

Surface Properties of Gluten Investigated by a Fluorescence Approach

Elena Sironi,^{1,2} Nicoletta Guerrieri,¹ and Paolo Cerletti¹

ABSTRACT

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The surface properties of glutes isolated from a durum wheat cultivar (Capeiti) and two bread wheats (Riband and Hereward) were investigated using intrinsic and extrinsic fluorescence. Intrinsic fluorescence decreased on increasing protein concentration and increased after urea addition. The extrinsic fluorescence was evaluated by a titration with 8-anilino-1-naphthalene sulphonate (ANS), an hydrophobic probe. The saturating con-

centration for ANS and its dissociation constant (Kd) were determined. The hydrophobicity of durum and bread wheat gluten showed a different behavior increasing the protein concentration: Capeiti was not influenced, but there was a change on the gluten surface for Riband and Hereward. The significance in understanding gluten structure and the relevance of the surface properties are discussed.

Gluten, the cohesive viscoelastic residual mass obtained after exhaustive washing of a dough with water or diluted salt solutions, is the main protein component of wheat flour. It is formed by the association of gliadins and glutenins, and together with starch, it is the basis of rheological properties of dough and of loaf characteristics. Gluten behavior depends on the interaction of the protein molecules themselves and much has been done for a better understanding of its molecular structure and constituent proteins (Shewry et al 1992; Shewry and Tatham 1997). However, little attention has been paid to the surface properties of the gluten, which regulate the behavior of gluten with other flour components, nor to its role within flour.

The protein association in gluten is directly related to the functional properties of flour, which is not the case of the isolated protein fractions extracted from flour. A molecular approach to obtain information on the surface properties of the gluten complex could be useful to relate the dough modification during the technological process.

The hydrophobic dyes 8-anilino-1-naphthalene sulphonate (ANS) and 2-p-toluidynaphthalene-6-sulphonate (TNS) in a polar solvent are fluorescent probes when interacted with the hydrophobic regions of gluten proteins (Greene and Kasarda 1971; Chen 1973; Weegels et al 1994; Guerrieri et al 1996; Sinha et al 1997). The gluten binding sites have good or poor affinity for ANS (Guerrieri et al 1996) which gives a measure of the hydrophobicity on the surface of the gluten. Because the binding constant and fluorescence emission differ from site to site, fluorescence studies can be used to characterize the surface of gluten itself.

We studied the surface behavior of gluten proteins by measuring the intrinsic fluorescence of the aromatic residues and the extrinsic fluorescence of ANS bound to gluten, increasing the concentration of protein and urea. Bread wheat (*Triticum aestivum*) cultivar Capeiti has good breadmaking quality (bq): HMW glutenin 20 and LMW glutenins type 2, including γ 45, a marker of good baking quality (Autran and Galterio 1989; Boggini and Pogna 1989; Kovacs et al 1993). Cultivars Hereward (good bq) and Riband (poor bq) were also examined.

MATERIALS AND METHODS

Materials

All chemicals were analytical grade. Capeiti (good bq) (Pasqui et al 1994) was supplied by the Istituto di Ricerca per gli Alimenti e la Nutrizione (I.R.A.N.), Rome, Italy. Bread wheats Hereward and

Riband were supplied by the Campden & Chorleywood Food Research Association (U.K.).

Gluten of Capeiti was prepared according to Approved Method 38-10 (AACC 2000) using 30 min of resting time. Gluten of Riband and Hereward was prepared using a Glutomatic (Perten Instruments, Huddinge, Sweden). Capeiti contained 79% protein, Riband 64%, and Hereward 81%. All samples were frozen in liquid nitrogen, lyophilized, ground, sieved (60 mesh), and dry-stored at 4°C.

Protein Determination and Separation

The total protein of the gluten samples was determined with a Carlo Erba NA 1500 automatic nitrogen analyzer, with an atropine standard; the conversion factor was 5.7 (Tkachuk 1969). Soluble proteins were quantified spectrophotometrically with a colorimetric assay as described by Eynard et al (1994).

Gluten proteins were extracted using 10 mL of 0.05N acetic acid added to 4.0 mg of lyophilized gluten. Extraction was done for 1 hr at room temperature under magnetic stirring. The acetic acid extract (AAE) is the supernatant after 30 min of centrifugation at 11,300 \times g at 25°C. The yield of extracted protein was 94.5 \pm 14.4, checked by both procedures. In experiments with high protein concentrations, the ratio of the three glutes to acetic acid was changed from 0.1 to 1.0 mg protein/mL. For spectroscopic studies the AAE was used immediately after extraction.

Fluorescence Measurements

Intrinsic fluorescence spectra of AAE (0.1–1.0 mg of protein/mL) were recorded at 25°C with a luminescence spectrometer (LS50B, Perkin Elmer) using FLWinLab software (Perkin Elmer) with 280 nm excitation wavelength, 120 nm/min scan speed, slit width for emission and excitation 2.5 nm. Emission was recorded at 300–500 nm.

The ANS bound to the protein was evaluated by titration: increasing amounts of the probe, 2–4–6–8–10 μ L of 1 mM and 10 mM ANS in acetic acid were added to 3 mL of sample, 0.1–1 mg of protein/mL. Multiple additions of the probe in the same cuvette were done up to saturation (constant fluorescence response). ANS concentration in the sample after every addition is given in the figures. The developed fluorescence was measured at the wavelength of the maximal excitation and emission, λ excitation 402 nm for Capeiti and Riband, 400 nm for Hereward, λ emission 480 nm for all, slit with 2.5 nm. Operations were performed at 25°C with magnetic stirring. The titration curve as described previously (Guerrieri et al 1996), showed a positive cooperation for the probe. Curve analysis by Hill equation ($n > 1$) showed two types of sites with high and low affinity for the ANS, the deconvolution of the curve confirmed this behavior. The curve giving the best fit to titration values using Peakfit software (Jandel Scientific, Erkrath, Germany) was deconvoluted into two components corresponding to reactivity at high or low ANS concentration. The two components as described by Guerrieri et al (1996) were linearized by the Lineweaver Burk plot (low ANS concentration) and the Hill equation (high ANS concentration) (Cantor and Schimmel 1980). This allowed the

¹ Dipartimento di Scienze Molecolari Agroalimentari (DISMA), Università degli Studi di Milano, via Celoria 2, 20133 Milano, Italy.

² Corresponding author. E-mail: sironi6@utenti.unimi.it Fax: 39-02-58356801.

determination of maximal fluorescence for both the low and high affinity sites in gluten, the ANS concentration producing it, and the dissociation constants (K_d) of high and low affinity sites of the ANS-gluten complex. An ovalene standard in metacrylate was used to produce comparable data over the time to avoid problems of lamp decay. Multiple assays (two or three) were performed for each experimental condition and averages were considered; the confidence intervals were always >99%.

Denaturation by Urea

The AAE with added urea was kept 15 min at 25°C. The increase in volume, due to urea addition, was considered to calculate the final urea and protein concentrations.

RESULTS

Intrinsic Fluorescence

The intrinsic fluorescence spectra of native Hereward and Riband glutes and of native and urea-treated Capeiti gluten are shown in Fig. 1. The emission peak in the 350 nm region indicates that the fluorophores consisted mainly of exposed tryptophan residues and the shoulder at 310 nm indicated the presence of tyrosine residues quenched tryptophan emission (Schmid 1989). The emission intensity slightly differs in the three glutes but the maxima are located at the same wavelength. This was probably due to different amounts of tryptophan and tyrosine residues and in their location in the gluten structure. Urea ($\leq 3M$) caused a minor fluorescence increase in Capeiti as it did for gluten of other cultivars (Stevenson and Preston 1994). A significant change occurred at 6M urea (Fig. 1C and insert): the peak shifted from 348 to 354 nm. This indicates an exposure of tryptophan residues to a polar medium.

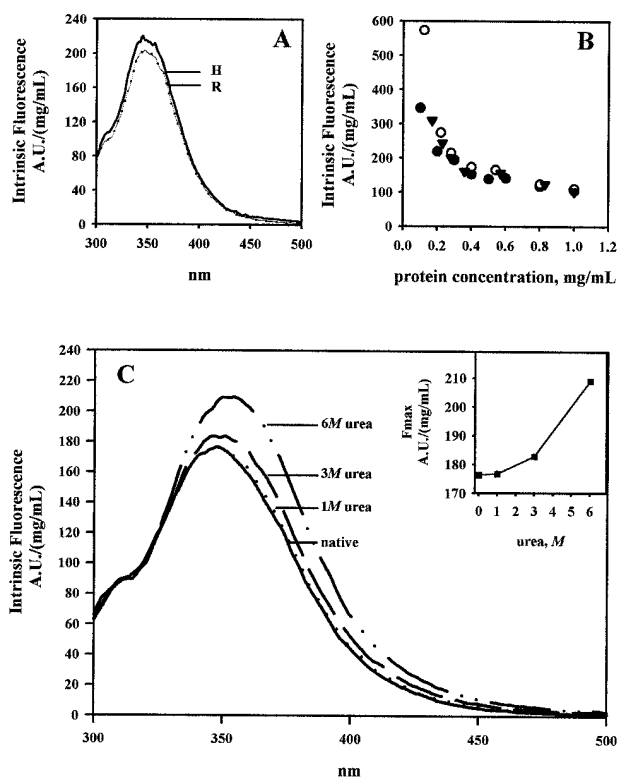


Fig. 1. A, Intrinsic fluorescence spectra of Hereward (H) and Riband (R) glutes (0.25 mg/mL). B, Maximal intrinsic fluorescence of Capeiti (●), Hereward (○), and Riband (▼) glutes at various protein concentrations. C, Intrinsic fluorescence spectra of Capeiti gluten native and denatured at various urea concentrations. Insert: maximum intrinsic fluorescence emission (F_{max}) at increasing urea concentration. Fluorescence given as arbitrary units/mg of protein/mL.

The effect of protein concentration on the intrinsic fluorescence of gluten was practically the same in the three wheat cultivars (Fig. 1B). Increasing the protein concentration the spectra remained unmodified (not shown) and there was at first a rapid, nearly linear, quenching in tryptophan emission (≤ 0.4 mg protein/mL) followed by a more gradual decrease up to the highest concentrations measured (1 mg/mL) (Fig. 1B). The quenching of tryptophan fluorescence may be attributed to the protein-protein association that modifies the fluorescence emission of tryptophan residues.

Extrinsic Fluorescence

The effects of ANS and protein concentration on the extrinsic fluorescence of the ANS-gluten complex were studied. Figure 2 describes the titration with ANS on increasing gluten concentration in the three cultivars: the fluorescence developed was measured. The fluorescence intensity in the three glutes is different: H > C > R, especially at low protein concentrations where it was noticeably high. The fluorescence emitted depends on the protein concentration in the assay; indeed, a similar quenching in the three glutes was evidenced on increasing protein concentrations. At first the effect was quite rapid (≤ 0.4 mg protein/mL) but then became more gradual.

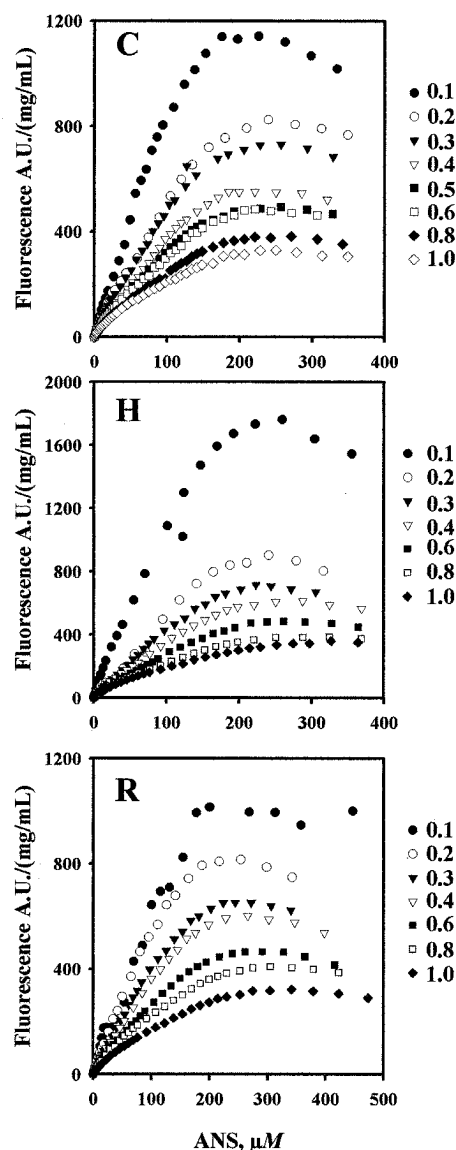


Fig. 2. Extrinsic fluorescence emission of Capeiti (C) Hereward (H) and Riband (R) glutes titrated with increasing amounts of 8-anilino-1-naphthalene sulphonate (ANS). Titration (mg/mL) at different protein concentrations. Fluorescence given as arbitrary units/mg of protein/mL.

TABLE I
Dissociation Constant (Kd) of ANS^a Bound to Gluten

Protein Concentration (mg/mL)	Hereward		Riband		Capeiti	
	Kd ^b , μ M	Kd ^c , <i>M</i>	Kd, μ M	Kd, <i>M</i>	Kd, μ M	Kd, <i>M</i>
0.1	106.9	33.9	86.9	25.1	116.4	0.1
0.2	91.2	234.0	130.4	2.4	63.2	12.6
0.3	163.3	5.5	136.1	2.0	59.0	4.0
0.4	122.5	21.7	130.0	14.7	67.1	4.8
0.5	114.10	91.8	94.6	6.9	57.1	4.7
0.6	nd ^d	nd	nd	nd	63.9	14.3
0.8	91.3	95.7	104.9	61.1	68.8	55.6
1.0	82.8	44.9	119.7	183.7	67.9	94.3

^a 8-anilino-1-naphthalene sulfonate (ANS).

^b Double exponential component.

^c Logistic component.

^d Not determined.

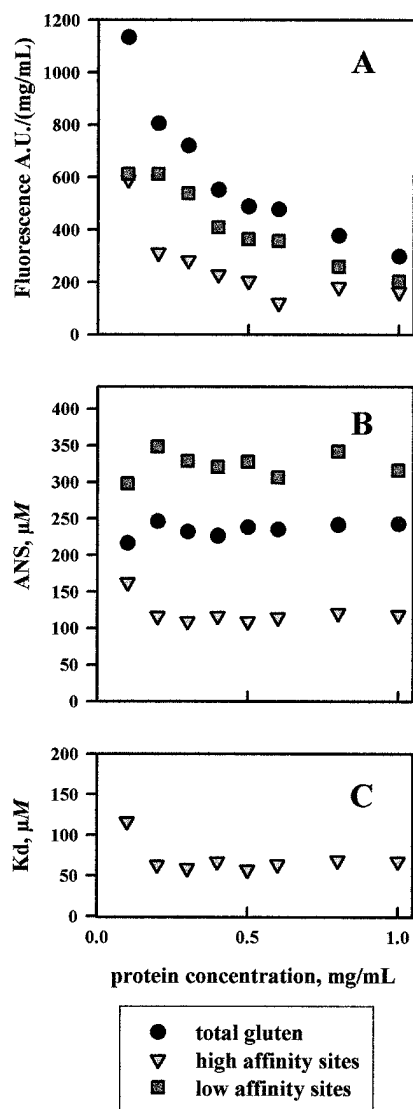


Fig. 3. Effect of protein concentration on Capeiti gluten. A, Maximal extrinsic fluorescence. B, 8-anilino-1-naphthalene sulphonate (ANS) concentration calculated at maximal fluorescence. C, Dissociation constant (Kd, μ M) of high affinity sites for ANS.

The fluorescence emitted by the high and low affinity sites was quite similar in the three glutes with a biphasic quenching (Fig. 3A). Values for high and low affinity sites were derived by the deconvolution of the titration curves (Guerrieri et al 1996). The fluorescence emitted by the ANS gluten complex was higher than those

pertaining to high and low affinity sites (Fig. 3A), while the amount of ANS bound in total gluten is intermediate to the amount of the high and low affinity sites (Fig. 3B). This is true for all the protein concentrations assayed. Increasing the protein concentration does not influence the behavior of the high affinity sites in Capeiti gluten (Fig. 3C), a difference was observed only at low protein concentration (0.1 mg/mL).

The amount of ANS bound in total gluten measured at maximal fluorescence increased moderately with protein concentration, and more sharply but to a similar extent in Hereward and Riband, more gradually in Capeiti (Fig. 4A). The behavior of the high and low affinity sites differs considerably: the ANS bound to gluten in high affinity sites was less affected by protein concentration for Capeiti, unlike Hereward and Riband, there was a scattered effect (Fig. 4B), whereas in low affinity sites, there was a moderate increasing of the ANS bound to gluten with increasing protein concentration (Fig. 4C). The Kd of Hereward and Riband high affinity sites is higher than Capeiti (Table I), showing less affinity for the probe. These two cultivars showed a Kd dependence on the protein concentration.

Urea addition significantly reduced the fluorescence of ANS bound to the gluten (Fig. 5A), the decrease was more pronounced in the low affinity sites. It was nearly linear for the high affinity sites while in the low affinity sites it was at first rapid, nearly complete at 3M urea; above this concentration, urea had poor influence on residual fluorescence (Fig. 5A). Increasing urea concentration the ANS bound to gluten and to low affinity sites increased nearly linearly, while the effect was minimal in high affinity sites (Fig. 5B). The Kd of the high affinity sites increased with the urea concentration (insert Fig. 5B), indicating loss of affinity for the hydrophobic probe. The fluorescence decrease was completed during the denaturation time used for the experiments. The urea kinetics on ANS fluorescence in Capeiti gluten were followed by time drive-scans up to 10 min of denaturation; the change was extremely rapid, nearly complete after urea addition within the instrument time. Two-phase behavior with increasing urea was observed; the change was at 3M urea concentration (Fig. 5C).

DISCUSSION

The modifications in intrinsic fluorescence (aromatic residues) and in extrinsic fluorescence (ANS-gluten interaction) correspond to changes on protein conformation and on gluten surface properties. The intrinsic fluorescence in the three glutes denotes similar surface structure, indicated by a similar exposure of tryptophan residues and aromatic residues, with minor differences in the amount of residues in the three glutes. Their maximum at 350 nm suggests that tryptophan lies in a hydrophilic surrounding (Schmid 1989). Fluorescence decrease with protein concentration indicates a quenching of exposed tryptophan residues, which is expected in associating molecules. Urea addition enhanced the exposure of tryptophan due

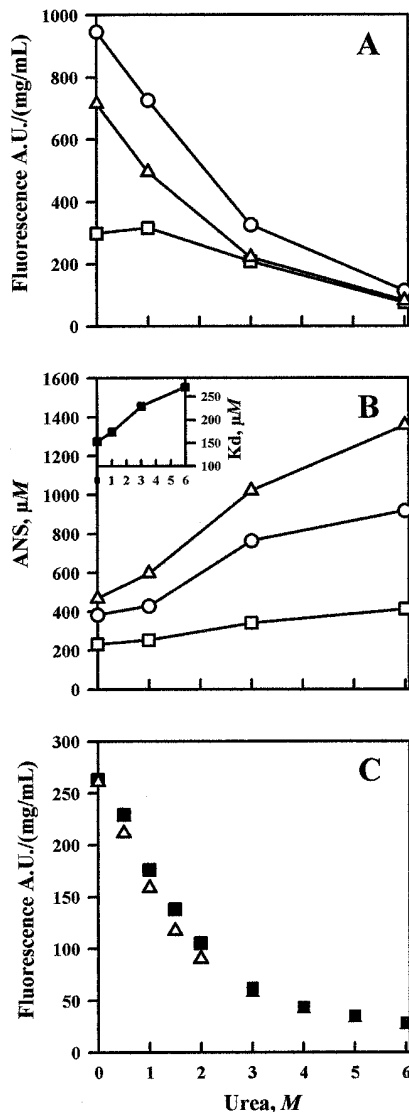


Fig. 4. 8-Anilino-1-naphthalene sulphonate (ANS) concentration calculated at maximal fluorescence for Capeiti, Hereward, and Riband glens (A), and for high (B) and low (C) affinity sites.

to unfolding of the gluten complex. Increase in tryptophan fluorescence upon urea denaturation was observed for lysozyme by Edelhoch and Steiner (1962) and Churchich (1964, 1966).

Two types of reactive sites for ANS (hydrophobic) are confirmed in gluten, with high or low affinity for the probe (Guerrieri et al 1996). Different quantum yield of ANS depending on the micro-environment and on its orientation on the protein is described in the literature (Chen 1973). A more limited number of high affinity sites may depend on precise steric and chemical correspondence requirements, whereas low affinity areas can be obtained with more randomized fitting. Weak hydrophobicity may also derive from the coalescence of polypeptides localized on the surface of the gluten complex that easily undergo modifications. The amino acid composition of the gluten differs from other seed proteins. It is rich in glutamine, proline, cysteine, phenylalanine, and leucine and poor in basic amino acids (Shewry and Tatham 1997). This structural basis may explain the peculiar surface behavior of the gluten complex.

Different fluorescence emission in the three glens may indicate a different number of hydrophobic sites or distribution on the protein surface. The amount of ANS bound in the three glens, and in their high and low affinity sites, as well as the dissociation constant values indicate that reactivity for the probe (hydrophobicity) is not the same in the glens. The durum wheat Capeiti shows greater affinity for the probe than the bread wheat cultivars Hereward and Riband.

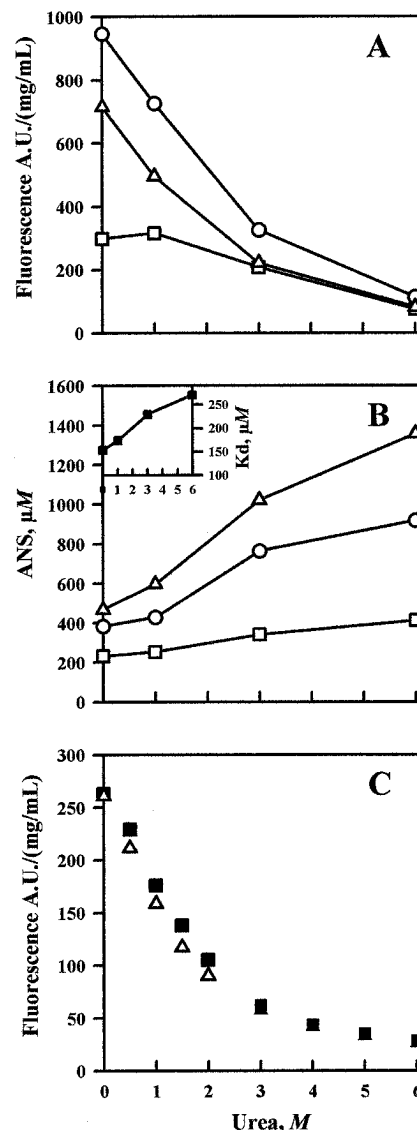


Fig. 5. Extrinsic fluorescence of Capeiti gluten denatured by urea. A, Maximal fluorescence. B, 8-Anilino-1-naphthalene sulphonate (ANS) concentration calculated at maximal fluorescence. Insert: dissociation constant of high affinity sites (K_d , μM). Gluten (○), high (□) and low (△) affinity sites. C, Denaturation kinetics of Capeiti gluten. Extrinsic fluorescence was followed by time-drive scans, values 0 sec (■) or 10 min (△) after urea addition at room temperature are given.

The decrease in fluorescence emission with increasing protein concentration, is probably due to gluten association that obliterates part of the exposed surface hydrophobic sites with low or high hydrophobicity. The ANS affinity and K_d of the durum wheat Capeiti indicate that association phenomena have an effect on the low affinity areas that are less specific and widespread than on the high affinity ones that retain high specificity. ANS fluorescence of the two bread wheats shows a concentration dependence in the low and high affinity sites. This behavior indicates a different distribution of the hydrophobic areas on the gluten complex of the bread wheat and of the durum wheat Capeiti.

Dissociation of the complex or unfolding by urea affect the exposed hydrophobic areas of gluten, decreasing fluorescence emission and affinity for ANS, thereby increasing the concentration of ANS needed for saturation of the available binding sites. The 3M urea has been used for the separation of gliadins from glutenins in purification processes (Huebner and Rothfus 1971; Skerrit et al 1996). The change at this urea concentration on intrinsic fluorescence and on the ANS fluorescence suggests that the gluten complex structure is loosened, giving greater freedom to the gliadin molecules.

Fluorimetric procedures have been developed to monitor conformational changes of a wide variety of proteins because the techniques are sensitive and reproducible. The intrinsic and extrinsic fluorescence provides complementary information on the structural properties of the proteins. The technological performance of the flours depends on the content and on the assembly of the polypeptides which are different among cultivars. The fluorescence techniques and in particular the surface hydrophobicity measure the different reactivity of the gluten complex and give information on its stability, that could be correlated with the technological properties. The behavior of the hydrophobic regions of the gluten is an important parameter that could be related to the interactions among proteins and with lipids or aromatic molecules.

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