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A MALDI-TOF based study of the in-vivo assembly of glutenin polymers of durum wheat

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ABSTRACT

Wheat grain is recognized as the most suitable raw material for bread and pasta making due to the unique viscoelasticity of its storage proteins, and in particular its glutenin polymers. During grain development, low molecular weight (LMW) and high molecular weight (HMW) glutenin subunits (GS) gradually assembled through inter-chain disulfide bonds. Despite the impact of the final glutenin polymer size distribution on wheat technological quality, little is known concerning the oxidative folding of GS. In particular, which of their cysteine (Cys) residues participate to GS inter-chains remains putative. In this study GS from immature *Triticum durum* wheat grain were separated (1D SDS-PAGE) and digested into peptides, which were analyzed by MALDI-TOF/TOF. Differential alkylation of free and disulfide bonded Cys allowed us to identify the oxidative folding state of five types of LMW-GS and of the 1Bx20 HMW-GS. GS as monomers or as part of small oligomers carried a high number of free cysteine residues. In addition, some Cys residues, hitherto assumed as involved in intra-chain disulfide bonds, appeared simultaneously in free and oxidized forms. The last result could be assigned to their partial-blocking by glutathione. We concluded that the complete oxidative folding of LMW-GS is a late event, subsequent to GS inter-chain pairing.

1. Introduction

Amongst the cereal grain species produced and consumed by human beings, wheat is the only one easily transformed into pasta and leavened baked products because of the unique viscoelastic properties of its storage proteins. The latter includes monomers (gliadin) and polymers (glutenin) with a roughly 50:50 weight distribution. Polymeric structure of glutenin arises from the disulfide bonding of low molecular weight (LMW) and high molecular weight (HMW) polypeptides, herein referred as glutenin subunits (GS). GS typically carry 2 to 8 cysteine (Cys) residues including two to three that would form inter-chain linkages. In contrast, Cys of gliadin polypeptides are in an even number and all involved into stable intra-chain disulfide bonds.

The formation of inter-chain disulfide bonds between GS is a post-translational event essential for their assembly into polymers. Nevertheless in wheat, the relationship between the functionality and structure of these polymers is still poorly understood. Cereal chemists agree that the number and position of Cys within the polypeptide primary sequence should impact GS ability to form inter-chain disulfide bonds, whereas relative proportion of the different GS should affect the final polymer structure. Hence, the number of Cys per GS able to form inter-chain disulfide bonds will decide on the linear or branched structure of glutenin polymers. Despite the impact of the glutenin polymer size distribution on wheat technological quality (Naeem & MacRitchie, 2005; Popineau et al., 1994), the arrangement of inter-chain pairing of cysteine and their position in the GS sequences are largely ignored.

Unraveling the intimate architecture of glutenin polymers is a challenging goal since GS include numerous polypeptides sharing strongly homologous sequences. Indeed, while HMW-GS include a small number

Abbreviations: 1D SDS PAGE, One dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis; 4-VP, 4-Vynil pyridylethyl; ACN, Acetonitrile; Cys, Cysteine; Cys-CAM, Carbamidomethyl cysteine; Cys PAM, Propionamide-cysteine; Cys-PE, 4 Vynil pyridylethyl-cysteine; Cys-NEM, N-ethylmaleimide-cysteine; DHB, Dihydroxybenzoic acid; HCCA, a-Cyano-4-hydroxycinnamic acid; HMW-GS, High molecular weight glutenin subunits; IAM, Iodoacetoamide; INRA, National Institute for Agricultural Research; LMW-GS, Low molecular weight glutenin subunits; MALDI-TOF, Matrix assisted laser desorption ionization time of flight; M_r, Relative molecular mass; MS, Mass spectrometry; NEM, N-ethylmaleimide; PMF, Peptide mass fingerprinting; PSH, Protein sulfhydryl groups; RP-HPLC, Reversed phase-high performance liquid chromatography; SA, Sinapinic acid; SE-HPLC, Size exclusion high performance liquid chromatography; TFA, Trifluoroacetic acid.

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of easily identifiable polypeptides of high molecular weight (70,000–90,000), LMW-GS consists of a multigenic family of numerous proteins of smaller polypeptides (20,000–45,000) that overlap gliadin, making difficult their proper identification. The allopolyploïd nature of the wheat genome which leads to orthologous loci further complicates the scenario.

The most detailed investigations about the mapping of GS disulfides bonds were carried out by H. Wieser and collaborators (Keck et al., 1995; Köhler et al., 1993; Müller et al., 1998). They submitted isolated glutenin from mixed water/dough to partial hydrolysis, hence identified cystine containing peptides by differential Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) analysis under reduced or non-reduced conditions, and finally submitted them to automated Edman degradation. The amino-acid sequences of peptides were then assigned to known GS sequences. From this huge work, maps of Cys pairing were proposed and disulfide bond formation assumed as highly targeted (Müller et al., 1998). In particular, it was established that LMW-GS contain two Cys that are likely to be involved in an inter-chain disulfide bond, i.e. the first (C^b) and the seventh (C^x). Amino-acid sequence analysis showed that these residues are surrounded by flexibility areas, which could promote the glutenin polymerization (D'Ovidio & Masci, 2004; Masci et al., 1998). The other six Cys, common to most of LMW-GS are expected to be involved in intra-chain disulfide bonds.

To the best of our knowledge, since the 2000s and the introduction of modern proteomic technology, no additional data has been released on that topic, despite the powerfulness of mass spectrometry (MS) for establishing disulfide bond mapping in proteins (Alvarez et al., 2009; Bloom et al., 2002; Foley et al., 2008; Schnaible et al., 2002; Zhang & Kaltashov, 2006).

Another approach addressing glutenin disulfide pairings concerns the study of native or modified GS expression in transgenic plants (Shani et al., 1992). The expressed polypeptides were characterized by SDS-PAGE under reduced or non-reduced conditions, and then detected by using GS specific anti-sera (Lombardi et al., 2009; Shani et al., 1992, 1994). Several oligomer configurations were observed and they concluded that inter-chain disulfide pairings of GS lack of specificity. Additionally, an intra-chain disulfide bonds between two highly conserved Cys (C^c–C^{f1}) of LMW-GS was found mandatory to prevent aberrant GS aggregation (Lombardi et al., 2009).

More recent approaches focused on the biochemical events accompanying glutenin polymerization during wheat grain development (Ferreira, Martre et al., 2012; Ferreira, Samson et al., 2012; Rhazi et al., 2003; Shewry et al., 2009). Protein sulfhydryl groups (PSH) from immature grains were labeled by specific fluorescent probes and storage proteins were identified by SDS-PAGE and RP-HPLC (De Gara et al., 2003; Ferreira et al., 2009; Gobin et al., 1997; Rhazi et al., 2003). Only glutenin, in both monomeric and polymeric form, was labeled indicating that gliadin polypeptides readily undergone oxidative folding.

For *Triticum aestivum*, the relative amount of fluorescent labeled GS highly increased during the enlargement phases of grain development. The fluorescence associated to GS rapidly decreases after the onset of the desiccation phase. At that time, the rate of glutenin polymer formation further increased but above all large SDS-insoluble glutenin polymers (De Gara et al., 2003; Gobin et al., 1997; Rhazi et al., 2003). In contrast, at the first phases of *Triticum durum* grain development, glutenin polymer accumulation increased as PSH content dropped. Furthermore, polymer accumulation coincidently stopped with the onset of the grain desiccation phase, during which only slight increase in the mean size of glutenin polymers was observed (Ferreira, Samson et al., 2012; Ferreira et al., 2009).

These contrasted results point to different glutenin polymer assemblies for *T. aestivum* and *T. durum*. Both species differed in respect to their LMW-GS/HMW-GS ratio, in *T. durum*, LMW-GS accounts for 80 to 90% of the total glutenin, compared with 55 to 75% in *T. aestivum*. Due to their tetraploid nature, durum specie includes a lower number of GS variants and can be considered as prime candidate to establish the dynamic of LMW-GS assembly into glutenin polymers.

In the present study we focused on the redox state of nascent LMW-GS and in particular on the intra-chain disulfide bonds. The rapid oxidative folding of GS would leave in the reduced state only the Cys involved in inter-chain disulfide bonds. Hence, a directed GS inter-chain pairings could be inferred. To gain a deep insight into the dynamic of LMW-GS assembly into polymers, a Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) in tandem analysis was used to investigate the redox state of storage proteins from immature durum wheat grains. Based on the differential alkylation of the glutenin Cys, the mass spectrometry analysis allowed, not only the identification of storage proteins and the oligomers composition, but also the redox status of cysteine from newly synthesized GS. Glutenin polymer formation is discussed in relation to the oxidative folding of the nascent subunits.

2. Material and methods

2.1. Plant materials

Cultivar Orlu (*Triticum turgidum* L. var. durum Desf.) possessing HMW-GS 1B 20x + 20y and expressing LMW-GS type 2 was grown in INRA experimental fields (Mauguio, Southern France). Thermal time accumulation (°Cd) was calculated from daily average temperature hourly recorded. Several immature spikelets were harvested at 330 and 470 °C days after anthesis (°Cd). Grains were sampled, freeze-dried and protein sulfhydryl groups (PSH) (typically about 3 μmol/g grain) was determined as described in Ferreira, Samson et al. (2012b).

2.2. Strategy

An overview of the strategy used to identify the PSH status of GS and oligomers is given in Fig. 1. Total or sequentially extracted protein from immature grains were alkylated with iodoacetamide (IAM) allowing the conversion of reduced cysteine to Cys-CAM. Protein were sequentially fractionated in various solvents and separated by SDS-PAGE under non-reducing condition. Protein bands of interest were submitted to in-gel reduction and alkylation with 4-vinyl-pyridine (4-VP) to label the cysteine released from the disulfide bonds (Cys-PE). After trypsin digestion, cysteine-containing peptides (Cys-CAM and Cys-PE) were identified by MALDI-TOF/TOF through mass changes associated with the different alkylating agents used.

2.3. PSH alkylation and extraction of proteins

All steps were carried out in dark and at room temperature (RT). Centrifugations were performed at 10,600 ×*g* during 10 min at 20 °C unless otherwise specified. Modification of PSH was performed at 100 mg/mL on 20 mg of ground grains placed within Eppendorf tubes.

2.3.1. PSH alkylation

Alkylation was performed using large molar excess of reactants, i.e. 185 mM iodoacetamide (IAM) in 80 mM Tris–HCl buffer (pH 8.0) or 20 mM N-ethylmaleimide (NEM) in 80 mM sodium phosphate buffer (pH 6.8) eventually supplemented with 2% SDS. Samples were shaken for 60 min. Alkylated samples were subjected to a sequential extraction, whereas SDS soluble samples were used to the total protein extraction. Based on the different effectiveness, IAM was used at a 9.5 greater concentration than NEM (Rogers et al., 2006).

2.3.2. Extractions

Alkylated samples were immediately centrifuged to recover the total SDS-soluble proteins (in the presence of 2% SDS) or the buffer soluble protein fraction. For the latter, pellets were suspended with 200 μL of 70% (v/v) ethanol (EtOH) and agitated for 15 min. EtOH soluble proteins were then recovered by centrifugation. The remaining pellet was