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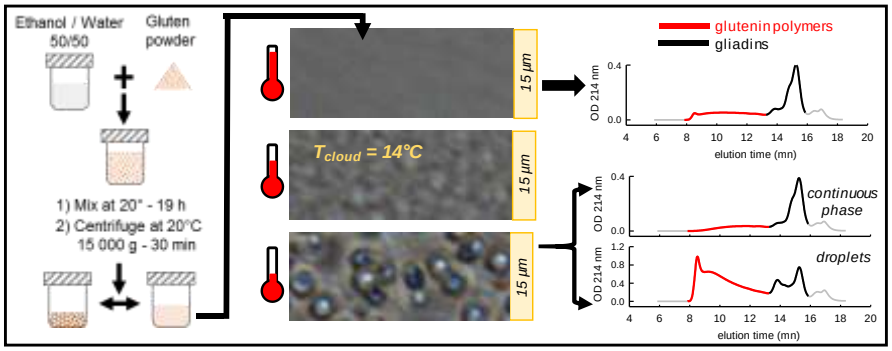
Highlights (85 characters including spaces)

- An ethanol/water extract containing 30% glutenin and 60% gliadin was obtained from gluten
- The extract (45 g/L) underwent liquid-liquid phase separation (LLPS) below 14°C
- Glutenin polymers were fractionated by LLPS according to their molecular weight
- LLPS delivered fractions with large range in glutenin/gliadin weight ratios (up to 2.5)
- Flory-Huggins lattice model properly reflected gluten proteins partitioning by LLPS

Abstract (250 words)

Wheat gluten includes two major proteins classes, gliadin (25-65 kg/moles) and glutenin polymers (100 to >2,000 kg/mol) each comprising several polypeptides routinely identified by size-exclusion chromatography and electrophoresis. Gluten proteins are rich in glutamine (30%) and contain several repeated sequences, linking them to the wide class of intrinsically disordered protein (IDP). Here we showed that an ethanol/water (EtOH/W, 50/50, v/v) extract of an industrial gluten comprising 1/3 of glutenin polymers and 2/3 of gliadin, underwent liquid-liquid phase separation (LLPS) below 14°C, leading to two coexisting phases, respectively rich and poor in protein. As the quenching depth increased, proteins of lower and lower molecular weight joined the rich phase, akin to what would have been obtained for a polydisperse polymer sample. Within the rich phase the mass ratio of glutenin over gliadin shifted from 2.5 to 0.5 when the temperature decreased from 14°C to -0.8°C. Concomitantly the concentration in glutenin polymers increased until 9°C and then stopped to evolve, suggesting that the LLPS line intersected the gelation line below 9°C. We showed that the partitioning of the glutenin polymers in the coexisting phases obeyed the Flory-Huggins (FH) lattice model, with different partial specific volumes in the coexisting phases and that the enthalpy of mixing of all gluten proteins with ethanol/water was size-independent. However, between 14°C and 5°C, some gliadins joined the rich phase, whereas on a strict thermodynamic basis and owing to their small-size they should not. The feature exemplified their specific interactions with glutenin polymers through weak forces.

Graphical abstract



Keywords: Liquid-liquid phase separation, Intrinsically disordered protein (IDP), gluten, glutenin polymers, Flory-Huggins interaction parameter

Abbreviations

HMW-GS: high molecular-weight glutenin subunit, LLPS: liquid-liquid phase separation, LS-glu: large-size glutenin polymers (>700 kg/mol), LMW-GS: low molecular-weight glutenin subunit, MS-glu: medium-size glutenin polymers (80 to 700 kg/mol), SDS: sodium dodecyl sulfate v/v: volume per volume, wb: wet basis,

1-Introduction

The unique bread making potential of wheat flour is based on the physicochemical properties and composition of its grain storage proteins. They are water-insoluble, but in the course of flour-water mixing they associate into a hydrated viscoelastic mass called gluten. Gluten is available as a dry powder, being produced at an industrial scale, and used in several food sectors as a texturing agent. It includes approximately equal masses of two protein classes, referred to as gliadin and glutenin. Following the pioneering works of T.B. Osborne (1907), gliadin and glutenin are each regarded as complex blends of several polypeptides, sharing similar solubility in specific solvents (Osborne, 1907, 1924). Gliadin, whose distinctive feature is to be freely soluble in 40-80% aqueous ethanol, comprises dozens of polypeptides classified as ω, γ, α/β-gliadin, according to their decreasing molecular weight (from 65 to 25 kg/mol). Glutenin is difficult to extract from flour, even when using solvents containing urea and ionic surfactants for instance. When fractionated by high performance size-exclusion chromatography (HPSEC) in sodium dodecyl-sulfate (SDS) phosphate buffer, the molar mass distribution of glutenin spreads from several hundred to thousands kilograms per mole (Morel, Dehlon, Autran, Leygue & Bar-L'Helgouac'h, 2000). Glutenin is made up of several distinct polypeptides (subunits) covalently linked through disulfide bonds and is commonly referred to as glutenin polymers. After chemical reduction of the disulfides, low- (25-50 kg/mol) and high- (68-90 kg/mol) molecular weight glutenin subunits (LMW-GS and HMW-GS) are released, as assessed by SDS-PAGE. The subunits carry several cysteine residues; two to three of them are supposed to be involved in inter-chains disulfide pairing. This would give the glutenin polymers a branched molecular structure as recently attested by dynamic light scattering and small-angle X-ray scattering measurements (Dahesh, Banc, Duri, Morel & Ramos, 2014). Gliadin and glutenin show similar amino-acids

compositions, being rich in glutamine (35-40%) and proline (12-27%) and poor in charged residues (5-6.5%), the majority of which being basic (basic/acidic ratio, 1.2-2.5). As another specific feature, gluten polypeptides comprise several repeated sequences of 6 to 8 amino-acids, made of glutamine, proline and phenylalanine (LMW-GS and gliadins) or glutamine, proline, glycine and tyrosine (HMW-GS) (Wieser, 2007). On the grounds of these features gluten proteins belongs to the class of intrinsically disordered proteins and should display extensive structural plasticity (Rauscher, Baud, Miao, Keeley & Pomes, 2006).

The way the gliadin and glutenin interact in the gluten viscoelastic mass is still poorly understood. It is likely that subtle balances of hydrogen, hydrophobic and ionic forces are at play, but this remains experimentally difficult to establish. At the origin of the problem is first the complexity of gluten composition, with several dozens of different components. Secondly, the lack of solubility of gluten protein in water has limited the use of classical scattering methods, especially because establishing the 3D structure of a protein dispersed in a denaturing solvent (to maintain solubility) is unlikely to provide relevant information about functionality. Assimilating gluten proteins to intrinsically disordered proteins (IDPs) opened up a new world of thought by challenging the classical structure–function paradigm. It allows one to choose the solvent solely on the basis of thermodynamic grounds. Thus, while 50% ethanol in water induces denaturation and aggregation of most globular proteins, it can be assimilated to a good to theta solvent of gluten proteins, been known for a long time to be well suited to maintain them into solution (Beckwith, Nielsen, Wall & Huebner, 1966; Jackson, Holt & Payne, 1983; Huebner & Bietz, 1993). In addition, a century ago, Dill and Alsberg (1925) showed that aqueous ethanol (55% to 70%, v/v) extracts of wheat gluten obtained at ambient temperature underwent liquid-liquid phase separation (LLPS) when temperature was lowered; the phenomena being fully reversible and repeatable at will (*ibid*; Dill 1927). The solubility of polymers in a solvent is driven by thermodynamic considerations and relies on null to negative change of Gibbs free energy of mixing ($\Delta G_{mix}= \Delta H_{mix}-T\Delta S_{mix}$). In the case of moderately bad or theta solvent, the polymer transfer into a solvent phase is endothermic, and the solubility entirely relies on entropic gain. Mixing is always accompanied by a gain in combinatorial entropy ($\Delta S_{mix}>0$), whose amplitude is inversely proportional to the polymer to solvent molar volume ratio. Thus, irrespective of chemical composition, large polymers are always less soluble than their small counterparts. They are the first to undergo phase separation when the temperature decreases, causing the Gibbs free energy of mixing to become positive except for the two coexisting phases where solvent-polymer or polymer-polymer interactions are privileged.

Recently, some of us (Boire, Menut, Morel & Sanchez, 2013) took advantage of the LLPS of a wheat flour protein extract (in 0.5 mM NaCl in 55% ethanol) to recover a soluble fraction including mainly gliadin (less than 10% of glutenin polymers) following a quench at 2°C,. They studied the LLPS behavior of this fraction and found that the glutenin polymers partitioning between the two coexisting phases was consistent with the Flory-Huggins lattice model developed for polymer solutions. In addition, some of us investigated the potential of LLPS to deliver protein samples of given glutenin/gliadin ratio, starting from an ethanol/water (50/50, v/v) wheat gluten extract. Using the same binary solvent and in the dilute regime, multi–angle light scattering analysis revealed that a balanced blend of gliadin/glutenin polymers displayed the typical features of Gaussian chains in a good solvent (Dahesh et al., 2014). In the semi-diluted to the concentrated regime (100-400 g/L) and by using a combination of various scattering techniques, large protein assemblies of radius of gyration ~120 nm were identified (*ibid*). More recently we showed that these protein assemblies comprised glutenin polymers and specific ω-gliadins, interacting together through weak bonds (Morel et al., 2020). The dynamic of the phase separation, the rheological and gelling properties of these gliadin/glutenin samples have been recently investigated (Dahesh, Banc, Duri, Morel & Ramos, 2016; Banc et al., 2016, 2019; Ramos et al. 2021). Here we present in details the main experimental parameters governing the extraction yield of gluten protein in ethanol/water and driving their fractionation by LLPS, highlighting the underlying thermodynamic causes.

2 Materials and methods

2.1 Chemicals

Deionized water and ethanol 99.9% of analytical grade (CarloErba) were used to prepare the water/ethanol solvent solutions. Other chemicals were of ACS grade. Industrial gluten powder batches were courtesy of TEREOS-SYRAL (Aalst, Belgium). Gluten protein content was estimated by Kjeldahl analysis (AACC-Method 46-10). Most experiments, unless otherwise specified, were performed using a gluten batch including 5.89% water (wb) and 74.5% protein (wb) and comprising 42.5%, 43.8% and 13.6% glutenin polymers, gliadin and albumin/globulin, respectively.

2.2 Extraction and liquid-liquid fractionation of gluten protein in 50/50 (v/v) ethanol/water

Typically, gluten (20 g) was placed in a 250 mL propylene Nalgene bottle, in which 200 mL of water/ethanol (50/50, v/v) solvent was added. The bottle was closed and vigorously shaken by hand for 60 s to disperse gluten. The bottle was then agitated for 19 h at 20°C, using a rotary shaker (Reax 2, Heidolph) rotating at 60 rpm. After 30 min of centrifugation (15 000 g, 20°C) the supernatant (parent extract, Par) was recovered, weighted and placed in a clean bottle, while the pellet (gluten gel, Gel) was sampled for high performance size-exclusion chromatography (HPSEC) analysis and then discarded. The bottle containing the parent extract was immersed in a stirred bath regulated in temperature (from for 12.5°C to -0.8°C) to trigger phase separation. The temperature quenching rate was about -0.5 °C/min. After one hour in the thermostated bath maintained at a quench temperature Tq, the bottle was centrifuged (3 000 g) for one hour at Tq. Then the upper (poor phase, P phase) and bottom (rich phase, R phase) phases were collected and weighted. Routinely 6 replicate bottles were run in parallel.

2.3 High Performance Size-Exclusion Chromatography (HPSEC)

HPSEC analysis of the proteins present in the gluten powder and in the collected Gel, Par, P et R phases was performed as described by Morel et al. (2000). Gluten powder (20 mg at 1g/mL) was first extracted at 60°C in sodium phosphate 100 mM, pH 6.9; SDS 1% (sample buffer below) by rotary shaking (Reax 2, Heidolph at 60 rpm for 80 min). The supernatant was recovered by centrifugation (30 min, 18 000rpm) for analysis of SDS-soluble proteins. The pellet was further extracted using 5 mL of the sample buffer applying a sonication step (Vibra Cell sonificator at 20 kHz for 90s) allowing one to recover the SDS-insoluble glutenin polymers into solution through mechanical disruption. The gluten gel (Gel) and the ethanol/water parent extract (Par), poor (P) and rich (R) phases (cf. 2.2) were simply diluted 201, 31, 41 and 201 times in the sample buffer supplemented with 6M urea before analysis. Routinely, 20µL was injected onto a size exclusion column TSK-G 4000-SW (Merck, France) (7.5 mm x 30 cm) coupled to a TSK 3000-SW (Merck, France) guard column (7.5 mm, 7.5 cm). Elution was performed with 0.1 M sodium phosphate buffer, 0.1% SDS and protein detection was monitored at 214 nm. The HPSEC UV trace was converted into protein mass concentration using a specific extinction coefficient of 18.51 L/cm/g (Morel & Bar-L'Helgouac'h, 2000). From the protein concentration, the volume of each samples (Gel, Par, P and R) was calculated considering the weighted mass and the densities of gluten protein and solvent (respectively, 1.32 and 0.923). Then the total mass of protein in each extract was calculated from the protein concentration deduced from HPSEC and the calculated sample volume. The apparent molecular weight of the eluted species was assessed after column calibration with known protein standards saturated with SDS, as described in Redl, Morel, Bonicel, Vergnes & Guilbert(1999).

2.4 SDS-PAGE

Freeze dried samples were suspended at 3 mg/mL in a loading buffer including 0.5 M Tris-glycine buffer (pH 6.8), 2% SDS (w/v), 5% beta-mercaptoethanol (v/v), 20% glycerol (v/v) and traces of pyronin Y. After 2 h shaking at ambient temperature, sample tubes were heated for 2.5 min in boiling water. The discontinuous SDS-PAGE buffer system of Laemli was used with a running gel (T=15%, C=0.5%) and a stacking gel (T=5%, C= 10%). Samples loading was 10 µL per well (comb of 10 wells). A home-made vertical gel (160 x 180 x 1.5 mm) was run at 20 mA/gel at 18°C and stopped 1 h after the

tracking dye (pyronin Y) had reached the bottom of the gel. The gels were stained in 12.5% (w/v) trichloroacetic acid, 0.01% (w/v) Coomassie Brilliant Blue R250, and destained with distilled water before being scanned using a Biorad GS-700 gel scanner. Densitometry profiles of each sample track were extracted from inverted scanned images, using FiJi software and a macro computing the sum of pixels' grey level from a box of one pixel high and of the track width.

3 Results & discussion

3.1 Impact of solid/solvent ratio and ethanol content on gluten protein extraction

Solid/solvent[a]	Par/Gel	% of gluten	[total protein][b]		[Glutenin][b]		Par[c]		Gel[c]	
(g/100mL)	volume ratio	prot. in Par	Par	Gel	Par	Gel	%glu	%gli	%glu	%gli
10.0	4.5	45.5	47.5	259.3	14.0	129.8	29.4	59.3	50.1	33.1
12.5	2.9	47.3	57.7	241.9	19.1	110.5	33.1	56.0	45.7	33.5
15.0	2.4	42.6	69.7	218.2	23.6	102.5	33.9	55.4	47.0	33.2
17.5	2.1	40.7	86.0	223.0	30.0	105.7	34.9	54.5	47.4	34.6
20.0	1.7	42.1	91.7	241.7	32.2	110.0	35.1	54.3	45.5	34.8

Table 1: Gluten extraction with ethanol/water (50/50, v/v) at variable solid/solvent ratios.
[(a)] Gram of gluten powder (84.5% protein, wb) per 100 mL of added solvent (50/50, ethanol/water, v/v). [b] concentration in g/L. [c] in mass percent of the total sample protein content. (glu) stands for glutenin polymers, (gli) for gliadins.

A gluten powder including 84.5% protein (wb) of which 89% were soluble in SDS-phosphate buffer, one of the best extracting solvent for gluten protein, was used for studying the impact of solid/solvent ratio on the protein extraction yield. Extraction was performed for 19 h at 20°C, using aqueous ethanol (50/50, v/v) for solid/solvent ratios ranging from 10 g to 20 g/100 mL, in order to recover the soluble proteins fraction (thereafter parent fraction, Par) according to figure 1a. The percentage of gluten proteins recovered in Par averaged around 44±3% (Table 1). The protein concentration in Par increased proportionally to the solid/solvent ratio, while the volume ratio of Par over the remaining insoluble gel fraction (thereafter gel fraction, Gel) tended to decrease (Table 1). Attempts to further increase the protein concentration in Par by rising the solid/solvent ratio beyond 20 g/ 100 mL was not feasible because more and larger clumps formed, which contrarily to what was obtained at lower ratios, were impossible to disperse, even after prolonged shaking. HPSEC analysis of the proteins present in the Par and Gel fractions revealed that even if Gel included a majority of glutenin polymers it also comprised one third of gliadins, whereas Par included one third of glutenin polymers (Table I, Figure 1b). As already noted by several authors (Beckwith et al., 1966, Jackson et al., 1983) a clear cut separation of gliadin and glutenin polymers could thus not be obtained by using aqueous ethanol for gluten protein extraction.

Whatever the solid/solvent ratio, the Gel was enriched in protein (concentration 236± 16 g/L), while its volume ranged from 14 to 33% of the total sample volume, as the solid/solvent ratio doubled. Typically, the Gel consisted in a pale greyish cohesive mass of elastic consistency (Figure 1c). This elastic mass was highly solvated (solvent volume fraction φ = 0.8±0.1). Interestingly, it was significantly more solvated than typical gluten gel extracted from flour doughs (φ = 0.66 to 0.74, depending on the flour baking quality) (McCaig & McCalla, 1941). This higher swelling ratio could be due to the better solvent quality of aqueous ethanol for gluten proteins as compared to pure water. The Gel displayed stable protein composition and concentration whatever the solid/solvent ratio. This may not be surprising, considering that hydrated gluten is a critical gel (Ng & McKinley, 2008). Replacement of water by aqueous ethanol, a better solvent for gluten proteins simply shifted the sol-gel equilibrium of gluten, increasing the gel swelling capacity, while promoting gliadin and glutenin solubility.

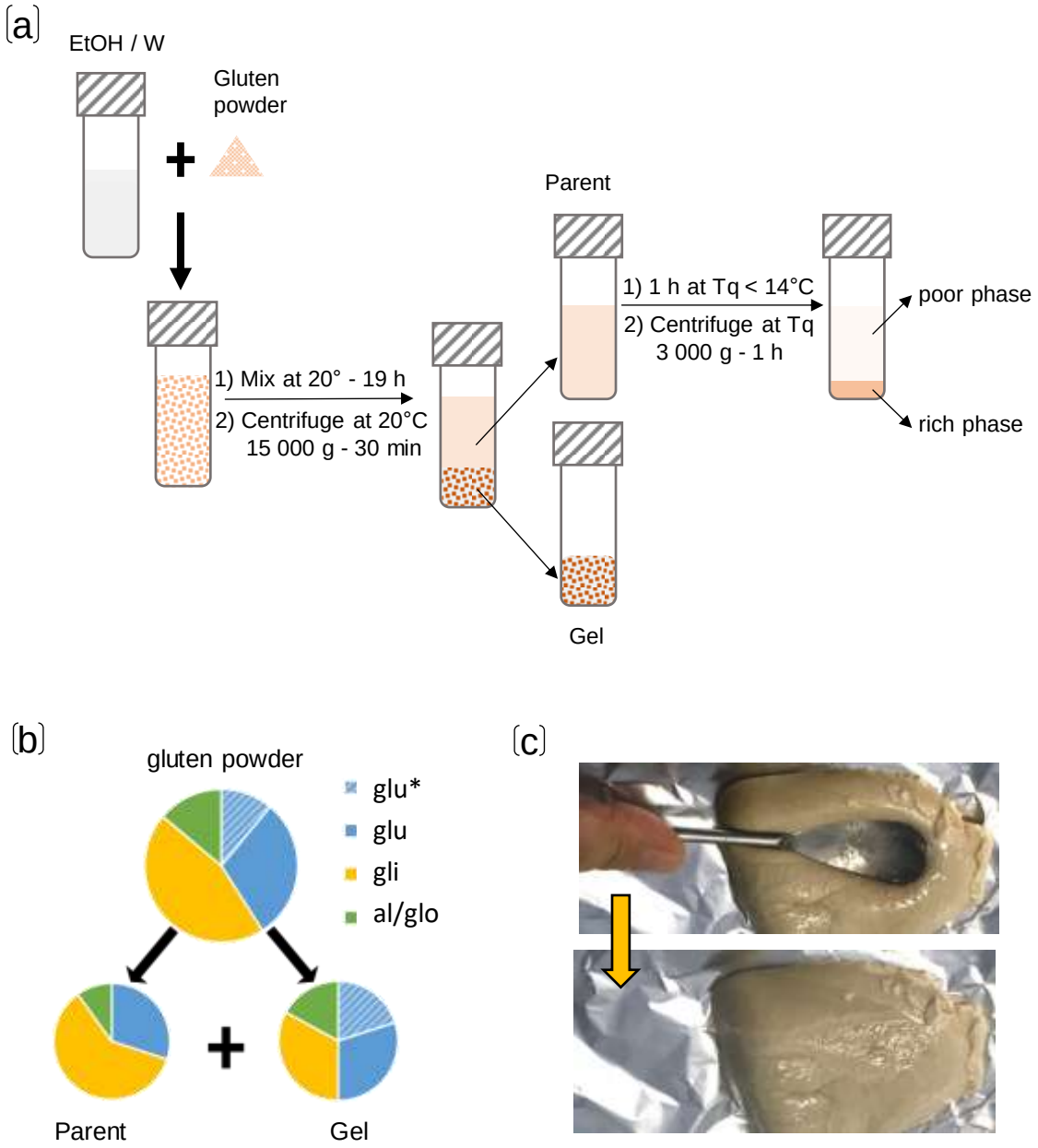


Fig. 1ab: Sketche of the extraction procedure (a) and protein composition of the gluten powder, parent and gel phases (b), physical aspect of the Gel (c).

(a) Gluten powder extracted with 50/50 ethanol/water (v/v) at 10 g/100 mL (cf.2.2). (b) Protein composition of gluten powder and of the Parent and Gel fractions as determined by HPSEC allowing to quantify the content in SDS-soluble (glu) glutenin, gliadin (gli), albumin/globulin (al/glo) and SDS-insoluble glutenin (glu *) (cf. 2.3). (c) Pictures of the Gel, deformed with a spatula (top) and relaxed after removing the spatula (bottom).

Because our ultimate aim was to fractionate the gluten proteins according to their molecular weight by liquid-liquid phase separation (LLPS), in the following we selected the lowest solid/solvent ratio (10 g/100 mL), as it gave the lowest supernatant concentration. Indeed, the partitioning efficiency between small and large polymers is better when starting from very diluted polymer solutions (Flory 1944). According to the seminal work of Dill & Alsberg (1925) the cloud point temperature of gliadin suspended in ethanol/water mixtures is highly dependent on ethanol content. Isobaric cloud point temperature curves of gliadin follow a U-profile, nearly flat between 55 and 65% (v/v) ethanol where the solvent quality is the best possible one. This prompted us to examine the impact of the ethanol

content in the solvent. As shown in figure 2, an increase of the ethanol/water ratio from 48/52 to 66/34 (v/v) slightly decreased the protein extraction yield. It also shifted the protein composition at the benefit of gliadin leading to a drop in the glutenin/gliadin ratio from 0.5 to less than 0.4 when the ethanol content increased. Since our objective was to obtain by LLPS samples especially enriched in glutenin polymers, the soluble protein extract obtained using 50/50 ethanol/water (v/v) was adopted in the following.

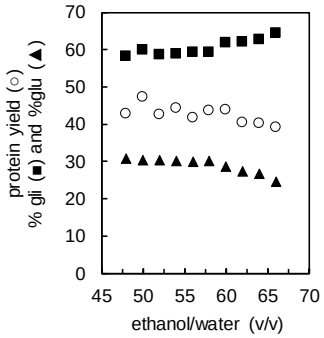


Fig. 2: Impact of solvent composition on gluten protein extraction yield and supernatant composition.
Extraction was performed using 10% of gluten in solvents of various ethanol contents (v/v), according to 2.2.

3.2 Impact of the quench depth on gluten fractionation by LLPS

The experiments reported hereafter were performed using the parent fraction obtained as described in 2.2. On average, 49 ± 3 % of the gluten proteins were recovered in the Parent (96 extractions), at a concentration of 46 ± 2 mg/ml (with 57 ± 6% gliadin, 31 ± 3% glutenin polymers and 11 ± 1.4 % albumin/globulin).

The Parent remained stable at ambient temperature for days but became cloudy when cooled below the cloud temperature, T_{cloud} = 14°C, while subsequent sample warming reversed the phenomena. Phase Below T_{cloud}, Parent demixing into well delineated bottom and upper phases (thereafter called rich and poor phases) could take hours, especially when the quench temperature (Tq) was closed to T_{cloud}. To recover the demixed phases, samples, once tempered for one hour at a given quench temperature (Tq with $Tq < T_{cloud}$), were submitted to mild centrifugation (1 hour at 3 000g) at Tq. We verified that extended tempering (from 1 to 4 hours) or centrifugation did not change the respective volumes or compositions of the recovered phases (see Fig. 5ab, below).

We plot in figure 3a the protein concentration of the rich and poor phases as a function of the quench depth $\Delta T=T_{cloud}-Tq$. Figures 3b and 3c report the partitioning of the parent volume and protein content between the demixed rich and poor phases as a function of ΔT.

Below a quench depth ΔT=5°C the protein concentration in the poor phase remained almost constant (41±2 mg/ml), close to that of the Parent one (46±2 mg/ml). Concomitantly, the protein concentration in the rich phase increased substantially from 84±7 to 173±27 mg/mL because of the large imbalance in the coexisting phases volumes (Fig. 3b). At a quench depth of 1.5°C, the protein concentration in the rich phase was already nearly twice as much as in the Parent phase (84 vs 46 mg/mL, Fig. 3a). Ultimately, at the highest quench depth tested (ΔT=14.8°C), about 80% of the Parent proteins joined the rich phase (Fig. 3c) whose protein concentration reached 376±32mg/mL (Fig. 3a).

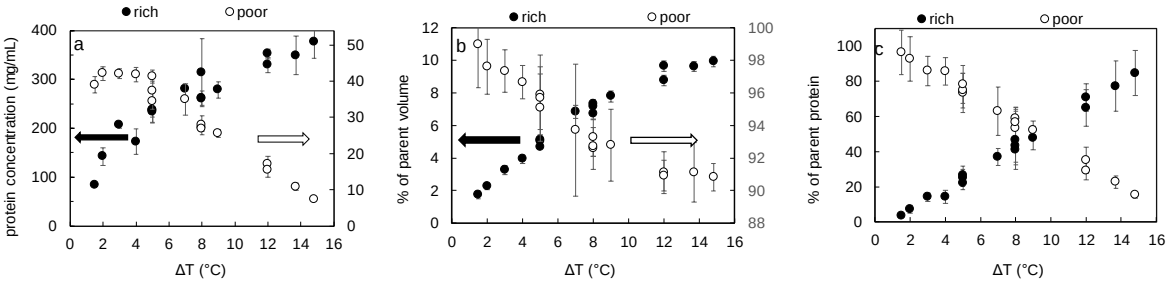


Fig. 3abc: Protein concentration (a), percentage of the Parent volume (b) and percentage of the Parent protein (c), recovered in the rich and poor phases following a quench of depth ΔT.
Standard errors from 6 replicate bottles.

3.3 Quantitative partitioning of gluten protein types depending on the quench depth

Fig. 4 shows the HPSEC profiles of proteins from parent (yellow) and demixed phases (blue and orange for poor and rich phases, respectively) obtained at different quench temperatures. Profiles were expressed on a weight basis to reflect the partitioning of the parent proteins into rich and poor phases. Typically, looking at the parent phase (yellow profile), glutenin polymers appeared first for elution times between 8.0 to 13.2 min. The first fraction between 8.0 and 8.9 min, demarcated by vertical bars on Fig.4, coincides with the column void volume and includes the glutenin polymers of the largest size (LS-glu polymers).

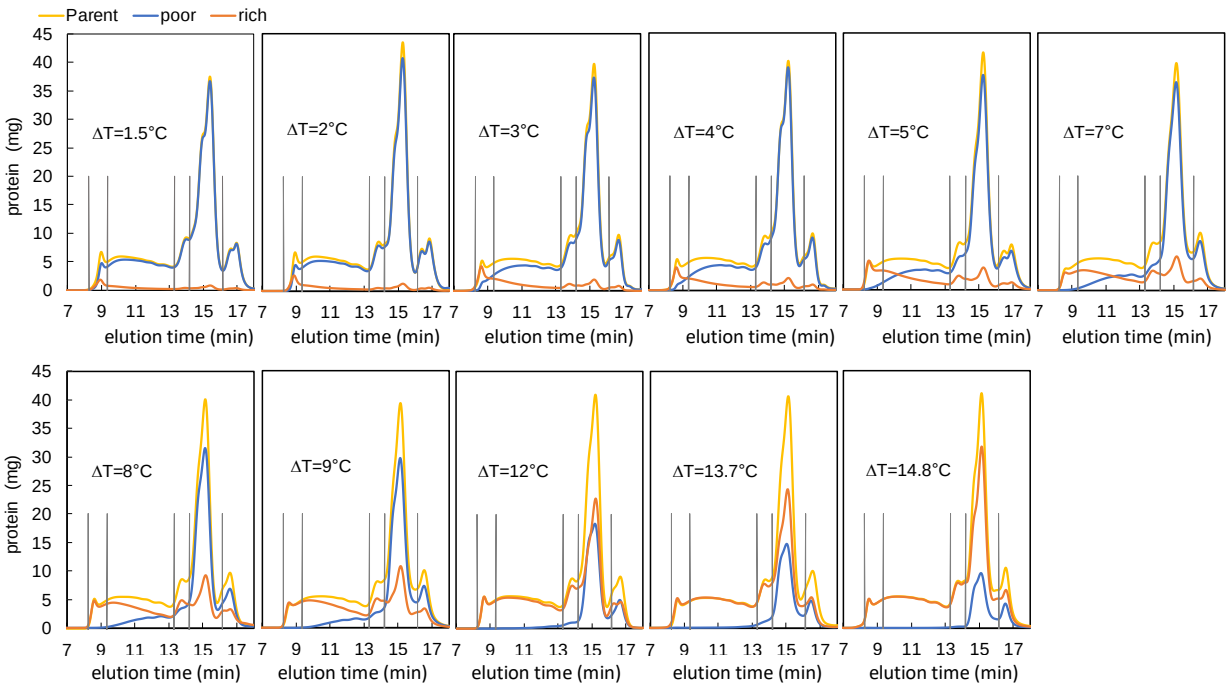


Fig. 4. Protein size distribution profiles of Parent and demixed phases obtained at different quench depths.
Quench depth ($\Delta T=T_{cloud}$ -Tq) is indicated in each graph. Averaged HPSEC-UV trace from 6 replicates was converted into protein concentration and then into protein mass taking the volume of the phases into consideration. Results are given for a total of 4.57 g of proteins in 100 mL of parent phase. (Yellow) Parent; (Orange) rich phase; (blue) poor phase.

The molecular weight of the LS-glu polymers could not be estimated precisely but should be comprised between 700to more than 2,000 kg/mol. Next, glutenin polymers ranging from 700 to 80

kg/mol were recovered (medium size, MS-glu polymers). The following small shoulder (13.2-14.2 min) corresponded to ω-gliadin with molecular mass of about 60 kg/mol. The high peak between 14.2 and 16.1 min included other types of gliadin (γ, β and α; from 45 to 18 kg/mol). Lastly albumin and globulin were eluted (16.1-17.80 min).

Just below T_{cloud}, following a quench depth ΔT=1.5 °C, the rich phase included almost exclusively LS-glu polymers. At ΔT=5°C, all LS-glu polymers from Parent joined the rich phase, as exemplified by the overlapped HPSEC profiles of Parent and rich phases, at the location of the exclusion peak. For deeper quenches, in addition to LS-glu polymers, medium-sized glutenin polymers joined the rich phase. Gliadins started to join the rich phase beyond a quench depth of 5°C, as revealed by the down shift of the main peak of the blue profile (poor phase) compared to the Parent one. At ΔT=14.8°C the rich phase included almost all proteins, the poor phase being diluted (protein concentration 7.4 mg/mL) and almost exclusively composed of γ-, α/β-gliadins (92%) supplemented by albumin/globulin.

HPSEC analysis revealed that as the quench depth increased, gluten proteins tended to join the rich phase in sequence according to their molecular weights, from the largest to the smallest species. As a result, the composition of the rich phases progressively shifted towards decreasing proportion in glutenin (Fig. 5a and b). Thus, the liquid-liquid phase separation of an ethanol/water extract of gluten proteins allowed one to obtain protein extracts with a tunable proportion of glutenin from 0.6 to 65%. Note that ultimately extracts richer in glutenin polymers than the Gel fraction could be obtained (Table 1).

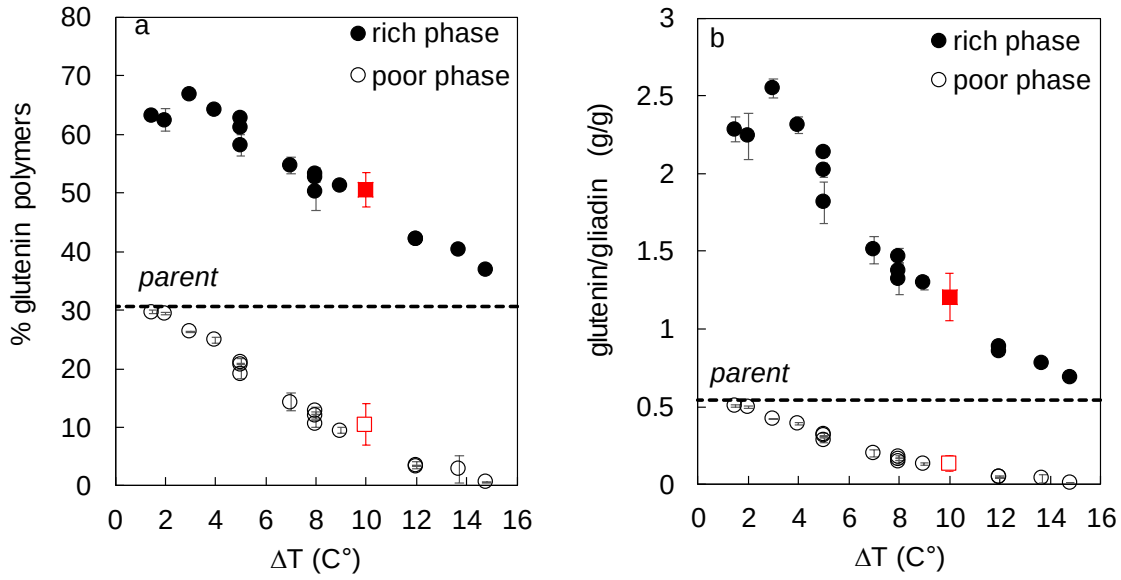


Fig. 5ab. Proportion in glutenin polymers (in % of total protein mass) and glutenin/gliadin mass ratio in the rich and poor phases recovered at different quench depths.

Filled and empty black symbols are from the present study, while red squares are from a previous study of our group (Dahesh et al. 2014, 2016) obtained using another batch of gluten and letting the phase separation to proceed naturally at ΔT=10°C during 24H without centrifugation.

3.4 Polypeptides composition of parent and demixed phases

Figure 6 shows a picture of the SDS-PAGE obtained for gluten, Parent and some of the poor and rich phases recovered after applying quench depths, ΔT = 5°, 8° and 12°C. On the right hand side of the picture, the densitometry profile of the gluten protein sample is presented. High-molecular weight subunits (HMW-GS) and ω-gliadins were easily identified in the upper part of the gel while the patterns of the other gliadins and of the low-molecular weight glutenin subunits (LMW-GS) overlapped, preventing a specific band assignment. At the bottom of the gel, a protein doublet was identified as belonging to albumin/globulin proteins. Comparison of the rich and poor patterns

showed that the 4 bands corresponding to the HMW-GS behaved as one, joining together the rich phase. The situation was more contrasted when considering the ω-gliadins pattern. The two upper bands (of higher molecular weight) disappeared from the poor phase at ΔT=8°C, while the two other seemed rather unaffected. Despite, their close molecular weight, ω-gliadins demonstrated different ability of to undergo phase separation.

The contrast between the different patterns were quantified by gel densitometry (Table 2). In the co-existing phases, the higher the relative proportion in LMW-GS/γ-gliadin, the lower was the proportion in HMW-GS. The ω-gliadin did not follow the same trend as the LMW-GS/γ-gliadin since it was preferentially enriched in the rich phases, like the HMW-GS.

As anticipated, a linear relationship (R^2=0.996) was obtained between the content in glutenin polymers of the samples, determined from HPSEC analysis, and the proportion of the HMW-GS measured by densitometry (glutenin polymer[a] and HMW-GS*, Table2).

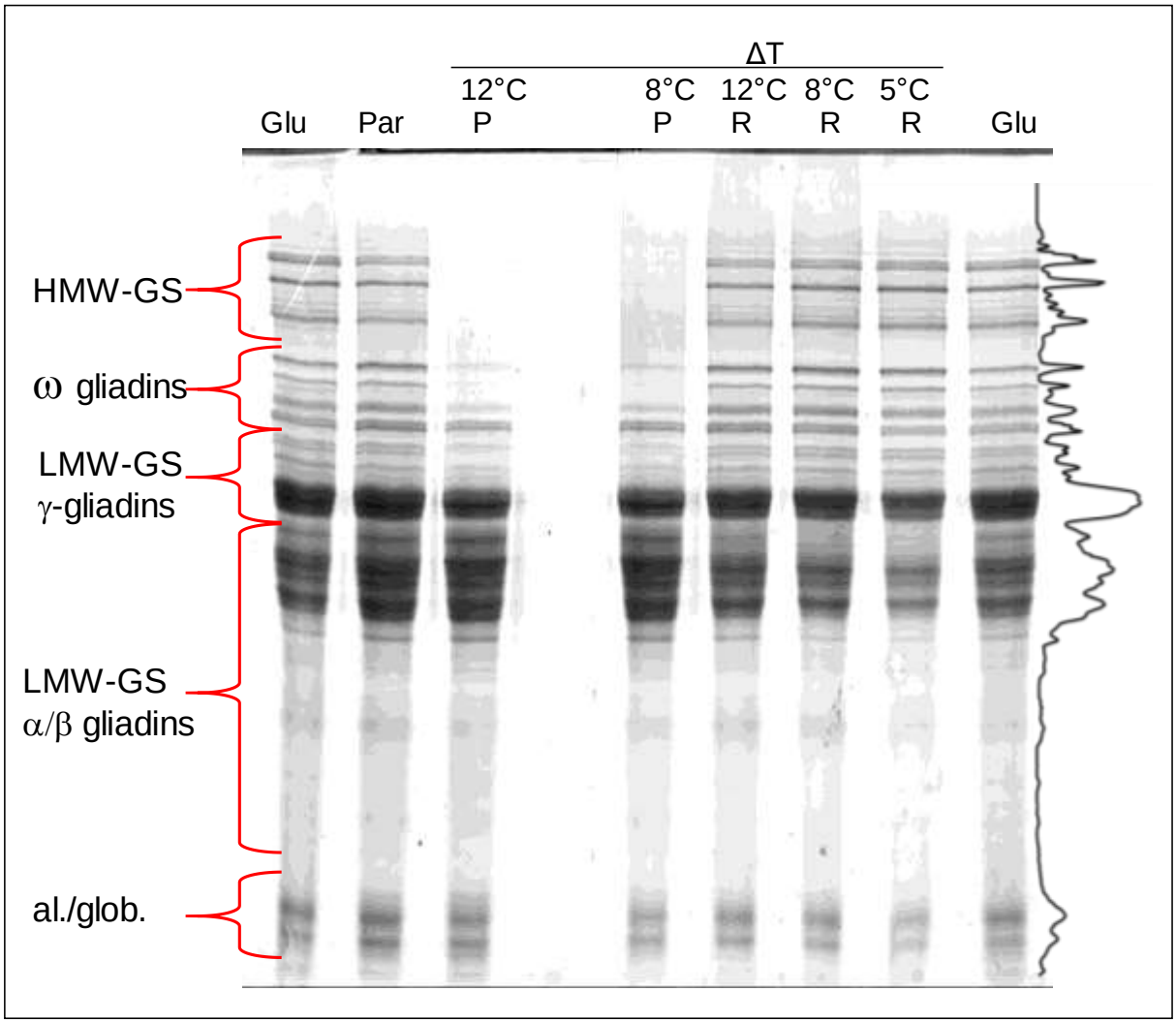


Fig. 6. SDS-PAGE analysis of gluten, parent and demixed phases.
Electrophoresis in reducing conditions, 30 µg of protein per well. (Glu) gluten powder, (Par) parent phase, (P) poor and (R) rich phases. The quenching depth (ΔT) is indicated on the top of the picture of the gel. (HMW-GS, LMW-GS) high- and low-molecular-weight glutenin subunits, respectively. On the right of the picture the densitometry profile of the gluten sample is given as a typical example.

Considering the total glutenin polymers content determined from the HPSEC profiles of the samples (Fig. 4 and 5a) and assuming that it coincided to the sum of the HMW- and LMW-glutenin subunits

contents determined from gel densitometry, one could estimate the LMW-GS content. This assumption neglected the possible involvement of atypical gliadins within the glutenin polymer chain architecture. Comprising an odd number of cysteine residues, the atypical gliadins were found covalently linked to glutenin polymers through disulfide bonds (Vensel, Tanaka & Altenbach, 2014). They may act as terminator of the glutenin polymer chain propagation and therefore might be over-represented in the small glutenin polymers, as compared to larger ones.
The HMW-GS/LMW-GS glutenin subunits ratio for gluten, parent and rich phases were of the same order of magnitude, around 0.5, well within the typical range of wheat flours (HMW-GS/LMW-GS ratio about 0.46±0.05) as rigorously assessed by reverse-phase HPLC analysis (Wieser & Kieffer, 2001). Hence, even if the rich phases differed in their glutenin polymers contents (Fig. 5a) and size distributions (Fig. 4), they would include polymers of relatively identical subunits composition. By contrast the poor phases obtained at ΔT=8°C and in particular at ΔT =12°C, showed low to very low HMW-GS/LMW-GS ratios (0.34 and 0.05, respectively). The small glutenin polymers remaining in these two poor phases (see blue profiles in Fig. 4) would be enriched in gliadin terminators.

Table 2: Proportions in gluten protein types estimated from SDS-PAGE densitometry (Fig. 6) as compared to HPSEC analysis.

Samples	HMW-GS*	ω-gli*	LMW-GS γ-gli*	LMW-GS α/β-gli*	al/glo*	Glutenin polymers[a]	HMW-GS*/LMW-GS ratio
Gluten	13.67	8.20	40.07	29.94	8.12	42.50	0.47
Par	9.55	9.69	46.63	26.36	7.78	31.25	0.44
p-ΔT 12°C	0.17	3.22	60.78	25.88	9.95	3.37	0.05
p- ΔT 8°C	3.26	4.86	58.27	25.86	7.75	12.86	0.34
r- ΔT 12°C	14.15	10.22	41.16	27.08	7.39	42.21	0.50
r- ΔT 8°C	17.80	11.53	36.30	26.60	7.78	52.60	0.51
r- ΔT 5°C	20.33	10.36	33.79	27.14	8.37	62.87	0.48

(*) Relative amounts deduced from gel densitometry. ([a]) results from HPSEC analysis (Fig.4 and 5a). LMW-GS = [glutenin polymers[a] – HMW-GS*].

3.5 Coexisting concentration curves

Large supra-molecular protein assemblies (~ 120 nm) displaying an apparent molecular size of 26,000 kg/mol were previously identified in the rich phase recovered at ΔT=10°C (Dahesh et al., 2014). These assemblies were successfully isolated from gliadins and free glutenin polymers by asymmetrical flow field-flow fractionation in ethanol/water (50/50, v/v) (Morel et al., 2020). After HPSEC analysis in a SDS-buffer, which disrupted all the weak interactions, they were found to be composed of glutenin polymers and of fast eluting ω-gliadins (likely the two ones of higher molecular weight, identified by SDS-PAGE in Fig.6).
The gluten protein assemblies, owing to their huge molecular size should be the first to phase separate as temperature is lowered, followed by free glutenin polymers and then free gliadins, in decreasing order of size and so ascending order of combinatorial entropy of mixing.
Fig. 7ab presents the coexistence concentration curves for glutenin polymers (black), α/β and γ-gliadins (red) and ω-gliadin (green). Continuous lines are drawn as guide for the eyes to delineate the rich (full lines and symbols) and poor (dotted lines and empty symbols) branches arising from the liquid-liquid phase separation of the parent phase. For the glutenin polymers, a large concentration gap was observed between the rich and poor phases, even at a shallow quench depth (ΔT =1.5°C) (Fig. 7a), a feature theoretically consistent with the large size of the gluten protein assemblies and their low concentration in the Par extract (Flory, 1944).

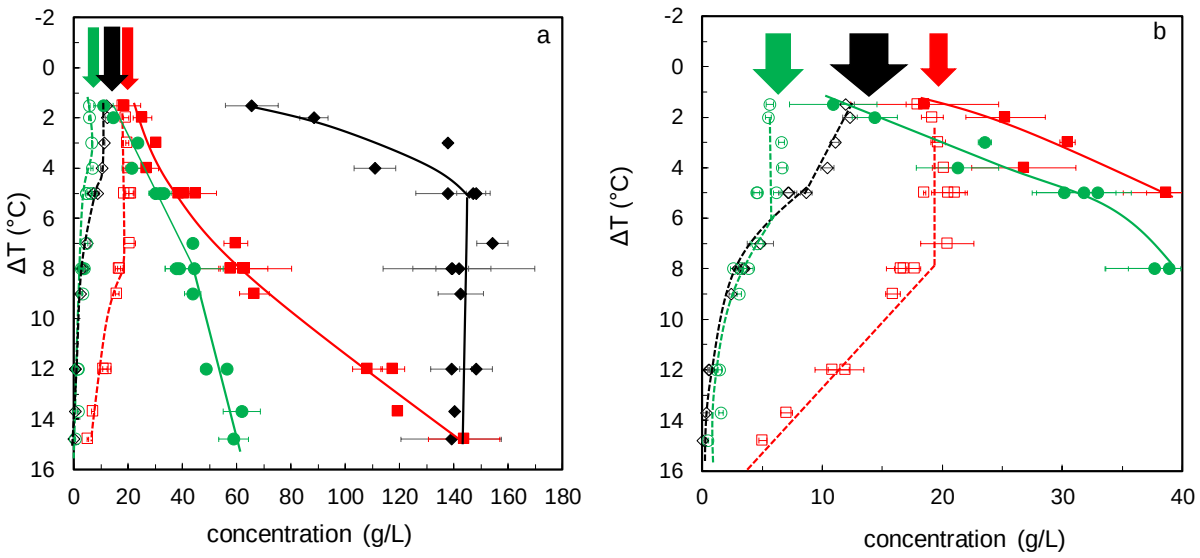


Fig. 7ab. Coexistence curves for glutenin polymers (black, diamond), α/β and γ-gliadins (red, square) and ω-gliadin (green, circle).
(Empty and filled symbols) poor and rich phases, dotted and continuous lines are guides for the eye to delineate the poor and rich branches, respectively; arrows (width in proportion with standard error) indicated proteins concentration in the parent phase. The concentration in the different proteins were calculated from the HPSEC profiles taking into account the following time limits: glutenin polymers (8.0-13.2 min), ω-gliadin (13.2-14.2min) and α/β and γ-gliadins (14.2-16.1).

Compared to the glutenin, the increases with ΔT of α/β and γ- gliadins concentration within the rich phases were first modest before intensifying beyond quench depths ΔT = 8-9°C (Fig. 4). Concomitantly, the gliadin poor branch showed an inflection point at ΔT=8-9°C (Tq=5°C) which may signal the onset of an additional demixing event. In support to this assumption, the demixing temperature of an almost pure sample of α/β and γ-gliadin, used in the same concentration range, was found to be about Tq=5.1±1.0 °C (Morel et al., 2020). For shallow quenches (0°C<ΔT<8°C) the accumulation of α/β and γ-gliadins in the rich phase would t arise from their specific interaction with the glutenin polymers rather than from a decrease in solvent quality.
Poor branches of glutenin polymers and of ω-gliadin also showed an inflection point in between ΔT = 5-6°C (Fig.7b), a feature that could signal the exhaustion of the gluten protein assemblies from the Par phase. Thereafter, for deeper quenches, free glutenin polymers and the two remaining ω-gliadins of smaller molecular weight identified above by SDS-PAGE, would begin to join the rich phase. Whether they will form new dense protein droplets or merge with the former ones is undecidable. However, multiphases coexistence is a hallmark of complex systems comprising several non-interacting components (Feric et al. 2016). In the case of blends of multiple species (A and B) undergoing spinodal decomposition, as the quench depth increases, creation of inclusions of species B within an already formed droplet enriched in A, or alternatively successive trains of spinodal decomposition forming rich droplets of variable composition in A and B can be speculated (Dignon, Best & Mittal, 2020).
At ΔT= 5°C, the concentration in glutenin polymers within the rich phase, stopped growing (vertical black branch) suggesting that the LLPS line could intersect the gelation line. Gelation of gluten protein samples, deriving from rich phases obtained after variable ΔT (4°C to 12°C) and suspended at 237 g/L in ethanol/water (50/50, v/v) was found to vary with the protein composition (Banc et al., 2019). The samples comprising 120 and 145 g/L of glutenin polymers (*ibid,* Glu 57% and 66%) showed the typical mechanical spectra of gels, while the sample including only 99 g/L glutenin remained liquid (*ibid,* Glu

44%). In another study, the gluten proteins from the rich phase recovered at ΔT=10°C (red dot in Fig. 5ab) demonstrated spontaneous gelation at room temperature; in several hours to days depending on concentration (Dahesh et al, 2016). The critical concentration for gelation, extrapolated in the limit of vanishing gelling time, was found to be 250±10 g/L (*i.e*., 125±5 g/L in glutenin, *ibid)*. Based on these investigations, we propose that beyond a quench depth of 5°C and as the glutenin concentration within the rich phase attained 143 ± 6 g/L of glutenin, a sol-to-gel transition would occur. Whether gelation can precede, accompany or follow spinodal decomposition is well documented for synthetic polymers and for proteins in a binary solvent (Bansil, Lal & Carvalho, 1992; Tanaka, Swislow & Ohmine, 1979; Kawanishi, Komatsu & Inoue, 1987).

3.6 Thermodynamic insights of phase separation

For polymers carrying multiple sites for solvent interaction the balance between the enthalpy cost of mixing and the entropic gain is in favor of the former as the molecular size of the polymer grows and the temperature decreases. This property is at the basis of the fractionation of polydisperse polymers according to their size by liquid-liquid phase separation (LLPS). The lattice model for binary blends provides a simple formalism for fractionation of polymers by LLPS according to their molecular weight (Flory, 1944):

$$\ln\left(\varphi'_{(i)}/\varphi''_{(i)}\right) = -\alpha x_{(i)} \quad (1)$$

where, prime and double prime refer to poor and rich phase, respectively and index *(i)* refers to individual molecular species, φ is the polymer volume fraction, α is the partitioning coefficient and $x_{(i)}$ is the molar volume ratio of polymer (*i*) to the solvent:

$$x_{(i)} = \frac{M_{w(i)} \times \bar{V}_{seg}}{M_{seg} \times \bar{V}_{sol}} \quad (2)$$

In the frame of the lattice model, $x_{(i)}$ stands for the number of adjacent lattice sites a polymer molecule of size $M_{w(i)}$ occupies while a solvent molecule occupies one lattice site. Here $M_{w(i)}$ is the polymer (*i*) molecular weight, $\bar{V}_{sol}$ and $\bar{V}_{seg}$ are the molar volumes of the solvent and the polymer segment while M_{seg} is the molecular weight of the polymer segment unit.

Using $\varphi'_{(i)} = C'_{(i)}\,\nu'$ and $\varphi''_{(i)} = C''_{(i)}\,\nu''$ with $C_{(i)}$ the mass concentration of the molecular species (i) (in g/L) and ν' and ν'' the partial specific volume of glutenin polymers in the poor and rich phases, leads to :

$$\ln\left(\frac{C'_{(i)}}{C''_{(i)}}\right) = -\alpha x_{(i)} - \ln\left(\frac{\upsilon'}{\upsilon''}\right) \quad (3)$$

Equation (1) applies for binary systems, composed of one solvent and one type of polydisperse polymer. For more complex systems involving several types of polymers or binary solvent, the Flory interaction parameter will not capture the multiple interactions at play. In our case, we found that the parent, poor and rich phases had the same ethanol content (checked by infrared spectroscopy, results not shown). Thus with respect to gluten proteins, the ethanol/water solvent would behave like a monomolecular solvent. Accordingly, its molar volume was calculated considering ideal mixing rules on a molar fraction basis.

According to Eqs. 1 and 3 the partitioning of polymers between a rich phase and a poor phase follows an exponential law with their molecular weight; *i.e.* larger polymers are first to join the rich phase as temperature is lowered below the cloud temperature (T_{cloud}). Figure 8ab shows that Flory assumption well applies here since the logarithm of the phases concentration ratio linearly scales for glutenin polymers of molecular weights ranging from 80 kg/mol to 700 kg/mol. Beyond this size limit, elution of

the glutenin polymers at the void volume of the HPSEC column, precluded accurate molecular weight determination.
As expected from lattice theory, the fitted slopes (α) increased with the quench depth (Fig. 8c) (Flory, 1944).
According to equations 1 and 3, the origin of the linear fit should be zero, as long as the partial specific volume of the glutenin polymers is the same in the poor and rich phases ($\nu' = \nu''$). This was not the case in our experiments, especially when the quench depth increased. Fig. 8c shows that the ratio of the partial specific volumes of the glutenin polymers, as estimated from Eq. 3, increased up to 8 times when ΔT increased from 1.5 to 9 °C. Similar deviation from a zero intercept has already been reported for the fractionation of polydisperse polystyrene polymers in cyclohexane and was attributed to a coil to globule collapse of the polymer chains as they settled in the rich phases (Breitenbach & Wolf, 1967). Gluten protein assemblies detected in diluted suspensions of gluten proteins in ethanol/water (50/50, v/v) were identified as highly swollen with an apparent density of 5 to 7 mg/mL (Dahesh et al. 2014, Morel et al. 2020). Their swelling factor is likely to depend on the osmotic pressure and transfer within the rich phase would lead to their shrinkage.

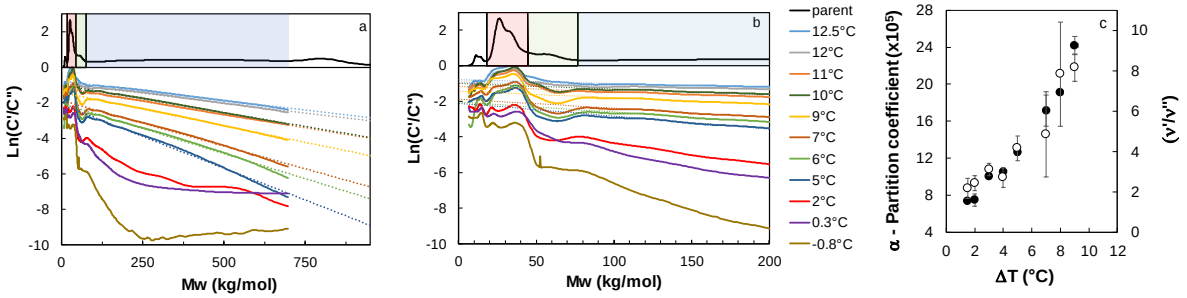


Fig. 8abc: Partitioning of proteins in rich and poor phases according to quenching temperatures (a,b) and parameters fitted according to Eq. 3 for glutenin polymers (c).
(8a, 8b) Continuous lines are experimental data and dotted lines are the best fit with Eq.3. The typical HPSEC profile of the parent phase (upper insert) is given to help identify the considered respective ranges of α/β and γ-gliadins (light red area), ω-gliadin (light green area) and glutenin polymers (light blue area). The ln(C'/C'') parameter is the logarithm of the average protein concentration ratio obtained from 6 replicates. Only the glutenin polymers with Mw ranging from 98.7 to 700 kg/mol ($2{,}519 < x_{(i)} < 17{,}760$) were considered for data fitting according to Eq.3 (dotted lines). (8c) average partitioning coefficient (α, black symbols) and polymer partial specific volume ratio (empty symbols) were calculated from Eq. 3, taking $\bar{V}_{sol}$ and $\bar{V}_{seg}$ equal to 27.6 and 79.2 mL/mol and M_{seg} to 96.4 g/mol. Only regressions giving R^2 value > 0.99 are considered (ΔT=1.5 to 9 °C).

As shown in figure 8b, the behavior of ω-gliadins (Mw ~ 60 kg/mol) is very different from that of the other gliadins. Their partitioning toward the rich phase was more pronounced than it would have been for a glutenin polymer of similar molecular weight, since the experimental data laid below the linear fit. However, the feature was consistent with the privileged interaction of the fast eluting ω-gliadins with the glutenin polymer assemblies (Morel et al, 2020). For α/β and γ-gliadins (18 < Mw < 49.8 kg/mol) the partitioning did not follow the trend found for glutenin polymers. In other words, their tendency to join the rich phase appeared far lower than it would had been for a theoretical glutenin polymer of same mass (see the offset from the linear tendency, Fig. 8b).

To account for the multiple possible interactions between segments of polymer chains and solvent molecules through van der Waals forces, a single polymer-solvent interaction parameter (χ) was introduced independently by Flory and Huggins (Flory, 1941; Huggins, 1941). This phenomenological parameter, was calculated for the glutenin polymers, considering the fitted partitioning coefficients α (Eq. 3, Fig. 8c) and equation 4 where:

$$\alpha = \varphi' \left(1 - \frac{1}{\bar{x}_n'}\right) - \varphi'' \left(1 - \frac{1}{\bar{x}_n''}\right) + \chi\{(1 - \varphi')^2 - (1 - \varphi'')^2\} \quad (4)$$

Where $\bar{x}_n$ is the number average molecular size of the polymers within the range of interest in the poor (') and rich phase ('') and χ is the Flory-Huggins (FH) interaction parameter.

In order to estimate the protein volume fractions, the partial specific volume of the gluten proteins within the rich phases (ν") was taken as the inverse of the gluten protein density (1/ρ, 0.758 ml/g). To account for the shift in the partial specific volume ratios between rich and poor phases (Fig. 8c), the partial specific volume of the polymers in the poor phases (ν') were taken as 0.758 ml/g multiplied by the ratio ν'/ ν".
The numerical values of χ (half-filled red dots) are plotted against the inverse of the quenching temperature in Fig. 9. They exceeded 0.5 and tended to grow linearly when the quenching temperature decreased, at least until ΔT =7°C. This finding confirmed that glutenin polymers demixing was enthalpy driven, linked to a decreasing contribution of the mixing combinatorial entropy to the free energy of mixing as the solvent quality decreased.

The partitioning behavior of the α/β and γ-gliadins could also be analyzed on the basis of the Flory-Huggins lattice model, considering that the proteins eluted between 14.2 and 15.8 min (Fig. 4) behaved like a single polymer species, because of an overall low polydispersity index (PI=1.04). In that case, the Helmholtz free energy of mixing the pure protein in the solvent (Δf_{mix}), expressed per lattice site, is given by (Flory, 1944):

$$\Delta f_{mix}(\varphi) = kT[\frac{\varphi}{x}\ln(\varphi) + (1-\varphi)\ln(1-\varphi) + \chi\varphi(1-\varphi)] \quad (5)$$

with φ=φ' or φ'' and x is the molar volume ratio of the polymer to the solvent (Eq. 2).
Phase separation occurs when the free energy of mixing, instead of being a convex function of the polymer volume fraction, shows two local minima at $\varphi = \varphi'$ and φ'', lying on a common straight line, whose slope is given by:

$$(\Delta f_{mix}/\delta\varphi_{(\varphi=\varphi')}) = (\Delta f_{mix}/\delta\varphi_{(\varphi=\varphi'')}) \quad (6)$$

where the first derivative of the Helmholtz free energy of mixing is given by:

$$(\delta\Delta f_{mix}/\delta\varphi) = \frac{1}{x}\ln(\varphi) + \frac{1}{x} - \ln(1-\varphi) - 1 + \chi(1-2\varphi) \quad \text{with } \varphi=\varphi' \text{ or } \varphi'' \quad (7)$$

Yielding:

$$\chi = \frac{\frac{1}{x}\ln\left(\frac{\varphi''}{\varphi'}\right) + \ln\left(\frac{1-\varphi'}{1-\varphi''}\right)}{2(\varphi''-\varphi')} \quad (8)$$

The volume fractions for both phases were calculated from the known mass concentrations taking 0.758 mL/g as the gluten protein partial specific volume. The numerical values of χ as computed with Eq. 8 are given in Fig. 9 (full and half-filled black symbols). They displayed a monotonic evolution with 1/Tq roughly followed along a convex trend; χ first decreasing as the quench depth increased before increasing as ΔT increased further. In the limit of deep quenches (ΔT>9 °C), the χ values for the gliadin appeared in line with the trend observed for the glutenin polymers. The feature supports that interestingly the partitioning of the α/β and γ-gliadins and of glutenin polymers follows the same trend, albeit their different molecular weights.

From a theoretical standpoint, χ was originally envisioned as purely enthalpic in nature, with negative value indicating favorable interactions between polymer and solvent while when χ> 0.5, polymer-polymer interactions are favored at the expense of polymer-solvent mixing. In practice it is considered as a free-energy parameter including, both enthalpic (χ_H) and entropic (χ_S) contributions; χ_S accounting for polymer conformational or solvent translational entropies. The enthalpic and entropic contributions of χ are estimated according to (Schuld & Wolf, 2001):

$$\chi = \chi_H + \chi_s \quad \text{with } \chi_H = T^{-1}(d\chi/dT^{-1}) \quad \text{and} \quad \chi_S \cong -\Delta S \tag{9}$$

Thus, the decrease of the χ values for the α/β and γ-gliadins, from T_{cloud} up to ΔT = 7°C, derived from a negative χ_H value counterbalanced by a positive χ_s. In this range of quench depth, the partitioning of the α/β and γ-gliadins into the rich phase would be driven by their specific interactions with glutenin polymers (negative χ_H) in parallel to an increase of ordering (positive χ_s). Only at deeper quenches the partitioning of the α/β and γ-gliadins within the rich phases would become enthalpy driven (positive χ_H) as the solvent quality decreased.
The χ_H and χ_s contributions to the Flory-Huggins (FH) interaction parameter were determined by fitting the χ values obtained for the glutenin polymers and the ones obtained for the gliadin beyond ΔT > 7 °C (half-filled symbols, Fig. 9). according to:

$$\chi = A/T + B \tag{10}$$

Best linear fit of the experimental data yielded χ_H= 0.72 ±0.07 (at T_{cloud} = 14°C) and χ_S = -0.21±0.08. Considering the negative value of χ_S, it can be concluded that the solubility of gluten proteins in ethanol/water relies on a significant entropic gain, acting against phase separation. The feature is common in the case of co-solvency brought by binary solvents, composed of highly miscible solvent molecular species (like water and ethanol) able to interact with each other but also with the polymer chains. In this way, the addition of polymers increases the degree of disorder of the solvent compared with its pure state, thereby providing an appreciable entropic gain (Zhang & Hoogenboom, 2015).

The critical value of χ (χ_c) below which glutenin polymers and solvent will be miscible over the entire concentration range and the corresponding critical temperature (T_C) below which phase separation will occur, can be calculated according to (Flory, 1953):

$$\chi_c = 0.5 \times \left(1 + 1/\bar{x}_n{}^{0.5}\right)^2 \tag{11}$$

and

$$T_c = (\chi_H)/(\chi_c - \chi_s) \text{ (in K)} \tag{12}$$

For the glutenin polymers, a theoretical critical temperature (T_c, Eq. 11) of 13.2 °C (red star, Fig. 9) was calculated, closed to the cloud point temperature of 14°C, visually detected. The feature indicated that the concentration the glutenin polymers in the parent extract was closed to their critical concentration. This would also be the case for the α/β and γ-gliadins, since the calculated critical temperature (5°C, black star, Fig. 9) was also close to the 5.1°C obtained for a gluten protein sample including almost pure gliadins (Morel et al., 2020). The enthalpy of mixing gluten proteins with the ethanol/water solvent appeared size-independent as long as we admitted that the partial specific volume of the gluten protein assemblies could vary during LLPS. Thus, within the poor phase it would increase from 1.6 to 6.2 ml/g, as the protein concentration decreased from 41 to 26 g/L, while the gluten protein assemblies reaching the rich phase would undergo full deswelling.

The formalism followed to determine the range of χ values for the α/β and γ-gliadins undergoing LLPS at different quench depths, cannot be applied for the ω-gliadins, since HPSEC did not allow to discriminate them according to their specific involvement in the gluten protein assemblies or not.

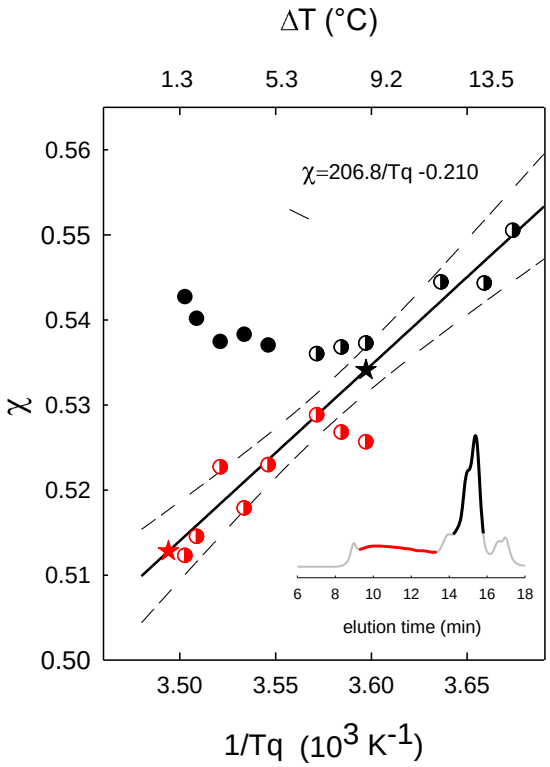


Fig. 9: Flory-Higgins interaction parameters for gliadins and glutenin polymers
(red symbols) glutenin polymers; (black symbols) α/β and γ-gliadins; (hall-filled symbols) data considered for the linear fit of the χ values (Eq. 10); (black continuous line) fitted linear regression; (short dashed lines): regression 95% confident intervals; (star symbols) critical temperatures calculated according to Eq. 11; (insert): parent HPSEC UV profile with red and black highlighted parts indicating the glutenin polymers and α/β and γ-gliadins ranges considered for χ calculation, average Mw for glutenin; 358.3 kg/mol (PI=1.44), average Mw for gliadin 31 kg/mol (PI=1.04).

Conclusion

Gluten, an industrial commodity derived from wheat flour, is mainly composed of gliadin and glutenin polymers that were found in the present study to be partly soluble (by 45-50%) in an ethanol/water solvent (50/50, v/v). Even if gliadin was the main class of wheat storage proteins solubilized, the extract also included 30% of glutenin polymers. Their molecular weight distribution range did not differ from the ones of the SDS-soluble glutenin polymers from wheat flours. The gluten/solvent ratio (10-20 g/100 mL) and the ethanol content (48 to 66 %, v/v) of the solvent did not significantly impacted the extraction yield and composition of the extract. Due to the low solid/solvent ratio chosen (10 g/100mL) the obtained soluble extract was not very concentrated (~45 g/L), a situation very favorable for polymer fractionation by liquid-liquid phase separation (LLPS) induced by a temperature quench. We showed that dense phases substantially enriched in glutenin polymers could be recovered by applying shallow temperature quenches, a few degrees below the cloud temperature of the extract (T_{cloud} = 14°C). Because of the very unbalanced volumes of the coexisting phases, fractions of high protein content (up to 384 g/L) and of tunable glutenin over gliadin ratio (from 0.5 to 2.5) could be recovered in a few hours. The fractionation method paves the way for a rigorous

assessment of the impact of glutenin polymers content on the rheological properties of gluten proteins.

Thermodynamic insights on gluten protein LLPS were examined at the level of each gluten protein classes. We found that the partitioning of the glutenin polymers between the rich and poor phases varied exponentially with their molecular weight, consistently with the lattice theory model introduced by Flory (Flory, 1994). Contrarily to what was observed for the α/β and γ-gliadins undergoing LLPS, the partial specific volume of the glutenin polymers varied whether they settled in the poor or in the rich phase and the difference widened when the quench depth increased. The change in the partial specific volume of the glutenin polymers during LLPS would be related to their involvement in highly swollen protein assemblies. It could even play a role in relation to the rising idea that de-solvation entropy drives liquid-liquid phase separation of proteins (Harmon, Holehouse & Pappu, 2018; Park et al., 2020).

Thanks to the analysis of the temperature dependency of the enthalpic and entropic contributions of the Flory-Huggins interaction parameters, we were able to predict the critical temperatures of the different gluten protein classes. We showed that interactions between α/β and γ-gliadins and glutenin polymers caused the accumulation of the former in the rich phase, while the quench temperature was above the critical temperature of gliadins. At lower temperatures, for deeper quench depths ($\Delta T>9°C$), we demonstrated that the α/β- and γ-gliadins remaining in the parent phase reached a metastable state and in turn phase separated, as individual sepcies. Thus within the rich phase droplets already formed at shallow quench depths, further decomposition might occur leading to the internal clustering of the gliadins. The glutenin concentration in the protein rich phase was found to plateau at about 143 g/L for quench temperature below 9°C. In our opinion, this would coincide to the critical glutenin polymer concentration leading to gelation, irrespective of the glutenin/gliadin ratio. Whether the transition from critical to viscoelastic gel occurred in the course of the present LLPS experiments, performed at a relatively slow quenching rate would deserve further exploration. Overall, fine-tuning of gluten protein LLPS in aqueous ethanol solutions might be a way to prepare micro-sized droplets with heterogeneous internal structure. This could open the gate to a better understanding of formation of cereal storage protein bodies, which may show (maize, sorghum) or not (wheat) a spatial gradient in the storage proteins distribution from the core to the periphery or even inner droplets of specific composition (Lending, Kriz, Larkins & Bracker, 1988). The interest in LLPS of storage proteins is a worthwhile cause, inasmuch that internal structure of protein bodies impact cereal protein digestibility (Duodu at al., 2002; Oria, Hamaker, Axtell & Huang, 2000).

Author contributions

M.-H. Morel: Supervision, Conceptualization, Methodology, Visualization, Resource, Formal analysis, Writing-original draft & editing J. Pincemaille: Investigation, Visualization L. Lecacheux: Investigation P. Menut: Funding acquisition, Supervision, Conceptualization, writing-reviewing. L. Ramos: Funding acquisition, Supervision, Conceptualization, Writing-reviewing. A. Banc: Conceptualization, writing-reviewing

Visualization : Preparation, creation and/or presentation of the published work, specifically visualization/ data presentation

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