The Effect of Lipid on the Solubility and Molecular-Weight Range of Wheat Gluten and Storage Proteins

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ABSTRACT

Extraction with 6M urea has been used to separate the more readily soluble gliadin and glutenin fractions of freeze-dried gluten, and of wheat storage protein, from the insoluble residue. The choice of this solvent has preserved the structure of this residue, allowing its microscopic examination as described in the article following. Much less protein was extractable from gluten than from storage protein, which had been prepared with organic solvents and was thus depleted in lipid. Reconstitution experiments which involved the wetting of storage protein in the presence of readded flour lipid suggested that the difference in protein solubility in the case of gluten is due to lipid-protein association during dough formation. The protein fractions involved have been partly characterized by gel filtration.

One of the major omissions in previous studies of wheat proteins has been a consideration of the relationship of the material being extracted to the morphological structure of the wheat grain, particularly the nature and source of the fraction remaining undissolved after treatment with commonly used protein solvents such as dilute organic acids or urea. Most research effort has bypassed this insoluble fraction, to concentrate primarily on the gliadin proteins and the easily extractable glutenin. The few studies made on the insoluble gluten residue have involved dissolving it with reasonably harsh solvents (1-4), which would disrupt its structure and organization to an unknown extent.

In the present work, particular attention has been paid to the use of solvent mixtures which are known to dissolve proteins through the disruption of specific bond types. On the basis of this study, a solvent has been selected (urea at 4 to 6M concentration) which disperses a high proportion of the protein without breaking covalent bonds, while leaving lipid-protein interaction products undissolved. The residue undissolved by this solvent has been characterized by transmission electron microscopy, leaving the way open for its controlled degradation by other, more vigorous, solvents, capable of cleaving covalent bonds of specific types. Examination of the fine structure of the urea-insoluble residue, described in an accompanying article (5), provides useful information about the origin and composition of this hitherto intractable portion of gluten.

The present communication describes the extraction procedures used in this approach, and characterizes the protein composition of the urea-soluble material. Classification according to molecular size (3) has been used to distinguish between groups of proteins previously defined on the basis of their solubility.

Furthermore, this article and the following one describe the use of this approach to distinguish more adequately between the storage protein as it exists between the starch granules in the mature grain, and gluten, formed by the interaction of wheat-flour components in the presence of water. By examination of both

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freeze-dried gluten and "storage protein", prepared from flour by organic solvents under nonaqueous conditions, further information has been obtained about lipid-protein association during dough formation.

MATERIALS AND METHODS

Preparation of Purified Storage Protein and Freeze-Dried Gluten

The flours used in this study were straight-run flours prepared by experimental Buhler milling from the Australian hard white spring varieties Timgalen and Falcon, and the soft white spring varieties Heron and Summit. These were pin-milled, air-classified, and solvent-fractionated as previously described (6), to yield purified storage-protein preparations containing 92 to 96% (dry weight) protein.

Gluten samples were prepared from each of the air-classified coarse fractions obtained above, by the normal hand-washing technique under 1% sodium chloride. The glutens so obtained were freeze-dried and ground in a hammer mill to yield products which contained 85% to 87% protein (dry weight).

Extraction of Purified Storage Protein and Gluten

In preliminary experiments, portions of freeze-dried gluten and purified storage protein, weighing approximately 100 mg., were extracted three times with 5-ml. volumes of one of the following aqueous solvents:

a) Urea of various concentrations (2 to 8M).
b) Urea (2 to 8M) and diethyl ether or *n*-butanol (2 ml.)
c) Urea (2 to 8M) containing 1% 2-mercaptoethanol or 0.1% Cleland's reagent (Dithiothreitol) (7).
d) Urea (2 to 8M) containing 1% mercaptoethanol and diethyl ether (2 ml.).
e) Acetic acid (0.1M), urea (3M), cetyltrimethylammonium bromide (CTAB, 0.01M) [solvent of Meredith and Wren (3)], with and without 1% mercaptoethanol.
f) Acetic acid (0.05 and 0.1M).

After shaking on an oscillating shaker for 10 min. in a stoppered tube, the samples were centrifuged (2,000 × g, 10 min.) and the extracts were decanted. The residues were washed four times with distilled water, then freeze-dried and weighed.

Protein determinations on dry samples were performed by the micro-Kjeldahl procedure; and in solution, by the Lowry method (8).

Reconstitution Experiments

Portions of storage protein (500-mg.) were moistened with sufficient distilled water to give a stiff "dough", which was freeze-dried prior to extraction with urea. Where lipid and storage protein were to be reconstituted, the former was obtained immediately before use by extracting an amount of flour (4.2 g.) equivalent to the weight from which the storage protein had been derived, with benzene-chloroform (15 ml., density 1.42), corresponding to the solvent in which the storage protein had been suspended during its preparation. Storage protein (500 mg.) was then mixed with the filtered lipid extract and the solvent removed on a rotary evaporator at 40°C. under reduced pressure. Sufficient distilled water was added to the solvent-free residue to give a stiff dough, which was freeze-dried prior to extraction with urea.

Preparative Scale Extraction of Storage Protein, Gluten, and Treated Storage Protein

In the preparative procedure finally adopted, 400-mg. portions of storage protein, gluten, and of storage protein subjected to the various water and lipid treatments described above, were gently dispersed into urea (4 or 6M; 10 ml.). After standing for 2 hr. at room temperature, the dispersions were centrifuged at 25,000 r.p.m. (110,000 X g) in a M.S.E. Superspeed 65 centrifuge for 60 min. The clear supernatant was decanted, and the residue was extracted twice with fresh urea solution. The amount of protein extracted into the third wash was less than 1% of that in the first urea extract. The final residue was washed four times with distilled water, freeze-dried, and dried to constant weight.

Gel Filtration

The combined extracts (4 or 6M urea) were used for gel filtration on Sephadex G-150 (Pharmacia, Uppsala, Sweden) in 6M urea. The column (2.5 X 35-cm. bed) was fitted with flow adapters for upward elution at about 20 ml. per hr. The sample (5 ml.) was applied through a three-way stopcock (9). Elution profiles of absorbance at 254 nm. were obtained using a LKB Uvicord. The proportions of protein size-groups were estimated on the basis of molecular-size distribution as suggested by Meredith and Wren (3): Glutenin, over 100,000 molecular weight; gliadin, 25,000 to 100,000; and albumin, 10,000 to 25,000. After the column was calibrated with proteins of known molecular weight, appropriate fractions were pooled and the proportions of albumin, gliadin, and glutenin were determined on the basis of their absorbance at 280 nm.

RESULTS AND DISCUSSION

Preliminary extraction experiments were performed to determine the most satisfactory solvent system for further work, using the procedure described under Materials and Methods.

For each concentration of urea used, a greater amount of storage protein than of freeze-dried gluten was dispersed. However, even at the highest urea concentration employed (8M), there was a small fraction (4%) of the storage protein which resisted dispersion unless a reducing agent was also present. Similar results were observed with the acetic acid-urea-CTAB solvent of Meredith and Wren (3), where it was found that the addition of 1% mercaptoethanol was essential for the complete dispersion of both gluten and storage protein. Contrary to expectation, the additional presence of an organic solvent such as ether or *n*-butanol was found to depress the solubility of gluten proteins in urea solutions. This may be due to an increase in hydrogen bonding under these conditions, and suggests that glycolipid bridges, if present (10), are not susceptible to disruption by organic solvents.

Acetic acid (0.05 or 0.1M) was not as effective a solvent as 2M urea, and it tended to yield flocculent residues which were very difficult to separate from the supernatant extracts. In the absence of reducing agent, the urea extracts were clear. However, in its presence they were turbid, due both to the presence of some high-molecular-weight material, and to contamination by oxidation products of mercaptoethanol. Even centrifugation at 110,000 X g for 60 min. failed to completely remove this turbidity. On dialysis, these extracts yielded a flocculent

precipitate of protein which readily redissolved in 4M urea containing 1% mercaptoethanol. More protein was extracted from gluten or storage protein by higher concentrations of urea both in the presence and absence of mercaptoethanol. Urea solutions of higher concentrations (6 to 8M), in the presence of solvent and reducing agent, tended to disperse residual starch, which made estimation of their solvent properties by the present method rather difficult.

Of all the solvents tested, however, 6M urea was preferred, and was used in all further work reported. This solvent gave reasonably efficient extraction of protein without the major modification of primary structure that would result from the addition of reducing agents, or the risk of disrupting membrane structures which accompanies treatment with detergent or lipid solvents. The observation that addition of reducing agents causes increased dispersion of protein provides an obvious basis for further investigation of the urea-insoluble fraction after its morphological characterization (5). This approach is at present being investigated.

Figure 1 presents a closer examination of the extraction, by 6M urea, of gluten and modified storage-protein preparations derived from a hard wheat flour (Timgalen) and a soft flour (Heron).

In each case microscopic examination showed that the residue insoluble in 6M urea consisted of a proportion of starch derived from the original preparation. In addition, there was a small proportion of membrane-derived protein in the residues of the storage-protein sample (a), and in the storage protein which had been treated with water, followed by freeze-drying prior to extraction (b). However, in the case of gluten (c), and the lipid-treated storage protein (d and e), additional insoluble material was recorded (shaded areas). This is possibly derived from interaction products between the lipid and protein present in the latter three cases. In the replicate extraction experiments averaged above, the weight distributions usually agreed within 10%. The marked differences illustrated in Fig. 1 were always observed with both varieties investigated.

It should be emphasized that the object of adding lipid back to the storage protein in this series of experiments was to replace material extracted during the preparation of the latter. This lipid is already present in gluten (5) by virtue of the interaction which takes place when normal flour is moistened with water and mixed to form a dough.

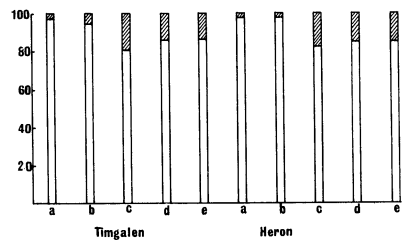


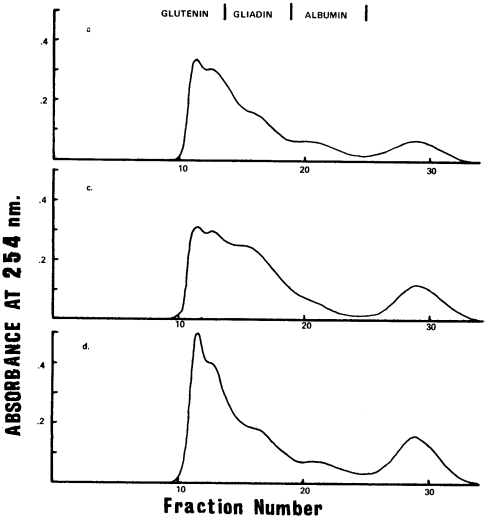
Fig. 1. Solubility in 6M urea of Timgalen and Heron a) storage protein, b) storage protein to which water has been added, c) gluten, d) storage protein to which Timgalen flour lipids have been added, and e) storage protein to which Heron flour lipids have been added. Results are the averages of three replicate extractions and are expressed as percentage total protein; estimated by a gravimetric balance of dissolved and residual material. Unmarked area = protein soluble in 6M urea; hatched = protein insoluble in 6M urea.

The behavior of only two varieties was compared in the present study (Fig. 1). Although there appeared to be significant varietal differences in the interaction of lipid and protein, clarification of this point will have to await further investigation involving more varieties.

The molecular-weight distribution and protein composition of the 6M urea extracts were investigated by gel filtration. Figure 2 shows the elution profiles obtained.

The calculated proportions of glutenin, gliadin, and albumin in the extracts shown in Fig. 3 are representative of four series of analyses. They indicate that gluten contained considerably less albumin proteins than did storage protein. The water washing used to prepare gluten would be expected to remove a larger proportion of the albumin compared with storage protein which has not had contact with aqueous solvents during its preparation. Since this difference affected the relative proportions of glutenin and gliadin, the ratio between the amounts of these two protein classes is listed in Table I, to facilitate comparison between them. The ratios show that gluten contained comparatively less glutenin than did storage protein. The difference in extractability between the two preparations is therefore due partly to the content of water-soluble albumin in the storage protein and partly to the extraction from this material of more glutenin.

All the elution profiles obtained indicated that there were two groups of glutenin proteins having different molecular-size ranges. One appeared at the column void volume ("large glutenin"), and a second in the molecular-weight range of about 300,000 to 600,000 ("medium glutenin"). Such a fractionation was also



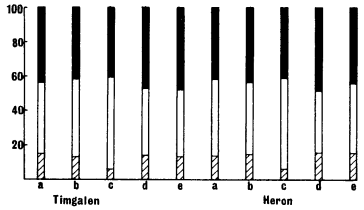


Fig. 2 (left). Elution profiles (absorbance at 254 nm.) for gel filtration of Timgalen gluten and storage-protein preparations, designated a, c, and d as in Fig. 1.

Fig. 3 (right). Proportions of albumin (hatched), gliadin (unmarked), and glutenin (black), determined by gel filtration, for gluten and storage-protein preparations designated a to e as in Fig. 1.

TABLE I. PROPORTIONS OF GLIADIN AND GLUTENIN FRACTIONS IN GLUTEN AND STORAGE-PROTEIN PREPARATIONS

	Storage Protein	Storage Protein + H_2O	Gluten	Storage Protein + Flour Lipids from	
				Timgalen	Heron
	TIMGALEN				
Glutenin to gliadin ratio	1.08	0.98	0.79	1.20	1.23
Large to medium glutenin ratio	1.14	1.08	1.13	1.24	1.26
	HERON				
Glutenin to gliadin ratio	0.99	1.05	0.82	1.33	1.05
Large to medium glutenin ratio	1.03	1.00	1.08	1.47	1.39

reported by Bushuk and Wrigley (11). The latter fraction may correspond to the medium-sized gluten proteins previously reported in this general size range (1,12,13). The two peaks were not sufficiently resolved to allow an estimate to be made of the relative amounts of each present, but an approximate indication for comparison was obtained by calculating the ratio between their heights in the elution profile (Table I). There was little difference in this ratio between gluten and storage protein, suggesting that both size ranges of glutenin are equally involved in the formation of the urea-insoluble residue in gluten.

To further investigate the difference in urea solubility between gluten and storage protein, the latter was mixed with water in the presence and absence of added lipid, in an attempt to simulate gluten formation. Water addition alone produced no change in urea solubility (Fig. 1, column b) and no change in any aspect of protein composition was detectable by gel filtration (Fig. 3; Table I). In the presence of added lipid, moistening with water reduced the urea solubility (Fig. 1, columns d and e), produced an increase in the content of high-molecular-weight material (Fig. 3), and considerably increased the proportion of large- to medium-sized protein (Table I).

However, the addition of water and lipid to storage protein did not exactly simulate gluten formation in untreated flour. The change in urea solubility was of the same order, but the changes in the molecular-weight distribution of the urea-soluble proteins were slightly different. Nevertheless, the results obtained by this treatment further confirm that interaction between lipid and protein occurs in wheat flour during dough formation, leading to the formation of high-molecular-weight material having the urea-solubility characteristics of glutenin.

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