

Accessibility of Amino Groups in Gluten Proteins Studied by a Combination of Chemical Labeling and Immunochemical Detection

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ABSTRACT

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The accessibility of primary amino groups to an external probe in durum wheat gluten proteins in the flour itself, and after the extraction and separation of gluten proteins, was studied by labeling the free amino groups with the hemisuccinate of 2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl) propanol (FF18) and evidencing the label immunochemically. Within

the flour, the amino groups were less available to the probe than after extraction, and gliadins were less accessible than glutenins, differences that decrease after solubilization and separation of the proteins. Data are discussed in relation to the structural organization of proteins within gluten.

The surface properties of gluten proteins are important in breadmaking as they play a determining role in the interactions of these proteins among themselves and with other components of flour, starch for instance, and with water in the formation of dough and during heat treatment at baking (Payne 1987, Popineau et al 1994, Gupta et al 1995). We, and other authors, have tried to make a profile of the distribution of hydrophobic amino acids on the gluten surface by following their interaction with molecules like 8-aniline-1-naphthalene sulfonate (ANS, Guerrieri et al 1996) and 2-p-toluidinylnaphthalene-6-sulfonate (TNS, Greene and Kasarda 1971). The surface distribution of the terminal amino groups of polypeptide chains and lysine amino groups is little known. Most of the information available concerns the amino acid amount and the distribution in polypeptide chains. From the published polypeptide sequence of S-rich prolamins (Shewry and Tatham 1997), it appears that lysine residues, in γ -type gliadins and LMW glutenin subunits, are predominantly located within the C-terminal domain of the polypeptides in from the C-terminal end of the domain. These amino groups may be important because of the salt bridges they could form, therefore influencing molecular interactions and solubility. It is well known that in cereal prolamines, the lysine level is especially low, resulting in limited nutritional effectiveness: 1.1–1.8 mol% in HMW glutenins and 0.6–1.0 mol% in LMW glutenins (SWISS-PROT Data Bank release 31, Seilmeier et al 1987), 0.3–0.7 mol% in α - and γ -type gliadins, and 0.6 mol% in ω gliadins (Wieser et al 1987). For comparison, the content of lysine in flour albumin is 3.2 mol% (Eynard et al 1994) and in conalbumin 8.4 mol% (Williams et al 1982).

In the present research, our aim was to spot available amino groups in the assembled gluten in conditions as similar to native ones as possible and in isolated gluten proteins. For this purpose, we assumed that an assay based on chemical labeling, followed by immunochemical detection, was the most appropriate to provide information without damaging gluten structure within flour. We therefore studied the interaction of the hemisuccinate of 2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl) propanol (FF18) with gluten proteins. The availability of FF18 specific antiserum allowed us to detect the FF18-labeled proteins immunochemically.

MATERIALS AND METHODS

Analytical grade chemicals were from Fluka. The flour sample was remilled semolina from the durum wheat (*Triticum turgidum* L. durum) cultivar Capeiti (Pasqui et al 1994). It was supplied by Pasqui and Carcea of the Italian National Institute of Nutrition.

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General Methods

Total nitrogen was analyzed using an automatic nitrogen analyzer (NA 1500 from Carlo Erba, Milano, Italy), interfaced with a personal computer, software EAGER, using an atropine standard. The protein conversion factor was 5.7 (Tkachuck 1969). Soluble proteins were quantified spectroscopically as described by Eynard (1994).

The gliadins were extracted from the flour according to the sequential extraction described by Capelli et al (1998). After removal of the gliadins, the residual sediment contained glutenins. For electrophoresis, the gliadins were vacuum-dried and suspended in SDS-PAGE sample buffer (31 mM Tris-HCl, pH 6.8, 1.25% 2-mercaptoethanol, 1.9% SDS, 24% glycerol, and 0.06% bromophenol blue) and the glutenins were directly suspended in the SDS-PAGE sample buffer. The protein amounts used for SDS-PAGE and acid-PAGE, and for SDS-PAGE followed by western blot were 100 and 10 μ g, respectively.

The 2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl) propanol (FF18), the conjugate conalbumin FF18 containing 5 mol FF18/mol conalbumin and an anti BSA-FF18 serum were prepared as described by Forlani et al (1992) and were stored in a lyophilized state.

Activation of FF18 and Labeling of Flour

The formation of an amide bond by reacting the FF18 carboxyl group with protein amino groups was achieved by the mixed anhydride technique.

FF18 was activated by adding sequentially to 6.9 mg of FF18: 200 μ L of anhydrous dioxane (99% purity, water <0.01%), 2.8 μ L of isobutylchloro formiate, 5 μ L of tri-*n*-butylamine, and then stirring 1 hr at room temperature (RT). This solution was then diluted in dioxane to give final FF18 concentrations from 9.5 μ M to 89.27 mM, which were added (20 μ L) to 30 mg of flour suspended in 1 mL of 0.05M Tris-HCl, pH 8.5, 0.5M NaCl (Tris/NaCl) with 30 min stirring at RT. Gliadins and glutenins were then extracted from labeled flour following the procedure for the sequential extraction of flour proteins described above. Unreacted FF18 was discarded, together with globulin- and albumin-rich fractions. The composition of the extracted material was checked by SDS-PAGE. In the controls, dioxane did not contain FF18, or else the flour was substituted with 3.5 mg of conalbumin. Conalbumin was labeled as described for flour and, after labeling, was submitted to ultra dialysis against 0.015M KH₂PO₄, 0.15M NaCl, pH 7.4 (PBS) in Centricon 30 (Amicon, USA) microconcentrators with centrifugation at 5,000 \times g.

Glutenin Labeling

The glutenins extracted from 0.6 g of flour were labeled in Tris/NaCl solution with or without SDS.

The glutenins (27 mg) were suspended in 20 mL of Tris/NaCl. The suspension was sonicated twice at melting ice temperature in a Uniprep 150 MSE equipment, average medium power, for 30 sec with a 5-min pause so as to prevent overheating the suspension. Aliquots (1 mL) were added with 20 μ L of activated FF18 (from

9.5 μM to 89.27 mM) and labeling was done as described for flour. Unreacted FF18 was removed by washing once in Tris/NaCl and twice in water.

For labeling with SDS, the solution for suspending the glutenins was Tris/NaCl containing also 2% SDS (Tris/NaCl/SDS). Other procedures were as above. Because glutenins appear soluble in Tris/NaCl/SDS, unreacted FF18 was dialyzed off overnight against Tris/NaCl/SDS.

Gliadin Labeling

Gliadins were sequentially extracted from flour and labeled in Tris/NaCl or in 70% ethanol solution.

A 12.5-mL aliquot of the ethanol extract containing 14 mg of gliadins was vacuum-dried in Speed Vac (Savant) at low temperature. The dry powder was suspended in 15 mL of Tris/NaCl. Aliquots (1 mL) were added with 20 μL of activated FF18 (from 9.5 μM to 89.27 mM) and labeling was done as described for the labeling of flour, except that the sediment washed with water was directly solubilized in 0.75 mL of 70% ethanol and stored at -20°C .

For labeling in ethanol solution, aliquots (0.75 mL) of the ethanol extract, adjusted to 1 mL with 70% ethanol were slowly added with 20 μL of activated FF18 (from 9.5 μM to 89.27 mM). After 30 min of incubation at RT, ethanol was evaporated in the Speed

Vac and the residue was suspended in 1 mL of Tris/NaCl, with 1 hr of stirring. After centrifuging for 15 min at $10,000 \times g$, the sediment was washed with water, centrifuged again, resuspended in 0.75 mL of 70% ethanol and stored at -20°C . The pH of the 70% ethanol solution was 8.2.

Electrophoresis

SDS-PAGE was performed in reducing conditions (0.17M 2-mercaptoethanol) according to Laemmli (1970) either on 12% polyacrylamide gels or on a gel consisting of a 8–15% gradient of acrylamide. When the proteins separated on polyacrylamide were transferred to a nitrocellulose (0.45 μm) filter, trans blot equipment (Bio Rad, Richmond, CA) working in 25 mM Tris, 0.2M glycine, and 20% methanol transfer buffer was used.

Proteins separated by SDS PAGE were stained with Coomassie brilliant blue R-250 or, after transfer to nitrocellulose filter, with fast green (Reinhart and Malamud 1982) or red Ponceau S. The immunochemical determination of protein-bound FF18 was done by western blot using a 3,000-fold diluted anti BSA-FF18 serum. The apparent molecular mass of the separated polypeptides was determined by comparison with the standard mixture of LMW proteins from Pharmacia (trypsin inhibitor, α -lactalbumin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, lysozyme).

Acid-PAGE was performed according to Khan et al (1985) with modifications. Polyacrylamide gels (8%, acrylamide to bisacrylamide in a 24:1 ratio) were used. Ethanol was removed from the gliadin fraction by evaporation of the extract, and the sediment was suspended in 0.05M acetic acid and then mixed 1:1 with a solution containing 17 mM lactic acid, pH 3.1, 30% glycerol, and 0.03%

TABLE I
Relative Amounts of Glutenin Subunits Determined Either on Coomassie Blue Stained 8–15% Gradient Acrylamide Gel or, After Blotting, on Fast Green Stained Nitrocellulose Membrane

Bands	% Staining Intensity	
	Coomassie Blue	Fast Green
glute1	16.0	16.6
glute2	10.8	7.3
glute3	6.1	5.7
glute5	32.1	36.2
glute6	12.0	11.9
glute7	23.0	22.3

TABLE II
Calculated LC_{50} Values^a

Band	M_r	LC_{50} (μM)			
		A ^b	B ^c	C ^d	
Glutenins					
HMW	glute1	94,800	101	17	nd ^d
	glute2	91,000	119	18	nd
	glute3	86,300	120	17	nd
HMW albumin	glute4	64,700	122	15	1
LMW	glute5	52,300	100	21	nd
	glute6	48,800	99	25	nd
	glute7	43,700	110	18	3
	glute8	36,800	99	16	1
	glute9	35,200	101	20	0.9
	glute10	31,200	125	15	nd
	Gliadins				
ω	glia1	62,500	562	100	82
α - and γ -type	glia2	49,300	239	<14	13
	glia3	47,400			
	glia4	43,800	226	9	3
	glia5	40,300	200	40	4
	glia6	39,100			
	glia7	34,200			
	glia8	32,200	190	7	3
	glia9	30,600			

^a Label concentration that yields a response halfway between maximum and minimum.

^b In Tris-HCl flour suspension.

^c In Tris-HCl suspension of isolated prolamin fraction.

^d In Tris-HCl and 2% SDS solution of isolated glutenin fraction or in 70% ethanol solution of isolated gliadin fraction.

^e Not determined.

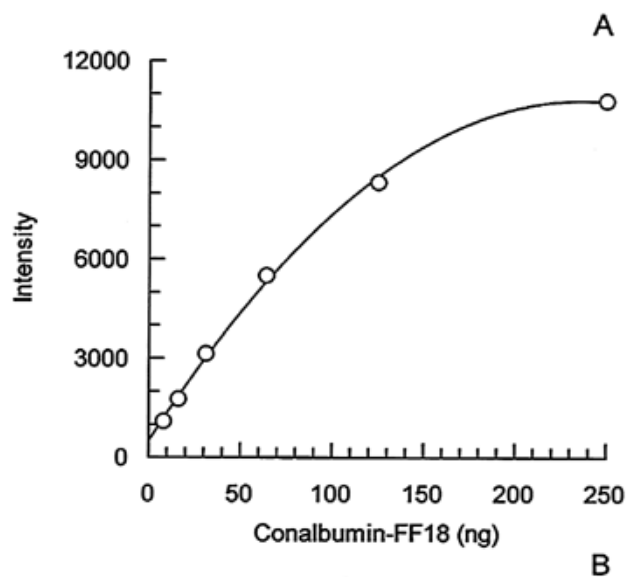
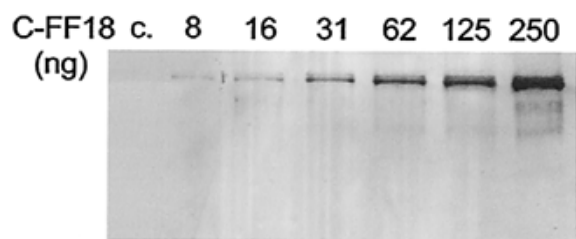


Fig. 1. Immunochemical detection of conalbumin-FF18. **A**, Amount (ng) of conalbumin-FF18 (C-FF18) run on SDS-PAGE then transferred to nitrocellulose filter where FF18 was detected immunochemically. c. = unlabeled conalbumin (250 ng). **B**, Intensity of immunochemical determination of samples in A calculated on digitized image of nitrocellulose filter.

pyronin G. Electrophoresis was run at 500V at 10°C. The prerun was for 30 min. The separation took twice the run time of the tracking dye (pyronin G). Blot of proteins separated by A-PAGE used 7% acetic acid for transfer.

SDS-PAGE and A-PAGE gels and nitrocellulose membranes were acquired using a video camera connected to a computer system, software Cream (Kem-en-Tech, Copenhagen, Denmark). Only representative western blots are reported for each experiment. The intensity values of the bands produced in the various samples were evaluated using software (Grafit, Erithacus Software Ltd) and were plotted with reference to the activated FF18 concentration. The plots yielded a sigmoid curve; the label concentration (LC₅₀) that yields a response halfway between maximum and minimum was evinced by applying the logistic equation reported by Rodbard and McClean (1977). This equation is used to describe the dose-response relation in immunoassays (ELISA, RIA, EMIT). The coefficient of variation of LC₅₀ values was <20%.

RESULTS

Detection of Conalbumin-FF18

The efficiency of western blot in detecting bound FF18 was measured in an experiment where increasing amounts of the conjugate conalbumin-FF18 with 5 mol bound FF18 per mol conalbumin had been run in SDS-PAGE, transferred to nitrocellulose, and then immunodetected by anti BSA-FF18 serum (Fig. 1A). The minimum amount of detected label was in 8 ng of conjugate. The response had a nearly linear increase up to 125 ng of conjugate (Fig. 1B), indicating that the antiserum operating concentration was, in these conditions, large enough.

Interaction with Gluten Proteins

Gluten proteins were labeled with FF18 within the flour suspended in Tris/NaCl (pH 8.5) and were then extracted separately and reacted with anti BSA-FF18 serum. Labeling was also done on gluten proteins after their extraction and separation. In this case,

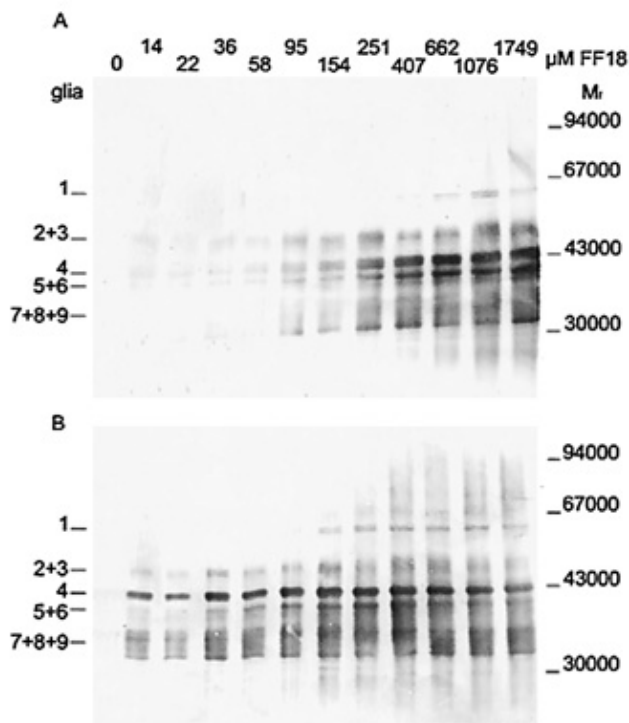


Fig. 2. Western blot after SDS-PAGE separation of labeled gliadins. Labeling reaction used activated FF18 at concentrations indicated at top in flour suspended in Tris-HCl (A) or in Tris-HCl suspension of isolated gliadin fraction (B). Gliadin bands identified and numbered in left-hand column.

the gluten proteins were solubilized (gliadins in 70% ethanol, glutenins in Tris/NaCl/SDS) or were finely dispersed in Tris/NaCl. The alkaline pH adopted is the most favorable for FF18 reactivity with protein amino groups using the mixed anhydride technique (Wong 1993).

Gluten proteins tagged with FF18 had the same SDS-PAGE behavior as the native proteins (not shown). SDS-PAGE separation is governed by molecular size, and the M_r of FF18 is small enough (272 Da) not to significantly change the size of the molecule to which it is bound. The separation of labeled gliadins in A-PAGE evidenced smeared bands reinforced at the level of the untagged gliadins. Western blot of such A-PAGE displayed an immunoreactivity limited to a smear. This behavior indicated that gliadin tagged with FF18 have a change in electrophoretic mobility due to the blocking of amino groups; the protein remaining unreacted retained the same mobility as the untreated sample. This can be explained by FF18 blocking the amino groups available at the protein surface, such groups being involved in the electrophoretic migration. Nonetheless, the answer did not allow a clearcut resolution of the various gliadins, and A-PAGE was not further used.

The transfer to the nitrocellulose filter of HMW glutenin subunits separated on SDS-PAGE gels of constant acrylamide concentration was not satisfactory because the glutenins remained partially on the gel. As shown in Table I, the transfer was satisfactory in separations run on acrylamide gradient gels, probably because the cross-linking was more favorable to the migration of HMW components during transfer.

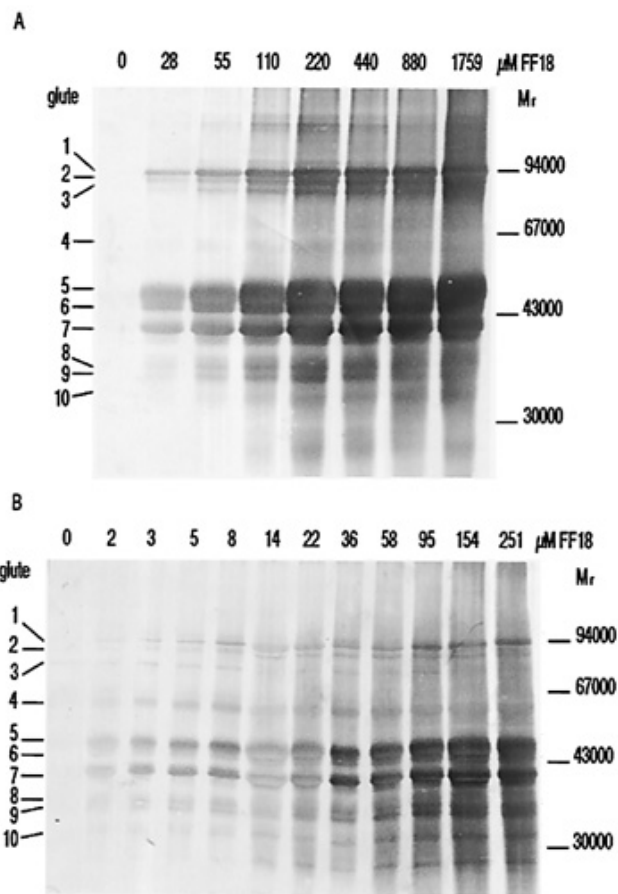


Fig. 3. Western blot after SDS-PAGE separation of labeled glutenins. Labeling reaction used activated FF18 at concentrations indicated at top in flour suspended in Tris-HCl (A) or in Tris-HCl suspension of isolated glutenin fraction (B). Glutenin bands are identified and numbered in the left-hand column.

The transfer of glutenins labeled with different amounts of FF18 (28/420 μM) was not affected by labeling (not shown).

The reactivity of FF18 with gliadins and glutenins in flour and after purification, assayed under various conditions, is shown in Table II. Proteins labeled within the flour had higher LC_{50} values than the isolated proteins, as if shielded by a component that interferes with interaction with FF18. In flour, the proteins required a more concentrated activated FF18 to form visible bands (Figs. 2A and 3A for gliadins and glutenin, respectively). The sigmoid increase was sharper than for the gliadin solubilized in ethanol (Fig. 4). The higher reactivity of the gliadins and the glutenin subunits after extraction is apparent in Figs. 2B and 3B. For glutenins solubilized in Tris/NaCl/SDS, an FF18 concentration of 0.5 μM was enough (not shown). The decrease of LC_{50} value (the increased protein affinity for FF18 on passing from the flour to purified conditions) is apparent in Table II. The two protein groups show a similar increase in affinity, suggesting that quenching in flour is due to some component acting in the flour rather than to a separate effect on each of the proteins, which could be expected to differ depending on the type of proteins considered.

On the other hand, the LC_{50} values in flour are higher for gliadins than for glutenins (Table II) indicating that, in flour, FF18 generally had easier access to the glutenins. The difference in the LC_{50} value of proteins labeled in flour or labeled after extraction is greater for gliadins than for glutenins. It is as if extraction improved amino group availability in gliadins more than in glutenins. The lower LC_{50} values displayed by gliadins reacted in ethanol solution may indicate a better reactivity of the amino groups in this solvent, where gliadins are soluble while they are not in Tris/NaCl. It seems possible that the binding of FF18, an aromatic molecule, to the protein amino groups may alter the surface polarity of the gliadins and further decrease solubility, gliadins not being very soluble in water media. The reactivity of ω gliadins with FF18 is poor in both flour and after isolation.

For glutenins, the presence of SDS in the suspension medium presumably improves solubilization considerably, and thereby the FF18 access to amino groups, indicating a weakening of the conformational stability and structure loosening due to SDS.

DISCUSSION

Activated FF18 interacts with primary amino groups of protein (terminal amino group of polypeptides and lysine ϵ -amino groups) only when deprotonated (Wong 1993). Interaction with arginine is doubtful, the pK value of amine within the guanidyl group was >12 . In our labeling conditions, the terminal amino groups are nearly all deprotonated (90%, pK 7.6–7.8); lysine ϵ -amino groups are scarce and partly deprotonated at the pH of our experiments (pK_a 10.0–10.2, 5% deprotonated). Therefore, their contribution is comparable to that of terminal amino groups. Thus the changes in the FF18 labeling are mainly due to the availability of the above mentioned groups (pK_a values are taken from Wong 1993).

Significantly, our results indicate that amino group availability to FF18 increases in conditions where the gliadins or glutenins are finely dispersed or solubilized, the size of the effect differing in the two protein fractions. This suggests that the higher requirement of FF18 within flour suspension is not due to some competitive group reacting in flour and suggests that some other component in flour interacts with gluten proteins, mainly with gliadins, and directly or indirectly blocks access to amino groups. The polysaccharide component of flour is the most abundant. A preferential interaction of gliadins with other flour components has been observed by Guerrieri et al (1997), who described a preferential effect of gliadins (among gluten proteins) in reducing the activity of amyloglucosidase on polysaccharide substrates.

An alternative hypothesis is that amino group availability is impeded as these groups are shielded by the polypeptide network within the complicated structure of gluten. In such a structure, the

LMW subunits are reciprocally disulfide bonded and bonded to HMW subunits (the latter interconnected by disulfide bridges), while the α -type, most γ -type, and ω gliadins interact noncovalently with the β spiral domain of HMW subunits (Shewry et al 1999).

Also interchain hydrogen bonds are important in gliadin and glutenin interactions and in the interaction of the individual glutenin polymer (Shewry et al 1998). Indeed, FT-IR spectroscopy of purified HMW subunits shows that increased hydration results in an extended structure (Shewry et al 1999), and this may induce exposure of the polypeptide segments containing amino groups. However, lysines in γ -type gliadins and LMW S-rich prolamines lie in close proximity to conserved cysteine residues that, by forming intrachain disulfide bridges, stabilize the folded conformation of the polypeptide (Shewry and Tatham 1997). Therefore, these lysine residues are not so liable to changes in protein structure. Thus it seems unlikely that modified FF18 availability depends on conformation changes in these proteins after extraction from flour. It is interesting to note that α -type S-rich prolamines have a different lysine distribution, and the amino acid is not located near cysteine residues (Shewry and Tatham 1997). Such a situation would allow more relevant conformational modifications, thereby resulting in greater FF18 affinity than in the γ -type gliadins and LMW glutenins. Also in HMW glutenins, the lysine position in relation to the cysteines is not equally binding as in the mentioned proteins; the lysine residues do not benefit from the stabilizing effect. In both cases, change in FF18 accessibility based on conformational modifications at separation from the flour should be more relevant than in γ -type gliadins and in LMW S-rich prolamines. The modifications occurring that do not satisfy this scheme may be attributed to the behavior of terminal amino groups of polypeptides. We suggest that amino group accessibility improves when protein is extracted from flour, because of the removal of some quenching molecule (coverage by starch). Shielding by other proteins (globulins and albumins) is probably limited because these proteins are well dissolved during the labeling step and not aggregated to the gluten proteins. However, the gluten protein network may also come into play. Some aspects of the second type, namely conformational modifications, cannot be fully excluded.

Within flour, glutenins appear somewhat more exposed to FF18 than gliadins but the difference practically vanishes after extraction. This indicates a different protein assemblage within gluten and modification upon extraction. The difference evidenced within the

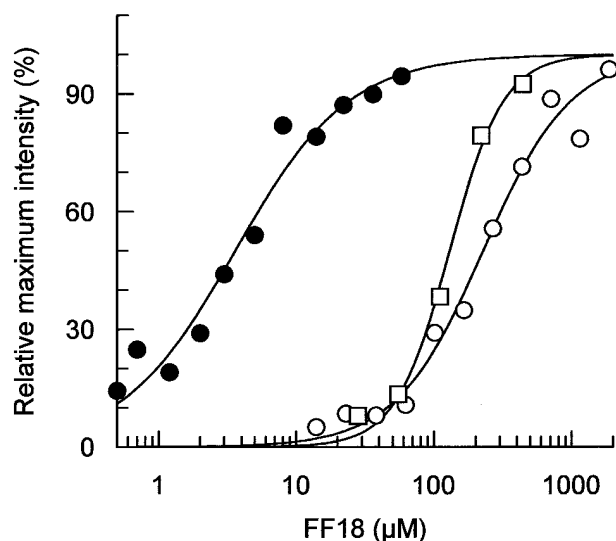


Fig. 4. Effect of concentration of activated FF18 on labeling immunodetection response (relative intensity): gliad4 (○) and glute4 (■) evidenced by labeling in flour suspended in Tris-HCl buffer and gliad4 (●) labeled in a 70% ethanol solubilized gliadin fraction. Logistic curve calculated LC_{50} value according to Rodbard (1977).

flour indicates that chemical labeling by an immunodetectable molecule like FF18 is a valid approach to study the molecular arrangements of proteins in a complex food material.

The study of the distribution of protein-bound amino groups may serve as a probe for polar sites on the surface of the gluten protein, as does titration with ANS (Guerrieri et al 1996) for hydrophobic regions. Both approaches may be of considerable help in studying the modifications of gluten during heating or in relation to the effects of reducing conditions.

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