch</td>
<td>0.72</td>
<td>75</td>
<td>17.0</td>
<td>440</td>
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<tr>
<td>Dry reaction,</td>
<td></td>
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<tr>
<td>undried starch</td>
<td>0.56</td>
<td>75</td>
<td>25.7</td>
<td>675</td>
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</table>

* Concentration, 10 g./100 ml.

b Ratio of 24-hour value to 1-hour value.

Data for the “dry” reaction products indicate that predrying of starting material results in a product having higher intrinsic viscosity, lower alkali number, and higher setback than when the starting material contains equilibrium moisture. The differences in intrinsic viscosity and alkali number indicate that predrying the starch results in a larger polymer and increases resistance to alkali. The latter property is probably due to more complete conversion of aldehyde groups to acetals.

Amylograph curves indicated that these three products commenced pasting at 69°C. The “percolation” product reached maximum viscosity in 8 minutes; both “dry” products reached maximum viscosity in 12 minutes.

Acknowledgment

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Literature Cited

C4251, C6330, and C4740 respectively in that paper. Flour D6391 was milled from a Gabo-Mentana-Kenya crossbred wheat grown at Winton, northwest Queensland; flour D5177, Dural, was a durum-type wheat grown at the Waite Agricultural Research Institute, South Australia. Full details of their total nitrogen content, percentage pyrophosphate-soluble nitrogen, and baking behavior are reported in Table I of that paper. Diethylaminoethyl-(DEAE-) cellulose was obtained from Eastman Kodak Co., Rochester, New York.

**Experimental Methods and Results**

Nitrogen was estimated by a micro-Kjeldahl procedure.

*Extraction of Pyrophosphate-Soluble Flour Proteins and Their Chromatography on DEAE-Cellulose.* The procedure described by Coates and Simmonds (4) was followed throughout except that all buffer solutions were pumped through the chromatography columns by means of a bank of micro-pumps (16). In the experiments comparing the protein composition of different flour samples, six chromatography columns (1.8 × 15 cm.) were set up simultaneously around two fraction collectors, each column receiving buffer via a separate pump from a common reservoir. A gradient of increasing salt concentration was then introduced into this reservoir (3 liters in volume) by connecting it to a second adjacent reservoir of similar capacity filled with the limit buffer. In this way the salt gradient was varied in an identical manner for all six columns simultaneously, and inter-comparison of the effluent patterns became meaningful. A plot of effluent absorbance at 280 m\(\mu\) against fraction number for six of the ten flours examined in this manner is shown in Fig. 1.

An attempt was made to compare the relative proportions of the various peaks present in six of the flours examined, by summing the absorbance of each peak and expressing this as a percentage of the total absorbance. These results are summarized in Table I and show

<table>
<thead>
<tr>
<th>FLOUR No.</th>
<th>PEAK A</th>
<th>PEAK D</th>
<th>PEAK E</th>
<th>PEAK F</th>
<th>PEAK J</th>
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<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
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<td>25.8</td>
<td>21.7</td>
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<tr>
<td>2</td>
<td>19.1</td>
<td>11.8</td>
<td>27.2</td>
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<td>3.5</td>
</tr>
<tr>
<td>3</td>
<td>16.4</td>
<td>8.5</td>
<td>28.2</td>
<td>20.5</td>
<td>3.8</td>
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<td>4</td>
<td>16.8</td>
<td>10.3</td>
<td>29.8</td>
<td>27.19</td>
<td>2.1</td>
</tr>
<tr>
<td>5</td>
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<td>9.1</td>
<td>33.0</td>
<td>27.3</td>
<td>4.6</td>
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<tr>
<td>6</td>
<td>19.3</td>
<td>9.6</td>
<td>26.9</td>
<td>20.9</td>
<td>4.2</td>
</tr>
</tbody>
</table>

**TABLE I**

PROTEIN COMPOSITION OF B.R.I. FLOURS

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Fig. 1. Chromatography of pyrophosphate extracts of six different flour types on DEAE-cellulose. Column dimensions: 1.8 x 15 cm. Column loading: 22-28 mg of protein nitrogen contained in 150 ml of original pyrophosphate extract. Columns and samples were equilibrated against 0.006M glycine buffer at pH 9.5. Gradient to 0.3M sodium chloride in 0.006M glycine buffer of pH 9.5 started at arrow marked NaCl; 0.05M acetic acid and 0.1M NaOH introduced at arrows marked AcH and NaOH respectively. For details or flour samples loaded see "Materials" section. Temperature: 15°C.
that the main difference between different flours lies in the relative proportions of peak D present. Unfortunately, the accuracy of this method is limited by the incomplete separations obtained between those components emerging in the salt gradient.

Relative proportions of peaks A, D, E, F and J present in pyrophosphate extracts of flours numbers C4266 (flour 1), C4251 (flour 2), C6330 (flour 3), C4740 (flour 4), C4747 (flour 5), and C4753 (flour 6) are shown in Table I. For further details of the physical, chemical and baking characteristics of these flours, see Bell and Simmonds (2). The amount of each peak present is expressed as a percentage of the total amount recovered from the column.

**Large-Scale Preparation of Peaks D, E, and F:**

1. Extraction of pyrophosphate-soluble proteins from flour samples C4266 (Tichborne ex Sydney University) and D5178 (Gabo ex Waite Agricultural Research Institute). Portions of each flour weighing 500 g. were blended with 1000 ml. of water-saturated n-butanol. After 20 minutes' stirring the suspension was centrifuged and the supernatant decanted. The residue was extracted twice in each case with 1,500 ml. of 0.01M sodium pyrophosphate of pH 7.0. The two extracts were combined. The solution containing 1.4 g. of nitrogen was dialyzed against six changes of 0.006M glycine buffer of pH 9.5 for 4 days at 4°C. The final volume of dialyzed solution in each case was approximately three liters and its pH was 9.0. Each dialyzed extract was centrifuged for 20 minutes at 2,000 r.p.m. to remove a small amount of precipitated protein.

2. Chromatography of extracts on DEAE-cellulose. The prepared solutions were loaded onto DEAE-cellulose columns 6.5 cm. in diameter and 28 cm. high, previously equilibrated at pH 9.5 with 0.006M glycine, and set up in the cold room at 4°C. In the case of flour C4266, peak A was collected into a beaker; for separation of the remaining peaks, a fraction collector was used and 20-ml. fractions were collected. In the case of flour D5178, the outlet from the chromatography column was arranged to flow continuously through a short length of silica tubing (1 cm. diameter, 4 cm. high) set up in the light path of a Uvispek spectrophotometer (Hilger and Watts Ltd., London, England). Absorbance measurements at 280 mμ were taken at intervals of time varying from 15 minutes to 3 hours, depending on the rate of change of absorbance of the effluent. The spectrophotometer was set up outside the cold room and the outlet from the flow cell was led into a flask cooled in ice water. The flask was changed at the points noted on the effluent diagram of Fig. 2. After approximately 7 liters of 0.006M glycine had passed through the column, peak A had been completely
removed. The gradient to 0.3M sodium chloride in 0.006M glycine was then started (Fig. 3: arrow 1) with 17 liters of 0.006M glycine in the mixing reservoir and 20 liters of 0.3M sodium chloride in 0.006M glycine in the limit reservoir. The pH throughout was 9.5. At arrows 2 and 3 the eluting solutions were changed to 0.05N acetic acid and to 0.1N sodium hydroxide respectively. Both flours gave essentially the same elution pattern, that from the Gabo flour (D5178) being shown in Fig. 2.

![Flow diagram](image)

**EFFLUENT VOLUME - LITERS**

Fig. 2. Effluent pattern obtained from large-scale separation of pyrophosphate-extracted proteins of Gabo flour D5178. Column: DEAE-cellulose, 6.5 cm. x 28 cm., equilibrated at pH 9.5 with 0.006M glycine. For loading and eluant changes, refer to text.

Fractions were collected as indicated in Fig. 2. The combined solutions were freeze-dried to yield light fluffy powders containing varying amounts of glycine and sodium chloride.

3. Rechromatography of peak D on DEAE-cellulose. Freeze-dried peak D was dissolved in 50 ml. 0.006M glycine (pH 9.5) and dialyzed overnight against the same buffer. It was then loaded onto a column of DEAE-cellulose 3.2 cm. in diameter and 21 cm. high, equilibrated with 0.006M glycine in the usual manner. The column was washed with 500 ml. of 0.006M glycine before the gradient to 0.3M sodium chloride was started with 1 liter of each buffer in the mixing and in the limit reservoirs. Fractions 15–20 ml. in volume were collected by means of a fraction collector operated every 10 minutes. The chromatography was carried out at room temperature (about 15°C.). Figure 3, A, shows the effluent pattern obtained. The contents of the tubes from the section marked D were combined and freeze-dried and the residue was dissolved in 25 ml. of 0.006M glycine (pH 9.5) and rechro-
matographed on a column of DEAE-cellulose (1.8 × 15 cm.) to give the symmetrical elution pattern shown in Fig. 3, B.

4. Rechromatography of peak E on DEAE-cellulose. Freeze-dried peak E from the large-scale experiment of Fig. 2 was mixed with the small amount of peak E material recovered from the rechromatography of peak D (Fig. 3, A, tubes 33–43). The mixture was dialyzed for 2 days against two changes of 0.006M glycine buffer and chromatographed under the usual conditions on a 3.2 × 18 cm. column of DEAE-cellulose. Tubes 87–117 of the slightly unsymmetrical effluent curve shown in Fig. 4, F, were combined and freeze-dried. A similar but
somewhat more symmetrical elution pattern was obtained from the first rechromatography of peak E from flour C4266.

Since end group determinations (see later) showed only one N-terminal amino acid to be present in this preparation of peak E, it was not considered necessary to submit it to a second rechromatography.

5. Rechromatography of peak F on DEAE-cellulose. Freeze-dried peak F from the large-scale separation of flour D5178 gave a symmetrical electrophoretic pattern (Fig. 5, C) and was not rechromatographed.

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Fig. 5. Electrophoresis of purified fractions from flours C4266 and D5178. Buffer: glycine-sodium hydroxide, ionic strength 0.1, pH 9.5; ascending limb on left, descending limb on right. A, peak D, flour C4266 after 3.5 hours; field strength 3.78 volts/cm.; pH 9.42. B, peak E, flour C4266 after 2.75 hours; field strength 3.79 volts/cm.; pH 9.16. C, peak F, flour D5178 after 1.5 hour; field strength 4.03 volts/cm.; pH 9.16. D, peak F, flour C4266 after 4.25 hours; field strength 3.90 volts/cm.; pH 9.11.
However, preparation of peak F from flour C4266 gave a nonsymmetrical pattern on electrophoresis and it was accordingly rechromatographed on a 3.2 × 27 cm. column of DEAE-cellulose in a manner similar to that already described. A single but rather skew elution peak was obtained (Fig. 4, F) which still yielded two peaks on electrophoresis (Fig. 5, D). This material also appeared to be heterogeneous when subjected to qualitative identification of its N-terminal residue.

Homogeneity of Peaks D, E, and F. 1. Electrophoresis studies. Moving-boundary electrophoresis was carried out using a Perkin Elmer apparatus (Model 38A) according to the methods described by Coates and Simmonds (4). Figure 5 shows the patterns obtained in both ascending and descending limbs for the final purified samples of peaks D, E, and F, together with the mobilities calculated for each electrophoretic peak. In the case of peak F prepared from flour C4266, two peaks were obtained (Fig. 5, D); peak F, prepared from flour D5178, gave a single symmetrical peak (Fig. 5, C).

2. Qualitative identification of N-terminal amino acid residues. The N-terminal amino acid residues contained in peaks D, E, and F were examined by the DNP-method of Sanger (15) and co-workers. Prior to reaction with fluorodinitrobenzene, the freeze-dried proteins were dissolved in water and dialyzed against several changes of distilled water until the diffusate gave a negative test for free glycine with ninhydrin. The protein solution was then freeze-dried and 10-mg. portions of the residue were reacted with fluorodinitrobenzene according to procedure 1 described by Fraenkel-Conrat, Harris, and Levy (7). After hydrolysis with 0.6 ml. constant-boiling hydrochloric acid at 105°C, for 8 hours, the free DNP-amino acids were extracted with ether. The ether extracts were evaporated to dryness, and the residue transferred to a sheet of Whatman No. 1 filter paper, 17 in. × 22.5 in. Chromatography in toluene:pyridine:2-chloroethanol:ammonia, followed by 1.5M phosphate buffer of pH 6 (3), was used to separate the DNP-derivatives.

Peaks D and E yielded, in addition to yellow areas corresponding to dinitrophenol and dinitro aniline, a single spot identified as dinitrophenyl serine. The picture given by peak F, prepared both from flours C4266 and D5178, showed the presence of approximately equivalent amounts of DNP-serine and DNP-threonine, together with lesser quantities of DNP-aspartic acid and DNP-glutamic acid, DNP-leucine or DNP-methionine, together with DNP-alanine, di-DNP-lysine and di-DNP-tyrosine also appeared to be present in trace amounts.

The relevant chromatograms are shown in Fig. 6.
Discussion

The effluent curves of Fig. 1 show a clear resolution of five components by the sodium chloride gradient. This confirms the earlier recognition by moving-boundary electrophoretic studies of five com-
ponents in this group (4). The improved resolution compared with earlier work (4) appears to be due to the higher temperature at which the present chromatographic separations were conducted, and also to the use of pumps which have enabled a smooth gradient to be maintained throughout. Denaturation of the proteins at this higher temperature does not seem to be serious, since the height of peak K eluted by 0.1N sodium hydroxide does not seem to have significantly altered.

Flours D6391 and D5177 clearly differ from the remaining flours in Fig. 1. The chromatographic separations on these two flours were carried out at a different time and it was felt that any quantitative assessment of the relative proportions of the peaks present could not therefore be compared with the results from the flour listed in Table I, all of which were treated simultaneously and had the same fraction size. Although these flours have a very high total nitrogen content, and fit into the normal nitrogen distribution pattern described by Bell and Simmonds (2), they exhibit very poor baking behavior. This is more probably related to differences in the gluten or acetic acid-extractable proteins (17) in these flours than to those proteins discussed here. However, it would be of considerable interest to see whether any, or all, of the proteins A to H would have a deleterious effect on the baking behavior if added back to a normal flour.

This approach has been utilized by Finney (6), Pence and co-workers (9–11) and others (1,14) in attempt to assess the effect of various flour constituents on baking quality. Pence, Elder, and Mecham (10) reported that addition of total buffer solubles to gluten-starch doughs resulted in an increase in loaf volume in all except the durum flour examined. In later work Pence, Weinstein, and Mecham (12) developed a method for determining the ratio of the albumin to globulin proteins present in the buffer-soluble fraction, and explored the possible relationship between the composition of this fraction and the baking quality of the flour (13). The ratio of albumin to globulin was found to be correlated with baking quality beyond the 1% significance level, the albumin fraction appearing to improve baking performance whereas the globulin fraction affected it adversely. However, in these experiments, individual protein components were not isolated and added back to the flours. The chromatographic method here described allows this possibility for the first time.

Peaks D, E, and F have now been obtained in a state approaching chromatographic and moving-boundary electrophoretic homogeneity. In addition, peaks D and E give a single amino acid, serine, as their
N-terminal residue, making it probable that the two major components of the buffer-soluble proteins of normal flours have been obtained in a reasonable state of homogeneity.

It is, however, possible that when more sensitive methods of separation, such as starch-gel electrophoresis, are applied to these preparations, additional contaminating components may be revealed.

Acknowledgments

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Literature Cited