Dye-Binding Capacity and Amino Acid Content of Wheat-Protein Gel-Electrophoresis Bands[1]

JOHN M. LAWRENCE[2], SU-CHIN LIU[3], and D. R. GRANT[4],
Washington State University, Pullman, Washington

ABSTRACT

The ratio of bound amido black dye to the actual protein content has been determined for seven groups of wheat flour protein bands separated by polyacrylamide gel electrophoresis. The starting materials used for the electrophoresis were aluminum lactate buffer extracts of flours from two wheat varieties, fractions from carboxymethyl cellulose chromatography, and more-or-less purified flour protein preparations. The bound dye was represented by densitometric peak areas and compared to the protein content as determined by amino acid analysis of the hydrolyzed gel bands. Some of the faster-moving proteins bound almost three times as much dye as the least mobile group. Corresponding band groups from different sources showed considerable variation in dye-binding capacity. Examination of the amino acid analyses shows that both mobility and dye-binding strength are positively correlated to the arginine and lysine content and negatively correlated to the glutamic acid content of flour proteins.

The proteins of wheat flour are readily separable into a large number of bands as a result of electrophoresis in starch or polyacrylamide gels; the bands are made visible by treatment of the gels with a dye which is bound by the proteins. Densitometry gives a graphic picture of the bands in the case of polyacrylamide gels, and the area under the peaks can be measured. However, densitometric measurement of the protein-bound dye does not allow a ready calculation of the actual protein content of the bands. Different proteins have very different capacities for binding dyes. Strickland and co-workers (1) have demonstrated that among 11 serum proteins, the range of dye-binding capacity for amido black 10B[5]

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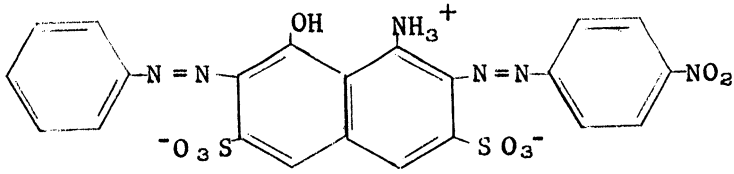
[2] Department of Agricultural Chemistry, Washington State University, Pullman, Washington.

[3]Department of Agricultural Biochemistry, University of Idaho, Moscow, Idaho.

[4]Department of Chemistry and Chemical Engineering, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

[5]This dye is known by many other names, among which are amidoschwarz 10B, naphthylamine black 10BR, naphthalene black 12B, aniline blue black, naphthol blue black, acid black 1, pontacyl blue black SX. It has been designated with C.I. No. 20470 and Schultz No. 299.

was from 0.21 to 0.39 mg. of dye per mg. of protein. This is an acidic dye with the chemical formula as shown below, so that one can expect the amount bound to be



related to the number of free basic groups of the protein (2). Since the amino acid compositions and, in particular, the lysine contents of different flour proteins are known to differ (3,4), there is likely to be at least as much variation in the dye-binding strength of wheat proteins as in serum proteins. Hence, any quantitative method for the analysis of the proteins in wheat flour that involves dye-binding to the proteins requires the use of conversion factors based on the amount of dye bound by the various proteins. The work described in this report was undertaken in order to complete the requirements for such an analytical method, based on polyacrylamide gel electrophoresis (5). The results yielded not only a measure of the real protein content of the electrophoretic bands, but also data of considerable interest on the amino acid compositions of wheat proteins of different mobility.

Determining these conversion factors is not a simple matter, particularly when the gels contain urea. Methods based on the ninhydrin reaction are not practical, since large amounts of ammonia are liberated from the gel during hydrolysis, and both urea and ammonia react with ninhydrin to give the characteristic color. Attempts to use the Folin-Lowry (6) method were frustrated by problems of turbidity. Ultraviolet absorption and biuret methods (7) were not sufficiently sensitive, and suffered from interferences. The method finally adopted was use of acid hydrolysis of gel pieces corresponding to protein bands, removal of most of the liberated ammonia by aeration, and summation of the amino acid constituents of the bands as determined with the amino acid analyzer.

MATERIALS AND METHODS

Proteins from Federation and Thatcher wheat flours were used. Federation is a soft white spring (SWS) wheat, and Thatcher is a HRS variety. The Federation sample was grown in a growth chamber; two samples of Thatcher were used, one grown in a growth chamber, the other grown in the field at Lind, Washington. The flours were extracted by stirring overnight at about 4°C. with 2.5 vols. of the 0.1 ionic strength aluminum lactate buffer described by Jones et al. (8), followed by ultracentrifugation. In such extracts, certain of the proteins are present in relatively small quantities. To obtain more accurate data, solutions containing a high proportion of these proteins were desirable. Such solutions were obtained by the carboxymethyl cellulose column fractionation methods of Gehrke and co-workers on the 0.005M acetic acid-soluble proteins (9) and the 0.1M NaCl-soluble proteins (10) of Federation flour. Although our elution curves did not match theirs particularly well, we obtained fractions which appeared to correspond to fraction 10 of their 0.005M acetic acid solubles and to fractions 4 and 6 of their 0.1M NaCl

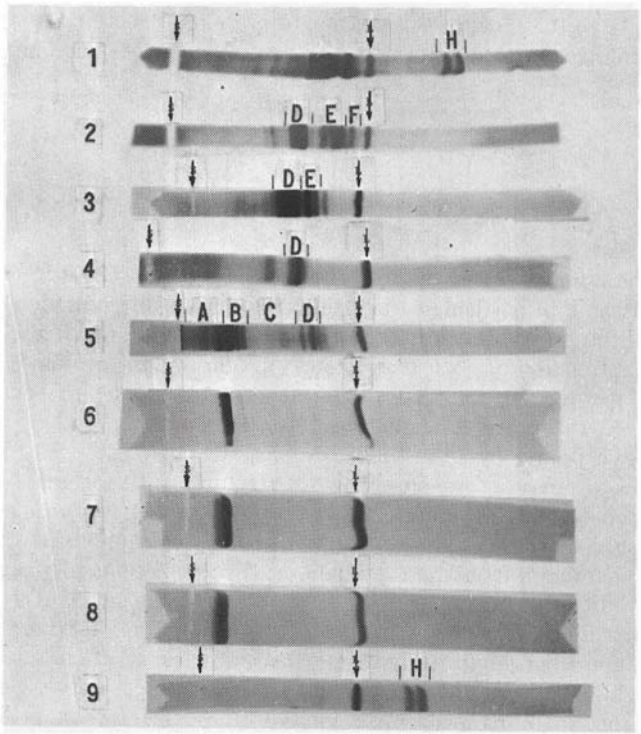


Fig. 1. Identification of gel electrophoresis strips: 1) Fraction IIIa from carboxymethyl cellulose chromatography of a 0.1M NaCl extract of Federation flour; 2) fraction IIIb from a similar procedure; 3) fraction II from a similar procedure; 4) fraction I from carboxymethyl cellulose chromatography of a 0.005M acetic acid extract; 5) extract of Thatcher flour; 6) alpha-gliadin; 7) beta-gliadin; 8) gamma-gliadin; and 9) free film electrophoresis fraction (13). Explanation of symbols: S with vertical arrow, sample starting slot; L with vertical arrow, lysozyme band.

solubles. We have named our fractions I, II, and III (a and b), respectively. The fractions were dialyzed against deionized water, lyophilized, and redissolved in aluminum lactate buffer. The concentrations were adjusted to give densitometry peaks of sufficient height to permit accurate measurements. The gel-electrophoretic patterns of the fractions used are shown in Fig. 1. Because of the uncertainty in the identification of individual bands in a gel, determination of the dye-binding capacity of such bands appeared impractical. Instead, the bands were grouped as indicated in Fig. 1, and a factor was determined for each group. The system used for designating the band groups has been discussed in the previous paper (5).

Through the courtesy of J. S. Wall, Northern Utilization Research and Development Division, Peoria, Illinois, samples of alpha-, beta-, and gamma-gliadin (11,12) from Ponca flour were obtained. A free-film electrophoresis fraction consisting of essentially only the two fast-moving bands of group H was kindly supplied by C. C. Nimmo of the Western Utilization Research and Development Division, USDA, Albany, California. The polyacrylamide gel electrophoretic patterns obtained for these samples are shown in Fig. 1.

The solutions in aluminum lactate buffer of whole flour proteins, of the

fast-band preparation, and of the fractions from carboxymethyl cellulose chromatography were mixed with lysozyme as an internal standard, and were subjected to polyacrylamide gel electrophoresis using 1.5 in. wide gel strips (5). The gels were cut lengthwise into equal halves, and one-half of each was dyed with amido black. The undyed halves were covered with polyethylene film and refrigerated until the dyed halves had been washed long enough to make the bands distinct (at most overnight). Both halves were then cut into sections as illustrated in Fig. 1. Peak areas were determined on the reassembled dyed portions with the position of the cuts showing up clearly on the densitometer recorder charts. Flour protein peak areas were corrected for variation in the volume of the mixture that had been placed in the slot by comparison with the peak area of the lysozyme internal standard, as described in the accompanying paper (5).

Corresponding undyed gel sections were pooled, homogenized, and hydrolyzed with 8 vols. of 5.6N HCl in an autoclave at 110°C. for 32 hr. The hydrolyzed material was cooled to about 5°C. and filtered. A suitable amount of norleucine was added to the filtrate and washings to serve as an internal standard. The combined solution was evaporated to dryness under vacuum, then redissolved; the pH was adjusted to 3.2, and the precipitate which formed was filtered out. The solution was then made alkaline, and nitrogen was bubbled through it at pH 9 to remove the ammonia. It was concentrated under vacuum to a suitable volume for amino acid analysis, the pH was adjusted to about 2, and the sample was added to the Technicon automatic amino acid analyzer.

Considerable beta-alanine, some glycine and alpha-alanine, and very small amounts of most of the other amino acids were found in the hydrolysate of blank gels. The protein amino acid analyses were corrected for the quantity of each amino acid derived from the gel.

The sum of the amino acids for a particular band group section was taken as the protein content without further correction, as it was assumed that water added by hydrolysis would approximate the amino acid destruction. The protein contents of the sections were then added together, and the results were compared with the amount of protein placed in the gel slots, as determined by Kjeldahl nitrogen analysis of the flour extract. Recoveries, after correction for norleucine loss, varied from 88 to 112% and averaged 102%. The recovery figure for each experiment was used as a correction factor for the protein content of each of the band group sections.

The alpha-, beta-, and gamma-gliadins were also dissolved in aluminum lactate buffer and subjected to gel electrophoresis. In these instances, all the protein was found in a single band or close group of bands, so that the protein content could be considered that of the solution placed in the slot as determined by Kjeldahl analysis. The dye absorption was determined in the usual way (5) by averaging three scans of the whole dyed gel.

RESULTS AND DISCUSSION

Results of the determinations of ratios of dye absorption to real protein content are given in Table I. Of course, the measure of dye color will be entirely dependent on the equipment used for densitometry, but since the peak areas are based on those of known amounts of added lysozyme, other workers should be able to obtain comparable results. The method for determining the real protein content of

TABLE I. DENSITOMETRIC PEAK AREAS[a] OF DYED GELS PER mg. OF FLOUR PROTEIN

Band Group	Source of Flour Protein						
	Extract from Federation Flour	Extracts from Two Thatcher Flours		Fractions from CMC Chromatography			Free Film Electrophoresis Fraction[b]
				I	II	III	
A	140	145	95				
B	220	215	140				
C	240	320	220		275		
D	330	300	325	280	310		
E	390					275[c]	
F						380[c]	
H						240[d]	320

[a]The peak areas are relative to an area of 14.7 for 14.3 γ of lysozyme N (by Kjeldahl) or 85.6 γ of lysozyme (Sigma, Grade 1).

[b]From C. C. Nimmo.

[c]From fraction IIIb.

[d]From fraction IIIa.

the band groups is not highly precise, but is probably as good as is justified in view of the fact that the proportion of individual proteins within a band group will vary from one flour sample to another. Furthermore, the proportions in fractions from chromatography are likely to deviate considerably from those of the corresponding groups from a whole-flour extract. Indications of such deviations can be seen in Fig. 1. If it is assumed that some variation in dye-binding capacity exists among the proteins within a group, considerable variability can be expected in the average dye-binding capacity for that group. Such variability is present in the ratios cited in Table I. Band group D, the base proteins of Oh and Gehrke (9), consists of a relatively small number of proteins, two of which are clearly predominant, and in this case the results are fairly consistent.

It is clear that some variation in dye-binding capacity exists within some of the groups that have been selected. Our first attempt at carboxymethyl cellulose chromatography of a 0.1M NaCl extract resulted in an elution pattern quite different from what has been reported (10). However, one fraction, IIIa, showed three strong bands in the area of the E and F groups, as well as a strong group H pair. The exact identification of the former three is uncertain, but ratios of densitometer peak areas per mg. of flour protein found (in order of increasing mobility) were 335, 600, and 345. In later chromatographic runs, we did not reproduce the same elution pattern, so that these figures could not be confirmed. With another type III chromatographic fraction, IIIb, ratios were estimated on each of the two strong group D bands separately, and figures of 180 and 315 were found for the slower and faster bands, respectively.

The beta- and gamma-gliadin samples fall in band group A, and alpha-gliadin is in band group B (Fig. 1). The peak area per mg. protein ratios we found were 75 for beta-gliadin, 90 for gamma-gliadin, and 100 for alpha-gliadin. These are distinctly lower than the ratios found for groups A and B as a whole. The explanation for the difference may be that additional proteins appear to be present in these band groups. We have on occasion detected as many as eight bands in group A; Woychik

et al. (11) report four components for beta-gliadin, and one each for gamma- and omega-gliadin. Gamma-gliadin has been resolved into three components, but not by gel electrophoresis (13). Band group B usually shows four components; alpha-gliadin is reported to have two (11) or, more recently, three components (14).

One can note in Table I that there is almost a threefold difference in dye-binding strength between the group A proteins and the groups E and F proteins. Also, one may observe a regular progression of increasing dye-binding strength with increasing mobility of the proteins, most clearly shown with Federation flour. This could reasonably have been expected, since the dye-binding strength (2) and electrophoretic mobility at pH 3.2 are both directly related to the number of free basic groups on the protein. The mobility is also inversely related to the size of the protein molecules, and this probably accounts for some of the deviation in proportionality between mobility and dye-binding power. In particular, the fast-moving group H proteins do not have an exceptionally high dye color per mg. of protein. Strickland et al. (1) found quite large standard deviations in the dye-binding capacity of corresponding serum proteins from six different persons. They took this as evidence of differences between the proteins. Analogous varietal differences in dye-binding capacity of flour proteins may be a factor here, in addition to the expected variation in the proportion of individual proteins within a band group.

It is apparent that no single value is adequate as a conversion factor to determine the real protein content of a band group, and the data here are insufficient to establish an average value. However, it is also clear that it is seriously misleading to consider the density of a band as a direct measure of its protein content, and the use of approximate conversion factors (5) will give a much improved estimation.

The amino acid composition of the proteins of the different band groups is given in Table II. Groups E and F are omitted because the results were of low

TABLE II. AMINO ACID COMPOSITION[a] OF GEL ELECTROPHORESIS BAND GROUPS

	Protein Band Group				
	A	B	C	D	H[b]
Aspartic acid	3.5	13.3	7.9	8.3	8.8
Threonine	5.4		5.9	6.2	9.6
Serine	2.7		5.7	6.8	6.0
Glutamic acid	44.8	35.5	22.0	14.1	6.8
Proline	10.8	12.4	5.7	6.3	3.6
Glycine	2.9	4.3	6.4	5.9	6.6
Alanine	2.6	3.5	6.5	6.8	7.0
Valine	3.5	5.9	5.7	7.1	2.6
Cystine	0.7	2.8	2.6		
Methionine	0.9		1.5	2.7	
Isoleucine	3.7	4.5	4.0	3.2	0.8
Leucine	6.2	7.4	7.0	7.5	7.4
Tyrosine	4.0	1.1	4.9	8.3	
Phenylalanine	3.0	3.2	2.5	3.7	
Lysine	1.2	1.3	3.8	4.4	9.8
Histidine	1.4	2.0	2.5	1.6	3.2
Arginine	2.3	3.0	6.1	7.8	11.7

[a]Percent of total.

[b]Free film electrophoresis fraction received from C. C. Nimmo.

precision because of the small amount of material. The primary purpose of these analyses, of course, was to determine the total amino acid content, rather than a precise determination of individual amino acids. The results for cystine and methionine would probably have been improved by a performate oxidation step. Those for tyrosine and phenylalanine are inexact because of interference by beta-alanine derived from the polyacrylamide during hydrolysis.

There have been no published amino acid analyses of fractions of wheat flour proteins that can be compared exactly with the gel-electrophoresis fractions analyzed here. Woychik et al. (3) have reported analyses of beta-, gamma-, and omega-gliadin, which would be included in our fraction A; of alpha$_2$-gliadin (later referred to as alpha$_1$ + alpha$_2$ (11)), which would be included in our fraction B; and of water-solubles, which would be distributed in some manner among our more mobile groups. Bernardin et al. (14) have analyzed a purified alpha-gliadin (alpha$_1$ + alpha$_2$) preparation, and Huebner et al. (13) have analyzed three purified gamma-gliadin preparations. The gliadin recently reported analyzed by Ewart (4) would presumably include both of our groups A and B. Sissakian and Markossian (15) have determined the amino acid content of gliadin, albumin (probably most similar to our groups C plus D), and globulin (presumably primarily our group H). Nimmo and co-workers have reported analyses on fractions from chromatography of water-soluble flour proteins (16), and on the rapidly migrating "globulin" preparation similar to that supplied to us, but more highly purified (17).

The most consistent and striking result of these analyses is the observation that with increasing mobility of the protein groups, the lysine, histidine, and arginine content increases, while the glutamic acid content—actually the glutamine content (4,17)—decreases. This is shown in Table III, which includes the corresponding figures for the most comparable preparations from the literature recalculated to percent of the total amino acids. It is clear that both mobility and dye-binding capacity are correlated with the basicity of the proteins derived from the basic amino acid content.

TABLE III. SUMMARY OF ARGININE, HISTIDINE, LYSINE, AND GLUTAMIC ACID CONTENTS[a] OF FLOUR PROTEINS

	Arginine	Histidine	Lysine	Glutamic Acid
A	2.3	1.4	1.2	44.8
β-Gliadin (3)	1.9	1.5	0.7	40.0
γ-Gliadin (13)	1.7-2.0	1.5-1.7	0-0.7	43.2-45.1
B	3.0	2.0	1.3	35.5
α-Gliadin (14)	2.7	2.6	0.6	42.5
α_2-Gliadin (3)	2.9	2.4	0.7	37.7
C	6.1	2.5	3.8	22.0
D	7.8	1.6	4.4	14.1
Albumins (15)	7.5-7.7	6.2-6.6	3.1-3.4	13.9-14.9
H	11.7	3.2	9.8	6.8
"Globulin" (17)	14.5	1.3	12.6	5.1

[a]Percent of total amino acids.

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