Comparison of Peptides from Wheat Gliadin and Glutenin[1]

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ABSTRACT

Peptic hydrolysis of gluten proteins is rapid at pH 1.8. Concomitant increases in alpha-amino groups suggest that peptides from gliadin and glutenin have respective average molecular weights (MW) of 1,990 and 1,150. Gel filtration, however, shows that the bulk of each digest contains peptides with apparent MW over 2,000. Size distribution of glutenin peptides is different from that of gliadin peptides. Evidently larger peptide segments occur between pepsin-susceptible bonds in glutenin than in gliadin. Additional pepsin-susceptible bonds are exposed in S-aminoethylgliadin, but reduction and S-aminoethylation produce little change in the digestion of glutenin. Similarities between peptide maps from cation- and anion-exchange chromatography of digests of gliadin, glutenin, and their derivatives suggest that most gluten proteins contain segments of polypeptide sequence that are similar or identical. Gliadin peptides eluted from cation-exchange resins at pH 1.8 are especially rich in glutamic acid and proline but are noticeably deficient in lysine, arginine, cystine, and methionine. Corresponding peptides from glutenin are also rich in glutamic acid and proline; in addition, they contain significant amounts of glycine and cystine. The greatest difference between gliadin and glutenin may reside in peptides that are largely ninhydrin-negative.

The prominence of wheat gluten proteins in human diets and their implication in certain unusual alimentary disorders have prompted several investigations (1,2,3,4) of products from enzymatic digests in attempts to correlate the physiological activity of gluten fragments with chemical structures. Other studies (5,6,7,8) involving enzymatic digestions of gluten and its fractions, glutenin and gliadin, have been directed toward elucidating reasons for the unique physical properties of wheat flour proteins.

In terms of commercial uses, the relationship of glutenin to gliadin is especially interesting because the protein fractions exhibit extremes of the viscoelastic properties that render whole gluten useful (9). Preliminary chromatographic studies by Rohrlich and Schulz (10) on peptic hydrolysates showed no characteristic differences between the alcohol-soluble (gliadin) and alcohol-insoluble (glutenin) proteins. Considerable similarity was also demonstrated by Ewart (11), who compared digests of glutenin and gliadin by the "fingerprint" technique.

Subsequent investigations corroborated these observations, but also emphasized the possibility of structural differences between glutenin and gliadin (12,13,14). Immunodiffusion studies by Escribano et al. (15) showed that reduced glutenin contains an antigen not present in gliadin. More recently, Rothfus and Crow (16) isolated from glutenin a fraction that represented 22% of the protein and that had chromatographic properties and an amino acid composition different from those of gliadin components.

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The evidence of unique components in glutenin prompted us to compare enzymatic digests of glutenin and gliadin and their derivatives by gel filtration and column chromatography on both cation- and anion-exchange resins in search of fragments that might identify structures which make the proteins different.

MATERIALS AND METHODS

Gliadin and glutenin were isolated from Ponca HRW wheat flour by acetic acid extraction of gluten and fractionation of the extract in 70% alcohol as described by Jones et al. (17). Low-molecular-weight (MW) glutenin was removed from gliadin by chromatography on Sephadex G-100 (Pharmacia, Inc., Uppsala, Sweden) as described by Beckwith et al. (18). Pepsin, twice crystallized, was a product of Worthington (Freehold, N.J.). Spectrophotometric-grade pyridine was used in chromatographic buffers; some lots were refluxed with ninhydrin (0.2% w./v.) for 1 hr. (19) and then distilled to remove ninhydrin-positive contaminants. Ninhydrin and hydrindantin were obtained from Pierce Chemical Co., Rockford, Ill. All other chemicals and reagents were analytical grade from commercial sources. Deionized water was used throughout.

Cation-exchange chromatography was performed at 38°C. on a 100 X 0.6-cm. column of Chromobeads P, a strongly acidic sulfonic acid cation-exchange resin (Technicon Corp., Tarrytown, N.Y.). Chromatograms were developed at 0.5 ml. per min. (300 to 400 p.s.i.) with a pyridine-acetate gradient (Table I, gradient 1) (19) produced by a nine-chambered Autograd, and were complete within 21 hr.

AG1-X8 (200- to 400-mesh, Bio-Rad Laboratories), a strongly basic resin containing quaternary ammonium groups, was used in a 0.9 X 60-cm. column for anion-exchange chromatography of peptides. The gradient developed for this system (Table I, gradient 2) incorporated a nonlinear decrease in pH and change of solvent from 0.2M pyridine to 50% acetic acid.

Column effluents were continuously monitored by a Technicon peptide analyzer

TABLE I. GRADIENTS USED FOR ION-EXCHANGE CHROMATOGRAPHY[a]

	Gradient 1 (Chromobeads P)			Gradient 2 (AG1-X8)				
Chamber No.	Buffer 1[b]	Buffer 2	Buffer 3	Buffer 4	Buffer 5	Buffer 6	Buffer 1	Buffer 7
1	90			90				
2	90			90				
3	60	30			90			
4	20	70			90			
5		90				90		
6		40	50			90		
7			90				90	
8			90				90	
9			90					90

[a]Values represent volume of buffers (ml.) in each chamber to produce gradient.

[b]Composition of buffers is as follows: 1) pH 3.1 pyridine-acetate, 0.20M pyridine; 2) pH 5.10 pyridine-acetate, 2.0M pyridine; 3) pH 6.5 pyridine-acetate, 2.0M pyridine; 4) 0.20M pyridine; 5) pH 6.5 pyridine-acetate, 0.20M pyridine; 6) pH 5.10 pyridine-acetate, 0.20M pyridine; 7) 50% acetic acid.

essentially as described by Catravas (20). The effluent was divided so that norleucine produced equal absorbance with ninhydrin with and without alkaline hydrolysis in 2.5M NaOH at 94°C. for 40 min. In some experiments, fractions of column effluent were collected, pyridine-containing buffers were removed by lyophilization, and recovery of peptide nitrogen was determined by semimicro Kjeldahl analysis.

Gel-filtration chromatography was performed on Sephadex G-25 (fine) (1.9 X 147 cm.); samples (10 to 15 mg.) were eluted with 1% acetic acid. Aminoethylation of gluten proteins was carried out as described by Rothfus and Crow (16). Peptide maps on paper were prepared as described by Katz et al. (21), but with a pH 1.9 electrophoresis-buffer (22). Peptides were located with ninhydrin-collidine reagent (23) and modified starch-iodide reagent (24). Amino acid analyses were performed as described by Spackman et al. (25) on a Phoenix Model K-8000 analyzer, modified for accelerated operation; results were computed automatically (26).

RESULTS

Hydrolysis of Gluten Proteins with Pepsin

At present, polypeptide structures of proteins are most readily compared in terms of products produced by enzymatic hydrolysis. Ideally, this digestion should yield fragments large enough to maintain some of the unique sequences present in the proteins, yet small enough to be easily resolved by available methods. In terms of specificity, number of peptides produced, and ease of peptide separation, pepsin was equal or superior to trypsin, chymotrypsin, papain, or pronase in digesting gliadin and glutenin.

Figure 1 shows the digestion of gliadin (0.1 mg. per ml. pH 1.8 HCl) by pepsin (2% w./w.) at 38°C. as followed with the Technicon peptide analyzer to detect an increase in ninhydrin color from free amino groups. Initially, there were 6.3 mmol. of free amino groups per 100 g. gliadin. After 24 hr., free amino groups had increased eightfold, and the reaction was essentially complete. Doubling enzyme concentration or reaction time had slight effect on the extent of reaction. Free

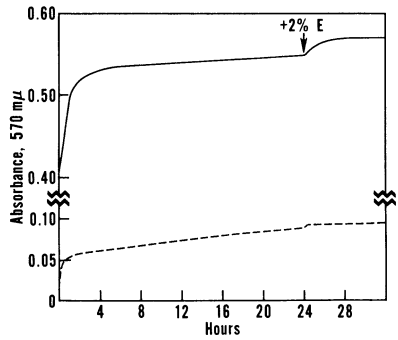

Fig. 1. Digestion of gliadin by pepsin. At 24 hr., pepsin concentration was increased to 4%. Solid line represents ninhydrin reaction after hydrolysis; broken line represents reaction with ninhydrin without hydrolysis. This convention is used in all figures.

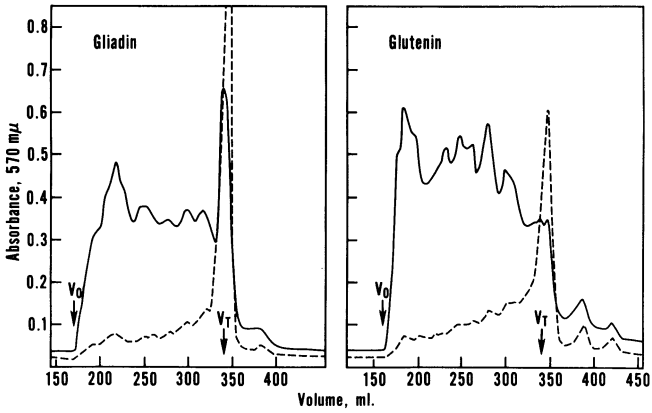

Fig. 2. Chromatographic separations of gliadin and glutenin peptic digests on Sephadex G-25. Exclusion volume (V_0) and bed volume (V_T) of the column are indicated on all G-25 chromatograms.

amino groups doubled at the initial rate in 3 min., and the digestion reached half completion in less than 1 hr. The rate after 24 hr. was less than 0.5% of that initially. The apparent increase in total amino groups with time (Fig. 1) indicates more complete alkaline hydrolysis in the analyzer as the peptides become smaller.

Difficulty in dissolving glutenin under these conditions prevented a similar detailed study of its initial rate of digestion, but additon of pepsin to a glutenin dispersion, as described above, solubilized the entire fraction within 15 min. The pH of these digests was monitored periodically, and adjusted when necessary with dilute HCl.

The extent of digestion of glutenin and gliadin and the average molecular weight for the peptides were estimated from the final concentration of free alpha-amino groups. On this basis, the average MW of glutenin peptides was 1,150, whereas for gliadin peptides it was 1,990. Digestion of S-aminoethylated (AE)-glutenin was similar to that of glutenin in terms of average MW of peptides in the digest. In contrast, the average MW of peptides from AE-gliadin was reduced to 1,550.

Comparison of Unmodified Proteins

The size distributions of peptides from gliadin (12.8 mg.) and glutenin (13.0 mg.) on Sephadex G-25 are indicated in Fig. 2. Exclusion volume (V_0) and bed volume (V_T) of the column were determined with gliadin and alanine, respectively. Both digests contain peptides distributed throughout the elution patterns. The apparent MW of peptides at V_0 may be more than 5,000 (27), whereas fragments that emerge near V_T are small (MW less than 1,000) and may include free amino acids. Presumably, alkaline destruction of some materials causes the hydrolyzed color to be less intense than the unhydrolyzed color. Assuming a linear relationship between elution volume and log MW (28), apparent MW of 1,270 to 4,160 can be calculated for gliadin peptides eluted before V_T, and 1,350 to 4,760 for peptides from glutenin.

Both Sephadex G-25 chromatograms are relatively complex, and there is no

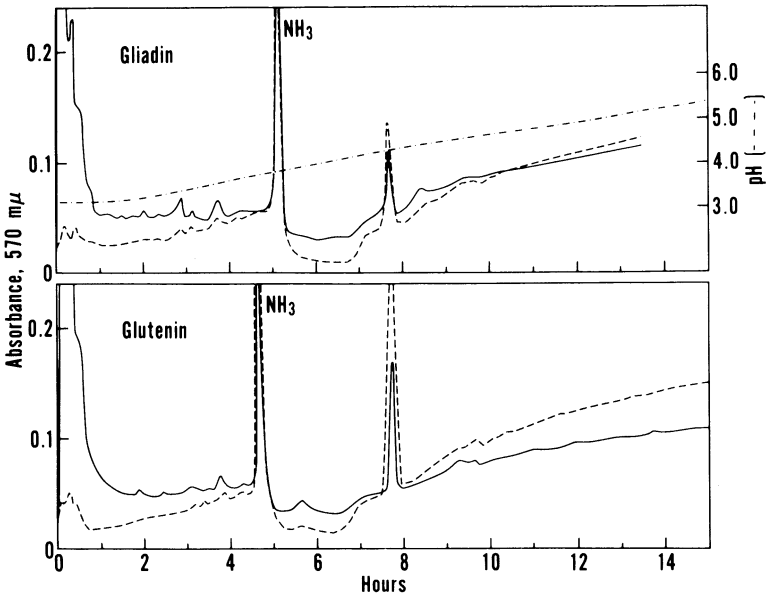

Fig. 3. Chromatographic separations of gliadin and glutenin peptic digests on Chromobeads P.

obvious correspondence between peaks in the two patterns. In both digests, substances retarded by the gel and which may contain aromatic amino acids (27) are present; some of these may be peptides, as indicated by increased reaction with ninhydrin after alkaline hydrolysis.

Figure 3 shows cation-exchange chromatograms of gliadin and glutenin peptic digests (3 mg. each) and the gradient used to produce them. The advantages of peptide mapping by column chromatography have been reviewed by Hill and Delaney (29). Distribution of peaks is similar in the chromatograms of both protein digests; several occur in corresponding positions. It is likely that peaks with similar elution times contain peptides with similar charge densities and adsorptive properties; intensity differences are probably due to variations in number of peptides per peak. Peptide mapping on paper has shown that peaks often contain more than one component. In addition to the similarities, there are notable differences between the elution patterns. For example, peaks are unique at 0.8, 1.2, 1.5, 6.45, and 8.4 hr. in the gliadin chromatogram and at 5.65, 11.0, 11.9, and 13.75 hr. in the glutenin elution pattern. The large peak between 4.5 and 5.5 hr. is ammonia. Blank analyses suggest that it is primarily a buffer contaminant. Base-line drift in the latter half of each tracing is caused by changes in buffer composition, which can be unequal in the hydrolyzed and unhydrolyzed streams. With the exception of minor differences due to variations in reagents or analyzer operation, all chromatograms are reproducible. Prolonged elution with 0.2M pyridine released no additional peptides, and recovery from the resin was quantitative when estimated by nitrogen determination.

As indicated by the tracings for hydrolyzed effluent in Fig. 3, a large part of each digest was eluted during the first hr. Peptides in this fraction are either highly acidic

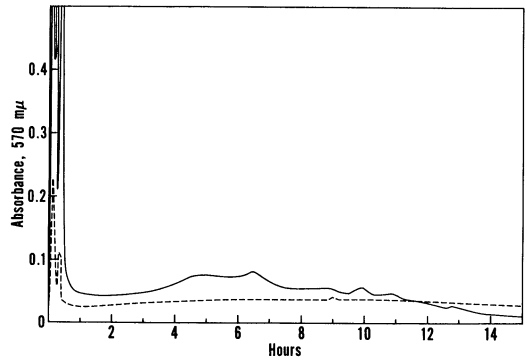

Fig. 4. Chromatographic separation on AG1-X8 of gliadin peptides eluted from Chromobeads P during the first hr.

or uncharged at pH 3.1, and may include N-pyrrolidone carboxyl peptides (4). Comparison of the ninhydrin color before and after hydrolysis shows that these fragments contain some free amino groups and are either large or rich in side-chain amides. It is unlikely, however, that they were excluded from the resin because of size, since even undigested gliadin and AE-glutenin can be fractionated by this method.

Nitrogen determinations indicated that 50 and 55%, respectively, of the total nitrogen in the gliadin and glutenin digests were eluted during the first hr. These nitrogen values, however, include side-chain amide as well as peptide nitrogen and may not give a true picture of the distribution of peptides in each chromatogram.

The gliadin peptides eluted during the first hr. from Chromobeads P (Fig. 3) were partially resolved by anion-exchange chromatography (Fig. 4). This fraction contains an initial region of uncharged peptides, followed by a series of acidic ninhydrin-negative peptides.

Anion-exchange chromatograms of gliadin and glutenin peptic digests (10.0 mg. each), and the gradient used, are shown in Fig. 5. The chromatograms are very similar: 15 peaks, of the 16 and 20 occurring in the glutenin and gliadin chromatograms, respectively, have similar elution times. The similarity between chromatograms is more apparent when the tracings for unhydrolyzed effluent are compared. Differences in peak intensity in corresponding positions may be due to variation in number of peptides per peak.

Comparison of S-Beta-Aminoethylated Proteins

Peptic digests of AE-gliadin (14.0 mg.) and AE-glutenin (13.3 mg.) were also compared by Sephadex G-25 chromatography (Fig. 6). Like the patterns of digests of the unmodified proteins (Fig. 2), there is no obvious equivalence between sizes of peptides indicated in these two complex chromatograms. Apparent MW's of 1,150 to 4,180 can be calculated for peptides eluted before V_T from gliadin, and 1,180 to 4,700 for those from glutenin.

Cleavage of AE-gliadin by pepsin is much different from digestion of unmodified gliadin (Fig. 2). In the AE-gliadin chromatogram, peptides eluted at 189, 214, and 223 ml. (apparent MW 3,050 to 4,180) are present in much smaller quantities, and

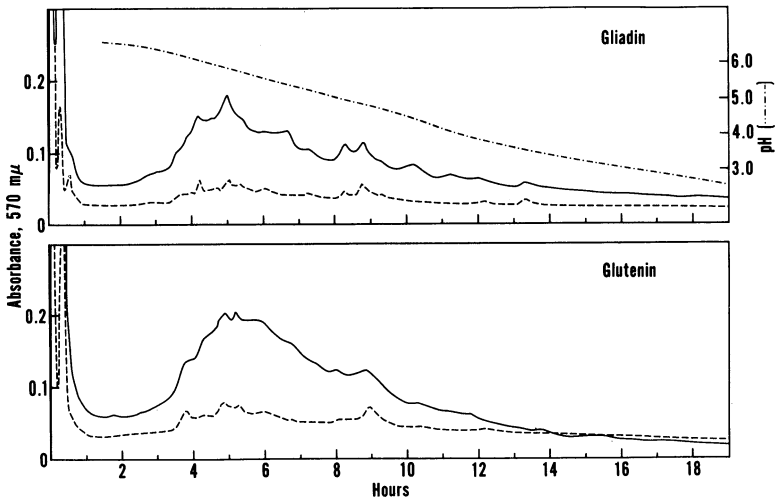

Fig. 5. Chromatographic separations of gliadin and glutenin peptic digests on AG1-X8.

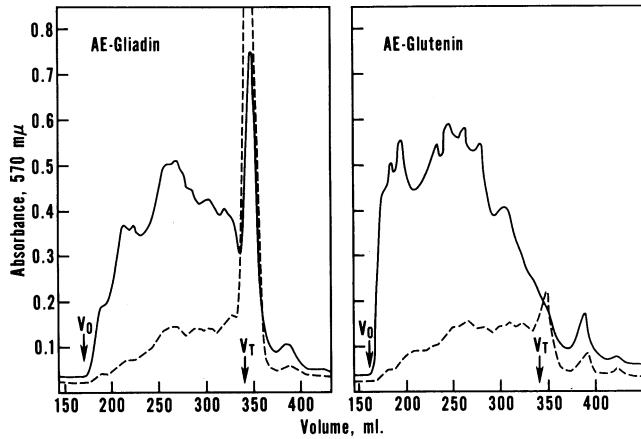

Fig. 6. Chromatographic separations of AE-gliadin and AE-glutenin peptic digests on Sephadex G-25.

peptides of MW 1,450 to 2,240 (elution volumes of 255, 268, 285, and 301 ml.) are present in greater amounts than in digests of unmodified gliadin.

Changes in the action of pepsin on AE-glutenin as compared to unmodified glutenin are smaller than with the analogous gliadin proteins. In the AE-glutenin chromatogram, peaks at 178 and 186 ml. (apparent MW's 4,350 and 4,700) were reduced slightly and those with apparent MW's of 2,130 and 2,450 at 247 and 262 ml. increased only slightly. An obvious difference between the two patterns in Fig. 6 is the lesser amount of material eluted near V_T in the AE-glutenin digest. Elution

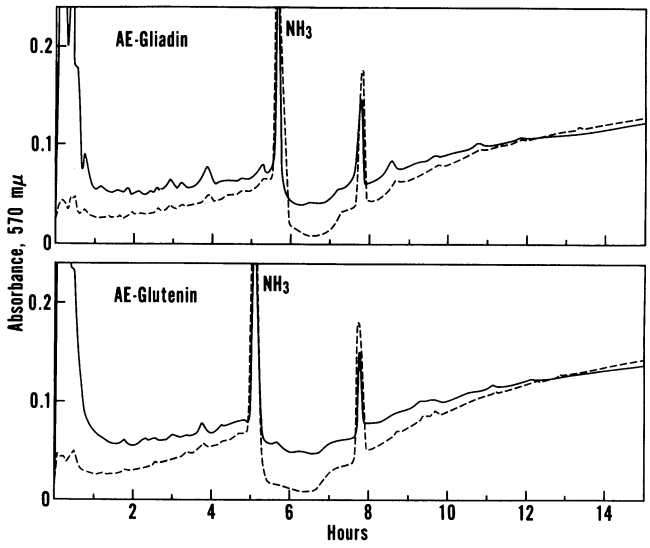

Fig. 7. Chromatographic separations of AE-gliadin and AE-glutenin peptic digests on Chromobeads P.

patterns from chromatography of other peptic digests of AE-glutenin have often contained a large peak at V_T, yet in other respects have been quite similar to the pattern in Fig. 6. Until the cause of this disparity can be identified, it would be premature to attribute any significance to differences at V_T between the patterns for AE-glutenin and those for AE-gliadin.

The relation between AE-gliadin and AE-glutenin digests (3.0 mg.) can also be seen from the cation-exchange chromatograms in Fig. 7. Like the patterns of unmodified gliadin and glutenin digests (Fig. 3), these chromatograms are very similar in number and position of peaks. However, peaks not occurring in chromatograms of peptides from the unmodified proteins are now present. In AE-gliadin new peaks occur at 0.75, 2.6, 5.3, 10.75, 11.9, and 13.3 hr., and in AE-glutenin at 2.6, 6.25, and 8.7 hr. The greater change noted in the AE-glutenin digest is additional evidence of the significantly different digestion of unmodified and modified gliadin by pepsin (Fig. 6).

Acidic Peptide Fractions

Cation-exchange resins fail to adsorb some gliadin and glutenin peptides even at low pH's. These peptides were separated on Chromobeads P by elution with pH 1.8 acetic acid. Amino acid compositions of the "pH 1.8" fractions are given in Table II. The peptides are enriched in glutamic acid and proline. Together these amino acids account for 78 and 63%, respectively, of the total residues in the pH 1.8 gliadin and glutenin fractions. In the same respective acidic fractions, glycine contents are 2 and 12%. Cystine-containing peptides absent in the acidic fraction from gliadin are present in this fraction from glutenin. On a nitrogen basis, 32% of the gliadin digest and 31% of the glutenin digest are in the acidic fractions. Two alternative methods, which consider side-chain amide nitrogen, support this

TABLE II. AMINO ACID ANALYSES OF PEPTIDE FRACTIONS FROM GLUTEN PROTEINS[a]

Amino Acid	Gliadin			Glutenin	
	Whole Digest	pH 1.8 Fraction	Ninhydrin-Negative	Whole Digest	pH 1.8 Fraction
Lysine	0.7	0.1	0.0	1.2	0.1
Histidine	1.8	0.2	0.0	1.4	0.2
Arginine	1.9	0.1	0.0	2.3	0.2
Aspartic acid	3.2	0.7	0.6	2.7	0.5
Threonine	2.5	1.2	1.6	3.3	2.1
Serine	4.1	2.9	3.7	7.0	6.7
Glutamic acid	37.0	60.4	57.0	33.5	45.4
Proline	14.7	17.6	15.2	13.0	17.4
Glycine	3.7	1.9	2.5	9.6	12.2
Alanine	3.3	0.8	1.0	3.8	1.5
1/2-Cystine	2.0	0.0	0.0	1.5	3.3
Valine	4.6	1.5	1.6	4.1	0.0
Methionine	1.2	0.0	0.1	1.3	0.3
Isoleucine	5.0	1.7	2.2	2.9	1.3
Leucine	8.2	5.1	8.7	6.4	3.4
Tyrosine	2.3	0.8	0.5	3.1	2.1
Phenylalanine	3.9	5.1	5.3	3.0	3.3

[a]Values represent percentage composition on a molar basis.

estimate of how material in each digest is distributed. Amino acid analyses show ammonia equal to 56.5% of total glutamic acid (on a molar basis) in the pH 1.8 fraction of gliadin and 67.5% of total glutamic acid in the pH 1.8 fraction of glutenin. If this ammonia represents the total glutamine content of the fractions, it can be calculated that 32.7 and 32.0% of the gliadin and glutenin digests, respectively, are eluted at pH 1.8. Alternatively, if the glutamic acid residues in the pH 1.8 fractions and the remainder of the digests are assumed to be either 0 or 100% glutamine, the limits of the proportions of the digests occurring in the pH 1.8 fractions are 29.1 to 36.6% for gliadin and 27.1 to 34.4% for glutenin.

Peptide maps of the pH 1.8 gliadin fraction on paper reveal several ninhydrin-negative peptides, which do not migrate at pH 1.9 upon electrophoresis but separate by chromatography. These peptides are separated from the rest of the pH 1.8 gliadin fraction by paper electrophoresis. Amino acid analyses (Table II) show that the ninhydrin-negative peptides are noticeably deficient in lysine, histidine, arginine, aspartic acid, cystine, methionine, and tyrosine but are particularly rich in glutamic acid and proline; the last two amino acids account for 62% of all residues. Anion-exchange and Sephadex G-25 chromatograms of this ninhydrin-negative peptide fraction are shown in Figs. 8 and 9, respectively. The fraction contains several peptides of varying size and charge. Presumably the ninhydrin-positive peaks are ammonia or traces of free amino acid contaminants acquired during separation of the fraction. The relatively low intensity of the hydrolyzed effluent at V_T in Fig. 9 makes it doubtful that significant amounts of free pyroglutamic acid are present.

DISCUSSION

The diverse physical properties displayed by gliadin and glutenin suggest, *a priori*,

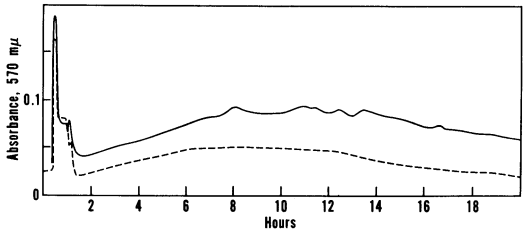

Fig. 8. Chromatographic separation on AG1-X8 of ninhydrin-negative peptide fraction from a gliadin peptic digest.

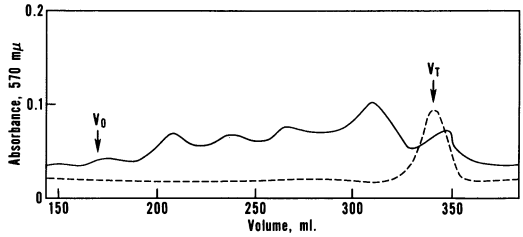

Fig. 9. Chromatographic separation on Sephadex G-25 of ninhydrin-negative peptide fraction from a gliadin peptic digest.

that these proteins contain significantly different chemical structures. Indeed, some of the differences have been attributed to different disulfide bond structures (12). Physical studies suggest that gliadins are fairly compact single-chain molecules (30), whereas glutenins appear to be multichain complexes stabilized by disulfide bonds (13). Our data, which show that pepsin digests AE-gliadin more readily than unmodified gliadin, offer additional evidence of a compact conformation in native gliadin. Because pepsin demonstrates little preference for AE-glutenin over unmodified glutenin, either glutenin proteins have amino acid sequences different from those in gliadin or else glutenin proteins are maintained, through some unusual disulfide structure, in conformations that render them more susceptible to peptic digestion.

Considering the present state of knowledge, some credence probably should be given to both these possibilities. Certainly if digests of AE-glutenin contain larger fragments than occur in digests of AE-gliadin (Fig. 6), there is evidence for differences in polypeptide structure. Nevertheless, structural similarity which has been demonstrated in many families of proteins (31) by minor differences between peptide maps and by amino acid sequence analysis, is also apparent in gliadin and glutenin. The correspondence among peptide maps from ion-exchange chromatography of digests of gliadin, glutenin, and their derivatives (Figs. 3, 5, and 7) suggests that most gluten proteins contain segments of polypeptide sequence that are similar if not identical, as hypothesized by Ewart (11). Differences between peptide maps could arise from segments in which amino acid sequences vary between polypeptide chains. A comparison of peptide maps from individual gliadin

components (32) is consistent with the general structural concept, which allows individual gluten components some unique identity. Given structures that contain both variant and invariant sequences, it is apparent why gluten proteins are difficult to resolve. It is also conceivable how Rothfus and Crow (16) could isolate a unique fraction accounting for 22% of glutenin, but we were unable to find a large proportion of fragments obviously different from those in gliadin.

Peptide size is the most striking difference between glutenin and gliadin that can be attributed to structures other than disulfide bonds (Figs. 2 and 6). At present, it is uncertain what type of peptide structure is responsible for the relatively large fragments in digests of AE-glutenin. Whether they are due to entirely unique proteins or to some novel cross-linking of proteins that are like gliadin will be decided by further investigation. Several observations suggest that the difference in fragment size is due to variations in the ninhydrin-negative peptides. First, the large peptides in Fig. 6 are essentially ninhydrin-negative. Second, peptide maps from anion-exchange chromatography of glutenin and gliadin digests differ most in their tracings for hydrolyzed effluent. Third, peptides eluted during the first hour of cation-exchange chromatography (Fig. 3) give distinct tracings for hydrolyzed ninhydrin color but very little unhydrolyzed color when chromatographed on the anion-exchange column (Figs. 4 and 8). Fourth, ninhydrin-negative peptides are distributed throughout a broad range of sizes by Sephadex (Fig. 9) and, thus, could affect the gel-filtration patterns of glutenin and gliadin digests. In the light of these considerations, the amino acid compositions of pH 1.8 fractions from cation-exchange columns are especially interesting. About one-third of both gliadin and glutenin are represented in these fractions, yet the peptides from gliadin are noticeably deficient in cystine and methionine, whereas glutenin peptides contain cystine and large amounts of glycine. This different distribution of cystine appears significant in terms of the greater tendency of glutenin to form intermolecular disulfide bonds (13). It may also be responsible for the differences that are apparent in chromatograms of AE-glutenin and AE-gliadin digests (Fig. 7). Most changes produced by aminoethylation are consistent with the possibility of different cysteine peptides in glutenin and gliadin. The new peak that appears at 2.6 hr. in both digests suggests, however, that at least one cysteine fragment may occur in both groups of proteins.

Acknowledgments

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