On the Solubility of Gliadinlike Proteins. IV. Characterization of Some Gamma-Gliadins[1]

PETER MEREDITH, Wheat Research Institute, Christchurch, New Zealand

ABSTRACT

Extracts of commercial wheat gluten have been fractionated by chromatography on carboxymethylcellulose to yield separated beta- and gamma-gliadin components. At least nine gamma-gliadins were observed, and each had one characteristic band in starch gel-urea electrophoresis. The triplet nature of the separated gliadins could be a reflection of the three genomes of the genetic constitution of bread wheat. Gamma-one gliadins are characterized by a high tyrosine:tryptophan ratio. Gamma-two and -three gliadins have distinctive phase separation behavior in aqueous salt-acid media.

Previous studies (1) showed a tendency of the gliadinlike proteins of wheat flour to form two liquid phases in aqueous acid-salt solutions. Phase diagrams plotted for such systems are complex, as the example in Fig. 1

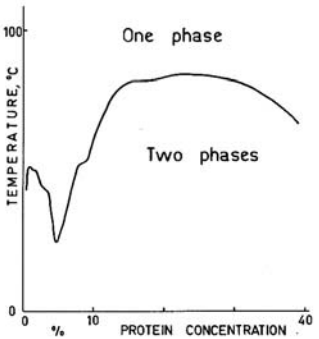

Fig. 1. Diagram of protein concentration against temperature for incipient phase separation in the system hydrochloric acid 20 m*M*/liter, sodium chloride 30 m*M*/liter, water, protein, for an acid-soluble preparation.

shows, and a variety of explanations for this complexity were suggested. It was concluded that further fractionation of the multicomponent protein mixture is necessary to distinguish between some of the possible explanations of the solubility behavior.

Gluten proteins may be fractionated by solubility, by ion-exchange chromatography, by gel-exclusion chromatography, and by various forms of electrophoresis. Electrophoresis has generally proved more useful as an analytical tool than as a method of separation *per se*. Solubility methods have enabled rough, large-scale groupings of components to be made, though fine separations would be impractical.

Thus some form of chromatography, so far, offers the best possibility

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of separating components of wheat gluten. The pioneer work of Woychik *et al.* (2) used carboxymethylcellulose (CMC) as an ion-exchanging adsorbant, with stepwise lowering of pH to elute distinguishable fractions. Similar adsorbant was used by Simmons and Winzor (3), but elution was done by increasing salt concentration in buffer containing dimethylformamide (DMF). Wrigley (4) further modified the technique by use of high temperature to reduce molecular interactions.

The present work has applied this last method of ion-exchange chromatography to fractions of gluten proteins obtained by solubility techniques. The success of the chromatographic fractionation has been assessed by starch-gel electrophoresis. Peak material obtained by chromatography has been examined by the phase-diagram technique, in an attempt to establish the reasons for the maxima in the diagrams.

To avoid confusion, in this paper the term *fraction* will be reserved for preparations by solubility and precipitation techniques, *peak* will denote chromatographically distinct material, and *band* will be used to describe material distinguishable by starch-gel electrophoresis. Rather than add a further system of nomenclature to the already confused literature, components will be described according to the Peoria system (5,6).

MATERIALS AND METHODS

A bulk of commercial dry glutens of good baking quality was fractionated crudely by techniques developed in earlier papers of this series (7,8). The glutens were manufactured in New Zealand from bakers'-quality flours derived from the two wheat varieties Aotea and Hilgendorf.

Acid-soluble fraction was prepared by gently stirring 1 kg. dry gluten with 6 liters 0.01*N* formic acid for 0.5 hr., then centrifuging. The supernatant was neutralized with sodium hydroxide, kept at 2°C. overnight, and decanted. The precipitate was redissolved by acidifying with *N* formic acid, dialyzed against several changes of distilled water, and freeze-dried.

Precipitated fraction was prepared by refluxing 200 g. of the acid-soluble preparation with 400 ml. methanol and 600 ml. chloroform. The clear filtrate was diluted with additional methanol. The first precipitate, at 52% methanol, was discarded. The second precipitate, at 80% methanol, was retained, washed with methanol and acetone, and vacuum-dried.

Properties of such preparations in terms of Tiselius electrophoresis patterns in aluminum lactate buffer (5) have been described previously (8).

Reagents were of analytical grade. All solvents were redistilled after appropriate treatments. DMF was purchased from L. Light and Co. and was redistilled. Starch for electrophoresis was from Connaught Laboratories, Toronto. CMC was Whatman CM 70.

Chromatography was carried out by the method of Simmons and Winzor (3), mainly as modified by Wrigley (4) but further modified by using a concave salt gradient initially. This gives better discrimination for that part of the elution pattern in which we are particularly interested. The column, 25 × 700 mm., maintained at 40°C., contained 90 g. CMC and handled 5 g. protein. A three-chamber eluant mixing system was used, the

first two chambers containing 6 liters and 3 liters of salt-free buffer; the third chamber contained 2 liters of buffer having 0.2*M* concentration of sodium chloride. Buffer was 0.01*M* sodium hydroxide neutralized with lactic acid to pH 4.1 and containing 1 molar DMF. The gradient was concave from 0 to 0.15*M* sodium chloride; thereafter a linear gradient from 0.15 to 0.35*M* sodium chloride was applied. A final gradient to 0.10*M* sodium hydroxide was used. Successive samples of approximately 15 ml. were collected on a time basis at about 1 ml. per min.

The elution pattern was determined by measurement of light absorption of samples at 280 mμ. In peaks 17 through 24 there was some turbidity, and readings were made after adjustment to pH 10–11 to give clear solutions. For fractions at maxima of the elution curve, absorption at 294 mμ was also measured. From absorption at the two wave lengths an apparent tyrosine:tryptophan ratio has been calculated (9). Some eluted samples contained yellow-brown pigment. The amount of this was determined by photometry at 350 mμ in alkaline solution.

Salt concentrations in effluent from the chromatographic columns were determined by flame-photometric analysis of sodium content.

Eluted samples were combined according to the elution pattern, neutralized, and concentrated by pervaporation. They were dialyzed against water, against 0.01*N* formic acid, and against many changes of distilled water, at 2°C., then freeze-dried.

Electrophoresis in starch gels was carried out in a plastic tray similar to that described by Cluskey (10). The gel technique, using 2*M* urea in aluminum lactate buffer, was carried out in the manner of Graham (11). Runs were made for 30 or 60 hr. at measured potential difference along the gel of 1.65 volt/cm. Staining with a dilute solution of water-soluble nigrosin was done according to Elton and Ewart (12). A very old sample of the dye gave results much superior to those given by one recently purchased. Photometry of the stained gels was carried out with a Chromoscan instrument (Joyce, Loebl and Co. Ltd., Gateshead-on-Tyne, England).

Phase diagrams were plotted by the technique previously described (1), with buffer 20 m*M* in hydrochloric acid and 30 m*M* in sodium chloride. Protein concentrations in excess of 40% were not studied.

Critical examination of the phase diagrams was made using partial dilution with a solution containing 30 m*M* hydrochloric acid and 30 m*M* sodium chloride, then continuing the dilution with either 30m*M* hydrochloric acid or 30 m*M* sodium chloride or water in order to judge the separate effects of acid, salt, and water at different protein concentrations.

RESULTS

Two chromatographic separations were made; the first of "acid-soluble fraction" and the second of "precipitated fraction" (Fig. 2). Eluted peak samples were also classified on the basis of their apparent tyrosine:tryptophan ratios (Fig. 3).

As so many peaks had been observed in the separation, it was obviously desirable to apply some other form of separation to the eluted peak material

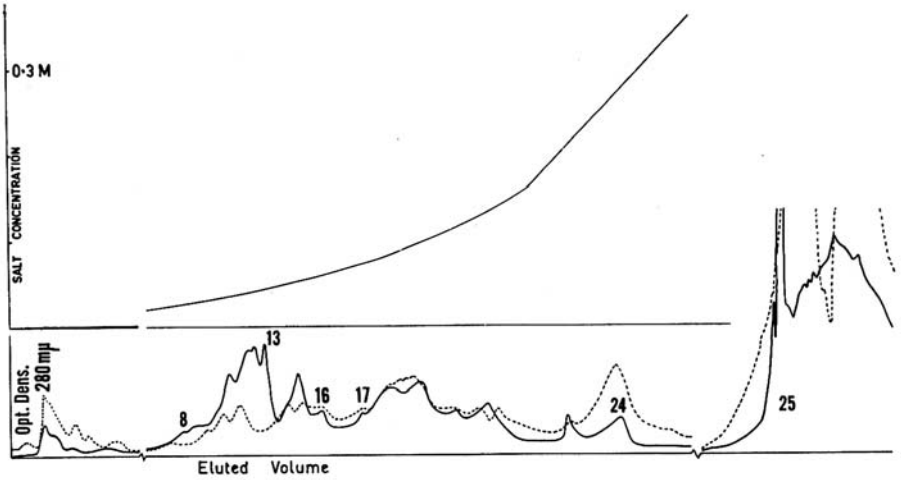

Fig. 2. *Lower,* elution curves of two preparations from CMC column; dotted curve, acid-soluble fraction; full curve, precipitated fraction. *Upper,* sodium chloride concentration in effluent. The final elution was by a gradient to 0.1*M* sodium hydroxide.

as a check on how nearly the peaks approached single components. Gel electrophoresis is one of the more sensitive methods at present available, and was used to follow the pattern of purification from the acid-soluble extract to a single peak of the chromatographic separation. Photographs of such starch

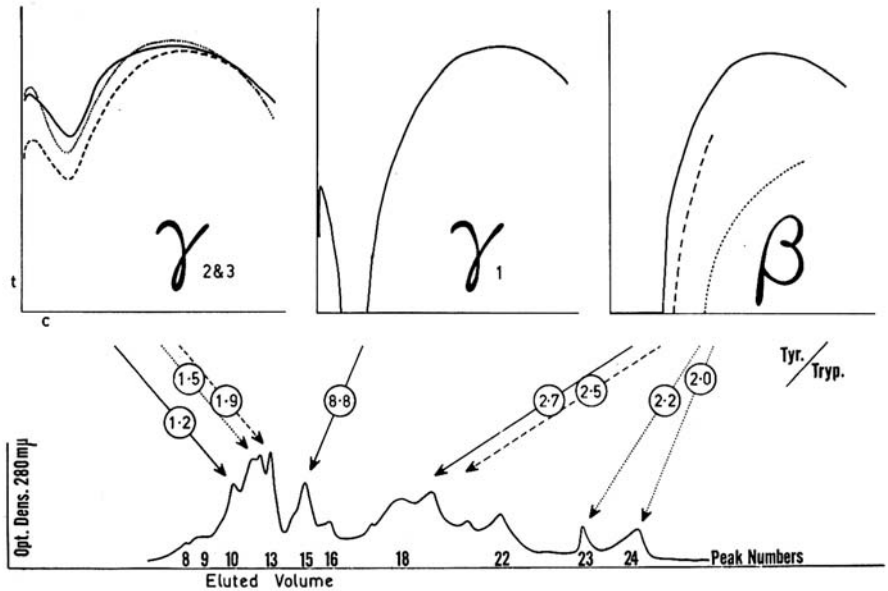

Fig. 3. *Lower,* elution curve of precipitated fraction from CMC column. *Middle,* apparent tyrosine:tryptophan ratios of peaks indicated. *Upper,* phase diagrams for material of peaks indicated.

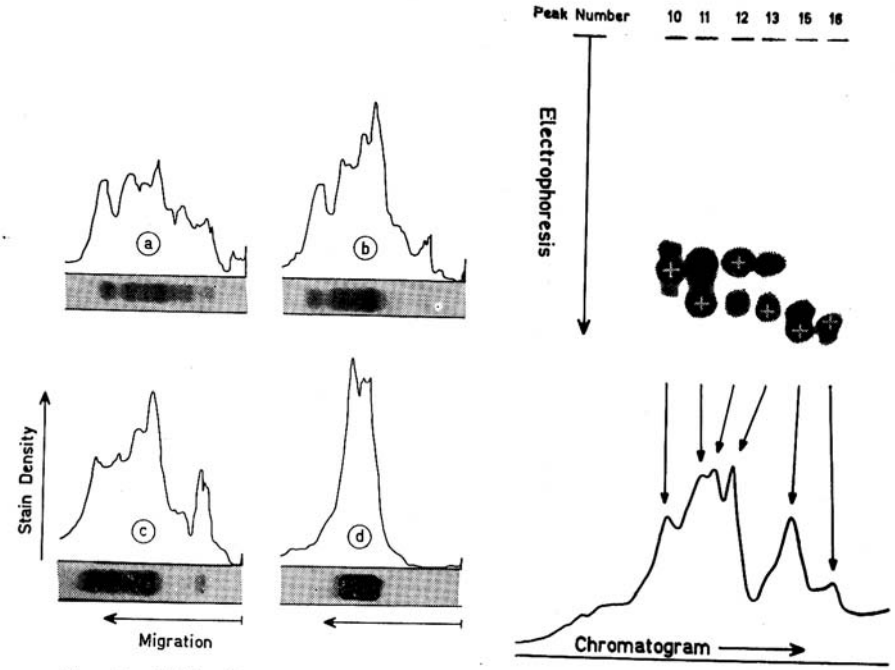

Fig. 4. (left). Starch-gel-electrophoresis patterns at 30 hr., and corresponding photometer tracings. Migration from slots at right-hand edges. (a) "acid-soluble fraction"; (b) fraction soluble in methanol-chloroform; (c) "precipitated fraction"; (d) material of peak 11.

Fig. 5 (right). *Lower,* portion of elution curve showing gamma-gliadin peaks. *Upper,* starch-gel-electrophoresis patterns, at 60 hr., corresponding to the chromatographic peak materials. Migration from slots at top. The band characteristic for each peak is marked with a white cross.

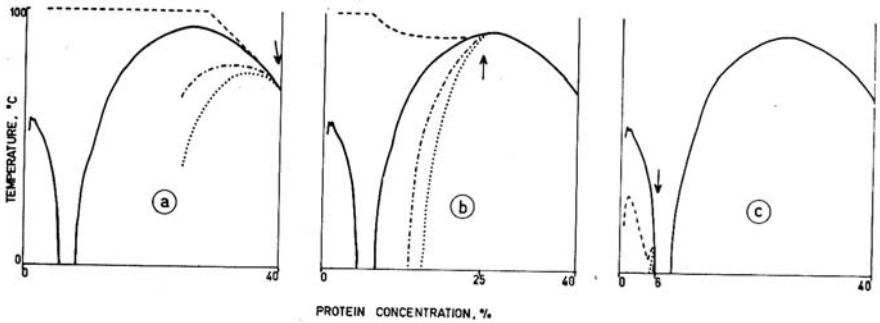

Fig. 6. Phase diagrams for precipitated fraction. Solid line, entire dilution with 30 m*M* hydrochloric acid, 30 m*M* sodium chloride per liter solution. Dashed line, dilution from arrow continued with 30 m*M* sodium chloride only. Dotted line, similar dilution with 30 m*M* hydrochloric acid only. Dot-and-dash line, similar dilution with water only. In a, b, and c, modified dilution begins at differing protein concentrations indicated by arrows.

gels and corresponding photometer tracings are shown in Fig. 4. Electrophoresis patterns of some chromatographic peaks are shown in Fig. 5.

Phase diagrams of peak materials from chromatographic separations are shown in Fig. 3. Effects on phase curves of separate additions of acid, salt, and water at different protein concentrations are shown in Fig. 6.

Eluted Pigment

Some eluted samples were pigmented, the color intensity being pH-sensitive. The color elution nearly coincides with peak 24 of the protein elution, but comparisons of absorption at several wave lengths for selected samples suggest that the elution of protein and pigment is coincidental rather than the two being in combination. Such brown pigment has been present in at least trace quantities in all protein preparations we have studied. It is not an artifact of heating during gluten manufacture, since it is found in freeze-dried laboratory preparations. All New Zealand wheat varieties exhibit this pigment, which is not removed by exhaustive defatting and is possibly polyphenolic in nature. It absorbs markedly in the region 350 to 400 mμ, the absorption being much greater in alkaline solution.

The pigment bears obvious relationship to the indicator pigment observed by Kent-Jones and Herd, and others, and discussed by Markley and Bailey (13). An indicator pigment with similar absorption characteristics was studied in this institute by Bird in 1951 (unpublished); he considered it a characteristic component of wheat germ tissue.

DISCUSSION

Fractionation of Gliadins

The elution curves of Simmons and Winzor (3) and of Wrigley (4) showed excessive elution of proteins near the start of the linear salt gradient. Better resolution obtained with a concave salt gradient is shown by the results of Fig. 2.

"Precipitated fraction" is enriched in gamma-gliadins, as demonstrated by Tiselius electrophoresis patterns (8). Thus comparison of the two elution curves of Fig. 2 suggests that the gamma-gliadins are contained in peaks 8 through 16. For other regions there is either no difference in peak area or a decrease when "precipitated fraction" is compared with "acid-soluble fractions." The increase of peak areas 8–16 is less than the decrease of peak areas 25 onward because of considerable difference in specific UV absorption of the two kinds of protein.

Starch-gel electrophoresis of similar protein preparations fractionated by the technique of Woychik *et al.* (2) and the present chromatographic technique, and further comparison with Tiselius patterns, have enabled identification of the material of peaks 8 through 16 with gamma-gliadins, peaks 17 through 24 with beta-gliadins, and peaks 25 onward with alpha-gliadins. Peaks 23 and 24 have been grouped here with the beta-gliadins because they exhibit two-liquid-phase behavior whereas the definite alpha-gliadins of peak 25 onward do not. Glutenins have been largely excluded from the protein

preparations by the gentle extraction with acid, but some alpha-gliadins may be considered soluble forms of glutenin.

The chromatograms of Fig. 2 and the electrophoretic patterns of Fig. 5 suggest three groups of gamma-gliadins, each group containing three individuals. Thus peaks 8, 9, and 10 form one group. Peaks 11, 12, and 13 form another group, their close resemblance being well seen in the electropherograms. Clearly peaks 14, 15, and 16 form yet another group, identified by their high apparent tyrosine:tryptophan ratios (Fig. 3) as the gamma-one gliadin described by Huebner *et al.* (6). Distinction between the groups labeled beta, gamma-one, and gamma-two-three is also obvious from the different types of phase diagrams.

Figure 5 shows a more detailed resolution of the bands shown in Fig. 4, which is due to longer time of separation. Thus comparison of Fig. 5 with the purification stages of Fig. 4 will show the relation of the gamma-gliadin bands to the pattern for the entire soluble fraction of gluten.

The chromatographic resolution within the group of gamma-gliadins is imperfect. Therefore cross-contamination will occur, and we should expect several electrophoretic bands in addition to the characteristic one from each peak. Indeed, each peak does show several electrophoretic bands, the balance of intensity of bands varying from one peak to another. A possible interpretation showing the characteristic band for each peak has been marked on Fig. 5. Although this interpretation may be doubted in detail, it is nevertheless obvious that the order of mobility of gamma-gliadins in starch-gel electrophoresis does not strictly correspond to their order of elution from the chromatographic column. It is also obvious that these bands, satisfactorily resolved only after lengthy electrophoresis, could not have been so resolved had they not first been fractionated chromatographically.

Since there is a correspondence of one band to each peak in this gamma-gliadin region of the chromatogram, we may reasonably assume that, given better chromatographic resolution, each of the peaks would contain just one electrophoretic component. It seems worthwhile to pursue separation by an even shallower salt gradient in the hope of separating completely a few single components for structure determination. However, additional criteria of purity should be applied to such components. Undoubtedly, as Fig. 5 shows, a very useful degree of purification has been attained.

Gehrke, Oh, and Freeark (14) applied a similar chromatographic system to an acid extract of flour with similar results. They also applied starch-gel electrophoresis under similar conditions, except that higher voltage for a shorter time was used. The proteins in which we are interested at present seem to fall into their classes of "base proteins" and "slow globulins." Their electropherograms show a relative lack of resolution in this region. Little more detail is exhibited in a subsequent publication (15). Undoubtedly the present work has achieved higher resolution in this region by both chromatography and gel electrophoresis.

Graham (11) also has applied this method of starch-gel electrophoresis to the chromatographic fractions of Simmons and Winzor (3). Resolution of

the chromatogram has been insufficient, so that multiple electrophoretic bands appear in each fraction.

The methods used have perhaps not been well chosen. The chromatographic separation depends principally on molecular charge; titration studies of eluted protein fractions in general confirmed this. The pattern of electrophoresis in gel mainly corresponds to that of electrophoresis in free solution, so that apparently this method also is based on molecular charges. Application of another technique with a different basis is needed to confirm that single components are being obtained.

Phase Diagrams

A phase diagram plotted for a crude protein preparation (Fig. 1) shows more fine structure than the three maxima originally described (1). In this typical example, maxima, or inflections suggesting maxima, were observed at about 1, 2, 3, 9, 15, and 25% protein concentrations. These maxima have been observed for several different preparations of mixed proteins. This finding rules out the idea that the maxima represent differing ionic forms of the proteins.

Single peaks of the chromatographic separation gave differing phase diagrams, suggesting that different components are responsible for different maxima. A progressive change in the shapes of the curves was observed with elution order from the chromatogram. However, in peaks 10 through 15, our area of immediate interest, there are always two maxima, at high and low protein concentrations (Fig. 3), showing that this pattern of two maxima is due to some other factor than a multiplicity of components. It seems to be a property of these proteins and the solvent system in which they have been examined. In this system salt and acid concentrations were constant with respect to water and varied with respect to protein.

Beyond the range of peaks for which phase diagrams are presented, peak material is either entirely soluble or mainly insoluble as solid phases at all concentrations, so that this two-liquid-phase behavior is characteristic of a narrow spectrum of gliadinlike proteins. It is conceivable that such behavior may be of great importance in the plastic properties of dough and gluten through a plasticizing interaction with the gellike glutenin proteins. Two kinds of gliadinlike proteins may be distinguished: the beta-gliadin type that undergoes phase separation which is due to increasing salt at about 25% protein concentration but not due to increasing salt plus acid at 2% protein concentration; and the gamma-gliadin type that is separated at 2% protein concentration by salt-plus-acid solution.

The Role of Salt and Acid in Precipitation

To further examine the properties of the two-phase system, varying water at constant ratio of salt or acid to protein was tried. The results suggest that the phase diagrams might be considered artifacts; two kinds of precipitation are clearly occurring in the two major regions of phase maxima. The maximum at about 25% protein concentration is a result of precipitation by salt as seen in Fig. 6, a, this precipitation being opposed at greater protein

dilution by acid (Fig. 6, b). The maximum at about 2% protein concentration, however, seems (Fig. 6, c) to be an effect of a joint precipitation by both acid and salt acting in unison.

It seems reasonable to assume that the separation at 25% protein that is a property of both beta- and gamma-gliadins is due to a hydrogen-bonding interaction of amide groups and others in concentrated solutions. The effect of salt, then, may be to increase dissociation of the solvent and so reduce its association with the protein molecules. A counter-ion effect on the charged proteins resulting in decreased mutual repulsion is an alternative explanation. Acid, on the other hand, induces charges on the basic groups of the proteins, increasing the electrostatic repulsion between similarly charged molecules. These are the phenomena normally observed as isoelectric precipitation and salting-out, though these particular proteins seem ionically very sensitive. This is probably a reflection of the very small number of ionizable groups that they carry (16,17). The titration curves of the gliadins (18–20) do not suggest the presence of any abnormal ionizations. Beckwith *et al.* (21) found that in 30 m*M* acetic acid, the salt concentration required to precipitate gluten proteins increased with greater substitution of methoxyl for amide.

When precipitation occurs at about 2% protein we are not effectively altering the concentrations of acid and salt in the water, and the protein is already moderately dilute. Assuming 10 basic groups per molecule of about 30,000 molecular weight, the precipitation conditions are such that at 25% protein there are 0.27 equivalents each of salt and acid per protein basic group, whereas at 2% protein there are 4.5 equivalents of each per basic group. This does not suggest simple protein salt formation, though such a possibility cannot be excluded. Edestin, which has only a small number of acidic and basic groups, forms an insoluble hydrochloride (22). Whatever explanation is provided for the acid-plus-salt precipitation of the gamma-gliadins must take into account that the beta-gliadins, of rather similar composition, are not precipitated under these conditions.

Interaction among gliadins has been shown by Winzor (23), who used gel-exclusion chromatography. He interpreted the reversible interaction in 10 m*M* sodium acetate-20 m*M* sodium chloride at pH 4.1 and protein concentration up to 1% as a "chemical reaction, presumably hydrogen or hydrophobic bonding." Wu and Dimler (24) showed that tyrosine and tryptophan are not involved in interactions with other groups between pH 3 and 10 in 3*M* urea solution.

General Discussion

The beta- and gamma-gliadins are multicomponent. We can distinguish two kinds of gamma-gliadin as those having high or low tyrosine:tryptophan ratios. The low-ratio kind (gamma-two and -three) is chromatographically separable into six components, and the high-ratio kind (gamma-one) into three components. Each chromatographic peak of the gamma-gliadins corresponds to only a single gel-electrophoretic band. The apparently triplet nature of gamma-gliadins could be a result of the peculiar genetic constitution of bread wheat that bears three effective sets of chromosomes (25). Further

work is being carried out with genetically defined material to test this suggestion.

No explanation for the characteristic precipitation at low concentration of the gamma-gliadins can so far be offered. This precipitation condition does, however, offer a possible technique for critical separation of the gamma-gliadins in quantity. Similar precipitation at, say, 7% protein should allow removal of gamma-one from gamma-two and -three proteins. Such solvent fractionation by precipitation from relatively dilute solution should be more effective than that from concentrated solution, since a greater dilution condition is thermodynamically more satisfactory.

Gamma-two and -three gliadins are a considerable part of gluten structure. Their relation to water is critical, and other work yet to be described shows that they seriously modify the ion-sensitive swelling and shrinking properties of the gluteninlike protein that we consider to be the skeleton of gluten and dough structure (26).

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