Acid-Souble Proteins of Wheat Flours. II. Binding to Hydrophobic Gels[1]

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ABSTRACT

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Binding of acid-souble proteins from untreated and defatted good (Shawnee, C.I. 14157) and poor (Chiefkan/Tenmarq, KS501097) baking quality flours to Phenyl-Sepharose CL-4B was examined by batch-elution and column-elution techniques. When a mixture of hydrophobic gel and acid-soluble flour proteins was eluted batchwise with five solvents, total absorbance at 280 nm of the eluates was higher for the poor than for the good baking quality flour. Acid-soluble proteins of defatted good and poor baking flours differed little from those of nondefatted flours in hydrophobic binding capacity. Glutenins from isopropanol-defatted poor baking quality flour (KS501097) were less hydrophobic, and gliadins were more hydrophobic, than glutenins and gliadins from the good baking quality flour. The difference in apparent hydrophobic interaction was more pronounced for glutenin than for gliadin. The relation between the amount of protein eluted and the amount of protein adsorbed by the hydrophobic gel varied with protein sources and their concentrations. Among protein eluting agents, 1% sodium dodecyl sulfate in $0.01N$ acetic acid was most effective. The results indicate that acid-soluble proteins from good and poor bread-making flours differ in apparent hydrophobic properties.

The structure of wheat proteins and the interactions of the proteins in the gluten matrix have been studied for many years so that properties of proteins might be correlated with flour-baking quality (Huebner 1970, Orth and Bushuk 1972, Pomeranz 1968, Pomeranz et al 1970, Wall and Beckwith 1969). We observed recently (Chung and Pomeranz 1978) that the effect of defatting wheat flours on protein extractability was greater for the good baking quality flour (good flour), Shawnee, than for the poor baking quality flour (poor flour), KS501097. The experimental results also explained that the stable protein solubility of the poor flour after delipidation was due to either a high concentration of available charged polar groups or less interaction between lipids and proteins than in the good flour.

To correlate physicochemical properties of acid-soluble proteins with baking quality, the hydrophobic binding properties of those proteins were examined by using alkylated and arylated Sepharose CL-4B. Properties of the commercially available Sepharose gels are described in the technical literature (Anonymous 1975). Those Sepharose gels contain hydrophobic groups (Hjerten et al 1974), which interact with others on the surface of proteins (Tanford 1973). Hence, strongly hydrophobic proteins should bind firmly to the gel, with the result that little protein should be present in the eluates.

Although there is experimental evidence for the existence and importance of hydrophobic bonds in dough (Elton and Fisher 1968, Pomeranz et al 1966, Ponte et al 1967, Wehrli and Pomeranz 1970), hydrophobic binding properties of wheat proteins have not been studied directly nor has anyone to date reported the hydrophobic interactions of flours that differ in bread-making quality.

In this study, a hydrophobic gel was used to characterize some of the physical properties of proteins in bread-making flours.

MATERIALS AND METHODS

Flour samples, delipidation with isopropanol of flours, and extraction and fractionation of acid-soluble proteins on Sephadex G-100 have been described (Chung and Pomeranz 1978). A nonionic hydrophobic gel, Phenyl-Sepharose CL-4B from Pharmacia Fine Chemicals Co. (Uppsala, Sweden), was washed with deionized water to remove sodium azide and was equilibrated with eluting agent before use.

Eluants were all analytical reagents: electrophoretic purity, sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories, Richmond, CA); spectrograde methanol (Analabs Inc., North Haven, CT); propylene glycol (Matheson Coleman Bell Co., East Rutherford, NJ); and acetic acid and glycine (Eastman Chemical Co., Rochester, NY).

Batch Experiments

To determine optimum conditions for column chromatography, batch fractionation experiments were made at different ionic strengths and pH. Equilibrated Phenyl-Sepharose CL-4B gel was filtered, and 2-g sublots of the wet gel were mixed with 0.5 ml of acid-soluble proteins (4 mg protein/ml $0.05N$ acetic acid). After resting for 30 min at room temperature, the mixture was stirred for 10 sec with 4 ml of eluant on a vortex stirrer (Thermolyne, Sybron Corp., Dubuque, IA), and the suspension was filtered through a coarse sintered glass filter that was connected to a constant vacuum line. In experiments to determine the effect of protein concentration on degree of interaction, 1 ml of a solution containing 1, 2, 3, 4, or 5 mg of protein was used and was followed by the same procedure as described above.

The volume of filtrates was then made up to 4.5 or 5.0 ml for the first elution (volumes for protein samples, 0.5 or 1.0 ml, plus 4.0 ml of eluant) and 4.0 ml for the rest of elutions with each eluant, respectively.

Absorbance at 280 nm of the volume-adjusted filtrate was measured with a Cary 118C spectrophotometer for estimation of protein uptake by the gel. As a blank, 0.5 or 1.0 ml of $0.01N$ acetic acid containing no protein was subjected to the same procedure as the protein solutions. Total absorbance was obtained by multiplying volumes with the absorbance differences between sample and blank.

Column Chromatography

A laboratory column (K 16/20 jacketed, Pharmacia Fine Chemicals Co., Uppsala, Sweden), filled with 7.0 g Phenyl-Sepharose CL-4B, was equilibrated at room temperature with $0.01N$ acetic acid. Twelve milligrams of protein in 3 ml of $0.01N$ acetic acid was applied and eluted at a flow rate of 20 ml/hr. Eluants used sequentially were 50 ml $0.01N$ acetic acid, 150 ml 1%

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SDS in 0.01N acetic acid, 50 ml 0.1M glycine-NaOH, 100 ml 50% propylene glycol in 0.005M glycine-NaOH, and 50 ml 30% methanol in 0.005M glycine-NaOH.

Elution profiles were obtained from the 280 nm absorbance of 40-drop fractions of column eluates collected with a fraction collector; in addition, the total absorbance of pooled fractions was measured. It is recognized that absorbance of proteins at 280 nm varies widely depending on the concentrations of aromatic amino acids, and can therefore be used only for the semi-quantitative assessment of the sample (Jankiewicz and Pomeranz 1965). All experiments and assays were made at least in duplicate.

RESULTS AND DISCUSSION

The binding capacities of Octyl- and Phenyl-Sepharose CL-4B gels were first examined in preliminary experiments to compare the hydrophobic interactions of acid-soluble proteins. In batch experiments (not reported here), no major differences were observed in flour protein binding capacities between Phenyl- and Octyl-Sepharose CL-4B (with the same degree of substitution: 40 μmol/ml gel bed). Hydrophobicity of the phenyl group of Phenyl-Sepharose CL-4B was intermediate between those of n-butyl and n-pentyl groups (Anonymous 1975). Phenyl-Sepharose, which is less hydrophobic than Octyl-Sepharose, was selected as a suitable matrix for the hydrophobic chromatography of acid-soluble flour proteins. A possible specific interaction (π—π interaction) of Phenyl-Sepharose CL-4B with aromatic groups of proteins was not examined.

Eluting conditions were studied in preliminary batch experiments by use of 2 g of Phenyl-Sepharose CL-4B and 0.5 ml of acid-soluble protein extracts from the untreated poor flour (KS501097). Absorbance values at 280 nm of the eluates under various conditions are given in Table I. Absorbance (at 280 nm) of the protein eluates of 1 plus 2 was higher under acidic than under neutral or basic conditions; absorbance values of eluates 3 and 4 were highest under basic conditions. To determine protein absorbance at different pH values, equal amounts of acid-soluble flour protein extract were added to eluants of different pH. Both solubility and absorbance of the protein solutions differed under these conditions:

a. Protein dissolved well in 0.05N acetic acid and all eluting agents that were 0.05N in acetic acid.

b. Protein dissolved well also in 1% SDS and in 50% propylene glycol under neutral and basic conditions.

c. Absorbance of 1 mg acid-soluble protein in 1 ml 50% propylene glycol was 0.99, 0.85, and 0.71 under basic, neutral, and acidic conditions, respectively.

Consequently, eluants 1 and 2 in Table I were used under acidic conditions (pH 3.2). Gel pH was then made basic by gel equilibration with 0.1M glycine-NaOH (pH 9.8) for 30 min, and eluants 3 and 4 were used under mild basic conditions (pH 9.8). Differences in absorbance under various conditions may result, in part at least, from differences in protein solubility. In addition, pH changes may affect protein apparent hydrophobic properties. This should not detract, however, from comparison of absorbance values of eluates of the various flour proteins.

Apparently most acid-soluble flour proteins are strongly bound to the hydrophobic matrix, even when eluants containing high chaotropic agents (low salting-out effect) are used at low ionic strength (Von Hippel and Schleich 1969). Ionic strengths of acid and base (below those in Table I) were lowered when we used strong eluting agents. Those agents included detergents (nonionic Tween-20, Triton X-100, and anionic SDS) and agents that lower eluant polarity (for example, ethylene glycol and prolylene glycol) in attempts to desorb the proteins on the basis of differences in strength of their hydrophobic interactions with the matrix (Hjerten et al 1974).

Elution with Tween-20, Triton X-100, urea, and guanidine HCl was unsatisfactory because they absorbed strongly at 280 nm and interferred with the measurements.

Under the batch elution conditions given in Table II, proteins

TABLE I

Absorbance (at 280 nm) of Protein[a] Eluates from Phenyl Sepharose CL-4B Under Various Conditions of Batch Elution

Eluant	0.05N Acetic Acid (pH 3.2)	0.05M Tris-Acetate (pH 7.0)	0.05M Glycine-NaOH (pH 9.8)
1) Starting eluant[b]	0.01	0.02	0.10
2) 1% Sodium dodecyl sulfate in 1)	0.74	0.60	0.60
Subtotal	0.75	0.62	0.70
3) 50% Propylene glycol in 1)	0.05	0.17	0.28
4) 30% Methanol in 1)	0.03	0.01	0.01
Subtotal	0.08	0.18	0.29
Total absorbance	0.83	0.80	0.99

[a]0.5 ml acid-soluble proteins (4 mg/ml of 0.05N acetic acid) from untreated KS501097 flour.
[b]0.05N acetic acid, 0.05M Tris-acetate, or 0.05M glycine-NaOH under acidic, neutral, and basic conditions, respectively.

TABLE II

Absorbance (at 280 nm) of Protein[a] Eluates from Phenyl Sepharose CL-4B for Different Flours

Eluant	Shawnee—Good Baking Flour		KS501097—Poor Baking Flour	
	Control	Defatted	Control	Defatted
1) 0.01N CH_3COOH	0.11	0.02	0.15	0.01
2) 1% Sodium dodecyl sulfate in 1)	0.56	0.61	0.62	0.65
3) 0.1M Glycine-NaOH	0.04	0.04	0.10	0.15
4) 50% Propylene glycol in 0.05M glycine-NaOH	0.16	0.18	0.17	0.18
5) 30% Methanol in 0.005M glycine-NaOH	0.02	0.05	0.04	0.05
Total absorbance	0.89	0.90	1.08	1.04
Yield, %[b]	57.1	57.7	69.2	66.7

[a]0.5 ml of acid-soluble protein solutions (4 mg protein/ml of 0.01N acetic acid) per 2 g of Phenyl-Sepharose CL-4B.
[b]Based on an absorbance of 1.56 for 2 mg of acid-soluble flour proteins/ml of 0.01N acetic acid.

from the good flour (both untreated and defatted) showed higher hydrophobic interaction (less protein eluted) than did proteins from the poor flour. There was little difference in total absorbance (and apparent hydrophobic interactions) between defatted and untreated flours for either the good or the poor baking flour. The five solvent systems extracted different (but reproducible) amounts of proteins. The order of solvents applied was dictated by the viscosity and difficulty of handling the detergent solutions and by the advisability to use, sequentially, solvents with increasing capacity to break hydrophobic bonds. The identities of the proteins extracted by the various solvent systems will be the subject of a future communication.

The relatively low yield of eluted proteins (57.1–69.2%, Table II) points to the presence of substantial amounts of strongly hydrophobic gluten proteins that are retained by the gel. They could not be eluted by available eluants recommended in the literature (Anonymous 1975). More complete elution would probably require harsh and destructive solvents.

Results of eluting proteins from untreated flours on a Phenyl-Sepharose CL-4B column are compared in Fig. 1. After the elution profiles and volumes for the eluting agents had been examined individually, the column was eluted with a series of eluting agents. When proteins were eluted from the column with 1% SDS and 50% propylene glycol, the volume of each eluate droplet was about one-third less than that from the 0.01 *N* acetic acid washing. Therefore, at the beginning of 1% SDS elution, eluate was collected in increments of 60 drops rather than 40 drops. The decrease in droplet volume seemed to be caused by the high surface tension of the solvent. Although elution patterns were identical, the intensities of elution peaks from the Sepharose column were different for the proteins from the untreated poor and good bread-making flours (Fig. 1). The eluate in about tube 22 was slightly turbid and immediately preceded a clear protein fraction eluted by 1% SDS. Therefore, the apparent difference of the second peak's intensity for the proteins from the untreated flours may have been related to the degree of turbidity. On the other hand, there was little difference for this fraction between the defatted good and poor flours. Although the turbid aggregation of protein can be brought about by the high content of protein in a solvent that has limited solubility, the turbidity from acid-soluble proteins either in acetic acid (0.01 *N*) or in 1% SDS in 0.01 *N* acetic acid was not observed unless there was a direct modification of, or an interfering compound on, the

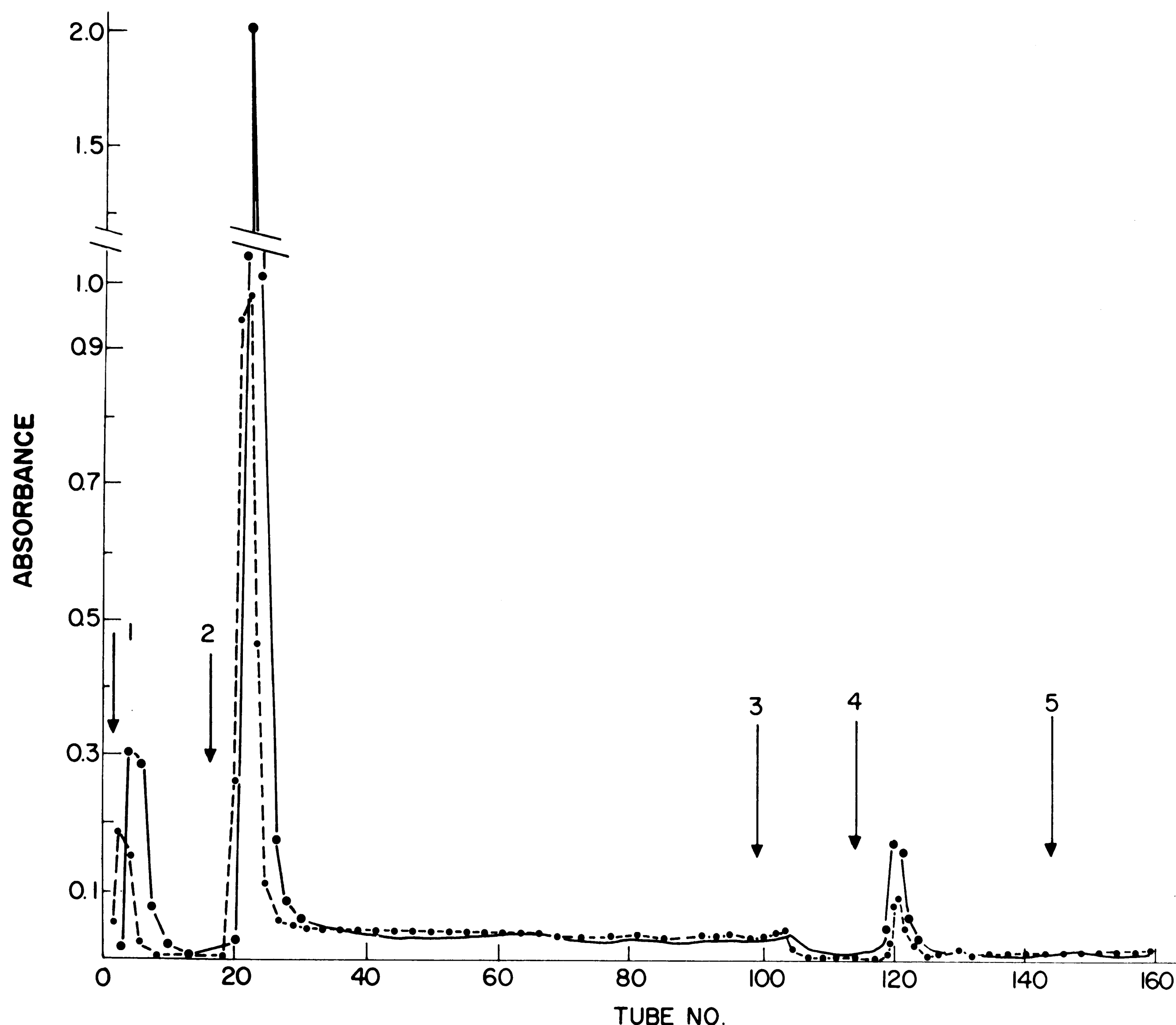

Fig. 1. Elution of acid-soluble proteins (3 ml of a solution containing 4 mg protein/ml 0.01 *N* acetic acid) from untreated KS501097 (——) and Shawnee (--------) flours. The 1.6 × 20-cm column, packed to a height of 4 cm, contained 7.0 g Phenyl-Sepharose CL-4B. Elution was at a rate of 20 ml/hr with: **1)** 50 ml 0.01 *N* acetic acid; **2)** 150 ml 1% sodium dodecyl sulfate in 0.01 *N* acetic acid; **3)** 50 ml 0.1 *M* glycine-NaOH; **4)** 100 ml 50% propylene glycol in 0.005 *M* glycine-NaOH; and **5)** 50 ml 30% methanol in 0.005 *M* glycine-NaOH.

proteins. For this reason, the turbid fractions were pulled together and concentrated further for detection of lipids by thin layer chromatography (TLC). A lipid (presumably glycolipid) was present in these turbid fractions (as shown by TLC). This indicates that lipids from protein fractions of the untreated flours were liberated by exchanging protein hydrophobic group interactions from weaker lipids to the stronger hydrophobic gels. Consequently, the presence of a more turbid (apparently, lipid containing) fraction in the poor than in the good bread-making flour pointed to stronger lipid binding in the good than in the poor flour.

We determined (Chung and Pomeranz 1978) the apparent hydrophobic interactions of glutenins and gliadins that had been separated by Sephadex G-100 gel chromatography from the proteins of the two defatted flours; the results are given in Table III. Glutenin proteins showed stronger hydrophobic binding and also greater difference between poor and good flours than did the corresponding gliadin proteins. Among the eluting solvents, 1% SDS in 0.01*N* acetic acid eluted the highest amount of proteins. About 70% gliadin and 40% glutenin (as percentage of total eluted proteins) were eluted by 1% SDS in 0.01*N* acetic acid (Table III). The recommended alcohol (Anonymous 1975), 30% methanol in 0.005*M* glycine-NaOH, was an ineffective eluant, and better eluants are needed both for more complete elution and reuse of the gel.

To confirm the differences between the apparent hydrophobic interactions of the glutenins and gliadins from the two flours (Shawnee and KS501097), we examined various concentrations of Sephadex-fractionated glutenin and gliadin (1, 2, 3, 4, and 5 mg of protein/ml) with a fixed amount (2 g) of hydrophobic Sepharose gel. As shown in Fig. 2, the glutenin fraction from the poor flour showed less hydrophobic interaction with the gel than that from the good flour, as indicated by higher absorbance at 280 nm of the eluates. Except for components eluted with 0.01*N* acetic acid, other glutenin components were bound stronger in good than in poor quality flour. There were differences between the two flours in both slopes and intercepts of the five linear elution lines. Nevertheless, the patterns were generally similar for the two flours. The fractional elution of proteins by the first four eluants (listed in Tables II and III) indicated a wide range in apparent hydrophobic interactions among glutenins. On the other hand, gliadin proteins were eluted mainly by 1% SDS and some by 50% propylene glycol (Fig. 3). Although overall apparent hydrophobic interaction of gliadins from the poor flour was slightly higher than that of the good flour (as indicated by absorbance of the 1% SDS eluate), gliadins from the two flours showed similar elution patterns (Fig. 3) in their intercepts and slopes. The differences in elution patterns of glutenins and gliadins from a hydrophobic gel confirm basic differences in those proteins (Huebner and Rothfus 1971).

To examine the effect of protein concentration on noncovalent binding, we determined the absorbance of the eluates after allowing protein-gel and protein-protein associations, then calculated the ratios of the absorbances per milligram protein. Table IV shows that for all proteins (gliadins or glutenins from poor or good flour), binding capacity to the gel per milligram protein increased with protein concentration. Furthermore, the glutenin from good baking flour, Shawnee, had the lowest amount of protein eluted from the gel at all levels of concentration, compared with other proteins. On the other hand, eluate absorbance per milligram protein for gliadins from both flours and glutenins from the poor flour showed similar trends, with increase in protein concentration.

The noncovalent binding experiments were based on the premise that the proteins would interact mainly with the hydrophobic gel at low concentration (1 to 2 mg) and might have intermolecular association at high concentrations after the hydrophobic gel had been saturated. The general trends of eluate absorbance per milligram protein at the saturated hydrophobic gel could, therefore, provide additional information on some properties such as degree of hydrophobicity and molecular structure of the acid-

TABLE III

Absorbance (at 280 nm) of Protein[a] Eluates from Phenyl Sepharose CL-4B for Different Proteins

Eluant	Glutenin[b] Shawnee	Glutenin[b] KS501097	Gliadin Shawnee	Gliadin KS501097
1) 0.01*N* Acetic acid	0.08	0.07	0.04	0.03
2) 1% Sodium dodecyl sulfate in 1)	0.26	0.34	0.70	0.64
3) 0.1*M* Glycine-NaOH	0.10	0.07	0.02	0.06
4) 50% Propylene glycol in 0.005*M* glycine-NaOH	0.18	0.24	0.15	0.16
5) 30% Methanol in 0.005*M* glycine-NaOH	0.04	0.06	0.08	0.06
Total absorbance	0.66	0.78	0.99	0.95
Yield, %[c]	42.3	50.0	63.5	60.9

[a]0.5 ml protein solution (4 mg/ml of 0.01*N* acetic acid) per 2 g of Phenyl-Sepharose CL-4B.
[b]The glutenin and gliadin of two defatted flours had been separated on Sephadex G-100 from other proteins.
[c]Based on an absorbance of 1.56 for 2 mg of acid-soluble proteins/ml of 0.01*N* acetic acid.

TABLE IV

Absorbance (at 280 nm) of Glutenins and Gliadins from Two Flours in Eluates from Phenyl-Sepharose CL-4B

	Shawnee—Good Baking Flour					KS501097—Poor Baking Flour				
	1[a]	2	3	4	5	1	2	3	4	5
Total absorbance[b]										
Glutenins	0.50	0.76	1.05	1.36	1.64	0.71	0.98	1.25	1.56	1.83
Gliadins	0.80	1.14	1.47	1.80	2.15	0.75	1.11	1.38	1.69	2.00
Total absorbance/mg of protein[c]										
Glutenins	0.50	0.38	0.35	0.34	0.33	0.71	0.49	0.42	0.39	0.37
Gliadins	0.80	0.57	0.49	0.45	0.43	0.75	0.56	0.46	0.42	0.40

[a]Number of milligrams protein/ml 0.01*N* acetic acid used with 2 g Phenyl-Sepharose CL-4B gel.
[b]Total Absorbance is the sum of the absorbance of eluates from five eluants.
[c]Total absorbance values divided by weight of protein samples used.

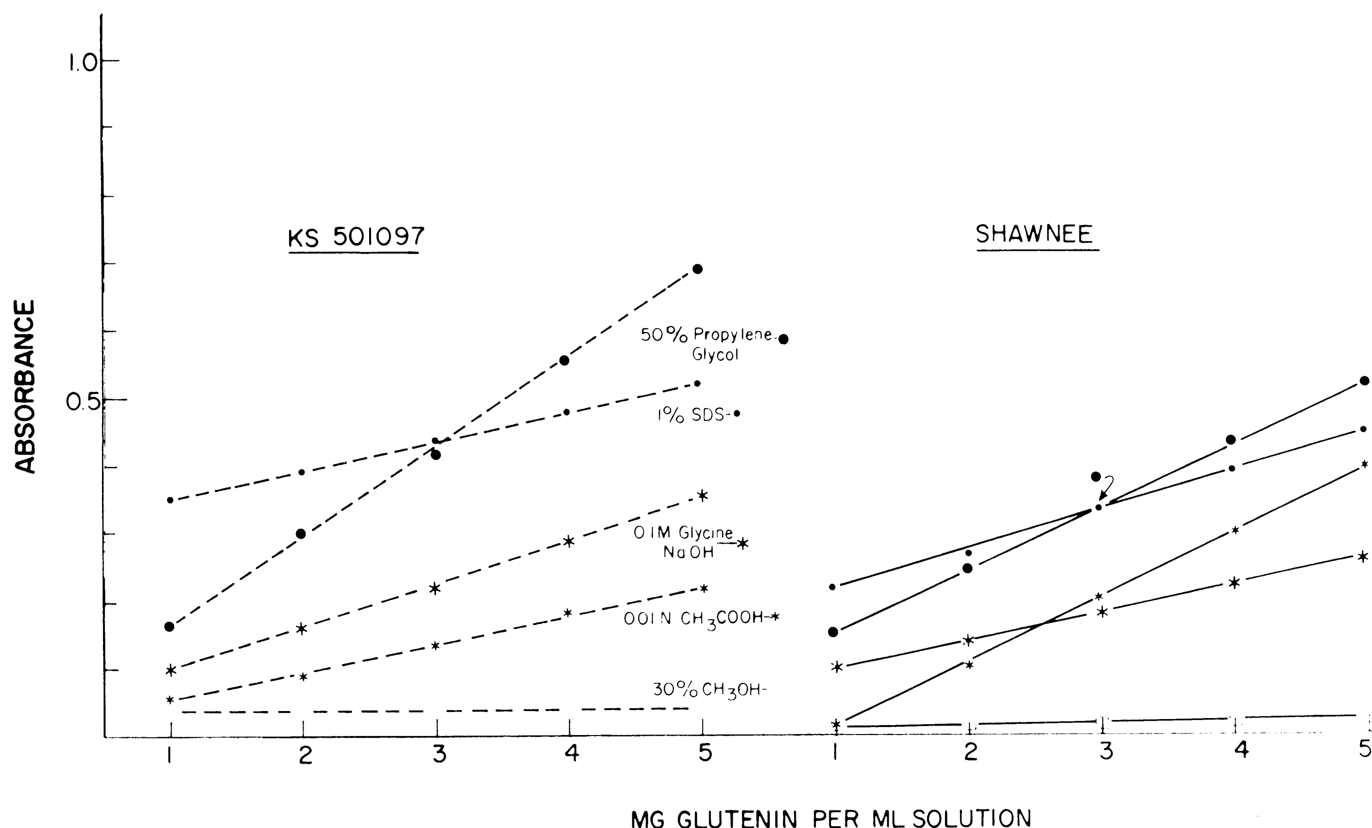

Fig. 2. Absorbance at 280 nm of proteins in eluates (as in Fig. 1) from 2 g Phenyl-Sepharose CL-4B gel mixed with 1 to 5 mg glutenins (Sephadex fractionated from defatted flours).

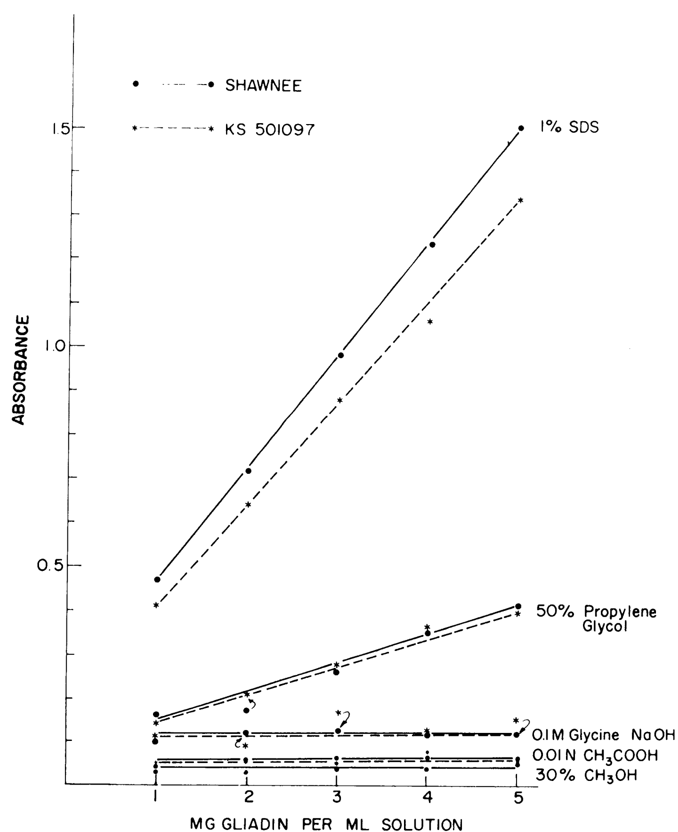

Fig. 3. Absorbance at 280 nm of proteins in eluates (as in Fig. 1) from 2 g Phenyl-Sepharose CL-4B gel mixed with 1 to 5 mg gliadins (Sephadex fractionated from defatted flours).

soluble wheat proteins. In fact, at low concentrations, the glutenins from the good flour showed the greatest interaction with the gel, as indicated by the absorbance of the eluates per milligram protein. At high concentrations, however, all samples gave similar results by showing practically no increase in binding when the total protein concentration was increased from 3 to 5 mg (Table IV).

The adsorbance of proteins on the hydrophobic gel could be influenced in a different manner by the larger molecular weight glutenins than by the gliadins as a result of differences in localized hydrophobic interactions. Among glutenins with large molecular weights, however, binding properties could be affected both by the proportion of hydrophobic sites per unit length of the total polypeptide chain and by the arrangement of nonpolar groups (nonpolar groups inside or outside the molecules). Thus, glutenin fractions from wheats can differ in degree of hydrophobic binding as reported here. At the same time, their functionalities rendered by the different degrees of hydrophobic interactions between proteins and lipids (nonpolar hydrocarbon groups) may play an important role in overall bread-making performance, including mixing.

Glutenins are known (Pomeranz et al 1970) to contribute to physical dough properties (mixing time and mixing tolerance). Optimal dough development is a prerequisite to optimal bread quality. Results of our study show that the contribution of glutenin to dough properties may involve hydrophobic interactions. Our previous studies (Chung and Pomeranz 1978) on protein solubility before and after delipidation of wheat flours indicated that proteins from a good baking flour bound more strongly with lipids than did proteins from a poor baking flour. In addition, glutenin bound more lipids than did gliadin. Thus, the previous and present studies give similar information.

Whether the interactions involved in dough development (as related to optimal mixing time) and in mixing tolerance involve the same mechanism remains to be determined. It may be that since dough development (mixing time) is mainly influenced by the sum of all the forces among the wheat proteins, mixing tolerance is

affected by the location of noncovalent binding sites on the protein molecules (binding site location would determine the interactions among the proteins and the interactions with other flour components). If so, a long mixing time would not necessarily be associated with a good mixing tolerance. Our study of Sephadex-fractionated gliadin and glutenin showed that they interact in the dough. The mode of that interaction is complicated by the presence of other flour components. The involvement of nonprotein components is clearly indicated by the interaction of gluten proteins with lipids.

The difference in apparent hydrophobic interactions between glutenins of good and poor bread-making flours may explain, in part, differences in mixing times or mixing tolerances of the flours. It does not follow, however, that differences in hydrophobicity are always causatively related to loaf volume potential. Reconstitution studies (Hoseney et al 1969a,b) have shown that gliadin proteins control the loaf volume potential of wheat flour. Similar reconstitution studies have shown that the glutenin fraction governs the mixing requirement of a wheat flour. The interaction of glutenins with gliadins and other flour components, however, quite likely contributes to the quality of the baked bread.

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