Fractionation of Acetic Acid-soluble Proteins from Wheat Gluten by Hydrophobic Interaction Chromatography: Evidence for Different Behaviour of Gliadin and Glutenin Proteins

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Acid-soluble proteins extracted from defatted gluten were fractionated by hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B. The hydrophobicities of gliadin and glutenin components were compared directly on the basis of their respective adsorption properties. No difference was observed between the adsorption properties of the gliadin components of defatted gluten and those of the gliadin components of non-defatted or partially defatted glutes. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed that unreduced glutenin aggregates were present in several chromatographic fractions; this suggests that they differ with respect to their surface hydrophobicities as do the gliadin components. Some glutenin aggregates displayed hydrophobicities similar to those of gliadins but others were significantly more hydrophobic. Differences in subunit composition were observed among glutenin aggregates differing in surface hydrophobicity.

Introduction

Fractionation of gliadins by hydrophobic interaction chromatography (HIC) has demonstrated the importance and variability of their surface hydrophobicities^{1, 2}. Other investigations have also shown that gluten components differ with respect to their hydrophobic properties³. Gliadin-like subunits tend to aggregate through hydrophobic interactions but high molecular weight glutenins possess an even higher surface hydrophobicity⁴. This observation agrees with previous studies that have indicated the importance of hydrophobic interactions in the structure and the aggregation state of glutenin^{5, 6}. Chung and Pomeranz⁷ studied the binding of acid-soluble proteins to a hydrophobic gel, but their method did not provide a clear fractionation and the fractions were not identified. As we had developed an HIC method for gliadin fractionation², we also applied it to the acetic acid-soluble proteins of wheat gluten to compare the surface hydrophobicities of the gliadin and glutenin components.

Abbreviations used: HIC = hydrophobic interaction chromatography; AUC = acetic acid-urea-cetyltrimethylammonium bromide; A-PAGE = polyacrylamide gel electrophoresis at acid pH; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Experimental

Flour

Proteins were extracted from a bread wheat flour (cv. Capitole) milled on a Chopin-Dubois laboratory mill (extraction rate : 60%).

Defatting of the flour

The sample was stirred for 3 h at room temperature in hexane (flour/solvent ratio 1 : 10). The flour was separated from the hexane by filtration, resuspended in chloroform (flour/solvent ratio 1 : 10) and stirred for 16 h at 4 °C. After filtration, residual solvent was evaporated from the flour at room temperature under reduced pressure.

Gluten extraction

Gluten was extracted from the defatted flour by manual washing of a dough sample under tapwater until no starch was released. The gluten ball was freeze-dried and ground in a refrigerated grinder (IKA . A . 10S).

Extraction of proteins soluble in acetic acid

Samples of dry gluten (250 mg) were stirred gently in 0.01 M acetic acid (10 ml) for 60 h at 4 °C. The suspension was then centrifuged (38,000 g, 20 min) and the soluble and insoluble fractions were collected. An aliquot of each fraction was taken for protein determination and the remainder was freeze-dried.

Fractionation by gel filtration on Sepharose CL-4B

The acetic acid-soluble proteins were fractionated on a column of Sepharose CL-4B^s using acetic acid-urea-CTAB (AUC-0.1 M acetic acid, 3 M urea, 0.01 M cetyltrimethylammonium bromide) as eluant. Protein samples (75 mg) were suspended in AUC (10ml), stirred for 16 h, centrifuged (38,000 g, 10 min), and the clear supernatants were loaded on to the column (1.6 × 90 cm). Elution was performed at a flow rate of 15 ml/h. Proteins in the column effluent were estimated from the absorbance at 280 nm and the volume of the fractions. Fractions were combined as appropriate, dialysed exhaustively against water and then against 0.01 M acetic acid and freeze-dried.

Hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B

Protein samples (300 mg) were dissolved in 0.028 M aluminum lactate buffer (pH 3.6) (20 ml) and loaded on to the column (1.27 × 28 cm) equilibrated in the same buffer. Elution was performed using a linear ethanol concentration gradient in 0.02 M ammonia as described previously². The pH of this elution buffer is about 9 and analysis of the separated polypeptides by techniques such as electrophoresis has given no indication of any degradation of polypeptides under these conditions. Fractions from the Phenyl-Sepharose columns were pooled as appropriate, dialysed against 0.01 M acetic acid and freeze-dried.

Polyacrylamide gel electrophoresis at acid pH (A-PAGE)

Gliadins were identified in chromatographic fractions by vertical A-PAGE (apparatus GE 2/4 L.S. Pharmacia) in 0.188 M acetic acid-glycine buffer⁹, pH 3.1, using gel concentrations of 7%

FIGURE 1. Fractionation of acetic acid-soluble proteins of gluten by gel filtration on Sepharose CL-4B in AUC. V_0 = Void volume; V_t = total volume.

(Bis/Acrylamide = 5%) and polymerisation with H_2O_2 . The migration distance of the fastest α -gliadin was about 16 cm after 4 h 30 min at 450 V. Other conditions and the nomenclature of gliadin components were as described by Bushuk and Zillman¹⁰.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE)

The subunit compositions of the acetic acid-soluble proteins, and of the chromatographic fractions (1.5 mg in 150 μ l), were determined by SDS-PAGE, after reduction of disulfide bonds with 2-mercaptoethanol (25 μ l; 1 min at 100 °C), using a discontinuous buffer system¹¹ and a gel concentration gradient (10–20%). Molecular weight standards were α -lactalbumin (14,400), trypsin inhibitor (20,000), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (67,000) and phosphorylase b (94,000).

Lipid content determination

The lipid contents of flour and gluten samples were determined as described by Drapron¹².

Results

Characterisation of defatted gluten and of acetic acid-soluble proteins

The protein content ($N \times 5.7$) of the defatted gluten (90.6%, w/w dry basis) was higher than that of the normal gluten (82.9%, w/w, dry basis). This difference was due mainly to extraction of lipids: the lipid contents of glutens prepared from undefatted and defatted flours were 5.41 and 0% respectively. Defatting the flour resulted in a very small decrease in the solubility of the gluten in acetic acid (84.0 vs. 86.3%). We did not observe major changes in solubility on defatting as reported previously¹³. Rather our results tend to confirm those of Patey and Shearer¹⁴, who showed that defatting at room temperature,

FIGURE 2. SDS-PAGE of fractions of acetic acid-soluble proteins of gluten separated by gel filtration on Sepharose CL-4B. Fraction numbers correspond to those shown by bars in Fig. 1. MW = Molecular weight standards.

under mild conditions, did not decrease gluten solubility. From the unmodified solubility of gluten after defatting, we can expect that no major modification of the aggregation state of the proteins occurred.

Figure 1 shows the elution pattern of acetic acid-soluble proteins from gluten on Sepharose CL-4B. Four fractions were separated. The excluded peak (fraction 1) accounts for 12% of the total protein in the profile and peaks 2, 3 and 4 for 25, 61 and 2% respectively. Huebner and Wall¹⁵ indicated that the apparent molecular sizes of the first three fractions correspond approximately to mol. wts of over 10^7 (fraction 1), between 10^7 and 10^5 (fraction 2) and below 10^5 (fraction 3). The last fraction (4), which was eluted after the total volume of the column, probably consisted of adsorbed glutenins as suggested by its subunit composition (Fig. 2).

SDS-PAGE showed that fractions 1 and 2 comprised subunits corresponding to those of glutenin as reported previously^{8, 15}. Fraction 3 contained mainly subunits with mol. wts of 35,000 and 15,000. According to the elution volume, these components correspond to α -, β - and γ -gliadins (35,000) and to albumins (15,000). Fraction 4, as noted above, consisted primarily of subunits similar to those in fractions 1 and 2, suggesting that fraction 4 comprised adsorbed glutenin.

FIGURE 3. Fractionation of acetic acid-soluble proteins of gluten by HIC on Phenyl-Sepharose CL-4B. At 'a', the column was eluted with aluminum lactate buffer. At 'b', a linear gradient of ethanol in 0.02 M ammonia (the slope is indicated by the dashed line) was initiated. At 'c', the column was eluted with 0.02 M ammonia-3 M urea-60% ethanol.

Fractionation of acetic acid-soluble gluten proteins by HIC on Phenyl Sepharose CL-4B

The elution pattern of the acetic acid-soluble proteins on Phenyl-Sepharose CL-4B (Fig. 3) is similar to that of gliadin under the same conditions². Nevertheless, in contrast to gliadin, not all of the acid-soluble proteins were bound to the gel under acid conditions. The unbound fraction (1) accounts for 16% of the collected proteins. The fractions that were eluted between ethanol concentrations of 0-15% (fractions 3-6), 20-40% (fractions 7-11), 40-46% (fractions 12-13), 46-60% (fraction 14) and by urea addition (fractions 15-17) account for 34, 16, 13, 8 and 13% of the recovered protein, respectively. The ranges of ethanol concentrations were chosen for convenience, as representing the main regions of the elution pattern, i.e. unbound proteins, proteins eluted at the beginning of the ethanol gradients, proteins eluted before the two main fractions, the two main fractions (12 and 13), proteins eluted after the two main fractions and proteins eluted by urea. Furthermore, some proteins were probably tightly bound to the gel, for the recovery of protein from the column was only about 70%. Compared with gliadin², the components of the acid-soluble proteins that were eluted between ethanol concentrations of 0 and 46% were quantitatively slightly less important, and those that were eluted at

FIGURE 4. A-PAGE of fractions of acetic acid-soluble proteins of gluten separated by HIC on Phenyl-Sepharose CL-4B. Fraction numbers correspond to those shown by bars in Fig. 3. P = Total acetic acid-soluble proteins of gluten; G = total gliadin.

ethanol concentrations greater than 46% were more important. It appears from this that the acid-soluble proteins comprised some components of higher surface hydrophobicity than gliadin.

A-PAGE (Fig. 4) revealed the nature of the components present in the HIC fractions. The fraction not bound (fraction 1), and the fraction corresponding to the beginning of the peak that was eluted by 0.02 M NH_4OH (fraction 3), contained no gliadin component. However, the compositions of fractions 4–14, obtained by HIC of the acid-soluble gluten proteins, were similar to those found previously for gliadin extracted from several varieties of bread wheat using a water/dioxane procedure¹⁶. ω - and α -gliadins displayed the lowest hydrophobicities whilst γ -gliadins were significantly more hydrophobic than most α -, β - and ω -gliadin components. It should be noted that polyacrylamide gel electrophoresis provided a better identification of the components in these fractions than did starch gel electrophoresis. For example, A-PAGE allowed differentiation between the main components of fractions 12 and 13, and showed that each one contained a γ -gliadin component unique to that fraction (γ -44 in fraction 12 and γ -46 in fraction 13). These components probably differ in their surface hydrophobicities.

The presence of glutenin was indicated by the dark staining of many of the sample wells on A-PAGE plates (Fig. 4). SDS-PAGE of the main fractions was used to investigate their subunit compositions (Fig. 5). Fraction 1 had a high content of subunits

FIGURE 5. SDS-PAGE of fractions of acetic acid-soluble proteins of gluten separated by HIC on Phenyl-Sepharose CL-4B. Fraction numbers correspond to those in Fig. 3. MW = Molecular weight standards; P = total acetic acid-soluble proteins of gluten.

with mol. wts between 39,000 and 43,000, but subunits with mol. wts of 82,000, 94,000, 100,000, 140,000 and 145,000 were also present. Polypeptides corresponding to gliadin were absent, as expected from A-PAGE results. Thus the fraction not adsorbed to Phenyl-Sepharose CL-4B comprised only glutenin. Important differences in subunit compositions were observed among the four fractions that were eluted as a result of the increased pH (fractions 3–6). Subunits having mol. wts of about 14,000 were present in fraction 3 together with subunits having mol. wts of 67,000 and 69,000. These higher mol. wt subunits were not ω -gliadins as indicated by their absence on analysis by A-PAGE (Fig. 4). Fraction 4 comprise gliadin polypeptides together with some lower mol. wt albumin/globulin components, fraction 5 comprised mainly gliadins and fraction 6 contained relatively high amounts of higher mol. wt subunits (67,000–100,000). Fractions 9, 10 and 11, which were eluted by moderate concentrations of ethanol, contained only small amounts of high mol. wt subunits (82,000, 94,000, 100,000); fraction 11 was enriched in polypeptides having mol. wts of about 43,000, corresponding either to subunits of glutenin or to the gliadin γ -42 identified by A-PAGE (Fig. 4). Only trace amounts of subunits with mol. wts greater than 60,000 appeared in fractions 12 and 13. Fraction 12 contained several polypeptides, which are probably gliadins (32,500–

35,000 mol. wt), as well as one of 39,500 mol. wt and one of 41,500 mol. wt. Fraction 13 contained low mol. wt glutenin subunits (39,000–45,000) and two major lower mol. wt polypeptides (31,500 and 35,000). The apparent mol. wts of purified gliadins γ -44 and γ -46 have been estimated by SDS-PAGE to be 41,000 and 35,000 respectively (unpublished results). Therefore, it was deduced that the 35,000 mol. wt polypeptide of fraction 13 was γ -46 and the 41,500 mol. wt polypeptide of fraction 12 was γ -44 (compare Figs 4 and 5). The 39,500 mol. wt polypeptide of fraction 12 was probably a glutenin subunit. Fraction 14 comprised mainly glutenin subunits (mol. wts between 39,000 and 43,000) as did the most strongly adsorbed fractions (16 and 17) (subunits with mol. wts of 39,000–100,000).

Discussion

HIC Separations of the gliadin components extracted from undefatted, pentane-defatted^{2, 16, 17} and hexane plus chloroform-defatted gluten samples (this study) did not differ significantly. Therefore, defatting of gluten did not modify the surface hydrophobicities of gliadins, as shown recently by Bietz *et al.*¹⁸, even though lipids may promote gliadin aggregation^{19, 20}. This was confirmed by several HIC separations of gliadins extracted from normal, hexane-defatted and hexane plus chloroform-defatted glutes (unpublished results). Neither did the presence of glutenin alter the surface hydrophobicities of gliadins. Therefore, we consider that surface hydrophobicity, as determined by HIC, is an intrinsic property of gluten protein macromolecules.

Separation of the components of the γ -gliadin doublet (γ -44 and γ -46) confirmed that HIC is a powerful purification method. Recent results from reversed-phase HPLC^{21, 22} have also emphasised the resolving power of fractionation methods based on surface hydrophobicity.

A fraction of acetic acid-soluble proteins that was not adsorbed on to Phenyl-Sepharose CL-4B at acid pH was observed previously but not characterised⁷. We found that this fraction consisted of glutenin. It may comprise aggregates having surface hydrophobicities too low for adsorption to the gel, but this does not agree with recent results that show glutenins to be very hydrophobic⁴. It is more likely (though not certain) that this fraction comprised high mol. wt aggregates that were excluded from Sepharose CL-4B (mol. wt > 10⁷) (Fig. 1), since these two fractions accounted for the same proportion of the acid-soluble proteins and since their subunit compositions were similar. If proteins are excluded from the gel matrix, only a small number of phenyl group is accessible, and adsorption does not occur. Some glutenins were fractionated by HIC as evidenced by SDS-PAGE (Fig. 5). This indicated that intermediate mol. wt (below 10⁷) glutenin comprised several types of aggregate that differed in their surface hydrophobicities. Subunits of 67,000 and 69,000 mol. wt were adsorbed at acid pH but, as soon as the pH was increased, they were eluted in the same fraction as the low mol. wt polypeptides corresponding to albumins (fraction 3). These subunits have been shown previously to have amino acid compositions that are different from those of gliadins and glutenins, but that are similar to those of soluble flour proteins^{23, 24}. According to Bietz and Wall²⁵, these could be membrane globulins; their hydrophobic properties would support this hypothesis.

Some glutenin aggregates displayed surface hydrophobicities comparable with those of gliadins. Using γ -gliadins as references, aggregates enriched in subunits with mol. wts over 60,000 (fractions 6 and 9) had lower hydrophobicities, whereas those aggregates rich in subunits with mol. wts of 39,000 and 43,000 (fraction 14) had higher hydrophobicities. Glutenin aggregates rich in subunits having mol. wts of 39,000 and 43,000 correspond closely in subunit compositions to high mol. wt gliadins²⁵ or to aggregated gliadins²⁶. The similarities in hydrophobicities between this fraction and the γ -gliadins underlined the relationship between those two groups of proteins, even though most aggregated gliadin polypeptides do have a unique N-terminal amino acid sequence²⁶.

HIC fractions 15–17 had higher apparent surface hydrophobicities than preceding fractions, but comprised subunits having the same mol. wts. This may have been due to aggregate size or solubility, to the structure or to intrinsic properties of the subunits, which may differ in physicochemical properties other than mol. wt²⁷.

HIC on Phenyl-Sepharose CL-4B thus permitted the separation of several glutenin complexes, suggesting that variation in the hydrophobic properties of the glutenins may be larger than suggested previously⁴. These observations are in good agreement with observations on the different solubility properties of the glutenins in solutions containing fatty acid salts²⁸, and they have important implications for the role of hydrophobic interactions in the structure and properties of gluten. Optimisation of conditions for the fractionation of glutenin by HIC, and further characterisation of purified fractions, are necessary in future investigations.

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