Immunochemical Comparisons of Antigenic Proteins of Durum and Hard Red Spring Wheats[1]

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ABSTRACT

Protein components of a hard red spring and a durum wheat were compared by immunochemical methods, including immunoelectrophoretic analysis (IEA). Each protein component of one wheat type showed reaction of identity with a protein component of similar migration rate in the other type. Among the gliadins and salt-soluble proteins examined, no components unique to one type were found. Agarose gels at pH 8.1 (barbital buffer) and pH 3.1 (aluminum lactate buffer), with or without 3*M* urea, were used for IEA. Interrupted-trough IEA was the most useful method in comparing the components. Results of antibody absorption tests and modified Osserman IEA supported the conclusions. The results suggest that the proteins of the two wheat types are qualitatively matched; they also support others' conclusions that baking-quality differences among wheats must be related to factors other than individual protein differences.

In studies of the relation of bread quality to protein composition of flour, many analytical approaches have been used. Among those methods that attempt to view the protein components individually, analytical electrophoresis, of free-boundary type or in stabilizing media (gels), has been used extensively. Both qualitative and quantitative differences in migrating components were considered in comparing and contrasting flours of different baking quality, in studies by Elton and Ewart (1), Koenig *et al.* (2), and Coulson and Sim (3, 4).

In qualitative and quantitative comparisons of plant proteins by electrophoresis, of either gel or free-boundary type, basic assumptions are made that peaks or bands of identical mobility represent proteins of comparable composition, and, conversely, that protein mixtures whose protein peaks or bands do not match are not identical in protein composition. Although the risk may be small, within species particularly, evidence from such sources must be considered circumstantial until molecular compositions (amino acid composition, physical constants) can be more directly compared.

Immunochemical methods can provide a separate criterion for determining whether or not two protein components in question have similar composition. Rigorous molecular specificity cannot be assumed, but reactions of nonidentity are generally accepted as proof of difference and reactions of identity can usually be considered to mean that the molecules are similar. Fundamental studies of wheat proteins, with the use of immunoelectrophoresis, were made by Grabar (5, 7) and Benhamou-Glynn (6) and their co-workers. Hall (8) has also used the technique with antibody absorption, to show that there are proteins in rye that cross-react with those in wheat, and that a rye-wheat hybrid contained essentially the sum of the protein specifici-

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ties found in the parent grains. For varieties of barley, there are cross-reactions among proteins soluble in dilute salt solution (9).

The immunochemical work most directly concerned with comparison of wheat varieties and species was that of Elton and Ewart (10) and Ewart (11). From evidence derived from the use of Ouchterlony double diffusion, they concluded that there were reactions of identity between four gluten components of the various wheats, particularly for *Triticum vulgare* and *T. durum*. They concluded that major components of gluten from the different wheats studied may be similar in composition, with baking quality dependent on quantitative variations or other unrecognized factors.

Application of immunoelectrophoretic analysis to the gliadin and the salt-soluble proteins appeared to offer the possibility of comparison of protein components of similar mobility, or, by means of adjustments in the electrophoretic origin, those of different mobility. The work was started with comparison of durum and common wheat, with the idea that differences would be more likely to appear than in comparisons within the common wheat class. Furthermore, baking quality difference is relatively large. Most of the results are concerned with direct comparison of antigenic components of Mindum and Selkirk varieties.

MATERIALS AND METHODS

Wheat

Durum and hard red spring wheats of known variety and history were supplied by the North Dakota Agricultural Experiment Station, through K. A. Gilles. Review of breeding history of varieties available (12) led to selection of Selkirk (HRS) and Mindum (durum) as likely to show component differences as they existed between durum and common wheats. For other comparisons mentioned later in the text, commercially milled, unbleached, unmalted HRS and durum flours were used.

Extraction

The Selkirk and Mindum wheats were extracted by two different schemes to give three preparations. For one preparation, later referred to as *acetic acid extract solids,* a single extraction with 0.1*M* acetic acid, 10 ml. per g. of wheat, was made. A second preparation, *buffer-soluble components,* was made by extraction with 0.10*M* sodium barbital, 0.028*M* HCl, pH 8.1, three times, with 5 ml. of buffer per g. of wheat. The residue from the buffer extraction was extracted once with the same buffer containing 3*M* urea, to obtain *buffer-insoluble proteins.* In each case, the wheat or solid residue was comminuted in a Serval Omnimixer at 8,000 r.p.m. and centrifuged at 30,000 $\times$ *g*. The extracts were dried from the frozen state and stored at –15°C.

The second and third preparations were made to obtain materials similar to that used by Benhamou-Glynn *et al.* (6) for studies of gluten components of wheat. On gel electrophoresis (aluminum lactate, pH 3.1), the buffer-insoluble proteins showed only gliadin bands; the buffer-soluble components showed bands over the entire range normally seen by this method, including some in the gliadin region. The component overlap should not be

a problem, because the principal aim was to have all extractable components represented among the preparations rather than to have clear fractionation, although the latter would be desirable for greater ease of interpretation.

Preparation of Antiserums

For each wheat type, three rabbits were hyperimmunized by subcutaneous injection of the solids from the acetic acid extracts. The first three injections were in Freund's complete adjuvant, and contained 1 mg. of protein; later injections used 10 mg. protein suspended in distilled water. Serum samples were tested periodically, and at 6 weeks the antiserum for use in the work was collected. Antiserums giving the strongest response were pooled for use in the tests. Serums from each rabbit before immunization showed no precipitation reaction with the solids used for injection. These serums were prepared by Antibodies, Inc., Davis, Calif.

Immunochemical Procedures

Antigen-antibody precipitation reactions were used throughout. Antibody absorption was carried out as described by Hall (8). Immunoelectrophoretic analyses were on the micro scale, with templates over the gel, as described by Crowle and Lueker (13). Agarose (Marine Colloids, Inc.) was used to form 1% gels; it shows little or no electroendosmosis and does not precipitate with gluten components. Barbital buffer (0.05*M* sodium barbital, 0.014*M* HCl, pH 8.1) was used for results shown in all figures. For other results described more briefly, the agarose gels were formed in aluminum lactate-lactic acid (pH 3.1, ionic strength 0.05), or in the barbital buffer containing 3*M* urea. When these latter systems were used, the gel strips were soaked after electrophoresis in the simple buffer solution for about 30 min. to adjust pH or remove urea, before antiserum was added. The visible precipitin reaction will not occur at the low pH or at the high urea concentration.

Two procedures were used for identity determinations in conjunction with immunoelectrophoresis. The Osserman technique (14), which was originally described for determining the position of a purified protein in the electrophoretic pattern of a source-mixture, was used to a limited extent. More successfully used for comparison of the complex mixtures was the interrupted-trough comparison of Clausen and Heremans (15). For this, the Crowle template was modified to have three short slots instead of one long one. The short slots were located so that comparison of all of the precipitin arcs could be made by using the template in two positions, reversed lengthwise.

Gel Electrophoresis

Starch-gel electrophoresis was used in conventional systems, as designated in figure captions, on a horizontal, water-cooled apparatus.

RESULTS AND DISCUSSION

Gel Electrophoresis

Starch-gel electrophoresis of Selkirk and Mindum wheat extracts (Fig. 1) showed definite differences in "band" location, notably in the gliadin (slow)

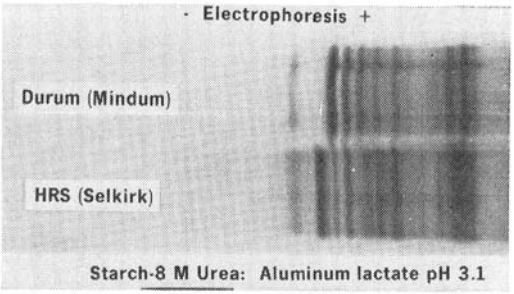


Fig. 1. Comparison of acetic acid extract solids of Selkirk and Mindum wheats by starch-gel electrophoresis (18% starch, 8*M* urea, aluminum lactate-lactic acid buffer of pH 3.1, $\Gamma/2 = 0.1$).

region, in a prominent band for Selkirk which had no counterpart in the diagram for Mindum, and in some differences in the minor bands. Such differences supported the idea that immunochemical distinctions might be found.

Immunochemical Results

Antibody absorption tests and three variations of immunoelectrophoresis, as described below, were used to try to find distinctions in components.

Antibody Absorption Tests

Addition of increasing amounts of the antigen proteins of the acetic acid extract of Selkirk wheat to the anti-Mindum serum caused successively less available antibody action, to final elimination of visible precipitation. The reverse combination, Mindum antigen and anti-Selkirk serum, showed the same effect. From this it was concluded that the two wheat types had immunologically common antigens. The anti-Selkirk serum was demonstrably weaker, requiring about one-third as much Mindum extract solids to eliminate visible precipitation, as was the case for the reverse combination.

Immunoelectrophoretic Analysis

In all four combinations of use, the two anti-serums and the two extract solids gave generally similar precipitation arc diagrams. The anti-Selkirk serum did not give as distinct lines as did the anti-Mindum serum. For most of the work, a mixed antiserum was used, as in the typical diagram of Fig. 2

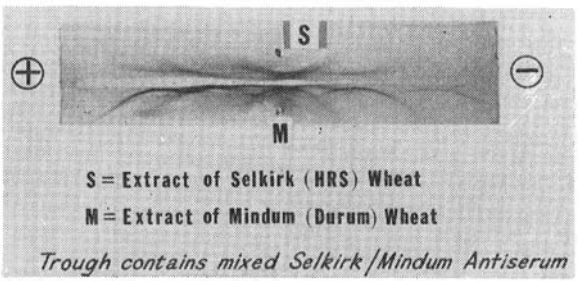


Fig. 2. Simple immunoelectrophoretic comparison of buffer-soluble components of Selkirk and Mindum wheats (1% agarose, 0.05*M* sodium barbital, 0.014*M* HCl, pH 8.1).

for soluble proteins. There are differences in intensities of the precipitation arcs, but the qualitative nature appears to be the same, with the possible exception of the most cathodic arc for the Mindum sample.

When the procedure with agarose gels containing barbital buffer and 3*M* urea was used, and urea was removed by leaching with the buffer after electrophoresis (6), both antiserums reacted with buffer-insoluble proteins from the other wheat type (Fig. 3). What appeared to be three nearly con-

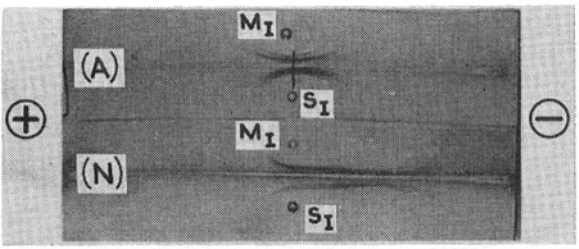


Fig. 3. Comparison of IEA of buffer-insoluble proteins in agarose and Noble agar. M_I, S_I, extracts of Mindum and Selkirk wheats with barbital buffer–3*M* urea (see text). A = agarose; N = Noble agar. Conditions as for Fig. 2.

centric arcs were formed, only slightly cathodic from the origin in A. Qualitatively similar results were obtained when this buffer was used without urea. The small movement from the origin appears to agree with results reported for Ionagar (9). When Difco Noble agar was used for the gels (N), pronounced migration toward the cathode was evident, similar to that found by Benhamou-Glynn (6). Presumably the difference in migration was caused by the difference in electro-osmotic flow, agarose having practically none.

When aluminum lactate buffer (pH 3.1) was used, with pH adjustment by soaking in barbital buffer, three long arcs, nearly superimposed, moved to the cathode. Both this and the urea-barbital method, used directly in this way, are considered to show the buffer-insoluble proteins, those not in the salt-soluble class, the latter being removed from reaction by the leaching step. Visible cloudiness formed at the buffer-insoluble protein sites during the leaching step, but strong precipitation arcs were formed against the antiserum.

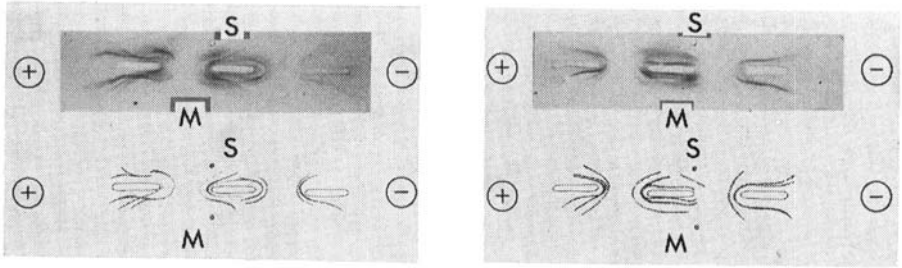
From this information it was concluded that cross-reaction between the proteins of these two wheat types is extensive for immunochemically precipitating components. For direct comparison of the relationships between components, systems using identity testing reactions were used as described below.

Osserman Tests

This method was used for comparison of the two wheat types, as an identity-reaction test. Because both antigen systems were complex, there was considerable difficulty in tracing the immunochemical reaction lines of individual components. However, from careful examination it was concluded that cross-reaction was complete, or at least very extensive.

Interrupted-Trough Tests

This method was the most effective of those used. From the information represented by Figs. 4 and 5, for buffer-soluble components, it appears that



Figs. 4 (left) and 5 (right). Interrupted-trough IEA of buffer-soluble components, showing results with alternate position of short slots. Conditions as for Fig. 2; S = extract of Selkirk (HRS) wheat; M = extract of Mindum (durum) wheat.

each of the visible precipitation arcs from one wheat type is confluent with a line of roughly corresponding mobility from the other type. The diagrams were made by tracing of photographs and direct inspection of the original slides; this method allowed more detail to be shown than is visible in the photographs. Figure 4 differs from Fig. 5 in the relative position of the origin holes and the antiserum wells, as will be evident by inspection. This difference, obtained simply by reversal of the templates, allowed better observation of confluence of opposing precipitin lines by proper positioning of the gaps between antiserum wells.

The same confluency is shown for the buffer-insoluble proteins, extracted by 3*M* urea, in Fig. 6. In these tests the gels did not contain urea as

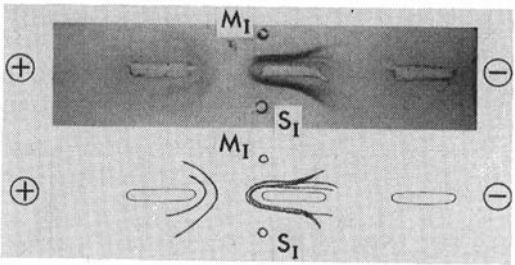


Fig. 6. Interrupted-trough IEA of buffer-insoluble proteins. Conditions as for Fig. 2. M_I = proteins extracted by 3*M* urea from Mindum; S_I = proteins extracted by 3*M* urea from Selkirk.

mentioned above, similar test results were obtained, actually with somewhat greater clarity because extra diffusion in the leaching step was avoided. One slot position only is shown; the other position was used and the same conclusion drawn.

Selkirk and Mindum were considered typical varieties of HRS and

durum wheats. However, no baking tests were made with these particular varietal samples, because of limited supply. In order to have information on wheats for which baking quality had been determined in this laboratory, one HRS and one durum flour, commercially milled, unbleached and unmalted, and of typical baking quality for the type, were extracted with acetic acid (see "Methods"). The extract solids were subjected to interrupted-trough-electrophoresis comparisons. Figure 7 shows results typical of these

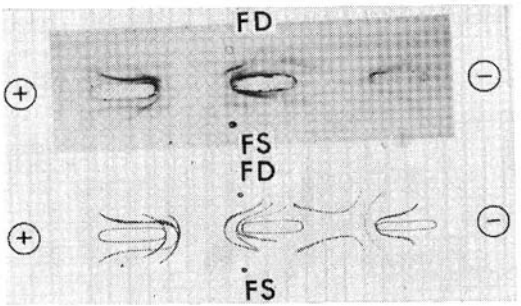


Fig. 7. Interrupted-trough IEA of acetic acid extract solids of flours. Conditions as for Fig. 2. FS = extract of commercial (HRS) flour; FD = extract of commercial durum flour.

tests (one-slot position), again leading to the same conclusions as for comparison of the individual varieties. The two apparently nonconfluent arcs were a result of slot position and were shown to be confluent when the other slot position was used.

When the starch-gel electrophoresis method (which has become a standard of reference for wheat proteins) was used, there appeared to be differences, in migration of both major and minor protein components, between the Selkirk and Mindum wheats. From this it might be concluded that there are distinctions in protein components of the two wheat types. The evidence from the IEA did not support this concept of differences, showing identity of components of similar migration rate in the agarose-barbital buffer system, and also showing no evidence of distinctive components.

Glutenin has probably not been directly accounted for (11) in this work. It has shown immunochemical reaction with antigliadin serums (6), and from this relationship it could be concluded that glutenin preparations from the two wheat types would probably show immunochemical identity reactions.

Quantitative differences in protein components may be responsible for differences in baking quality. However, the results available at this time from gel and free-boundary electrophoresis are only roughly quantitative and have furnished no real explanation of variation in bread quality among wheats. The results of this immunochemical work support the idea expressed by Elton and Ewart (10), that variation in baking quality does not seem to be the result of major change in protein structure from one variety to another, and that other factors may be involved. It may be that minor differences in protein structure, insufficient to affect immunochemical relation-

ships, or in type or quantity of nonprotein constituents, are the factors responsible. Such ideas have been expressed in the past; this work gives it further support from the immunochemical standpoint.

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