FRACTIONATION AND ZONE ELECTROPHORESIS OF PROTEINS OF WATER-SOLUBLE MATERIALS OF FLOUR[1]

C. C. NIMMO, MARY T. O'SULLIVAN, ALI MOHAMMAD, AND J. W. PENCE

ABSTRACT

Zone electrophoresis applied to water extracts of flour using poly-acrylamide gels at pH 6.0–6.1 and either cacodylate or phosphate buffers revealed 15 to 17 apparent protein components. Paper electrophoresis was much less effective. Starch gel electrophoresis showed a serious discontinuity caused by a separable fraction containing beta amylase activity.

Fractionation by means of diethylamino-ethylcellulose ion-exchanger permitted separation of several fractions. The bands shown by gel electrophoresis were accounted for in the fractionation. Substantial concentration of several components was achieved, and amino acid composition was determined for five of the fractions obtained. One fraction, eluted by relatively high ionic strength, had about one-fourth the "half-cystine" value of the others. Other differences were smaller.

The effect of soluble proteins in baking processes has been investigated in several laboratories (4,12,13,15). More recently, fractionation methods for these proteins have included separations on ion-exchange cellulose, and recognition of components has been extended from moving-boundary and paper electrophoresis methods (11,12) to the apparently more sensitive gel electrophoresis methods (2,3,5,20). The association of carbohydrate materials such as pentosans with fractions from soluble proteins has been shown by means of preparative and analytical moving-boundary electrophoresis (7,17a,17b,18), as well as by work primarily concerned with pentosans (10). A recent contribution (6) has provided correlation between staining and immunodiffusion techniques as a means of detection of protein components in extracts of wheat, wheat flour, and barley.

The work reported here concerns the materials soluble in a single extraction with a small amount of water, in an effort to determine number, approximate quantity, and composition of proteins soluble under these conditions. Fractionation has been carried out with anion-exchange cellulose. Electrophoresis has been done with polyacrylamide gels, which have been used with better results than starch gels. Amino acid compositions obtained for the fractions are discussed.

Materials and Methods

Flour. Except where otherwise stated, the flour used for this work was a commercial baker's patent from hard red spring wheat obtained from Montana in 1958 and stored in friction-top cans at −23°C.

Extraction. All results reported are from material extracted by water, two parts by weight to one part of flour, at 2°C. After about 1 min. of mixing at low speed in a blender (vortex prevented with an inserted beaker) and 10 minutes' holding in an ice bath, the mixture was centrifuged at 27,000 × g for 20 min. at 1°C. The clear supernatant solution was frozen and then dried at 0.1 mm. Hg pressure with a finishing shelf temperature of about 35°C. The dried material was held over anhydrous calcium sulfate until used.

The single-extraction procedure was chosen in order to work rapidly with extractable material available to a relatively small amount of water. Electrophoresis results not given here indicated that second and third successive water extractions yielded relatively less of the components shown at both ends of the electrophoretic pattern. Possibly this is caused by change in electrolyte concentration or exhaustion of some of the components in the material subjected to the extraction.

Electrophoresis. Horizontal electrophoresis was used, in gels 28 × 7 × 0.6 cm. in an apparatus provided with water cooling plates. Polyacrylamide gels were made up at 5% concentration, based on acrylamide monomer, according to the procedure described by Raymond (14), in a casting form enclosing trays to mold the gel. The buffer used predominantly was sodium cacodylate-sodium chloride, pH 6.1, ionic strength, 0.01. Clear and generally similar patterns were obtained with $0.01M$ phosphate buffers at pH 6.1 and 6.9, cacodylate buffer (pH 6.1) at an ionic strength of 0.05, and tris-citrate, pH 8.6 ($0.08M$ tris). Glycine-NaOH ($0.01M$, $0.1M$), pH 9.5, gave a less intense pattern. Aluminum lactate, pH 3.2, ionic strength 0.1, gave nine or ten bands moving rapidly to the cathode.

The polyacrylamide gels were washed before use by soaking in three changes of approximately ten volumes of the buffer to be used

for electrophoresis. Although the patterns were not different between washed gels and freshly prepared ones, the washing caused reduced and stabilized current during electrophoresis and removed substances other than the polymer and the buffer concerned. For the aluminum lactate buffer gels, the polymer was cast in water alone, then equilibrated to the buffer with changes of solution. Possibly such a procedure would be desirable for any buffer because it would permit formation of the gel itself under the same conditions each time.

Insertion of sample was made with filter paper strips, soaked in 2 to 15% solutions of the material to be investigated, in a transverse cut at an appropriate point in the gel. Most insertions were made somewhat anodic to the middle of the gel. Effective voltage gradient was 7 to 8 volts per cm.; duration of runs was 1.5 to 4 hr.

Bands were detected by staining with 1% naphthylamine black 10 BR in methanol:water:acetic acid (5:5:1) and destaining in this solvent. Polyacrylamide gels were stained and destained in the original thickness; when sufficiently cleared of unbound dye, they were photographed (panchromatic film, Wratten A filter, back light). The gels were impregnated with aqueous 10% glycerol in 5% acetic acid, dried in air on greased glass plates, and wrapped in transparent film for preservation for future reference.

Starch gel electrophoresis was tried early in the work and abandoned because of a discontinuity in the pattern similar to that described by Elton and Ewart (5) as a "fringe." They have established that the "fringe" is caused by beta-amylase action. A similar effect of rat serum in electrophoresis on starch gels has been mentioned by Beaton (1). Our own work showed that the "fringe" can be removed or intensified by fractionation, and that beta-amylase activity is present when a fraction producing the "fringe" is run. Although it is possible to inhibit this action (5), starch gels were not used for this work.

Column Chromatography. The diethylaminoethyl-cellulose (0.9 meq./g., Brown Co., Keene, N. H.) used was pretreated as described by Mandeles (8). Protein-containing solutions were dialyzed 15–20 hr. (2°C.) against 0.01M glycine, and loaded on the column equilibrated to 0.01M glycine. Some unadsorbed material was washed through with the glycine solution, as observed also by Coates and Simmonds (3). Gradient elution was carried out with 0.1M phosphate buffer, pH 6.7 in 0.01M glycine, as described for Fig. 2. The fractions obtained were dialyzed against distilled water and dried from the frozen state.

Amino Acid Analysis. The Beckman Spinco adaptation of the method of Moore, Spackman, and Stein (9) was used. Twenty milli-

grams of sample were hydrolyzed in $6N$ HCl at $110°C$. for 22 hours in capped, evacuated tubes. Tryptophan was determined separately by the direct method of Spies and Chambers (16).

Results and Discussion

Electrophoretic Patterns for Water Extracts. Zone electrophoresis on polyacrylamide gels at pH 6.1 showed many bands in direct runs on water extract solids. Figure 1 (1) shows patterns representative of the results with the cacodylate buffer, using four flours (Rio, Burt, Omar, and Brevor varieties) from Pacific Northwest wheats with considerably different baking characteristics. All figures show the anode end on the right.

There are reproducible differences between the varieties. Omar and Brevor, for example, lack bands in the most cathodic portion of the pattern where some bands are evident for the other flours. Further, the Omar pattern contained a distinct extra band in the anodic portion. Since Omar is a club wheat, distinctly different genetically from the others, such differences are of interest. There are many bands toward the cathode from the insertion. Electro-osmotic effect has not been charted. From comparison of the results with other reports (11) it is safe only to say that the positively charged components at pH 6.0 are probably limited to the group shown in the middle strip of Fig. 1 (section 3).

An electrophoretic pattern similar to those in Fig. 1 (1) is evident for the commercial hard red spring wheat flour used for the fractionation experiments, and it is shown as the control "original" pattern on each of sections 3 through 6, Fig. 1.

Column Chromatography. The detail afforded by the electrophoresis pattern on polyacrylamide gel led us to re-examine fractionation procedures on anion-exchange cellulose, along lines which had been explored earlier in this laboratory when paper electrophoresis was used for monitoring. The elution system used (glycine-phosphate, pH 6.8) differs somewhat from that used by Coates and Simmonds (glycine-chloride, pH 9.5) (3) or by Chang (phosphate only, pH 7.0) (2).

The elution diagram of Fig. 2 presents the essential picture encountered in these runs. An unadsorbed portion was carried through by the $0.01M$ glycine initially used. This material is apparently high in carbohydrate (3,10) and contained about one-third of the protein fed to the column. When the unadsorbed portion was clearly through, gradient elution was begun as shown, continuing through tube 65. The ion-exchange process resulted in a rise in effluent pH to 8.5–9.5
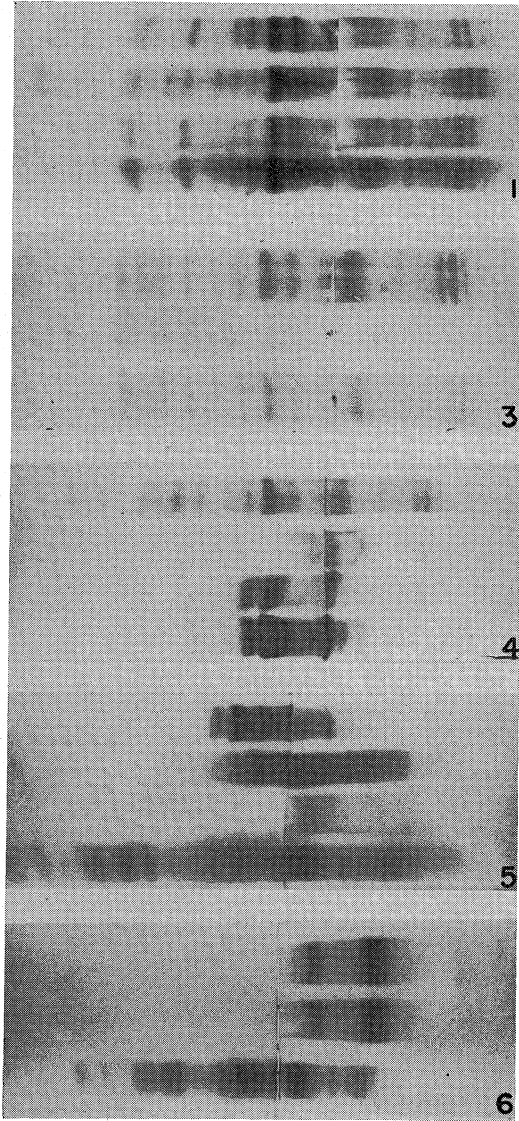
Fig. 1. (1) Polyacrylamide gel electrophoresis of water extracts of varietal wheat flours. Buffer: Sodium cacodylate-sodium chloride, pH 6.1, ionic strength = 0.01. Voltage gradient: 7–8 volts/cm. Wheat varieties, top to bottom: Rio, Burt, Omar, Brevor. Anode at the right for all electrophoresis. (3) Acrylamide gel electrophoresis of unadsorbed portion (middle pattern). Outer patterns are of original extract. (4) Acrylamide gel electrophoresis of eluted fractions represented in Fig. 1. Patterns, top to bottom: original extract; fraction 1, 2, and 3; fraction 4; and fraction 5. (5) Acrylamide gel electrophoresis of eluted fractions represented in Fig. 1. Patterns, top to bottom: fraction 6, fraction 7, fraction 8, original extract. (6) Polyacrylamide gel electrophoresis of eluted fractions represented in Fig. 1. These bands are from elution of fractions 10–11.
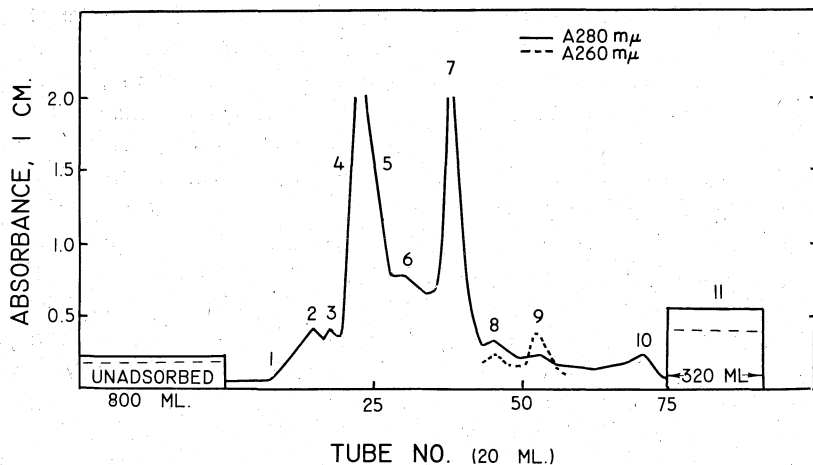
Fig. 2. Chromatography of water-soluble protein material from a commercial hard red spring wheat on DEAE-cellulose. Column size: 1.9 × 24 cm. 300 mg. protein N applied to column. Flow rate: 2 ml./min. Tube volume: 20 ml. Unadsorbed eluted with 0.01M glycine. Linear gradient elution, tubes 1–65; mixer, 0.01M glycine; reservoir, 0.01M glycine, 0.1M potassium phosphate, pH 6.8. At tube 65, reservoir KH_2PO_4 increased to 1M. At tube 75, 1M KH_2PO_4 fed directly to column, collection in one portion.

in the region from tubes 10 to 37 (to the peak of fraction 7). Thereafter, the pH dropped within a few tubes to 6.9. It is likely that the material of fraction 7 was eluted largely by being carried just ahead of breakthrough of buffer of the original pH value. A yellow-colored band was also sharply eluted in this peak. This effect can be avoided by holding the eluant pH higher, as in the report of Coates and Simmonds (3). However, there were other fractions of sufficient interest to present the result as it stands.

Eluant buffers of increased phosphate concentration at tubes 65 and 75 were used to drive off material which had been shown to be not eluted otherwise. This may be similar to the material eluted by acetic acid in the Coates and Simmonds report, although comparison is difficult because amino acid analysis for that fraction was not given, and it apparently differs considerably from other fractions (see below).

Rechromatography of several fractions was carried out particularly for the peaks labeled 4 and 5. Some improvement in concentration was made, but there was no clean separation of individual components.

Fraction Electrophoresis. The progressive elution of material corresponding to the total picture given by electrophoresis for the original water extract solids is illustrated in Fig. 1 (3 to 6). In Fig. 1 (3), the bands corresponding to the unadsorbed material are clearly the

most cathodic. Even though the amount of protein in this material is small, it accounts for several bands. Fractions contained in the first 50 tubes (fractions 3 to 8) are represented in the electrophoretic patterns of Fig. 1 (4 and 5). Fraction 9 was not included in this series.

In Fig. 1 (6) are shown the patterns corresponding to material eluted from tube 65 on, from two successive portions of the eluant. Combined fractions 10 and 11 showed an electrophoretic migration corresponding almost entirely to the group of three bands found close together at the anode end of the pattern for the whole extract. The multiple nature may be an artifact; this suspicion has been supported by the discovery of three exactly similar bands of amylolytic activity, as determined by direct application of starch substrate. Analyses of combined fractions 10 and 11 for beta-amylase activity have shown it to be a concentrated source of the enzyme. Previous work on a prepared albumin has shown this enzyme activity to be in the alpha group described by Pence (13), similar to the present results.

Amino Acid Composition. Analysis of the amino acids present in the predominant fractions is shown in Table I.

TABLE I
AMINO ACIDS IN PROTEIN FRACTIONS

AMINO ACID	AMINO ACID N AS PERCENT OF TOTAL N					AMINO ACIDS [b]				
	Fr. 4[a]	Fr. 5	Fr. 6	Fr. 7	Frs. 10–11	Fr. 4	Fr. 5	Fr. 6	Fr. 7	Frs. 10–11
	%	%	%	%	%	mmol.	mmol.	mmol.	mmol.	mmol.
Lysine	4.6	5.2	6.3	6.7	6.4	26.2	30.4	36.2	39.2	36.3
Histidine	3.0	4.7	3.6	4.7	6.1	11.4	18.3	13.8	18.3	23.4
Arginine	9.8	16.0	16.2	14.0	13.7	28.0	46.1	45.5	40.6	39.1
Ammonia	8.1	7.3	7.1	7.1	6.6	92.0	85.0	81.5	82.0	75.9
Aspartic acid	4.0	4.5	4.8	5.3	6.9	45.6	51.8	54.5	61.0	78.9
Glutamic acid	8.3	8.7	8.8	8.9	8.8	95.0	100.8	100.8	102.8	101.0
Alanine	6.0	7.0	7.6	6.5	6.7	67.9	80.7	87.5	75.9	68.1
Half-cystine	3.1	3.9	4.4	3.8	1.2	35.6	45.5	50.3	44.4	14.3
Glycine	4.7	5.8	6.4	6.2	6.6	53.9	66.5	72.9	72.0	75.9
Isoleucine	1.4	1.6	2.2	2.3	2.7	16.5	18.7	22.6	26.4	30.8
Leucine	3.8	4.9	5.2	5.7	5.9	43.8	56.2	60.0	65.9	67.2
Methionine	0.8	1.3	1.5	1.3	1.4	9.1	15.4	16.8	15.2	15.8
Phenyl-alanine	1.0	1.1	1.4	1.7	2.9	11.2	12.6	15.7	19.6	32.4
Proline	4.6	5.3	5.4	5.1	4.0	52.8	61.2	62.1	59.5	45.4
Serine	3.4	3.7	4.1	4.0	3.6	38.5	43.3	45.3	46.1	40.8
Threonine	2.1	2.0	2.4	2.9	2.4	23.6	23.3	27.0	33.9	27.4
Tryptophan	2.1	2.9	2.8	2.4	2.5	11.7	16.7	16.1	13.5	14.4
Tyrosine	1.6	2.3	2.4	2.7	2.6	17.8	26.4	27.8	31.0	29.2
Valine	3.9	5.2	5.4	5.4	5.2	44.3	60.4	62.0	62.9	59.0

[a] Fraction 4, etc.
[b] Millimoles per 16 g. total N.

Fractions 4 and 5, two slopes of the first large peak in Fig. 2, were analyzed separately. Fraction 4 contained 10.5% nitrogen, as compared with 15.2% for fraction 5. Humin precipitate formed during hydrolysis of fraction 4 contained about 11.5% of total N; fraction 5 correspondingly had about 2% of the nitrogen as humin and the other zones much less. Three of the five fractions analyzed, which were eluted close together with the system used, showed very similar composition, considering relative amino acid content. Fraction 4 showed some differences, even though it was taken as first half of the large peak. There was, however, an evident change in the excess of dicarboxylic acid equivalence over that of ammonia, largely accounted for by the change in aspartic acid. Combined fractions 10–11, relatively difficult to elute from the column, had a distinctly different "half-cystine" value, with a considerable difference also in phenylalanine. There are other less marked differences. In view of the importance of sulfur bonding in baking processes, the differences in the cystine-cysteine values may be of particular interest.

No attempt was made to compensate for expected losses of serine or threonine. However, ammonia derived from the partial destruction of these amino acids in the 22-hour acid hydrolysis may contribute as much as 10% to the ammonia values obtained.

Presentation of the amino acid results is in two forms, for comparison with the results obtained by Coates and Simmonds (3) and with the gluten-water solubles results of Woychik et al. (19). The values are in substantial agreement with the earlier results and with the alpha- and beta-components of Waldschmidt-Leitz and Hochstrasser (18). In our results, the ammonia fell considerably short of accounting for the dicarboxylic acids as amides, in agreement with Coates and Simmonds (3) and with the albumin amide values of Pence (12). As fractions 4, 5, and 6 carry the largest part of the protein and have the analyses which show closest agreement with the prior work, their composition is in accord with amide:tryptophan relations reported for the albumin proteins (12).

From these results it appears that the liquid phase in the 1:2 flour-water mixture contains at least 15 components which can be resolved by electrophoresis. Studies based on single extraction with this amount of water (or less, as in doughs) may have an advantage, as part of a study of wheat proteins in bakery use, of providing components in more nearly the mixture present during at least the initial stages of dough or batter preparation. This approach may also be inferred from the reports of Holme (7) and Strobel and Holme (17a,17b).

Complexity of the pattern on electrophoresis seems to show that the fractionation methods are not yet adequate for separation into individual components for this system. However, the separations based on preparative electrophoresis and column chromatography do provide components and groups of components from which any significant contrasts in effects on baked products can be determined, before further resolution is attempted.

Acknowledgment

Literature Cited

1. BEATON, G. H., SELBY, A. E., and WRIGHT, A. M. Starch gel electrophoresis of rat serum proteins. I. Procedure and designation of components. J. Biol. Chem. **236**: 2001–2004 (1961).

2. CHANG, L. E.-H. The amino acid and protein composition of certain varieties of wheat. Doctoral dissertation. Cornell Univ., Ithaca, N.Y. (1960).

3. COATES, J. H., and SIMMONDS, D. H. Proteins of wheat and flour. Extraction, fractionation, and chromatography of the buffer-soluble proteins of flour. Cereal Chem. **38**: 256–272 (1961).

4. DONELSON, D. H., and WILSON, J. T. Effect of the relative quantity of flour fractions on cake quality. Cereal Chem. **37**: 241–262 (1960).

5. ELTON, G. A. H., and EWART, J. A. D. Starch gel electrophoresis of cereal proteins. J. Sci. Food Agr. **13**: 62–72 (1962).

6. GRABAR, P., BENHAMOU, N., and DAUSSANT, J. Etudes électrophorétiques et immunochimiques de quelques protéines de l'orge et du blé. Arch. Biochem. Biophys. (suppl.) **1**: 187–199 (1962).

7. HOLME, J. Characterization studies on the soluble proteins and pentosans of cake flour. Cereal Chem. **39**: 132–144 (1962).

8. MANDELES, S. Use of DEAE-cellulose in the separation of proteins from egg white and other biological materials. J. Chromatography **3**: 256–264 (1960).

9. MOORE, S., SPACKMAN, D. H., and STEIN, W. H. Chromatography of amino acids on sulfonated polystyrene resins. Anal. Chem. **30**: 1185–1190 (1958).

10. NEUKOM, H., KUENDIG, W., and DEUEL, H. The soluble wheat flour pentosans. Cereal Sci. Today **7**: 112, 114, 125 (1962).

11. PENCE, J. W. Approximate isoelectric pH's of albumins from wheat flour. Cereal Chem. **30**: 328–333 (1953).

12. PENCE, J. W., and ELDER, ANGELINE H. The albumin and globulin proteins of wheat. Cereal Chem. **30**: 275–287 (1953).

13. PENCE, J. W., ELDER, ANGELINE H., and MECHAM, D. K. Some effects of soluble flour components on baking behavior. Cereal Chem. **28**: 94–104 (1951).

14. RAYMOND, S., and WANG, Y.-J. Preparation and properties of acrylamide gel for use in electrophoresis. Anal. Biochem. **1**: 391–396 (1960).

15. SOLLARS, W. F. Evaluation of flour fractions for their importance to cookie quality. Cereal Chem. **33**: 121–128 (1956).

16. SPIES, J. R., and CHAMBERS, D. C. Chemical determination of trytophan in proteins. Anal. Chem. **21**: 1249–1266 (1949).

17a. STROBEL, R. G., and HOLME, J. Curtain electrophoretic separation of the water-soluble constituents of bleached cake flour. Cereal Chem. **40**: 351–360 (1963).

17b. STROBEL, R. G., and HOLME, J. Chemical composition of the water-soluble constituents of bleached cake flour. Cereal Chem. **40**: 361–372 (1963).

18. WALDSCHMIDT-LEITZ, E., and HOCHSTRASSER, K. Über die Albumine aus Gerste und Weizen. Z. Physiol. Chemie **324**: 243–249 (1961).

19. WOYCHIK, J. H., BOUNDY, JOYCE A., and DIMLER, R. J. Amino acid composition of proteins in wheat gluten. J. Agr. Food Chem. **9**: 307–310 (1961).

20. WOYCHIK, J. H., BOUNDY, JOYCE A., and DIMLER, R. J. Starch gel electrophoresis of wheat gluten proteins with concentrated urea. Arch. Biochem. Biophys. **94**: 477–482 (1961).