ISOLATION AND CHEMICAL COMPARISON OF DIFFERENT GAMMA-GLIADINS FROM HARD RED WINTER WHEAT FLOUR[1]

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ABSTRACT

By a combination of ion-exchange and gel-filtration chromatography, gamma-gliadin has been separated into three different components. Elution of gliadin from sulfoethyl cellulose (SEC) columns with a nonlinear sodium chloride gradient (0.01–0.3*M*) contained in 2*M* dimethylformamide-0.01*M* acetate buffer (pH 2.1) gave three peaks, each of which contained a component having a mobility in the range ascribed to gamma-gliadin on starch-gel electrophoresis. Contaminating beta-gliadins contained in two of the peaks were eliminated by subsequent chromatography on SEC in 5*M* urea at pH 2.5 and by gel filtration through a 200-cm. column of Sephadex G-50 in 0.05*M* acetic acid. The behavior of gamma-gliadin during SEC chromatography was due to heterogeneity of the protein fraction rather than to molecular aggregation. Separated gamma-gliadins migrated at slightly different rates upon electrophoresis in starch gel and differed significantly in content of lysine, tryptophan, tyrosine, phenylalanine, glutamic acid, and proline.

Classic gliadin, like the albumin, globulin, and glutenin fractions from wheat flour, is now generally considered to be composed of several proteins that have similar physicochemical properties. Jones *et al.* (1) first used moving-boundary electrophoresis to resolve wheat gliadin into four electrophoretically distinct components, alpha, beta, gamma, and omega, which were shown to have different amino acid compositions (2) but similar molecular weights (3).

In subsequent experiments, Woychik and co-workers (4) separated the respective alpha- and beta-gliadin fractions into two and four components by zone electrophoresis on starch gel. When the gamma-gliadin fraction was isolated, it appeared to be homogeneous by gel electrophoresis and end group analysis, but the molecular weight (MW) determined by approach to sedimentation equilibrium was higher than the minimum molecular weight calculated from the amino acid composition (5). In an investigation of the source of this disparity, recent improvements (6) in chromatography of wheat gliadin on sulfoethyl cellulose (SEC) made it possible to isolate three components from the gamma-gliadin fraction. These purified components are distinct in amino acid composition and differ slightly in electrophoretic mobility.

Materials and Methods

Reagents. Gliadin was prepared by acetic acid extraction of gluten from hard red winter wheat (variety Ponca) and fractionation of the extract in neutral 70% ethanol according to the method of Jones *et al.* (1). High-MW

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components in the gliadin were eliminated by gel filtration on Sephadex G-100 as described by Beckwith *et al.* (7).

N,N-Dimethylformamide (DMF) (Matheson Co., East Rutherford, N. J.) was used without purification. Urea (analytical reagent, J. T. Baker, Phillipsburg, N. J.) was further purified by solution in 0.01*N* acetic acid and filtration through a short column of SEC.

Ion-Exchange Chromatography. In the sodium form, SEC (Bio-Rad Laboratories, Richmond, Calif., 0.2 meq./g.) was freed of fines by being suspended in water and decanted three to four times. Chromatographic columns were packed with SEC in the sodium form and were equilibrated with initial buffers before the samples were applied. Chromatography on SEC was carried out as previously described (6) in 2*M* DMF, 0.03*N* acetic acid, 0.015*N* HCl pH 2.2 (buffer I), or 5*M* urea, 0.03*N* acetic acid, 0.005*N* HCl pH 3.5 (buffer II). Each buffer was prepared immediately before use. Nonlinear gradients of NaCl in these buffers were generated with a Buchler Varigrad mixer. Column effluent absorbance was monitored automatically at 280 mμ by a Vanguard recording flow spectrophotometer. Fractions were collected with a Technicon fraction collector equipped with a drop counting device. Alternatively, these operations were performed simultaneously with the aid of a Beckman Model 130 Spectrochrom, an automatic chromatographic apparatus.

Contents of tubes under each peak were combined and dialyzed exhaustively against 0.01*N* acetic acid before lyophilization.

Gel Filtration. Protein fractions were subjected to gel filtration on a 2.8-cm. $\times$ 200-cm. column of Sephadex G-50 in 0.05*N* acetic acid at room temperature with a flow rate of 2.5 ml./hr./cm.^{2} Protein, 50 to 100 mg., was dissolved in 2 ml. of buffer and applied to the column. Fractions of 7.5–8 ml. were collected mechanically without flow analysis to minimize mixing of components that migrated at only slightly different rates. The absorbance of each fraction was measured at 280 mμ with a Beckman DU spectrophotometer, and the desired fractions were combined and lyophilized. On a weight basis, the recovery was 95 to 100%.

Starch-Gel Electrophoresis. Starch-gel electrophoresis was carried out in 3*M* urea, 0.005*M* aluminum lactate, and 0.02*M* lactic acid buffer as described by Woychik *et al.* (4) and modified by Beckwith *et al.* (7). The buffer was kept at 4°C. and discarded if unused after a week. Portions (about 25 μl.) of 1–2% solutions of protein in the electrophoresis buffer were absorbed on small squares of filter paper and inserted into the gel for electrophoresis.

Amino Acid Analysis. Triplicate samples (about 1.5 mg.) of protein, thoroughly dialyzed against 0.01*N* acetic acid and lyophilized, were placed in 2-ml. hydrolysis vials, dissolved in 2 ml. of twice-distilled, constant-boiling HCl, frozen in solid carbon dioxide-acetone, evacuated, and allowed to melt *in vacuo* to remove dissolved air, flushed with nitrogen, re-evacuated, and sealed. The respective samples were then heated at 110°C. $\pm$ 1°C. for 24, 72, and 96 hr., after which they were evaporated to dryness, dissolved in water, evaporated twice more, and finally diluted with pH 2.2 citrate buffer. Analyses were carried out on a Phoenix Model K-8000 automatic amino acid analyzer

modified with the gradient elution system of Piez and Morris (8). Where ammonia prevented an accurate estimation of lysine, the basic amino acids were measured on a 15-cm. column by the method of Spackman *et al.* (9). Tryptophan was estimated by the colorimetric procedures of Spies and Chambers (10) and Opieńska-Blauth *et al.* (11).

N-Terminal Amino Acids. Ten milligrams of each gamma-gliadin sample was dinitrophenylated (DNP) as described by Fraenkel-Conrat *et al.* (12), then hydrolyzed, and the DNP-amino acids were identified by high-voltage electrophoresis in a Gilson Model D high-voltage electrophorator (Gilson Medical Electronics, Middleton, Wis.) on paper (Whatman No. 3) at pH 4.2 in 0.1*M* sodium acetate, 2,000 volts for 1.5 hr.

Experimental Work and Results

γ_1-*Gliadin.* Previous work demonstrated that wheat gliadin can be separated into several fractions by ion-exchange chromatography on SEC (6). In general, the components of gliadin were eluted in sequence from gamma-gliadin through the alpha-gliadins, but material that migrated like gamma-gliadin upon gel electrophoresis was also eluted with the beta-gliadins. A distribution of this sort might be due to aggregation or nonspecific association of the proteins; however, the consistency with which this distribution could be reproduced, even in the presence of higher concentrations of urea, makes such an explanation untenable. Most likely, gliadin contains more than one component that migrates with the mobility ascribed to gamma-gliadin during starch-gel electrophoresis. Accordingly, the fractions from chromatography on SEC that contained gamma-gliadin were analyzed individually.

Figure 1 shows the elution pattern when 1.5 g. of gliadin was chromatographed in buffer I on the Spectrochrom 130 with a nonlinear gradient

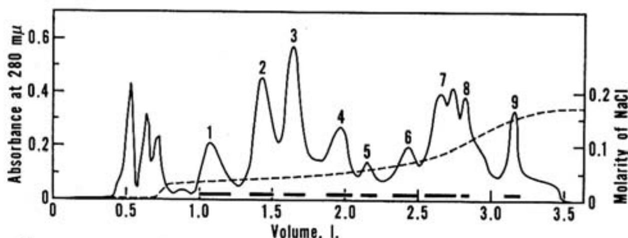

Fig. 1. Chromatographic serapation of gliadin on sulfoethyl cellulose (SEC). Column, 3.9 × 50 cm.; temp., 30°C. Buffer: 2*M* dimethylformamide (DMF), 0.03*N* acetic acid, 0.015*N* HCl, pH 2.2. Broken line shows salt gradient. Straight, dark lines along the abscissa indicate fractions that were pooled.

from 0.03*M* to 0.16*M* NaCl over 3.5 liters. Subsequent starch-gel electrophoresis (Fig. 2) showed that fractions 1–3 (Fig. 1) all contained material which migrated like gamma-gliadin. For convenience, these materials have been designated γ_1-, γ_2-, and γ_3-gliadin.

Since the protein in fraction 1 was not further resolved by additional attempts at fractionation, it was analyzed directly. Fractions 2 and 3 were purified further.

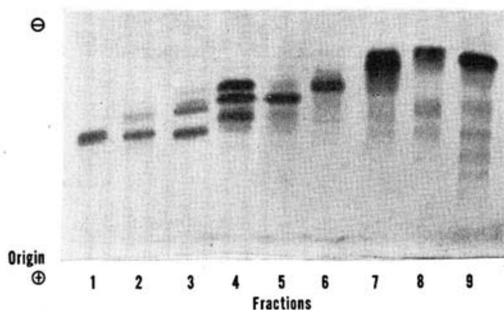

Fig. 2. Starch-gel electrophoretic pattern of gliadin fractions 1 to 9 from chromatography of gliadin on SEC (Fig. 1).

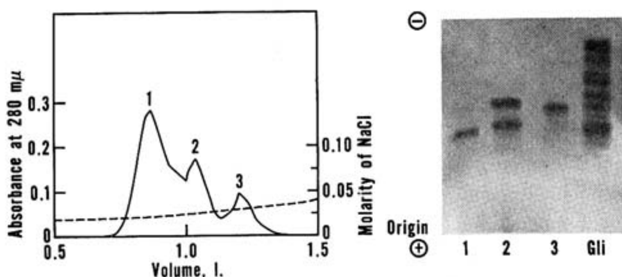

Fig. 3. Left, chromatographic separation of fraction 2 (Fig. 1) on SEC. Peaks numbered 1 to 3. Column, 2.8 × 50 cm. Buffer: 5*M* urea, 0.03*N* acetic acid, 0.005*N* HCl, pH 3.5. Broken line is salt gradient. Right, starch-gel electrophoretic pattern of fractions.

γ_2-Gliadin. Several variations in chromatography and gel filtration were tested before a successful method of resolving the gamma- and beta-gliadins in fraction 2 was obtained. Although gel filtration on Sephadex G-50 gave partial separation, better results were obtained by rechromatography of 400-mg. samples of fraction 2 on SEC at room temperature in buffer II with a nonlinear gradient from 0.017*M* to 0.05*M* NaCl over 1,600 ml. Figure 3 shows the separation obtained under these conditions. γ_2-Gliadin emerged first, followed by mixtures of γ_2- and beta-gliadins.

γ_3-Gliadin. In contrast to fraction 2, rechromatography of fraction 3 (Fig. 1) on SEC in buffer II failed to separate γ_3-gliadin from the beta-gliadins with which it was mixed. Upon gel filtration, however, the beta-gliadins were retarded enough to allow resolution of these proteins (Fig. 4c). Satisfactory separation of 50–100 mg. of protein was achieved in 0.05*N* acetic acid on a 2.8-cm. × 200-cm. column of Sephadex G-50. Resolution was improved by collecting small fractions (7.5–8 ml.) and using a slow flow rate (2.5 ml./hr./cm.^{2}) to allow complete equilibration of the proteins throughout the column. Higher flow rates failed to resolve the proteins.

Gel Filtration and Electrophoresis of Gamma-Gliadins. Gel filtration of

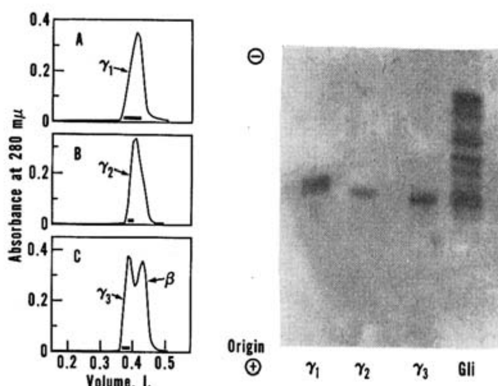

Fig. 4. Left, Sephadex G-50 filtration of (A) γ_1-gliadin, (B) γ_2-gliadin, and (C) a mixture of γ_3- and beta-gliadins. Dark line at bottom of peak shows fractions that were pooled before electrophoresis. Column, 2.6 × 200 cm. Buffer: 0.05*N* acetic acid. Right, starch-gel electrophoresis of purified gamma-gliadins and whole gliadin.

γ_1-gliadin, γ_2-gliadin, and a mixture of γ_3- and beta-gliadins on Sephadex G-50 produced the elution patterns given in Fig. 4. The γ_1-gliadin emerged from the column slightly later than γ_2- and γ_3-gliadin; however, the difference in mobility was not great enough to separate them when they were applied together. The similar behavior of the gamma-gliadins on Sephadex G-50 suggests that they may have similar molecular dimensions.

Differences in the ionic states of the three gamma-gliadins were more apparent. The gamma-gliadins exhibited different affinities for SEC and migrated at different rates during starch-gel electrophoresis when the electrophoresis was carried out for an extended period (Fig. 4). This difference in

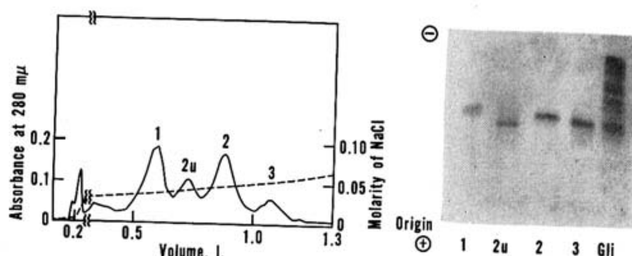

Fig. 5. Chromatography of three gamma-gliadins on SEC. Column, 2.8 × 50 cm. Buffer: 2*M* DMF, 0.03*N* acetic acid, 0.015*N* HCl, pH 2.2. Left, elution pattern; broken line shows salt gradient. Right, electrophoresis pattern.

electrophoretic mobility could be demonstrated with the three individual components, but was not usually seen with whole gliadin because separation was obscured by diffusion and by other proteins which migrated in the gamma- and beta-regions (fractions 7 to 9, Fig. 2).

Cole and Mecham (13) reported that cyanate in urea solutions produced artifacts during gel electrophoresis of proteins. The change in ionic state pro-

duced by carbamylation of side-chain amino groups caused a significant change in the mobility of the proteins. A similar formation of artifacts in urea solutions was also encountered with the gliadin proteins. Figure 5 presents the results of rechromatography of a mixture of γ_1-, γ_2-, and γ_3-gliadin on SEC in buffer I. Peaks 1, 2 and 3 represent, respectively, γ_1-, γ_2-, and γ_3-gliadin. Peak 2u, which arose after lyophilization of a solution containing urea, is the same as peak 2 by amino acid analysis. Apparently its charge had been changed sufficiently to allow it to separate both on the ion-exchange column and on the starch gel.

Although Stark *et al.* (14) pointed out that acid treatment removes cyanate from urea solutions, this method affords little control over ionic strength, a factor that is critical in the chromatography of wheat gliadin. Generally, work performed with freshly prepared DMF and urea buffers gave results with the same reproducibility, whereas old buffers more often produced artifacts or variable results. It would seem imperative that work involving proteins in urea buffers be performed in fresh, highly purified solutions, preferably at low temperatures.

N-Terminal Amino Acid. Previous work (5) showed aspartic acid to be the principal N-terminal amino acid in gamma-gliadin. In this work aspartic

TABLE I

Comparative Amino Acid Compositions of Gamma-Gliadins

Amino Acid	Residues per Minimal Molecular Weight					
	Calculated[a]			Nearest Even Integer		
	γ_1	γ_2	γ_3	γ_1	γ_2	γ_3
Lysine	trace	1.1	1.0	0	1	1
Histidine	2.1	2.0	2.2	2	2	2
Arginine	2.0	2.0	1.9	2	2	2
Aspartic acid	4.0	2.9	2.7	4	3	3
Threonine	2.7	2.9	3.4	3	3	3
Serine	7.5	7.6	7.0	8	8	7
Glutamic acid	55.5	56.5	61.7	56	57	62
Proline	21.3	26.8	29.4	21	27	29
Glycine	3.4	4.4	4.2	3	4	4
Alanine	4.7	4.4	5.2	5	4	5
½ Cystine	2.9	3.2	3.0	3[b]	3[b]	3[b]
Valine	6.1	6.8	6.5	6	7	7
Methionine	1.1	2.4	1.7	1	2	2
Isoleucine	6.5	7.4	7.1	7	7	7
Leucine	9.9	10.8	10.0	10	11	10
Tyrosine	3.9	1.0	0.6[c]	4	1	1
Phenylalanine	5.1	7.4	8.8	5	7	9
Tryptophan	0.3	1.1	1.2	0[b]	1	1
Minimal molecular weight				16,000	17,000	18,000

[a] The calculated figures represent at least triplicate analyses at three hydrolysis times: 24, 72, and 96 hr. Threonine and serine have been extrapolated to zero time. For valine and isoleucine only the 72- and 96-hr. hydrolysates were used. Values are molar ratios determined from amino acid analyses as described in the text.

[b] See text for explanation.

[c] From 24-hr. hydrolysates.

acid proved to be the N-terminal group in γ_2- and γ_3-gliadin. Under the same conditions, however, γ_1-gliadin failed to yield any DNP-amino acid.

Amino Acid Analysis. The relative amino acid compositions of the three gamma-gliadins are compared in Table I in terms of residues per minimum molecular weight. Serine and threonine, which were partially destroyed during prolonged hydrolysis, were estimated by extrapolation to zero hydrolysis time. Conversely, optimum values for valine and isoleucine were obtained only after hydrolysis for 72 and 96 hr. The residue values calculated for each gamma-gliadin were obtained by dividing averaged analysis results by a minimum weighted mean determined from the amounts of lysine (methionine in γ_1-gliadins), histidine, arginine, aspartic acid, glycine, alanine, leucine, and phenylalanine in each set of data. The levels of histidine, arginine, threonine, cystine, and isoleucine were the same in each of the three gamma-gliadins, but substantial variation was apparent in the other amino acids, particularly lysine and the aromatic amino acids. γ_1-Gliadin was devoid of lysine, whereas γ_2- and γ_3-gliadin each contained at least one residue per minimum calculated molecular weight.

There also was a similar distribution of tryptophan in the gamma-gliadins. On the basis of the procedure of Spies and Chambers (10), γ_2- and γ_3-gliadin each contained one residue of tryptophan per minimum MW. Under the same conditions, however, γ_1-gliadin gave an atypical yellow color. Since lesser amounts of this yellow color also developed in test solutions that did not contain *p*-dimethylaminobenzaldehyde, the chromophoric reagent of the Spies and Chambers method, it is doubtful that the 590-mμ absorbance observed with γ_1-gliadin should be attributed to tryptophan. Zein, which lacks tryptophan (15), gave a slight yellow color under the same conditions. An independent measurement of the tryptophan content of the three gamma-gliadins according to the procedure of Opieńska-Blauth *et al.* (11), with zein as the blank, corroborated results obtained with the Spies and Chambers method. Tryptophan values reported in Table I are averages of these two determinations.

Values for the ½ cystine content of the gamma-gliadins appeared anomalous in view of the demonstration by Beckwith *et al.* (16) that gliadin contains no free sulfhydryl groups. Possibly during hydrolysis there is a greater destruction of the sulfur-containing amino acids than can be accommodated by extrapolation of the data to zero hydrolysis time. Accordingly, two residues of cystine per minimum molecular weight would not be impossible. Alternatively, the value of only three ½ cystines may be evidence of a mixed disulfide in wheat gliadin. This discrepancy should be resolved by further structural analysis.

Also, some independent means may be necessary to demonstrate the significance of smaller differences between serine, glutamic acid, proline, alanine, leucine, and phenylalanine in γ_2- and γ_3-gliadin.

The similarity of the minimum MW's derived from the amino acid analyses (Table I) is consistent with the ultracentrifugal homogeneity of gamma-gliadin observed by Woychik and Huebner (5), and the similar behavior of the three gamma-gliadins during gel filtration on Sephadex G-100 and Sephadex G-50 (Fig. 4).

Discussion

In the absence of specific biological activities, the purification of the storage proteins from cereal grains has been assessed by following changes in physical dimensions or electrophoretic and chromatographic properties. An additional parameter, amino acid analysis, has become more useful with the advent of rapid automatic analytical techniques. Analysis of the gliadin components initially obtained from Ponca wheat flour gluten showed that each contained all the common amino acids, but that there were differences in relative composition (2). The results presented in this paper demonstrate that there are qualitative as well as quantitative differences in the amino acid composition of proteins from the gamma-gliadin fraction.

There may also be other differences in the primary structures of the wheat proteins. For example, the apparent lack of N-terminal aspartic acid in γ_1-gliadin is evidence of variation in the N-terminal region of these proteins. It remains for further investigation to determine whether this difference is the result of cyclization of an N-terminal residue of glutamine to pyrrolidone carboxylic acid, or the result of simple acylation of the terminal amino group as observed in some mammalian (17,18) and virus (19) proteins.

Woychik and Huebner (5), while studying the physical state of whole gamma-gliadin, obtained a value of 26,000 for the MW of the whole fraction and established that reduction and cyanoethylation of the proteins caused no change in this value. The minimal MW's based on amino acid composition of 16,000, 17,000, and 18,000 for γ_1-, γ_2-, and γ_3-gliadin, respectively, determined in the present studies, are not consistent with the MW of whole gamma-gliadin determined by Woychik and Huebner (5). However, the use of partial specific volumes determined from amino acid composition in calculating MW's from sedimentation data requires that both ultracentrifuge and composition analyses be obtained on the same material. The availability of pure gamma-gliadin proteins of known amino acid compositions will facilitate further studies of the physical properties of these proteins.

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