THE EXTRACTION AND ION-EXCHANGE CHROMATOGRAPHY OF BUFFER-SOLUBLE AND GLUTEN PROTEINS OF WHEAT FLOUR

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ABSTRACT

Methods for the extraction and chromatographic separation of wheat flour proteins have been developed as part of a comprehensive study of cereal proteins. Proteins were extracted by treating flour successively with four portions of pyrophosphate-ethylenediaminetetraacetate buffer (at pH 7.0) followed by four portions of 0.01N formic acid. This extraction procedure solubilizes 95% of the total flour nitrogen. The buffer extracts were fractionated on carboxymethyl (CM) cellulose at pH 5.5. Much of the buffer-soluble protein is not adsorbed on CM cellulose under these conditions. This unadsorbed protein was fractionated on diethylaminoethyl (DEAE) cellulose at pH 7.0. The gluten proteins were fractionated on CM cellulose at pH 4.0 with eluant solutions which contained the protein dispersant dimethylformamide. Six varieties of vulgare wheat and two varieties of durum wheat have been studied with these techniques. The vulgare wheat protein chromatograms are characterized by a marked intervarietal similarity, although there are intervarietal differences in the total amounts of buffer-soluble and gluten proteins. On the other hand, one buffer-soluble protein which is prominent in vulgare wheats was not found in durum wheats.

The literature of cereal chemistry contains numerous reports of the extraction and separation of wheat flour proteins. A procedure of successive extraction of flour with buffer and dilute acetic acid was described by Coates and Simmonds (1) in 1961. This procedure provides for the separation of wheat proteins into two main fractions, buffer-soluble and acid-soluble or gluten proteins. The separation of the individual components of these two main fractions was carried out by means of ion-exchange chromatography (1,2) on columns of substituted cellulose. There have also been a number of other reports of the fractionation of wheat proteins by means of ion-exchange chromatography.

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The chromatography of proteins soluble in either water or buffer has been carried out under a variety of conditions (1,3–6). On the other hand, the chromatography of gluten proteins has been restricted to acidic conditions (2,7,8) because of the limited solubility of these proteins. In each of the studies cited only one set of chromatographic conditions has been used for the fractionation of either major group of proteins. This is a serious limitation for chromatography of the buffer- or water-soluble proteins, because these proteins are quite heterogeneous in their acid-base character. Therefore, during chromatography at near neutral pH conditions (3,6), a significant portion of the protein is not adsorbed onto a single ion-exchange column and is thus not fractionated in a useful way. At more extreme pH conditions (1,4,5) a larger portion of the total protein is adsorbed onto a single column. However, extreme pH conditions are generally unfavorable, since ion-exchange fractionation of proteins is usually assumed to be most efficient at pH values close to the protein isoelectric points (2) where charge differences between individual proteins in a mixture are likely to be greatest. Extreme pH conditions may also contribute to enzyme inactivation.

The present investigation was therefore undertaken with the intention of improving the previous methods of wheat protein fractionation. The major deficiencies of the previous fractionations of buffer-soluble proteins have been overcome by conducting the chromatography of crude buffer extracts of wheat flour on carboxymethyl (CM) cellulose, a cation exchanger, at pH 5.5. Protein not adsorbed onto the ion-exchange column under these conditions has been fractionated on diethylaminoethyl (DEAE) cellulose, an anion exchanger, at pH 7.0. A number of other improvements in the extraction and fractionation of both buffer-soluble and gluten proteins are also described. Some of the protein fractions isolated have been partially identified on the basis of enzymatic activity and carbohydrate content. Finally, varieties of wheat grown in Canada and not previously examined by these techniques have been utilized in the present study.

For clarity of presentation the paper has been divided into three parts. Part I describes the extraction of protein from the flour; Part II the chromatography of buffer-soluble proteins; and Part III the chromatography of the gluten proteins. A general discussion of the whole investigation follows Part III.

**Materials**

The wheats used in this study were selected to include varieties commonly grown in western Canada as well as varieties of vulgare
wheat displaying a wide range of baking performance. The vulgare wheats are represented by Pembina, Thatcher, Prairie Pride, RL 2520, Kenhi, and Lemhi; durums are represented by Stewart and Mindum. Pembina, Thatcher, Prairie Pride, and RL 2520 are hard red spring wheats. Pembina and Thatcher have high baking quality and are licensed for commercial production in Canada. Prairie Pride and RL 2520 have low baking quality and are not licensed for commercial production. The soft white spring wheats Kenhi and Lemhi and the spring-habit durums Stewart and Mindum are grown commercially in Canada. Samples of all the varieties except Mindum were milled into flour in this laboratory. The Mindum sample was milled into semolina. The milled products were stored in a cold room at 2°–3°C. The chemicals were of reagent grade unless otherwise noted.

Part I
Extraction of Flour Proteins

Methods

The protein extraction was conducted in a cold room at 2°–3°C.; flour and solutions were conditioned to that temperature. Buffer-soluble proteins were extracted with a solution which contained 0.0100M sodium pyrophosphate plus 0.00250M disodium ethylenediaminetetraacetate (EDTA) and was adjusted to pH 7.0 with hydrochloric acid. Toluene (0.1 ml./liter) was added as a preservative.

Flour (75 g., 14% moisture basis) was mixed in a Waring Blender under nitrogen for 5 min. with 210 ml. of the sodium pyrophosphate solution. The resulting slurry was centrifuged in the cold at approximately 8,000 × g for 15 min.; the supernatant was collected and the residue mixed as before with another portion of the pyrophosphate buffer. The flour was thus subjected to four successive extractions with 210, 135, 130, and 125 ml. respectively of pyrophosphate buffer. The four extracts were combined and the nitrogen content was determined by the Kjeldahl method.

After the fourth pyrophosphate extraction the residue was extracted similarly with four successive portions (210, 135, 130, and 125 ml.) of 0.0100M formic acid. The nitrogen content of the combined formic acid extracts was also determined. A few drops of octanol were added to each extraction mixture to prevent excessive foaming.

A thorough dialysis to equilibrate the protein extract with starting buffer is an essential preliminary step in all ion-exchange chromatography experiments. In some early experiments it was noted that a considerable portion of the buffer-soluble nitrogen is dialyzable. Therefore, the losses of nitrogen from several extracts during dialysis have
been measured. For this purpose portions of the extracts were dialyzed exhaustively in the cold against 0.05M acetic acid. This operation was carried out in a rocking dialyzer which provides for agitation of the sample and the passage of about 20 liters of dialyzing fluid in 20 hr. Acetic acid solution was used instead of distilled water to keep the gluten proteins in solution. The nitrogen content of each extract was determined by the Kjeldahl method before and after dialysis.

Results and Discussion

The extraction of nitrogenous material from the eight wheats is summarized in Table I. In this table and throughout the paper, protein is taken as Kjeldahl \( N \times 5.7 \). The amount of protein in the extracts has been calculated on both a flour basis and a total protein basis to facilitate interpretation of the results.

**TABLE I**

**Extraction of Buffer-Soluble and Formic Acid-Soluble Proteins from Wheat Flour**

<table>
<thead>
<tr>
<th>Flour</th>
<th>Flour Protein</th>
<th>Pyrophosphate-Soluble Protein</th>
<th>Formic Acid-Soluble Protein</th>
<th>Total Protein Extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Pembina</td>
<td>14.1</td>
<td>14.9</td>
<td>2.11</td>
<td>79.5</td>
</tr>
<tr>
<td>Thatcher</td>
<td>14.3</td>
<td>15.9</td>
<td>2.27</td>
<td>77.2</td>
</tr>
<tr>
<td>Prairie Pride</td>
<td>12.7</td>
<td>19.4</td>
<td>2.48</td>
<td>74.8</td>
</tr>
<tr>
<td>RL 2520</td>
<td>14.9</td>
<td>20.5</td>
<td>2.24</td>
<td>79.5</td>
</tr>
<tr>
<td>Lemhi</td>
<td>11.0</td>
<td>18.3</td>
<td>2.01</td>
<td>74.5</td>
</tr>
<tr>
<td>Kenhi</td>
<td>11.7</td>
<td>20.3</td>
<td>2.38</td>
<td>73.9</td>
</tr>
<tr>
<td>Stewart</td>
<td>13.4</td>
<td>15.9</td>
<td>2.12</td>
<td>79.4</td>
</tr>
<tr>
<td>Mindum</td>
<td>14.2</td>
<td>15.3</td>
<td>2.17</td>
<td>75.0</td>
</tr>
</tbody>
</table>

The present extraction procedure differs slightly from the procedure of Coates and Simmonds (1). Ethylenediaminetetraacetate (EDTA) has been included in the pyrophosphate buffer to augment the chelating properties of the pyrophosphate (9) and thereby deal with the possible metal contamination resulting from the use of the Waring Blendor. Formic acid has been used in place of acetic acid since Cunningham, Geddes, and Anderson (10) have shown it to be a somewhat more effective dispersant than acetic acid for a wide range of cereal cohesive proteins. The ratio of the total volume of extractant to flour weight is the same as that used by Coates and Simmonds (1); however, the present use of a larger proportion of the solution in the first extraction should help to reduce mechanical losses arising from a high concentration of protein in the first extract. These latter two factors
could account for the approximately 10% higher extraction of flour nitrogen in the present work as compared with the results of Coates and Simmonds (1).

Bell and Simmonds (11) have shown that over a wide range of flour nitrogen content, the amount of buffer-soluble nitrogen is relatively constant. Changes in total flour nitrogen are reflected mainly by changes in the amount of nitrogen soluble in 0.05M formic acid. The results in Table I agree in general with the work of Bell and Simmonds (11), although the range of flour protein content is much smaller.

The recovery of nitrogen in the extracts, relative to total flour nitrogen, was 93–95% for all samples except Mindum, which yielded only 90%. This sample was milled into semolina rather than flour, and the reduced solubility of the protein could be due to the larger particle size of the semolina. The sample was used in this study because the authenticity of the variety of the sample was certain, and also because this variety is the standard for durum quality in Canada.

Table II shows the losses of nitrogen which occurred when the flour extracts were thoroughly dialyzed. These results are included in this paper to help indicate the amount of dialyzable nitrogen and non-dialyzable protein in proper relation to each other. Also, since dialysis is an essential first step in all ion-exchange chromatography experiments, it is important to determine the dialysis loss in order to determine the recovery of protein after chromatography. Furthermore, the losses of dialyzable nitrogen calculated as protein, although as high as 1.4% of flour, have not been reported in any other recent publication of this nature.

**TABLE II**

**Loss of Nitrogen from Flour Extracts during Dialysis**

<table>
<thead>
<tr>
<th>FLOUR</th>
<th>Pyrophosphate Extract</th>
<th>Formic Acid Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extract Nitrogen Basis</td>
<td>Flour Protein Basis*</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Pembina</td>
<td>28</td>
<td>4.2</td>
</tr>
<tr>
<td>Thatcher</td>
<td>30</td>
<td>4.8</td>
</tr>
<tr>
<td>Prairie Pride</td>
<td>30</td>
<td>6.0</td>
</tr>
<tr>
<td>RL 2520</td>
<td>29</td>
<td>4.3</td>
</tr>
<tr>
<td>Lemhi</td>
<td>26</td>
<td>4.6</td>
</tr>
<tr>
<td>Kenhi</td>
<td>27</td>
<td>5.3</td>
</tr>
<tr>
<td>Stewart</td>
<td>31</td>
<td>4.8</td>
</tr>
<tr>
<td>Mindum</td>
<td>34</td>
<td>5.2</td>
</tr>
</tbody>
</table>

*Calculated as Kjeldahl N × 5.7.
Part II
Ion-Exchange Chromatography of Buffer-Soluble Wheat Proteins

Methods

Apparatus. A schematic diagram of the apparatus is shown in Fig. 1. The gradient device consists of four identical cylindrical plastic vessels in hydrostatic equilibrium interconnected by small-bore rubber tubing, and is identical in principle to devices described by Peterson and Sober (12). Tygon tubing (1/16-in. i.d.) carries the buffer from the gradient vessels to the pump and from the pump to the rest of the apparatus. The glass-fiber plugs in the Tygon line provide a slight back-pressure which is necessary to maintain a constant flow rate from the pump and also to prevent the formation of air bubbles in the effluent solution cuvet. The positive-displacement piston-type buffer pump was adjusted to give a flow rate of 1.25 ± 0.005 ml./min. before each experiment. The temperature of the columns was controlled at 30.0°C.

![Diagram](image)

Fig. 1. Schematic diagram of the ion-exchange chromatography apparatus.

A Vanguard Ultraviolet Analyzer equipped with 0.66-cm. quartz cells and a diffraction grating set at 280 m\(\mu\) was used to estimate protein in the column effluent. The recorder chart speed was 0.5 in./hr. The fraction collector was actuated at 20-min. intervals, thereby collecting 25-ml. samples.

Solutions. Sodium malonate and sodium phosphate solutions were used for chromatography on CM and DEAE cellulose respectively. The
malonate solutions were prepared from Matheson-Coleman-Bell disodium malonate and titrated to pH 5.5 with malonic acid solution. The phosphate solutions were prepared from monosodium phosphate and titrated to pH 7.0 with freshly prepared sodium hydroxide solution. All solutions for column chromatography contained $1.00 \times 10^{-4} M$ EDTA and 0.1 ml./liter toluene in addition to other indicated constituents.

Cellulose. Carboxymethyl and DEAE cellulose were obtained from Eastman Kodak Co. and Matheson, Coleman, and Bell respectively. Each sample of new cellulose was washed alternately with 0.1N HCl and 0.1N NaOH at least five times or until no more colored material was leached out. Fine material was removed by decantation after the major portion of the cellulose had settled from a dilute suspension.

In practice the cellulose was used for many experiments, and from time to time portions of new cellulose were added during the regeneration procedure to make up for small losses. After each ion-exchange experiment the column (2 cm. × approximately 35 cm.) was washed with 2 liters of 0.1N NaOH, followed by 0.5 liters of 0.0500M buffer of the same type as that to be used for the next experiment. This treatment provides for removal of residual protein and equilibration of the cellulose to the pH of the next experiment. The cellulose was then withdrawn from the column, diluted to 1 liter with water, and allowed to settle. After the fines were removed by decantation, the suspension was diluted to 0.7 liters with water, and dissolved gases were removed by stirring under vacuum for a few minutes. The degassed, dilute suspension was poured into the column with the aid of a 40-cm. extension tube. The settled cellulose was washed first with 0.25 liters of 0.0500M buffer and finally with 1.7 liters of starting buffer.

Sample Preparation and Chromatography. A portion of the buffer extract equivalent to 450 mg. protein was prepared for column chromatography by thorough dialysis against three changes of 0.00500M disodium malonate starting buffer, followed by centrifugation to remove the small quantity of insoluble material which precipitates out during dialysis. The clear solution was allowed to run into the cellulose column under gravity. When all the protein solution had run into the cellulose, the top of the column was rinsed with starting buffer. The headspace was filled with starting buffer, and the same buffer was pumped through the column until all the unadsorbed protein was removed from the column. The pump intake was then connected to the gradient device and the desired gradient of increasing ionic strength was pumped through the column to elute the adsorbed protein.
TABLE III
COMPOSITION OF GRADIENTS USED FOR THE CHROMATOGRAPHY OF BUFFER-SOLUBLE WHEAT FLOUR PROTEINS

<table>
<thead>
<tr>
<th>Experiment a</th>
<th>Vessel Number b</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Disodium malonate M</td>
<td>0.00500</td>
<td>0.0405 c</td>
<td>0.00500</td>
<td>0.182</td>
<td></td>
</tr>
<tr>
<td>B Monosodium phosphate M</td>
<td>0.00500</td>
<td>0.0500</td>
<td>0.00500</td>
<td>0.600</td>
<td></td>
</tr>
</tbody>
</table>

a Experiment A: pyrophosphate extract on CM cellulose pH 5.5. Experiment B: material from pyrophosphate extract unadsorbed on CM cellulose on DEAE cellulose pH 7.0.

b Vessel numbers are the same as in Fig. 1.

c 0.0500M disodium malonate was used for experiments with Kenhi, Stewart, and Mindum only.

Each vessel of the gradient device (see Fig. 1) contained 500 g. of the solution indicated in Table III. Each charge of gradient solution was degassed by stirring under vacuum before it was poured into the appropriate vessel.

The material unadsorbed on CM cellulose was collected and dialyzed thoroughly against 0.00500M sodium phosphate pH 7.0, then fractionated in the same manner on DEAE cellulose with a gradient of phosphate. The composition of the phosphate gradient is also shown in Table III.

When the buffer in the gradient device was nearly exhausted, the intake of the pump was switched to a supply of 0.1N sodium hydroxide to remove any residual protein from the column.

**Acid Phosphatase Activity.** Acid phosphatase activity was determined by measuring the increase in absorbance due to the liberation of salicylic acid from o-carboxyphenyl phosphate. Additional details of the procedure were described previously (13).

**Amylase Activity.** Amylase activity was determined by allowing the enzyme to react with soluble starch at 20°C. The formation of reducing sugar was measured by the dinitrosalicylic acid method. The amylase assay procedure was the same as that of Tipples and Tkachuk (6), except that the starch digestion time was 5 instead of 3 min.

**Carbohydrate Analyses.** The phenol-sulfuric acid method of Dubois, Gilles, Hamilton, Rebers, and Smith (14) was used to provide an approximate assay of the carbohydrate content of the various fractions. For each test a 0.5-ml. sample of effluent was mixed with 1.5 ml. of 0.66% phenol solution and 5 ml. of conc. sulfuric acid in an 18 × 150-mm. test tube. The absorbance of each tube was measured at 490 mμ with a Coleman Junior colorimeter which was previously adjusted to a blank which contained only phenol and sulfuric acid.

**Results**

Figures 2 and 3 show the chromatograms which were obtained when
Fig. 2. Chromatography of buffer-soluble wheat proteins on CM cellulose. Continuous line, no points: absorbance (O.D.) at 280 μμ, 0.66 cm., of column effluent as recorded by the automatic apparatus. Solid circles: sodium ion concentration, moles/liter. Open circles: effluent pH. Open squares: carbohydrate test absorbance (O.D.); see text for details. Open triangles: phosphatase activity Δ O.D./min.; see text for details.

Note: In this and the following graphs (Figs. 3–7), "O.D." and "absorbance" are used synonymously.
Fig. 8. Chromatography of buffer-soluble wheat proteins on CM cellulose. Continuous line, no points: absorbance at 280 m\textmu, 0.66 cm., of column effluent as recorded by the automatic apparatus. Solid circles: sodium ion concentration, moles/liter. Open circles: effluent pH. Open squares: carbohydrate test absorbance (O.D.); see text for details. Open triangles: phosphatase activity \Delta O.D./min.; see text for details.
the pyrophosphate extracts were fractionated on CM cellulose with a gradient of disodium malonate at pH 5.5.

Figures 4 and 5 show the results of the chromatography of the buffer-soluble proteins which were unadsorbed on CM cellulose, on DEAE cellulose at pH 7.0 with a phosphate gradient.

The gradients, as calculated from the data of Peterson and Sober (12), which were used to elute the proteins, are shown as curves drawn through solid points at the top of each figure. Furthermore, the positions at which the gradients were started in each experiment have been aligned, and the flow rate was virtually the same in all experiments. In this way similarities or differences between the various wheats can be readily observed, and the approximate salt concentration required to elute a particular protein can be determined. The continuous line without plotted points is the continuous trace of the absorbance at 280 m\(\mu\) of the column effluent obtained from the recorder.

The effluent pH is indicated by open circles below the corresponding chromatogram. Also, in Figs. 2, 3, 4, and 5, the results of the carbohydrate tests are indicated by open squares below the corresponding chromatograms. The carbohydrate test results are expressed as absorbance per 0.5 ml. of effluent according to the procedure described under "Methods." Under these conditions 0.1 mg. glucose in 0.5 ml. water gave an absorbance of 0.8.

The results of the phosphatase determinations are indicated by open triangles in Figs. 2 and 3 as the change in absorbance at 300 m\(\mu\) per min. per 0.5 ml. of effluent according to the previously described procedure.

In Figs. 4 and 5, the results of amylase determinations are shown by center dot circles as mg. of maltose liberated by 1.0 ml. of effluent, although normally less than 1 ml. of effluent was used in the actual test.

During the experiment with Thatcher in Fig. 3 the flow rate of the pump decreased and the protein peaks are displaced slightly to the right. Mechanical difficulties made it impossible to record the peak eluted by sodium hydroxide in the RL 2520 experiment in Fig. 3. In the RL 2520 experiment in Fig. 5, the flow rate was increased during the sodium hydroxide elution, and the protein peak is thus much narrower than in other similar experiments.

The recovery of protein during the chromatography of a buffer extract on CM cellulose has been determined. The column effluent was concentrated under vacuum in a rotary evaporator, and the concentrate was subsequently dialyzed against 0.05M acetic acid prior to nitrogen determination. The results are presented in Table IV.
Fig. 4. Chromatography on DEAE cellulose of buffer-soluble wheat proteins not adsorbed on CM cellulose. Continuous line, no points: absorbance at 280 m\(\mu\), 0.66 cm., of column effluent as recorded by the automatic apparatus. Solid squares: phosphate ion concentration, moles/liter. Open squares: carbohydrate test absorbance; see text for details. Open circles: effluent pH. Center dot circles: amylase activity mg. maltose/ml. effluent; see text for details.
Fig. 5. Chromatography on DEAE cellulose of buffer-soluble wheat proteins not adsorbed on CM cellulose. Continuous line, no points: absorbance at 280 m
, 0.66 cm., of column effluent as recorded by the automatic apparatus. Solid squares: phosphate ion concentration, moles/liter. Open squares: carbohydrate test absorbance; see text for details. Open circles: effluent pH. Center dot circles: amylase activity, mg. maltose/ml. effluent; see text for details.
**TABLE IV**

**Recovery of Protein after Chromatography of Buffer Extract on CM Cellulose**

<table>
<thead>
<tr>
<th>Protein</th>
<th>mg.</th>
<th>% original</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss during dialysis</td>
<td>149</td>
<td>33</td>
</tr>
<tr>
<td>Residue after centrifugation of dialyzed sample</td>
<td>30</td>
<td>7</td>
</tr>
<tr>
<td>Not adsorbed plus eluted by gradient</td>
<td>217</td>
<td>48</td>
</tr>
<tr>
<td>Eluted by sodium hydroxide</td>
<td>40</td>
<td>9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>436</td>
<td><strong>97</strong></td>
</tr>
</tbody>
</table>

**Discussion**

The most prominent over-all characteristic of the protein chromatograms, resulting from the fractionation of pyrophosphate extracts on CM cellulose, is the similarity between the six vulgare wheats, Pembina, Prairie Pride, Kenhi, Thatcher, RL 2520, and Lemhi. These chromatograms are shown by the first three experiments in Figs. 2 and 3. There are some small differences in the shape of the unadsorbed protein peaks, and there are some minor peaks eluted soon after the gradients were started which differ from one variety to another. The significance of these differences is questionable, since these peaks represent protein that is extremely lightly bound by the cellulose. Variations in sodium ion content and the pH of the buffers, as well as variations in the cellulose equilibration which are within normal experimental reproducibility, could be expected to give rise to most of these differences. The small peak which occurs at an elution volume of about 250 ml., just prior to the first major peak, may be significant since it occurs after the salt gradient has taken effect. This component, which is most prominent in the soft white spring wheats Kenhi and Lemhi, appears to be much reduced or absent in the durums Stewart and Mindum.

The first major protein peak eluted by the gradient at a volume of 450–600 ml. appears to be partly an artifact. Evidence for this is provided partly by the sharp drop in effluent pH which coincides with this peak and partly by the occurrence of a trace of carbohydrate in the peak. Also, in two experiments, a trace of amylase was found in the vicinity of the leading edge of this peak. The latter result is not recorded in the figures. The association of artifact fractionations with variations in effluent pH during ion-exchange chromatography has already been discussed thoroughly (13). The presence of carbohydrate and the amylase in this peak seems to indicate that it is similar to material which is unadsorbed, since a considerable quantity of carbohydrate and amylase is found in the unadsorbed peak when it is fractionated on DEAE cellulose. This first adsorbed peak usually takes the
form of a double peak composed of a leading edge spike and a normally eluted peak. This is most obvious in the Kenhi experiment in Fig. 2. The present evidence indicates that the leading edge and spike peaks are more or less artifacts similar in composition to the unadsorbed material; whereas the trailing edge represents a protein eluted in the normal way. Other data recorded in Figs. 2 and 3 show that the protein eluted normally contains acid phosphatase activity. This is the major acid phosphatase component, but there also appear to be some other relatively minor components eluted later as shown by the Prairie Pride experiment in Fig. 2.

The most significant result of the chromatography of the buffer-solubles on CM cellulose seems to be the absence in the durum wheats Stewart and Mindem of the major component eluted at approximately 900 ml. in experiments with vulgare wheats. This point has been thoroughly investigated, and this component was not found in additional authentic samples of the North American durum varieties Ramsey and Carleton, or in the middle eastern variety known as PI 94701.

After chromatography of the pyrophosphate extract on CM cellulose, the unadsorbed proteins were fractionated on DEAE cellulose as shown in Figs. 4 and 5. These proteins contain most of the wheat amylase. The adsorption of amylase-active wheat protein onto DEAE cellulose under similar conditions of near neutral pH has been observed in this Laboratory by Tipples and Tkachuk (6); and also by Nimmo, O'Sullivan, Mohammad, and Pence (3). The present work indicates that wheat amylase is the most acidic of all the pyrophosphate-soluble proteins. The results of Tipples and Tkachuk (6) indicate that there are chromatographically separable amylases in wheat. In comparison with the present work, Tipples and Tkachuk used a higher loading of protein on their DEAE cellulose column and their eluants were less effectively buffered. Furthermore, different extraction procedures have been used. For these reasons, it is not possible to either confirm or contradict the observation of multiple amylase components on the basis of the present evidence.

The material which is unadsorbed on either CM or DEAE cellulose appears to be mainly carbohydrate with very little protein associated with it. However, a glycoprotein is definitely present at an elution volume of about 300 ml. It appears to be somewhat better resolved in the experiments with durum wheats than with vulgare wheats. The amylase is essentially free of carbohydrate.

Although there is some acid phosphatase activity present in the

\[2^{Clayton, J. W., unpublished results.}\]
pyrophosphate extract which is not adsorbed on CM cellulose (Figs. 2 and 3), none was detected in the fractions obtained when this material was separated on DEAE cellulose. The reason for this loss of activity is not clear. It is not due to a general instability of the enzyme, since even in the dilute fractions obtained from CM cellulose, the half-life of the enzymatic activity was of the order of months when stored in the cold room.

The results (Table IV) show that there is no irreversible adsorption of pyrophosphate-soluble protein onto CM cellulose and that all the nitrogen taken from chromatography can be accounted for.

**Part III**

**Ion-Exchange Chromatography of Wheat Gluten Proteins**

**Methods**

The same apparatus and general methods were used for the chromatography of both buffer-soluble and formic acid-soluble (gluten) proteins. However, a completely different set of solutions containing the protein dispersant dimethylformamide (DMF) was required for the chromatography of these proteins. These solutions were buffered at pH 4.0 with sodium lactate-lactic acid. Lactate-DMF solutions were also used for cellulose equilibration.

A stock solution of 1.00M sodium lactate was prepared by the careful titration of a 0.500M sodium carbonate solution to pH 5.0 with lactic acid. The solutions required for column chromatography were prepared from this stock solution plus other reagents indicated in Table V, and then titrated to pH 4.0 with lactic acid in order to maintain a known cation concentration. These solutions also contained $1.00 \times 10^{-4}M$ EDTA and 0.1 ml./liter toluene.

<table>
<thead>
<tr>
<th></th>
<th>Vessel Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Sodium lactate</td>
<td>M</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>M</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>M</td>
</tr>
</tbody>
</table>

*Vessel numbers as in Fig. 1.*

For each chromatography experiment a portion of the formic acid extract equivalent to 430 mg. of protein was taken for dialysis against 0.00400M sodium lactate, 1.0M DMF starting buffer.
Results and Discussion

Figures 6 and 7 show the elution gradients which were applied and the protein chromatograms obtained during the separation of the gluten proteins. The ragged appearance of the absorbance trace at 280 m\(\mu\) in the early part of some chromatograms is due to a slight instability of the base line of the recorder with this particular buffer system. The chromatograms are presented with the starting points of the gradient elution aligned, similarly to the buffer-soluble protein chromatograms, to facilitate the comparison of various chromatograms. Mechanical difficulties prevented the recording of a protein peak eluted by sodium hydroxide in the Thatcher experiment in Fig. 7.

The similarity of the chromatograms of gluten proteins from the vulgare wheats is quite striking. The chromatograms in Figs. 6 and 7 show no significant differences among the six vulgare wheats which are widely different in baking quality. The two durum wheats appear to contain all of the protein components which are observed in the vulgare wheats, but the relative amounts of the various components are not the same. It is apparent that there is still considerable crowding of the components in these chromatograms. In experiments not recorded here, the use of 5M dimethylformamide in the gradient as well as different ionic strength gradients gave no significant improvement in the resolution of the various protein components. The slight rise in the effluent pH in the early part of the chromatograms is probably a result of the ion-exchange process which occurs during the adsorption of the protein onto the cellulose. The relative constancy of the pH during the elution of the major protein fractions indicates that these fractions are not likely to be artifacts arising from fluctuations in pH. Normally a higher buffer concentration would have been used, but this was not practical because of the high ultraviolet absorbance of lactic acid solutions. The advantages of dimethylformamide as opposed to the more widely used protein dispersant urea have already been discussed by Simmonds and Winzor (2).

The recovery of protein during the chromatography of formic acid-solubles on CM cellulose has been determined. The tubes of effluent were concentrated under vacuum using a rotary evaporator, and the concentrate was thoroughly dialyzed against 0.05M acetic acid prior to nitrogen determination. The results, which are summarized in Table VI, indicate that all of the protein applied to the ion-exchange columns can be accounted for and that there is no significant irreversible adsorption.

Also, Table VI shows that a significant amount of protein is not eluted by the salt gradient and is finally removed by sodium hydroxide.
Fig. 6. Chromatography of formic acid-soluble wheat proteins on CM cellulose. Continuous line, no points: absorbance at 280 mμ, 0.66 cm., of column effluent as recorded by the automatic apparatus. Solid triangles: dimethylformamide (DMF) concentration, moles/liter. Solid circles: sodium ion concentration, moles/liter. Open circles: effluent pH.
Fig. 7. Chromatography of formic acid-soluble wheat proteins on CM cellulose. Continuous line, no points: absorbance at 280 mμ, 0.66 cm., of column effluent as recorded by the automatic apparatus. Solid triangles: dimethylformamide (DMF) concentration, moles/liter. Solid circles: sodium ion concentration, moles/liter. Open circles: effluent pH.
TABLE VI
RECOVERY OF PROTEIN AFTER CHROMATOGRAPHY OF FORMIC ACID EXTRACT ON CM CELLULOSE

<table>
<thead>
<tr>
<th>Protein</th>
<th>mg.</th>
<th>% original</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss during dialysis</td>
<td>73</td>
<td>17</td>
</tr>
<tr>
<td>Residue after centrifugation of dialyzed sample</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>Not adsorbed plus eluted by gradient</td>
<td>276</td>
<td>64</td>
</tr>
<tr>
<td>Eluted by sodium hydroxide</td>
<td>77</td>
<td>18</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>440</strong></td>
<td><strong>102</strong></td>
</tr>
</tbody>
</table>

The proportion of the protein eluted by sodium hydroxide after gradient elution of the formic acid-soluble fraction is similar to that recorded by other workers (2,7).

**General Discussion**

The present extraction procedures solubilize almost all the wheat protein. This facilitates interpretation of the subsequent chromatography results, since one may consider the portions of flour extract taken for chromatography as representative of all the wheat protein. This appears to be a significant improvement over other reports which deal with only a part of the total flour protein.

During the course of a number of preliminary chromatography experiments not recorded here, it was observed that the ion-exchange chromatographic conditions of the previous work (1,2) could be changed in a number of ways to improve the separation of wheat proteins. Very early in this work it was found that the resolution of individual components was greatly improved by operating the column at 30°C. instead of at 2°–3°C. or even at room temperature. This effect has subsequently been reported by Wrigley (7) and Simmonds (5). This observation is at variance with what is often mentioned in reviews of ion-exchange chromatography (15).

Another important feature of the present work is the separation of soluble proteins at near neutral pH. This seems more likely to preserve enzymes in an active condition than the alkaline conditions which have been used previously (1,5). Furthermore, the desirability of conducting ion-exchange separations of proteins at pH values close to the isoelectric point has already been discussed by Simmonds and Winzor (2). It seems likely, considering the data of Pence (16), that the present experiments at pH 5.5 and 7.0 were conducted much closer to the isoelectric points of most of the buffer-soluble proteins than the experiments of Coates and Simmonds (1) and Simmonds (5) at pH 9.5.

The gradients of ionic strength used to elute proteins from the ion-
exchange cellulose in this work are all of the concave nonlinear type. In previous work (1–3, 5–7), linear gradients have been used which frequently led to crowding of readily eluted components into a large first peak followed by extensive trailing of the more tightly bound constituents (17). The present concave nonlinear gradients help to overcome these problems.

The present work also recognizes the possibility that artifact separations may occur if the eluant solutions are not well buffered (13). This point has not been considered in many previous publications, although Nimmo and co-workers (3) did report rather extensive variations in the effluent pH when water-soluble proteins were fractionated on DEAE cellulose. The possibility of artifact separations in the present chromatography of buffer-soluble proteins has been minimized by use of elution gradients composed entirely of buffer salts. Furthermore, these buffers have pK values very close to the pH of the eluant solutions. In the case of the formic acid extracts where the buffer was lactate-lactic acid, it was not practical to use high concentrations of this buffer substance because it has a high ultraviolet absorbance. However, the results of the effluent pH determinations shown in Figs. 6 and 7 indicate that there were no large or sharp deviations from the nominal pH of the eluant solution during chromatography of formic acid-soluble proteins.

A final important feature of the present work is the absence of any significant differences in protein distribution among wheats of the same class. It would appear that any methods which do reveal varietal differences should be thoroughly checked to eliminate the possibility of artifact fractionations.

Conclusions

The results of this investigation have shown that the present methods provide for the extraction and fractionation of almost all the protein in wheat flour. The most serious shortcoming of the present work lies in the fact that some of the wheat nitrogen is either lost through dialysis or is not eluted from the ion-exchange columns by the various salt gradients. While these nitrogenous constituents have been accounted for, the present methods do not provide any useful way of fractionating these components. On the other hand, by far the major proportion of the wheat protein has been separated into many fractions which can be readily isolated for further study. The possibility that these fractions are artifacts has been investigated by following the pH of the ion-exchange column effluent. According to this analysis only one component is likely to be an artifact and this has
been adequately described. Finally, the present comprehensive methods allow each protein component to be viewed in proper perspective relative to all the wheat protein.

Acknowledgment

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Literature Cited