JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Review

pubs.acs.org/JAFC

Role of Non-prolamin Proteins and Low Molecular Weight Redox Agents in Protein Folding and Polymerization in Wheat Grains and Influence on Baking Quality Parameters

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ABSTRACT: The various enzyme systems and low molecular weight (LMW) redox agents are related to the folding and polymerization of prolamins in the ripening wheat grains and the formation of baking quality. Protein disulfide isomerases (PDIs) and cyclophylins accelerate "correct" folding of prolamins, which is most likely necessary for the subsequent formation of the macromolecular structure of the gluten protein matrix. PDIs are also involved in the polymerization of prolamins, catalyzing the oxidation of protein sulfhydryl groups. Molecular chaperone binding BiP protein facilitates folding of prolamins, with its role increasing in the stressful conditions. Reducing systems of thioredoxin and glutaredoxin, LMW redox pairs GSH/GSSG and Asc/DHAsc, thiol oxidases, and lipoxygenases (LOXs) regulate redox balance and the rate of polymerization of prolamins at the different stages of grain ripening. Additionally, LOX is probably involved in the protein−starch−lipid interactions between the starch granule and the protein matrix, mediated by puroindolines, determining the formation of grain texture. It is assumed that the high variability of baking quality in different environmental conditions is due to the interaction of labile enzyme systems with the storage proteins in the developing wheat caryopsis.

KEYWORDS: *grain texture, maturation, polymerization glutenins, SS/SH redox regulation, stability quality, wheat*

INTRODUCTION

Wheat is one of the most important cereal crops for human nutrition because of storage proteins (prolamins), which, when hydrated, form gluten. Wheat gluten is characterized by viscous−elastic properties, and in combination with other flour components, wheat gluten ensures the characteristic texture of bread and other flour products. Breadmaking quality traits vary on the basis of the genotype, the environment, and the interactions between these two factors.[1−4] In recent years, the study of the stability of gluten quality and other traits of baking quality has been the focus of wheat investigations.[5]

In comparison with other wheat grain proteins, wheat prolamins, such as monomeric gliadins and polymeric glutenin, are well-characterized. Comprehensive reviews describe their biochemical, genetic, and molecular characteristics, post-translational processing, assembly and compartmentalization, and various aspects of wheat grain use in human nutrition.[5−9] After 7−10 days after anthesis (DAA), the prolamins are determined in wheat caryopses and mainly accumulate during the physiological ripening that occurs between 12 and 35 DAA. During grain desiccation, storage protein accumulation virtually stops.[10,11] The high rate of protein synthesis results in high storage protein concentrations in the endoplasmic reticulum (ER) lumen and promotes protein aggregation within the ER.[12] The nascent polypeptides are cotranslated and cotranslocated into the rough ER lumen, which is the place where many post-translational processes occur, including folding, the formation of intramolecular disulfide (SS) bonds, and polymerization via the formation of intermolecular SS bonds. During the ripening phase, protein bodies are integrated and enlarged, and by the beginning of caryopsis dehydration, these protein bodies form a continuous protein matrix.[12−14] At the dehydration stage, large, spherical glutenin particles form in the grain.[15] Grain texture formation is related to lipid−starch−protein interactions between the gluten matrix proteins and the lipids of starch grain membranes, which are mediated by granule-bond starch synthase, gliadin, low molecular weight glutenin subunits (LMW-GS), serine protease inhibitors, α-amylase inhibitors, and puroindolines.[16,17]

The polymerization of glutenin subunits is the most important step in the formation of the gluten protein network. Shewry et al.[11] used size exclusion high-performance liquid chromatography to demonstrate that caryopses already contain high molecular weight (high M_r) and low molecular weight (low M_r) polymers at 14 DAA; the proportion of these polymers (high M_r/low M_r) rapidly increases during grain desiccation. Rhazi et al.[10] showed that during the cell division and cell enlargement phase, glutenin subunits and particularly LMW-GS have a large amount of free SH groups and become oxidized during grain dehydration, which coincided with the formation of SDS-unexractable polymeric protein (UPP). According to Abony et al.[18] gluten formation was first observed only at 20−25 DAA, and its amount increased rapidly during the next period of grain development. Research results of Shewry et al.,[11] Rhazi et al.[10] and Abony et al.[18] agree with the model proposed by Hamer and van Vliet,[19] which presumes the existence of two distinct levels of storage protein aggregation

Received: August 11, 2012
Revised: November 20, 2012
Accepted: November 21, 2012
Published: November 21, 2012

during grain ripening. The first level concerns the formation of exractable polymeric protein (EPP) during cell division and cell enlargement and depends on the genetically determined composition of glutenin subunits. A second level of aggregation concerns the formation of UPP by entanglement, stabilized by hydrogen bonding and additional disulfide bridges, and would lead to the formation of a protein network.[10,19] This level of aggregation is induced by the dehydration of the grain. Carceller and Aussenac[20] showed that the quantity and molecular size distribution (i.e., polymerization index) of level 2 aggregates is largely influenced by the composition of level 1 aggregates. Moreover, according to Rhazi et al.[10] the alkylation of free glutenin SH groups before the desiccation induces an increase in the SDS solubility of the polymeric proteins by reducing both their molecular weight distribution and their compactness.

Thus, not only the composition of glutenin subunits but also the conditions of their polymerization on the first and second levels of aggregation determine the distribution of the molecular masses (MWD) of protein polymers, which is known to be the molecular basis of gluten viscous−elastic properties.[21,22] By estimates of Lemelin et al.,[23] 60−84% of the stability values of the loaf volume and of breadmaking score were explained by the stability of only a few quality parameters, among which the molar masses of the polymers played the major role. Variance analysis revealed a significant effect of environmental factors on molar masses of the polymeric fraction of wheat flours obtained at grain maturity. Lemelin et al.[23] concluded that the stability of MWD parameters among the varieties will be influenced by the physiological events during grain maturation, mostly due to environmental variations but to a lesser extent to the relative amounts of HMW-GS and their allelic composition.

Non-prolamins also influence the processing and rheological properties of wheat flour. Wheat genotypes of different quality have demonstrated diverse proteomic profiles of non-prolamin proteins, including albumins and globulins; approximately 80% of these proteins were identified in the developing grain as enzymes with different physiological functions.[24] Primo-Martin et al.[25] investigated changes in the quantity, quality, and viscoelastic properties of the glutenin macropolymers by the addition of enzymes: pentosanase, glucose oxidase, laccase, and their combinations. They found that glucose oxidase gave the least extensible and most resistant dough and that pentosanase/glucose oxidase resulted in dough with improved extensibility. It is known also that some peptidases,[26] xylanase,[27] and amylase[28] affect the quality of the bread. At the same time, the effect on the parameters of baking quality by the endogenous enzymes, involved in the folding of newly synthesized prolamins and their polymerization in the ripening wheat caryopsis, is studied to a lesser extent. Among the albumins and globulins identified in proteomic studies by Merlino et al.[29] and Hurkman et al.[30] were proteins involved in protein folding (22%) and in stress response (28%). Synthesis of these proteins is increased at the early stages of ripening and during the maturation of grains at high temperature. Apparently, environmental effects on the baking quality mediated the labile systems of enzymes that regulate the folding of polypeptides and redox SS/SH balance of polymeric proteins, as well as starch−lipid and lipid−protein interactions.

This review focuses on studies of the impact of non-prolamin proteins and low M_r agents on the folding and polymerization of storage proteins in ripening caryopsis and the viscous−elastic properties of gluten and wheat grain texture.

STORAGE PROTEIN FOLDING AND HOUSEKEEPING PROTEINS OF THE ER LUMEN

In the ER lumen, the prolamins can interact with foldases, such as protein disulfide isomerase (PDI, EC 5.3.4.1) and peptidyl-prolyl *cis*−*trans* isomerase (PPI, EC 5.2.1.8), to catalyze the rate-limiting steps of protein chain folding, which involve a thiol−disulfide exchange reaction (PDI)[31] and isomerization of a peptide bond, which precedes proline (PPI).[32] A typical wheat PDI is a glycoprotein of the thioredoxin family with a molecular mass of 60 kDa, and its polypeptide chain contains 515 amino acids and 2 thioredoxin-like Cys-Gly-His-Cys domains.[33,34] In recent studies, PDI and PDI-like proteins containing a thioredoxin site were frequently combined into a superfamily of thiol oxidoreductases. The C-terminal end of these PDI proteins has a Lys-Asp-Glu-Leu sequence (512−515), which is characteristic of all proteins residing in the ER and most likely prevents the secretion of enzymes from the ER. Residues 26−150 and 386−489 correspond to the first thioredoxin domain and the second thioredoxin-like domain, respectively. Residues 68−71 and 412−415 form redox-active disulfide bonds.[33] The PDI activity includes isomerase and chaperone-like activity.[35] It is assumed that PDI operates primarily as a chaperone in the early stages of folding, blocking the incorrect association of polypeptide chains, and as an isomerase at later stages, implementing the regrouping of thermodynamically strained, "incorrect" SS bonds into the configurations that are characteristic of the native proteins (Figure 1).[35]

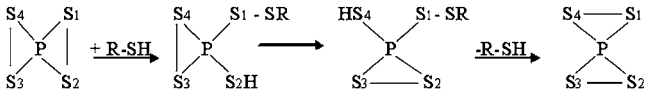

Figure 1. PDI catalyzes the isomerization of the protein disulfide bonds. P, protein; R-SH, low molecular weight thiol.

Wheat PDI compartmentalization in the ER and endosperm protein bodies suggests that this enzyme is involved in the assembly of storage proteins both in the ER lumen and in protein bodies.[33] Direct evidence of PDI participation in protein deposition was obtained by Takemoto et al.,[36] who revealed a connection between the absence of PDI expression and abnormal protein deposition in the rice mutant *esp2*. Johnson and Bhave[37] developed markers for the genes of the PDI family demonstrating orthology between the loci of rice and wheat PDI and found connections between loci involved in protein deposition and PDI loci.[38] The nine PDI and PDI-like sequences of wheat were located in chromosomal regions that shared synteny with the regions found in rice.[39] The effects of endogenous PDI family proteins on the properties of gluten proteins in dough during breadmaking were determined using bacitracin, an inhibitor of PDI.[40] Dramatic decreases in SDS-insoluble glutenin macropolymer after the addition of bacitracin suggest that the endogenous PDI family proteins in flour suppress the depolymerization of glutenin macropolymer.[40] In flour, the activity level of endogenous PDI weakly correlated with baking qualities; however, in tests with the addition of ascorbic acid (Asc), four of the wheat species studied in this work showed a positive correlation between the level of PDI activity and baking parameters.[41] The authors suggested that

PDI was involved in the Asc improver effect in baking through the following reactions, which produce larger gluthenin polymers that are associated with good bread quality:

$$\text{Asc} + \frac{1}{2}O_2 \xrightarrow{\text{AOX}} \text{DAsc} + H_2O$$

$$\text{DAsc} + \text{reduced PDI} \rightarrow \text{Asc} + \text{oxidized PDI}$$

$$\text{oxidized PDI} + 2\ \text{gluthenin-SH} \rightarrow \text{reduced PDI} + \text{gluthenin-SH} - \text{gluthenin}$$

AOX represents ascorbate oxidase and DAsc, dehydroascorbic acid.

A level of endogenous PDI >50 U/g of flour is significant for the catalysis of ascorbic acid enhancing effect.[41]

The second enzyme identified is a foldase, peptidyl-prolyl *cis*−*trans* isomerase. PPIs catalyze the *cis*−*trans* isomerization of peptide bonds preceding proline (Figure 2) and accelerate the

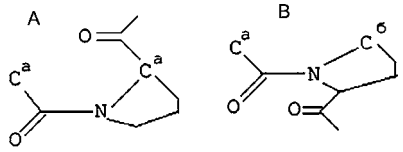

Figure 2. *cis* (A) and *trans* (B) configurations of the peptide bond of the fragment Xaa-Pro. Xaa, any amino acid; Pro, proline.

creation of the peptide bond configurations needed for the "correct" folding. These enzymes are found in all intracellular compartments, including inside the ER.[42] The ubiquity of PPIs and their inherent foldase and chaperone activities draw attention to these enzymes in relation to the wheat storage protein folding[43−46] due to their proline-rich nature.[6] In wheat caryopsis, two families of PPIs were found, FKBP[32] and cyclophylins.[43−45] PPIs are related to the prolamin folding of wheat caryopses through cyclophylins because the A-type cyclophylin genes expressed in the wheat endosperm are localized in the same region as the genes encoding quality parameters, which are located on the short arms of the sixth homeological group of chromosomes.[44] The A-type cyclophylins as PDI have a higher accumulation at early stages of grain development.[47] Wu et al.[45] identified *CypB* genes encoding an ER-localized cyclophilin from common wheat (*Triticum aestivum* L.). Using the sequence variations, the common wheat genes were localized to chromosomes 7AL, 7BL, and 7DL. Certain quantitative trait loci (QTLs) related to protein quality occur at the rice and wheat *CypB* loci. The results support this enzyme being a strong candidate for regulating storage protein quality in wheat.[45] PPIase activity, measured at different stages of grain development, showed variability with both the developmental stage and cultivar. Principal component analysis revealed the association of PPIase activity with either gliadin or total proteins, suggesting their significant role in the deposition of storage proteins in wheat.[46]

On the basis of the data presented, foldases interact with storage proteins in the ER lumen and inside protein bodies. These protein bodies are formed through immediate protein deposition in the ER lumen, which happens when the speed of synthesis and the concentration of storage proteins are high. PDIs and PPIs accelerate folding of prolamins in these conditions; most likely this is necessary for the subsequent formation of the macromolecular structure of gluten. PDI is also involved in the polymerization of prolamins.

At high temperatures (37/28 °C day/night), the expression of heat shock proteins (Hsp) increases during wheat growth.[30] One of the well-characterized members of the Hsp70 protein family is an ER-localized molecular chaperone binding protein (BiP). Using immunochemical and biochemical methods, Li et al.[48] showed that BiP is localized on the surface of protein bodies of rice that were formed by aggregated prolamins, and BiP forms complexes with free prolamins and nascent chains of prolamins in polyribosomes. The authors hypothesized that BiP functions to keep prolamins in the ER lumen to accelerate their folding and assembly inside protein bodies. In the developing wheat caryopsis, the BiP level increased between 5 and 9 DAA. This level remained high until 17−21 DAA and decreased simultaneously with the ripening and dehydration of caryopses.[33] It follows that BiP, as well as foldase, facilitates folding of prolamins, and its role increases in stressful conditions.

REGULATION OF STORAGE PROTEIN POLYMERIZATION BY LOW M_r REDOX AGENTS AND OXIDOREDUCTASES

The key role of intermolecular SS bonds in the formation and stabilization of protein polymers of wheat grain suggests that the process of glutenin polypeptide polymerization during the ripening of caryopsis is regulated by thiol oxidoreductases and various cellular redox agents.

Low M_r Redox SS/SH Agents of the Developing Wheat Endosperm. The two low M_r redox pairs that are most frequently involved in various processes of plant development are reduced glutathione/oxidized glutathione (GSH/GSSG) and ascorbic acid/dehydroascorbic acid (Asc/DAsc).

It is generally accepted that the redox environment in the ER is more oxidizing than in the cytosol and that the ratio of reduced to oxidized glutathione ([GSH]/[GSSG]) is optimal for disulfide bond formation.[49] This environment facilitates the formation of native disulfide bonds in the ER through a complex process, which is catalyzed by PDI and involves disulfide bond formation and the isomerization of non-native disulfide bonds. Stress leads to an integrated stress response that increases the production of glutathione by increasing amino acid metabolism. Glutathione has integral roles in native disulfide bond formation and in balancing redox reactions that protect the cell from oxidative stress.[49] In response to stress, increases in the GSH concentration of the ER lumen and accumulating organelles of developing grains can negatively affect the rate of polymerization of glutenin subunits.

In the cells of mature wheat endosperm, glutathione is present both in the free state and in the protein-bound state (PSSG).[50−52] Li et al.[51] found that the total contents of free and protein-bound glutathione in 36 wheat species were 358 ± 41 and 190 ± 10 nmol/g, respectively. Hütter and Wieser evaluated the distribution of the endogenous glutathione in Osborne fractions from the distribution of radioactive glutathione, added in trace amounts together with water to the dough and to the gluten. About 65% of glutathione was found in the water-soluble fraction (this was mostly not associated with protein glutathione), 6−7% was found in each of the globulin and gliadin fractions, and 21% was found in the glutenin fraction. This distribution suggests that glutathione blocks reacting SH groups of protein polymers and hinders polymerization processes. Indeed, Rhazi et al.[53] applied liquid

chromatography on a reversed-phase support for two species of soft wheat that differed in their composition of high M_r glutenin subunits and showed that the glutathione reductase activity decreases, the GSSG/GSH proportion increases, and mixed disulfide protein−glutathione (PSSG) forms 33 DAA during grain dehydration, a time point which coincides with UPP formation. Approximately 85% of PSSG in ripe grains was present in the polymer proteins bound with glutathione (PPSSG). Li et al.[52] demonstrated that a significant amount of PSSG was negatively correlated with the molecular masses of polymers; the PSSG level in the gluten of low-quality flour was always higher and significantly different from the PSSG level in flour of high baking quality. According to Rhazi et al.[53] and Li et al.,[52] the formation of PPSSG during grain dehydration might play a decisive role in the degree of polymerization, induce an increase in polymer protein solubility, and reduce the molecular mass and branching of polymers. During the development and ripening of caryopses, the redox balance of the glutathione pool (GSH/GSSG) compared with ascorbate changed more slowly in the direction of the oxidized form and amounted to approximately 1−1.5 until 28 DAA; the GSH/GSSG subsequently decreased to 0.4.[54] During ripening, the total ascorbate content (Asc + DAsc) increased gradually from 13 to 21 DAA, remained constant during the next 7 days, and then reduced to low values in ripe grain;[54] the redox balance of the ascorbate pool significantly changed. At early stages of caryopsis development, the reduced form of ascorbate prevailed; in the following ripening period, the Asc/DAsc proportion decreased; and in ripe grain, dehydroascorbic acid was the only form of vitamin C present. Every et al.[55] experimentally demonstrated that reduced glutenin SH groups can be oxidized by dehydroascorbic acid through the mediation of PDI. Taken together, these data prove that a tight relationship exists between the balance of redox pairs of ascorbate and glutathione and the SS/SH redox status of storage proteins. A pool of reduced glutathione and ascorbic acid in caryopsis is maintained by glutathione reductase and dehydroascorbate reductase; the level of activity of these enzymes remains high prior to grain desiccation, and after desiccation, the level decreases.[54] Reinbold et al.[56] showed that in sulfur-deficient wheat, the concentrations of total glutathione and cysteine (Cys) in flour were proportional to the amount of sulfur supplied during growth. The low concentrations of GSH and Cys in flour from sulfur-deficient wheat had a similar effect on the technological properties as the altered composition of gluten proteins.[56] Thus, a low M_r redox agent essentially affected the process of protein polymerization during the formation of the protein matrix in maturing grain.

Reducing Systems of Thioredoxin (Trx) and Glutaredoxin (Grx). A key role in the maintenance of intracellular SS/SH redox balance belongs to the proteins of the superfamily of thiol oxidoreductases, thioredoxins, and their functional analogues, glutaredoxins, which catalyze the reaction of dithiol−disulfide exchange or the removal of glutathione from the mixed disulfide protein−GSH.[57,58]

In the context of this review Trx *h* is of interest as a central regulatory protein in seeds, reducing SS bonds of diverse seed proteins, including storage proteins, enzymes, and enzyme inhibitors. Kobrehel et al.[59] have shown that gliadin and glutenin can be the target proteins for the reducing system NADPH/Trx-reductase/Trx *h*, which functions in the starchy endosperm as a signal in germination to enhance metabolic processes such as the mobilization of storage proteins. Ren et al.[60] have demonstrated that down-regulation in wheat of Trx *h*9 leads to a reduction in the incidence of preharvest sprouting. Trx *h* is localized in the cytosol[61] and can potentially regulate the polymerization of storage proteins in the later stages of caryopsis development. Increased transcripts of three Trx *h* isoforms were observed at the beginning of seed desiccation in developing caryopses of wheat.[62] However, according to Serrato et al.,[63] the level of NADPH-dependent Trx-reductase, which is required for the regeneration of Trx, is low in ripening wheat caryopsis, and this low level apparently restricts the role of Trx *h* in the formation of gluten protein networks. Moreover, according to the available data, Trx *h* specifically reduces intramolecular SS bonds of wheat storage proteins but is not efficient in the reduction of intermolecular SS bonds,[64] which is why the activity of Trx *h* in ripening caryopsis does not seem to produce a significant effect on storage protein polymerization. In the in vitro tests, the addition of components of the Trx reducing system (Trx *h*, NADPH, and Trx reductase) into flour resulted in dough strengthening, enhancement of bread volume, and improvement of its structure.[65] The impact of the Trx reducing system on dough strengthening is explained by the breaking of intramolecular SS bonds that leads to the recombination of storage proteins in the gluten protein matrix and to the formation of new intermolecular SS bonds. The newly formed structure fosters dough strengthening because the newly formed intermolecular SS bonds are not affected by Trx *h*.[65] In the reduction of intramolecular gliadin SS bonds, Trx *h* decreased its immunoreactivity by >50% and did not significantly affect dough rheological properties.[66,67] Immunoreactivity in the gliadin fraction also decreased in wheat lines with an increased expression of Trx *h*.[68]

The reducing system of glutaredoxin includes NADPH-dependent glutathione reductase, GSH, and Grx.[57,58] For GRXs there are two main catalytic mechanisms: a dithiol reaction that leads to the reduction of protein disulfide bonds and a monothiol reaction involving the reduction of protein−GSH mixed disulfides (deglutathionylation). Grxs in most organisms can be classified by their active site sequence motif into either the CPYC (Cys-Pro-Tyr-Cys motif) or the CGFS (Cys-Gly-Phe-Ser motif) class. CPYC GRXs can catalyze both reactions, but CGFS GRXs are limited to only monothiol mechanisms. Thus, GRXs participate in regulation of protein−protein or protein−GSH interactions. Grxs of wheat and plant Grxs as a whole remain an understudied protein class. Analysis of Grx expression in wheat caryopsis based on expressed sequence tags (ESTs) showed that 67% of the 152 Grx ESTs identified in the caryopsis have the CPYC motif in the active site and 33% of Grx have the CGFS motif.[69] Therefore, we assumed that Grxs may be involved in the regulation of SS/SH redox balance and the polymerization of the prolamins.

We managed to extract and purify a GSH-dependent protein, thiol:protein-disulfide oxidoreductase (TPDO, EC 1.8.4.2), from wheat grain;[70] this protein catalyzed the cleavage of disulfide bonds in acetic-soluble gluten proteins (Figure 3) and reduced their capacity to aggregate.[71] The enzyme was

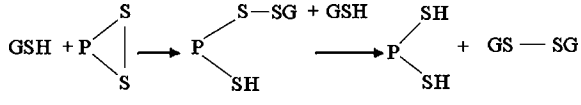

Figure 3. TPDO catalyzes the break of the protein disulfide bonds using GSH. P, protein; GSH, reduced glutathione.

classified as a Grx-like protein because the molecular masses of the native TPDO protein (approximately 167 kDa) and its two subunits (77 and 73 kDa) significantly exceeded the molecular mass of typical glutaredoxins (12−14 kDa)[70] and typical wheat PDI (60 kDa).[33] The maximum level of TPDO activity occurred between the third and fifth weeks after anthesis (WAA), in a period of maximum synthesis of storage proteins in wheat kernels. Some wheat varieties maintained high-activity TPDO until the stage of full ripeness.[71] The study revealed a negative correlation between TPDO activity in the phase of milky ripeness of grain (3 and 4 WAA) and dough stiffness ($r = -0.84$, $P < 0.05$). The addition of exogenous TPDO to flour significantly increased dough extensibility (from 17 to 49% for cultivars with different gluten qualities), which implies the ability of the enzyme to disrupt SS bonds in high molecular weight gluten polymers.[71] Mapping of the activity level of endogenous TPDO was performed using an International Triticeae Mapping Initiative mapping population and revealed chromosomal loci (QTLs) associated with TPDO activity and dough physical properties, such as flour strength, elasticity, and water-absorbing capacity.[72] The optimum temperature for TPDO activity was established at 36 °C.[70] Gluten weakening was observed in wheat growing at high temperatures (37/28 °C, day/night)[73,74] and might be connected to the activity of enzymatic systems that are similar to those of TPDO.

Thiol Oxidase and Lipoxygenase (LOX) of Wheat Caryopsis. Thiol-oxygen oxidoreductases (thiol oxidase, EC 1.8.3.2) oxidize SH groups of thiol compounds and proteins via the direct transfer of electrons from thiol to molecular oxygen according to the scheme

$$2GSH + O_2 \rightarrow GSSG + H_2O_2$$

H_2O_2, formed during these reactions, might also oxidize SH groups of cysteine.[75] Trufanov et al.[76] examined the endogenous thiol oxidase activity in the ripening grain of soft wheat species grown in different soil−climatic conditions for three years; the authors showed that this activity was positively correlated with the flour strength, dough stability, and valorimetric assessment (a generalizing farinograph parameter, determined as the size of the farinogram area calculated using a special device, a valorimeter) and was negatively correlated with the dough dilution. The combined use of fungal thiol oxidase and low ascorbic acid levels could improve both fresh and frozen dough baking.[77]

SH groups of storage proteins might be oxidized by hydroperoxides of fatty acids that form because of the activity of lipoxygenase (linoleate:oxygen reductase, EC 1.13.11.12, LOX), which is present in significant amounts in wheat seeds[78] and is expressed in the developing grain[79] (Figure 4). We showed that the activity of endogenous LOX in wheat caryopsis increased the water-absorbing capacity of flour and decreased dough extensibility. Flour strength, tenacity, and dough mixing properties were positively connected among each other. LOX impact on these parameters (positive or negative) depended on the level of enzyme activity. At a low level of LOX activity, its correlations with these parameters were positive, whereas they were negative at high activity level.[80]

The improving effect of lipoxygenase on gluten and dough is apparently pronounced because the enzyme blocks the excess of rheologically active thiol groups in storage proteins, which contributes to gluten strengthening. Nevertheless, high-quality gluten requires an optimal range of LOX activity, and high activity might cause the excessive formation of disulfide bonds,

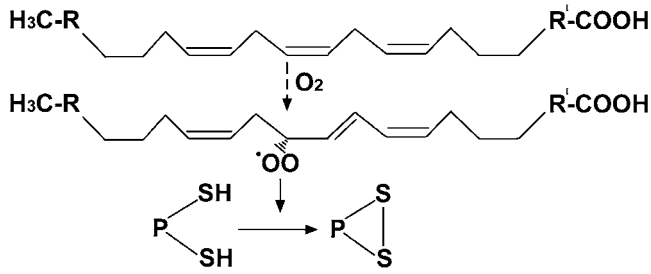

Figure 4. Lipoxygenase catalyzes the incorporation of molecular oxygen in the unsaturated fatty acid to form fatty acid hydroperoxide. Hydoperoxide radical oxidizes SH groups in protein, which results in disulfide bond formation.

a reduction in dough extensibility, and the excessive strengthening of gluten and dough.[80] Moreover, the impact of different lipoxygenases on grain technological parameters is connected possibly with its participation in the regulation of lipid−protein interactions at the interface between the starch granule surface and the protein matrix, which is related to kernel hardness.

PUROINDOLINES AND LIPID−PROTEIN INTERACTIONS AT THE INTERFACE BETWEEN STARCH AND THE PROTEIN MATRIX

The texture of ripe endosperm is one of the most important parameters of wheat grain quality and is determined by the adherence of the protein matrix to starch granules. The differences in grain hardness is present as early as 5 days after flowering[81] and was affected by the environment.[2]

The lipoprotein family of puroindolines is considered a molecular genetic marker of grain softness. They are basic and cysteine-rich proteins with a molecular mass of approximately 13 kDa and contain five disulfide bonds.[16,81,82] There are many data suggesting that functioning of puroindolines on the surface of starch could be lipid mediated.[81,83,84] It was shown that puroindolines decreased lipid breakdown during wheat seed development[85] and increased polar lipid content at seed maturity.[86] Puroindolines interact with lipids of starch granule membranes via a unique amphiphilic tryptophan-rich domain.[87,88] Puroindolines are surface-active proteins that adsorb spontaneously at air−water interfaces[89] and form a very stable foam.[83]

Puroindolines are composed by two isoforms, puroindoline-a (PINA) and puroindoline-b (PINB), with about 60% nucleotide similarity and distinction in the tryptophan-rich domain. In PINA the lipid binding site consists of five tryptophan residues and three basic residues (Trp-Arg-Trp-Trp-Lys-Trp-Trp-Lys), whereas in PINB there are three tryptophan residues (Trp-Pro-Thr-Trp-Trp-Lys).[90] Both isoenzymes have shown structural and functional properties similar to those of wheat thionins, lipid transfer proteins (LTP), and α-amilyse inhibitors. Puroindolines expressed specifically in developing seeds of *T. aestivum* L., but not in *Triticum durum*. Puroindoline homologues are also found in barley, oat, and rye but not in maize, rice, and sorghum.[16,83,91]

Studies of allelic variation of puroindolines have shown that the expression of genes encoding the wild type forms of PINA and PINB results in soft grain texture, whereas hardness is associated with two types of mutations. These are either "null" mutations, which result in the silencing of the genes encoding

PINA and/or PINB, or mutations affecting the amino acid sequence of the PINB protein.[16,81,92]

The lipid-binding capacity of puroindolines was found to have a positive impact on the bread-baking process[93] and plant resistance to pathogens.[94,95] Puroindolines positively affect dough tenacity and the texture of baked products and negatively affect dough extensibility. Unique surface-active properties of puroindoline proteins help stabilize the liquid lamella, which separates the gas cells in wheat dough and thus improves the volume and structure of bread.[88,96,97]

Earlier it was demonstrated that flour functionality is related to the interaction between gluten proteins (gliadin and glutenin) and the starch surface.[98] During gluten formation the majority of flour polar lipids located on the surface of starch granules become incorporated into the gluten phase of dough and are likely to be associated with gluten proteins through both hydrophobic interactions and hydrogen bonds.[99] The puroindoline proteins also are removed from the starch surface. Sroan and MacRitchie demonstrated that endogenous polar lipids (phospho- and galactolipids) and proteins act synergistically to stabilize the gas cells during the process of breadmaking. By contrast, wheat nonpolar lipids appear to destabilize gas cells and decrease bread volume.[97]

Marion et al. noted that absorption of puroindolines can affect the activity of lipases and lipoxygenases that work at these interfaces.[83] Descriptions of the effects of LOX on a dough rheology and foaming were the same as for the puroindoline proteins,[78,80] as well as their antimicrobial and antifungal properties.[100]

We believe that the functioning in the protein−starch interface of polar lipids, lipid-transfer proteins, and lipid-degrading enzymes can be connected to their common physiological function, which is probably related to the plant defense against pathogens. This results in the formation of a specific structure of the endosperm, which affects the rheological parameters of dough and loaf volume. These substances are likely to act synergistically to improve the baking quality. As for LOX, in our opinion its ability to bind polyunsaturated fatty acids (PUFA), either free or in monoglycerides, can lead to a decline in the nonpolar lipid level, thereby reducing the deleterious effects of nonpolar lipids. Moreover, the enzyme can also oxidize the PUFA in phospho- and galactolipids transported by puroindolines and initiate further metabolism of these lipids.

Unfortunately, the joint action of lipids, puroindolines, and LOX on the surface of starch has not yet been proven, but some of the available genomic data confirm our assumption. It is well-known that the principal locus controlling grain hardness is the *Ha* locus at the end of the short arm of the 5D chromosome. The structural genes encoding PINA and PINB have been mapped in the classical *Ha* locus, which also includes the *Gsp-1* gene encoding the protein called grain softness protein. It is also well-known that one of the genes regulating the level of free polar lipids (*Fpl-2*) is tightly linked with the *Ha* locus.[16,101,102] Recently we have found that the QTL associated with lipoxygenase activity in the growing grain is located in the same region of the 5D chromosome (unpublished data). Lee et al. mapped the gene of thaumatin (*Tha3*) that is known to be the product of the LOX metabolic pathway, also located at the distal end of the 5DS chromosome.[103]

Now it is considered that grain hardness is associated mainly with allelic variation of puroindolines in the *Ha* locus. However, it is possible that other genes carried on the short arm of chromosome 5D in hexaploid wheat are also important in regulating grain hardness. Therefore, further study of corresponding genes is very significant for the usage, in breeding programs, of improvement of breadmaking quality.

In conclusion, strong variability in the baking quality of wheat, grown in different environmental conditions, is most likely due to the different conditions of polymerization and folding of storage proteins, as well as differences in the protein−starch−lipid interactions. Many labile enzyme systems influence the formation of the protein polymer network and regulate the interactions between the molecular components of the caryopsis and dough (during baking); therefore, it is difficult to identify the critical factor responsible for the stability of the baking quality of the genotype in the changing environment. It seems that genomic approaches can be useful for a better understanding of the relationship between the stability of the baking quality and the molecular components of the grain. This would include the study of colocalization in the chromosomes of wheat QTL/genes associated with baking quality parameters and non-prolamin proteins.

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Funding

This work was supported by the State Project of the Siberian Institute of Plant Physiology and Biochemistry SB RAS no. 6.8.3.1

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

DAA, days after anthesis; ER, endoplasmic reticulum; EST, expressed sequence tag; Grx, glutaredoxin; LOX, lipoxygenase; QTL, quantitative trait locus; PDI, protein disulfide isomerase; PPI, peptidyl-prolyl-*cis*−*trans*-isomerase; PUFA, polyunsaturated fatty acids; TPDO, thiol:protein-disulfide oxidoreductase; Trx, thioredoxin; WAA, weeks after anthesis

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