Surface Hydrophobicity of Gliadin Components

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ABSTRACT

Gliadin components were fractionated, depending on their accessible (surface) hydrophobicity, by hydrophobic interaction chromatography (HIC) on phenyl- and octyl-Sepharose CL 4B. Hydrophobicity was also estimated by measuring heptane-binding capacity. Several HIC procedures were compared, using modified pH and eluting solvent polarity. The adsorption of gliadins to hydrophobic gels depended on the nature of the ligand attached to the matrix; the binding was stronger on phenyl- than on octyl-Sepharose, but nonspecific hydrophobic interactions played the leading role in the adsorption, even though aromatic stacking obviously occurred on phenyl-Sepharose. Fractionation patterns on phenyl-Sepharose were different for proteins solubilized in aluminum lactate buffer and those solubilized in acetic acid, due to conformational changes in the macromolecules. On octyl-Sepharose, the behavior of gliadins also varied with the eluant, but the cause was not clearly established. The combination of a pH increase and a linear gradient of ethanol concentration gave a satisfactory fractionation of gliadins on phenyl-Sepharose. Electrophoretically defined gliadins are not composed of proteins of identical hydrophobicity, and some components were eluted as several distinct HIC fractions. Hydrophobicity of gliadins was generally high but variable. No relation was observed between HIC results and hydrophobicity calculated from amino acid compositions of fractions, but correlation with heptane binding was good except for ω-gliadins. A rough comparative estimation of the surface hydrophobicity of gliadin components was made. The relationship between gluten structure and the functional properties of gliadins is discussed.

The structure and properties of gluten depend on the state of aggregation of its components, ie, on the interactions that control the conformation of protein macromolecules and their ability to associate through hydrogen and ionic linkages, disulfide bridges, and hydrophobic interactions.

The role of hydrogen and ionic linkages was reported by Pomeranz (1971) and Bloksma (1971). Gluten resistance is increased by D_2O (Tkachuk and Hlynka 1968), and a strong gluten has more hydrogen bonds than a weak one (Wakar et al 1975). Only a small fraction of thiol groups and disulfide bonds influence rheological properties of gluten (Bloksma 1972, 1975). Many workers (Bigelow 1967; Chothia 1976; Tanford 1962, 1978) have shown the role of hydrophobic interactions in the structure of proteins. The solubility of wheat gluten and its characteristic high content of apolar amino acids indicate that hydrophobic interactions are important in determining its properties. This would occur essentially in protein-protein and lipid-protein interactions. Hydrophobic bonding is important in the association of lipids with gluten proteins (Chung and Pomeranz 1978, Chung and Tsen 1975, Chung et al 1978, Hoseney et al 1970, Olcott and Mecham 1947). The hydrophobic nature of some protein-protein bonds is also indicated by the fact that alkanes decrease gluten solubility and extensibility (Ponte et al 1967). The occurrence of these interactions in glutenins was established by Godon (1969) and Wakar et al (1975). Their characteristic properties can probably be explained in terms of hydrophobic interactions, disulfide bridges, and hydrogen bonds (Bernardin 1978; Kahn and Bushuk 1978, 1979). Thus, general indications of hydrophobic interactions are found in gluten proteins, but very few specific studies have been made, except by Greene and Kasarda (1971) on α-gliadin. They showed that apolar areas exist on the surface of the molecules and that hydrophobicity relies upon conformational changes.

Therefore we decided to study hydrophobic interactions by hydrophobic interaction chromatography (HIC) and by determining alkane binding. HIC fractionates substances according to the importance of hydrophobic zones on the outer shell of a protein molecule that interact with apolar groups grafted onto a gel matrix. In this technique, only surface hydrophobicity of the protein is involved because the inner apolar regions of proteins are not accessible to the apolar groups on the gel. The strength of the interaction depends on three factors: the surface hydrophobicity of the proteins, the hydrophobicity of the gel, and the composition of the eluant. Elution is generally achieved by modifications of the eluant composition to weaken hydrophobic interactions between the proteins and the gel. Reviews on HIC were recently published by Shaltiel (1975), Hofstee (1976), Ochoa (1978), and Yon (1978).

Hofstee (1975a) used this technique to confirm the existence, previously indicated by Klotz (1970), of apolar areas on the surface of protein macromolecules. First results on gluten proteins showed that gliadins can be fractionated by this technique and thus do not all possess the same surface hydrophobicity (Popineau and Godon 1978). Caldwell (1979) found HIC to be a suitable and powerful method for the purification of gliadins and used it to isolate a β-gliadin component very simply.

Chung and Pomeranz (1979) related the adsorption of glutenins to hydrophobic gels to the bread-making quality of wheats.

The measurement of alkane binding gives a quantitative estimation of the accessible hydrophobicity of proteins with respect to their molecular surroundings and has been used to study protein hydrophobicity and its variation with conformational changes (Aso et al 1974; Ishino and Okamoto 1975; Mohammadzadeh-K et al 1967, 1969a, 1969b).

In the present work we used these two complementary methods to study how gliadins are involved in gluten structure and to determine whether their hydrophobicity could be related to some of their functional properties.

MATERIALS AND METHODS

Gluten Preparation

The wheat variety Capitole was milled to flour in a Chopin-Dubois laboratory mill. Flour yield was about 65%. Gluten, extracted manually by washing a dough-ball with tap water, was freeze-dried, ground, and defatted with pentane before extraction of gliadins.

Extraction of Gliadins

Gliadins were extracted by solubilization in a water-dioxane mixture. Following a suggestion of De Deken and Mortier (1955), Lefebvre[1] found that a mixture of water and dioxane (60:40, v/v) extracts gliadins from gluten exhaustively and more specifically than ethanol. Furthermore, from a practical point of view, a solution of proteins in water-dioxane can be directly freeze-dried without previous dialysis.

Gluten was suspended in a water-dioxane (60:40, v/v) mixture and extracted by stirring gently for 16 hr at 5°C. The soluble

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[1]Personal communication.

fraction, mainly composed of gliadins, accounted for 50–60% of the initial gluten.

Preparation of Purified ω-Gliadins

These water:dioxane-soluble proteins (WDSP) were fractionated on Sephadex G-100 in aluminum lactate buffer (0.028*M* aluminum lactate, 0.018*N* lactic acid, pH 3.6) in a 100 × 2.5-cm column. The first two peaks were collected. The first contained ω-gliadins and some soluble glutenins; the second contained α- and β-gliadins and γ-gliadins. ω-Gliadins were obtained free of soluble glutenins from the first peak by selective precipitation (Bietz and Wall 1975).

HIC

Because the application of HIC to gluten proteins is a new development of this technique, we had to adjust experimental conditions. Several types of gels can be used (Yon 1978). We chose neutral phenyl-Sepharose and octyl-Sepharose CL 4B gels (from Pharmacia) to avoid ionic effects between proteins and the gel matrix. These are cross-linked agarose gels to which phenyl or octyl groups are attached by reaction with corresponding glycidyl-ethers (Hjerten et al 1974). Theoretically octyl-Sepharose should develop stronger hydrophobic interactions than phenyl-Sepharose. However, phenyl-Sepharose is known to develop additional specific interactions with aromatic amino acids (Anonymous 1975).

The low solubility of gliadins in many common buffers limited the choice of eluting conditions. In the absence of denaturing agents such as urea or detergents, the use of salt concentration gradients was impossible because of salting out of gliadins even at very low salt concentrations. The elution processes used were therefore to increase pH and to decrease eluant polarity.

Preliminary experiments showed that the following chromatographic conditions were satisfactory. A column (diameter, 12.7 mm; length, 180 mm) was packed with phenyl- or octyl-Sepharose CL 4B and equilibrated with aluminum lactate buffer (0.028*M* aluminum lactate, 0.02*N* lactic acid, pH 3.6) or 0.01*N* acetic acid. Protein was dissolved and applied to the column in the same buffer (100–300 mg of protein in 10–30 ml of buffer). When a decrease of eluant polarity was used to elute gliadins in an acidic medium, desorption was promoted by progressively increasing the 2-propanol concentration from 10 to 50% in steps of 10%. The column was finally rinsed with 50% 2-propanol and 50% buffer (0.015*M* Tris HCl, pH 8.6, and 3*M* urea).

When pH modification was used, the column was first washed with about 60 ml of acid buffer. The elution was then performed with a linear gradient of up to 60% ethanol in 0.02*N* ammonia, to give a total eluted volume of 480 or 600 ml, depending on the weight of protein applied to the column (100–300 mg). The column was finally washed with 60% ethanol:0.02*N* ammonia:3*M* urea. In all procedures, the flow rate was 20 ml/hr. The eluate was collected in 4-ml fractions, and proteins were detected by measuring absorbance at 280 nm. Yields were determined from the absorbance of the fractions and from their dry weight after freeze-drying.

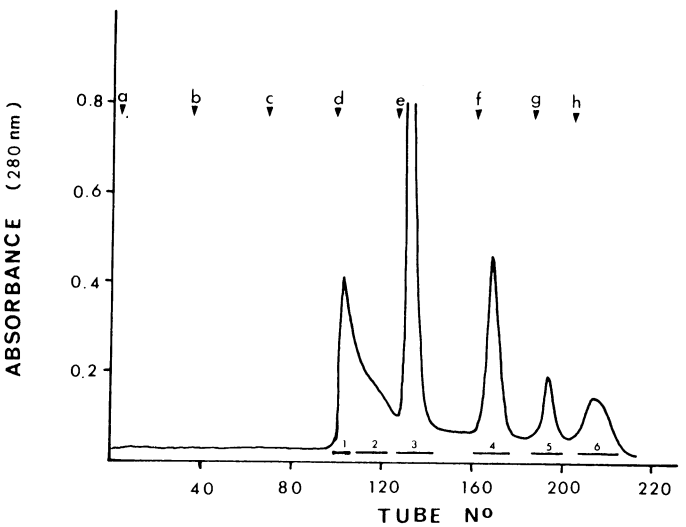

Fig. 1. Elution profile of gliadin fractions **1–6** separated by hydrophobic interaction chromatography on phenyl-Sepharose CL 4B. Elution performed by decreasing eluant polarity. **a–f,** Aluminum lactate buffer (0.028*M*, pH 3.6) alone (**a**) and with 2-propanol at 10% (**b**), 20% (**c**), 30% (**d**), 40% (**e**), and 50% (**f**); **g,** buffer with 50% 2-propanol and 2*M* urea; **h,** Tris buffer (0.015*M*, pH 8.6) and 3*M* urea with 50% 2-propanol.

Starch Gel Electrophoresis

Fractions were analyzed by starch gel electrophoresis (starch concentration 12%) in an aluminum lactate (pH 3.2)-3*M* urea buffer. The method is derived from that of Autran and Bourdet (1975). After staining with nigrosine and destaining with ethanol:water, gliadins were identified according to their mobility (the fastest α-gliadin band was assigned a mobility of 100). The relative amount of each band was determined by measuring transmitted light, using a recording densitometer (PH I 6 Vernon), with a coefficient of variation of about 10%.

Alkane Binding

Alkane binding was measured by a method adapted from Mohammadzadeh-K et al (1967). Protein solution in 2.4 ml of aluminum lactate buffer (pH 3.6, protein content 0.5–1.0 mg/ml) was equilibrated with *n*-heptane (1.8 ml) in a stoppered test tube by stirring gently for 16 hr at 25° C to avoid formation of an emulsion. The two phases (aqueous and alkane) remained separate during the whole equilibration process, and binding of alkane occurred only at the water-alkane interface. The upper *n*-heptane layer was then discarded. The heptane bound to the proteins was extracted from the protein solution with an undecane (1 ml of equilibrated protein solution and 5 ml of undecane) by stirring gently for 90 min and using *n*-nonane as internal standard. The quantity of bound heptane was measured by gas chromatography. The alkane mixture (about 1 μl) was injected into a column (diameter, ⅛ in.; length, 210 cm) packed with Chromosorb (80/100 mesh W A W) coated with 10% silicone SF 96. The temperature of the injection port was 180° C and of the column, 120° C. The Girdel 3000 chromatograph was equipped with a flame ionization detector connected to an integrator. The direct injection of protein solution (Mohammadzadeh-K et al 1969a) was less successful than use of the internal standard (*n*-nonane), which improved the accuracy of the determinations (coefficient of variation = 5%).

Hydrophobicity Calculated from Amino Acid Composition

Three relationships regarding polarity or hydrophobicity of proteins were calculated from amino acid compositions reported previously (Popineau and Godon 1978). These were: polarity (p) = volume of polar residues/volume of nonpolar residues (Fisher 1964), the nonpolar side chain content (NPS) = content of (Trp + Ile + Tyr + Phe + Pro + Leu + Val)/total number of residues (Waugh 1954), and the average hydrophobicity ($H\phi_{ave}$) = total hydrophobicity of amino acid side chains/total number of residues (in calories per residue) according to Bigelow (1967).

The precise meaning of those three relationships was discussed by Bigelow (1967). Briefly, Waugh (1954) indicated that the hydrophobicity of the molecule partly determines the structure of the protein, and Fisher (1964) established a relationship between p, the molecular weight, and the most probable shape of the molecule. Bigelow (1967) regards $H\phi_{ave}$ as a more precise estimation of hydrophobicity than NPS; to calculate $H\phi_{ave}$ he attributed to each amino acid a distinct value of hydrophobicity, corresponding to the energy required to solubilize the amino acid in ethanol (Tanford 1962). Waugh (1954) distinguished only two classes of amino acid. For our study, the main importance of those relationships was that they provided an alternative way of estimating hydrophobicity fundamentally different from HIC or alkane binding. In fact, these relationships measure a total, and a rather theoretical, hydrophobicity due to all the amino acids of the molecule, whatever their position in the molecule may be.

RESULTS AND DISCUSSION

In this article, hydrophobicity refers to surface or accessible hydrophobicity, unless otherwise specified.

Fractionation on Phenyl-Sepharose and Octyl-Sepharose CL 4B

Gliadins in aluminum lactate buffer were eluted from phenyl- and octyl-Sepharose by decreasing eluant polarity with increased amounts of 2-propanol at constant pH (Figs. 1 and 2). The results suggest that adsorption of gliadins on both gels is mainly due to hydrophobic interactions, although the elution profiles were different. Elution of gliadins from phenyl-Sepharose required higher concentrations of 2-propanol than elution from octyl-Sepharose. Some gliadins were not bound on octyl-Sepharose: in aluminum lactate buffer without 2-propanol, all gliadins were adsorbed to phenyl-Sepharose but 30% were not bound on octyl-Sepharose. This was not due to different binding capacities of the gels because the columns were not saturated. This nonadsorbed fraction (on octyl-Sepharose) had a composition different from that of the total extract; few γ-gliadins were present and some α-gliadins were missing. Although the relative hydrophobicity of octyl-Sepharose was greater, the strength of the interaction between the gliadins and the gels was higher on phenyl- than on octyl-Sepharose. Specific binding occurred between aromatic groups of phenyl-Sepharose and proteins. However, previous results on phenyl-Sepharose (Popineau and Godon 1978) showed that the most strongly adsorbed gliadins had a similar phenylalanine content but a much lower tyrosine content than the gliadins eluted first. This suggests that nonspecific hydrophobic interaction plays the leading role in adsorption, even on phenyl-Sepharose. The gliadins that are not adsorbed on octyl-Sepharose are obviously not very hydrophobic and must be bound to phenyl-Sepharose mainly by aromatic linkages.

On phenyl-Sepharose, gliadins were separated into six fractions. A 30% concentration of 2-propanol was necessary to start elution, and elution of the last two fractions required the addition of urea and an increase of pH (Fig. 1). In elution order, the fractions accounted for 16, 15, 35, 21, 7, and 7%, respectively, of the proteins eluted (yield 100%), and ω-gliadins comprised 82% of the first fraction (Fig. 3). In fraction 2, each type of gliadin is present, but more faster ω-gliadins are present that slower ones. Fewer γ-gliadins were found than in the total WDSP: γ-71 was absent, as were two α-gliadins (α-93 and α-100). In fraction 3, ω-gliadins and γ-74 were totally absent, but γ-71 and all β- and α-gliadins were present. The last three fractions had a faint electrophoretic pattern, but the presence of β- and γ-gliadins was clear in fractions 4 and 6. Albumins were present only in fractions 1 and 2.

On octyl-Sepharose, gliadins were separated into eight fractions. In elution order, the fractions accounted for 8, 13, 10, 26, 25, 12, 1, and 6%, respectively, of the proteins eluted (yield 91%). Some gliadins were eluted in aluminum lactate buffer without 2-propanol (Fig. 2). Fraction 1 consisted of traces of ω-gliadins. Fraction 2 was mainly composed of ω-gliadins (particularly ω-24, ω-27, ω-41, and ω-44) and β-gliadins (Fig. 4). Very few γ-gliadins were observed and only one α-gliadin (α-97). Fraction 3 was composed mainly of β-gliadins, but γ-74, γ-65, and a very small quantity of γ-71 were present. Fraction 4 was principally composed of three bands (γ-65, α-97, and α-100, 60% of the fraction) and β-gliadins (25% of the fraction). γ-Gliadins (γ-60, γ-65, and γ-71, but no γ-74) accounted for 66% of fraction 5. β-Gliadins were principally represented by β-84, and α-97 was the most important of the α-gliadins.

The electrophoretic pattern of the three last fractions was very faint; however, some γ-gliadins were visible in fraction 6, some proteins too large to enter the gel seemed to be present in fractions 7 and 8, and some albumins were present in fractions 2 and 3.

A first classification of gliadins, according to their surface hydrophobicity was thus established: ω-gliadins appear to have a low hydrophobicity and γ-gliadins (except γ-74) a high one, while α- and β-gliadins are more heterogeneous in hydrophobicity. However, this type of fractionation does not give a very effective separation of the gliadins because every fraction is composed of several electrophoretic bands. The main difference between the two gels lies in the strength of their interactions with the gliadins, and octyl-Sepharose permits the separation of a fraction (fraction 3) more enriched in β-gliadins.

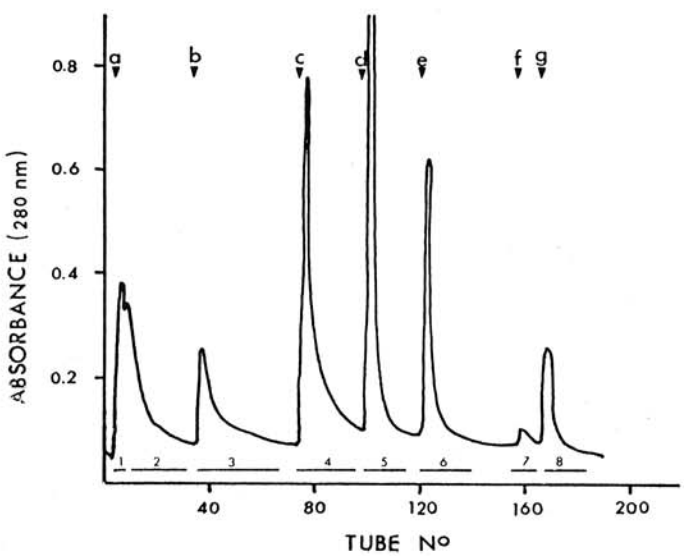

Fig. 2. Elution profile of gliadin fractions **1–8** separated by hydrophobic interaction chromatography on octyl-Sepharose CL 4B. Elution performed by decreasing eluant polarity. **a–f,** Aluminum lactate buffer (0.028 *M*, pH 3.6) alone (**a**) and with 2-propanol at 10% (**b**), 20% (**c**), 30% (**d**), 40% (**e**), and 50% (**f**); **g,** Tris buffer (0.015 *M*, pH 8.6) and 3 *M* urea with 50% 2-propanol.

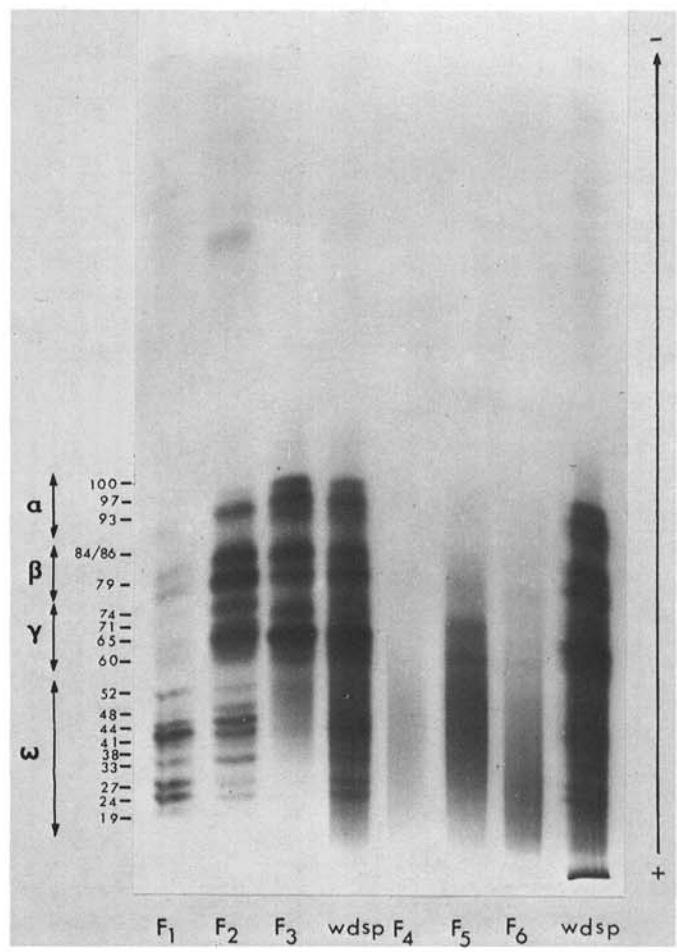

Fig. 3. Electrophoresis pattern of gliadin fractions separated by HIC on phenyl-Sepharose CL 4B using decreasing eluant polarity. Fraction numbers correspond to those of Fig. 1; **wdsp** = water:dioxane-soluble proteins. Mobilities of α-, β-, γ-, and ω-gliadins are shown.

Elution by Aluminum Lactate Buffer vs Acetic Acid

Phenyl-Sepharose. Gliadin binding to phenyl-Sepharose was stronger in acetic acid than in aluminum lactate. Without urea (Table I), 86% of the gliadins were eluted in aluminum lactate but only 24% in acetic acid. This difference cannot be explained by the different ionic strengths, because a higher ionic strength generally reinforces hydrophobic interactions and adsorption on neutral gels (Porath et al 1973, Rosengren et al 1975, Yon 1978). The opposite phenomenon was observed here. Furthermore, substitution of lactate buffer for acetic acid (alcoholic concentration being equal) was sufficient to elute bound gliadins. The conformation of gliadin molecules is probably different in the two media. A viscometric study of peak 2 from Sephadex-G 100 (composed of α-, β-, and γ-gliadins) proved that expansion and flexibility of gliadin molecules are high in 0.01*N* acetic acid and that partial aggregation occurs in aluminum lactate buffer; differential spectrophotometric measurements showed that the proportion of chromophores exposed to solvent was greater in acetic acid than in lactate buffer, although it is large in both cases (Lefebvre[2]). In acetic acid, the accessibility of a larger number of hydrophobic areas (and their stronger binding to the gel) can probably be explained by the greater expansion of molecules. Whatever the solvent may be, a high level of chromophore exposure (aromatic amino acid residues) is consistent with a great number of accessible hydrophobic areas and a strengthening of the binding of the proteins to phenyl-Sepharose by linkages between aromatic groups. These results agree with those of Greene and Kasarda (1971) concerning the modifications of α-gliadin hydrophobicity by conformational changes. As in aluminum lactate buffer, ω-gliadins were eluted first in acetic acid, but fractionation of α-, β-, and γ-gliadins was very poor. These were eluted almost all together by alumimum lactate buffer and 2*M* urea with 50% 2-propanol after 0.01*N* acetic acid:2*M* urea with 50% 2-propanol (Table I).

TABLE I
Quantitative Estimation of Gliadin Fractions Progressively Separated on Phenyl- and Octyl-Sepharose CL 4B by Decreasing Eluant Polarity: Results from Two Eluants

Eluant	Protein Recovery (percent of total) on Phenyl-Sepharose	Octyl-Sepharose
Aluminum lactate (0.028*M*, pH 3.6)	0	21
Plus 2-propanol		
10%	0	10
20%	0	26
30%	31	25
40%	35	12
50%	21	1
Plus 2*M* urea	7	ND[a]
Tris HCl (0.015*M*, pH 8.6) plus 50% 2-propanol and 3*M* urea	7	6
Acetic acid (0.01*N*)	0	53
Plus 2-propanol		
10%	0	7
20%	0	8
30%	8	10
40%	9	0
50%	7	0
Plus 2*M* urea	4	ND
Aluminum lactate (0.02*M*) plus 50% 2-propanol and 2*M* urea	64	ND
Tris HCl (0.015*M*, pH 6.8) plus 50% 2-propanol and 3*M* urea	8	23

[a]Not done.

Octyl-Sepharose. On octyl-Sepharose, more gliadins were bound in aluminum lactate buffer than in acetic acid (Table I), but whereas gliadins were progressively and almost completely eluted (yield 95%) with aluminum lactate buffer, only 77% of applied gliadins were washed out of the column with acetic acid. In acetic medium, gliadins appeared to separate into three groups (Fig. 5).

The first consisted of gliadins that were more weakly adsorbed in acetic acid than in lactate buffer (fractions 1 and 2). Their electrophoretic pattern (Fig. 6) showed that they were composed of α-, β-, γ-, and ω-gliadins, but not in the same proportions as in the whole WDSP, and that α-gliadins and γ-65 were noticeably less important.

The second group consisted of fraction 6, eluted with Tris buffer: urea:50% 2-propanol and composed of α-, β-, and γ-gliadins.

The third group (fractions 3–5) was characterized by the following points: fraction 3 had a high content of β-gliadins; in fraction 4, β-gliadins were less important than α-gliadins; and fraction 5 contained γ-65 and a faster γ-71 that was missing in previous fractions. Only one β-gliadin (β-84) and three α-gliadins could be found. Thus, the three fractions have a composition similar to those eluted for the same 2-propanol content in aluminum lactate buffer (Figs. 2 and 4).

For the gliadins of groups 1 and 2, the differences between lactate

[2]Personal communication.

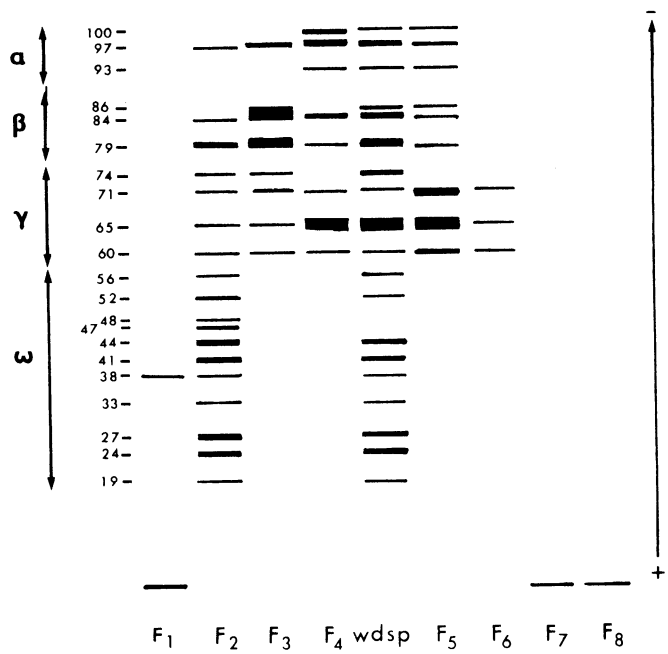

Fig. 4. Diagram of the electrophoresis pattern of gliadin fractions separated by HIC on octyl-Sepharose CL 4B using decreasing eluant polarity. Fraction numbers correspond to those of Fig. 2. **wdsp** = water:dioxane-soluble proteins. Mobilities of α-, β-, γ-, and ω-gliadins are shown.

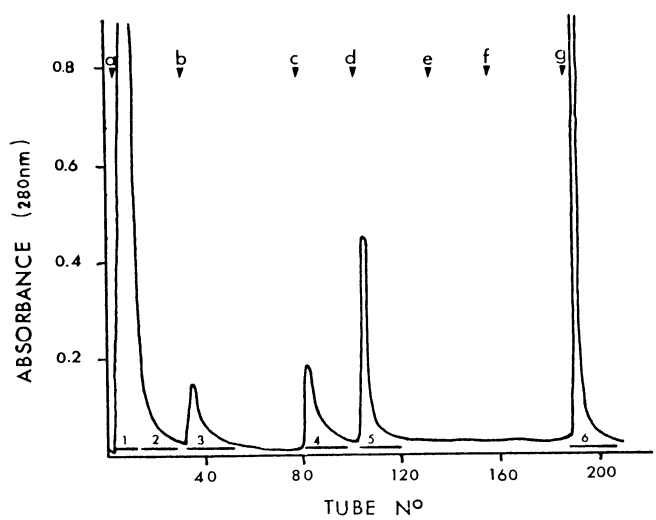

Fig. 5. Elution profile of gliadin fractions **1–6** separated by HIC on octyl-Sepharose CL 4B. Elution by decreasing eluant polarity by successive stages. **a–f,** Acetic acid (0.01*N*) alone (**a**) and with 2-propanol at 10% (**b**), 20% (**c**), 30% (**d**), 40% (**e**), and 50% (**f**); **g,** Tris buffer (0.015*M*, pH 8.6) and 3*M* urea with 50% 2-propanol.

and acetic acid elution on octyl-Sepharose are difficult to explain in the same way as those on phenyl-Sepharose.

The gliadins of groups 1 and 2 were not equally affected by the composition and ionic strength of the eluant. For the first group, the effect of ionic strength seemed to be predominant in the absence of specific interactions between aromatic groups: ie, the higher the ionic strength, the stronger the adsorption. The degree of expansion of the molecules did not play a big role in binding. The binding of the gliadins of the second group did not follow the general rule of ionic strength; it seemed to be largely compensated for by the greater expansion of the molecules in acetic acid. Further investigations are necessary to understand this point, for HIC behavior of gliadins obviously varies largely with the eluants used. This effect could explain why Caldwell (1979) found all gliadins adsorbed to octyl-Sepharose in ammonium acetate buffer and why the yields reported for gliadins by Chung and Pomeranz (1979) are rather low. These results could be related to conformational changes of proteins.

Elution Using Increasing pH Before a Linear Gradient of Alcohol Concentration

Preliminary experiments using a gradient of pH from 3.6 to 4.9 and then a step increase to 8.6 before a linear gradient of 2-propanol concentration at pH 8.6 indicated that a better fractionation of the gliadins was likely if the pH jump preceded the gradient of alcohol, although the resolution of the different fractions was still not satisfactory. The technique using ammonia and ethanol was therefore adopted. Figure 7 shows the elution profile on phenyl-Sepharose of the WDSP that were separated by a pH increase followed by a linear gradient of ethanol concentration. The first peak, eluted in the void volume, consisted of nonprotein material. Three protein fractions were then eluted by ammonia when pH was increased from 3.6 to 9.0 and six fractions by the ethanol gradient. A tenth fraction was eluted with 0.02*N* ammonia:60% ethanol:3*M* urea. These fractions represent 4, 37, 12, 3, 4, 8, 9, 6, 4, and 13%, respectively, of the proteins eluted (yield 80%).

Figure 8 shows the electrophoretic patterns of the 10 fractions. Fraction 1 tended to precipitate. It consisted of 50% albumins and 50% gliadins, as determined by densitometry. The slowest ω-gliadins and one α-gliadin were present. Fractions 2 and 3 consisted of all types of gliadins but in different proportions from those of the total extract, γ-gliadin content being much lower. Fraction 3 had a noticeably low content of α-gliadin and a different composition of ω-gliadins. Moreover, a large amount of γ-74 was found, whereas the content of other γ-gliadins was very low. No albumins were in the fractions eluted by the ethanol gradient. These fractions consisted of α-, β-, and γ-gliadins in variable proportions. ω-Gliadins were absent or were present in low concentration in all those fractions. Fractions 4 and 5 (eluted with 25–33% ethanol) were not fully separated, but high concentrations of β- and γ-gliadins were obvious. The main fractions (6 and 7) eluted with 37 and 40% ethanol, respectively, consisted almost entirely of β- and γ-gliadins. In fraction 6, two gliadin components (γ-60 and γ-65) amounted to 68% and two β (β-79 and β-84) to about 30% of the total. The presence of a γ-gliadin (γ-71) and the low content of

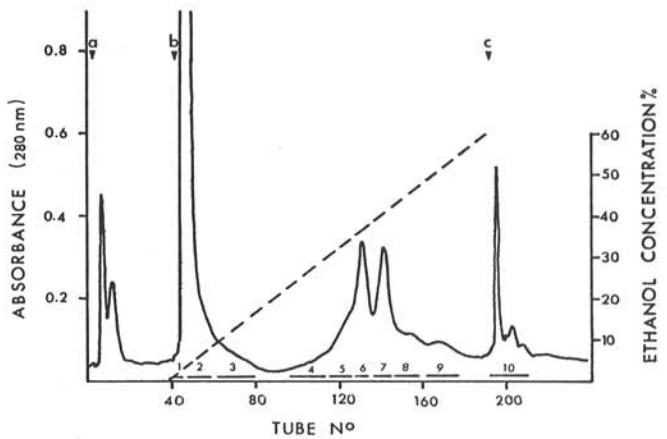

Fig. 7. Elution profile of gliadin fractions **1–10** separated by HIC on phenyl-Sepharose CL 4B. Elution performed by increasing the pH and decreasing eluant polarity. **a,** Aluminum-lactate buffer (0.028*M*, pH 3.6); **b,** linear gradient of ethanol in 0.02*N* ammonia (slope indicated by dashed line); **c,** 0.02*N* ammonia and 3*M* urea with 60% ethanol.

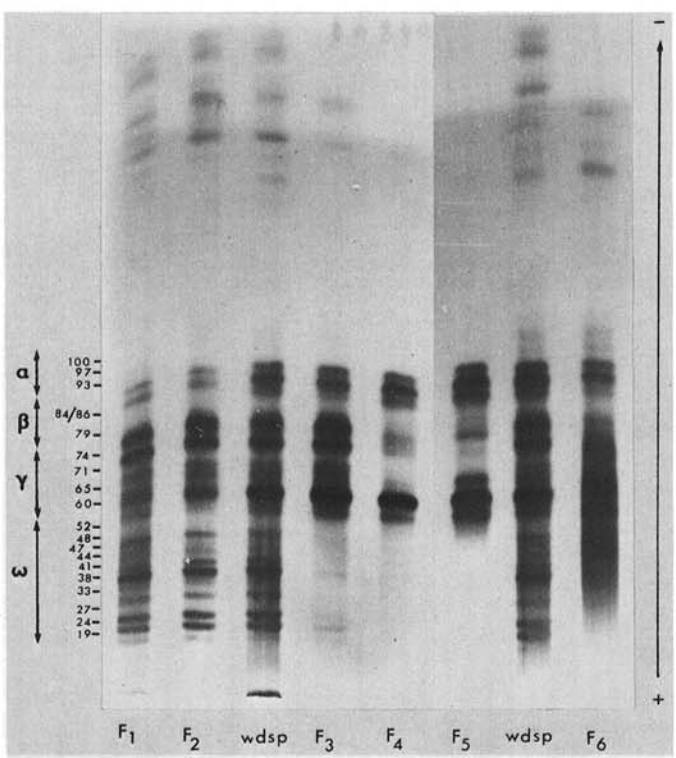

Fig. 6. Electrophoresis pattern of gliadin fractions separated by HIC on octyl-Sepharose CL 4B. Elution in 0.01*N* acetic acid performed by decreasing eluant polarity. Fraction numbers correspond to those of Fig. 5. **wdsp** = water:dioxane-soluble proteins. Mobilities of α-, β-, γ-, and ω-gliadins are shown.

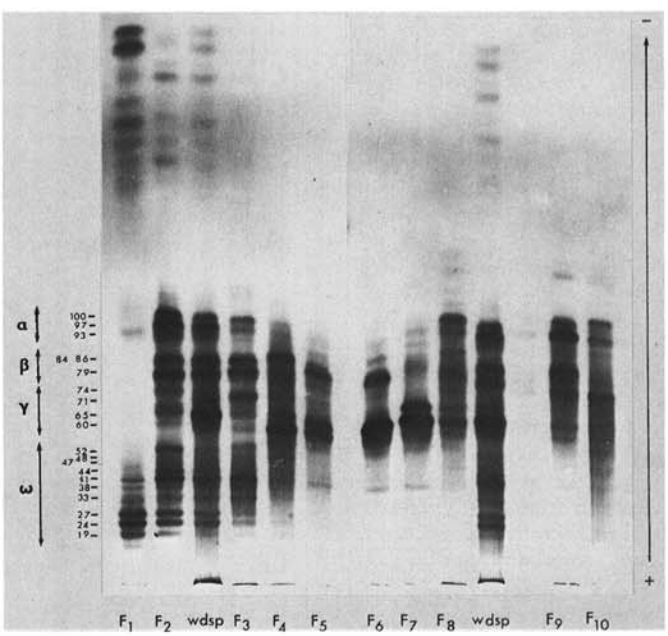

Fig. 8. Electrophoresis pattern of gliadin fractions separated by HIC on phenyl-Sepharose CL 4B using increasing pH and decreasing eluant polarity. Fraction numbers correspond to those of Fig. 6. **wdsp** = water:dioxane-soluble proteins. Mobilities of α-, β-, γ-, and ω-gliadins are shown.

β-gliadins distinguishes fraction 7 from the previous one. The following fractions (8 and 9), eluted with ethanol concentrations above 40%, were rich in α-gliadins (fraction 8) and β-gliadins (fraction 9). In fraction 9, α-97 concentration was very high, and in fractions 8–10, an α-gliadin of higher mobility than 100 was found.

Of all the methods used for HIC, this last elution scheme gave the best results, judging by the separation of the gliadin components. Some complementary investigations were made on the fractions.

The amino acid composition of the main fractions separated on phenyl-Sepharose have already been reported (Popineau and Godon 1978). Fraction 1 has a high lysine content because of the presence of albumins. The main fractions eluted by the ethanol gradient (6 and 7), composed mainly of γ-gliadins, had lower tyrosine and total aromatic amino acid contents than did fractions 1 and 2, and on octyl-Sepharose (with identical chromatographic conditions), two similar fractions were strongly adsorbed to the gel. This confirms that the strong binding of fractions 6 and 7 to the gel was due to nonspecific hydrophobic interactions, rather than to interactions between aromatic groups of the gels and the proteins.

The polarity and hydrophobicity ratings calculated from amino acid composition and the heptane binding results are shown in Table II for the main fractions obtained by HIC. The values of NPS and $H\phi_{ave}$ correspond to those of globular proteins. They are in a range in which Bigelow (1967) found more than 50% of the 150 proteins he studied (1,000–1,200 calories per residues). According to the "limiting law" established by Fisher (1964) and Bigelow (1967), the values of p and $H\phi_{ave}$ of the gliadin fractions also indicate that their components belong to the group of globular proteins with a "hydrophobic core" rather than to the group of proteins with a great excess of hydrophobic groups.

An obvious lack of correlation exists between the calculated hydrophobicity relationships and the surface hydrophobicity suggested by HIC results. This is not surprising because, as already pointed out, hydrophobicity relationships do not depend upon a protein's three-dimensional structure. Those relationships do not take into account the positions of the amino acid side chains, ie, whether they are exposed to the solvent or are buried in the "core" of the molecule. On the contrary, HIC behavior is related only to the apolar residues exposed to the solvent (not to all the apolar amino acids of the polypeptide chain) and to the accessibility of those hydrophobic surfaces to the ligand fixed on the gel.

On the other hand, heptane binding is closely related to the chromatographic properties of the fractions. This agrees with the results of Keshavarz and Nakai (1979), who found a good relationship for several proteins between surface hydrophobicity estimated by HIC and partition-phase coefficients but no significant relationship with $H\phi_{ave}$. Thus many hydrophobic areas of gliadins appear to be accessible, and, according to their heptane binding, gliadins are more hydrophobic than the most hydrophobic proteins studied by Mohammadzadeh-K et al (1967, 1969a, 1969b). Our findings confirm the results of Puri et al (1976), who showed from sorption isotherms that gliadins and glutenins have a large total specific area (ie, they are very extended molecules) and can be distinguished from the other vegetable proteins by the size of their hydrophobic surface. Those authors think that the very high proline content reduces their helical content, and this allows many apolar side chains to be exposed to solvent. Heptane binding of gliadins (Table II) also confirms that hydrophobicity of their components is variable. The electrophoretic pattern of the HIC fractions (Fig. 7) shows that components with different hydrophobicity are found within the gliadin groups, as defined by Woychik et al (1961), according to their electrophoretic mobility. This is particularly noticeable among α-, β-, and γ-gliadins. The ω-gliadin group seems more homogeneous and less hydrophobic than other gliadins, in spite of their high proline and phenylalanine content (Charbonnier 1974). Caldwell (1979) thinks that the poor accessibility of hydrophobic areas is caused by steric hindrance due to the high molecular weight of these gliadins. But, in contast to HIC, our heptane binding determinations proved that ω-gliadins purified by selective precipitation according to Bietz and Wall (1975) are able to bind four times more heptane than a total extract of gliadins, or a mixture of α-, β-, and γ-gliadins, obtained by molecular sieving on Sephadex G-100. Two explanations can reconcile these seemingly conflicting results. First, because of the molecular conformation of ω-gliadins, apolar side chains are not accessible to immobilized hydrophobic groups (eg, those grafted on the gel matrix) but are accessible to small, free hydrophobic molecules such as heptane in solution. If this hypothesis is verified, the estimation of accessible hydrophobicity strongly depends on the method used. The degree to which the hydrophobic areas are exposed to the solvent could be investigated by comparing the results given by HIC and by the small-molecule binding in solution. Second, ω-gliadins contain no disulfide bridge. These molecules could thus unfold easily. Opening and spreading of the molecules may occur at the protein solution-heptane interface during the equilibration phase, exposing additional hydrophobic regions (which constituted the "core" of the protein in the native state) and enabling a very large degree of heptane binding. This phenomenon is probably much less important when the structure is partly locked by disulfide bridges (eg, α-, β-, and γ-gliadins). The successful extensive use of HIC for enzyme purifications shows that this chromatographic procedure does not significantly alter the molecule structure if drastic eluants are not used. Indeed, time-dependent conformational changes and unfolding of proteins due to their binding to hydrophobic gels (not to the eluant) were only reported by Yon (1978) when the time of contact was very long. Thus the unfolding of gliadins during binding on phenyl-Sepharose is much less probable under our experimental conditions than the opening of ω-gliadins molecules at the aqueous buffer-alkane interface.

In other circumstances, different HIC fractions may contain components that migrate similarly on starch gel electrophoresis. This heterogeneity is not "apparent" as termed by Hofstee (1974, 1976), ie, due to different ways of binding to the gel shown by molecules having the same hydrophobicity. The differences of hydrophobicity are effective, as proved by the heptane binding of the fractions and because our fractionation results are reproducible regardless of the protein-gel ratio. Furthermore Wrigley (1970) showed the existence of over 40 gliadin components by combining electrophoresis and electrofocusing.

In our starch gel-lactate buffer system, a single electrophoretic band could obviously contain more than one protein and, conversely, proteins migrating identically could be different.

Although starch gel electrophoresis does not sufficiently characterize the chromatographic fractions, we can estimate the relative surface hydrophobicity of some gliadin components, considering each electrophoretic band individually.

According to HIC results reported here, ω-gliadins seem less hydrophobic than the other gliadins; they eluted mainly in peaks 1 and 2. Among them, the slower components appear to be less hydrophobic than the faster components. Most γ-gliadins (eluting in fractions 4–7) are very hydrophobic, but one γ-gliadin (eluting in fraction 2 and migrating at mobility 74) is clearly less hydrophobic

TABLE II
Hydrophobicity of Main Hydrophobic Interaction Chromatography (HIC) Fractions of Gliadin Extract (WDSP)[a]

Hydrophobicity Indices	WDSP	HIC Fractions[b] of WDSP 1	2	6	7
Calculated[c]					
Polarity	1.19	1.15	1.25	1.12	1.11
Nonpolar side chain content	0.39	0.39	0.40	0.39	0.39
Average hydrophobicity (calories per residue)	1,067	1,084	1,068	1,067	1,063
Experimentally determined Heptane binding[d]	179	86	115	360	329

[a] Water:dioxane-soluble proteins extracted from glutens.
[b] Fraction numbers correspond to those of Fig. 6.
[c] From amino acid composition.
[d] Grams of heptane per 10^4 g of protein.

than the other γ-gliadins. α-Gliadins elute in several fractions but can be divided into two groups; the first one (in fractions 1 and 2) consists of slightly hydrophobic proteins (particularly α-97), and the other (in fractions 8–10) consists of very hydrophobic proteins. The large hydrophobicity of α-gliadins was observed first by Greene and Kasarda (1971). The hydrophobicity of β-components is generally variable, and β-gliadins are present in almost all fractions except the first one.

General Considerations Regarding the Method

Several HIC techniques were used to fractionate gliadins on phenyl-Sepharose CL 4B (aromatic ligand) and octyl-Sepharose CL 4B (aliphatic ligand). Hydrophobic interactions clearly play a leading role in the binding of proteins to the gels.

The strength of protein binding to the gel depends on the characteristics of the eluant (particularly pH and polarity). This is why some systems gave poor fractionations and why many proteins eluted together when modifications of the eluant were too drastic. Another factor affecting binding is the modification of protein conformation induced by the solubilizing and eluting medium. For several proteins, conformational changes involved modifications of their surface hydrophobicity (Axelsson 1978, Mohammadzadeh-K et al 1969b, Wishnia and Pinder 1964). Likewise Greene and Kasarda (1971) reported an increase of binding of a hydrophobic probe by a purified α-gliadin when the pH of the solution was lowered from 5.0 to 3.1.

The determination of the surface hydrophobicity by HIC thus depends on the media used for the fractionation. In our experiments we observed differences depending on the type of gel and the elution conditions, but results were not incompatible. Nevertheless our results are not in complete agreement with those of Caldwell (1979), who did not observe very strongly bound α-gliadins. For the other components, the comparison is difficult, for electrophoresis is not sufficient to identify unequivocally separated fractions. But, like Caldwell (1979), we found that γ-gliadins eluted mainly at an ethanol concentration between 35 and 40%. The high hydrophobicity of this group of proteins seems well established.

We think that an estimation can be made of surface hydrophobicity of native proteins by HIC by using elution conditions that do not alter the molecular structure. This could be done by using a sequence of gels of increasing hydrophobicity as Hofstee recommended (1975b), for in this system the binding strength is minimal and no drastic elution condition is required. Our results confirm that gliadins have an important surface hydrophobicity, which is not identical for all the components. This is important for the study of their functional properties and their role in the properties of gluten and dough, because their surface hydrophobicity is partly responsible for their capacity to associate with other proteins and lipids. The most recent model of glutenin molecular association (Kahn and Bushuk 1979) involves subunits of small molecular weight (68,000 and lower) bound to subunits of higher molecular weight by hydrophobic interactions, which can be dissociated by the action of soap solutions (Kobrehel and Bushuk 1977). Following this hypothesis, gliadins, or at least the most hydrophobic components, could also bind to glutenin molecules by hydrophobic interactions. The cohesiveness and viscoelastic properties of gluten will vary with gliadin content, composition, and hydrophobicity, because noncovalent binding is responsible for viscous flow (Kahn and Bushuk 1979). A preliminary study has shown differences in surface hydrophobicity between gliadins of poor and good bread-making and spaghetti-making wheats (Godon and Popineau 1981, Popineau et al 1980), although no study has yet found specific characteristics for good quality wheats gliadins as opposed to bad quality ones.

Besides having a role in protein-protein interactions, surface hydrophobicity of gliadins plays a role in lipid-protein binding. In the present work, we did not verify the presence of lipids in the gluten or in the chromatographic fractions. We only defatted gluten with pentane, and this treatment does not remove strongly bound lipids. The lipid content of the fractions must then be checked to fully identify the fractions. This point is now under investigation. But the differences of surface hydrophobicity of the different gliadin components indicate that binding of nonpolar lipids on gliadins molecules (Chung and Tsen 1975, Chung et al 1978) will vary with the components. The involvement of gliadins in lipid binding is thus variable and is probably different from one wheat to another. This could partly explain the differences between bread-making qualities; Ponte et al (1967) showed important modifications of rheological properties of gluten through binding on nonpolar molecules.

Finally, surface hydrophobicity of gliadins influences their surfactant properties, which are due to the amphiphile character of the protein molecule. The relationship between the surface hydrophobicity and the interfacial tension of protein solutions with oil has been clearly demonstrated by Keshavarz and Nakai (1979). Thus, surfactant properties of gliadins would be as variable among their components as is their surface hydrophobicity. First results of a study now being developed in our laboratory confirm this hypothesis.[3]

[3]D. Pepin and J. Lefebvre. Personal communication. 1981.

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