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**SDS-insoluble glutenin polymer formation in developing  
grains of hexaploid wheat : role of the HMW-GS/LMW-GS ratio  
and the drying rate during ripening.**

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ABBREVIATIONS USED : DAA = days after anthesis ; DTT = dithiothreitol ; HMW-GS = high molecular weight glutenin subunits ; LMW-GS = low molecular weight glutenin subunits ; RP-HPLC = reversed phase high performance liquid chromatography ; SE-HPLC = size exclusion-high performance liquid chromatography ; SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis ;

### Abstract

The accumulation of polymeric proteins and the changes in molecular size distribution of these proteins were followed during grain filling and/or premature desiccation. The accumulation behavior of polymeric proteins and their constituent polypeptides (high molecular weight and low molecular weight glutenin subunits) was determined by RP-HPLC using a NaI/propanol purification procedure. By using this new extraction and separation procedure, we have demonstrated that there was a coordinated initiation of storage protein biosynthesis even if the accumulation rate varied greatly between the two main classes of proteins (i.e., monomeric and polymeric fraction). Moreover, the glutenin subunit composition was largely modified during glutenin accumulation. Both the HMW-GS/LMW-GS and HMW-GS-x/HMW-GS-y ratios increased significantly during the whole cell enlargement phase (from 16 to 37 DAA). By applying premature grain desiccation during this physiological phase, we demonstrated that the polymerisation index (SDS-insoluble polymers/total polymers) of the glutenin polymers was closely related to the HMW-GS/LMW-GS ratio of these proteins. An increase in the relative proportion of HMW-GS in glutenins caused the proportion of SDS-insoluble polymers to rise during grain desiccation. From these studies, it appears that the modification of the desiccation rate (grain desiccation at a constant temperature with variable relative humidity levels) induced a parallel modification of the glutenin insolubilisation rate but did not affect the polymerisation index of the glutenins at maturity.

## Introduction

The polymeric glutenin fraction constitutes about 35-45 % of the total proteins in wheat kernels. These proteins are formed by polypeptide chains linked by disulfide bonds (Shewry and Tatham 1990), and lead to large molecules with molecular weights ranging from a few hundred thousand to many millions (Shewry *et al.* 1986 ; Pomeranz 1988). It is now understood that this polymeric fraction governs most of the technological parameters of wheat flours (Jia *et al.* 1995 ; Gupta *et al.* 1992), and particularly the strength and the elasticity of dough (Wall 1979 ; Bietz and Huebner 1980). The quantities (Gupta *et al.* 1992), the size distribution (Gupta *et al.* 1993) and the SDS-solubility of the polymers (Jia *et al.* 1996) determine the breadmaking quality of flours. The size distribution of these glutenin polymers is controlled to a large extent by the genotype with respect to the composition in high and low molecular weight glutenin subunits (HMW-GS and LMW-GS) of glutenins (Gupta and MacRitchie 1994 ; Cornec 1994), leading to a differential solubility of the polymers, in relation to processing quality (Jia *et al.* 1996). The solubility of the glutenin fraction in various extracting systems (acetic acid, SDS...), and more particularly the proportion of the unextractable polymeric fraction represent major criteria for wheat quality.

Although correlations between these polymers and breadmaking quality are well known now, only a few papers have reported information about the SDS-insoluble glutenin polymer formation during grain filling (e. g. Bénétrix *et al.* 1994 ; Johansson *et al.* 1994 ; Gupta *et al.* 1996 ; Stone and Nicolas 1996) and furthermore, no definite conclusion has been reached about the insolubilisation mechanism. However, in a previous paper (Carceller and Aussenac 1999a), we found a close correspondence between the formation of the SDS-insoluble polymers and the rapid loss of water (grain desiccation) both in natural and artificial conditions. Indeed, by

applying premature grain desiccation during the cell-enlargement phase, we demonstrated that grain dehydration induces the deposition of the SDS-unextractable polymers whatever the genotype studied. These results contradict the hypothesis of Gupta *et al.* (1996) according to which SDS-insoluble polymer formation would be induced only when a certain amount of total polymers was accumulated, i.e. 60-75%. In the same study, we demonstrated that the molecular-size distribution of the polymers, which could be used to differentiate genotypes, was highly correlated with the percentage of high molecular weight glutenin subunits (HMW-GS) in glutenins present in kernels when desiccation occurred. These results, which show the quantitative importance of the HMW-GS for the SDS-insoluble polymer formation, are in total agreement with the observations made by Gupta and MacRitchie (1994), Gupta *et al.* (1995) and Cornec (1994), from isogenic lines and translocations. Nevertheless, even though as we have already proved (Carceller and Aussenac 1999) grain dehydration induces the deposition of the SDS-unextractable polymers whatever the genotype studied, this phenomenon is not constant during the grain cell-enlargement phase (i.e. the quantity of SDS-unextractable polymers is not directly in correlation with the quantity of glutenins present at the time of dehydration). The question arises as to whether or not, for a genotype, these variations can be explained by the rate of synthesis and incorporation of HMW subunits. Furthermore, could modifications (i. e. modification of the desiccation rate) or dehydration conditions of the grain lead to variations in the level of the content in SDS-unextractable polymers ?

In order to answer these different questions, we followed the present work SDS-insoluble glutenin polymer formation during the cell-enlargement phase by applying artificial desiccation of the kernels to determine the role of the HMW-GS/LMW-GS

ratio for the glutenin insolubilisation mechanism. A new purification procedure for the glutenins (Fu and Kovacs 1999) was applied for HMW-GS and LMW-GS separation and quantification by RP-HPLC. At the same time, we studied the specific role of the drying rate of the kernels during ripening (i. e. grain desiccation at a constant temperature with variable relative humidity levels) in the SDS-insoluble polymer formation rate and in the molecular size distribution of the glutenin polymers by SE-HPLC.

## Material and methods

### *Plant material*

The wheat cultivar used in this study was Soissons, possessing *Glu-D1* subunits 5+10. This cultivar was grown at the experimental farm of ESAP Toulouse, France (harvests of 1998 and 1999). At the first day of anthesis, each spike was tagged and dated. Subsequently, 40 spikes were collected from the 5th DAA up to the 53rd DAA. The fresh weight of 40 grains was taken and then grains were oven dried at 80°C to a constant weight after which the dry weight was taken. The difference between these two weights gave the water content in mg.1000 kernels<sup>-1</sup>. The fresh grains were freeze-dried and ground (200µm) in a Janke A10 grinder.

For applying artificial desiccation of the kernels (harvest 1998), eight samples (15, 18, 21, 24, 27, 31, 34, and 38 DAA) were used. 40 fresh spikes were oven-dried at 30°C (a temperature normally recorded during the grain desiccation phase) until the grains reached a constant humidity and a constant relative loss of mass. The desiccated grains were freeze-dried and ground.

Samples harvested in 1999 were used to study the specific role of the drying rate of the kernels during ripening on the SDS-insoluble polymer formation rate and on the molecular size distribution of the glutenin polymers. Spikes were collected from 5 to 51 DAA and grains were treated as previously described. At 29 DAA, 250 g of fresh spikes (approximately 65 spikes) were desiccated in controlled environment chambers at 30°C above 40% (56.8% R.H.) or 20% (88.5% R.H.) (v/v) H<sub>2</sub>SO<sub>4</sub> solutions or over forced air (35% R.H. at 30°C) according to Gibson *et al.* (1998). H<sub>2</sub>SO<sub>4</sub>, which is strongly hygroscopic, was used to control the rate of water loss from the grain. For the H<sub>2</sub>SO<sub>4</sub> drying treatments, spikes were placed on perforated, porcelain plates above 1 L of H<sub>2</sub>SO<sub>4</sub> (ACS grade) solution enclosed in 250-mm i.d.

glass desiccators. Spikes dried with forced air were placed in a tray on the chamber floor. Spikes from each treatment were weighed each day to determine the rate of moisture loss and five spikes were collected, freeze dried and ground as previously described.

#### *Extraction and purification of the glutenins subunits*

Two different isolation procedures for HMW-GS and LMW-GS were tested.

In procedure 1 (Fu and Sapirstein 1996), ground kernel samples (0.5 g) were stirred for 1 h at room temperature (25°C) with 2.25 mL of 0.080M Tris-HCl buffer (pH 7.5) containing 50% (v/v) 1-propanol. Extraction was followed by centrifugation at 15 900 x *g* for 30 min at 15°C. Soluble glutenins in 50% 1-propanol were precipitated by the addition of 1.5 mL of 1-propanol to bring the 1-propanol concentration of the supernatant to 70% (v/v). The mixture was agitated and allowed to stand at room temperature for 1 h. After centrifugation (26 000 x *g*, 15 min at 15°C), the supernatant (mainly monomeric proteins) was discarded. The residue (polymeric proteins) was then extracted with 2.25 mL of the 0.080 M Tris-HCl buffer (pH 7.5) containing 50% (v/v) 1-propanol and 1% (w/v) DTT by continual stirring for 1 h at 60°C. The supernatant obtained after a centrifugation at 15 900 x *g* for 30 min at 15°C was filtered through a 0.45 µm filter.

In procedure 2 (Fu and Kovacs 1999), ground kernel samples (0.1 g) were stirred for 1 h at room temperature (25°C) with 2 mL of 0.3 M sodium iodide (NaI) in 7.5% (v/v) of 1-propanol. Extraction was followed by centrifugation at 15 900 x *g* for 15 min at 15°C. The supernatant, containing the monomers, was discarded or kept for SDS-PAGE analysis. The glutenins remained unextractable in the sodium iodide solution. The pellet was washed twice with the solvent of extraction and glutenins



were then extracted with 2 mL of the 0.080 M Tris-HCl buffer (pH 7.5) containing 50% (v/v) 1-propanol and 1% (w/v) DTT by continual stirring (60 rpm) for 1 h at 60°C. The supernatant obtained after centrifugation at 15 900 x g for 15 min at 15°C was filtered through a 0.45 µm filter.

#### *Separation and quantification of glutenin subunits by RP-HPLC*

Glutenin purified extracts (600 µL) (procedure 1 or 2) were alkylated with 40 µL of 71% (v/v) 4-vinylpyridine in 50% (v/v) 1-propanol buffered with 0.080 M Tris-HCl (pH 7.5) at 60°C for 20 min just prior to RP-HPLC analysis. Samples were then analysed using a PC 1000/Spectranet data and chromatography control station (Spectra Physics analytical software, San Jose, CA, USA) incorporating a solvent degasser, a solvent delivery system, an auto-sampler, and a heated column compartment maintained at 50°C for analyses. A Zorbax 300 SB-C8 (Supelco Inc., Bellefont, PA, USA) column (30 nm pore size, 5 µm particle size, 15 cm x 4.6 mm i.d.) was used in conjunction with a Zorbax 300 SB-C8 cartridge guard column (1.25 cm x 4.0 mm i.d.). Solvents for RP-HPLC were : A, deionized water filtered (0.1 µm); and B, acetonitrile (ACN, HPLC grade, 0.45 µm filtered). Both solvents contained 0.1% (v/v) trifluoroacetic acid (HPLC Grade, Sigma). Solvents flow rate was maintained at 1.0 mL/min. After an initial 3 min isocratic condition at 23% solvent B, reduced and alkylated glutenin subunits were eluted in a 82 min linear gradient from 23 to 44% solvent B. The column was equilibrated at 23% solvent B for 8 min between runs. The column effluent was monitored at 214 nm using a Spectra 2000 detector. Injection volumes were 30 µL in conjunction with a multiple 5 µL injection technique (Marchylo *et al.* 1989).

*Modeling the accumulation of the different protein fractions*

The quantification of total proteins was obtained by the extraction of 0.1 g of flour with 2 mL of 50% 1-propanol in Tris-HCl buffer (0.08 M, pH 7.5) containing 1% DTT (w/v) for 1 h at 60°C. The supernatant obtained after centrifugation contained total proteins and was submitted to RP-HPLC after alkylation asfor RP-HPLC. The quantity of monomers was determined by the difference between total proteins and glutenin polymers (in arbitrary units per kernel).

Total protein, polymer, and monomer content datas were fitted to logistic regression analysis (Equations 1, 2 and 3).

$$y = y_0 + \frac{a}{1 + \left(\frac{x}{x_0}\right)^b} \quad (1)$$

$$y' = \frac{-a}{\left[1 + \left(\frac{x}{x_0}\right)^b\right]^2} \times \frac{b}{x_0} \times \left(\frac{x}{x_0}\right)^{b-1} \quad (2)$$

$$y'' = \frac{-ab}{x_0} \times \left(\frac{x}{x_0}\right)^{b-2} \times \frac{1}{\left[1 + \left(\frac{x}{x_0}\right)^b\right]^2} \times \left[ \frac{-2b\left(\frac{x}{x_0}\right)^b}{1 + \left(\frac{x}{x_0}\right)^b} + (b-1) \right] \quad (3)$$

The calculation of the first derivative ( $y'$ ) allows us to determine the accumulation rate of the total protein, polymer and monomer content in the kernels. The calculation of the second derivative ( $y''$ ) gives us the number of days after anthesis at which the maximum accumulation rate was reached. This maximum correspond to the

theoretical value when  $y''=0$  that is  $(x_0 \times \left(\frac{b-1}{b+1}\right)^{\frac{1}{b}})$ .

*Quantification and measurement of relative size distribution of polymeric proteins by SE-HPLC*

Extraction of total proteins and separation by SE-HPLC was carried out as described by Carceller and Aussenac (1999a). The total polymeric protein was separated into two size fractions based on its extractability in 2% (w/v) SDS-phosphate buffer (without sonication), i.e. extractable (Fs) and unextractable (Fi) fractions. Ground kernel samples (0.01 g) were suspended in 1 mL of 0.1 M sodium phosphate buffer (pH 6.9) containing 2 % (w/v) SDS and stirred for 2 h at 60 °C. These extractions were followed by centrifugation during 30 min at 12 500 x g at 20°C to obtain a supernatant (Fs or extractable protein). The unextractable fraction (Fi) was then sonicated for 15 s at 50 % power (output 25 W, 23 kHz) in 2% SDS-phosphate buffer (1 mL) using a stepped microtip probe (3 mm diameter) to solubilize the remaining protein (unextractable proteins). Both extracts were filtered through 0.45 µm filters and then injected (20 µL) onto a TSK G 4000 SW (TOSHAAS, Germany) size exclusion analytical column (7.5 x 300 mm, 45 nm). The HPLC apparatus was a Spectra SYSTEM LC (Thermo Separation Products S.A., Les Ulis, France) consisting of an online degasser (model SCM 400), an isocratic pump (model P4000), a variable auto-injector (model AS3000) and a variable wavelength detector (model UV 2000). Sodium phosphate buffer (0.1 M, pH 6.9) containing 0.1% (w/v) SDS was used as eluent with a flow rate of 0.7 mL/min. During the fractionation, the column was thermostated at 25 °C. For the extractable fraction, the elution profile was divided into two main peaks corresponding to polymeric protein and monomers respectively according to Gupta *et al.* (1993). Since the polymeric protein fraction contained mainly glutenin (Jia *et al.* 1995), it was referred to previously as glutenin

and the monomer fraction as majority gliadins. The percentage of unextractable polymeric protein in total polymeric protein was calculated as follows :

$$\frac{\text{peak 1 area (extractable)}}{(\text{peak 1 area (extractable)} + \text{peak 1 area (unextractable)})} \times 100$$

The quantities of the different fractions (polymeric extractable proteins, polymeric unextractable proteins and monomers) were estimated by the absolute area of the fraction. There is indeed a very strong linear relationship between the amount of total protein in flour estimated by the Dumas method (AOAC 7.024) and the total area of the chromatogram for each flour sample ( $r^2=0,9$  ;  $n=165$ ) (Jia *et al.* 1996).

## Results

### *Accumulation of total proteins, polymeric and monomeric fractions in kernels*

Accurate quantification and characterization of glutenins require the isolation of total glutenins in samples without monomeric protein contamination. Propanol has been widely used as a solvent for monomeric flour proteins, usually at 50% concentration (Marchylo *et al* 1989 ; Singh *et al* 1991). However, under those conditions, a significant amount of glutenin is also removed along with the monomeric proteins and can be precipitated by increasing the 1-propanol concentration to 70% (Fu and Sapirstein 1996). Even if all the glutenins can be recovered by using this procedure (procedure 1 in Material and Methods), a small amount of monomeric proteins, mainly  $\omega$ -gliadins and some albumins and globulins, coprecipitate with the polymeric glutenins in 70% 1-propanol. This coprecipitation can be visualized by RP-HPLC (Fig. 1a and c).

A recent study (Fu and Kovacs 1999) demonstrated that there is a synergistic effect between NaI and 1-propanol in removing the contaminating  $\omega$ -gliadins from the glutenins. This synergistic effect is probably due to their different effects on the interactions between proteins ; 1-propanol would be expected to disrupt mainly hydrophobic interactions, while NaI would interfere with both hydrophobic and electrostatic interactions among proteins. The RP-HPLC (Fig. 1b) results showed that the NaI/propanol insoluble fraction (procedure 2 in Material and Methods) contained mainly polymeric proteins. Thus, the NaI/propanol method provides better separation of monomeric from polymeric proteins by single-step extraction. Consequently, the NaI/propanol procedure was retained in conjunction with RP-HPLC separations to follow the accumulation of the HMW-GS and LMW-GS in kernels.

To study the accumulation of total protein, polymeric and monomeric fractions in developing grains, we defined three characteristic growth phases according to Wang and Gifford (1995) : the cell division phase (from anthesis to 15-16 DAA), the cell enlargement and dry matter accumulation phase (from 16 to 37 DAA), and the grain dehydration or maturation phase (from 38 DAA to maturity). Figures 2*a*, *b* and *c* show the accumulation profiles of total proteins, monomer and polymer fractions respectively, obtained by RP-HPLC. The total proteins (Fig. 2*a*) regularly accumulated from anthesis to 38 DAA and remained almost constant at the end of the cell enlargement phase till maturity. The end of the total protein accumulation coincided with the beginning of the rapid loss of grain water (dehydration phase). During the grain filling period, an important variation of protein accumulation was observed. The maximum accumulation rate, derived from equations 2 and 3, was reached during the cell enlargement phase (20-21 DAA). After 19-20 DAA, the protein accumulation rate decreased slowly until maturity. These results are in total agreement with our previous results (Carceller 2000). After an initial lag phase (from anthesis to 10 DAA), the monomeric protein content (gliadins, albumins, and globulins) (Fig. 2*b*) increased gradually and reached a maximum level around 30 DAA (7 days before the end of the cell enlargement phase). For this fraction, the maximum accumulation rate, derived from equations 2 and 3, was reached some days after the maximum accumulation rate of the total proteins (i.e. 22 DAA). On the other hand, even if the accumulation of polymeric proteins (glutenins) began early in the grain development (from 10 DAA), it amplified during the last days of the cell enlargement phase (37-39 DAA) (Fig. 2*c*). This amplification of the accumulation pattern resulted in a tripling of the polymeric content. For this fraction the maximum accumulation rate derived from equations 2 and 3, was reached at the end of the cell

enlargement phase (around 35 DAA). Even if all the different proteins (monomeric and polymeric) were concurrently synthesized as early as 10 DAA in the grains, the accumulation of these fractions was highly asynchronous. There is indeed a highly significant difference between the two maximum rates of accumulation (monomers vs. polymers).

#### *Accumulation of HMW and LMW glutenin subunits*

It was then interesting to study the possible variation of glutenin composition during grain development by following the accumulation of the different types of glutenin subunit (HMW-GS and LMW-GS). RP-HPLC patterns of propanol-DTT extracts of NaI-propanol purified glutenins revealed that the HMW-GS (A subunits) and LMW-GS (B+C subunits) were synthesized concurrently as early as 10 DAA (Fig. 3a). These measurements indicated a general increase in the absolute amounts of LMW and HMW glutenin subunits with the grain development until the end of the cell enlargement phase (about 39 DAA). Maximum glutenin subunit accumulation rate was reached at 35 DAA in association with maximum accumulation rate of the polymeric fraction.

At the same time, the HMW-GS/LMW-GS ratio increased dramatically from about 10% to 35% during the cell enlargement phase (Fig. 3a) and reached a maximum at about 30 DAA (40%). After 30 DAA the proportion of high molecular weight glutenin subunits decreased down to 40 DAA (25%) and then remained constant to maturity. These results are in agreement with previous work (Carceller and Aussenac 1999, Zhu and Khan 1999). These results demonstrate that, for a genotype, the glutenin subunit composition did not remain constant during their accumulation in the grains. The accumulation of the HMW-GS was faster than that of the LMW-GS during the

cell division and enlargement phases. Moreover, we noticed significant variations between the relative proportion of the x and y HMW-GS types during the glutenin accumulation (Fig. 3b). The ratio x-type/ y-type increased gradually during the cell enlargement phase (from 16 to 37 DAA) and reached a maximum value at the beginning of the desiccation phase. The behavior of the two glutenin subunits HMW-x-5 and HMW-y-10 served to illustrate this phenomenon. The percentage of the HMW-GS-x-5 indeed increased from 17% to 27% during the cell enlargement phase while, during the same period, the percentage of the HMW-GS-y-10 decreased from 24% to 16%.

#### *SDS-insoluble polymer formation under pre-mature desiccation*

We demonstrated previously (Carceller and Aussenac 1999), that there is a close correspondence between the formation of the SDS-insoluble polymers and the rapid loss of water (grain desiccation) in both natural and artificial conditions. Indeed, by applying premature grain desiccations during the cell enlargement phase, we found that grain dehydration induced the deposition of the SDS-unextractable polymers whatever the genotype studied. The results obtained in the present study confirmed this. Fig. 4 shows the accumulation of SDS-insoluble glutenins in the kernels in relation to early desiccation compared to controls. Grain dehydration by ventilation at low temperature (30°C) led to the insolubilisation of part of the accumulated glutenins, whatever the stage of development of the kernels. The molecular size distribution of the polymeric fraction produced by artificial desiccation and estimated by the SDS-extractability criterion was not stable during the cell enlargement phase. Actually, the SDS-insoluble polymers/total polymers ratio increased regularly between 15 and 31 DAA from 17.6% to 30.4%. This SDS-extractability criterion



obtained at 38 DAA for early desiccation was not significantly different from the natural index at maturity (51 DAA) (30% versus 27% respectively). A relationship could be found between the HMW-GS/LMW-GS ratio reached naturally in grain and the polymerisation index obtained by applying artificial desiccation. Premature desiccation of kernels which were characterized by a variable HMW-GS/LMW-GS ratio (from 15 to 40%) indeed, led to a variable glutenin molecular size distribution (SDS-insoluble polymers/total polymers ratio from 17 to 30%).

#### *SDS-insoluble polymer formation under different rates of grain dehydration*

As we have seen before (Carceller and Aussenac 1999), the loss of water from the grain plays an important role in the mechanisms of the glutenin insolubilisation. It was then interesting to determine if the SDS-insoluble polymer formation could be modified by the rate of water loss from wheat kernels. For this experiment, we applied premature desiccation at 29 DAA at a constant temperature (30°C) with variable relative humidity to control the rate of water loss from the grain (see Material and Methods). At 29 DAA, wheat kernels were characterised by the absence of SDS-insoluble polymers under natural conditions. Figure 5a presents moisture loss (i. e. relative loss of mass) from excised spikes of the wheat cultivar Soissons under different humidity conditions. Figure 5b shows the evolution of the polymerisation index (SDS-insoluble polymers/total polymers) as a function of the number of days of dehydration for the three treatments. As previously observed, grain desiccation induced SDS-insoluble polymers formation which led to an increase of the polymerisation index. Furthermore, the SDS-insoluble polymer formation rate was closely related to the grain dehydration rate. Indeed, for each treatment, the polymerisation index was directly proportional to the kernel dehydration level. At the

same time, whatever the treatment applied (i.e. whatever the rate of water loss from kernels), the grain desiccation led to a constant polymerisation index (about 35%) even if this value was reached after different periods. Figure 5c shows the evolution of the polymerisation index as a function of the relative loss of water during the desiccation phenomenon. In this figure, all treatments are comparable if one leaves aside the differences of dehydration rates. SDS-insoluble polymer formation, resulting from the insolubilisation of a great part of the glutenins accumulated in the kernels, seems to be directly proportional to the water content (i.e. humidity percentage). For a given relative loss of mass, indeed, the different treatments (air dried, 56.8% R.H., and 88.3% R.H.) were characterized by the same SDS-insoluble polymers/total polymers ratio. For example, whatever the treatment, a relative loss of grain mass of about 30% induced in all cases the formation of the same amount of SDS-insoluble polymers.

## Discussion

In previous work (Carceller and Aussenac 1999), we demonstrated that in natural growth conditions, the insolubilisation of a great part of the glutenin fraction was closely related to the rapid loss of water from the grains. Therefore, this phenomenon can be simulated by applying premature desiccation whatever the physiological stage of the kernels and whatever the genotype studied. Finally, in the same studies, we showed that the molecular size distribution of the glutenin polymers (i.e. SDS-insoluble polymers/total polymers) which can be used to differentiate wheat genotypes was highly correlated with the percentage of high molecular weight glutenin subunits in the glutenins present in kernels when desiccation occurred. In the present work, we have studied the accumulation of glutenin subunits (HMW-GS and LMW-GS) during grain development and the influence of the water loss rate under premature desiccation in order to clearly understand the mechanism which leads to SDS-insoluble glutenin polymer formation.

To carry out these studies, we used a purification and quantification procedure based on NaI-propanol extractions in conjunction with RP-HPLC separations. By applying this procedure, we have shown that there was a coordinated initiation of storage protein biosynthesis, even if the accumulation rate varied greatly between the two main classes of proteins (i. e. monomeric fraction and polymeric fraction), indicating differential regulation of protein biosynthesis in developing wheat kernels (Gupta *et al.* 1996). Among the grain storage proteins, the glutenins indeed reached a maximum accumulation rate at the end of the cell enlargement phase (at about 37 DAA). Moreover, the glutenin subunit composition was largely modified during glutenin accumulation. We thus demonstrated that the HMW-GS/LMW-GS ratio

increases significantly during all the cell enlargement phase (from 16 to 37 DAA) and reaches a maximum at about 35 DAA. These results, which are in total agreement with the observations of Seilmeier *et al.* (1990) and Gupta *et al.* (1996) show that during glutenin accumulation in kernels of a wheat genotype, the two main classes of glutenin subunits (HMW-GS and LMW-GS) can also be characterized by different accumulation rates. Finally, among the HMW-GS, the relative proportion of the x-type subunits gradually increases during the cell enlargement phase (from 16 to 37 DAA). All these observations demonstrate that large modifications of glutenin composition can occur during their accumulation in the kernels of a wheat grain.

At this stage, it is interesting to relate these modifications of glutenin composition to SDS-insoluble polymer formation. Indeed, by applying artificial grain desiccation at different physiological stages which were characterized by variable glutenin subunit composition as seen previously, we observe the influence of HMW-GS and LMW-GS on the polymerisation index (SDS-insoluble polymers/total polymers). Even if the amount of SDS-insoluble polymers can logically be related to the total glutenin amount accumulated before dehydration occurs, the polymerisation index is not constant throughout the physiological stages. This index gradually increases during the cell enlargement phase (from 16 to 35 DAA) and can be closely related to the modifications of the HMW-GS/LMW-GS ratio. An increase of the relative proportion of HMW-GS in glutenins induces an increase of the proportion of SDS-insoluble polymers during grain desiccation. These results, which show the qualitative importance of HMW-GS for the SDS-insoluble polymer formation, are in total agreement with the observations made by Gupta and MacRitchie (1994), Gupta *et al.* (1995), Cornec (1994) and Carceller and Aussenac (1999) from isogenic lines, translocations or varied genotypes. Even if LMW-GS can polymerize alone (Gao and

Bushuk 1993), a quantitative deficit in high molecular weight glutenin subunits significantly reduces the proportion of high-mass polymers (SDS-insoluble polymers). Moreover, even if in our study we could not identify the specific contribution of individual HMW-GS to the formation of the SDS-insoluble polymers, we can still indicate the significant variations of the x-type/y-type ratio in the HMW-GS. These results, which demonstrate the major role of HMW-GS and/or x-HMW-GS type in the formation of the SDS-insoluble polymers are consistent with the observations made by Lindsay and Skerrett (1998). These authors, using stepwise reduction to examine the structure of SDS-unextractable glutenins, suggested that these polymers have a well-ordered structure in which some HMW-GS (particularly HMW-GS 1Dx5) play a major role in forming the backbone to them. Kasarda (1999) has indeed proposed a hierarchical arrangement of the glutenin subunits that represents their relative potentials for glutenin polymer formation. This hierarchical arrangement of glutenin subunits in which HMW-GS-x are defined as the most important constituent, is based on a combination of two factors relating to (a) the potential of a subunit to form branched polymers, as opposed to linear polymers, and (b) the length of the repeating sequence domain.

During recent years, high temperature during grain filling (Blumenthal *et al.* 1991, 1995; Stone and Nicolas 1994, 1995; Uhlen *et al.* 1998) and rapid desiccation during kernel ripening (Gibson *et al.* 1998) have been evoked as major factors that alter the quality of wheat. Several hypotheses have been proposed to explain these effects (Blumenthal *et al.* 1998). In the present study, we studied the effects of the dehydration rate on the SDS-insoluble polymer formation. By applying premature desiccation at 29 DAA (when wheat kernels are characterized by the absence of

SDS-insoluble polymers under natural conditions) at a constant temperature (30°C) with variable relative humidity levels, we have demonstrated that the glutenin insolubilisation phenomenon (SDS-insoluble formation) was not modified by the different treatments. Indeed, even if the rate of the SDS-insoluble polymer formation is closely related to the rate of water loss from the grain, no significant differences can be observed both in the total amount and the polymerisation index (SDS-insoluble polymers/total polymers) of these polymers at the end of the desiccation experiments. From these results, it appears that a modification of the desiccation rate induces a parallel modification of the glutenin insolubilisation rate but does not affect the polymerisation index of the glutenins at maturity. We can thus conclude that if some quality alterations have been noted by some authors in response to natural modifications of the grain desiccation rate (Gibson *et al.* 1998), these alterations must be attributed to other chemical or physical relationships among the chemical constituents.

From our results, it appears that the polymeric distribution obtained at maturity is directly influenced both by the HMW-GS/LMW-GS ratio and the nature of the glutenin subunits (HMW-GS-x vs. HMW-GS-y) accumulated when desiccation occurs. Our results, which are in close agreement with previously published data (Seilmeier *et al.* 1990, Gupta *et al.* 1996, Zhu and Khan 1999), suggest that a switch in mechanism in the rate of synthesis and incorporation of HMW-GS to form polymers determines the size distribution of glutenin polymers confirming in this the hypothesis of Zhu and Khan (1999) whatever the genotype studied. Thus, at the end of this study it appears that all environmental factors which are susceptible to play a part in the synthesis and accumulation of glutenin subunits may be responsible for great modifications in

the molecular distribution of glutenins (i.e. polymerisation index). These include nitrogen and sulfur fertilizer levels (MacRitchie and Gupta 1993) and the effect of temperature during grain filling (Ciaffi *et al.* 1995, Blumenthal *et al.* 1998). However, as a result of this study it appears that even if grain desiccation induces the SDS-insoluble polymer formation in the grain, a modification of the water loss rate does not affect the polymerisation index obtained at maturity.

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## Captions

**Fig. 1** Reversed-phase high-performance liquid chromatography (RP-HPLC) of reduced and alkylated grain proteins of Soissons. (a) 50% 1-propanol insoluble glutenins and precipitated glutenins of 50 % 1-propanol soluble fraction obtained by increasing the 1-propanol concentration to 70 %, (b) NaI/propanol insoluble fraction (glutenin polymers), (c) NaI/propanol soluble fraction (monomeric proteins). Arrows indicate the contamination by some monomeric proteins.

**Fig. 2** Total protein (a) monomer (b) and total polymer (c) accumulation during the grain filling period. ( $\Delta$ ,  $\circ$ ,  $\square$ ) observed data for total protein, monomer, and total polymer accumulation respectively. Lines represent fitted data to logistic regression analysis (Equation 1). ( $\blacktriangle$ ,  $\bullet$ ,  $\blacksquare$ ) accumulation rate (Equation 2) for total proteins, monomers, and total polymers respectively. Bars are l.s.d. values at 5% probability.

**Fig. 3** Accumulation of the high (HMW-GS) and low molecular weight (LMW-GS) glutenin subunits (a), and x and y type of HMW-GS (b) per kernel as a function of the days after anthesis. ( $\bullet$ ) HMW-GS, ( $\blacksquare$ ) LMW-GS, ( $\Delta$ ) HMW-GS/LMW-GS ratio, ( $\square$ ) HMW-GS-1Dx5, ( $\circ$ ) HMW-GS-1Dy10, and ( $\blacktriangle$ ) HMW-GS-x/HMW-GS-y ratio. The vertical bars represent the standard deviation ( $n = 3$ ).

**Fig. 4** Quantity of unextractable polymers as a function of the day after anthesis. ( $\square$ ) unextractable polymers of control, and ( $\blacksquare$ ) unextractable polymers of desiccated. The numbers on the bars represent the polymerisation index (SDS-unextractable polymers/total polymers). Bar is l.s.d. value at 5% probability.

**Fig. 5** Modification of the formation of the SDS-unextractable polymers under premature desiccation at constant temperature (30 °C) with variable relative humidity (R.H.). (a) Relative loss of grain mass (%) as a function of relative humidity. (b) SDS-unextractable polymer accumulation as a function of relative humidity. (c) SDS-unextractable polymers accumulation as a function of the relative loss of grain mass (%). (●) air dried, (▲) 55% R.H., and (□) 86.5% R.H.. The vertical bars represent the standard deviation ( $n = 3$ ).

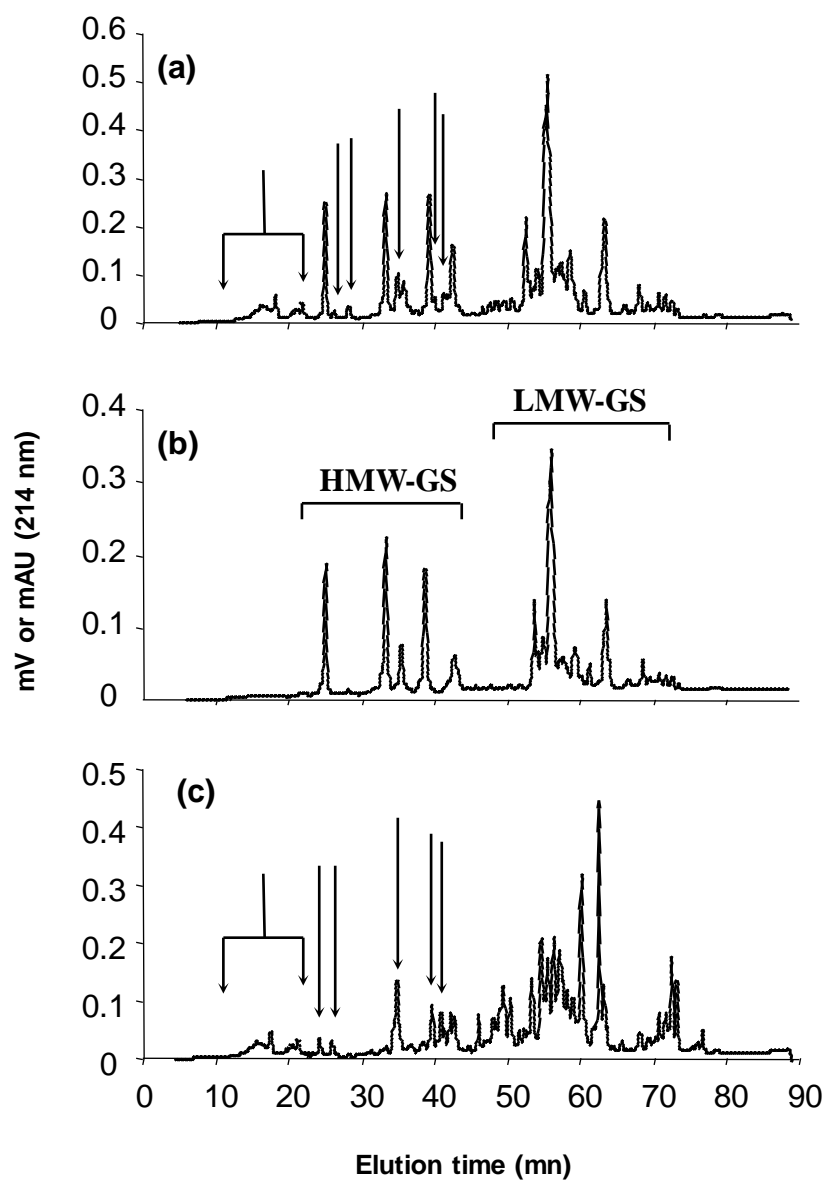


Fig. 1

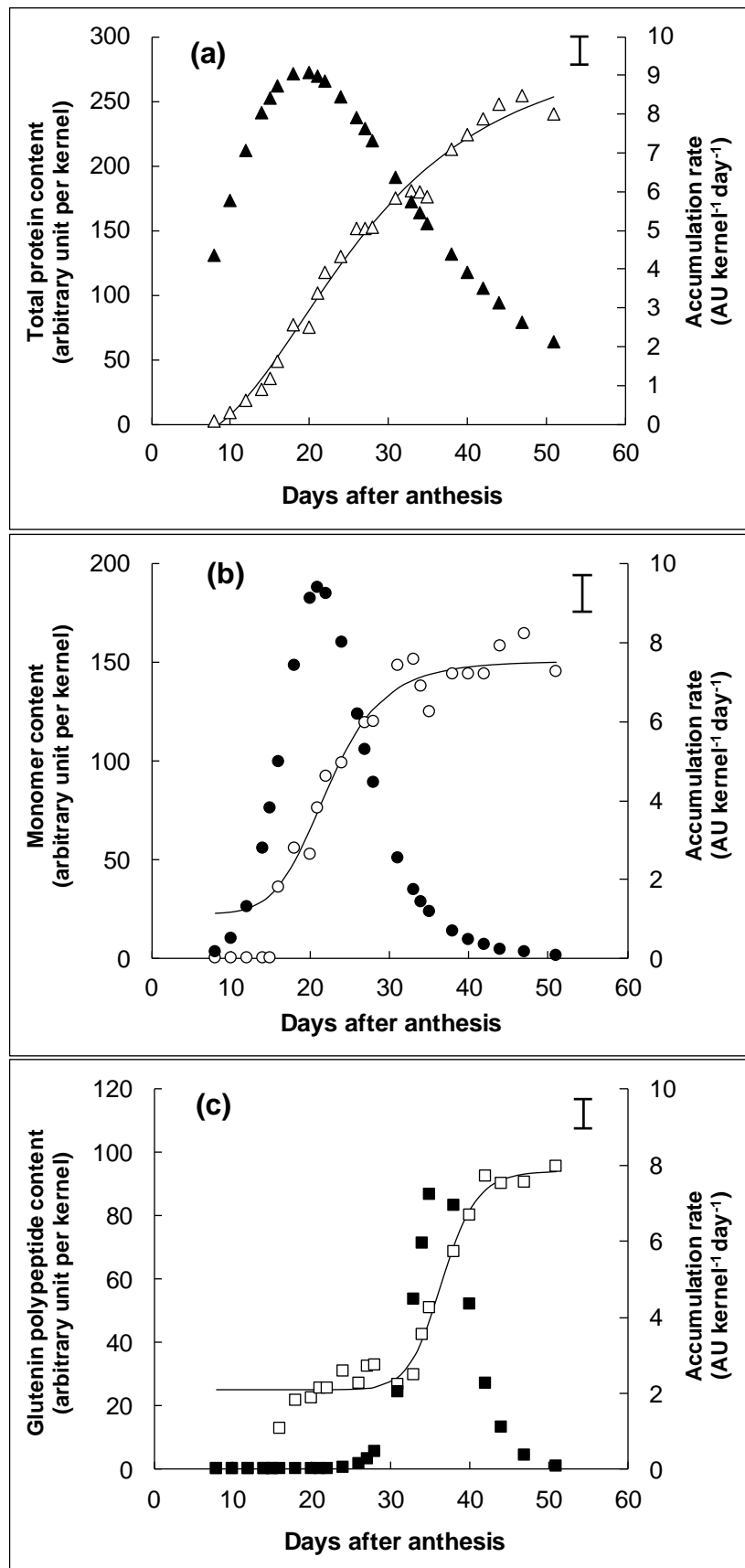
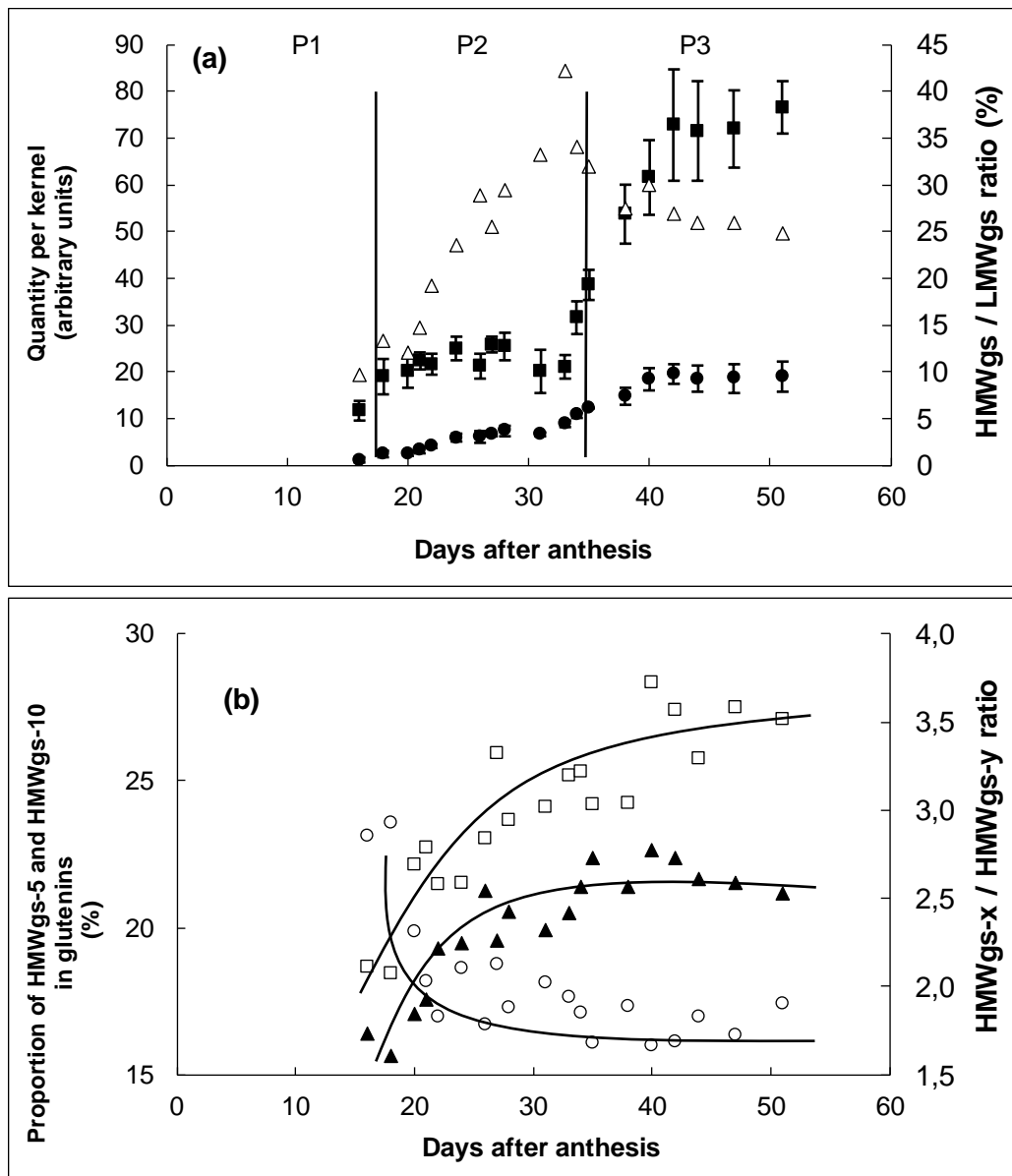


Fig. 2





**Fig. 3**

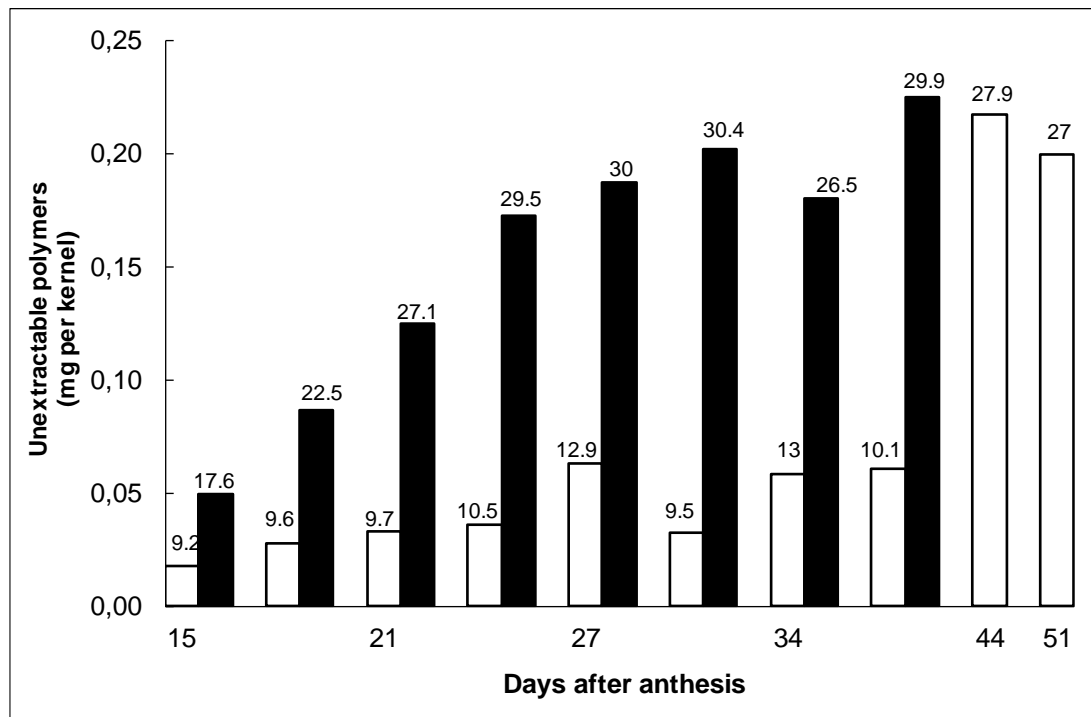


Fig. 4

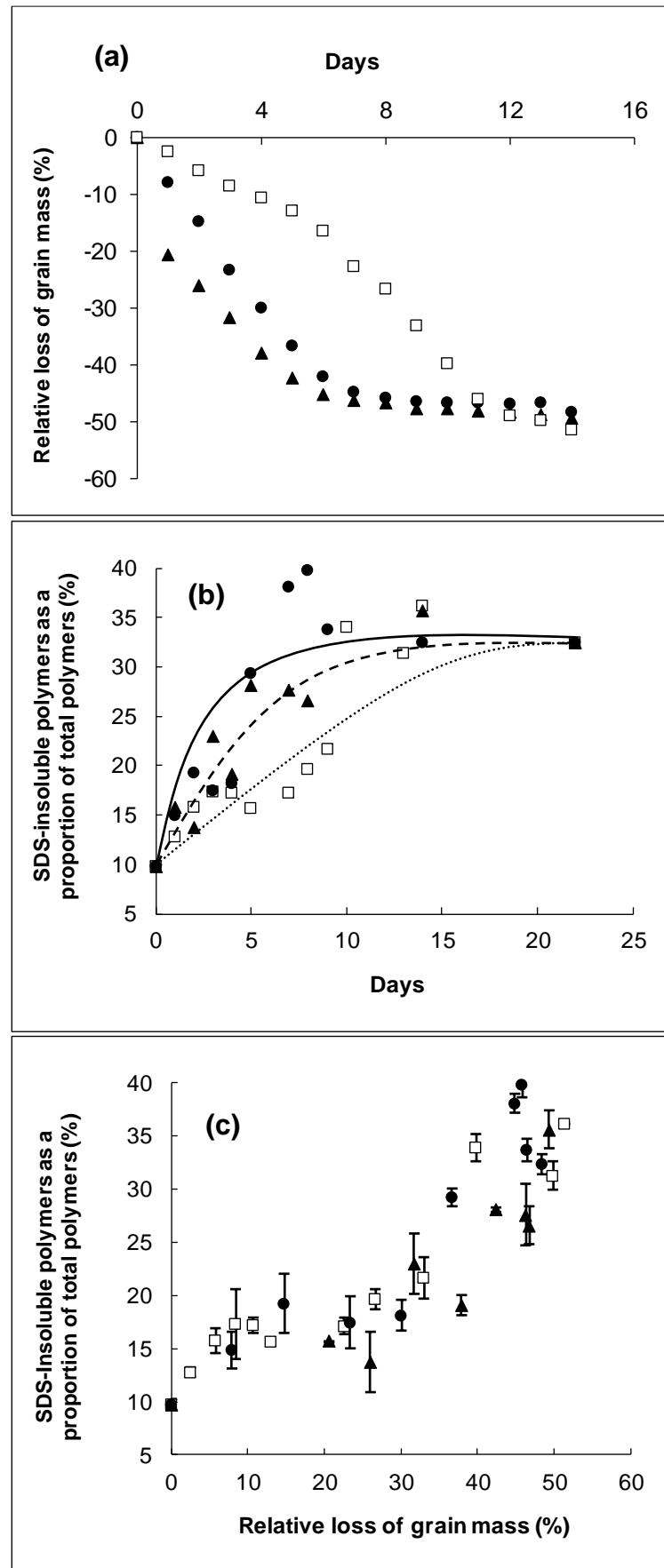


Fig.5