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Properties of the Protein and Carbohydrate Fractions in Immature Wheat Kernels

STEFANIA IAMETTI,[†,‡,*] FRANCESCO BONOMI,[†,‡] MARIA AMBROGINA PAGANI,[§] MARTA ZARDI,[§] CRISTINA CECCHINI,[⊥] AND MARIA GRAZIA D'EGIDIO[⊥]

Sezione di Biochimica and Centro Studi Celiachia, Dipartimento di Scienze Molecolari Agroalimentari, University of Milan, Via Celoria 2, I-20133 Milan, Italy; Sezione di Industrie Agrarie, Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, University of Milan, Via Celoria 2, I-20133 Milan, Italy; and C.R.A.- Istituto Sperimentale per la Cerealicoltura, Via Cassia 176, I-00191 Rome, Italy

The time course of compositional changes in the polysaccharide and protein fractions, and of changes in protein structural properties, was investigated in grains of two Italian durum wheat cultivars, *Ofanto* and *Duilio,* and the pattern of changes was found to be specific to each cultivar. The fructo-oligosaccharide content in ripening kernels was different between cultivars, as was the pattern of starch accumulation. Evaluation of gluten proteins by Western-blotting using broad-specificity anti-gliadin antibodies showed the sudden appearance of immunoreactive proteins during ripening. Protein surface hydrophobicity and thiol content and accessibility were evaluated in conditions apt at assessing changes in protein interaction and/or in aggregative patterns. Proteins in the two cultivars showed differences in their surface hydrophobicity and in their content of accessible thiols. The simultaneous evaluation of changes in the individual components indicates that use of immature grains for the production of functional foods will require accurate monitoring of the rapidly evolving biopolymer patterns, and careful choice of the cultivar.

KEYWORDS: Fructo-oligosaccharides; gluten proteins; protein surface hydrophobicity; protein thiol groups

1. INTRODUCTION

Changes occurring in the ripening kernel affect macromolecular components that are relevant to nutritional and health issues. Individual proteins are synthesized and accumulated in the developing grain at different times and at different rates, so that the protein composition of wheat grains is dependent on the state of grain maturity (*1*). Only marginal differences were reported in the nitrogen content in mature grains and in kernels at the milky stage, that is, between the second and third week after anthesis (*2*). Albumins and globulins are the dominant protein components all throughout the milky phase, when protein fractions related to the formation of the gluten network are absent (*3*). The nature, but not the overall amount, of the albumins and globulins changes during the first three weeks after anthesis, but their pattern stabilizes from week 4 through maturity (*4*). Gliadins have been found in kernels as early as 10 days after anthesis (DAA), and both high-molecular weight glutenin subunits (HMW-GS) and low-molecular weight glutenin subunits (LMW-GS) were shown to appear at 13 DAA (*5*). Production and accumulation of these proteins continues until physiological maturity (*6*). The dependence of these events on the particular cultivar and on agronomic and climatic conditions was extensively studied by many authors (*5*, *7*−*11*), as was their significance from a nutritional standpoint (*12*−*14*).

Oligosaccharides, and in particular fructo-oligosaccharides (FOS), are also present in kernels, and the appreciation of their nutritional relevance is increasing (*15*). Large quantities of FOS are stored in the stems and grains of wheat for much of its growing cycle, with a maximum accumulation during the milky phase and a rapid decrease thereafter (*16*).

The high level of FOS in immature wheat kernels could be exploited for the production of functional foods by harvesting wheat grains at the milky phase, when high levels of soluble sugars are accompanied by a low content of both starch and amylose (*17*). Given the concomitant absence of potentially toxic gluten proteins, immature grains could be processed into foods suitable for addressing the needs of sensitive individuals.

However, more information is needed for some biochemical properties of proteins in the immature grains, that may be relevant to their processing into foods when not using them in mixtures with either wheat or nonwheat ingredients. Biochemical studies on the protein components in wheat kernels during the

* Corresponding author. Tel.: +39-02-50316819; fax: +39-02-50316801, E-mail: stefania.iametti@unimi.it.
† Sezione di Biochimica, DISMA, University of Milan.
‡ Centro Studi Celiachia, University of Milan.
§ Sezione di Industrie Agrarie, DISTAM, University of Milan.
⊥ C.R.A.-Istituto Sperimentale per la Cerealicoltura.

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different phases of ripening have been limited to general chemical studies, to protein fractionation approaches, and to rheological studies. These approaches are not always sufficient to assess the interprotein interactions that contribute to the formation and stabilization of a network producing an acceptable food from a sensory standpoint.

Quite recently, we produced evidence that some molecular properties of grain proteins, such the number of accessible protein thiols and the surface hydrophobicity, relate to the capacity of proteins to establish and stabilize a protein network (*18*−*22*). The protocols developed in these studies allow the determination of these parameters on intact samples, without resorting to prior protein extraction or fractionation, that often call for conditions where the structure of individual proteins, or of the network that they establish between themselves and with other food components, may be lost.

Here, we have applied these methods to characterize some structural features of protein fractions during grain ripening in two different cultivars of durum wheat, namely *Duilio* and *Ofanto*, grown under identical agronomic and climatic conditions. We used immunochemistry to evaluate the time course of gluten protein appearance. On the same samples, we also evaluated changes in the pattern of starch and soluble sugars, with the aim of attaining a unifying vision of these materials, and to verify their suitability for transformation into food.

2. MATERIALS AND METHODS

2.1. Grain Samples. *Ofanto* and *Duilio* durum wheat cultivars were grown in the experimental fields of CRA-ISC, located in Rome (Italy). Grains from either cultivar were harvested at different ripening stages from 9 to 28 days after anthesis (DAA). To make milling possible, all the grains were dried at 35 °C for 24−30 h immediately after harvesting to reduce humidity from 65% to about 14%, and milled to whole-meal flour (particle size <0.5 mm) with a Fritsch Pulverisette mill (Fritsch GmbH, Idar-Oberstein, Germany).

2.2. Carbohydrate Characterization. The content of soluble sugars and fructo-oligosaccarides (FOS) was assessed by extraction of meals with water (2 h at 105 °C) preceded by an extraction with 96% ethanol in order to remove the mono- and disaccharides (*23*). α-Amylase activity was evaluated according to ICC Standard Method N*o*. 303 (1999) (*24*).

Total starch was evaluated according to the ICC Standard Method N*o*. 168 (1999) (*24*). Starch gelatinization properties were investigated by a Micro-Visco Amylograph (Brabender OHG, Duisburg, Germany). Fifteen grams of sample flour (14% moisture) was dispersed in 100 mL of distilled water and stirred in a Micro-Visco Amylograph at 250 rpm, heated from 50 °C to 95 °C at a heating rate of 6 °C/min, maintained at 95 °C for 5 min (first holding period), cooled from 95 °C to 50 °C, and then held at this temperature for another 5 min. When indicated, 1 mM silver nitrate was added to inhibit α-amylase activity (*25*).

Starch suspensions were placed on a microscope slide and directly observed with a light transmission microscope (Nikon Eclipse ME 600 Microscope, Nikon Co., Tokio, Japan). The images were taken with a Nikon camera interfaced to a personal computer.

2.3. Protein Characterization. *2.3.1. Total Protein Content.* Protein content was determined by the Dumas combustion method, using a Leco FP 428 analyzer (Leco Co., St. Joseph, MI) calibrated with an EDTA standard. The content in lysine and cysteine was determined according to AOAC (1990), Official Method 982−30 (*26*).

2.3.2. Protein Solubility and Protein Thiols. Protein solubility in native and denaturing conditions was determined by suspending 100 mg of whole-meal flour in 2 mL of buffer (50 mM phosphate, 0.1 M NaCl, pH 7.0), containing also 8 M urea and 10 mM DTT when required. The amount of protein dissolved after stirring the suspensions for 20 min at 25 °C was determined by a colorimetric method (*27*) on the supernatant of low-speed centrifugation of the suspension.

Accessible SH groups were measured directly on flour suspensions. An aliquot (100 mg) of whole flour was suspended in 5 mL of buffer (50 mM phosphate, 0.1 M NaCl, pH 7.0), containing 0.5 mM 5,5′-dithiobis(2-nitrobenzoate) (DTNB, (*28*)). The suspension was incubated at 25 °C for 60 min and centrifuged, and the absorbance of the clear supernatant was read at 412 nm. Total SH groups were measured by the same method but in the presence of 1% sodium dodecyl sulfate (SDS) in the suspension buffer.

2.3.3. Protein Surface Hydrophobicity. Protein surface hydrophobicity was determined by a ligand-binding approach according to published procedures (*29*). The method allows the determination of the amount of a hydrophobic fluorescent probe (1,8-anilinonaphthalenesulfonate, ANS) remaining bound to the buffer-insoluble protein fraction, and also the estimation of the dissociation constant of the complex formed by these proteins with the probe. In short, flours obtained from kernels at various ripening times were suspended at 20 mg/mL in 5 mL of 50 mM phosphate, 0.1 M NaCl, pH 7.0, containing 0.2 mM ANS. After overnight shaking at 25 °C, suspensions were centrifuged, and the amount of ANS remaining in the supernatant was measured spectrofluorimetrically by diluting an aliquot of the supernatant into 3 mL of aqueous 5% Triton X-100. Calibration was performed by addition of an internal ANS standard to the Triton X-100 solution.

2.3.4. SDS-PAGE and Immunoblotting. SDS-PAGE and immunoblotting were performed according to Berti et al. (*20*). A fixed volume of the supernatant obtained after urea/DTT treatment of the various meals was diluted 1/1 (v/v) with SDS-PAGE denaturing buffer (containing 2-mercaptoethanol) and denatured by boiling at 100 °C for 5 min. SDS-PAGE was carried out on a fixed porosity gel (12% monomer), using a MiniProtein apparatus (BioRad, Richmond, VA). Proteins in the gel were then transferred to a nitrocellulose membrane by using a semidry blotting apparatus (Sartorius) run at 50 mA for 60 min. The nitrocellulose membranes were incubated overnight in 10 mL of a 1% gelatin solution and then exposed to a rabbit anti-gliadin polyclonal antibody, that reportedly recognizes various gluten-related proteins with different specificity (*20*). Anti-rabbit peroxidase-labeled goat antibodies were used for the final detection step. Both the primary and secondary antibodies were from Sigma (St. Louis, MO) and were diluted 1:1000 (v:v) in 0.25% gelatin. Stained gels and membranes were scanned and analyzed by means of standard image analysis hardware and software.

2.4. Statistical Analysis. Measurements were replicated ($n \geq 3$). Statistical analysis was performed by using STATGRAPHICS PLUS, version 4.0. From an ANOVA data analysis, Fisher's least significant difference (LSD) and Pearson's correlation coefficient were estimated. Differences were considered significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Evolution of Carbohydrate Components during Ripening. The amount of individual carbohydrate fractions at different times after anthesis is presented in **Table 1**. Although a large variability was found between the two cultivars, each fraction showed a similar trend during kernel ripening. At the milky phase (i.e., ∼15 DAA, when kernels can be already harvested and milled with acceptable yields (*30*)), the amount of FOS accumulated in grains of the *Ofanto* cultivar was twice that in *Duilio* grains. In turn, at 28 DAA the FOS content of both cultivars was approximately the same (about 3% on a dry matter basis), that is, 5−10 times lower than the amount found at the beginning of milky phase.

The decrease in monosaccharides, disaccharides, and FOS during kernel ripening reflected their progressive conversion into storage polysaccharides. Starch content, in fact, increased from 15 to 20% at 9 DAA to about 50% at 28 DAA. It has to be noted that, within this particular time frame, changes affect not only the amount of starch but also its organization, as made evident by the micrographs in **Figure 1**. Grains in the milky phase were characterized by the presence of large-sized starch granules, whereas smaller starch granules only appeared in later phases of the ripening process.

Table 1. Content of Carbohydrate Fractions (% d.m.) in Meals from Kernels Harvested at Various DAA ($n = 4$)[a]

	Ofanto					Duilio			
carbohy drates	9 DAA	15 DAA	17 DAA	21 DAA	28 DAA	9 DAA	15 DAA	21 DAA	28 DAA
mono- and disaccharides	10.57 ± 0.17 d	10.80 ± 0.64 d	5.72 ± 0.06 c	2.86 ± 0.12 b	1.94 ± 0.05 a	15.77 ± 0.17 c	3.12 ± 0.04 b	1.88 ± 0.25 a	1.89 ± 0.05 a
FOS	29.73 ± 0.47 e	21.70 ± 1.31 d	14.25 ± 0.16 c	7.30 ± 0.29 b	3.12 ± 0.09 a	15.80 ± 0.82 c	8.49 ± 0.52 b	3.45 ± 0.19 a	3.15 ± 0.19 a
starch	22.52 ± 0.06 a	24.82 ± 0.55 b	37.64 ± 1.76 c	49.99 ± 036 d	53.26 ± 0.12 e	15.50 ± 0.62 a	31.88 ± 0.36 b	40.64 ± 0.81 c	46.74 ± 0.98 d

[a] Mean values in the same row with different letters are significantly different ($P < 0.05$).

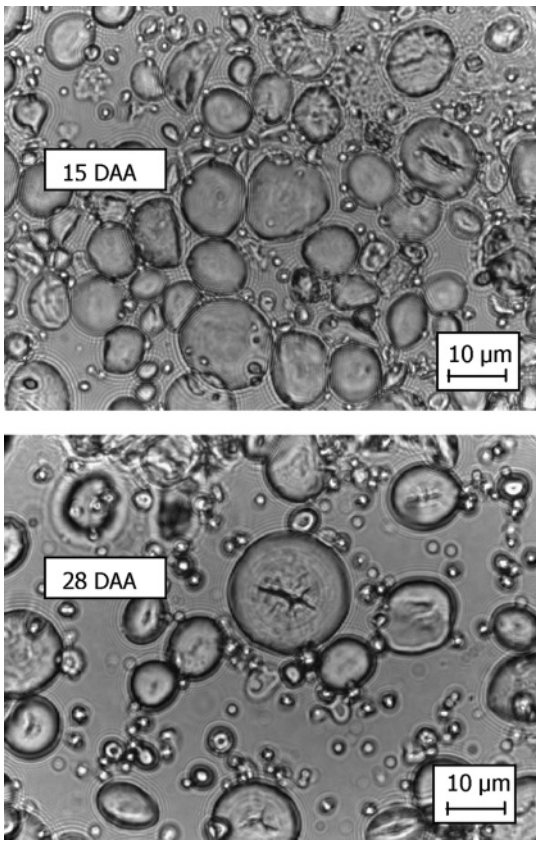

Figure 1. Light microscope images of starch granules in grains of the *Duilio* durum wheat cultivar at 15 and 28 days after anthesis.

The quantitative changes in the carbohydrate components were associated with relevant modifications of α-amylase activity (**Table 2**), in agreement with previous observations (*31*). The carbohydrate pattern reported in **Table 1** accounts for the trend of viscoamylographic indexes reported in **Table 2**, that compares data for kernels harvested at the milky phase (15 DAA) and at complete maturation (28 DAA). The viscosity after heating meals from immature kernels of both varieties was very low. This result was due not only to the low amount of starch being present at this time but also to the intense α-amylase activity in these samples, that caused a pronounced starch hydrolysis in the first minute of the test. This assumption was confirmed by the modifications observed in the viscoamylographic pattern when silver nitrate was added to inhibit α-amylase activity (not shown (*25*)). The predominant presence in immature grains of large starch granules (see **Figure 1**), having a lower gelatinization peak temperature in comparison to small starch granules (*32*), provides an explanation for the onset temperature of gelatinization phenomenon in immature grains being 2−3 °C lower than in the mature samples.

3.2. Evolution of Protein Fractions during Ripening. In the time frame considered in this study (from 9 to 28 DAA), the total protein content in immature grains (on a dry matter basis) showed only minor variations, remaining in the 14−17% range for the *Ofanto* cultivar and in the 12−15% range for the *Duilio* cultivar.

Solubility of immature grain proteins in phosphate/saline buffer in the absence and in the presence of denaturing and reducing agents at different ripening times is reported in **Figure 2**. The solubility of proteins in the solvent system with a different dissociating ability can be used to discriminate among different cereals (*20*) and to describe the effects of technological treatments on cereal-based foods (*22*). This approach also allows a coarse correlation between the physicochemical properties of proteins, as inferred from their aggregation state, and their behavior during food processing (*9*).

For both cultivars, addition of urea and DTT to the extraction buffer markedly increased the amount of extracted protein, in particular between 9 and 17 DAA. A decrease in the amount of albumins and globulins (empty symbols in **Figure 2**) was accompanied by a noticeable increase in the amount of proteins requiring urea and DTT to become soluble. This increase leveled off at 17 DAA. The amount of soluble proteins is clearly different between the two cultivars. Changes in the protein content and distribution occurred more gradually in the *Ofanto* cultivar than in the *Duilio* cultivar, as were the changes in the starch fraction discussed above.

Time-dependent changes in the protein fraction may stem either from modifications of the structural features of existing proteins (through the formation of aggregate species stabilized by interprotein disulfide bridges after the milky phase) or by changes in the nature of the proteins themselves, as a consequence of the degradation of a given set of proteins concomitantly to the synthesis of new protein species. In this frame, it has to be noted that the decrease in proteins soluble in the absence of denaturant was accompanied by a significant decrease of lysine content in the whole meal. The lysine content (on a dry matter basis) decreased from 4.55% at 9 DAA to 2.93% at 28 DAA in the *Ofanto* cultivar and from 5.0% to 3.3% in the *Duilio* cultivar.

To assess the pattern of proteins being present in kernels at different ripening times, proteins were also characterized by SDS-PAGE and immunoblotting, as shown in the different panels of **Figure 3**. Time-dependent changes in the protein pattern were evident in each cultivar, and are indicative of a different evolution of the protein pattern in the two cultivars. In the *Duilio* cultivar some high molecular mass bands (94000−48000 Da) were already evident at 15 DAA, whereas in the *Ofanto* cultivar they appeared only after 17 DAA.

Gluten-related proteins were identified by immunoblotting, using commercial anti-gliadin antibodies, following separation in SDS-PAGE. The immunochemical approach confirmed that the synthesis of the major gluten polypeptides is different in the two cultivars both in terms of the pattern of immunoreactive proteins and in terms of the time course of their appearance.

Table 2. Viscoamylographic Indices and α-Aylase Activity (IU/g d.m.) in Meals from Kernels Harvested at 15 and 28 DAA ($n = 3$)[a]

cultivar	DAA	α-amylase activity	onset gelatinization temperature (°C)	peak viscosity (BU)	peak viscosity temperature (°C)
Ofanto	**15**	7.5 ± 0.25 b	68.3 ± 0.21 b	73 ±1.20 a	79.8 ± 0.21 a
	28	3.7 ± 0.20 a	67.1 ± 0.18 a	200 ± 1.86 b	89.3 ± 0.52 b
Duilio	**15**	2.3 ± 0.06 b	67.6 ± 0.20 b	206 ±3.27 a	90.4 ± 0.58 a
	28	0.4 ± 0.02 a	66.2 ± 0.18 a	490 ± 4.45 b	94.9 ± 0.63 b

[a] Mean values in the same column with different letters are significantly different ($P < 0.05$).

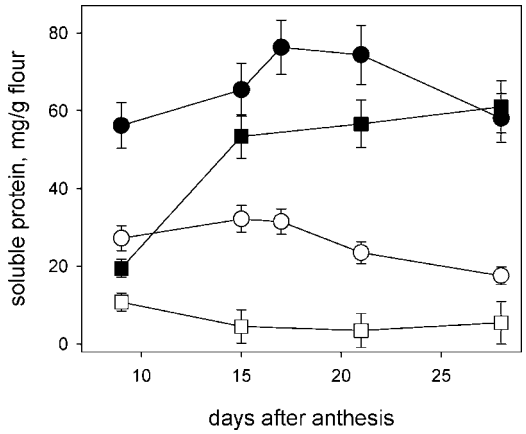

Figure 2. Time course of changes in protein solubility in plain buffer (open symbols) and in buffer containing urea/DTT (full symbols) for *Duilio* (squares) and *Ofanto* (circles) cultivars. Vertical bars indicate standard error.

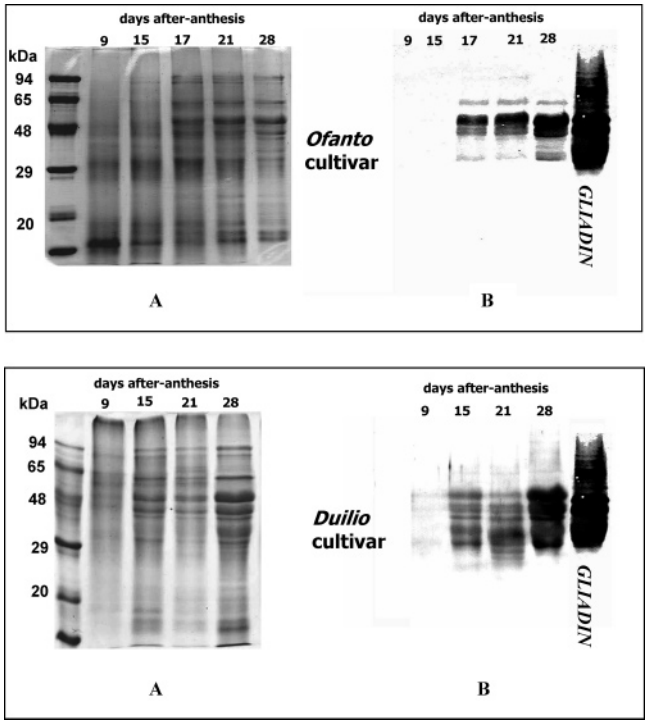

Figure 3. SDS-PAGE (A) and immunoblotting against commercial anti-gliadin antibodies (B) of proteins soluble in buffer containing urea/DTT at different ripening times in the *Duilio* (upper panel) and *Ofanto* (lower panel) cultivars.

In the *Ofanto* cultivar, gliadin synthesis was only noticeable from 17 DAA. No immunoreactive proteins were present at 9 or at 15 DAA in this cultivar. Appreciable amounts of immunoreactive proteins were already present at 15 DAA in the *Duilio* cultivar, indicating that the synthesis of immunoreactive, gluten-related proteins occurred earlier in the *Duilio* cultivar than in the *Ofanto* cultivar.

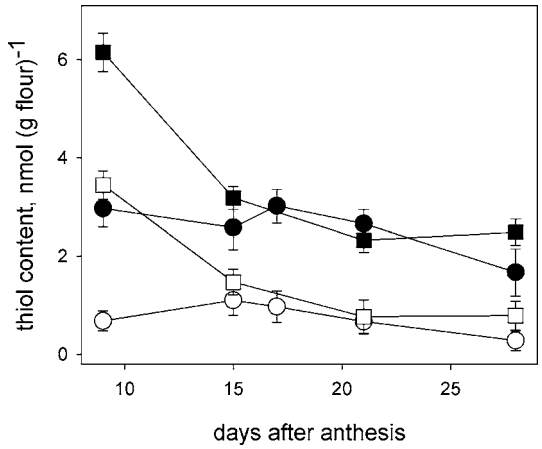

Figure 4. Time course of changes in the thiol content in the *Duilio* (squares) and *Ofanto* (circles) cultivars as detected in the absence (open symbols) or in the presence of 1% SDS (full symbols). Vertical bars indicate standard error.

All these approaches confirm that synthesis of the major gluten-related proteins occurs during the milky phase. The relatively sudden appearance of protein species recognized by anti-gliadin antibodies in a matter of few days, and the significant differences observed between cultivars may have great practical relevance if these grains were to be used as a starting material for foods to be consumed by gluten-sensitive individuals.

3.3. Structural Features of Proteins in the Developing Grains. Sulfhydryls (SH) and disulphides (S−S) play an important role in the structure and reactivity of food proteins and in assessing the technological properties of flours (*33*, *34*), since acquisition of a compact structure in individual proteins and formation of an inter-protein network depends on intra- and intermolecular S−S bonds. Here, quantification of SH accessibility (*19*) was used to better understand the nature and to evaluate the relevance of protein structural modifications that may occur during ripening of durum wheat grains.

The arrangement of thiols in immature grains proteins was studied by assessing the reactivity of SH groups toward the bulky thiol reagent, DTNB, under the different conditions reported in **Figure 4**. To estimate thiol accessibility, this determination was carried out in the absence and in the presence of 1% SDS at room temperature. Under these particular conditions, the detergent loosens parts of the protein structure but does not dissociate (and therefore does not make soluble) strong protein aggregates or disulfide-linked gluten proteins.

The results of these determinations indicated a marked decrease of total thiols and of thiol accessibility at the onset of gluten synthesis in the *Duilio* cultivar, that is, between 9 and 15 DAA, remaining stable afterward. Time-dependent variations in the content of accessible thiols were virtually absent in the *Ofanto* cultivar in spite of a marked increase in the cysteine

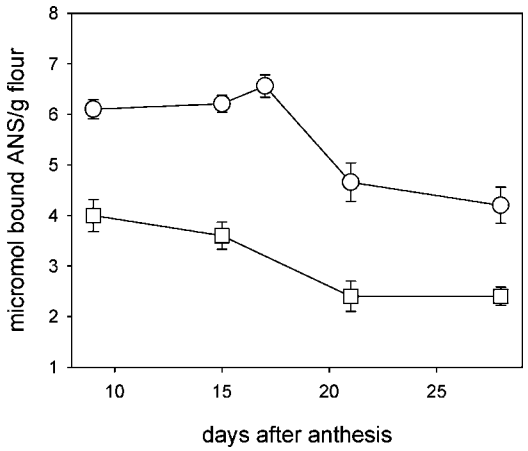

Figure 5. Time course of changes in the surface hydrophobicity of buffer-insoluble proteins in the *Duilio* (squares) and *Ofanto* (circles) cultivars. Vertical bars indicate standard error.

content of kernels in this cultivar (from 0.55 to 1.02 g cysteine/100 g protein), suggesting a progressive and continuous protein association via S−S bonds during kernel ripening in this cultivar.

This latter observation may be interpreted as due to the fact that cysteine may be involved in the formation of disulfide bonds, or be deeply buried in the protein interior thus escaping detection in mild denaturing conditions, that is, when using detergents at room temperature. In the *Duilio* cultivar, cysteine content increased only from 1.69 g/100 g protein in the milky phase to 2.1 g/100 g protein at final maturation.

Given that the total protein content in each cultivar does not change substantially in the time span considered here, the differences in thiol content and in thiol accessibility between the two cultivars appear related to the different nature and amount of proteins being present at each DAA in kernels of the two cultivars. This hypothesis is confirmed by a decrease in thiol accessibility, that may be defined as the ratio between the amount of thiols accessible in the absence of detergent and that detected in the presence of detergent, as presented in **Figure 4**). In both cultivars, thiol accessibility decreases sharply after the milky phase, suggesting that the proteins acquire a compact structure either through formation of intramolecular S−S bonds or through association into S−S-linked polymeric species.

To investigate further the factors involved in structural modifications of proteins during ripening, we also determined protein surface hydrophobicity (*19*, *29*). Hydrophobic interactions are relevant both to the folding of individual proteins and to their associative behavior. The approach used here allowed the determination of the amount of a fluorescent hydrophobic probe (1,8-anilinonaphthalenesulfonate, ANS) remaining bound to buffer-insoluble proteins as a function of the free probe, therefore providing information for the structural changes occurring in this otherwise elusive protein fraction without resorting to protein-denaturating extraction procedures.

As shown in **Figure 5**, accessibility of exposed hydrophobic regions on the surface of insoluble proteins dropped after 17 DAA in the *Ofanto* cultivar, suggesting that major compaction of the overall protein structure began at this time, as expected when gluten proteins became the most abundant protein component. The trend toward a decrease in the number of accessible surface hydrophobic sites was monotonic in the *Duilio* cultivar, consistent with the trend of changes discussed so far in terms of protein solubility, protein nature, and thiol content and accessibility in this particular cultivar.

4. DISCUSSION

Our results indicate that wheat kernels harvested at 12−15 DAA can represent a valuable ingredient for the production of functional foods, given the peculiar properties of both the polysaccharide and the protein fraction. The relevance of the elevated FOS content, the possibility of being ground into meals, and possible incorporation of these meals their into foods were already discussed in previous studies (*30*, *35*). Both the composition and structure of the carbohydrates in grains at the milky phase are of nutritional interest and reportedly did not impair further processing of these materials into foods (*30*, *35*).

Grains harvested at the milky phase have a total protein content very close to that at later ripening stage, with a predominance of albumins characterized by an equilibrated amino acid composition (*30*). As pointed out in this study, milky-phase grains do not contain gliadins but have proteins with thiol content and surface hydrophobicity properties compatible with their processing into foods. Therefore, they could represent a potential raw material for food to be consumed by gluten-sensitive individuals.

However, exploiting the features discussed above for the production of functional or healthy foods will require a careful choice of the cultivar and will also require suitable analytical approaches to monitor the rapidly changing protein pattern. Indeed, the data presented here indicate that differences between cultivars are relevant, and that quite sudden changes in composition of both the polysaccharide and the protein fraction may occur, resulting in completely different molecular patterns within a few days. These differences may go unnoticed if coarse compositional parameters are used for grain characterization. Therefore, further improvement and testing of the approaches presented here may be needed, in particular the structural approach to the characterization of the involved macromolecules.

A significant contribution to our understanding of these issues is expected from the ongoing development of powerful mass spectrometry techniques and of their application to food-related issues. These methodological tools will be particularly useful when the information they provide is put to work in interdisciplinary studies involving breeding and agronomic issues, traits relevant to food technology and nutrition, and the type of structural information taken into account in the present study.

ABBREVIATIONS USED

DAA, days after anthesis; FOS, fructo-oligosaccharides; SDS, sodium dodecyl sulfate.

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