ON THE SOLUBILITY OF GLIADINLIKE PROTEINS

III. Fractionation by Solubility[1]

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ABSTRACT

Fractionation of the acid-soluble proteins of gluten has been attempted with three different solvent-precipitant systems. Effectiveness of fractionation was assessed mainly from Tiselius electrophoresis patterns in aluminum lactate buffer.

Aqueous acid-salt solutions and dilute aqueous ammonia solutions separated gliadin from nongliadin proteins. Precipitation from methanol-chloroform solution by increasing concentrations of methanol fractionated the gliadin (beta and gamma) components and demonstrated four components in this group.

These methods are suitable for preliminary enrichments prior to use of the more exact chromatographic separations.

In the preceding paper of this series (1) it was concluded that, for further understanding of gluten solubility, fractionation of the mixture of proteins must be accomplished. In this, cereal chemistry is following several years behind the rubber, plastics, and paint industries, which have been faced with somewhat similar mixtures of polymers of various kinds and molecular weights and different degrees of cross-linking. The whole range of solubility effects, from complete miscibility through multiphase systems to limited swelling phenomena, may be encountered in such mixtures.

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There have been many attempts in the past to distinguish subfractions of gluten proteins on the basis of solubility. This was a favorite occupation of the 1930's, and indeed, long before that, the classification into gliadin, glutenin, and other proteins was based on solubility. Such attempts are still being made (2), though emphasis nowadays has largely turned to other physical methods such as electrophoresis and chromatographic procedures depending on size or charge of the molecules to effect a separation. The disadvantage of such elegant techniques is that they are not readily scaled up to yield larger amounts of material for easy study.

Separation of mixtures of high polymers is usually accomplished by fractional precipitation. To a dilute polymer solution at constant temperature, increments of precipitant may be added and the successive precipitates separated. The temperature of a dilute solution may be lowered in stages and, again, successive precipitates separated. Fractions of successively decreasing molecular weight are thus obtained. Fractional solution rather than fractional precipitation may be employed. Except for crystalline polymers, the precipitates are liquid phases richer in polymer than is the supernatant phase but containing large amounts of solvent. Polymer species are partitioned between the two phases, higher polymers occurring preferentially in the more concentrated phase though each fraction contains a wide distribution of species (3).

Although gluten as found in dough may be a complex molecule of interacting components, it seems clear that it can be dissociated into these many components by the breaking of only a few noncovalent links and that the components can be separated on the basis of their different solubilities. In this paper some exploratory attempts at such separations are described, using only one flour, and no final method is presented. We shall not be concerned with those proteins in which only swelling takes place (glutenin), but with the gliadinlike proteins which are freely soluble in dilute aqueous acid and in methanol-chloroform mixtures as previously described (1,4).

Materials and Methods

The flour used, from Canadian hard red spring wheat, has been described (5). It was defatted by percolation with ether and ethanol-ether as previously described (6). Gluten was hand-washed from the defatted flour and freeze-dried. An extract of the gluten in 0.01*N* formic acid was prepared by gentle stirring (4); the centrifugally clarified solution was dialyzed against distilled water and freeze-dried. A methanol-chloroform extract of this acid-soluble material was made

and, after evaporation, was taken up in 0.01*N* formic acid, dialyzed against distilled water, and freeze-dried. The "gliadinlike" product was 23.9% of the gluten or 3.01% of the flour.

Hydrochloric acid, methanol, ethanol, ether, and chloroform were purified and redistilled.

Electrophoresis was carried out in a Spinco Model H apparatus, using the aluminum lactate buffer of Jones *et al.* (7). Concentrations under the peaks were determined by counting Rayleigh fringes. Protein concentration was about 0.8% in buffer of pH 3.1 and 0.017*M*. Patterns presented are for about 2 hours' running at about 8 volts per cm. As symmetry of patterns from the two limbs was good, only the ascending limb pattern is shown, migration being toward the anode on the left-hand side. The mobility scale is marked in divisions of 2×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} (i.e., each two Tiselius units), from zero at the right-hand side where the delta boundary anomaly appears. The peaks in these diagrams have mobilities similar to those quoted by Woychik *et al.* (8), as shown in Table I, and for convenience of comparison, labels have been introduced at these positions on the diagrams.

TABLE I

COMPARISON OF ELECTROPHORETIC MOBILITIES OF MAIN PEAKS

	Mobility	
	Woychik[a]	Present
	cm.^{2} sec.$^{-1}$ volt^{-1} 10^5	*cm.^{2} sec.$^{-1}$ volt^{-1} 10^5*
Omega	2.3	2.3
Gamma	3.7	3.9
Beta	4.5	4.7
Alpha	5.9	6.2
Sigma (water-soluble)	10.8	11.1

[a] See reference 8.

Fractionation Methods. In this preliminary survey, three different solvent systems have been tried for fractionating power: aqueous hydrochloric acid-sodium chloride; aqueous ammonium hydroxide-ammonium sulfate; and methanol-chloroform mixtures. The aqueous acid has been used for both fractional solubility and fractional precipitation studies, and the methanol-chloroform mixture has been used for precipitation by both solvents. Aqueous ammonium hydroxide-ammonium sulfate was tried, after the analytical method so based by Tiunova (2). The only methods that have been used to assess the effectiveness of the fractionations are weight yields and Tiselius electrophoresis patterns.

Preliminary Separation of Gliadinlike Protein. Extraction of gluten with dilute acid leaves a gelatinous residue which is not soluble and

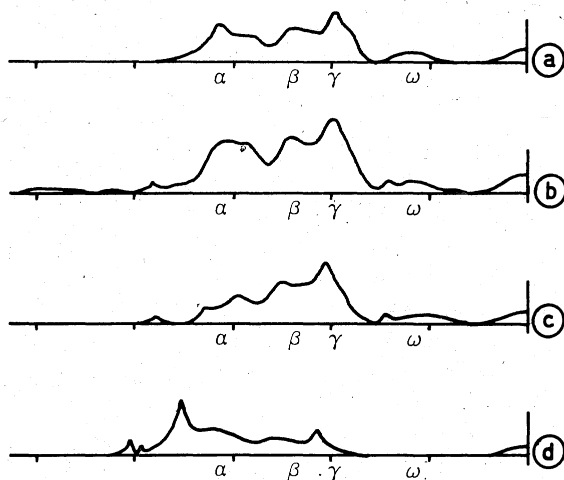

Fig. 1. Electrophoresis patterns of (a) gluten shaken vigorously with buffer and centrifuged; (b) acid-soluble protein (gliadinlike) fraction; (c) methanol-chloroform-soluble fraction of (b); (d) methanol-chloroform-insoluble fraction of (b).

therefore not determinable by simple electrophoretic methods. Gluten shaken vigorously with acid for some hours, however, gives a solution containing a considerable proportion of degraded gel, and the electrophoretic pattern does differ slightly from that of the acid-soluble protein obtained under gentler conditions (Fig. 1). Separation with methanol-chloroform yields two fractions shown by their electrophoretic patterns (Fig. 1) to be quite different. The acid-soluble material was 46.5% of the dry gluten and the methanol-chloroform-soluble material was 51.5% of the acid-soluble material.

Fractionation of Gliadinlike Protein by Acid-Salt Solutions. 1. Methanol-chloroform-soluble material was dissolved in aqueous acid-salt solution (hydrochloric acid 20 mM; sodium chloride 100 mM; protein 5% w/w) and heated briefly to 85°C. to produce complete solution. On cooling to 20°C. a separation into two phases occurred; that is, a liquid precipitate formed. After several hours the phases were separated by centrifuging and each was dialyzed against distilled water. The upper phase was retained for analysis (A100); the lower phase was freeze-dried, then used for a similar solution and precipitation with 20 mM HCl, 90 mM NaCl, and 5% protein concentration. This process of fractional solution was repeated several times, yielding fractions A90, A80, A70 through A10, and A0. The final fractionation left no residue.

2. Commencing with a 5% solution of methanol-chloroform-soluble material in 20 mM hydrochloric acid, successive additions of NaCl were

made. Heating, cooling, centrifuging, and separating were carried out for each cycle to imitate the conditions of the previous experiment but giving fractional precipitates. Pervaporation and dialysis were carried out every third cycle to maintain the protein concentration near to 5%. In this way fractions B0 to B100 were obtained corresponding to the A series.

Fractionation by Alkaline-Salt Solutions. Tiunova (2) had used dilute (0.08%) solutions of the magnesium form of the proteins for analytical precipitation, but for the present preparative precipitation a more concentrated solution was used, of partly purified material which was a bulk of fractions from the above experiment. This was dissolved in water to give a 5% w/v solution and diluted ammonia solution carefully added, with stirring, to give 0.2% v/v concentration (30 mM). After standing 2 hr. the two phases were separated by centrifuging, dialyzed against water, and then freeze-dried. The lower-phase material would not redissolve in water. The two fractions were markedly different in the dry state, the insoluble lower-phase material being of a fibrous texture compared with the powdery upper-phase material. Equal proportions of material were in each fraction.

Fractionation of the soluble material was attempted from 2% solution in 30 mM ammonia by incremental addition of ammonium sulfate solution. The precipitation curves were disappointing and will not be discussed.

It is concluded that dilute ammonia itself gives a definite separation of components but may alter some properties of the proteins. Ammonium sulfate did not, in this brief examination, seem of more use than ammonia.

Fractionation of Methanol-Chloroform Solutions. 1. To a clear solution of about 25 g. methanol-chloroform-soluble material in a mixture of 200 ml. methanol and 300 ml. chloroform were added successive 100-ml. increments of methanol. Not until the third increment (62% v/v methanol) was a measurable precipitate produced. Successive precipitates were separated by centrifuging, evaporated at low temperature to dryness, dissolved in dilute formic acid, and freeze-dried. The weight of each was recorded and its electrophoretic behavior determined. Each fraction was identified by the concentration of methanol (v/v) at which it was precipitated.

2. To a similar methanol-chloroform solution, successive incremental additions of chloroform were made. The separated protein-rich phases floated and were sufficiently coherent to be separated by decantation. Again they were dried, redissolved in acid, freeze-dried, weighed, and examined by the Tiselius technique.

Results

The weight yields of each acid-salt fraction are recorded in the histograms of Fig. 2, and the electrophoretic patterns of some series A fractions in Fig. 3, together with the pattern for a sample of flour albumins prepared according to Pence *et al.* (9) and for a mixture of two fractions. The amino acid composition of fraction A100 was determined and is compared in the histogram pattern of Fig. 4 with similar data for the starting material, for a sample of flour albumins, and for Woychik's (8) water-soluble protein fraction.

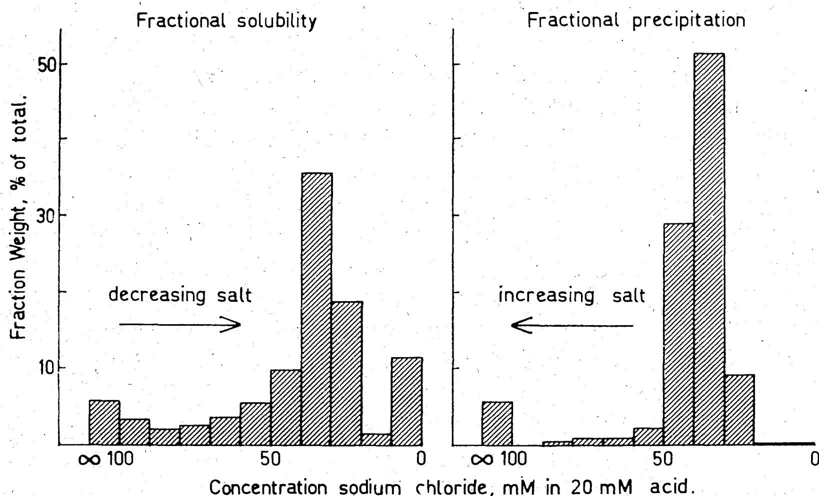

Fig. 2. Weight yields of successive fractions obtained by systematically varied concentrations of sodium chloride in dilute aqueous acid. Each fraction is soluble at the lower salt concentration but insoluble at the higher.

Electrophoresis patterns for the starting material and the two fractions obtained by dilute ammonia are shown in Fig. 5.

The cumulative recoveries of the two precipitations from methanol-chloroform are plotted on a common curve (Fig. 6) which almost coincides with the solubility curve for the same starting material. The Tiselius diagrams of the series of fractions are shown in Fig. 7.

Discussion

In view of the priority of Senti, Dimler, and their co-workers (10) in successful fractionation of the gluten proteins, it is convenient to discuss the present results in their terminology.

The two extreme fractions of the acid-salt separation represent two components of the protein mixture, sigma and omega, which are

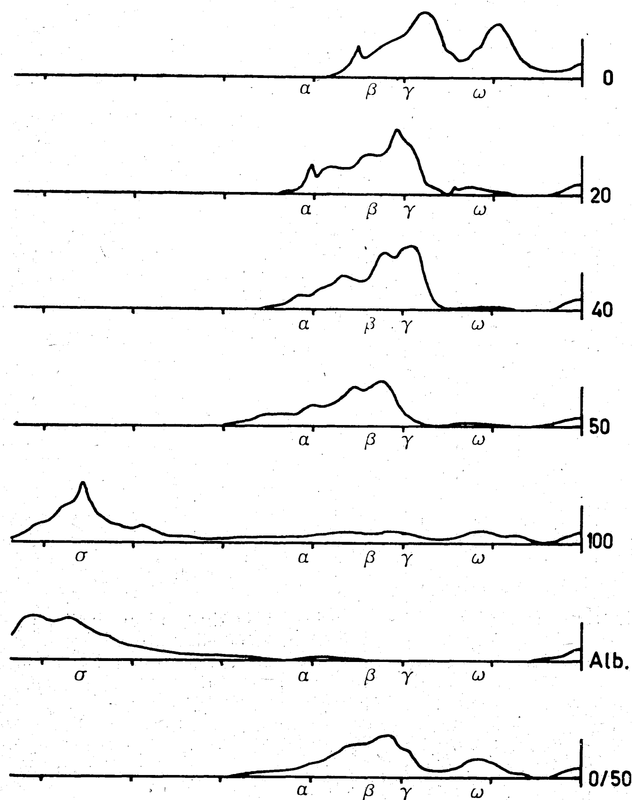

Fig. 3. Electrophoresis patterns of some fractions obtained by fractional solubility in aqueous acid-salt solutions. The numbers refer to the series A fractions shown in the left-hand part of Fig. 2. "Alb" is the pattern for a sample of flour albumins; 0/50 is for a mixture of equal weights of fractions A0 and A50.

clearly not related to the majority of the gliadin protein. Sigma, of fraction A100, is obviously similar to the albumins of wheat flour as shown by the high electrophoretic mobility and by the amino acid analysis. Woychik *et al.* (8) observed a water-soluble protein fraction during chromatographic fractionation of wheat gluten on carboxymethyl-cellulose columns and they concluded on the basis of mobility (8) and analysis (11) that this fraction was similar to the previously observed albumins and globulins. The yield of fraction A100 was about 5% of the methanol-chloroform-soluble material, and high-mobility components made up about 62% of A100. This implies about 3% of albuminlike components in the methanol-chloroform-soluble material, and the result agrees with a direct measurement by Tiselius pattern.

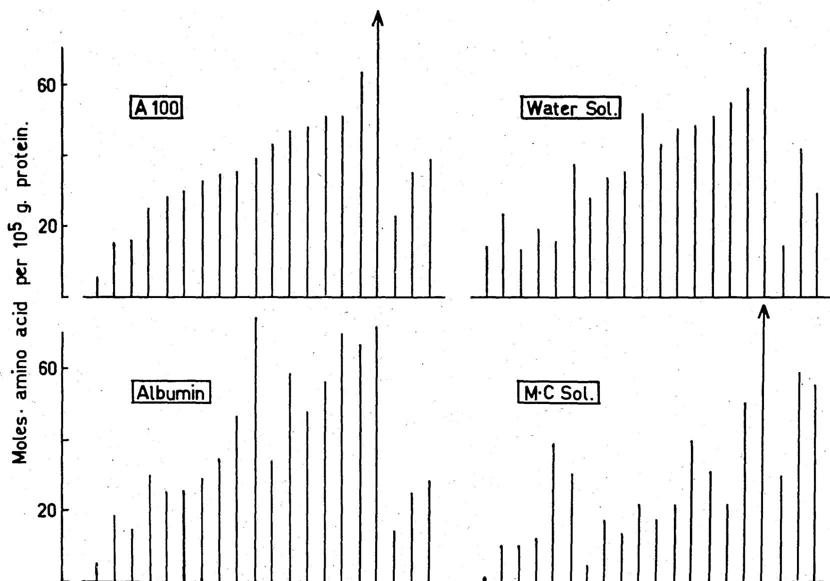

Fig. 4. Patterns of amino acid composition for three albuminlike preparations and the starting material of the fractionation. A100, first upper phase of acid-salt fractionation; "Water sol," the water-soluble fraction analyzed by Woychik *et al.* (11); "Albumin," a sample of albumins prepared from flour according to Pence *et al.* (9); "M-C Sol.," the methanol-chloroform-soluble starting material (6). From left to right the bars show the concentrations of tryptophan, histidine, methionine, tyrosine, phenylalanine, isoleucine, lysine, threonine, arginine, alanine, ½ cystine, aspartic acid, serine, valine, glycine, leucine, proline, proline, ammonia, glutamic acid. The last three are drawn one-fifth scale.

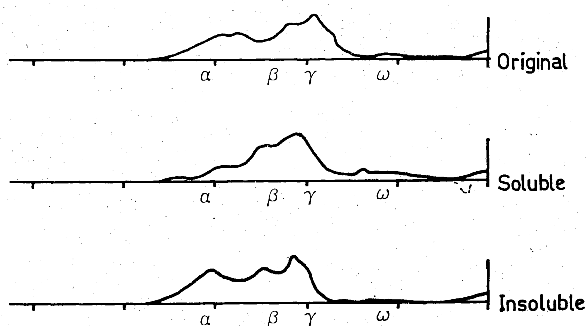

Fig. 5. Electrophoresis patterns showing effect of aqueous ammonia as a partial solvent.

The slower components of fraction A0, equally obviously, are not a part of the gliadin but are proteins of lesser solubility, possibly allied to the more soluble forms of the glutenin proteins. As seen in Fig. 2, the small amount of this protein in the starting material is not

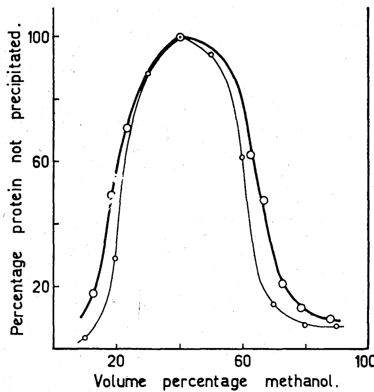

Fig. 6. Bold curve shows precipitation from a solution of protein in 40% (v/v) methanol in chloroform by dilution with either methanol or chloroform. Light curve, for comparison, is the corresponding solubility curve (Fig. 2A of ref. 4, replotted).

precipitated until the salt concentration is so high that gliadin proteins also are being precipitated. It is, however, left out of solution when the gliadin proteins are removed by fractional solubility.

Thus the acid-salt method is capable of removing contaminants of the albumin and glutenin types, but from the appearance of the yield curves has not fractionated the gliadin proteins. Although comparison of the electrophoretic patterns of fractions A20 and A50 (Fig. 3) does show a shift in the balance of the main components, beta and gamma, this shift does not seem sufficient to form the basis of a preparative method. The difference between fractional solubility and fractional precipitation is presumed due to coprecipitation effects.

Ammonia as a partial solvent may be useful (Fig. 5) for separation of the gliadin components from the rest and could well be investigated in more detail, though the use of ammonium sulfate seems to offer no additional advantage. Finlayson and McConnell noted (12), incidentally to another investigation, that gliadin was only partly soluble in dilute (25 mM) ammonia.

The solubility in methanol-chloroform mixtures and precipitation by dilution of either component of the solvent is a reversible process, as demonstrated by the similarity of the solubility and precipitation curves. The electrophoretic patterns show that the fraction separated at 79% methanol is highly enriched in only two gamma components compared with the 88% fraction. Both fractions are virtually devoid of the sigma and omega material considered above as contaminants, and are also low in alpha components. The fractions 73, 79, and 88 have typical gliadin properties.

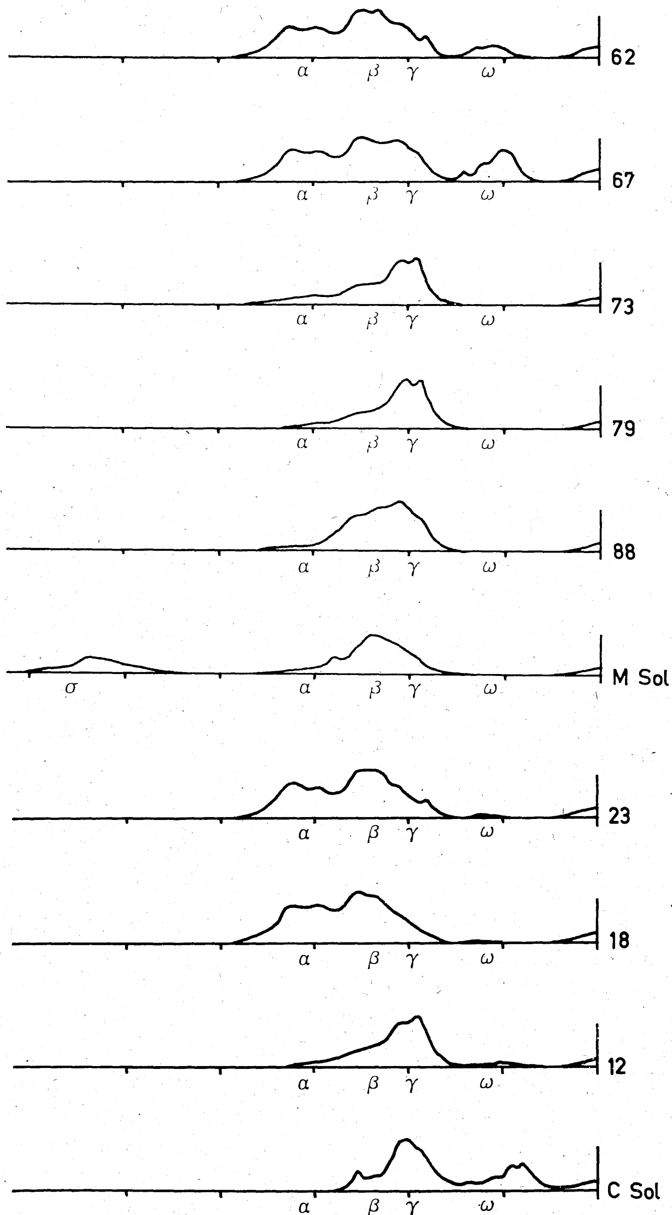

Fig. 7. Electrophoresis patterns of fractions obtained from methanol-chloroform solution. The numbers refer to the methanol concentration (percent by volume) at which each was precipitated. "M sol" and "C sol" are the materials remaining in solution at the ends of the fractionations.

We may now discuss these results in terms of the types of proteins present in gluten. The electrophoretic diagrams show four distinct groups of proteins. The fastest-moving components, sigma, are undoubtedly of the albumin type and are probably intrinsic contaminants of gluten. The next fastest group, alpha, occurs often concomitantly with the slowest group, omega. These seem to be of the glutenin type of proteins, of varying degrees of polymerization, and therefore of varying solubility. This is supported by the gel filtration studies of Jones *et al.* (13) where the alpha gliadin and glutenin components had opposite extremes of size. The slowest, omega, components tend to go with the gliadin components into solution in methanol-chloroform and, as we have seen, are not easy to separate. The faster, alpha, components, on the other hand, are predominantly insoluble in methanol-chloroform. The status of the two subdivisions, alpha one and alpha two, of Senti is not clear in this or in Senti's fractionations.

Finally, we have the group distinguished by two peaks, beta and gamma, in Fig. 1 which seem to be the gliadin components. That is, they are responsible for the gummy character of gluten and derived fractions and, as purification for the typical properties proceeds, these become the main components. They are the proteins which we desire to investigate physically if we can separate sufficient amounts, and they appear from fraction 88 of Fig. 7 to be separable into at least four components.

Jones *et al.* (7) attempted fractionations by precipitation with varying additions of alkali and salt to solutions in aluminum lactate buffer. Although clear shifts in the proportion of alpha components were obtained, there was no evidence of parting of the beta and gamma components. They noted that when the protein concentration was raised from 0.3 to 2%, very little fractionation was possible. This is in line with the common theory of fractionation of high polymers that efficiency of separation is greater in more dilute solutions. However, in the present investigation relatively concentrated (5%) solutions were used so that larger amounts of material might be handled. Under more dilute conditions better separations could be expected.

Lee *et al.* (14), Deschreider and Meaux (15), and Gehrke *et al.* (16) have fractionated gluten proteins by chromatography on CM-cellulose, as have also Woychik *et al.* (8), but the four groups used different elution systems. The last showed that their stepwise-eluted fractions corresponded in order to the electrophoretic peaks in aluminum lactate buffer.

To summarize, the gliadin-type proteins can be freed of contaminants by taking advantage of the relative solubilities in acid-salt solutions and in dilute aqueous ammonia solutions. Fractionation of the gliadin-type proteins is possible by precipitation with added methanol from methanol-chloroform solution. There are at least four components in this group. Whether fractionation by solubility is to be preferred to fractionation by chromatography on CM-cellulose depends on the scale of operations desired.

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