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1 **Structural consequences of the interaction of puroindolines with gluten proteins**

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3 Enoch T. Quayson^a, Alessandra Marti^{a,b,*}, Craig F. Morris^c, Mauro Marengo^b,
4 Francesco Bonomi^b, Koushik Seetharaman^d, Stefania Iametti^b

5
6 ^aDepartment of Food Science and Nutrition, University of Minnesota, 1334 Eckles Ave.,
7 St. Paul, MN, 55108, USA

8 ^bDepartment of Food, Environmental and Nutritional Sciences, Università degli Studi di
9 Milano, via G. Celoria 2, 20133, Milan, Italy

10 ^cUSDA-ARS Western Wheat Quality Laboratory, Washington State University, E-202 Food
11 Science & Human Nutrition Facility East Pullman, WA 99164, USA

12 ^dDeceased, formerly at the Department of Food Science and Nutrition, University of
13 Minnesota, 1334 Eckles Ave., St. Paul, MN, 55108, USA

14
15 *Corresponding author: Phone +3950316640. Fax +3950316672

16 E-mail alessandra.marti@unimi.it

19 **Abstract**

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

31

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

33

34 **Chemical compounds**

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

40

41

42

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

47 **1. Introduction**

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

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

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

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

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

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

87 To gather information on the type of possible interactions between PINs and gluten
88 proteins in flour, this study aims at investigating the effect of PINs on aggregation of gluten
89 proteins, on protein solvation, and on the exposure of reporter amino acid sidechains in gluten
90 proteins. Among the sidechains most relevant from a practical standpoint are those of
91 hydrophobic residues that re-organize in different fashion during mixing of dough from hard and
92 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**

116 Wheat cultivars (cvs) Alpowa (soft wheat, *T. aestivum* L.), hard kernel Alpowa (Hard Alpowa),
117 durum wheat (*T. turgidum* L., ssp. *durum*) cv Svevo, and soft kernel durum wheat (Soft Svevo)
118 were used in the study. Hard Alpowa (proteins: 14.8±0.1 g/100g d.b.; SKCS: 98) is a back-cross
119 of seven (BC₇) near-isogenic lines derived from soft wheat Alpowa lines (protein: 12.3±0.2
120 g/100g d.b.; SKCS: 16) that lacks the distal portion of the short arm of chromosome 5D (Morris
121 & King, 2008). Soft Svevo (protein: 14.8±0.2 g/100g d.b., SKCS: 17) was developed by back-
122 crossing durum wheat cv. Svevo (protein: 15.9±0.2 g/100g d.b., SKCS: 73) and a homologous
123 translocation line involving Langdon durum and the soft wheat cultivar Chinese Spring (Morris,
124 Simeone, King & Lafiandra, 2011). Alpowa and Hard Alpowa were grown in St. Paul (MN,
125 USA) in 2014. Svevo and Soft Svevo were grown in Pullman (WA, USA) in 2013. Wheat grains
126 were conditioned (14.5 g/100 g moisture for Alpowa and Soft Svevo; 15.5 g/100g for Hard
127 Alpowa; 16.5 g/100 g moisture for Svevo), prior to milling with a Quadrumat Junior (C.W.
128 Brabender Inc., South Hackensack, NJ, USA) flour mill. After milling, the refined flour from
129 each sample was collected and used for analysis.

130

131 2.2 Protein Aggregation

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

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

143

144 2.3 SDS-PAGE

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

160

161 2.4 Readily Accessible and SDS-Accessible Thiols

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

169

170 2.5 Protein Solvation Studies

171 Solid state tryptophan fluorescence in hydrated flour was measured at room temperature using a
172 front-face cell holder in a Perkin Elmer LS 55 Fluorescence Spectrometer (Perkin Elmer,
173 Llantrisant, UK). Solvation studies were performed by adding water to individual flour samples
174 (2.5 g each) to reach a final water content covering the 20-50% range in appropriate increments.
175 Samples were mixed in a beaker with a glass rod for 3 min as reported by Bonomi et al. (2004).
176 About 0.2 g of the resulting mixture were placed behind the quartz window of the measuring
177 cell, that was closed to spread the sample all across the measurement window. Tryptophan
178 fluorescence was monitored by taking emission fluorescence spectra from 350 to 450 nm with
179 excitation at 280 nm and emission and excitation slits set at 2 nm.

180

181 2.6 Protein Molecular Weight Distribution

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

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

193

194 2.7 Statistical Analysis

195 Protein solubility, thiol accessibility, and molecular weight distribution were analyzed in
196 triplicate. Three spectra were collected for each sample in front-face fluorescence spectroscopy
197 measurements. Analysis of variance (ANOVA) was performed utilizing Statgraphics XV version
198 15.1.02 (StatPoint Inc., Warrenton, VA, USA). Samples were used as factors. When a factor
199 effect was found significant ($p \leq 0.05$), significant differences among the respective means were
200 determined using Fisher's Least Significant Difference (LSD) test.

201

202 3. Results and Discussion

203 3.1 Protein Profiles and Protein Aggregation Behavior

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

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

210 As shown in Fig.1, the presence/absence of PINs did not affect – within a given species –
211 the polypeptide pattern of proteins solubilized from the various flour by media of different
212 dissociating ability and under conditions where protein association was differently affected (*vide*
213 *infra*). The SDS-PAGE profiles in Fig. 1 underscore the expected relevance of species-specific
214 proteins. Differences in the protein profile among the two wheat species appear most relevant in
215 the 40-50,000 Mr region. In particular, a band at Mr ~42,000 was evident in Alpowa and absent
216 in Svevo, whereas a band at Mr ~48,000 was present in Svevo and absent in Alpowa,
217 independently of the presence/absence of PINs. These differences in gluten protein profiles may
218 account for the contrasting results from previous studies on the relation between kernel texture
219 and SDS-protein solubility in various grain accessions (Bushuk, Hay, Larsen, Sara, Simmons &
220 Sutton, 1997; Hayta & Schofield, 2004; Kuktaite, Larsson & Johansson, 2004; Jazaeri et al.,
221 2015).

222 Cold-SDS protein extractability data from the various flour samples in the presence or
223 absence of DTT as a disulfide breaking agent are shown in Fig. 2. To the best of our knowledge,
224 this is the first time that these approaches have been used to investigate protein aggregation in
225 the same varieties in the presence or absence of PINs. PINs expression resulted in a significant
226 ($p \leq 0.05$) decrease in cold-SDS protein solubility in flour from *T. durum* grains (from 637 in
227 Svevo to 382 mg/g protein in Soft Svevo). In similar fashion, the 5DS distal end deletion resulted
228 in a significant ($p \leq 0.05$) increase in cold-SDS protein solubility in flour from *T. aestivum* grains
229 (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

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

268

269 3.2 Accessibility of protein thiols

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

275 The compactness of the protein organization in Alpowa - as indicated by the low protein
276 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 ($2.12 \pm$
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).

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

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

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

323 present, protein solvation occurs at sensibly lower water levels (solvation midpoints at 27.5 %
324 water in Soft Svevo and 28.5 % in Alpowa) than in the absence of PINs (solvation midpoints at
325 30.0 % water in Svevo and 33.5 % in Hard Alpowa). This confirms that the presence of PINs -
326 despite their low relative abundance - negatively affects the compactness of the protein network
327 in grains from different species, as also indicated by the molecular indices presented and
328 discussed in the previous subsections.

329

330 3.4 Size distribution of SDS-solubilized proteins

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

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

345 PINs also affect the aggregation of gluten proteins at mesoscopic level, as shown by the
346 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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526 **Figure 1.** SDS-PAGE of extractable proteins in various flour samples. 1: proteins solubilized in
527 buffered 0.85 % SDS and 60 mM 2-mercaptoethanol upon treatment at 100°C for 10 min; 2:
528 proteins solubilized in buffered 1% SDS upon treatment at 25°C for 60 min; 3: proteins
529 solubilized in buffered 1% SDS and 10 mmol/l DTT upon treatment at 25°C for 60 min. Equal
530 volumes of each extract (corresponding about 2 microgram protein, as calculated from the
531 protein content in each flour) were loaded in each lane.

532

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

537

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

543

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

547

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

Mr * 10⁻³

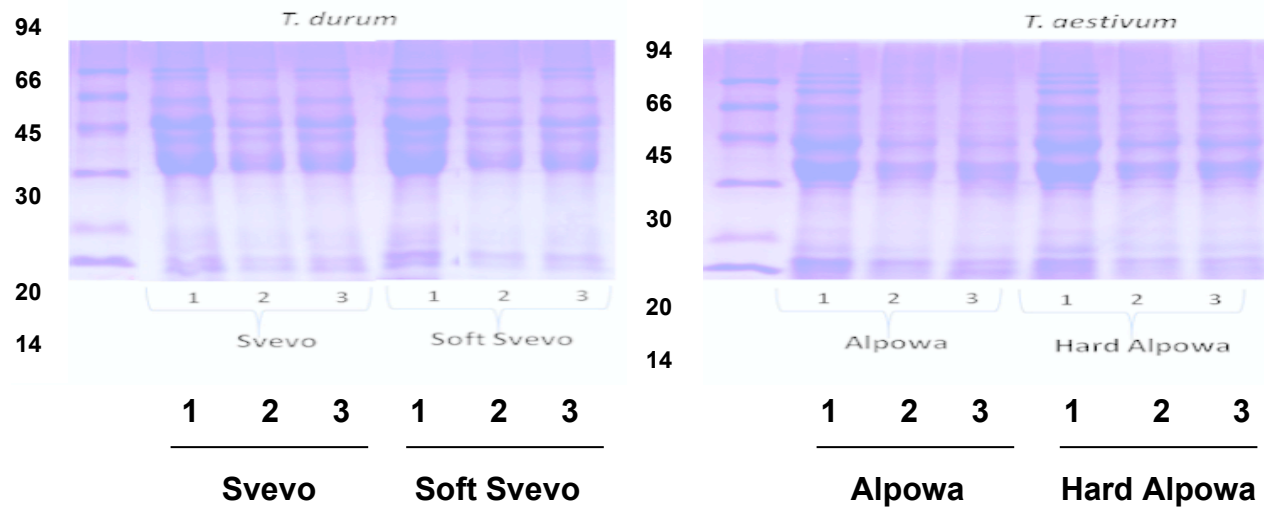


Figure 1.

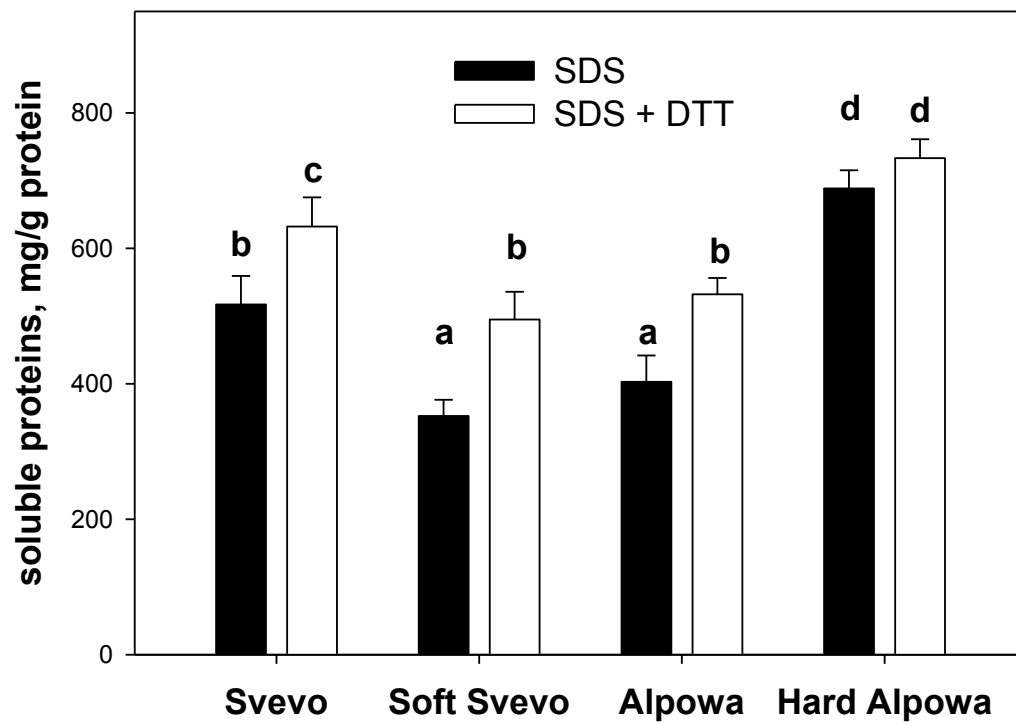


FIGURE 2

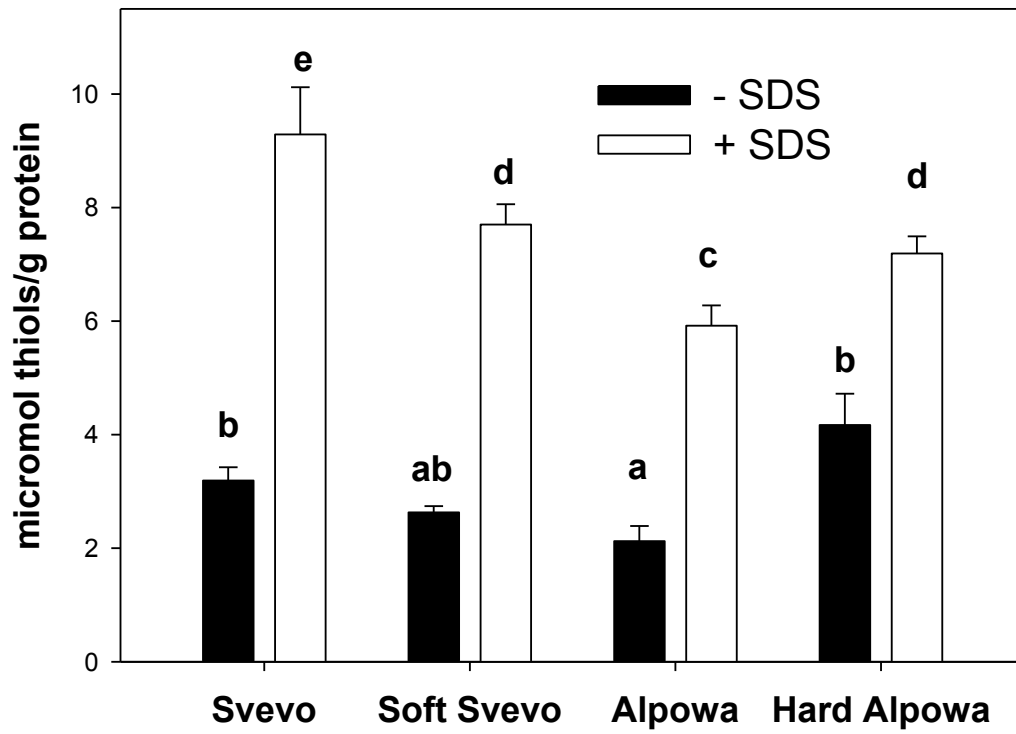


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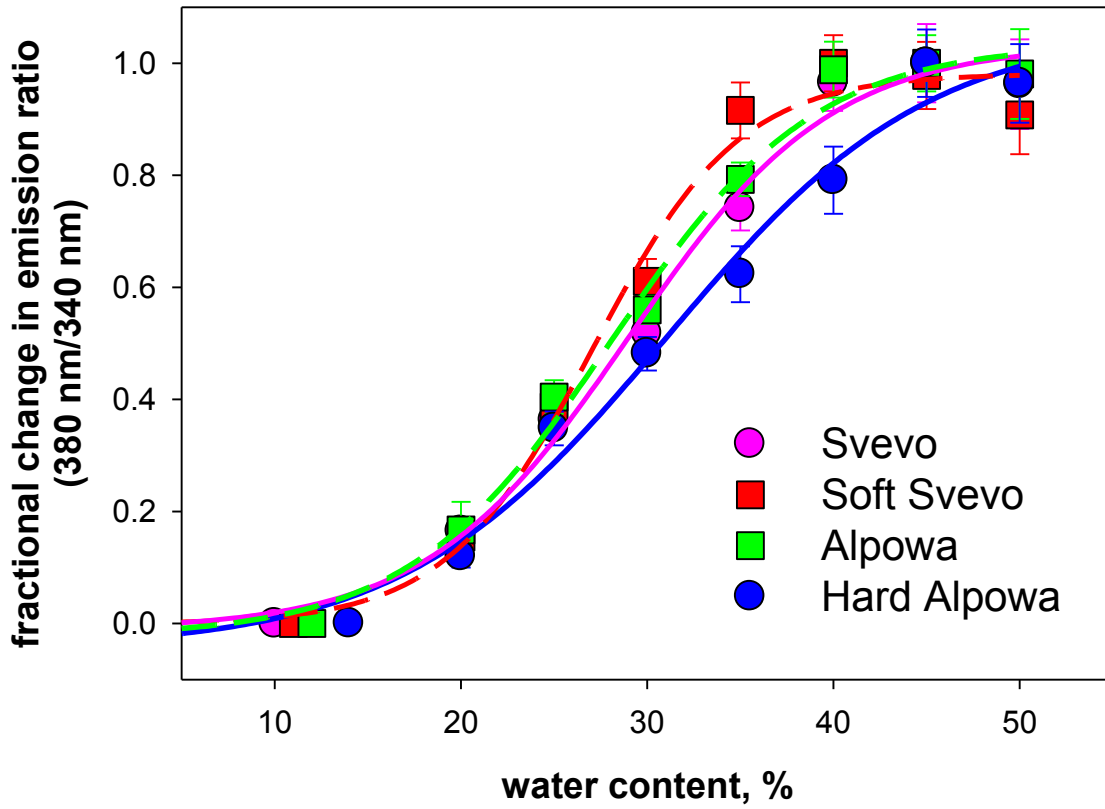


Figure 4.

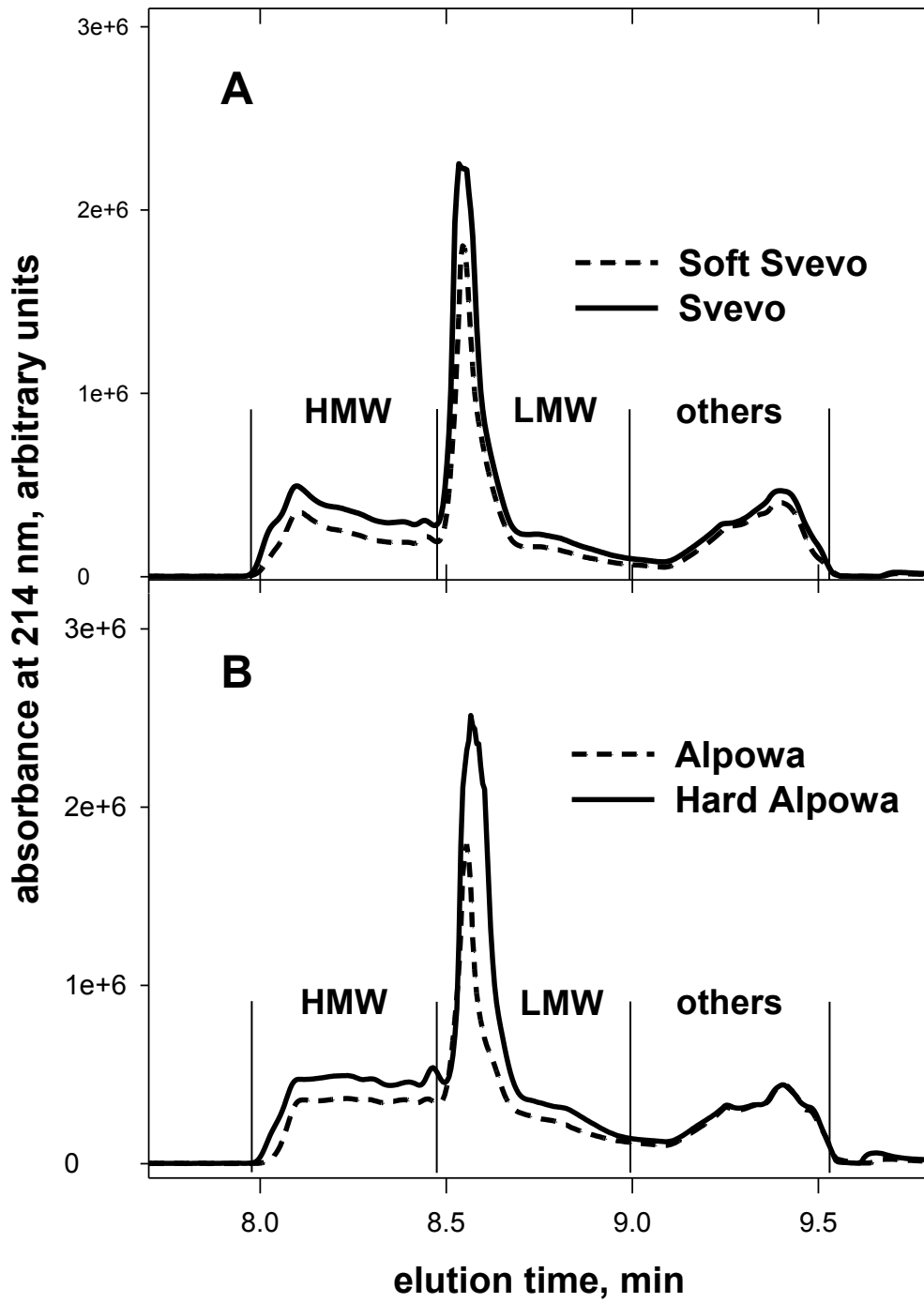


Figure 5.

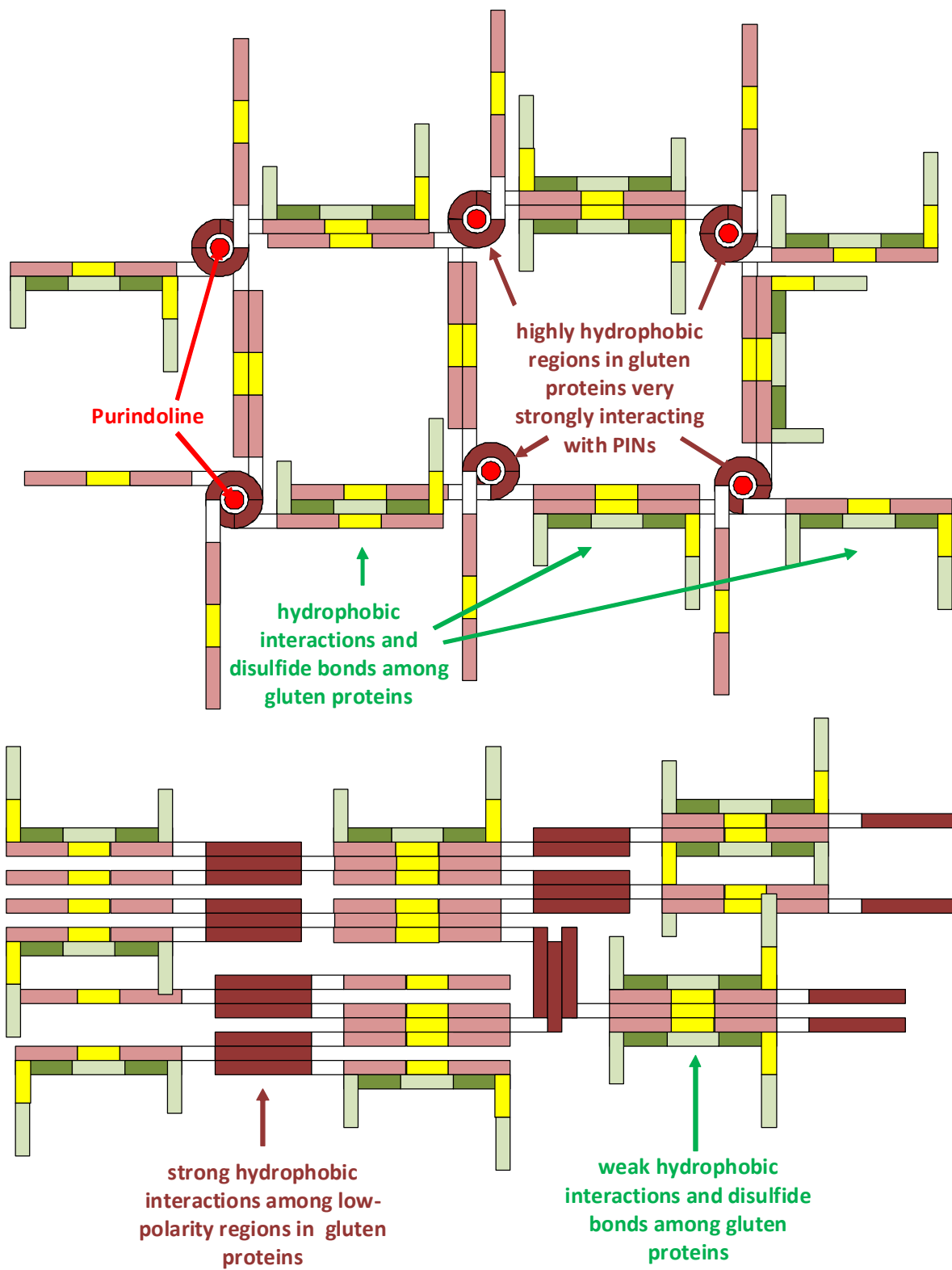


Figure 6