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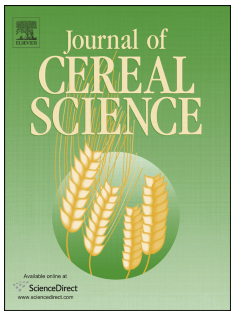

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Cloning, expression and functional analysis of HMW glutenin subunit *1By8* gene from Italy pasta wheat (*Triticum turgidum* L. ssp. *durum*)

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ABSTRACT

Cloning and functional analysis of high molecular weight wheat glutenin subunit (HMW-GS) *1By8* from Italy durum cultivar Simeto was carried out in this study. All HMW-GS from Simeto were separated and characterized by appropriate electrophoresis methods, reversed-phased high performance liquid chromatography (RP-HPLC) and mass spectrometry (MS). The complete gene encoding *1By8* subunit was amplified by allele-specific PCR primers, including an upstream sequence of 857 bp and an open reading frame (ORF) of 2166 bp encoding a mature protein of 720 amino acid residues. The promoter sequence, containing -300 element and enhancer was highly conserved among HMW-GS genes. Comparison with the sequence of subunit *1By9* from bread wheat demonstrated 99% identity with the main difference being that the *1By8* subunit possesses an additional insertion of 15 amino acid residues (QYPASQQQPA QGQQG) at position 342 and two residue substitutions at position 78 (leucine/proline) and 442 (arginine/glutamine). The molecular weight differences between MALDI-TOF-MS and deduced amino acid sequence of the coding gene revealed the possibility of some kinds of post-translational modifications present in *1By8* subunit. The protein subunit expressed in *E. coli* showed a very similar mobility to the endogenous *1By8* of Simeto on SDS-PAGE. The function of the isolated protein on wheat processing quality was determined by 10g Mixgraph analysis. Results demonstrated that addition of y-type HMW glutenin subunits into the base flour had significant positive effects on main mixing parameters and significant difference in effects were observed among different y-type subunits.

1. Introduction

Wheat glutenins, including high molecular weight glutenin subunits (HMW-GS) and lower molecular weight glutenin subunits (LMW-GS) are the major storage proteins that account for about 45% of the total protein in the seed endosperm. They link together and form heterogeneous mixtures of polymers by disulfide bonded linkages of polypeptides. The glutenin proteins, therefore, are among the largest protein molecules in nature with molecular weights up into tens of millions (Wrigley 1996). HMW-GS are the major determinants of gluten elasticity due to the formation of larger glutenin polymers that play an important role in bread-making quality (Gianibelli et al. 2001, Payne et al. 1984, Payne et al. 1987).

The HMW-GS are encoded by genes at the complex *Glu-1* loci located on the long arms of chromosomes 1A, 1B and 1D, namely *Glu-A1*, *Glu-B1* and *Glu-D1* locus, respectively (Payne, 1987). Each locus consists of two genes tightly linked together encoding a larger x-type subunit (80-88 kDa) and a smaller y-type subunit (67-73 kDa). The allelic variations at *Glu-1* loci have significant effects on wheat quality properties. Early work reported 11 alleles (14 different subunits) at *Glu-B1*, 6 alleles (8 different subunits) at *Glu-D1* and one or no allele (2 different subunits) at *Glu-A1* in about 300 bread wheat cultivars (Payne and Lawrence, 1983). In particular, the allelic pair *1Dx5+1Dy10* have been shown to be the best quality subunits with the highest score 4 while the *1Bx7+1By8* and *1Bx17+1By18* subunits are slightly lower with a quality score of 3 (Weegels et al. 1996; Gianibelli et al., 2001). The other subunits, such as *1Dx2+1Dy12*, *1Bx7+1By9* etc generally have lower quality scores and relate to poor quality performance (Shewry et al., 1992). A mathematical modeling approach to estimate the overall quality of a wheat line from its glutenin composition has been recently developed, taking into account not only the contributions of individual HMW-GS or LMW-GS but also their interactions (Bekes et al., 2006, Ma et al., 2005). The molecular mechanism behind these numeric models is related to the chemical structure of glutenin subunits.

The contribution of different protein types in the flour in determining the rheological properties of the wheat flour dough is caused by the different structural

features of the polypeptides (such as their size and the number and position of cysteine groups) and their role in the formation of the polymeric glutenin. The x-type subunits possess four conserved cysteine residues (3 in the N-terminal domain, 1 in the C-terminal domain), and the y-type subunits usually contain seven conserved cysteine residues (5 in the N-terminal domain, 1 in the repetitive domain and 1 in the C-terminal domain. Shewry et al. 1995).

Among the tetraploid species, durum wheat (*Triticum turgidum* L. ssp. *durum* Desf., 2n=4x=28, AABB) is the most cultivated, as an economically important cereal, mainly in the Mediterranean area and accounts for about 6-8% of the total wheat production in the world (Troccoli et al., 2000). The major use of durum wheat is for making pasta and semolina, particularly in European and North American countries. In some areas, it is also used for various types of bread-making. The pasta quality of durum wheat depends mainly on glutenin composition. Different HMW-GS and LMW-GS as well as their combinations may result in variations of gluten elasticity and strength. In bread wheat, the *1Bx7+1By8* subunits encoded by the *Glu-B1* locus (*Glu-B1b* allele) have also been shown to associate with high elastic recovery and gluten firmness (Pogna et al., 1990). This allele is widely distributed in durum, cultivated emmer, European spelt and bread wheat with the frequencies of 25.9%, 17.07%, 14.44% and 32%, respectively (Branlard et al., 1989; An et al., 2005; Li et al., 2006).

Until now, most studies have been focused on the x-type glutenin protein variations to explain functional difference of different alleles on quality. Little attention has been paid to the effects of y-type subunits. For example, the positive influence of *Dx5+Dy10* on quality was believed due to *Dx5*'s extra cysteine residue to form stronger gluten matrix (Gianibelli et al., 2001; Shewry et al., 1995; Lindsay et al., 2000); recently, a *Bx7* over expressed allele, *Bx7+By8**, has been found to be associated with high dough strength, which was believed to be caused by *Bx7* over expression even though the *By8* protein in this allele differs from the usual *By8* subunit. Additionally, there is only one *1By* gene, *1By9*, which has been cloned and characterized thoroughly (Halford et al 1987). This is perhaps due to two main reasons. Firstly, the x-type and y-type HMW-GS genes are tightly linked and it is difficult to dissect their genetic effects by

traditional approaches; secondly, single gene effect confirmation is still technically difficult in wheat due to its hexaploidy nature.

The development of genetic transformation systems provides the possibility for manipulating glutenin compositions and hence improving the technological properties of wheat grain (Shewry et al. 1995; Altpeter et al. 1996; Blechl and Anderson 1996; Popineau et al. 2001). Most of the work has focused on the transformation of HMW-GS *1Dx5+1Dy10* and *1Ax1* genes and has indicated that these genes can improve functional properties of bread wheat. Particularly, He et al. (1999) demonstrated that expression of the additional genes encoding HMW subunits in durum wheat resulted in increased dough strength and stability. The genetic transformation technology, therefore, can be used as a powerful tool for improving wheat quality. For rapid quality improvement, reserve genetics approach, ie, isolation, expression and functional studies of novel glutenin genes for good quality properties are highly important. Although more than ten major HMW glutenin subunit genes in bread wheat and related species have been cloned and sequenced (Shewry et al. 2003; Yan et al. 2004a; Li et al. 2004), genes for practical use are still limited. In this study, we used an integrated approach, ie, from gene to effects, to study the molecular structure, expression and functional properties of *1By8* subunit gene isolated from an Italian durum wheat cultivar Simeto.

2. Materials and methods

2.1. Plant materials

The Italian durum wheat cultivar Simeto, which was proved to have good technological characteristics (Galterio et al. 2001), was kindly provided by Dr. R. Acquistucci, Istituto Nazionale di Ricerca, per gli Alimenti e la nutrizione,. The bread wheat cultivar Chinese Spring was used as standard for HMW-GS identification.

2.2. One- and two-dimensional gel electrophoresis of HMW-GS

An improved procedure for extraction and sample preparation of HMW glutenin subunits, separation by one-dimensional sodium dodecyl sulphate (SDS)-, polyacrylamide gel electrophoresis (PAGE), acid (A)-polyacrylamide gel

electrophoresis (PAGE) and two-dimensional A-PAGE x SDS-PAGE were based on the protocols of Yan et al (1999, 2003a). Electrophoresis was performed with a Bio-Rad Mini-PROTEAN 3 system.

2.3. Acidic capillary electrophoresis (A-CE) and reversed-phase high performance liquid chromatography (RP-HPLC)

Sample preparation and A-CE analysis of the HMW-GS were carried out based on the method of Yan et al. (2003b, c; 2004b) with BioFocus 3000 instrument. RP-HPLC analysis was performed according to Burnouf and Bietz (1984).

2.4. Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS)

HMW glutenin subunit extraction, sample preparation and MALDI-TOF-MS analysis were carried out according to Zhang et al. (2006). The Shimadzu corporation A XIMA-CFRTM Plus MS apparatus was used and sinapinic acid (SA, 3, 5-Dimethoxy-4-Hydroxycinnamic acid) is the matrix in this study. Spectra were acquired in a linearity mode using a mass range of 10,000 to 100,000 Da and about 80 to 120 laser shots. Standard sample Albumin-Aldrase was used to perform two-dot calibration.

2.5. N-terminal amino acid microsequencing

Preparative SDS-PAGE gels were electroblotted to polyvinylidene difluoride (PVDF) membranes by using a mini Trans-Blot Electrophoretic Transfer cell (Bio-Rad). Proteins transferred to PVDF membranes were stained using 0.1% coomassie Brilliant Blue R-250 (CBB-R250) in 40% methanol for 40s, destained in 50% methanol until the background was light blue and allowed to dry. HMW-GS obtained were sequenced directly using conventional N-terminal Edman degradation microsequencing with PROCISE® cLC 491 protein sequence system (Applied Biosystems, Foster, USA).

2.6. DNA extraction and AS-PCR amplification

Two pairs of the allele-specific (AS) PCR primers, F1/R1 and F2/R2 (Yan et al., 2004a) were used to amplify the upstream and coding regions of *1By8* subunit gene respectively. They are:

F1(5'ACCACAGTTTGCTCATATTGTCTTG3') +

R1(5'ACGTCTACACTTCTGCAAACAATACC3')

F2(5'ATGGCTAAGCGGTTGGTCCT3') +

R2 (5'TCACTGGCTAGCCGACAATG3').

Total genomic DNA from dry seeds was isolated as described by Sun et al. (2004). PCR was carried out using a Perkin-Elmer Cetus DNA Thermal Cycler (PE Applied Biosystems, USA). Proofreading TAQ polymerase Takara LA Taq™ was used for all PCRs**.** The following PCR procedures were used: an initial denaturation step at 94^{o}C for 5 min followed by 35 cycles at 94^{o}C for 45 sec, an annealing step from 58^{o}C to 63^{o}C for 60 sec, an extension step at 72^{o}C for 120 sec concluded with a final extension step at 72^{o}C for 10 min. PCR products were analyzed with 1% agarose gel.

2.7. Cloning, sequencing and molecular analysis

The AS-PCR products of expected sizes were purified from the gel and cloned with the p^{GEM-T} plasmid vector (Promega, Madison, Wis.). The hybrid vector was used to transfer competent cells of *Escherichia coli* DH-5α strain. DNA sequences were obtained with three clones using primer walking and performed by TaKaRa Biotech. Multiple sequence alignment of nucleotide and protein sequences was completed by using Bioedit 7.0.1.1 software.

2.8. Expression in E. coli and Western-blotting

The ORF of *1By8* gene was reamplified using the PCR primers EX-1 (5'-ATAGAATTCATGGCTAAGCGGTTGGTCC-3') and EX-2 (5'-TATCTCGAGTCACTGGCT AGCCGACAAT-3'). Two restriction enzyme sites (*EcoR* I and *Xho* I) were introduced at the 5' and 3' end of PCR product, respectively. The fragment was ligated into the expression vector pET-28a (Novagen) and

re-sequenced to ensure no mistakes during PCR, then transformed into an expression host *E. coli* strain BLR (DE3) plysS. In order to express the HMW subunit protein, a single recombinant and a negative control bacterial colony were inoculated into 5 ml 2YT medium containing 50 μg ml^{-1} kanamycin and 34 μg ml^{-1} chloramphenicol. They were incubated for 4 hours at 37^{o}C until the OD600 reached 0.6. After removing 1ml of each culture, the remains were induced for 4 hours by the addition of IPTG at the final concentration of 1.2 mM. According to Lee et al (1999), the expressed *1By8* subunit protein was purified simply from the culture and detected by SDS-PAGE. The electrophoretic mobility of the *1By8* protein produced in the bacterial cells was compared to that extracted from the Simeto seeds. Western-blotting experiments by the polyclonal antibody specific for HMW-GS prepared in our laboratory were carried out based on Liu et al. (2003).

2.9. Protein purification and dough-quality testing

A 10-gram Mixograph was used to evaluate functional dough properties of *1By8* subunit according to Bekes et al. (2001). The protein subunits of *1By8* from Simeto, *1By9* and *1Dy10* from Zhongyou 9507 collected and purified by SDS-PAGE and RP-HPLC (60 mg each) were incorporated into 10 g of base flour of the bread wheat cultivar Jing411 (protein content 10.6%; HMWGS: Ax2*, Bx7+By8, Dx2+Dy12). The Mixograph assays were carried out in duplicate following the 54-40A AACC method (AACC 1995). Several parameters were measured, including mixing time (MT), stable time (ST), peak resistance (PR), peak width (PW) and width at 8min.

3. Results and Discussion

3.1. Separation and characterization of 1By8 subunit from Simeto

The HMW-GS extracted from durum wheat cultivar Simeto were separated and identified by different electrophoretic and chromatographic methods (Fig. 1). Compared to Chinese Spring, it is obvious that Simeto has the *1Bx7+1By8* subunits

at the *Glu-B1* locus and *null* allele at the *Glu-A1* locus. Two-dimensional A-PAGE x SDS-PAGE pattern clearly showed two protein dots, corresponding to *1Bx7* and *1By8* subunits (Fig. 1). RP-HPLC and acidic capillary electrophoresis (A-CE) analysis (Fig. 1) showed that the *1Bx7* and *1By8* subunits were well separated and readily identified based on the migration orders of HMW-GS established by Gianibelli and Solomon (2003) and Yan et al. (2003b; 2004b). The *1By8* subunit showed lower hydrophobicity than *1Bx7*. Different separation methods all demonstrated that the amount of *1By8* subunit was much lower than that of *1Bx7* (Fig 1).

In order to obtain accurate molecular mass, the HMW-GS of Simeto were further characterized by MALDI-TOF-MS. As shown in Fig. 1, two separate mass spectra with M_r 82974.5 Da and 77171.9 Da in the high molecular mass region were consistent with the molecular size of *1Bx7* and *1By8*, respectively. According to the gene sequence of *1Bx7* (Anderson and Greene 1989) and *1By8* subunits (see below), their deduced M_rs were 82873 Da and 75156.7 Da, generally corresponding well to the molecular weight of *1Bx* and *1By* subunit, respectively.

Twenty-eight residues (EGEASRQLQCERELQESSLEACRQVVDQ) of the N-terminal amino acid sequences of *1By8* subunit were obtained by microsequencing, which were identical to those of *1By9*, *1Dy10* and *1Dy12* subunits from bread wheat. This suggested that the N-terminal sequences of y-type subunits were highly conserved.

3.2. PCR and molecular analysis of 1By8 gene

In this work, different annealing temperatures from 58 ^{o}C to 65 ^{o}C were tested in order to obtain optimal PCR amplification results. As shown in Fig. 2, two single fragments of about 1.1 kb at 64 ^{o}C and 2.1 kb at 63 ^{o}C were amplified by primer pairs F1 + R1 and F2 + R2, respectively. According to previous results of y-type subunit genes (Halford et al. 1987; Yan et al. 2004a), they are consistent with the sizes of the 5' flaking upstream and coding region of *1By8* gene, respectively.

Both amplified products were cloned and sequenced by primer walking. The complete *1By8* gene sequence of 3020 bp was obtained by alignment of two amplified

sequences with 309 bp in overlapping. The complete gene contained 857 bp of upstream sequence and an intronless open reading frame (ORF) of 2166 bp encoding a mature protein of 720 amino acid residues. The comparisons of 5' flanking regions of *1By8* and other six HMW-GS genes (*1By9*, Halford et al. 1987; *1Ay*, Forde et al. 1985; *1Ax2**, Anderson and Greene 1989; *1Dx5* and *1Dy10*, Anderson et al. 1989; *1Dy12.1*^{t}, Yan et al. 2004a) were conducted and are shown in Fig. 3A. It is obvious that the 5' flanking regions of HMW-GS genes (about 750-850 bp from the initiator codon) have similar structures and contain some eukaryotic typical consensus, such as a TATA box and the –300 element (Forde et al. 1985) conserved in cereal storage protein genes, and a CCACC sequence that is highly conserved in eukaryotes (Kozak 1987). They may relate to tissue specific expression and efficient translation initiation of storage proteins (Anderson and Greene 1989). An enhancer sequence of HMW-GS genes identified by Thomas and Flavell (1990) is highly conserved and is considered as a major regulatory element for gene expression (Shewry and Halford, 2002). In addition, three CCAAT-like sequences (Messing et al., 1983) and three imperfect direct repeats (Halford et al., 1987) are present and also conserved in HMW-GS genes.

The deduced amino acid sequence of *1By8* subunit showed similar structural characteristics to other HMW-GS, including a signal peptide of 21 amino acid residues, an N-terminal sequence of 104 amino acid residues followed by a central repetitive domain of 553 residues and a C-terminal domain of 42 residues. The 28 N-terminal amino acid residues were identical to those obtained by microsequencing. The predicted molecular weight of *1By8* mature protein was 75156.7Da, 2015.2Da less than the molecular mass determined by MALDI-TOF-MS (77171.9Da) as shown in Fig. 1. The result is consistent with our unpublished data, which shows that discrepancy often exists between molecular weights deduced from sequence and measured with MALDI-TOF, based on analyzing a large number of germplasms. This suggests that some types of post-translational modifications, such as glycosylation or phosphorylation etc, may be present in the *1By8* subunit. This is consistent with the previous reports that some glycosylations and phosphotyrosine in HMW glutenin subunits were detected (Tilley et al. 1993; Tilley and Schofield 1995; Lauriere et al

1996). In particular, *1By8* subunit in genotype TAM105 was found to be extensively glycosylated by lectin binding and sugar analysis (Tilley et al. 1993).

The repetitive domain of *1By8* subunit possessed the typical characteristics of y-type HMW-GS. It consisted of tandem and interspersed repeats of 58 hexapeptide (consensus PGQGQQ) and 22 nonapeptide (consensus GYYPTSLQQ) motifs. Tripeptide (consensus GQQ), present in the x-type HMW subunit, was not present. The nucleotide and deduced amino acid sequences of *1By8* gene were deposited in the EMBL database under the accession number AY245797.

A comparison of the deduced amino acid sequences of the *1By8* subunit with other 6 y-type HMW-GS from different genomes is shown in Fig. 3B. Apparently, all subunits displayed high homology and 7 cysteine residues were highly conserved. In particular, *1By8* subunit showed the highest identity (99%) to *1By9* from bread wheat cultivar Cheyenne (Halford et al. 1987). Compared to the *1By9* subunit, the *1By8* subunit possessed an additional insertion of 15 amino acid residues (QYPASQQQPA QGQQG) at position 342. Consequently, the molecular weight of *1By8* was 1641.7 Da higher than that of *1By9* (73515.02Da). In addition, two residue substitutions occurred at positions 78 (leucine/proline) and 442 (arginine/glutamine) as indicated in Fig. 3B. The *1By8* contained 157 hydrophobic residues while *1Bx7* had 168 hydrophobic residues. Hence, *1By8* subunit showed less hydrophobicity than the *1Bx7* subunit, which is consistent with the migration order of RP-HPLC separation (Fig. 1).

3.3. Expression of 1By8 gene in E. coli

In order to analyze the effects of the *1By8* subunit on flour processing characteristics, the ORF of *1By8* gene was amplified and cloned into the expression vector pET-28a, and then transformed into an expression host *E. coli* strain BLR (DE3) plysS. The expressed protein was purified by extraction with 50% (v/v) propanol containing 2% DTT (w/v). The recombinant *1By8* subunit was separated by SDS-PAGE and the results are shown in Fig. 4. Apparently, the electrophoretic mobility of expressed protein is similar to that of the *1By8* subunit extracted from seeds of cultivar

Simeto. Their similarity was also confirmed by Western-blotting analysis. Both the bacterially expressed protein and endogenous *1By8* subunit of Simeto displayed strong reaction to the polyclonal antibody that was specific for HMW glutenin subunits.

3.4. Functional analysis of 1By8 subunit

The small-scale testing (2g or 10g Mixograph) of flour through single subunit incorporation with a reduction/re-oxidation procedure has been shown to be an effective method for investigating functional properties of HMW-GS (Bekes et al., 2001; Shewry et al., 2003). In this study, 60 mg subunit proteins of *1By8*, *1By9*, and *1Dy10* were incorporated into 10g of Jing411 base flour and their Mixograph properties were compared. As shown in Table 1 and Fig. 5, incorporation of y-type high molecular weight glutenin subunits can significantly alter Mixograph properties of base flour. Their impact on dough strength follows the order of *1Dy10* > *1By8* > *1By9*. Compared to *1By8* and *1By9* subunits, incorporation of subunit *1Dy10* resulted in increased mixing time, stable time and width at 8min. In contrast, incorporation of subunit *1By8* produced striking increases in peak resistance and peak width, and medium increases in MT, ST and width at 8min when compared to *1Dy10* and *1By9* subunits. While significant difference in effects on quality was observed between the two *By* subunits, their molecular weight or protein sequences are highly similar; ie, only a single 15 amino acid deletion within the repetitive domain and two single amino acid substitutions. This is consistent with previous findings that HMWGS subunits of different alleles usually share a high similarity in gene or protein sequences but differ widely in their effects on quality (Reviewed by Gianibelli et al., 2001; Ma et al., 2003; Butow et al., 2003; Ma et al., 2005; Lei et al., 2006; Liu et al., 2009). For example, a less desirable subunit Bx13 only differs in 100 daltons of molecular weight from Bx7 subunit (equal to 1 amino acid difference. Liu et al 2009). However, our results strongly demonstrate the importance of y-type subunits in wheat quality. The HMW-GS alleles usually consist of two genes or proteins, one x-type and one-y-type. Up to now, the functional differences between HMW-GS alleles have been usually interpreted by the

molecular and structural differences between x-type subunits. Little attention is paid to the variations of y-type subunits and their impact on quality parameters. Butow et al. (2003b) incorporated *1Dy10* subunit into transgenic lines and found that such incorporation significantly altered a range of quality parameters, including weaker dough and higher extensibility. The observed effect was attributed to the decrease of the *Dx*/*Dy* ratio after incorporating the *1Dy10* subunit. In contrast, our experiment provided direct evidence of functional difference between different y-type subunits. Molecular tools for differentiating and selecting different *By* subunits are now available (Lei et al 2006). The results obtained in this study may assist wheat breeders to utilize *By* subunit related variations in wheat quality improvement.

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Table 1 Effects of HMW-GS incorporations on Mixograph properties of dough

Parameters	Mix time (MT)	Stable time (ST)	Peak resistance (PR)	Peak width (PW)	Width at 8 min
Control（Jing 411）	1.900	1.850	43.250	17.000	4.250
1By8	4.325	5.100	70.250	34.000	20.500
1By9	3.000	5.900	47.500	29.000	17.500
1Dy10	4.925	7.900	49.250	22.500	24.000
$LSD_{0.05}$	0.319	0.353	6.418	3.539	2.454
$LSD_{0.01}$	0.529	0.585	10.644	5.870	4.070

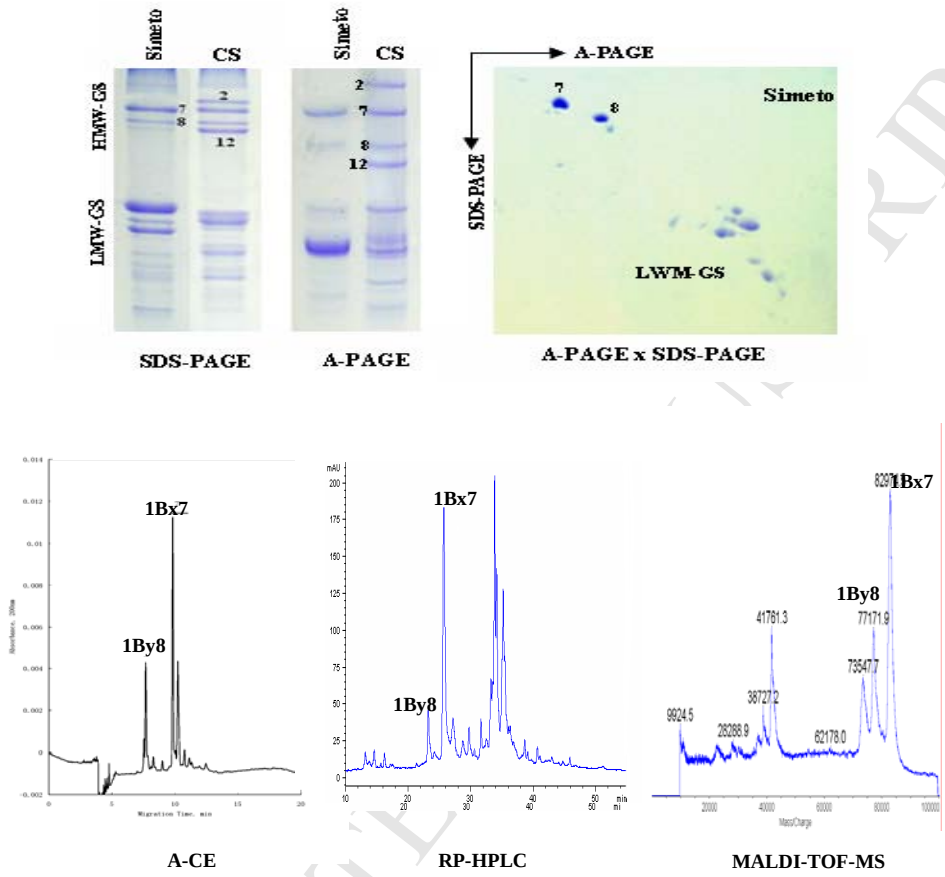


Fig. 1

Fig. 1. Separation and characterization of HMW-GS from Italy durum wheat cultivar Simeto by various separation methods.

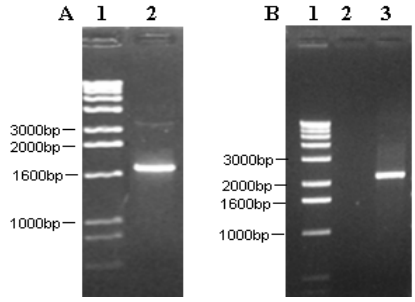


Fig. 2. AS-PCR products of *1By8* gene upstream and complete coding region from durum wheat cultivar Simeto.

A. Lane 1: 1kb DNA ladder Marker

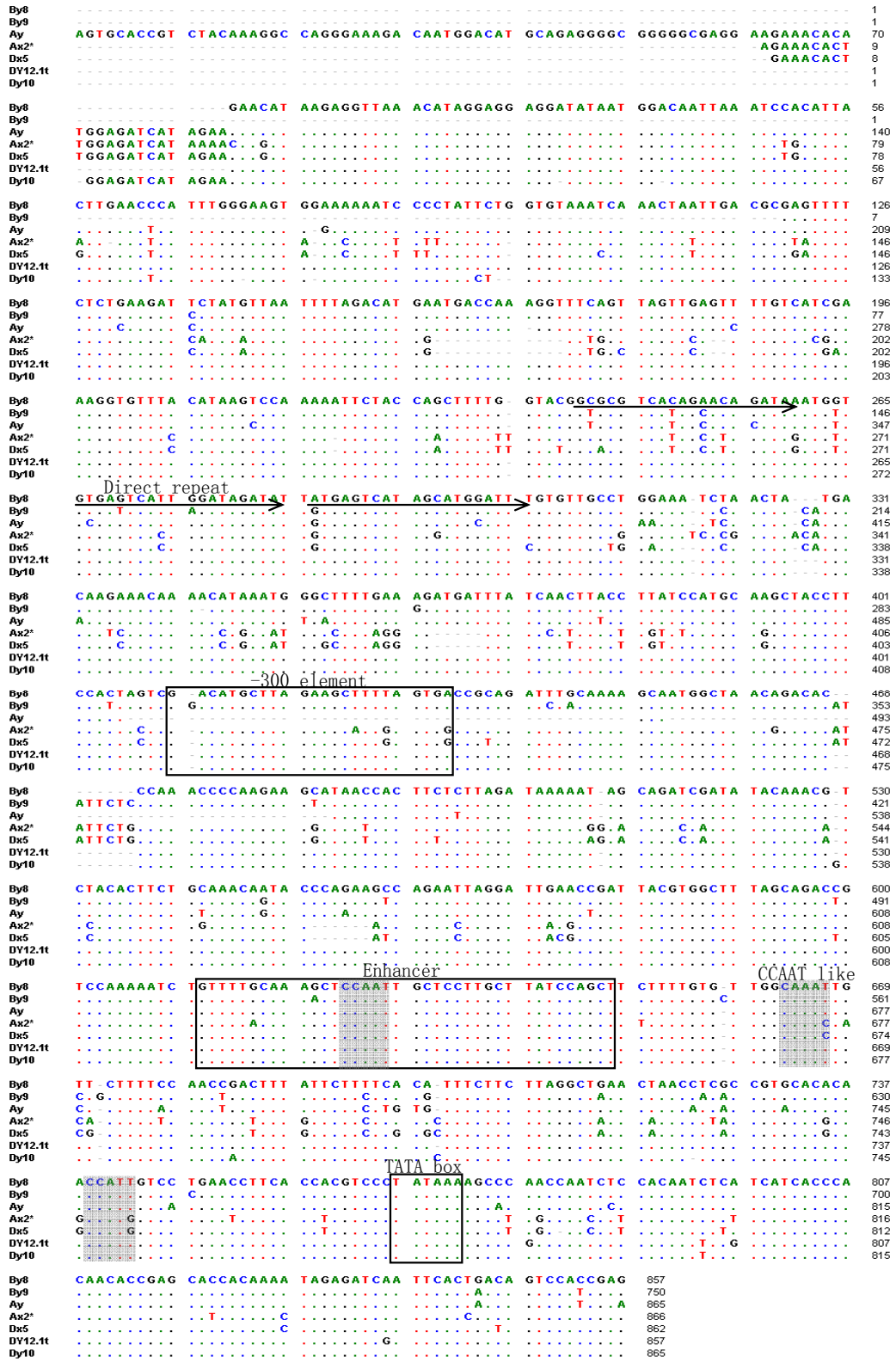
Lane 2: amplified product of *1By8* gene upstream

B. Lane 1: 1kb DNA ladder Marker

Lane 2: negative CK

Lane 3: amplified product of *1By8* complete coding region

A



B

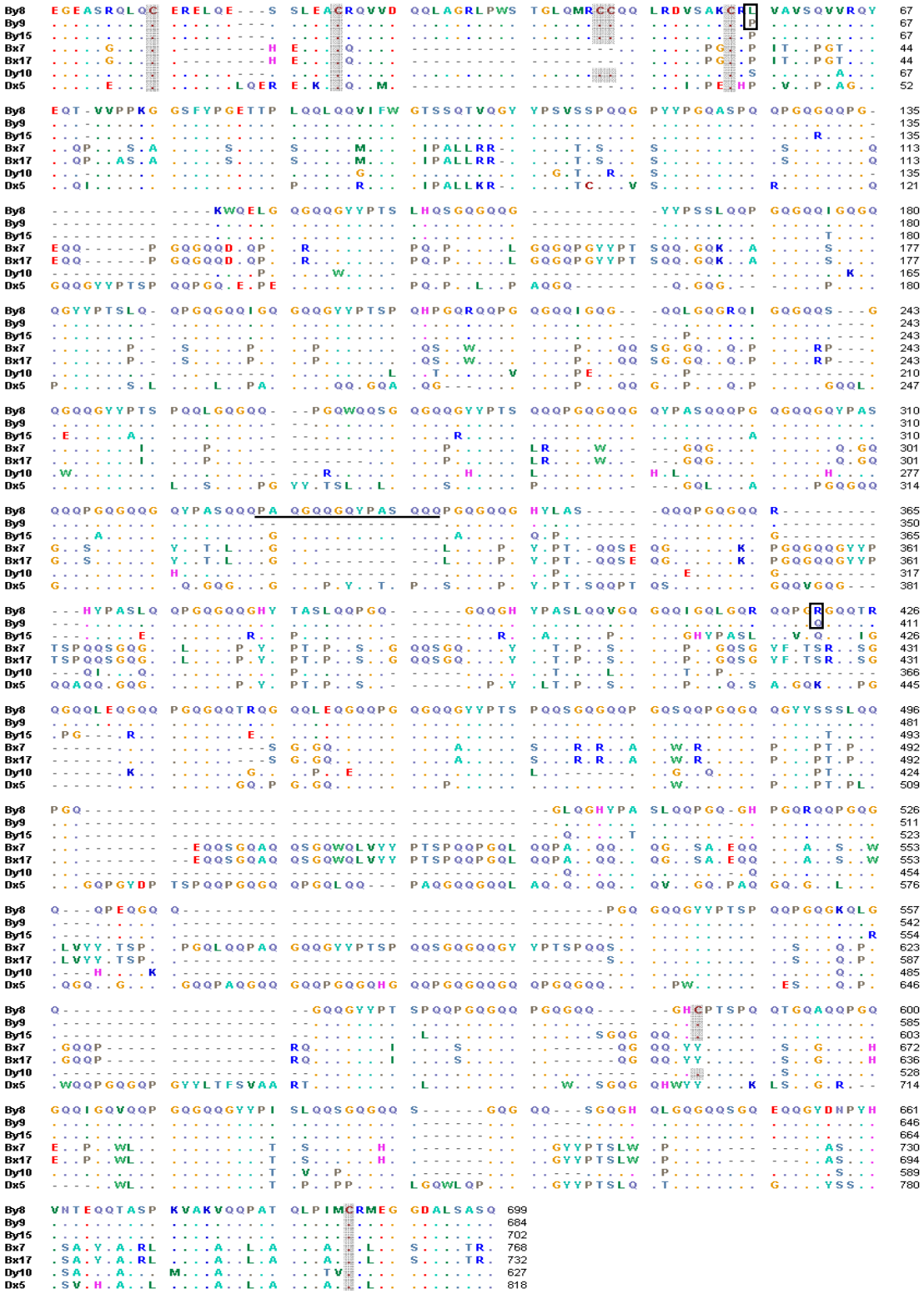


Fig 3.

Comparison of the 5' flanking DNA sequences and the deduced amino acid sequences of *1By8*

gene with those of other six HMW-GS gene. In the DNA sequences, *dots* indicate the consensus sequences of *1By8* and *dashes* represents deletions. Some typical sequences, including -300 element, enhancer, TATA box, CCAAT-like and three direct repeats were indicated. In the protein sequences, the cysteine residues and an additional insertion of 15 amino acid residues and two residue substitutions in 1By8 subunit were marked.

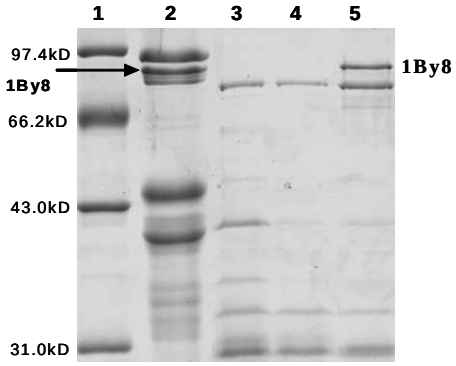


Fig. 4 Comparison of *1By8* subunit expressed in *E. coli* with that extracted from seeds of Simeto.

Arrow indicates the position of By8 subunit.

Lane 1: protein marker;

Lane 2: glutenin subunits extracted from of Simeto seed;

Lane 3: untransformed BLR-21(DE3) pLysS;

Lane 4: control bacterial culture transformed by *1By8* recombinant plasmid without IPTG inducing;

Lane 5: 1By8 recombinant plasmid lysate induced by IPTG.

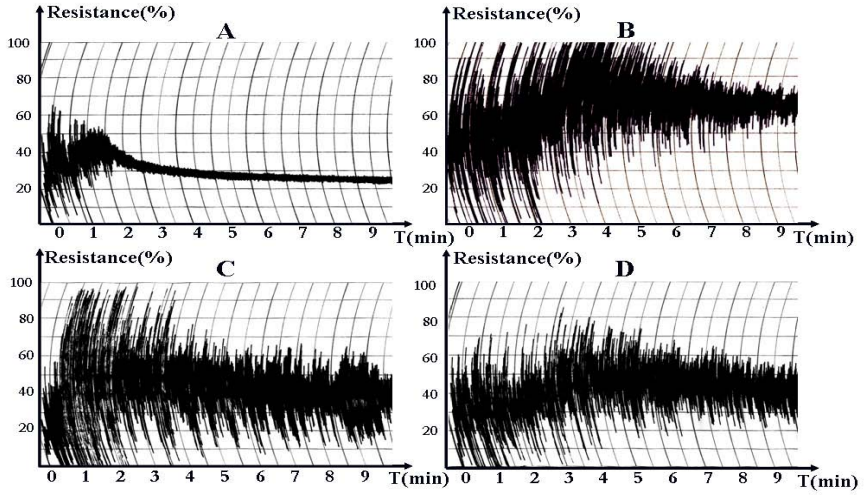


Fig. 5. Comparison of Mixograms of Control with those of incorporation into base flour of *1By8*, *1By9* and *1Dy10* subunits

A: Control (Jing 411)

B: *1By8* subunit

C: *1By9* subunit

D: *1Dy10* subunit