Theor Appl Genet (2006) 113:1295–1303
DOI 10.1007/s00122-006-0383-2

ORIGINAL PAPER

Functional properties of a new low-molecular-weight glutenin subunit gene from a bread wheat cultivar

H. Xu · R. J. Wang · X. Shen · Y. L. Zhao · G. L. Sun · H. X. Zhao · A. G. Guo

Received: 21 September 2005 / Accepted: 30 July 2006 / Published online: 24 August 2006
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Abstract Some allelic forms of low-molecular-weight glutenin subunit (LMW-GS) can greatly influence the end-use of wheat flours, understanding the function of each allele of LMW-GS is important to wheat quality breeding. A LMW-GS gene *XYGluD3-LMWGS 1*(AY263369) has been cloned from bread wheat cultivar Xiaoyan 6. The deduced protein contained nine cystine residues, one more than that in all other LMW-GSs reported previously, indicating that it is either a new gene or a new allele of a known LMW-GS gene. In this study, the gene was expressed in *E. coil* in large scale for the testing of its functional property. Reactive Red 120-Agarose resin was used efficiently to purify the expressed LMW-GS proteins from bacteria, with the lactic acid–sodium lactate buffer (pH 4.5) which contained low concentration SDS as elution solution. The purified protein (belonging to the LMW-m family, MW about 35 KDa) was supplemented into a base flour, the results of 10 g dough mixing test indicated that incorporation of the LMW-GS increased the strength of the dough, with significant increases in mixing time (MT) and peak width (PW), and decrease in breakdown in resistance (RBD) compared with the control. In addition, the dough with incorporation of the LMW-GS had more glutenin macropolyeric protein than the control, suggesting that the LMW-GS participated in forming larger glutenin polymers, and greatly contributed to dough strength. The changes in mixing parameters and the amount of glutenin macropolyeric protein were related to the quantity of incorporating subunits.

Communicated by M. Morell

H. Xu · R. J. Wang · X. Shen · Y. L. Zhao · H. X. Zhao (✉) · A. G. Guo (✉)
College of Life Sciences,
Northwest A & F University, Yangling,
Shaanxi 712100, People's Republic of China
e-mail: hxzhao212@yahoo.com.cn

A. G. Guo
e-mail: Guoaiguang@yahoo.com.cn

G. L. Sun
Department of Biology, Saint Mary's University,
Halifax, N S, Canada, B3H 3C3

H. X. Zhao · A. G. Guo
Key Laboratory of Agricultural Molecular Biology
of Shannxi Province, Yangling, Shaanxi 712100,
People's Republic of China

Introduction

The wheat endosperm is a major component of the human diet largely due to the unique physical properties of wheat flour water mixtures (Pomeranz 1988). The quality of wheat flour for bread making depends on the visco-elastic properties of the dough, which are influenced by the quantity and quality of the gluten-forming storage proteins of the endosperm. These proteins consist of two classes, gliadins, and glutenins. The gliadins are monomeric proteins, while glutenins are multimeric aggregates, which bind to each other forming polymers linked by disulphide bonds (Payne and Corfield 1979; Wrigley 1996).

After reduction of disulfide bonds, glutenin subunits can be divided into two main groups: high-molecular-weight glutenin subunits (HMW-GSs) and low-molecular-weight glutenin subunits (LMW-GSs), based on the relative mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The glutenin subunits are stabilized by intra- and inter-molecular

disulfide bonds (Wrigley 1996; Shewry and Tatham 1997; Shewry and Halford 2002). These glutenin subunits polymerized by intermolecular disulfide bonds are important to the rheological properties of wheat flour dough. The HMW-GS fraction determines gluten strength and elasticity (Weegels et al. 1996), and the relative amount and/or allelic forms of LMW-GS can influence the visco-elasticity of gluten dough, which are associated with dough resistance and extensibility (Gupta and Shepherd 1988; Gupta et al. 1989, 1991; Pogna et al. 1990; Metakovsky et al. 1990; Nieto-Taladriz et al. 1994; Sissons et al. 1998; Masci et al. 2003). Although HMW-GSs is the major group of gluten proteins that determine the bread-making characteristics of dough, LMW-GSs also play an important role in it. Some allelic forms of LMW-GSs showed even greater effects on these properties than HMW-GSs (Payne 1987; Gupta et al. 1989, 1994; D'Ovidio and Masci 2004). In particular, LMW-GS fractions have been found to be significantly correlated with dough extensibility (Gupta and Shepherd 1988; Gupta et al. 1989, 1991; Metakovsky et al. 1990) and increase dough-mixing time (Sissons et al. 1998). The distribution of cysteine residues available for intermolecular disulphide bonds (Shewry and Tatham 1997), as well as their overall involvement in non-covalent bonds (Bloksma and Bushuk 1988; Pomeranz 1988) is important in determining the rheological properties of dough. These properties are important to the food uses of wheat flour (Lee et al. 1999).

Understanding single glutenin functional property is important for wheat quality breeding. However, the purification of a single LMW-GS from wheat flour is much more difficult than a single HMW-GS, because typical LMW-GSs are encoded by multigeneic families located at *Glu-A3, Glu-B3,* and *Glu-D3* loci in bread wheat and there are 30–50 different allelic variations of LMW-GS in a cultivar (Lew et al. 1992). So far, the most efficient method for separating HMW-GS is RP-HPLC or SE-HPLC (Lew et al. 1992; Sissons et al. 1998; Ciaffi et al. 1999; Masci et al. 2003). More recently, dye-ligand chromatography (DLC) was used to separate the single high-molecular-weight glutenin subunit (Fisichella et al. 2002, 2003). This technique makes use of dye molecules, linked to an inert support, that interact with the protein sites by electrostatic and hydrophobic forces (Subramanian 1984; Denizli and Piskin 2001; Fisichella et al. 2003). This chromatography assures high specificity; purity and recovery in a single chromatographic step and shows the advantages in terms of economy, safety, and adsorbent capacity (Compagnini et al. 1993; Denizli and Piskin 2001). However, this method has not been used to purify and separate the LMW-GSs previously. The main reason was probably that there are 30–50 components of LMW-GS in single wheat cultivar, these proteins have similar hydrophobicity or isoelectric point (*pI*), and are difficult to be separated from each other by DLC from wheat flour.

The ability to express single polypeptides in heterologous systems, such as bacterial systems, has been of great value for the property analysis of LMW-GSs. Using the LMW-GSs produced from a bacterial expression system, small-scale mixing and extensibility tests can be conducted to study the functional behavior of the LMW-GSs in dough (Sissions et al. 1998; Lee et al. 1999; Wieser and Kieffer 2001; Patacchini et al. 2003). This approach can define cause–effect relationships between protein structure and final dough properties (Bekes and Gras 1992; Bekes et al. 1994a; Lee et al. 1999).

The bread-wheat cultivar Xiaoyan 6, derived from a progeny of *Triticum aestivum* crossed with *Thinopyrum ponticum* ($2n = 70$, $StStE^eE^bE^x$) (Zhang et al. 1993), has well balanced dough properties, which are suitable for making noodles and bread. Xiaoyan 6 was a main cultivar in Huanghe and Huaihe Wheat Zone of China for more than 10 years, and now is widely used as germplasm for good quality and disease-resistance wheat breeding programs in the winter wheat zone of Northern China. It has been known that the HMW-GSs in Xiaoyan 6 encoded by *Glu-A1*, *Glu-B1* and *Glu-D1* loci are 1, 14 + 15 and 2 + 12, respectively (Wang et al.1995; Fan and Guo 2000). The 14 + 15 HMW glutenin subunits are associated with good quality, and may partially account for the dough properties of Xiaoyan 6. However, the role of LMW-GS components contributed to the dough quality properties of Xiaoyan 6 remains to be studied. A better understanding of the functional properties of the individual alleles in the *Glu-3* locus in Xiaoyan 6 will help us to clarify the role of individual LMW-GSs.

Zhao et al. (2004) has cloned a new LMW-GS gene from Xiaoyan 6, designed as *XYGluD3-LMWGS1*, located on chromosome 1D, which contains a promoter region directing endosperm-specific expression. There are multiple LMW-GS genes present at each of the three LMW-GS loci, however, we do not know how highly expressed the cloned form is relative to the other genes present at the locus. The deduced amino acid sequence of this gene contained nine cysteine residues. All of the LMW-GS sequences previously published contain eight conserved cysteine residues (Ikeda et al. 2002), except *XYGluD3-LMWGS1* from *Triticum aestivum* (Zhao et al. 2004) and LAil from *Agropyron intermedium* (Xu et al. 2004), AeL2 and AeL5 from *A. elongatum* (Luo et al.

2005), which have nine cysteine residues. An additional cysteine residue in each of these four LMW-GS genes could lead to functional difference since there is correlation between the presence of cysteine residues available for inter-molecular disulphide bonds and polymerization capacity (Veraverbeke et al. 2000). However, this has not yet been demonstrated. In this study, we expressed the gene *XYGluD3-LMWGS1* in bacteria. The target protein was purified and its functional properties were tested by the 10-g micro-mixing tests and the measurement of glutenin macropolymeric proteins to identify the role of this LMW-GS gene in wheat quality.

Materials and methods

Bacterial expression of *XYGluD3-LMWGS1*

The LMW-GS gene *XYGluD3-LMWGS1* was isolated from bread wheat Xiaoyan 6 and cloned into the pGEM-T easy vector (Zhao et al. 2004).

The bacterial expression vector pET-32a(+) (Novagen) was used to express the *XYGluD3 -LMWGS1* in *E.coli*. The coding region of the mature proteins was amplified by PCR from the recombinant plasmid using the following two primers:

1. NdeI primer: 5′-GCG CAT ATG GAG ACT AGA TGC ATC CCT-3′,
2. EcoRI primer: 5′-GAT GAA TTC TTA TCA GTA GGC ACC AAC TCC -3′.

NdeI primer corresponds to the sequence encoding the N-terminus of the mature proteins (first 21 nucleotides in bold) downstream from a *NdeI* restriction site (underlined), including the ATG initiation codon. *EcoRI* primer is complementary to a region that corresponds to the end of the coding region (in bold) including the two final stop codons. It also contains an *EcoRI* site (underlined). The signal peptide was removed using *NdeI* and *EcoRI* restriction endonucleases, and the gene was ligated to liner vector pET-32a(+) to produce recombined plasmid pET-32a-LXY1, overnight at 4°C and transformed into competent cells of *E. coli* strain *Rosetta* (DE3)-plysS by $CaCl_2$ method following standard protocols. The recombinants were screened by Luria-Bertani (LB) solid culture medium with ampicillin (100 mg/l), and bacterial cell cultures were grown in LB liquid medium.

The transformants were inoculated in 5 ml LB broth with ampicillin (100 mg/l). After overnight incubation, this culture incubated in 300 ml of LB liquid medium in a 1 l flask with 1:100 ratio, totally, 1.5 l of LB liquid medium (in five flasks) were incubated. The cultures were incubated about 3 h until the cell density reached $A_{600} = 0.5–0.6$, the protein expression was induced by adding IPTG to a final concentration of 1.0 mM. The cultures were incubated at 37°C with shaking for 6 h. Then, the bacterial cells were harvested at 10,000 rpm at 4°C for 10 min and stored at −20°C for separation of LMW-GS.

Separation of expressed protein

The method for extracting LMW-GS proteins from bacteria on a large scale was modified from Ciaffi et al. (1999) and Lee et al. (1999). The cell pellets from each 150 ml cultures were re-suspended in 10 ml pre-cold TE solution (pH 8.0) with 10 mg/ml lysozyme in 50 ml centrifuge tube. After freeze–thaw in −20 and 37°C twice alternatively, the liquid was centrifuged in 10,000 rpm at 4°C for 15 min. The supernatants were discarded and the pellets were washed twice with 5 ml TE to remove the water-soluble cell proteins. The pellets were resuspended in 8 ml 50% (v/v) 1-propanol containing 1% DTT, incubated at 65°C for 2 h to extract the LMW-GSs. Then the liquids were centrifuged at 12,000 rpm for 10 min, the supernatants containing the proteins were stored at −20°C for SDS-PAGE analysis or further chromatography purification.

Purification of the expressed LMW-GS by Reactive Red 120-Agarose resins

The LMW-GS was purified from the crude protein extraction from the expressed bacteria. Two milliliters of the protein extraction was loaded onto a column (8 × 1 cm I.D.) with 5 ml Reactive Red-120 Agarose, which had been equilibrated with 0.01 M sodium lactate (pH 4.5) for about 2 h, then was eluted by using different SDS concentrations (0.01–0.05%) in 0.01 M sodium lactate buffer (pH 4.5) at a flow rate of 0.5 ml/min. Elutes were checked at 224 nm with a Jasco 1575 UV detector. The peak fractions were collected and freeze-dried. This preparation containing the LMW-GSs was assayed by SDS-PAGE ($T = 10\%$, $C = 2.67\%$) to check the purity of the extracted LMW-GSs. The large scale of protein used for micro-mixing tests was prepared with similar steps as the above except a larger column and more protein extraction used. Briefly, the peak fraction was collected, concentrated in a vacuum freeze concentrater, and then dialyzed in 1% (v/v) lactic acid solution using dialysis tubing (molecular weight cut off 12,000 Da, Sigma) for at least 72 h, the dialysis buffer was changed five times

to eliminate SDS, then freeze-dried and stored at −20°C until use.

N-terminal amino-acid sequencing

The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane after separation by electrophoresis, 10 mM CAPS (pH 4.5) was used as transferred buffer. The sequencing was performed using an Applied Biosystem amino-acid sequencer, Model 491 Procise, at the National Key Laboratory of Protein and Plant Genetic Engineering, Peking University, China.

Ten-gram mixing tests

Mixing tests were conducted with a 10 g Mixograph (National Mfg. Co.,Chicago, USA) following the procedure described by Zhen (1992). Mixing parameters were determined according to Zhen (1992) and Zhao et al. (1997). The parameters determined included the time taken to reach peak of the curve (dough development time, or mixing time, MT), peak dough resistance (PR), peak width (PW) and breakdown in resistance (RBD). MT, PR and PW are measurement of dough strength, while RBD is an inverse measurement of dough stability (Lee et al. 1999; Zhen 1992).

The purified LMW-GS was used for mixing test to examine its functionality. HMW-GS 1Bx14 and HMW-GS 1Dx2 purified directly from the flour of wheat cultivar Xiaoyan 6 (Kindly provided by Dr. Shen at our Lab) were used as two positive controls since HMW-GS 1Bx14 and HMW-GS 1Dx2 were assumed to be related to good and poor quality, respectively (Wang et al. 1995; Payne 1987; Lorenzo et al. 1987; Rogers et al. 1989). Flour of wheat cultivar Yuzhan 2000 was chosen as a base flour (control), in which the HMW-GSs encoded by *Glu-A1, Glu-B1,* and *Glu-D1* loci are 2*, 13 + 19, and 4 + 12, respectively. This cultivar does not contain HMW-GS 1Bx14 and 1Dx2 and is a weak-gluten cultivar with low protein content (12.4%, dry base), so the addition of the interested glutenin subunit to be tested in this base flour is expected to show clear effects on dough properties. A reversible reduction/oxidation procedure for incorporating the added polypeptides (50 mg of the LMW-GS, 1Bx14 or 1Dx2 subunit, or 100 mg of the LMW-GS for each mixing test) into glutenin was employed (Bekes et al. 1994b).

The 10 g base flour (in 14% moisture) and 50 or 100 mg additional proteins mixed with 6.09 ml of water (according to the protein content and moisture of the base flour, which determined by 1,241 type near-infrared optical spectrum transmission instrument of Foss company) and 0.6 ml water contained 1 mg/ml DTT for 30 s, reacted for 4 min. The reduced dough was then treated with 0.72 ml of oxidant solution containing 10 mg/ml of potassium iodate (KIO_3). Mixing was continued for 30 s, and reacted for 5 min, and then mixed for an additional 3.5 min. Mixing curves and parameters were recorded by Mixograph. Dough pieces after mixing were dropped into liquid nitrogen and freeze-dried and stored at −20°C for the following use.

Measurement of total glutenin polymeric proteins or glutenin macropolymeric proteins of dough

Total glutenin polymers free from monomeric proteins and glutenin macropolymeric proteins (50% 1-propanol insoluble fraction) of the dough resulted from the above were isolated according to a fraction procedure reported by Fu and Sapirstein (1996). The protein content of total glutenin polymer or glutenin macropolymer was determined by micro-Kjeldahl analysis.The amount of protein was estimated as N × 5.7.

Statistic analysis

The statistics test was carried out using SPSS 10.0 for Windows (SPSS 1999).

Results

Bacterial expression of *XYGluD3-LMWGS1*

Full length of the *LMW-GS* gene *XYGluD3-LMWGS1* was 1594 bp and contained an open reading frame from positions 606 to 1,520, which encoded a polypeptide of 304 amino acids, with MW 32.6 kDa, *pI* 8.76, as predicted using DNAman Version 3.0 (Lynnon Biosoft, Quebec, Canada). This gene was the first LMW-GS gene encoding for a LMW-GS with nine cystine residues that has been discovered in wheat so far (Zhao et al. 2004).

To investigate the role of the new gene in dough properties of Xianyan 6, we constructed the bacterial expressed plasmid pET-32a-LXY1. The recombinant plasmid only contained coding region of the mature peptide, which encoded a polypeptide with length of 284 amino acids excluding the signal peptide. SDS-PAGE was used to identify the expressed LMW-GS. The bacteria contained the plasmid pET-32a-LXY1 were used to express the target protein, while the bacteria contained the expression vector pET-32a(+) was used as control. The SDS-PAGE pattern of the protein extracted using 50% 1-propanol with DTT from the bacteria induced and not induced by 1 mM IPTG were

shown in Fig. 1a. As shown in the Fig. 1a, the bacterial extract contained a LMW-GS band about 35 kDa.

Comparing the SDS-PAGE patterns of the extract from wheat cultivar Xiaoyan 6 (The glutenin protein was extracted from wheat flour according to the method described by Fu and Kovacs 1999) and from the bacteria, we found a band in LMW-GS group of Xiaoyan 6 with the same mobility rate as the expressed LMW-GS in the bacteria, suggesting that the gene product XYGluD3-LMWGS1 was likely to be the corresponding band in Xiaoyan 6 (Fig. 1b), which was confirmed in the following steps.

Purification of the expressed protein by dye-ligand chromatography (DLC)

For the biochemical analysis and functional test in the next steps, it was necessary to further purify the expressed wheat LMW-GS. Fisichella et al. (2002, 2003) have developed a simple dye-ligand chromatographic method purifying wheat HMW-GS, but this method has not been used to purify and separate the LMW-GSs before.

The extracts of 1-propanol from the bacteria contained a few proteins because of the exclusion of all the water-soluble proteins with the major proportion belonging to the expected LMW-GS proteins. We tried to separate the bacterial expressed LMW-GS by Reactive-red 120 agarose. The 0.01 M sodium acetate (pH 4.5) containing 0.06–0.1% SDS had been used as elution system for HMW-GSs (Fisichella et al. 2002, 2003), but we found it was not suitable to our target protein, instead, 0.01 M sodium acetate (pH 4.5) contained 0.03% SDS was used. Two peaks were obtained after eluting with this buffer for 40 min, but the two peaks contained few proteins in SDS-PAGE profile (data not shown), the expressed LMW-GS could not be separated from other bacterial proteins.

Then, we chose a different elution system, 0.01 M sodium lactate (pH 4.5) containing 0.01–0.03% SDS, to separate the expressed LMW-GS, the results showed that this elution system was much more efficient. The peak eluted by 0.02% SDS contained the expressed protein (indicated by arrows in Fig. 2a), SDS-PAGE analyses showed the single band was separated (Fig. 2b).

N-terminal amino-acid sequencing

The N-terminal amino acid sequence of the purified proteins, sequenced by Edman degradation, was METRCIPGLE, and was same as the predicted sequence from the DNA, indicating that the purified protein was the expressed products, which belonged to the m-type of low-molecular-weight glutenin subunit (Lew et al. 1992). The cysteine residue of N-terminal could be involved in the formation of inter-molecular disulfide bonds (Gianibelli et al. 2001).

Ten-gram mixograph tests

The mixing tests were conducted with the base flour (control) and the base flour with addition of the interested

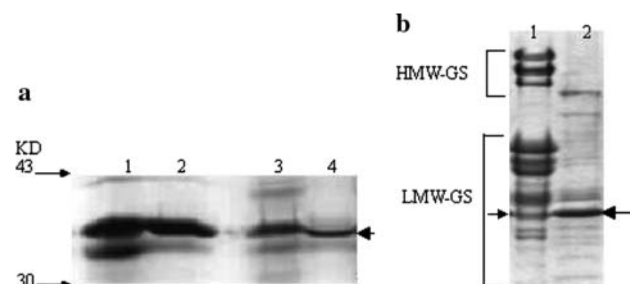

Fig. 1 SDS-PAGE profile of proteins extracted using 50% 1-propanol with DTT from the bacteria (**a**) and comparison of the proteins extracted from the bacteria and flour of Xiaoyan 6 (**b**). **a** *1, 2* Proteins from the bacteria contained the pET-32a(+) not induced and induced, respectively; *3, 4* Proteins from the bacteria contained the pET-32a-LXY1 not induced and induced, respectively. **b** *1, 2* Glutenin subunits from Xiaoyan 6 flour and total protein from the bacteria contained the pET-30a-LXY1, respectively. The *arrows* on the right side show the expressed LMW-GS band in the bacteria, numbers and *arrows* on the left side of **a** indicate standard molecular weight markers, and the *arrow* on the left side of **b** indicates the corresponding band in LMW-GS group of Xiaoyan 6

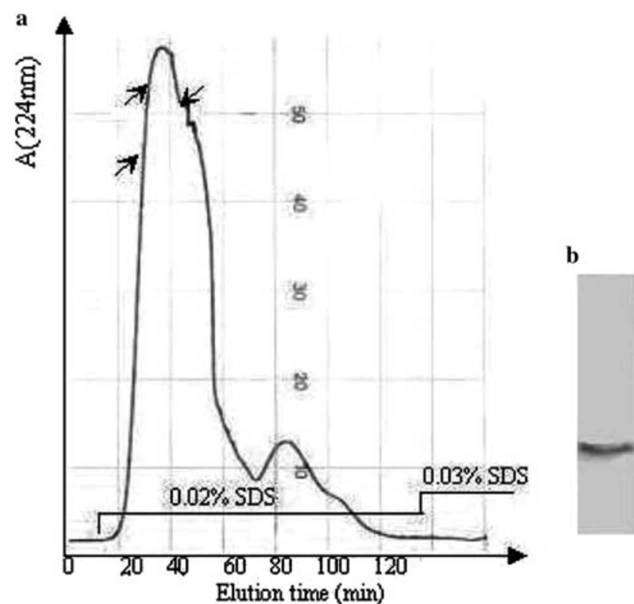

Fig. 2 The profile of the expressed LMW-GS eluted by sodium lacate system containing 0.02%–0.03%SDS from dye-ligand chromatography (DLC) on Red 120-Agrose (**a**) and SDS-PAGE pattern of peak fraction collected from the DLC indicated by *arrows* in Fig. 2a (**b**)

glutenin subunits. The means of four mixing parameters (mixing time, peak dough resistance, peak width and breakdown in resistance) from three replicate analyses are summarized in Table 1.

As indicated from the Table 1, when the LMW-GS or HMW-GSs were incorporated into the base flour, all of them caused significantly increase in MT and decrease in RBD values ($P < 0.01$), while all the PR values showed slight reduction, but not statistically significant. Strong HMW-GS 1Bx14 was included as one of positive controls, and showed the highest mixing time and peak width when 50 mg of alternative protein was incorporated, as expected. The LMW-GS was more effective in increasing peak width than weak HMW-GS 1Dx2, another positive control, while there were no significant differences in the other parameters between them.

It is notable that the effects of the LMW-GS on mixing time and peak width were largely strengthened when 100 mg of the protein was incorporated. Fig. 3 showed the comparison of these incorporations.

Table 1 The means of mixing time (MT), peak dough resistance (PR), peak width (PW), breakdown in resistance (RBD) of the dough of base flour (control) and the dough incorporated with 1Dx2, 1Bx14, LMW-GS, determined from triplicate mixing experiments

Item	Control	1Dx2 (50 mg)	1Bx14 (50 mg)	LMW-GS (50 mg)	LMW-GS (100 mg)
MT(s)	90 A	104 B	117 C	102 B	132 D
PR (A.U.)	575	564	566	564	549
PW (A.U.)	150 A	166 B	208 D	182 C	195 D
RBD (A.U.)	175 A	147 B	141 B	151 B	136 B

Means with different capital letters are significantly different at $P < 0.01$ level

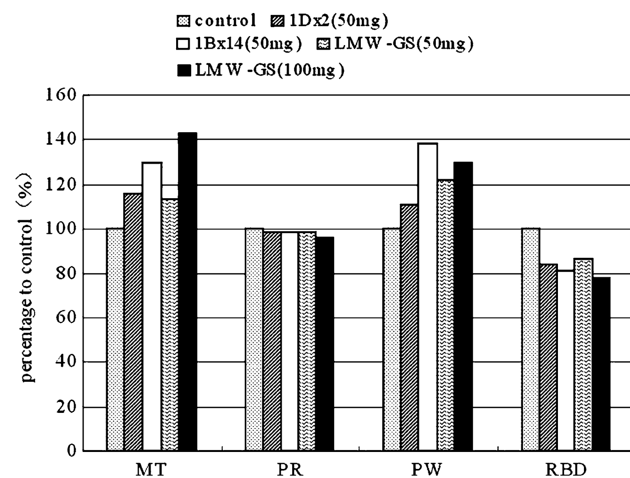

Fig. 3 A comparison of functional properties of different glutenin subunits

Table 2 The means of TPP, GMP or GMP/TPP of dough resulted from three replicate mixing experiments

Sample	TPP (%)	GMP (%)	GMP/TPP (%)
Control	5.00 A	3.44 a	68.80 A
+50 mg LMW-GS	4.92 A	3.38 a	68.70 A
+100 mg LMW-GS	5.41B	3.88 b	71.71 B
+50 mg HMW-GS 1Bx14	5.33 B	4.02 b	75.42 C

Means with different capital letters are significantly different at $P < 0.01$ level

Means with different small letters are significantly different at $P < 0.05$ level

Total glutenin polymeric proteins or glutenin macropolymeric proteins of dough

The incorporation of glutenin subunits (the LMW-GS and HMW-GS 1Bx14) into the glutenin polymeric protein of the base flour was confirmed by analyzing the quantity of the total glutenin polymeric proteins (TPP%), glutenin macropolymeric proteins (GMP%) of the dough, the results were shown in Table 2. It showed that TPP and GMP% of the dough mixed with 50 mg of the LMW-GS did not differ significantly from the control, however, TPP and GMP% of the dough mixed with 100 mg of the LMW-GS or 50 mg of HMW-GS 1Bx14 significantly increased than those of the control. Similar trends occurred in the ratio of glutenin macropolymeric protein to total glutenin polymeric protein (GMP/TPP), i.e. relative size distribution. This indicated that both the LMW-GS and HMW-GS 1Bx14, were incorporated into the large polymeric protein of dough.

Discussion

The primary structure of LMW-GS determines the functional property in end-use of flour, it is important to define all cysteine residues in the primary structure of LMW-GSs. Most of LMW-GS groups contain eight cysteine residues, which are conserved among all of the LMW-GS sequences previously published (Ikeda et al. 2002). Based on the distribution of cysteine residues, the LMW-GS proteins can be classified into three main different types: (1) those with one cysteine in the short N-terminal domain; (2) those with a cysteine residue in the repetitive domain replacing that in the N-terminus; and (3) those with eight cysteines in the C-terminal part of the protein (D'Ovidio and Masci 2004). The general structure of a typical LMW-GS and the locations of the conserved eight cysteines have been shown in Fig. 4a. Among the conserved eight cysteine residues, six devoted

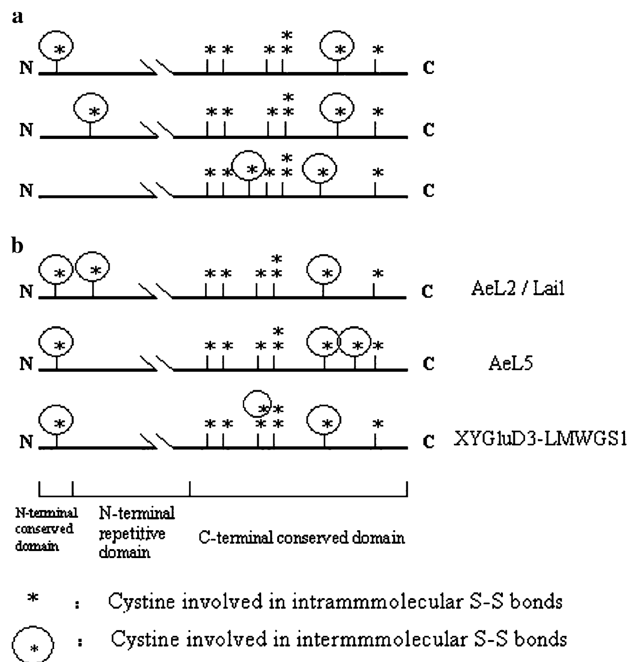

Fig. 4 The structure of the LMW-GS proteins. **a** The typical type of LMW-GS proteins published previously (D'Ovidio and Masci 2004), **b** Three new types of LMW-GS proteins with nine cysteine residues reported in recent 2 years (Xu et al. 2004; Zhao et al. 2004; Luo et al. 2005)

to intra-molecular disulfide bonds, and only two cysteine residues (the first and seventh) involved in inter-molecular disulphide bonds (Shewry and Tatham 1997; Anderson et al. 2001; Gianibelli et al. 2001; Orsi et al. 2001; D'Ovidio and Masci 2004). Recently, three new types of LMW-GS protein with nine cysteine residues have been discovered in *Agropyron intermedium* (Xu et al. 2004), *Triticum aestivum* (Zhao et al. 2004) and *Agropyron elongatum* (Luo et al. 2005). Three cysteine residues may be involved in forming inter-molecular disulfide bonds in these LMW-GS gene products (Fig. 4b).

On the basis of their structural characteristics, glutenin subunits can be considered as "chain branchers" when three or more cysteine residues are available to form inter-molecular disulfide bonds, as "chain extenders" when there are two cysteine residues available and as "chain terminators" when the presence of a single free cysteine residue which would halt the growing glutenin polymer (Shewry and Tatham1997; Kasarda 1989). HMW-GSs are likely to behave as chain extenders and/or branchers; while LMW-GS can be classified into two functional groups. One group, which includes the majority of the B-type subunits, acts as chain extenders of the growing polymers because of their ability to form two inter-molecular disulphide bonds. The second group, which includes most of the C and D-type LMW subunits having only one cysteine available to form an inter-molecular disulphide bond, has been proposed to act as chain terminators of the growing polymer (Kasarda 1989). The size distribution and composition of the polymers in the glutenin fraction are strongly correlated to flour technological characteristics (Southan and MacRitchie 1999; Wieser and Kieffer 2001). Our result suggested that *XYGluD3-LMWGS1* with three cysteine residues available to form inter-molecular disulfide bonds might act as a "chain brancher" and increase the probability of the protein joining into the glutenin network, forming larger glutenin polymers, which contribute to dough elasticity and high quality property in Xiaoyan 6 cultivar.

Some techniques for identifying the functional properties of single glutenin subunit have been developed, including expression of the interested gene in bacteria coupled with micro-mixing tests of wheat flour with addition of the gene products (Bekes et al. 1994a, b; Sissons et al. 1998), wheat genetic transformation (Altpeter et al. 1996; Barro et al. 1997), and development of recombinant inbred lines of glutenin subunit in wheat breeding (Nieto-Taladriz et al. 1994). All these are the effective approaches to investigate the functionality of a glutenin subunit, but the latter two methods take much longer time than the former one.

To examine the functional properties of the protein product of the *XYGluD3-LMWGS1* in wheat flour, a bacterial expression system was used to obtain sufficient quantities of the protein, and a reduction/oxidation protocol for incorporating the protein into flour in a reconstitution-type experiment was employed in this study. This approach had been proved being mostly reliable by the evidence that the properties of the Glu-1Ax1 and Glu-1Dx5 subunit proteins in reconstitution-type experiments corresponded exactly to their properties in vivo in transgenic wheats (Barro et al. 1997, 2003), and has been widely used in characterizing the HMW-GSs and LMW-GSs (Bekes and Gras 1992; Bekes et al. 1994a, b; Sissons et al. 1998; Lee et al. 1999). Two kinds of HMW-GS with known functionality (a strong HMW-GS 1Bx14 and a weak HMW-GS 1Dx2) were used as positive controls in the mixing experiment, making our results more reliable and accurate. Since the research on the functional properties of LMW-GSs was very limited, it is impossible to compare our findings with any LMW-GS with known functionality.

The result of 10 g Mixograph test in the present study indicated that the expressed LMW-GS was less effective in increasing mixing time and peak width than HMW-GS 1Bx14, as expected when 50 mg of the protein (HMW-GS 1Bx14, 1Dx2 or the LMW-GS) was incorporated, but it was as efficient as HMW-GS 1Dx2. In fact, 50 mg of the LMW-GS has about twice amount of molar of 50 mg of

the HMW-GS because of the different size of the polypeptides; it indicated that this LMW-GS had smaller effect on dough strength than any HMW-GS if the same amount of molar of protein were incorporated. In addition, the result of the mixing test also indicated that the effects of the LMW-GS on mixing time and peak width were largely strengthened when double amount of the glutenin subunit (100 mg) was incorporated. This suggested that the role of the LMW-GS in dough quality properties could be enhanced with increase of the protein quantity. Wieser and Kieffer (2001) found that the twice amount of LMW-GS was necessary to obtain the same dough resistance as they achieved with HMW-GS, as determined by rheological measurements and baking tests on a micro-scale. Our results supported this conclusion.

To understand the effects of the alternative glutenin subunits on dough mixing properties, we compared the quantity of total glutenin polymeric proteins and glutenin macropolymeric proteins of the dough without (control) and with the incorporation of the LMW-GS or HMW-GS 1Bx14. The result suggested that the LMW-GS, like HMW-GS 1Bx14, participated in the formation of larger glutenin polymers, and greatly contributed to dough strength and elasticity (Southan and MacRitchie 1999; Zhao et al. 2002). The similar result was obtained by using different LMW-GSs and base flour (Lee et al. 1999; Patacchini et al. 2003), and supported the hypothesis that *XYGluD3-LMWGS1* might act as “chain brancher” and enhance the protein to join into the glutenin network, forming larger glutenin polymers, which contribute to dough elasticity and high quality property of wheat cultivar Xiaoyan 6. To further confirm this hypothesis, site-specific mutagenesis of the extra cysteine residue coupled with the use of in vitro protein expression system and glutenin reconstitution technique is under way. In addition, the gene *XYGluD3-LMWGS1* can be used in transgeneic experiments in which it is over-expressed to investigate the functionality of this glutenin subunit and the interaction of different subunits.

Acknowledgments The authors thank Dr. Zhonghu He, Institute of Crop Science, Chinese of Agricultural Sciences for offering his lab for mixing test and Dr. Ming-Shun Chen, Department of Entomology, Kansas State University and Shengwu Hu, Northwest A & F University for the valuable suggestion on the manuscript. This work was financially supported by the grants from Ministry of Science and Technology of China (JY03-A-13-1) and Northwest A & F University (2005).

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