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## Structural consequences of the interaction of puroindolines with gluten proteins

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1	Structural consequences of the interaction of puroindolines with gluten proteins
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### 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 end of the short arm of chromosome 5D was deleted and PINs were not expressed (Hard 23 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 proteins, as detected by tryptophan fluorescence measurements carried out on minimally mixed 26 flour/water mixtures. We propose that PINs and gluten proteins are interacting in the grain or 27 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

# 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

### 47 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-60 61 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 62 63 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 64 could help increase its use both in traditional durum foods and unconventional ones, such as 65 66 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 67 decreased energy requirement for milling compared to durum wheat (Morris et al., 2015). The 68 69 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).

73 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 74 polar lipids. PINs are thought to bind to hydrophobic surfaces in the grain (either the starch 75 76 surface and/or the polar lipids) (Wall et al., 2010; Greenwell & Schofield, 1986) through a Trprich domain (Fiez, Wanjugi, Melnyk, Altosaar, Martin & Giroux, 2009; Alfredo, Palombo, 77 78 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 79 the exposed loops and helix surfaces of PINs. 80

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

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

96 Cysteine residues also are of paramount relevance in formation and stabilization of the gluten network through disulfide exchange processes. Accessibility of cysteine thiols in the 97 presence/absence of protein unfolding agents has been proposed as an index of network 98 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 of us have attempted to unravel the network of covalent and non-covalent interprotein bonds -102 103 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).

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

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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 (BC<sub>7</sub>) 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, USA) in 2014. Svevo and Soft Svevo were grown in Pullman (WA, USA) in 2013. Wheat grains 125 126 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

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 ( $\approx$  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  $\times$  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.

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  $3000 \times g$  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 gel (10% porosity) in a Mini-PROTEAN apparatus (Bio-Rad, Richmond, VA, USA), loading 155 about 2 microgram proteins per lane. Gels were stained with Coomassie Blue R-250. 156 157 Puroindoline-enriched fractions were obtained from individual flours essentially by following the 158 Triton<sup>®</sup> 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 $\times g$
167	for 5 min. The supernatant was filtered through a 10 $\mu$ m 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  $3,000 \times g$  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 15.1.02 (StatPoint Inc., Warrenton, VA, USA). Samples were used as factors. When a factor 198 199 effect was found significant ( $p \le 0.05$ ), significant differences among the respective means were

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200

### 202 **3. Results and Discussion**

203 3.1 Protein Profiles and Protein Aggregation Behavior

determined using Fisher's Least Significant Difference (LSD) test.

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

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

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 -210 211 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  $(p \le 0.05)$  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 ( $p \le 0.05$ ) 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 ( $p \le 0.05$ ) 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 mixing. Of course, this assumption does not rule out the possibility that these interactions may 246 247 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).

From our solubility results, it seems reasonable to assume that the differences in protein aggregation related to the presence/absence of PINs could involve more or less specific 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 Alpowa). The same reasoning may be applied to the results reported here from the Svevo durum 260 261 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 SDS-PAGE tracings in Fig. 1) may be playing a prominent role in explaining changes related to 264 the presence/absence of PINs. The nature of the gluten proteins relevant to the hypothetical 265 266 interaction with PINS and the molecular determinants of the interaction are currently being 267 investigated.

268

269 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

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

In all flours, the number of accessible thiols increased upon treatment with low SDS concentrations at room temperature. However, the SDS-dependent increase in thiol accessibility appears more pronounced in the presence of PINs. This behavior is particularly evident when comparing Alpowa and Hard Alpowa, and confirms the relevance of hydrophobic interactions as the major stabilizing element of interprotein interactions when PINs are present. Once again, it has to be noted that the differences in terms of readily accessible and SDS-accessible thiols that 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

The emission maximum of tryptophan fluorescence is indicative of the polarity of the chemical environment around the tryptophan side chains. The tryptophan emission maximum shifts towards higher wavelengths as the polarity of the environment increases. Front-face (solid state) fluorescence spectroscopy has proven useful in establishing the extent of contribution of hydrophobic interactions to the gluten protein network in dough and in defining the nature and extents of the structural rearrangements that accompany solvation of proteins in wheat-based 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.

306 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. 307 308 *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 309 310 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.

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.

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.

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 (Fig 5A and Fig. 2) (Veraverbeke et al., 2000a,b; Don et al., 2006). The 5DS distal end deletion 351 352 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 baking quality (Weegels, van de Pijpekamp, Gaveland, Hamar & Schofield, 1996), as reported 362 363 for Soft Svevo (Morris et al., 2015), and an increased concentration of HMW in proteins unextractable in cold-SDS has been reported to have a positive effect on baking quality (Don et 364 365 al., 2006).

366

## 367 4. Conclusions

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

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

377 No association or interaction of PINs with gluten proteins in flour was suggested in previous reports. We suggest here that PINs may associate in the grain also with gluten proteins, 378 379 promoting the formation of highly compact supra-macromolecular aggregates stabilized by local and very tight hydrophobic interactions. In this frame, and in consideration of the highly 380 381 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 proteins does not rule out a possible role of other flour components (either polysaccharides or 387 388 lipids (Wallet al., 2010) in the formation or stabilization of multi-component aggregates.

A possible view of the interactions occurring among PINs and other grain proteins in flour is hypothesized in the highly simplified scheme in Fig. 6. In the presence of PINs, the 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

view presented in Figure 6, our gel-permeation data suggest a prominent involvement of HMWcomponents 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 (Greenblatt & Schofield, 1986; Wall et al., 2010; Fiez et al, 2009; Alfredo et al., 2014). 402

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 structures of non-protein nature may be involved in PIN-mediated interactions, and of the 409 410 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.

From a more practical standpoint, we are currently taking advantage of recent
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	
422	References
423	Alfred, R. L., Palombo, E. A., Panozzo, J. F., & Bhave, M. (2014). The co-operative interaction
424	of puroindolines in wheat grain texture may involve the hydrophobic domain. Journal of
425	<i>Cereal Science</i> , 60,323-330.
426	Bhave, M., & Morris, C. F. (2008). Molecular genetics of puroindolines and related genes: allelic
427	diversity in wheat and grasses. Plant Molecular Biology, 66, 205-219.
428	Bonomi, F., Iametti, S., Mamone, G., & Ferranti, P. (2013). The performing protein:
429	Beyond wheat proteomics? Cereal Chemistry, 90,358-366.
430	Bonomi, F., D'Egidio, M. G., Iametti, S., Marengo, M., Marti, A., Pagani, M. A., & Ragg,
431	E. M. (2012). Structure-quality relationship in commercial pasta: A molecular
432	glimpse. Food Chemistry, 135,348-355.
433	Bonomi, F., Mora, G., Pagani, M. A., & Iametti, S. (2004). Probing structural features of
434	water-insoluble proteins by front-face fluorescence. Analytical Biochemistry, 329,
435	104-111.
436	Bushuk, W., Hay, R. L., Larsen, N. G., Sara, R. G., Simmons, L. D., & Sutton, K. H.
437	(1997). Effect of mechanical dough development on the extractability of wheat storage
438	proteins from bread dough. Cereal Chemistry, 74, 389-395.

- 439 Day, L., Bhandari, D. G., Greenwell, P., Leonard, S. A. & Schofield, J. D. (2006).
- 440 Characterization of wheat puroindoline proteins. *FEBS Journal*, 273, 5358-5373.
- 441 Dubreil, L., Meliande, S., Chiron, H., Compoint, J-P., Quillien, L., Branlard, G., & Marion,
- 442 D. (1998). Effect of puroindoline on the breadmaking properties of wheat flour.*Cereal*443 *Chemistry*, 75, 222-229.
- 444 Dubreil, L., Compoint, J. P., & Marion, D. (1997). Interaction of puroindolines with wheat
- flour polar lipids determines their foaming properties. *Journal of Agricultural and Food Chemistry*, 45,108-116.
- 447 Don, C., Mann, G., Bekes, F., & Hamar, R. J. (2006). HMW-GS affect the properties of glutenin
- 448 particles in GMP and thus flour quality. *Journal of Cereal Science*, 44,127-136.
- 449 Don, C., Lichtendonk, W. J., Plijter, J. J., van Vliet, T., & Hamer, R. J. (2005). The effect
- 450 of mixing on glutenin particle properties: aggregation factors that affect gluten451 function in dough. *Journal of Cereal Science*, 41, 69-83.
- 452 Fiez, L., Wanjugi, H. W., Melnyk, C. W., Altosaar, I., Martin, J. M, & Giroux, M. J.
- 453 (2009). Puroindolines co-localize to the starch granule surface and increase seed454 bound polar lipid content. *Journal of Cereal Science*, 50, 91-98.
- Finnie, S. M., Jeannotte, R., Morris, C. F., & Faubion, J. M. (2010a). Variation of polar
  lipid composition among near-isogenic wheat lines possessing different puroindoline
  halophytes. *Journal of Cereal Science*, 51, 66-72
- 458 Finnie, S. M., Jeannotte, R., Morris, C. F., Giroux, M. J., & Faubion, J. M. (2010b).
- 459 Variation of polar lipids located on the surface of wheat starch. *Journal of Cereal*460 *Science*, 51, 73-80.

- 461 Gautier, M-F, Cosson, P., Guirao, A., Alary, R., & Joudrier, P. (2000). Puroindoline genes
- 462 are highly conserved in diploid ancestor wheats and related species but absent in
  463 tetraploid *Triticum* species. *Plant Science*, 153, 81-91.
- 464 Giroux, M. J., and Morris, C. F., 1998. Wheat grain hardness results from highly conserved
- 465 mutations in the friabilin components puroindoline a and b. *Proceedings of the*
- 466 *National Academy of Sciences*, 95, 6262–6266.
- Greenwell, P., & Schofield, J. 1986. A starch granule protein associated with endosperm
  softness in wheat. *Cereal Chemistry*,63, 379-380.
- 469 Hayta, M., & Schofield, J. D. (2004). Heat and additive induced biochemical transitions in
- 470 gluten from good and poor breadmaking quality wheats. *Journal of Cereal Science*,471 40, 245-256.
- Huschka, B., Bonomi. F., Marengo, M., Miriani, M., & Seetharaman, K. (2012).
  Comparison of lipid effects on structural features of hard and soft wheat flour proteins
  assessed by front-face fluorescence. *Food Chemistry*, 133, 1011-1016.
- 475 Iametti, S., Marengo, M., Miriani, M., Pagani, M. A., Marti, A., & Bonomi, F. (2013).
- 476 Integrating the information from proteomic approaches: A "thiolomics" approach to
- 477 assess the role of thiols in protein-based networks. *Food Research International*, 54,478 980-987.
- 479 Iametti, S., Bonomi, F., Pagani, M. A., Zardi, M., Cecchini, C & D'Egidio, M. G. (2006).
- 480 Properties of protein and carbohydrate fractions in immature wheat kernels. *Journal of*
- 481 *Agricultural and Food Chemistry*, 54, 10238-10244.

Jazaeri, A., Bock, J. E., Bagagli, M., P., Iametti, S., Bonomi, F., & Seetharaman, K. (2015).
Structural modifications of gluten proteins in strong and weak wheat dough during
mixing. *Cereal Chemistry*, 92, 105-113.

485 Jolly, C. J., Rahman, S., Kortt, A. A., & Higgings, T. J. V. (1993). Characterization of the

- wheat Mr 15000 "grain softness protein" and analysis of the relationship between its
  accumulation in the whole seed and grain softness. *Theoretical and Applied Genetics*,
  86, 589-597.
- Kuktaite, R., Larsson, H., &Johansson, E. (2004). Variation in protein composition of
  wheat flour and its relationship to dough mixing behavior. *Journal of Cereal Science*,
  40, 31-39.
- Morris, C. F., Casper, J., Kiszonas, A. M., Fuerst, E. P., Murray, J., Simenone, M. C., &
  Lafiandra, D. (2015). Soft kernel durum wheat. A new bakery ingredient? *Cereal Foods World*, 60, 76-83.
- 495 Morris, C. F. (2002). Puroindolines: The molecular genetic basis of wheat grain hardness.
  496 *Plant Molecular Biology*, 48, 663-647.
- Morris, C. F., Simeone, M. C., King, G. E., & Lafiandra, D. (2011). Transfer of soft kernel
  texture from *Triticum aestivum* to durum wheat, *Triticum lundidurum* ssp. *durum*. *Crop Science*, 51, 114-122.
- 500 Morris, C.F., & King, G. E. (2008). Registration of hard kernel puroindoline allele near-501 isogenic line hexaploid wheat genetic stocks. *Journal of Plant Registration*, 2, 67-68.
- Murray, J.C., Kiszonas, A.M., Wilson, J.D., & Morris, C.F. (2016). The effect of soft
  kernel texture on the milling properties of soft durum wheat. *Cereal Chemistry*, 93,
- 504 513-517.

505	Quayson, E.T., Atwell, W., Morris, C.F., & Marti, A. (2016a). Empirical rheology and pasting
506	properties of soft-textured durum wheat (Triticum turgidum ssp. durum) and hard-textured
507	common wheat (T. aestivum). Journal of Cereal Science, 69, 252-258.

Quayson, E. T., Marti, A., Bonomi, F., Atwell, W., & Seetharaman, K. (2016b). Structural
modification of gluten proteins in strong and weak wheat dough as affected by mixing

510 temperature. *Cereal Chemistry*, 93, 189-195.

511 Veraverbeke, W.S., Larroque, O.R., Bekes, F., & Delcour, J. A. (2000a). In vitro polymerization
512 of wheat glutenin subunits with inorganic oxidizing agents. I. Comparison of single-step and
513 stepwise oxidations of high molecular weight glutenin subunits. *Cereal Chemistry*, 77, 582-

**514 588**.

- 515 Veraverbeke, W.S., Larroque, O. R., Bekes, F., & Delcour, J. A. (2000b). In vitro polymerization
  516 of wheat glutenin subunits with inorganic oxidizing agents. II. Comparison of single-step
  517 and stepwise oxidations of high molecular weight glutenin subunits. *Cereal Chemistry*, 77,
- 518 589-594.
- Wall, M. L., Wheeler, H. L., Smith, J. C., Figeys, D., & Altosaar, I. (2010). The tryptophan-rich
  domain of puroindoline is directly associated with the starch granule surface as judged by
  tryptic shaving and mass spectrometry. *Journal of Cereal Science*, 52, 115-120.
- 522 Weegels, P. L., van de Pijpekamp, A. M., Gaveland, A., Hamar, R.J. & Schofield, J.D.
- 523 (1996). Depolymerization and repolymerization of wheat glutenin during dough
- 524 processing. I. Relationship between GMP content and quality parameters. *Journal of*
- 525 *Cereal Science*, 23, 103-111.

526 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: 527 proteins solubilized in buffered 1% SDS upon treatment at 25°C for 60 min; 3: proteins 528 solubilized in buffered 1% SDS and 10 mmol/l DTT upon treatment at 25°C for 60 min. Equal 529 530 volumes of each extract (corresponding about 2 microgram protein, as calculated from the protein content in each flour) were loaded in each lane. 531 532 533 Figure 2. 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 534 535 DTT as indicated. Error bars refer to standard deviation (n=3). Different letters above each 536 column indicate a statistically significant difference ( $p \le 0.05$ ). 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 ( $p \le 0.05$ ). 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 to the actual data. Error bars refer to standard deviation (n=3). 546 547

Figure 5. Gel permeation profiles of proteins solubilized from the various flours upon incubation
for 60 min at 25°C in 50 mmol/l phosphate buffer, pH 6.8, containing 2% SDS in the absence of
DTT.

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Figure 6. A highly simplified schematic representation of the different organization of gluten proteins in the presence/absence of purindolines (red circles). The same number of two types of gluten proteins (identified by green and brown colors) is present in both the upper and the lower part of the scheme. In each protein, color intensity relates to the hydrophobicity of a given structural region. Cysteine-rich regions in gluten proteins are in yellow, but possible disulfides are not identified. Grain components other than proteins (and additional protein constituents) are not shown, for the sake of clarity.



Figure 1.



FIGURE 2



Figure 3.



Figure 4.



elution time, min

Figure 5.



Figure 6