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Abstract

The effect of puroindolines (PINs) on structural characteristics of wheat proteins was investigated in *Triticum turgidum* ssp. *durum* (cv. Svevo) and *Triticum aestivum* (cv. Alpowa) and in their respective derivatives in which PIN genes were expressed (Soft Svevo) or the distal end of the short arm of chromosome 5D was deleted and PINs were not expressed (Hard Alpowa). The presence of PINs decreased the amount of cold-SDS extractable proteins and the accessibility of protein thiols to specific reagents, but resulted in facilitated solvation of gluten proteins, as detected by tryptophan fluorescence measurements carried out on minimally mixed flour/water mixtures. We propose that PINs and gluten proteins are interacting in the grain or flour prior to mixing. Hydrophobic interactions between PINs and some of the gluten proteins modify the pattern of interactions among gluten proteins, thus providing an additional mechanistic rationale for the effects of PINs on kernel hardness.

Keywords: kernel texture, puroindoline proteins, gluten aggregation, protein thiols

Chemical compounds

Sodium dodecyl sulfate (PubChem CID: 3423265); Dithiothreitol (PubChem CID: 446094); Tris (PubChem CID: 6503); Bromophenol Blue (PubChem CID: 8272); 5,5'- dithiobis-2-nitrobenzoic acid (PubChem CID: 6254); Coomassie blue R-250 (PubChem CID: 23693030); Trifluoroacetic acid (PubChem CID: 6422); Acetonitrile (PubChem CID: 6342); 2-mercaptoethanol (PubChem CID: 1567)

43 **Abbreviations**

44 DTT, Dithiothreitol; HMW, high molecular weight; LMW, low molecular weight; PINs,

45 Purindolines; SDS, Sodium Dodecyl Sulfate; SKCS**,** Single-Kernel Characterization System

46

1. Introduction

Puroindolines (PINs) are wheat endosperm proteins that are present in nearly all taxa of the *Triticeae* and *Aveneae* tribes (Jolly, Rahman, Kortt & Higgings, 1993; Gautier, Cosson, Guirao, Alary & Joudrier, 2000). In spite of their low levels (0.1% in soft wheat (Dubreil et al., 1998)), PINs have been identified as determinants of wheat kernel texture (hardness) (Jolly et al., 1993; Morris, 2002; Bhave & Morris, 2008), i.e., of the force needed to crush the kernel. Kernel texture and protein content affect end-use characteristics.

PINs expression is controlled by two genes (*Pina-D1a* and *Pinb-D1a*) located on the distal end of the short arm of chromosome 5D (5DS), and encoding for Puroindoline A (PINA) and Puroindoline B (PINB), respectively. Expression of the two genes results in soft kernel texture, whereas the presence of only one functional gene or of mutations in either genes results in hard kernel texture. Durum wheat - a tetraploid with no D chromosome - has no PIN genes, and has higher kernel hardness than common wheat (Giroux & Morris 1998).

The effects of PINs expression or deletion on milling and rheological properties of soft-textured durum and hard-textured common wheat have also been investigated (Quayson, Atwell, Morris & Marti, 2016a; Murray, Kiszonas, Wilson & Morris, 2016). Presence of PINs delayed gluten protein aggregation, decreased dough stability and improved dough resistance, but had no effect on dough extensibility (Quayson et al., 2016a). The production of soft-textured durum could help increase its use both in traditional durum foods and unconventional ones, such as leavened products (Morris et al., 2015). Soft-textured durum is reported to have milling properties intermediate between soft wheat and hard wheat (Murray et al., 2016), resulting in decreased energy requirement for milling compared to durum wheat (Morris et al., 2015). The same study reported the successful use of soft-textured durum in the production of spaghetti and

bread that were of the same or better quality than the reference products. PINs also have shown to be relevant to gas cell stabilization and foam stability in baked products (Dubreil, Compoint & Marion, 1997).

Finnie, Jeannotte, Morris and Faubion (2010a) reported that wheat endosperm hardness involves a four-way interaction between the starch granule surface, storage proteins, PINs, and polar lipids. PINs are thought to bind to hydrophobic surfaces in the grain (either the starch surface and/or the polar lipids) (Wall et al., 2010; Greenwell & Schofield, 1986) through a Trp-rich domain (Fiez, Wanjugi, Melnyk, Altosaar, Martin & Giroux, 2009; Alfredo, Palombo, Panozzo & Bhave, 2014). Alfredo et al. (2014) also suggested the formation of PIN homo- or hetero-dimers/oligomers via ionic, polar, and/or hydrophobic interactions between residues on the exposed loops and helix surfaces of PINs.

During mixing, PINs supposedly detach from the starch granule surface and become incorporated in dough (Finnie, Jeannotte, Morris, Giroux & Faubion, 2010b) because - under mixing conditions - lipids and PINs may have higher affinity for gluten than for the starch granule surface (Finnie et al., 2010b). However, the type and manner of the association of PINs with gluten protein is unknown, and no information is available on whether this association may occur prior to mixing.

To gather information on the type of possible interactions between PINs and gluten proteins in flour, this study aims at investigating the effect of PINs on aggregation of gluten proteins, on protein solvation, and on the exposure of reporter amino acid sidechains in gluten proteins. Among the sidechains most relevant from a practical standpoint are those of hydrophobic residues that re-organize in different fashion during mixing of dough from hard and soft wheat (Jazaeri, Bock, Bagagli, Iametti, Bonomi & Seetharaman, 2015). The fluorescence of

93 tryptophan sidechains has been indicated as an useful "reporter" of the structural status also of
94 gluten proteins (Bonomi, Mora, Pagani & Iametti, 2004; Bonomi et al., 2012; Bonomi, Iametti,
95 Mamone & Ferranti, 2013).

96 Cysteine residues also are of paramount relevance in formation and stabilization of the
97 gluten network through disulfide exchange processes. Accessibility of cysteine thiols in the
98 presence/absence of protein unfolding agents has been proposed as an index of network
99 compactness in various cereal-based products (Bonomi et al., 2012, 2013; Iametti, Marengo,
100 Miriani, Pagani, Marti & Bonomi, 2013). By using conditions capable of dissociating weak
101 hydrophobic interactions in the presence/absence of a concomitant mechanical treatment, some
102 of us have attempted to unravel the network of covalent and non-covalent interprotein bonds -
103 and the kinetics of their formation - in wheat-based products at various stages of processing
104 (Jazaeri et al., 2015; Quayson et al., 2016a, 2016b).

105 The study presented here relies on the availability of lines of *Triticum turgidum* ssp.
106 *turgidum* ssp. *durum* (cv. Svevo) and *T. aestivum* (cv. Alpowa), and of their derivatives in which
107 PIN genes were expressed (Soft Svevo) or deleted (Hard Alpowa). The use of these simplified
108 models and of the molecular approaches outlined above should contribute to improve current
109 understanding of the role of PINs in determining the gluten structural characteristics in wheat
110 flour, paving the way for further detailed studies on the molecular determinants of reported
111 effects of PINs' presence.
112
113 **2. Materials and Methods**
114
115 2.1 Wheat Samples

Wheat cultivars (cvs) Alpowa (soft wheat, *T. aestivum* L.), hard kernel Alpowa (Hard Alpowa), durum wheat (*T. turgidum* L., ssp. *durum*) cv Svevo, and soft kernel durum wheat (Soft Svevo) were used in the study. Hard Alpowa (proteins: 14.8±0.1 g/100g d.b.; SKCS: 98) is a back-cross of seven (BC_7) near-isogenic lines derived from soft wheat Alpowa lines (protein: 12.3±0.2 g/100g d.b.: SKCS: 16) that lacks the distal portion of the short arm of chromosome 5D (Morris & King, 2008). Soft Svevo (protein: 14.8±0.2 g/100g d.b., SKCS: 17) was developed by back-crossing durum wheat cv. Svevo (protein: 15.9±0.2 g/100g d.b., SKCS: 73) and a homologous translocation line involving Langdon durum and the soft wheat cultivar Chinese Spring (Morris, Simeone, King & Lafiandra, 2011). Alpowa and Hard Alpowa were grown in St. Paul (MN, USA) in 2014. Svevo and Soft Svevo were grown in Pullman (WA, USA) in 2013. Wheat grains were conditioned (14.5 g/100 g moisture for Alpowa and Soft Svevo; 15.5 g/100g for Hard Alpowa; 16.5 g/100 g moisture for Svevo), prior to milling with a Quadrumat Junior (C.W. Brabender Inc., South Hackensack, NJ, USA) flour mill. After milling, the refined flour from each sample was collected and used for analysis.

2.2 Protein Aggregation

Protein aggregation in flours was investigated by a limited cold-solubilization approach, using low concentrations of sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) to break down hydrophobic interactions and disulfide bonds, respectively, as outlined by Quayson, Marti, Bonomi, Atwell and Seetharaman (2016b). Proteins were extracted in 0.05 mol/l sodium phosphate buffer, pH 7.0, containing 0.1 mol/l NaCl and 1% SDS (w/v) in the presence or in the absence of 10 mmol/l DTT as indicated. A 1 ml volume of the buffer was added to appropriate amounts of flour (≈ 1 mg protein, as estimated from the nitrogen content) and the suspension was

placed on a shaker for 60 min at 25˚C. After centrifugation at 3,000 × *g* for 30 min, the amount of protein in the supernatant was determined using the RC-DC (Reducing Agent and Detergent Compatible) Protein Assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard.

2.3 SDS-PAGE

SDS-PAGE was carried out as reported by Bonomi et al. (2012) with minor modifications. For assessing the overall protein profile, individual flour samples (15 mg) were suspended in a mixture of 0.2 ml of buffer (50 mmol/l sodium phosphate, 50 mmol/l NaCl, 1% SDS, pH 7.0) and 0.2 ml of SDS-PAGE reducing/denaturing buffer (0.125 mol/l Tris-HCl, pH 6.8, 50% (w/v) glycerol, 1.7% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.01% (w/v) Bromophenol Blue). The resulting suspension was heated at 100°C for 20 min, and clarified by centrifugation for 10 min at 3000 × *g* at room temperature. For characterization of the cold-SDS extracted proteins, soluble extracts containing approximately 1 mg protein (assessed colorimetrically as detailed in subsection 2.2) were diluted (1/1 v/v) with SDS-PAGE denaturing buffer, and the mixture was heated at 100˚C for 10 min. SDS-PAGE was carried out at 40 mA on a Mini-PROTEAN precast gel (10% porosity) in a Mini-PROTEAN apparatus (Bio-Rad, Richmond, VA, USA), loading about 2 microgram proteins per lane. Gels were stained with Coomassie Blue R-250. Puroindoline-enriched fractions were obtained from individual flours essentially by following the Triton® X-114 solubilization procedure outlined by Day, Bhandari, Greenwell, Leonard & Schofield (2006), and were analyzed by SDS-PAGE as reported above for cold-SDS extracts.

2.4 Readily Accessible and SDS-Accessible Thiols

Readily accessible thiols were determined by suspending 100 mg of flour in 5 ml 0.05 mol/l sodium phosphate buffer, pH 7.2, containing 0.1 mol/l NaCl and 0.5 mmol/l 5,5'- dithiobis-2-nitrobenzoic acid (DTNB). When assessing SDS-accessible thiols, the above mixture also contained 1% SDS (Iametti, Bonomi, Pagani, Zardi, Cecchini & D'Egidio, 2006). Suspensions were placed on a shaker at 25°C for one hour, and then clarified by centrifugation at 10,000 × *g* for 5 min. The supernatant was filtered through a 10 μm pore filter (Fisher Scientific, Pittsburg VA, USA) and read at 412 nm (S8000; Biochrom, MA, USA) against a DTNB blank.

2.5 Protein Solvation Studies

Solid state tryptophan fluorescence in hydrated flour was measured at room temperature using a front-face cell holder in a Perkin Elmer LS 55 Fluorescence Spectrometer (Perkin Elmer, Llantrisant, UK). Solvation studies were performed by adding water to individual flour samples (2.5 g each) to reach a final water content covering the 20-50% range in appropriate increments. Samples were mixed in a beaker with a glass rod for 3 min as reported by Bonomi et al. (2004). About 0.2 g of the resulting mixture were placed behind the quartz window of the measuring cell, that was closed to spread the sample all across the measurement window. Tryptophan fluorescence was monitored by taking emission fluorescence spectra from 350 to 450 nm with excitation at 280 nm and emission and excitation slits set at 2 nm.

2.6 Protein Molecular Weight Distribution

The molecular weight distribution of proteins in cold-SDS extracts from flour prepared in the absence of disulfide reducing agents was determined by Size Exclusion High Performance Liquid Chromatography (SE-HPLC), using a Prominence Shimadzu High-Performance Liquid

Chromatograph (C196-E061N), with UV/VIS Diode Array Detector (Shimadzu, Columbia, Maryland, US). Proteins were extracted from flour at room temperature by using 2% SDS in 0.05 mol/l sodium phosphate buffer, pH 6.8 essentially as indicated by Jazaeri et al. (2015). Flour suspensions were shaken for one hour at 25°C and centrifuged for 30 minutes at 3,000 × g at room temperature. The supernatant was filtered through a 0.2 µm Phenomenex cellulose membrane filter (St. Louis, MO, USA). An aliquot (60 µl) of the filtered extract was loaded on a Phenomenex Yarra 3µm SEC 3000 HPLC column run at 30°C with 0.05% trifluoroacetic acid in acetonitrile-water (1:1 v/v) at a flow rate of 1 ml/min. Elution was monitored at 214 nm.

2.7 Statistical Analysis

Protein solubility, thiol accessibility, and molecular weight distribution were analyzed in triplicate. Three spectra were collected for each sample in front-face fluorescence spectroscopy measurements. Analysis of variance (ANOVA) was performed utilizing Statgraphics XV version 15.1.02 (StatPoint Inc., Warrenton, VA, USA). Samples were used as factors. When a factor effect was found significant ($p \leq 0.05$), significant differences among the respective means were determined using Fisher's Least Significant Difference (LSD) test.

3. Results and Discussion

3.1 Protein Profiles and Protein Aggregation Behavior

The effect of puroindoline genes expression or of the deletion of the 5DS distal portion on the presence or absence of PINs was verified by analyzing the SDS-PAGE profiles of partially purified PINs from the grains used in this study. Data in the supplementary materials (Fig.S1) provide physical evidence for occurrence of the expected changes in the various grains used in

this study, namely the absence of PINs in Triton X-114® extracts from hard-kernel grains and the presence of PINs in extracts from soft-kernel grains, regardless of the species.

As shown in Fig.1, the presence/absence of PINs did not affect – within a given species – the polypeptide pattern of proteins solubilized from the various flour by media of different dissociating ability and under conditions where protein association was differently affected (*vide infra*). The SDS-PAGE profiles in Fig. 1 underscore the expected relevance of species-specific proteins. Differences in the protein profile among the two wheat species appear most relevant in the 40-50,000 Mr region. In particular, a band at Mr ~42,000 was evident in Alpowa and absent in Svevo, whereas a band at Mr ~48,000 was present in Svevo and absent in Alpowa, independently of the presence/absence of PINs. These differences in gluten protein profiles may account for the contrasting results from previous studies on the relation between kernel texture and SDS-protein solubility in various grain accessions (Bushuk, Hay, Larsen, Sara, Simmons & Sutton, 1997; Hayta & Schofield, 2004; Kuktaite, Larsson & Johansson, 2004; Jazaeri et al., 2015).

Cold-SDS protein extractability data from the various flour samples in the presence or absence of DTT as a disulfide breaking agent are shown in Fig. 2. To the best of our knowledge, this is the first time that these approaches have been used to investigate protein aggregation in the same varieties in the presence or absence of PINs. PINs expression resulted in a significant ($p \leq 0.05$) decrease in cold-SDS protein solubility in flour from *T. durum* grains (from 637 in Svevo to 382 mg/g protein in Soft Svevo). In similar fashion, the 5DS distal end deletion resulted in a significant ($p \leq 0.05$) increase in cold-SDS protein solubility in flour from *T. aestivum* grains (from 422 mg/g protein in Alpowa to 688 in Hard Alpowa).

230 Adding a reducing agent (DTT) to the SDS-containing buffer used for cold-extraction of
231 proteins resulted in a significant ($p \leq 0.05$) increase in protein solubility in all samples but Hard
232 Alpowa, where the observed increase was statistically not significant. The results obtained here
233 with cold-SDS as the dissociating agent used for breaking down non-covalent hydrophobic
234 interactions among aggregated proteins confirm the major role of interprotein disulfide bonds in
235 the stabilization of insoluble protein aggregates as observed with other chaotropes (Iametti et al.,
236 2006; Iametti et al., 2013; Bonomi et al., 2013). Some further considerations may be made in the
237 case of the Alpowa/Hard Alpowa system. The presence of PINs in Alpowa results in decreased
238 protein solubility in cold SDS (as also observed when PINs are expressed in Soft Svevo), and
239 brings back the sensitivity to DTT of protein solubility. Indeed, in the case of Hard Alpowa -
240 where purindolines are not present -, non-covalent interactions represent the most relevant
241 driving force in the formation and/or stabilization of the protein network.
242 It has to be noted that the solubility results discussed above were obtained on flour
243 suspensions, that is, in the absence of the mechanical unfolding steps associated with mixing
244 flour into a dough. Thus, interactions among PINs and gluten proteins may pre-exist in the grain
245 or flour itself, or may occur during the solvation step of proteins that occurs prior to dough
246 mixing. Of course, this assumption does not rule out the possibility that these interactions may
247 occur even if PINs are adhering to other types of macrostructures and /or macromolecules in the
248 kernel, as suggested in other previous studies (Wall, Wheeler, Smith, Figeys & Altosaar, 2010;
249 Greenblatt & Schofield, 1986).
250 From our solubility results, it seems reasonable to assume that the differences in protein
251 aggregation related to the presence/absence of PINs could involve more or less specific
252 interactions between PINs and those gluten proteins where specific functions are present. It

seems reasonable to assume that kernel hardness may somehow relate to the resilience or compactness of the protein network in the grain, as dictated by species-specific genetic factors. When the nature of gluten components and their structure make hydrophobic interactions among gluten proteins more relevant than disulfide bridges to the stabilization of inter-protein interactions (as in Hard Alpowa), the expression of PINs leads to an increased compactness of the protein aggregates, that in turn leads to a decreased cold-solubility in the presence of low SDS concentrations and in increased sensitivity to DTT of protein extractability (as observed in Alpowa). The same reasoning may be applied to the results reported here from the Svevo durum wheat, although in this case the contribution of disulfide bridges to interprotein interactions remains appreciable even when PINs are present. It is also reasonable to assume that some specific proteins or protein classes within individual grain species (as made also evident by the SDS-PAGE tracings in Fig. 1) may be playing a prominent role in explaining changes related to the presence/absence of PINs. The nature of the gluten proteins relevant to the hypothetical interaction with PINS and the molecular determinants of the interaction are currently being investigated.

3.2 Accessibility of protein thiols

The accessibility of cysteine thiols in the various flour samples is shown in Fig. 3. It has to be noted that the approach used for these studies is capable of detecting accessible thiols regardless of protein solubility, and has proven useful for indicating the compactness of a protein network in a number of food systems of different complexity whenever thiol-containing proteins are present (Iametti et al., 2006; Iametti et al., 2013; Bonomi et al., 2013).

The compactness of the protein organization in Alpowa - as indicated by the low protein solubility discussed in the previous subsection - is reflected in the low accessibility of cysteine

277 thiols observed in the absence of SDS. Conversely, the absence of PINs in Hard Alpowa makes it
278 possible to access – even in the absence of SDS - the thiol groups of cysteine residues that were
279 otherwise likely buried within protein aggregates. The content in readily available protein thiols
280 in Hard Alpowa (4.17 ± 0.55 micromol thiols/g protein) was indeed twice that in Alpowa (2.12 ±
281 0.55 micromol thiols/g protein). The same considerations may apply to Svevo and Soft Svevo,
282 where the effects of PIN presence/absence are less marked (3.19 ± 0.23 *vs* 2.6 ± 0.36 micromol
283 thiols/g protein).

284 In all flours, the number of accessible thiols increased upon treatment with low SDS
285 concentrations at room temperature. However, the SDS-dependent increase in thiol accessibility
286 appears more pronounced in the presence of PINs. This behavior is particularly evident when
287 comparing Alpowa and Hard Alpowa, and confirms the relevance of hydrophobic interactions as
288 the major stabilizing element of interprotein interactions when PINs are present. Once again, it
289 has to be noted that the differences in terms of readily accessible and SDS-accessible thiols that
290 are evident in Fig. 3 may relate to the different protein profiles in the two species (see Fig.1).
291

292 3.3 Protein solvation

293 The emission maximum of tryptophan fluorescence is indicative of the polarity of the chemical
294 environment around the tryptophan side chains. The tryptophan emission maximum shifts
295 towards higher wavelengths as the polarity of the environment increases. Front-face (solid state)
296 fluorescence spectroscopy has proven useful in establishing the extent of contribution of
297 hydrophobic interactions to the gluten protein network in dough and in defining the nature and
298 extents of the structural rearrangements that accompany solvation of proteins in wheat-based
299 materials (Bonomi et al., 2004; Huschka, Bonomi, Marengo, Miriani & Seetharaman, 2012).

In this study, front-face tryptophan fluorescence was used to understand the possible role of PINs on hydrophobic interactions in minimally mixed solvated flours. As pointed out in previous studies (Bonomi et al., 2004; Jazaeri et al., 2015), formation of an extended protein network in dough required a much higher level of mechanical stress than the one used here. Thus, the observed interactions reported here may be seen as representative of those occurring in solvated flour.

Before water was added to the various flours, expression of PINs had no relevant effects on the tryptophan emission maximum in *T. durum*, as did the 5DS distal end deletion in *T. aestivum* (see supplementary figure S1). In all cases, addition of water to flours resulted in protein "swelling" and in increased tryptophan exposure to the solvent, causing a rise in fluorescence intensity and a red-shift of the fluorescence emission maximum as water content of the minimally mixed flour increased (Bonomi et al., 2004; Huschka et al., 2012). The dependence of changes in tryptophan environment on the water content was evaluated by calculating the ratio between fluorescence intensities measured at wavelength typical of the water-exposed tryptophans (380 nm) and of those located in a non-polar environment (340 nm), as reported by Bonomi et al. (2004, 2012). In this regard, the 380/340 ratio takes into account both the shift in fluorescence emission maximum and the change in fluorescence intensity.

The calculated 380/340 ratios for the various samples at increasing moisture content are shown in Fig. 4. The different sensitivity of the 380/340 to increasing water content confirms previous reports on the different solvation behavior of protein in durum and common wheat (Bonomi et al., 2004). However, the expression of PINs has a remarkable effect on the sensitivity of the structural organization of proteins to increased water availability, that could be quantitated by estimating a solvation midpoint from the curves presented in Figure 4. When PINs are

present, protein solvation occurs at sensibly lower water levels (solvation midpoints at 27.5 % water in Soft Svevo and 28.5 % in Alpowa) than in the absence of PINs (solvation midpoints at 30.0 % water in Svevo and 33.5 % in Hard Alpowa). This confirms that the presence of PINs - despite their low relative abundance - negatively affects the compactness of the protein network in grains from different species, as also indicated by the molecular indices presented and discussed in the previous subsections.

3.4 Size distribution of SDS-solubilized proteins

Data in the previous subsections indicate that the presence/absence of PINs affects the aggregation state of gluten. Therefore, we attempted to verify whether the presence/absence of PINs affected the molecular weight distribution of cold-SDS-extractable proteins obtained from flour treated at room temperature in the absence of disulfide reducing agents. All the resulting chromatograms showed three prominent peaks that were designated as high molecular weight (HMW) components, low molecular weight (LMW) components, and other proteins, in analogy to that reported by Jazaeri et al. (2015). These fractions are identified by vertical thin lines in the two panels of Fig. 5.

Expression of PINs decreased the amount of SDS-extractable HMW and LMW, as indicated by the lower overall content of cold-SDS extractable proteins in Soft Svevo than in Svevo (Fig. 5A). Conversely, deletion of 5DS distal end resulted in higher cold-SDS extractable LMW and HMW in Alpowa than in Hard Alpowa (Fig. 5B). Thus, the results in Fig. 5 suggest that presence of PINs facilitates formation of compact large molecular weight aggregates, confirming the cold-SDS solubility data in Fig. 2.

PINs also affect the aggregation of gluten proteins at mesoscopic level, as shown by the effects of PINs absence/presence on the LMW-to-HMW ratio, as calculated from integration of

347 the corresponding chromatographic peaks. The values of this ratio were: 1.92; 2.15; 1.95; and
348 2.05 for Svevo, Soft Svevo, Alpowa, and Hard Alpowa, respectively. In the case of the
349 Svevo/Soft Svevo comparison, changes in this ratio were related to a decrease in the HMW
350 fraction, that was likely preferentially converted to non-extractable units in the presence of PINs
351 (Fig 5A and Fig. 2) (Veraverbeke et al., 2000a,b; Don et al., 2006). The 5DS distal end deletion
352 in Hard Alpowa facilitates the SDS-dependent breakdown of aggregates by, and the proteins
353 solubilized from Hard Alpowa under these conditions are characterized by an increase in their
354 LMW content with respect to HMW (Fig. 5B).

355 Don, Lichtendonk, Plijter, van Vliet and Hamer (2005) had demonstrated that the amount
356 of cold-SDS extractable LMW and HMW are directly related to the LMW and HMW in the so-
357 called Glutenin Macro-Polymer (GMP). Low molecular weight glutenin subunits (LMW-GS)
358 and high molecular weight glutenin subunits (HMW-GS) of GMP have been suggested to
359 associate within or between themselves to form large non-extractable aggregates (Veraverbeke,
360 Larroque, Bekes & Delcour, 2000a, 2000b; Don, Mann, Bekes & Hamar, 2006). From a practical
361 standpoint, increased levels of cold-SDS extractable proteins have been associated with good
362 baking quality (Weegels, van de Pijpekamp, Gaveland, Hamar & Schofield, 1996), as reported
363 for Soft Svevo (Morris et al., 2015), and an increased concentration of HMW in proteins
364 unextractable in cold-SDS has been reported to have a positive effect on baking quality (Don et
365 al., 2006).

366

367 **4. Conclusions**

368 The present study highlights that PINs have an impact on gluten protein interactions in flour.
369 PINs enhanced gluten protein aggregation, resulting in decreased SDS extractability, decreased
370 thiols accessibility, and increased LMW-to-HMW ratio in cold-SDS extractable fractions. PINs

371 also affected the interaction of gluten proteins with added water, as assessed through the solvent
372 accessibility of amino acid side chains that are considered as "reporters" of protein structural
373 organization. It is worth remembering here that this type of evidence was gathered on flour
374 aqueous suspensions in the absence of significant mechanical deformation of the relevant
375 proteins, suggesting that these interactions may occur in the grain and in the flour prior to
376 mixing.

377 No association or interaction of PINs with gluten proteins in flour was suggested in
378 previous reports. We suggest here that PINs may associate in the grain also with gluten proteins,
379 promoting the formation of highly compact supra-macromolecular aggregates stabilized by local
380 and very tight hydrophobic interactions. In this frame, and in consideration of the highly
381 hydrophobic character of PINs and of their low abundance with respect to gluten proteins, it is
382 tempting to speculate that PINs may provide some sort of “hydrophobic nucleus” for the
383 formation of protein aggregates of high compactness. It seems reasonable to assume that gluten
384 proteins should represent the most relevant constituent of these aggregates, and that their own
385 polypeptide composition (and, likely, structural features) should play a significant role in
386 determining the properties of the resulting system. Of course, the association of PINs and gluten
387 proteins does not rule out a possible role of other flour components (either polysaccharides or
388 lipids (Wallet al., 2010) in the formation or stabilization of multi-component aggregates.

389 A possible view of the interactions occurring among PINs and other grain proteins in flour
390 is hypothesized in the highly simplified scheme in Fig. 6. In the presence of PINs, the
391 hydrophobic interactions involving PINs and some gluten proteins lead to a localized
392 strengthening of the protein network. Although not accounted for in the necessarily schematic

393 view presented in Figure 6, our gel-permeation data suggest a prominent involvement of HMW
394 components in these interactions.
395 When PINs are absent, the same hydrophobic regions on gluten proteins become available
396 for interactions among gluten proteins themselves, thus stiffening the protein network. In other
397 words, in a more pictorial representation, the same amount of rope (gluten proteins) may be
398 organized as a net (i.e., loose, fluffy, and easy to access, but difficult to untangle) as opposed to
399 bundles (physically stiffer than a net, but allowing easier removal of individual lengths of rope).
400 Relating these concepts to the whole issue of grain hardness is far from straightforward, given
401 the fact that these relationships reportedly involve other macromolecular components of the grain
402 (Greenblatt & Schofield, 1986; Wall et al., 2010; Fiez et al, 2009; Alfredo et al., 2014).
403 It seems reasonable that proteins involved in interacting with PINs at the "structural knots"
404 hypothesized in Fig. 6 may be species-specific or even cultivar-specific. This hypothesis will
405 have to be verified by using some of the approaches presented here in studies on other types of
406 grains, including varieties that are characterized by a different PINs content, or that are known to
407 express (either exclusively or preferentially) one specific PIN isoform. Elucidating these aspects
408 will require further investigation, also in consideration of the possibility that components or
409 structures of non-protein nature may be involved in PIN-mediated interactions, and of the
410 additional possibility that PINA or PINB can have different sets of interactors. Addressing the
411 impact of PINs expression or 5DS deletion on the expression of specific protein fractions and/or
412 on the kinetics of protein synthesis and deposition in grains represents an another – and still non-
413 explored field of investigation.
414 From a more practical standpoint, we are currently taking advantage of recent
415 methodological developments (Quayson, Marti & Seetharaman, 2014; Quayson et al., 2016a) to

416 investigate how proteins in the different flours considered in this study behave when these same
417 flours are mixed into dough. Hopefully, these studies will also provide insights on the possible
418 impact of PINs on the structural modifications accompanying formation of a gluten protein
419 network upon mixing, that is, when mechanical unfolding of proteins and redistribution of polar
420 and non-polar components occurs.
421
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Figure 1. SDS-PAGE of extractable proteins in various flour samples. 1: proteins solubilized in buffered 0.85 % SDS and 60 mM 2-mercaptoethanol upon treatment at 100°C for 10 min; 2: proteins solubilized in buffered 1% SDS upon treatment at 25°C for 60 min; 3: proteins solubilized in buffered 1% SDS and 10 mmol/l DTT upon treatment at 25°C for 60 min. Equal volumes of each extract (corresponding about 2 microgram protein, as calculated from the protein content in each flour) were loaded in each lane.

Figure2. Protein aggregation in the various flour samples. Proteins were solubilized in 1% buffered SDS upon treatment at 25°C for 60 min in the presence/absence of 10 mmol/l DTT as indicated. Error bars refer to standard deviation (n=3). Different letters above each column indicate a statistically significant difference ($p \leq 0.05$).

Figure 3. Conditional accessibility of protein thiols in the various flour samples. Flour samples were incubated for 60 min at 25°C with 0.5 mmol/l DTNB in 50 mmol/l phosphate buffer (pH 7.2, containing 0.15 mol/l NaCl) in the presence/absence of 1% SDS as indicated. Error bars refer to standard deviation (n=3). Different letters above each column indicate a statistically significant difference ($p \leq 0.05$).

Figure 4. Changes in the front-face tryptophan fluorescence intensity at 340 and 380 nm occurring upon protein solvation in various flour samples. Curves are a polynomial best fit to the actual data. Error bars refer to standard deviation (n=3).

548 **Figure 5**. Gel permeation profiles of proteins solubilized from the various flours upon incubation
549 for 60 min at 25°C in 50 mmol/l phosphate buffer, pH 6.8, containing 2% SDS in the absence of
550 DTT.
551
552 **Figure 6.** A highly simplified schematic representation of the different organization of gluten
553 proteins in the presence/absence of purindolines (red circles). The same number of two types of
554 gluten proteins (identified by green and brown colors) is present in both the upper and the lower
555 part of the scheme. In each protein, color intensity relates to the hydrophobicity of a given
556 structural region. Cysteine-rich regions in gluten proteins are in yellow, but possible disulfides
557 are not identified. Grain components other than proteins (and additional protein constituents) are
558 not shown, for the sake of clarity.
559

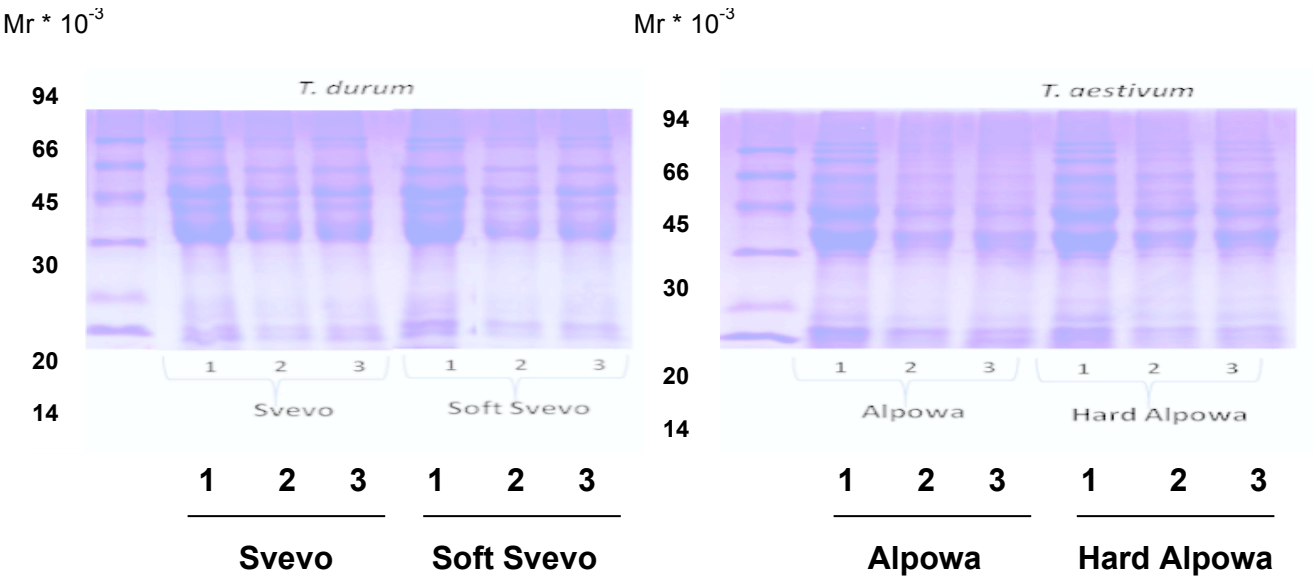
Mr * 10-3
94
66
45
30
20
14
T. durum
1 2 3 1 2 3
Svevo
Soft Svevo
Mr * 10-3
94
66
45
30
20
14
T. aestivum
1 2 3 1 2 3
Alpowa
Hard Alpowa
1 2 3 1 2 3 1 2 3 1 2 3
Svevo
Soft Svevo
Alpowa
Hard Alpowa

Figure 1.

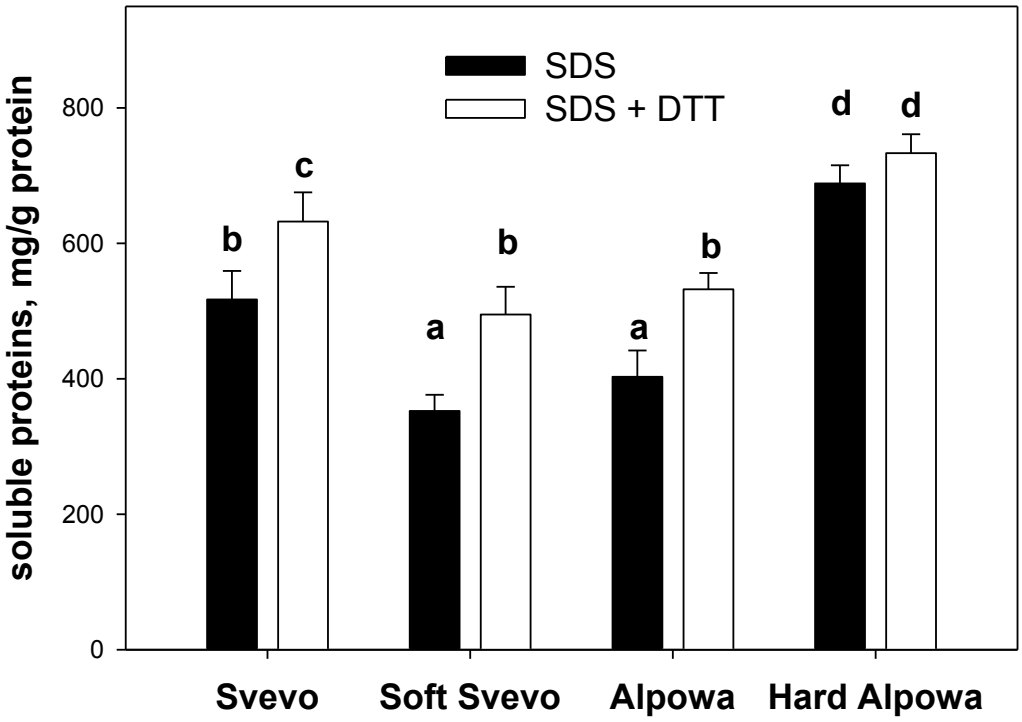
SDS
SDS + DTT
soluble proteins, mg/g protein
800
600
400
200
0
b
c
a
b
a
b
d
d
Svevo
Soft Svevo
Alpowa
Hard Alpowa

FIGURE 2

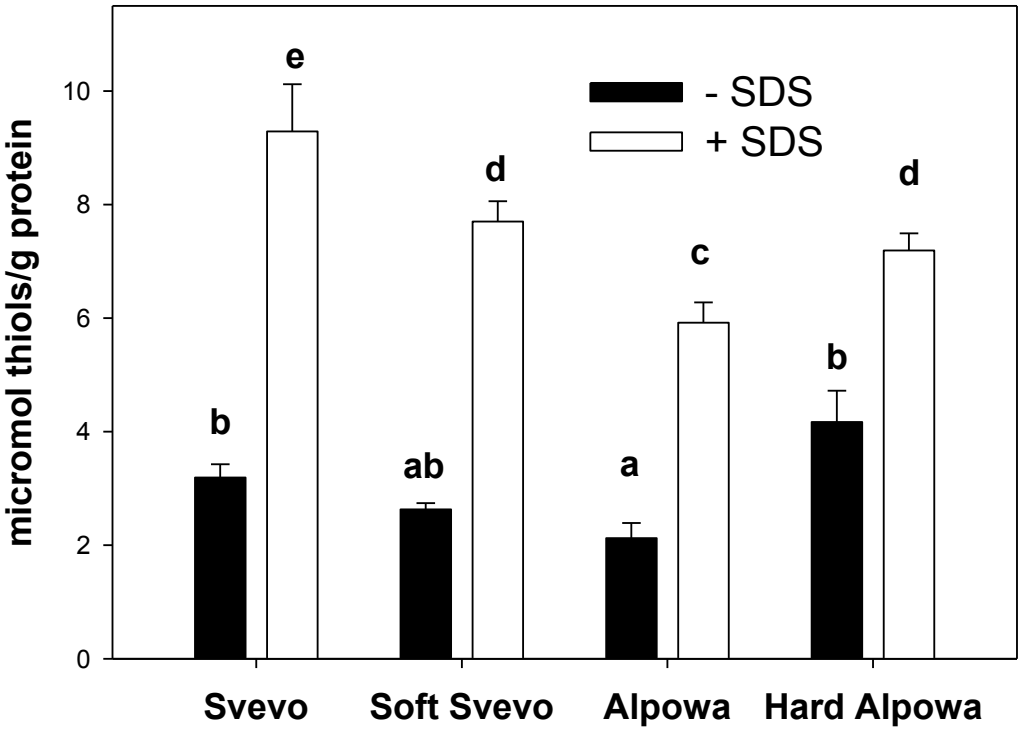
micromol thiols/g protein
0
2
4
6
8
10
- SDS
+ SDS
b
e
ab
d
a
c
b
d
Svevo
Soft Svevo
Alpowa
Hard Alpowa

Figure 3.

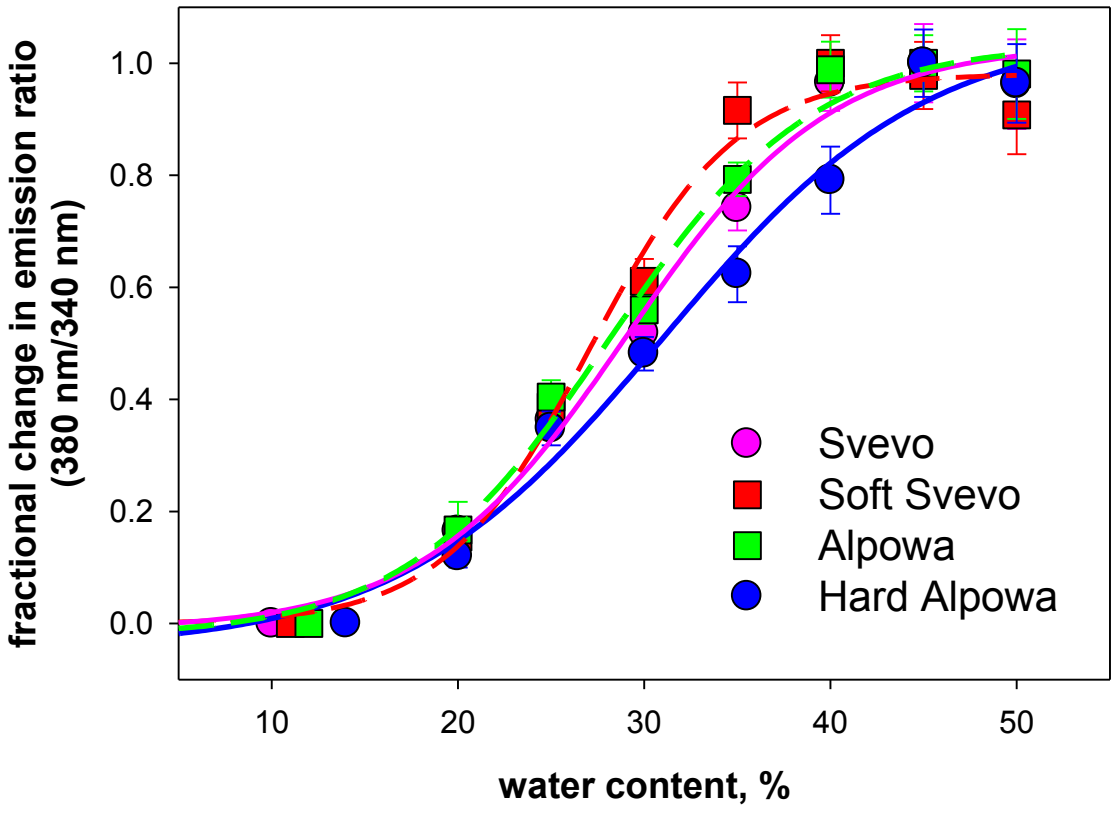
fractional change in emission ratio
(380 nm/340 nm)
1.0
0.8
0.6
0.4
0.2
0.0
10
20
30
40
50
water content, %
Svevo
Soft Svevo
Alpowa
Hard Alpowa

Figure 4.

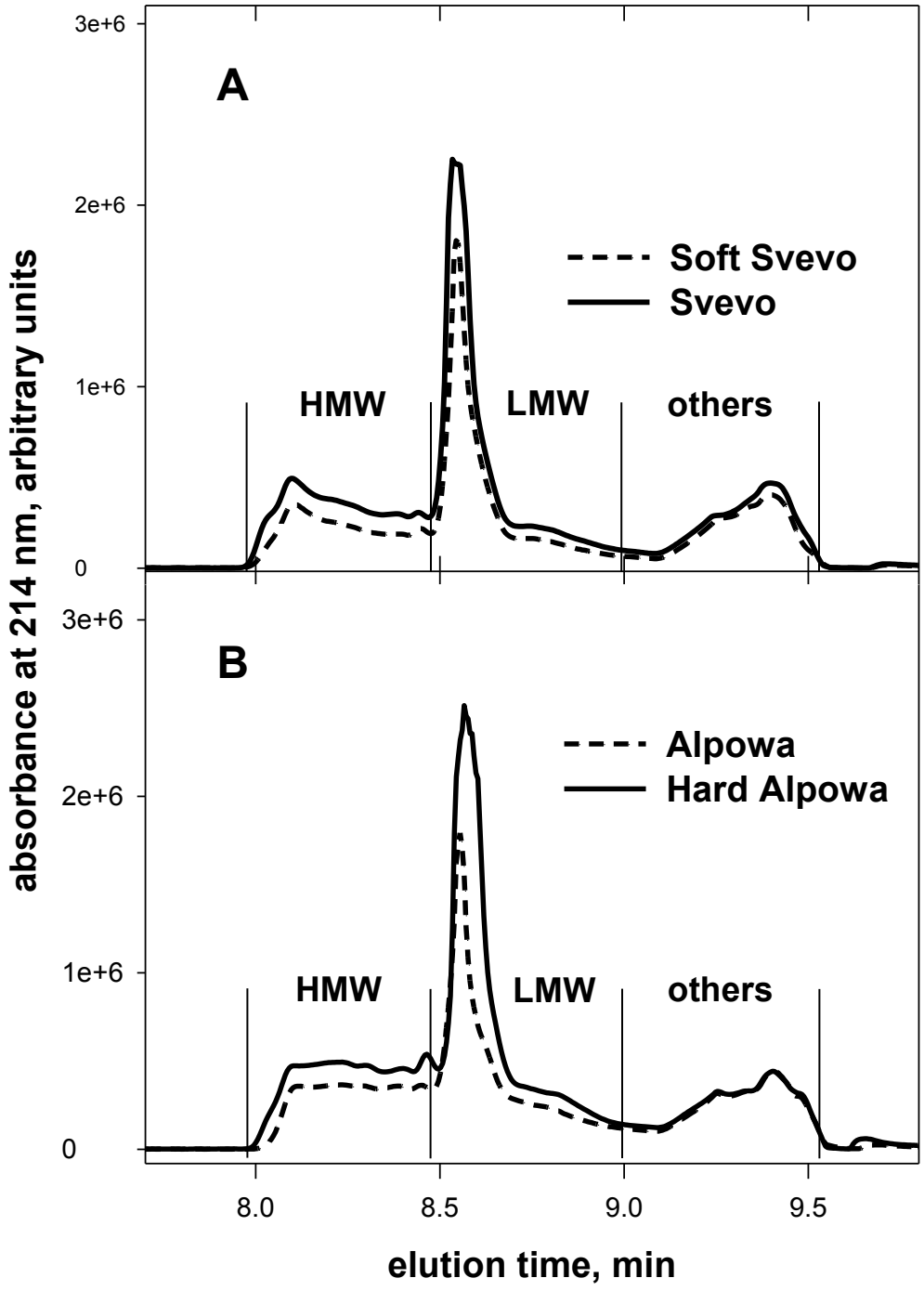
A
B
Soft Svevo
Svevo
Alpowa
Hard Alpowa
HMW
LMW
others
absorbance at 214 nm, arbitrary units
elution time, min
3e+6
2e+6
1e+6
0
8.0
8.5
9.0
9.5

Figure 5.

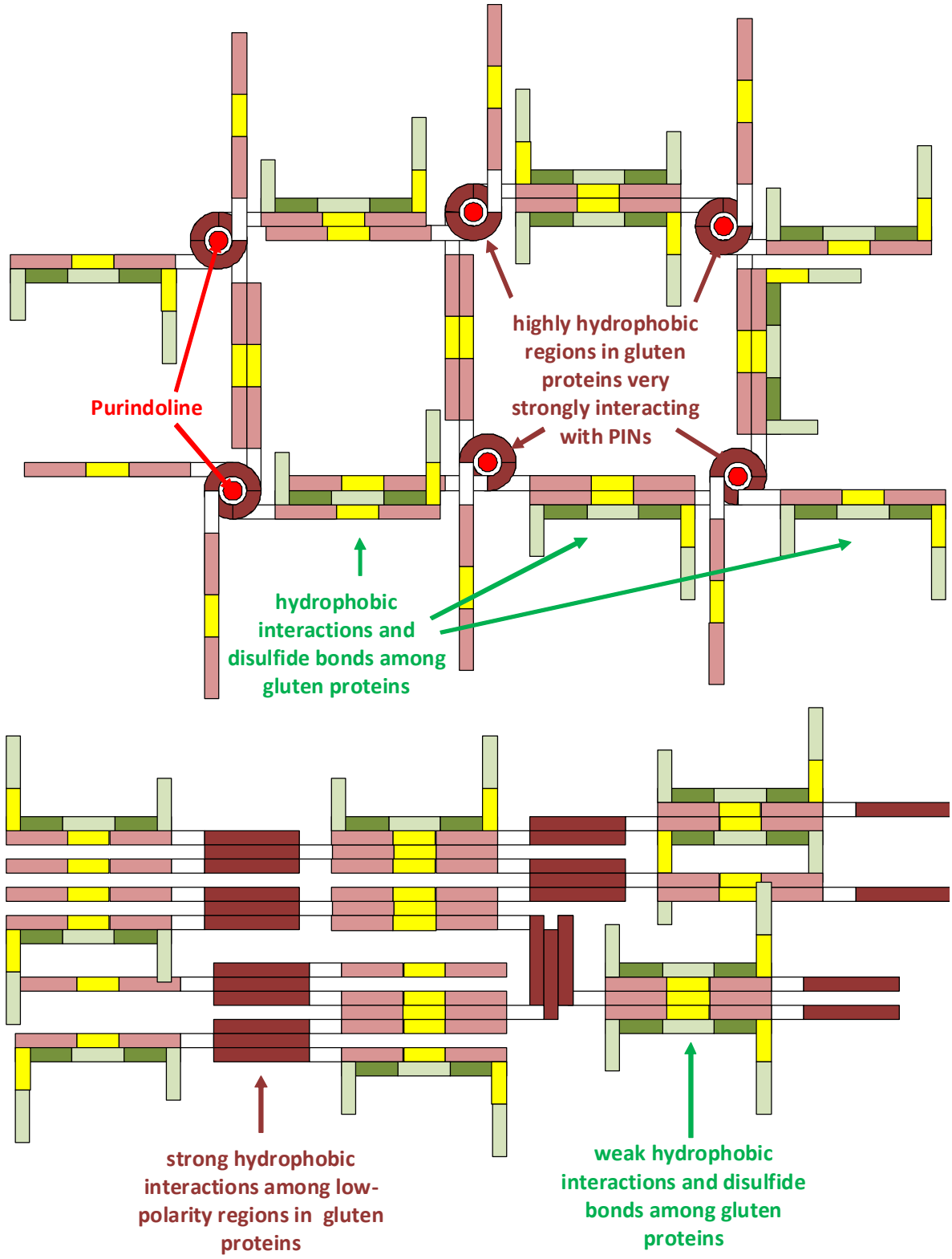
highly hydrophobic regions in gluten proteins very strongly interacting with PINs
Purindoline
hydrophobic interactions and disulfide bonds among gluten proteins
strong hydrophobic interactions among low-polarity regions in gluten proteins
weak hydrophobic interactions and disulfide bonds among gluten proteins

Figure 6