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Heterologous expression and purification of native and mutated low molecular mass glutenin subunits from durum wheat

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Abstract

Wheat technological properties are correlated with the size of glutenin polymers, consisting of high and low molecular mass glutenin subunits, linked together by disulphide bonds. In order to unravel glutenin polymer structure, we considered three LMW-GS genes, which differ in the number of cysteine residues and in the repetitive domain length. The three LMW-GS genes have been expressed in *Escherichia coli,* and purified with a yield of 40–100 mg/l of culture volume, depending on protein type. Single polypeptides are being used in re-oxidation and micro-mixographic experiments, in order to detect the influence of the differential structural characteristics on glutenin polymer formation.

Keywords: Heterologous expression; Purification; Low molecular mass glutenin subunits

1. Introduction

Wheat is the major food staple in many countries in the form of bread, pasta and biscuits. Gluten, the protein mass remaining after washing out starch and water-soluble components, is a viscoelastic protein complex responsible of the physical properties of wheat dough, such as elasticity and resistance. Gluten is mainly composed of two groups of proteins, the gliadins and the glutenins [1]. The gliadins are monomeric, whereas the glutenins consist of large polymers [2] of high-molecular-mass and low-molecular-mass glutenin subunits (HMW-GS and LMW-GS, respectively), linked together by disulphide bonds.

Both qualitative and quantitative differences within these groups of proteins contribute to inter-cultivar variation in quality. Correlative and incorporation studies have demonstrated that HMW-GS have a far greater effect than LMW-GS on dough strength in bread wheat [3,4], whereas in durum wheat LMW-GS have been found to be most determinant [5]. Although they represent ~80% of the durum wheat glutenin, the LMW-GS are less well characterised than the HMW-GS. Little is know about their structure–function relationships and only a small number of genes have been isolated [6–8].

Most typical LMW-GS are encoded by multigene families located on chromosome 1 of the A, B and D genomes of bread wheat or A and B genomes of durum wheat. Genes from the B genome play a major role in the quality of durum wheat, but they are the most difficult to isolate and characterise [8]. LMW-GS consist of a central repetitive domain containing irregular repeat motifs rich in proline and glutamine, a short N-terminal region and a long C-terminal domain, where all the intrachain disulfide bonds are located. LMW-GS contain two additional

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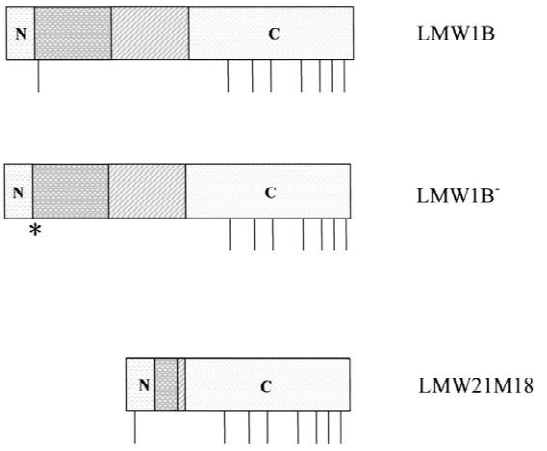

Fig. 1. Schemes representing the three LMW-GS genes that we have expressed in *E. coli*. The central dark zone stands for the repetitive domain rich in proline and glutamine and has been represented by two different grid styles in order to show which portion of the repetitive domain is deleted in pLMW21M18. Vertical lines represent cysteine residues and the asterisk represents the cysteine-arginine substitution in LMW1B$^-$.

cysteine residues available for the formation of interchain disulfide bonds, located in the N- and C-terminal regions.

Although the structural basis of the specific effects of the LMW-GS on glutenin polymer formation are unknown, data suggest that the number and position of cysteine residues available for the formation of interchain disulfide bonds are critical. Moreover, the numerous glutamine residues in the repetitive domain are supposed to form hydrogen bonds between the LMW-GS, contributing to gluten elasticity [9].

LMW-GS share sequence homologies and are structurally related to the gliadins. Therefore, it is difficult to obtain a single homogeneous protein from wheat. In addition, studying the structure–function relationships of the LMW-GS requires large amounts of single subunits. We used the *Escherichia coli* heterologous expression system to overproduce and purify large amounts of three different LMW-GS genes, two of which coded at the B genome, differing in the number of cysteine residues and in the length of the repetitive domain (Fig. 1).

2. Experimental

2.1. Bacterial strains, plasmids and growth conditions

The wheat lines, the strains and the plasmids used in this work are listed in Table 1. The strain used for heterologous expression was BL21Star (DE3) pLysS *E. coli* cells (Invitrogen) that offers high expression levels. It includes a mutation in the *rne* gene that encodes the endonuclease RnaseE, a key enzyme responsible for mRNA degradation. In this way mRNA is less susceptible to degradation and remains available for translation.

LMW1B and LMW1B$^-$ were cloned in the expression vector pDEST14 (Life Technologies) using the Gateway system (Life Technologies), based on specific recombination of sequences of the λ bacteriophage. The expression vector pDEST14 contains the promoter sequence for the bacteriophage T7 RNA polymerase, namely the T7 promoter, a pBR322 origin of replication, and an ampicillin resistance marker. LMW21M18 gene was cloned in the pET3a expression vector (Novagen) that contains T7 promoter and terminator regions, a pBR322 origin of replication and an ampicillin resistance marker.

Bacterial cells were grown in Luria-Bertani (LB) medium or on solid agar, obtained by addition of 1.5% agar, containing 1 mg/ml ampicillin (Ap) and 34 μg/ml chloramphenicol (Cm) at 37 °C.

Table 1
Wheat lines, bacterial strains and plasmids used in this work

	LMW1B	LMW1B$^-$	LMW21M18
Durum wheat line	Langdon	Langdon	Line 21
Bacterial strain	BL21Star (DE3)pLysS	BL21Star (DE3)pLysS	BL21Star (DE3)pLysS
Plasmid	pDEST14	pDEST14	pET3a

2.2. DNA techniques

Plasmid DNA was routinely isolated by the alkaline lysis method [10] or purified by using the QUIAGEN plasmid midi kit for sequencing and transformation of BL21Star (DE3) pLysS *E. coli* cells. PCR was carried out in a DNA thermal cycler (Perkin-Elmer) using Platinum *Pfx* DNA Polymerase (Gibco BRL). Sequencing was carried out using an ABI Prism 310 PE (Applied Biosystems) and a combination of universal and custom-synthesised primers.

2.3. Plasmid construction

The three LMW-GS genes that we studied were supplied by Professor Renato D'Ovidio: two (coding for LMW1B and LMW1B$^-$) cloned into pLRPT cloning vector and one (coding for LMW21M18) cloned into pET3a expression vector.

The LMW-GS genes coding for LMW1B and LMW1B$^-$ proteins were amplified using the PCR primers GWLMW1BF and GWLMW1BR amplifying the complete coding regions.

GWLMW1BF:

attB1

5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACCATGG
AGACTAGCCACATCCCT-3'

GWLMW1BR:

attB2

5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGTAGGCACCAACTCCGGT-3'

The attB sequences were added to the 5′-terminus of both primers allowing synthesis of a PCR product that is an efficient substrate for recombination with the donor vector in the presence of BP CLONASE enzyme mix.

Expression cloning was then performed according to the Gateway System manufacturer instructions. Briefly, the template DNA was denatured at the initial denaturation step at 94 °C for 2 min. The DNA was then amplified during 30 cycles of 15 s at 94 °C, 30 s at 55 °C and 1 min at 70 °C. The PCR products were then purified with PEG precipitation and cloned into pDONR201 donor vector (Life Technologies) using BP recombination reaction. Finally the inserts were transferred into pDEST14 expression vector using LR recombination reaction.

2.4. E. coli expression

BL21Star (DE3) pLysS derivatives containing LMW1B, LMW1B$^-$ and LMW21M18 genes cloned into expression vectors were grown in LB medium supplemented with Ap (1 mg/ml) and Cm (34 μg/ml) at 37 °C for 18 h under shaking at 250 rpm. These cultures were diluted to 1/50–1/100 in fresh LB medium containing Ap (0.04 mg/ml) and incubated at 37 °C under shaking at 250 rpm. When $O.D._{600}$ reached 0.3–0.4, the expression of the target proteins was induced by adding 0.5 m*M* isopropyl-β-D-thiogalactoside (IPTG). The growth was allowed to continue for 3–4 h at 37 °C. Cells were harvested by centrifugation at 6500 *g* for 15 min at 4 °C and stored at −20 °C until further use.

2.5. Purification of bacterially expressed LMW-GS

Cells from a 500-ml culture were resuspended in 50 ml of lysis buffer containing 20 m*M* Tris–HCl pH7.5, 10 m*M* EDTA, 1% Triton X-100. Cell suspensions were added to 100 μg/ml of lysozyme at 30 °C for 15 min and sonicated on ice until the solution was no longer viscous. Bacterial lysates were then centrifuged at 10 000 *g* for 10 min. The remaining pellets containing insoluble inclusion bodies were washed twice in 50 ml of lysis buffer. Finally, the inclusion body fractions were solubilized in 50% acetonitrile, 0.1% trifluoroacetic acid.

2.6. SDS–PAGE and Western immunoblotting

Whole cell lysates were analysed by resuspending 1 ml bacterial cell pellet from 1 ml culture in 100 μl of sample buffer (0.07 *M* Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.02% pyronine γ, 1% dithiothreitol). The mixtures were heated at 80 °C for 30 min and centrifuged at 15 800 *g* for 10 min. Electrophoresis was performed using a Mini Protean gel system (Bio-Rad) according to a modified Laemmli procedure [11] with $T=15$, $C=0.5$. Gels were stained with Coomassie Brillant Blue.

For Western immunoblotting, proteins were electrotransferred to a PVDF membrane (Bio-Rad) in Tris 48 m*M*, glycine 39 m*M*, SDS 1.3 m*M*. Proteins were visualised with alkaline phosphatase-conjugated

anti-rabbit polyclonal antibodies, according to standard protocol (Santa Cruz Technologies).

2.7. Protein assay

Protein concentrations were determined spectrophotometrically at 276 nm, using 0.6 as molecular extinction coefficient, on the basis of amino acid composition

3. Results and discussion

3.1. Expression of LMW-GS

LMW1B and LMW1B$^-$ genes have been isolated from genomic DNA of the durum wheat cultivar Langdon. The wild type gene, named LMW1B, codes for a polypeptide of 330 amino acids (MW 37 700) corresponding to a typical LMW-GS with eight cysteine residues, one located at the start of repetitive domain and seven located at the C-terminal domain. The other construct, named LMW1B$^-$, corresponds to the LMW1B in which the first cysteine residue has been replaced during PCR by an arginine residue to study the effect of its absence on gluten quality. The molecular mass of the corresponding polypeptide is 37 753.

In order to also analyse the effect of the length of the repetitive domain, we have expressed another LMW-GS gene (LMW21M18), isolated from a durum wheat line (Line 21) and whose encoded polypeptide is 232 amino acids long with a molecular mass of 26 219. This gene construct corresponds to a typical LMW-GS, but it is notably shorter than the other LMW-GS genes because it has undergone a deletion in frame of 102 nucleotides in the repetitive region during the cloning procedure. The corresponding protein, which possesses the regular eight cysteine residues, shows a deletion of 34 amino acids in the repetitive region. The deletion starts 15 amino acids after the end of the N-terminus domain and stops 12 amino acids before the start of the C-terminus domain. The repetitive domain of deleted protein is 56% shorter than that of the native protein and its molecular mass is 87% of that of the native protein.

LMW1B and LMW1B$^-$ genes have been cloned into pDEST14 expression vector through the Gateway system because they are difficult to clone using the classical T4 DNA ligase reaction.

LMW21M18 has been supplied by Professor Renato D'Ovidio already cloned in pET3a expression vector.

The heterologous expressions of the three LMW-GS genes are reported in Fig. 2. Heterologous polypeptides expressed in *E. coli* were identified on SDS–PAGE gels under reducing conditions by comparing the total cell protein of uninduced and induced bacteria carrying the LMW-GS genes. All three LMW-GS genes show a maximum heterologous expression level 4 h after induction with IPTG. Heterologous proteins, representing 14–50% of total cell proteins (as estimated by densitometric analysis), are expressed at very high levels; the highest levels have been detected for LMW21M18 followed by LMW1B$^-$ and LMW1B.

Electrophoretic mobility of the heterologous proteins indicated a molecular mass of ~31 000 for LMW21M18 and 44 800 and 43 000 for LMW1B and LMW1B$^-$, respectively, which are larger than those calculated from gene sequences; this behavior is already known for glutenin subunits because of their unusual conformational characteristics which cause anomalous mobility on a gel [12–14]. Attribution of the heterologous polypeptides to the LMW-GS family was performed by Western blotting analysis (Fig. 3).

Heterologous expression yield was very high, because we obtained ~200–300 mg protein per liter of culture medium, depending mainly on the type of gene and being greater for LMW1B$^-$ and lower for LMW1B and LMW21M18. The system used was also highly stable because high levels of expression were obtained up to 1 month after transformation.

3.2. Purification of the bacterially expressed LMW-GS

Because heterologous proteins are secreted into inclusion bodies (Fig. 2, lanes 10–11), we have exploited this characteristic in order to purify them with high efficiency. Inclusion bodies have been specifically extracted from the bacterial cells and then lysed in order to release their content, which consists almost exclusively of the heterologous pro-

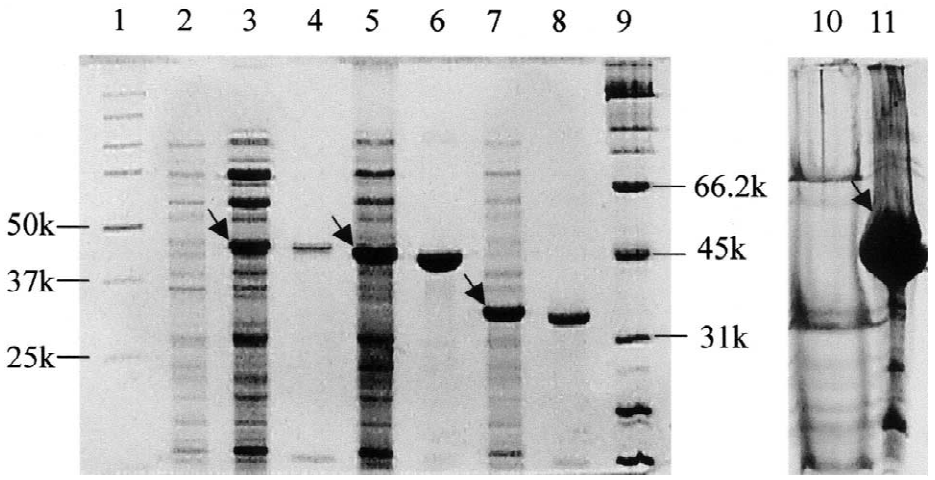

Fig. 2. SDS–PAGE showing heterologous expression and purification of LMW-GS. Lanes 1 and 9, SDS–PAGE molecular mass markers; lane 2, total cell proteins before induction; lanes 3, 5, 7, total bacterial proteins extracted after induction of heterologous expression of LMW1B, LMW1B$^-$, LMW21M18, respectively; arrows indicate the putative heterologous protein. Lanes 4, 6, 8, the purification of the heterologous protein is reported on the right side of each lane. Lanes 10 and 11 represent cellular proteins solubilised during extraction of inclusion bodies containing LMW1B$^-$ and proteins contained in inclusion bodies, respectively.

teins (Fig. 2). This procedure allowed us to purify ~40–100 mg protein with a purity of ~90% per liter of bacterial culture, with the lowest quantity obtained for LMW21M18. Besides the simplicity of purification, this method has the advantage of allowing the use of the heterologous LMW-GS for functional studies, such as micro-mixographic performances [15], extensibility tests [16], and re-oxidation experiments [17], because no reducing or chaotropic agents were used during the extraction procedure.

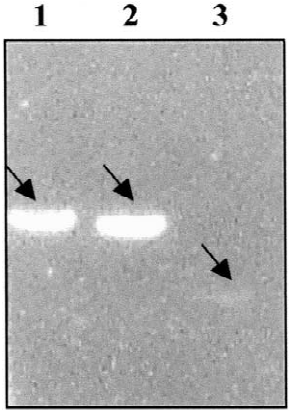

Fig. 3. Western blotting analysis to confirm identity of heterologous proteins. Lanes 1, 2, and 3, total cell proteins extracted from *E. coli* that express LMW1B, LMW1B$^-$, LMW21M18, respectively. Arrows indicates heterologous proteins.

4. Conclusion

Wheat is a source of a wide variety of products that requires dough with different characteristics of strength, elasticity and extensibility. Gluten proteins are mainly responsible for the technological characteristics of dough and, of these proteins, glutenin subunits contribute mainly to intercultivar variation of quality.

Because of their importance in determining dough physical properties, several studies have been carried out on the glutenin proteins [18], of which the LMW-GS are difficult to characterise. In order to determine the structure–function relationship of LMW-GS, we have expressed three different LMW-GS genes with peculiar structural characteristics in a heterologous system, with the aim of obtaining a large amount of protein to use for functional studies.

In the present study we have demonstrated that heterologous expression represents an easy and rapid tool to obtain a large amount of protein that can be

used for further studies. We are going to study the structure and function of different LMW-GS through micro-mixographic analyses, to evaluate the strength and the stability of a dough obtained by incorporating small amounts of purified protein in a base flour, and re-oxidation experiments to study their different behaviours in polymer constitution.

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