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ORIGINAL PAPER

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Similarities of omega gliadins from *Triticum urartu* to those encoded on chromosome 1A of hexaploid wheat and evidence for their post-translational processing

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Abstract The ω-gliadins encoded on chromosome 1 of the A genome were purified from *Triticum aestivum* L. (2*n*=6*x*=42, AABBDD) cv. Butte86, nullisomic 1D-tetrasomic 1A of cv. Chinese Spring (CS N1DT1A), and the diploid *T. urartu* (2*n*=2*x*=14, AA). Reverse-phase high-performance liquid chromatography combined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis of gliadin extracts from CS nullisomic-tetrasomic (NT) lines confirmed the assignment to chromosome 1A. The purified ω-gliadins were characterized by mass spectrometry and N-terminal sequencing. The 1A-encoded ω-gliadins were smaller than 1B- or 1D-encoded ω-gliadins. The N-terminal amino acid sequences for 1A ω-gliadin mature peptides were nearly identical to those for the *T. urartu* ω-gliadins and were more similar to 1D ω-gliadin sequences than to sequences for *T. monococum* ω-gliadins, barley C-hordeins, or rye ω-secalins. They diverged greatly from the N-terminal sequences for the 1B ω-gliadins. The data suggest that *T. urartu* is the A-genome donor, and that post-translational cleavage by an asparaginyl endoprotease produces those ω-gliadins with N-terminal sequences beginning with KEL.

Introduction

The protein composition of wheat flour is complex. Hexaploid wheat has three sets of genes from three different ancestors, represented by the A, B, and D genomes, and five major classes of gluten protein. Four classes, the high- and low-molecular-weight glutenin subunits (HMW-GS and LMW-GS) of the glutenin polymer and the γ- and ω-gliadins have been mapped to specific loci on homoeologous group 1 chromosomes (Dubcovsky et al. 1997; Lafiandra et al. 1984; Masci et al. 1991; Payne et al. 1984; Sabelli and Shewry 1991; Wrigley and Shepherd 1973). In this paper we refer to the ω-gliadins encoded at the *Gli-A1*, *Gli-B1* and *Gli-D1* loci at the distal end of the short arm of homoeologous group 1 chromosome 1A, 1B and 1D as the 1A, 1B and 1D ω-gliadins, respectively.

The ω-gliadins are unusual proteins in terms of their repetitive sequences rich in glutamine and proline, lack of cysteine and lack of alpha-helical structure. They represent 10% or less of total wheat flour proteins, but are reported to change in response to nitrogen (Daniel and Triboi 2000; Timms et al. 1981; Wieser and Seilmeier 1998) and sulfur availability (Wrigley et al. 1984), as well as in response to other environmental factors (Ciaffi et al. 1996). It is possible that changes in ω-gliadin amounts or proportions contribute to variability in flour quality. Also, ω-gliadins may have epitopes that are involved in the gluten-sensitive response of celiac patients (Denery-Papini et al. 1999; Ensari et al. 1999) and ω-gliadins are the principal target of an enzyme-linked immunoassay used to determine the degree of gluten contamination in "gluten-free" products for celiac patients (Skerritt and Hill 1991). A number of papers describe the unusual physical structure and behavior of purified ω-gliadins and the related C-hordeins and ω-secalins (DuPont et al. 2000; Field et al. 1986; Pézolet et al. 1992; Tatham and Shewry 1985, 1995; Tatham et al. 1989; Thomson et al. 1999; Wellner et al. 1996). However, many basic questions about the ω-gliadins need to be answered.

The 1A, 1B and 1D ω-gliadins were purified and characterized in terms of amino acid composition and N-terminal sequence (Kasarda et al. 1983; Popineau et al. 1986; Seilmeier et al. 2001; Tatham and Shewry 1995). Based on lactate gel electrophoresis and N-terminal sequence, ω-gliadins were categorized as ω-1, ω-2 and ω-5 types. The ω-1 and ω-2 types had similar amino acid compositions and N-terminal sequences and were associated with chromosomes 1A and 1D. Lactate gel electrophoresis of aneuploid and substitution lines was used to

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distinguish 1A from 1D ω-gliadins (Kasarda et al. 1983; Lafiandra et al. 1984; Metakovsky and Sozinov 1987; Wrigley and Shepherd 1973). The ω-5 types had higher glutamine contents and a different N-terminal sequence, were associated with chromosome 1B (Kasarda et al. 1983), and recently were characterized in some detail (DuPont et al. 2000).

Currently, there are only a few gene sequences for the ω-gliadins (Hsia and Anderson 2001; Masoudi-Nejad et al. 2002). The sequences encoded proteins of 30,000 Da to 39,000 Da, which are smaller than the masses of those 1B and 1D ω-gliadin proteins that have been characterized (DuPont et al. 2000). In this study, we identified, purified and characterized the 1A ω-gliadins from *T. aestivum* cv. Butte86 and CS N1DT1A, and compared them with ω-gliadins from *T. urartu*, suggested to be the donor of the A genome (Ciaffi et al. 1997; Dubcovsky et al. 1997; Dvorak et al. 1993; Galili et al. 2000; Waines and Payne 1987). Size and N-terminal sequence were similar to those predicted by the Hsia and Anderson (2001) ωG3 gene sequence. Other CS group 1 nullisomic-tetrasomic (NT) lines were examined to verify the assignment of the ω-gliadins to chromosome 1A.

Materials and methods

Grain and flour samples

Flour was prepared from grain of Butte86 grown as previously described (DuPont et al. 2000). Grain or flour samples also were obtained from the following lines of CS and CS NT lines that were originally developed by Sears (1954) and provided to us by Dr. Sears or Dr. A. Lukaszewski, University of California-Riverside: CS, CS N1AT1B, CS N1AT1D, CS N1BT1A, CS N1BT1D, CS N1DT1A, and CS N1DT1B. Grain from *T. urartu* was obtained from Dr. J. Giles Waines, University of California-Riverside.

Protein extraction

Milled white flour or grain samples crushed in a mortar and pestle were extracted with 50% (v/v) n-propanol (DuPont et al. 2000) to obtain a fraction highly enriched in gliadins. Small amounts of HMW-GS, LMW-GS, albumins and globulins also were eluted by this procedure, as previously reported (Fu and Sapirstein 1996). Alternatively, gliadins were prepared by extraction with 40% ethanol, followed by precipitation with 1.5 M NaCl (DuPont et al. 2000). Protein was determined by N analysis (DuPont et al. 2000), using a protein to N ratio of 5.7.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Freeze-dried protein samples were suspended in Novex NuPage sample buffer (Invitrogen, Carlsbad, Calif.) with 50 mM DTT by vortexing for 1 h at 22°C in a TOMY MT-360 Microtube Mixer (Tomy Seiko, Tokyo, Japan), then centrifuged for 10 min at 14,000 rpm in a microfuge. Five to 20 μl was loaded onto a Novex NuPAGE 4–12% acrylamide, Bis-Tris gel and separated using the Novex MES SDS running buffer (Kasarda et al. 1998). The Mark12 protein standard (Invitrogen) was used. Gels were stained overnight using Brilliant Blue G (Sigma, St. Louis, Mo.) and destained with water for less than 8 h to ensure retention of the ω-gliadins in the gels (Kasarda et al. 1998).

Gel filtration chromatography

Gliadins were dissolved in 0.1 N acetic acid, applied to a 2.5× 90-cm BioGel P-100 (BioRad, Hercules, Calif.) column equilibrated with 0.1 M acetic acid, eluted with 0.1 M acetic acid at a rate of 16 ml h^{-1}, and collected in 4-ml fractions (DuPont et al. 2000). Fractions were freeze dried and stored at −80°C. Fractions enriched in ω-gliadins were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pooled, freeze dried, and stored at −80°C.

Reverse-phase high-performance liquid chromatography

Freeze-dried proteins were dissolved at a concentration of 1 mg protein/ml in 6 M guanidine HCl adjusted to pH 8.0 with TRIS, plus 50 mM DTT, and 500 μl was applied to a Vydac (Hesperia, Calif.) 218TP C18 semipreparative reverse-phase high performance liquid chromatography (RP-HPLC) column (3-nm pore diameter, 10-mm ID, 250-mm length). A Hewlett Packard Series 1100 HPLC (Wilmington, Del.) was used to elute the proteins using gradient 1 or gradient 2. Gradient 1 was from 10% to 90% (v/v) acetonitrile in 0.05% trifluroracetic acid (TFA). The concentration was increased at a rate of 0.92% per min with a flow rate of 1.5 ml min^{-1} at 50°C (DuPont et al. 2000). Gradient 2 incorporated a 4-min delay for sample loading, followed by an increase from 10% to 50% acetonitrile in 0.05% TFA for 90 min, followed by an increase from 50% to 65% acetonitrile in TFA for 18 min. Peaks were collected, freeze dried, and stored at −80°C.

Mass spectrometry

The mass of the ω-gliadins was determined by electrospray ionization (ESI) mass spectrometry using a QSTAR Pulsar *i* quadruple time-of-flight (TOF) mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with a Proxeon Biosystems (Odense, Denmark) nano-electrospray source. Prior to analysis, freeze-dried ω-gliadin fractions were reconstituted in 8 M guanidine HCl and purified by RP-HPLC using a 1×150-mm Vydac C-18 column. A Hewlett Packard Series 1090 HPLC was used to elute the proteins using a gradient of 20% to 70% (v/v) acetonitrile in 0.1% TFA. The concentration was increased at a rate of 0.83% per min with a flow rate of 50 μl min^{-1} at 50°C (60 min). Five-microliter portions of the HPLC-purified ω-gliadins were loaded directly into New Objective offline PicoTip emitters (New Objectives, Woburn, Mass.). Protein concentration was estimated to be 40 pmol μl^{-1}. Spray voltage was set to 1,200 V, and the nano-electrospray capillary was advanced toward the orifice until spray commenced. Data were collected in the TOF mode over the range of 500–2,000 atomic mass units (amu). The mass spectra were reconstructed from the electrospray (mass/charge) spectra using the Bayesian Protein Reconstruct Tool supplied with the Analyst QS instrument software package.

Protein sequencing

Protein sequence determination of the ω-gliadins was carried out using a Perkin Elmer Applied Biosystems (Foster City, Calif.) Procise 492 equipped with an online HPLC system for phenylthiohydantoin-amino acid identification (DuPont at al 2000). In brief, samples were dissolved in a small volume of 50% acetonitrile containing 0.1% TFA and applied to a preconditioned, Biobrene (Applied Biosystems)-coated glass fiber filter, dried and subjected to Edman degredation. Data were interpreted using the supplied software algorithms and by visual inspection of the chromatograms.

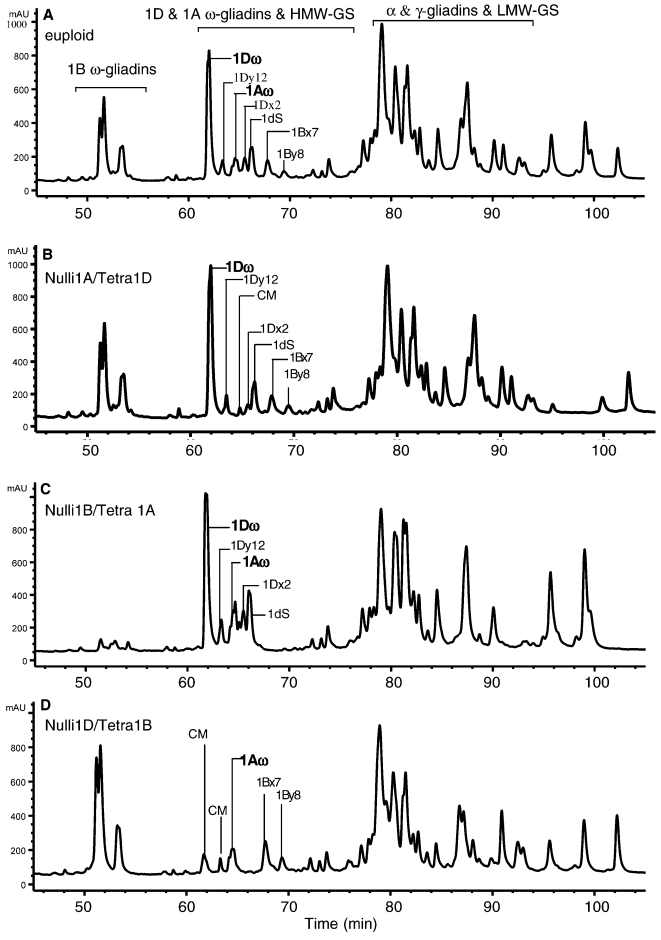

Fig. 1A–D RP-HPLC analysis of the proteins extracted with 50% propanol from flour of *Triticum aestivum* cv. Chinese Spring lines **A** CS, **B** CS N1AT1D, **C** CS N1BT1A, and **D** CS N1DT1B. The positions of the 1A, 1B, and 1D *ω*-gliadins are indicated (*1Aω*, *1Bω*, *1Dω*), as well as minor amounts of the 1Dx2, 1Dy12, 1Bx7 and 1By8 high-molecular-weight glutenin subunit (*HMW-GS*), a putative D glutenin-subunit (*1dS*) and a CM-type, alpha-amylase trypsin inhibitor. Analyzed using gradient 2

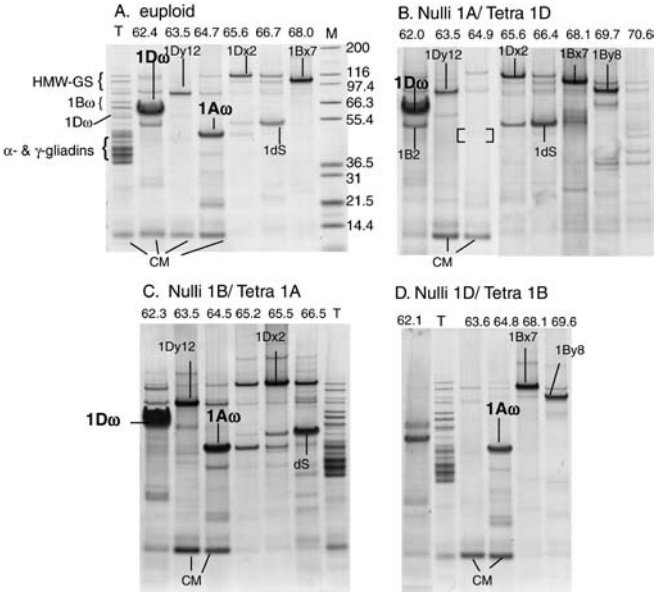

Fig. 2A–D SDS-PAGE analysis of the *ω*-gliadin peaks collected between 62 min and 70 min from the RP-HPLC analysis shown in Fig. 1. **A** CS, **B** CS N1AT1D, **C** CS N1BT1A, and **D** N1DT1B lines. The position of the 1A and 1D *ω*-gliadins is indicated (*1Aω*,*1Dω*), as well as the 1Dx2, 1Dy12, 1Bx7 and 1Bx8 HMW-GS, the D-subunit (*1dS*), and an alpha-amylase/trypsin inhibitor (*CM*). *Brackets* indicate the missing 1A *ω*-gliadin. *T* Total 50% propanol extract from the euploid line, *M* mass standards. Relative mass is indicated on *left* of A. Elution time is indicated in minutes

Results

Chromosomal localization of CS *ω*-gliadins

In order to purify the 1A *ω*-gliadins, it was essential to identify them in RP-HPLC elution patterns and in SDS-PAGE. Therefore, *ω*-gliadins from CS and CS group 1 NT lines were compared. Gliadin extracts were analyzed by RP-HPLC (Fig. 1), followed by SDS-PAGE of the RP-HPLC fractions (Fig. 2). The identifications of peaks, protein bands and chromosomal assignments were arrived at by interpretation of a larger data set combined with prior knowledge of the characteristic RP-HPLC elution patterns of the abundant gluten storage proteins and their mobilities in SDS-PAGE.

Proteins from the CS flour were separated by RP-HPLC into three distinct groups, distinguished by brackets in Fig. 1A. The first group of peaks eluting at 51–54 min mainly corresponded to the 1B *ω*-gliadins, along with minor amounts of unidentified proteins (DuPont et al. 2000). The 1B *ω*-gliadin peaks were missing from the N1BT1A line (Fig. 1C). The second group of peaks, between 61 min and 70 min, included 1D and 1A *ω*-gliadins, small amounts of the four HMW-GS (1Dy12, 1Dx2, 1Bx7, 1By9), a D glutenin-subunit (1dS), and an alpha amylase/trypsin inhibitor, otherwise known as a CM-type albumin (CM). The 1D *ω*-gliadin peak eluted at 62 min and was missing from the N1DT1B line (Fig. 1D). The 1A *ω*-gliadin peak eluted at 64–65 min, between the 1Dy12 and 1Dx2 peaks. The 1A *ω*-gliadin peak was present in all lines except the N1A/T1D line (Fig. 1B). The 1Dy12, 1Dx2 and 1dS peaks were missing from the N1D/T1B line (Fig. 1D), and the 1Bx7 and 1By8 peaks were missing from the N1B/T1A lines (Fig. 1C). Peaks that contained the chromosome 7-encoded CM (Singh and Skerritt 2001) are also indicated. The third group of peaks eluted between 77 min and 100 min and were mainly composed of *α*- and *γ*-gliadins, along with a small amount of LMW-GS, albumins and globulins. Details for the third group will not be discussed.

The individual RP-HPLC peaks were collected and analyzed by SDS-PAGE. Analysis of HPLC peaks collected between 62 min and 70 min (Fig. 2) revealed the putative 1A *ω*-gliadin with an apparent molecular mass of 47,000 that eluted at 64.7 min along with lighter bands of apparent molecular masses 17,000–37,000 that also were associated with chromosome 1A. The 1A *ω*-gliadin band

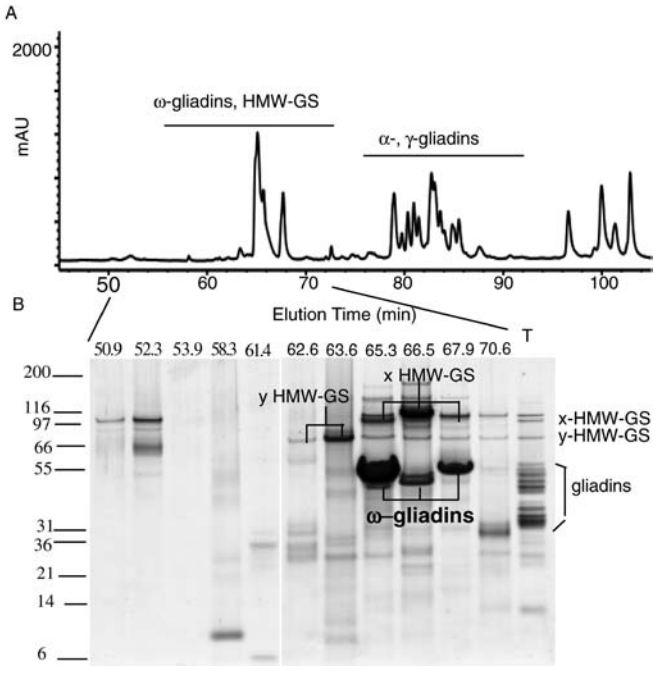

Fig. 3 **A** RP-HPLC analysis of the proteins extracted with 50% propanol from grain of *T. urartu,* analyzed using gradient 2. **B** SDS-PAGE analysis of the RP-HPLC peaks collected between 50 and 71 min. The positions of putative y- and x-type HMW-GS and the *ω*-gliadins are indicated. *T* Total 50% propanol extract. Mass of standards is indicated to left of B. Elution time is indicated in minutes

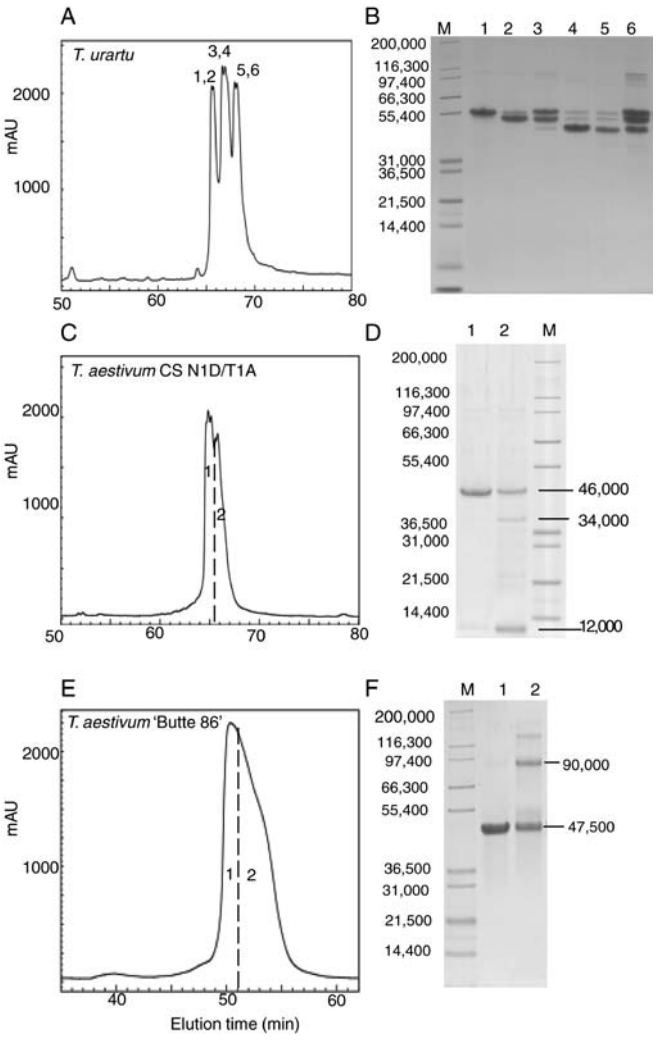

Fig. 4 RP-HPLC and subsequent SDS-PAGE analysis of purified *ω*-gliadins from *T. urartu* (**A**, **B**), CS N1DT1A (**C**, **D**), and Butte86 (**E**, **F**). Gradient 2 (**A**, **C**) or gradient 1 (**E**) was used for the RP-HPLC analysis of the purified *ω*-gliadins. *M* Molecular mass standards

was present in all lines except the N1AT1D line (Fig. 2B), indicating that it was associated with chromosome 1A. A dense, 1D *ω*-gliadin protein band with an apparent molecular mass of 57,000 (1D*ω*) eluted at 62.4 min. The 1D*ω*-gliadin band was present for all lines except the N1DT1B line (Fig. 2D), confirming its assignment to chromosome 1D. An additional band at 50,000 Da coeluted with the 1D *ω*-gliadin and appeared to be associated with chromosome 1B. Small amounts of the 1Dy12, 1Dx2, 1Bx7 and 1By8 HMW-GS are evident. The 1dS band, of apparent molecular mass of 52,000, eluted at 66.7 min and was present in all lines except the N1DT1B line (Fig. 2D), suggesting that it was associated with chromosome 1D. The 1dS band may be an example of a 1D-encoded D-glutenin subunit (Masci et al. 1993). The approximately 12,000-Da CM band eluted between 62.4 min and 64.7 min and was present in all lines.

Identification of *ω*-gliadins from *T. urartu*

Gliadin-rich fractions were prepared from *T. urartu* flour and separated by RP-HPLC (Fig. 3A). Only minor peaks eluted at 50–62 min, where the 1B and 1D *ω*-gliadins from *T. aestivum* would have eluted. The first large group of protein peaks emerged between 63 min and 68 min, exactly when the HMW-GS and 1A *ω*-gliadins from *T. aestivum* would have eluted. SDS-PAGE of the HPLC peaks (Fig. 3B) revealed at least three dense protein bands with an apparent molecular mass of 45,000–59,000 that eluted at 65.3–67.9 min and were likely to be *ω*-gliadins. In the same fractions, there were proteins with an apparent molecular mass of 70,000–116,000 that probably were x-type and y-type HMW-GS.

Purification and characterization of *ω*-gliadins

The *T. urartu* *ω*-gliadins were purified on a P100 column followed by RP-HPLC, which resolved them into three pairs of overlapping peaks (Fig. 4A). The six peaks were collected, repurified by RP-HPLC, and analyzed by SDS-PAGE, revealing a series of proteins with apparent sizes ranging from 45,000 Da to 59,000 Da (Fig. 4B). To purify the 1A *ω*-gliadin from CS, the N1DT1A line was used, to eliminate cross contamination from 1D *ω*-gliadins and the

1dS protein. The N1DT1A line is not shown in Figs. 1 and 2; the major peak in the vicinity of 64 min was the 1A *ω*-gliadin. The P100 column purification step was omitted; instead, the 1A *ω*-gliadin peak obtained by RP-HPLC was rechromatographed by RP-HPLC. The partially purified 1A *ω*-gliadins were resolved as a double peak that was collected in two fractions (Fig. 4C). SDS-PAGE revealed a band of 46,000-Da apparent mass in both fractions along with additional bands, including one at 12,000 Da in the trailing edge fraction 2 (Fig. 4D). The *ω*-gliadins from Butte86 were purified on a P100 column and then separated by RP-HPLC, using gradient 1. The 1A *ω*-gliadins were rechromatographed by RP-HPLC. They formed a broad peak with a trailing shoulder and were collected in two fractions (Fig. 4E). SDS-PAGE analysis revealed one band of apparent molecular mass 47,500 Da in both fractions, and additional bands of approximately 90,000 Da and 120,000 Da in the trailing fraction (Fig. 4F). We often observed these extra bands in gels of well-characterized, purified *ω*-gliadin preparations, and we suspect that they represent dimers and trimers formed by interactions between *ω*-gliadin monomers.

The elution patterns for the purified 1A *ω*-gliadin and the total gliadin fraction from Butte86 were compared in order to identify the 1A *ω*-gliadin peak in the RP-HPLC elution pattern (Fig. 5). The RP-HPLC pattern for the Butte86 gliadin extract was similar to that for CS and the purified 1A *ω*-gliadin formed a single peak that coincided with the fourth *ω*-gliadin peak in the total gliadin extract.

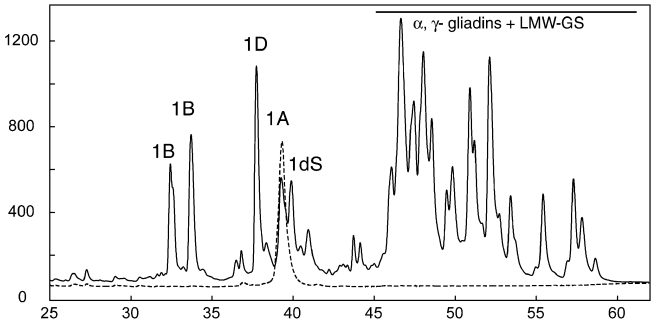

Fig. 5 RP-HPLC analysis of the total gliadin fraction (*solid line*) and the 1A *ω*-gliadins (*dotted line*) from Butte 86. The position of the 1B, 1D, and 1A encoded *ω*-gliadin peaks and the putative 1dS subunit are indicated. Analyzed using gradient 1

Mass spectrometry and N-terminal sequencing of 1A *ω*-gliadins

The masses of the *ω*-gliadins from *T. urartu* and the putative 1A *ω*-gliadins from Butte86 and CS N1DT1A were determined by ESI mass spectrometry (Table 1).

Table 1 Masses and N-terminal amino acid sequences of 1A *ω*-gliadins. *ESI* Electrospray ionization

Source	Molecular mass by SDS-PAGE	Mass[a] (amu)	ESI Proportions[b]	ΔMass[c] (amu)	Amino acid proportions[d]	N-terminal amino acid sequences[e]
Triticum urartu 1	58,000	43,363	0.50	-	0.4	ARQLNPS**NK**ELQSPQQS
		42,479	0.50	883	0.6	**K**ELQSPQQSFSHQQQPF
T. urartu 2	55,000	41,241	0.60	-	0.9	ARQLNPS**NK**ELQSPQQ
		40,358	0.27	883	0.1	**K**ELQ
T. urartu 3	58,000	42,145	0.29	-	0.3	ARQLNPS**NK**ELQSPQQS
	55,000	40,359	0.67	1,786	0.7	**K**ELQSPQQSFLHQQQPF
T. urartu 4	45,000	35,440	0.84	-	0.8	ARQLNPS**NK**ELQSPQQS
		34,559	0.15	881	0.2	**K**ELQSPQQSFSH?Q??F?
T. urartu 5	45,000	35,449	0.02	-	0.1	?RQ?NP?**NK**ELQ?PQ
		34,566	0.98	883	0.9	**K**ELQSPQQSFSHQQQPF
Triticum aestivum						
CS N1DT1A 1A1*ω*	46,000	39,347	0.68	-	0.8	ARQLNPS*KQ*ELQ*X*PQQ*L*
					0.2	RQLNPS*KQ*
CS N1DT1A 1A2*ω*	46,000	39,450	0.97		0.5	ARQL
					0.1	R?
CM contaminant	12,000	-	-	-	0.4	*TGQYCYAGMGLP*
Butte86 1A1*ω*	47,500	39,447	0.48	-	0.2	AR??N*P*?
		35,342	0.27	4,105	0.3	RQLNPS**NKEL**QSPQ
		34,530	0.25	812	0.5	KELQSPQQSFS?H?*P*
Butte86 1A2*ω*	47,500	39,345	0.46	-	0.2	ARQ?N
		35,334	0.24	4,011	0.3	RQLNPS**NK**ELQSPQQ
		34,523	0.30	811	0.5	**K**ELQSPQQSFSHQQQ

[a] Mass of the predominant protein(s) in a single HPLC fraction
[b] Proportions of the two or three protein species found in a fraction, based on the area in the ESI spectrum
[c] Difference in mass compared to the protein with the next highest mass in the same fraction
[d] Proportions of the two or three protein species found in a fraction, based on the proportions of the amino acids identified in the first sequencing cycle
[e] Putative asparaginyl endoprotease cleavage site is in *bold*, *X* indicates that both S and P were detected, *italics* indiates difference from consensus sequence

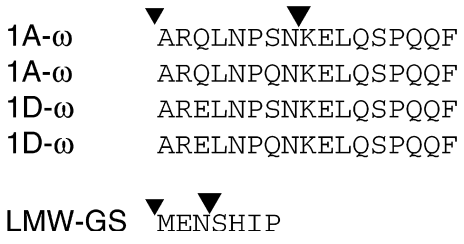

Fig. 6 Suggested cleavage sites for an asparaginyl endoprotease in the N-terminal sequences of the 1A and 1D *ω*-gliadins and in the predicted N-terminal amino acid sequence of a SHIP-type Low-molecular-weight (LMW)-GS

The fractions correspond to the subdivided HPLC peaks shown in Fig. 4. Mass spectrometry consistently detected two or three masses in fractions that had only a single band in SDS-PAGE. The masses were 15–28% less than the sizes estimated by SDS-PAGE. For each fraction, the proportion of protein associated with each mass was estimated based on the area of the ESI peak. The difference between the mass of each protein and the next smaller protein in the same fraction is also indicated (Δ-mass). Analysis of the *T. urartu* *ω*-gliadins from five of the six HPLC peaks in Fig. 4A, B detected ten major protein species, based on mass, ranging from 34,566 to 43,363. Two major protein species were determined in the CS 1A *ω*-gliadin fractions shown in Fig. 4C, D. The 1A1 fraction had a major component at 39,347, and the 1A2 fraction had a major component at 39,450. Mass spectrometry of the 1A *ω*-gliadins from Butte86 (Fig. 4E, F) revealed six protein species, with three in each subfraction, ranging in mass from 34,523 to 39,447.

N-terminal sequences also were determined (Table 1). Each fraction had two to three distinct N-terminal sequences, corresponding to the number of masses in the fraction. It was possible to distinguish between two sequences for as many as 17 sequencing cycles. The proportions of proteins with each N-terminal sequence were estimated based on the proportions of the N-terminal amino acids in the first sequencing cycle. Two sequences were obtained for each *T. urartu* peak, beginning with either ARQ or KEL. Two sequences also were obtained for the CS 1A1 fraction, beginning ARQ or RQL. The CS 1A2 fraction had two clear sequences, beginning ARQ and TGQ, and may have had a third sequence beginning with R. Comparison with protein sequence databases suggests that the sequence beginning TGQ is an alpha amylase/trypsin inhibitor that corresponds to the 12,000-Da band in Fig. 4D and the CM bands in Fig. 2. The CM protein did not coelute with the *ω*-gliadins in the final HPLC run prior to mass spectrometry, and thus, the mass was not determined. Three N-terminal sequences were obtained for each of the Butte86 sub fractions, beginning ARQ, RQL and KEL.

All N-terminal sequences for Butte86 1A *ω*-gliadins and *T. urartu* *ω*-gliadins began with, or were abbreviated from, the fundamental sequence of ARQLNP-SNKELQSPQQ. The fact that many of the fractions contained an ARQ/KEL pair or an ARQ/RQ/KEL triplet suggested that these pairs or triplets had highly similar interactions with the HPLC column and that their complete protein sequences were very similar. The differences in mass of 881 Da to 883 Da between the two proteins in four of five *T. urartu* *ω*-gliadin fractions corresponded to the mass of the ARQLNPSN segment by which the two sequences differed. Within the error of measurement, this was the difference predicted if the larger ARQ protein were cleaved between the N and K to produce the smaller KEL protein (Fig. 6). It is not known for sure which N-terminal sequence corresponded to which mass, but comparison of the proportions of the proteins based on mass and based on the first amino acid suggest that the ARQ type was the larger protein, and the KEL type was the smaller protein. If so, the difference between the proteins beginning ARQ and KEL may be the result of post-translational cleavage of the ARQ protein between the asparagine and the lysine at positions 8 and 9. The two Butte86 fractions each had three masses differing by 4,105 or 4,011 amu (equivalent to 35 to 36 amino acids) and 810 or 813 amu (equivalent to seven amino acids), the mass of the peptide RQLNPSN. Therefore, the KEL proteins may have originated from the RQL proteins by cleavage between the N and K at positions 7 and 8. The difference of 35 or 36 amino acids suggests that the protein beginning ARQ represented a separate gene product, or that an additional C-terminal cleavage occurred. For Butte86 1A *ω*-gliadins, there was not a clear relationship between the relative amounts of protein with the three different masses and the estimated amounts of A, R, or K in the first amino acid sequencing cycle. This is not surprising, given the increased difficulty of discriminating between three different sequences.

The N-terminal sequences for the CS 1A1 *ω*-gliadin differed from those of *T. urartu* and Butte86 by having KQ rather than NK at positions 8 and 9. Thus, CS 1A1 did not have the proposed cleavage site. CS 1A2 did not give very good sequence. No KEL-type sequence for CS N1DT1A was found.

Table 2 lists estimated masses and N-terminal sequences for other *ω*-gliadins, rye *ω*-1 secalins and barley C-hordeins. Published protein sequences for 1D *ω*-gliadins from Butte86 and CS began ARE and KEL, and differed by only one or two amino acids from those of the 1A *ω*-gliadin sequences. The reported masses of the Butte86 ARE and KEL 1D *ω*-gliadins differed by 777 amu, less than the mass of 881 amu needed to account for the eight-amino acid difference between the ARE and KEL N-terminal sequences. It is possible that the MALDI-TOF was out of calibration when those data were obtained and/or guanidine adducts broadened the protein peaks, decreasing the resolution. Published sequences for *ω*-2- and *ω*-1-type *ω*-gliadins of *T. durum* (AABB) were identical to the ARQ and KEL sequences for the *T. urartu* and Butte 86 1A *ω*-gliadins. Sequences for the *ω*-gliadins from *T. monococcum* (A^mA^m) differed by 6 of the first 20 amino acids and lacked the proposed NK cleavage site. Published C-1 hordein and *ω*-1 secalin N-terminal se-

Table 2 Published N-terminal amino acid sequences of ω-gliadins, ω-secalins, and C-hordeins

Source	Molecular mass by SDS-PAGE	Mass[a] (amu)	ΔMass[b] (amu)	N-terminal amino acid sequences[c]
T. aestivum			-	
Butte86′ 1D1 ω[f]	56,000	42,700	777	AR*E*LNP*Q***NK**EL
Butte86′ 1D2 ω[f]	56,000	41,923	-	**K**ELQSPQQSF
CS 1D ω-2[d]	-	-	-	AR*E*LNPS**NK**ELQSPQQSFS
CS 1D ω-1[d]	-	-	-	**K**ELQSPQQSFSHQQQPFPQQ
T. durum ω-2 E[d]	-	-	-	ARQLNP?**N**?E
T. durum ω-1 E[d]	-	-	-	**K**ELQSPQQSF?HQQQPFPQQ
T. durum ω-2 M[d]	-	-	-	ARQLNP?**N**?E
T. durum ω-1 M[d]	-	-	-	**K**ELQSPQQSF
T. monococum ω-1[d]	-	-	-	RQLNPS*DQ*ELQSPQQ*LYPQQPYPQQPY*
T. monococum ω-1[d]	-	-	-	ARQLNPS*DQ*ELQSPQQ*LYPQQPYPQQPY*
Hordeum vulgare C-1 hordein[e]	-	-	-	RQLNPS*SQ*ELQSPQQS*YLQQPYPQNPY*
Secale cereale ω-1 secalin[e]	-	-	-	RQLNPS*EQ*ELQSPQQ*PV*
T. aestivum	-	-	-	
Butte 86 1B1 ω[f]	65,000; 57,000	51,300; 50,100; 48,900	1,200	SRXL*SPG*-KEL*HT*PQ*EFQFP*QQQ G T
Butte 86 1B2 ω[f]	65,000; 57,000	51,500; 50,300; 49,085	1,200	SRXL*SPG*-KEL*HT*PQ*E*-Q*FP*QQQ*Q* G T

[a] Mass of the predominant protein(s) in a single HPLC fraction
[b] Difference in mass compared to the protein with the next highest mass in the same fraction
[c] Differences from the 1A ω-gliadin consensus sequence are *underlined*, putative asparaginyl endoprotease cleavage site is in *bold*, *X* indicates that L and M were detected
[d] Kasarda et al. 1983: *E T. durum* Edmore, *M T. durum* Mindum
[e] Shewry et al. 1981
[f] Dupont et. al. 2000

Table 3 N-terminal amino acid sequences and masses of ω-gliadins, ω-secalins, and C-hordeins predicted from gene sequences

Source	Mass[a] (amu)	N-terminal amino acid sequence[b]
T. aestivum		
Cheyenne ωG3[c]	39,210	ARQLNPS**NKEL**QSPQQSFSHQQQPFPQK
Cheyenne ωF20b[d]	30,460	AR*E*LNPS**NKEL**QSPQQSFS*Y*QQQPFPQ*Q*
CS A1[e]	46,090	AR*H*LNPS*DQ*ELQSPQQ*Q*F
H. vulgare C hordein[f]	36,000	ARQLNPS*HQ*ELQSPQQ*P*F*LKQQSYLQQPYPQQPY*
S. cereale ω-1 secalin[g]	37,000	ARQLNPS*EQ*ELQSPQQ*PVPKEQSYPQQ*

[a] Mass predicted for the mature protein
[b] Differences from the 1A ω-gliadin consensus sequence are *italicized*, putative asparaginyl endoprotease cleavage site is indicated in *bold*
[c] Hsia and Anderson 2001, GenBank accession number AF280606
[d] Hsia and Anderson 2001, GenBank accession number AF280605
[e] Masoudi-Nejad et. al. 2002, GenBank accession number AB059812
[f] Entwistle 1988, GenBank accession number AAA92333
[g] Hull et al. 1991

quences differed from the *T. aestivum* 1A ω-gliadin consensus sequence by only 4 of the first 17 amino acids and lacked the NK motif.

The published N-terminal sequences for 1B ω-gliadins of Butte86 differed from those of the 1D and 1A ω-gliadins by 8 of the first 20 amino acids, yet they too could be aligned with the other sequences if a space was inserted after amino acid 7 (Table 2). They did not have the NK motif. Butte86 had two sets of three 1B ω-gliadins that differed in mass by increments of 1,200 Da—equivalent to 11 amino acids. Because they had three different N-terminal amino acids, it is likely that separate genes encoded each of the 1B ω-gliadins.

There are few published gene sequences for ω-gliadins from *T. aestivum*; the predicted N-terminal protein sequences for those of which we are aware are included in Table 3. Hsia and Anderson (2001) did not determine whether the sequences for ωF20b and ωG3 were for 1A- or 1D-encoded ω-gliadins. However, their sequences encode proteins similar in size to the 1A ω-gliadins and smaller than the previously characterized 1D ω-gliadins. The sequence of ωG3 was identical to the consensus sequence for the 1A ω-gliadins described in this paper, and the predicted size of the encoded protein was within 200 Da of 1A ω-gliadins from Butte86 and CS. It is likely that ωG3 encodes a 1A ω-gliadin, though this must be determined by mapping. The sequence for ωF20b differed

by beginning with ARE rather than ARQ, as well as having a Y instead of an H at position 20, and is a pseudogene with a stop codon. Masoud-Nejad et al. (2002) published a sequence (A1) for an ω-gliadin encoded on chromosome 1A of CS based on DNA obtained by PCR of genomic DNA. A1 is a pseudogene with two stop codons and was not localized to the *Gli-1* locus. The sequence differs from the other 1A ω-gliadins at 4 of the first 20 amino acids. All three sequences encode a 19-amino acid signal peptide (not shown) prior to an N-terminal protein sequence beginning ARQ, ARE, or ARH. Predicted sequences based on cDNAs for a C-hordein and an ω-secalin (Table 3) differ from the consensus sequence for the 1A ω-gliadins by having HQ or EQ instead of NK at amino acids 9–10 and have additional differences after amino acid 17.

Discussion

The gluten storage proteins are food ingredients of great economic value because of their essential role in wheat flour quality. The size, sequence, structure and level of expression of the HMW-GS alleles are major determinants of quality for bread making. However, the proteins encoded by LMW-GS and gliadin alleles also vary in structure and level of expression and influence flour quality (Payne 1987). The ability to identify all of the 1A-, 1B- and 1D-encoded ω-gliadins in SDS-PAGE, RP-HPLC and 2D-PAGE patterns makes it possible to quantify the amounts of these proteins that accumulate in different genotypes, under different environmental conditions, in order to evaluate their impact on flour quality. Also, many gene sequences for gliadins and glutenins have been obtained, but long, repetitive sequences can make cloning difficult, sequence similarities add difficulty to mapping efforts, and many pseudogenes are present. Therefore, the proteomics approach of identifying gliadins and glutenins by their masses, N-terminal sequences and characteristic patterns in gels, and HPLC is invaluable for determining whether all the expressed genes have been sequenced.

In this paper, the identity of the 1A ω-gliadins was confirmed using HPLC, SDS-PAGE and N-terminal sequencing of ω-gliadins from Butte86 and CS NT lines. The great similarity of the N-terminal sequences to the *T. urartu* ω-gliadins supports the proposal that *T. urartu* is the donor of the A genome (Dvorak et al. 1993). The N-terminal sequences of the *T. urartu* ω-gliadins and *T. aestivum* and *T. durum* 1A ω-gliadins were nearly identical to those of the 1D ω-gliadins and similar to published sequences for the secalins and hordeins, indicating a common origin for these proteins. The N-terminal sequences, amino acid compositions and internal sequences indicate that the 1B-type ω-gliadins are more distantly related to the 1A and 1D types. It should be pointed out that different results were obtained by Seilmeir et al. (2001), who reported 1B ω-gliadin-type N-terminal sequences for ω-gliadins from the A^mA^m genome *T. monococcum*, and reported only 1B-, but not 1A-type, N-terminal sequences for ω-gliadins from the AABB genome *T. turgidum* ssp. *dicoccum*. Their results are inconsistent with previous N-terminal sequences for *T. monococcum* and *T. durum* (Kasarda et al. 1983) and with our results for *T. urartu.*

The masses of the 1A ω-gliadins were overestimated by SDS-PAGE, as was the case for the 1D and 1B ω-gliadins (DuPont 2000). It has been suggested that their highly repetitive internal sequence, rich in proline and glutamine, has an extended structure that retards the mobility of ω-gliadins in SDS-PAGE. Also, SDS may bind poorly because of the extremely low content of amino acids with positively charged side chains, leading to decreased electrophoretic mobility and overestimated size for the ω-gliadins.

Previous efforts to characterize ω-gliadins revealed three characteristic types, based on N-terminal sequences and mobility in lactate gel electrophoresis (Kasarda et al. 1983; Shewry et al. 1981). The ω-1 type began KEL, the ω-2 type began ARQ, ARE, XRQ, or RQ, and the ω-5 type began SRL. The ω-5 type corresponded to the 1B ω-gliadins, whereas the 1A and 1D ω-gliadins included both the ω-1 and ω-2 types. It was speculated that separate genes encoded the ω-1- and ω-2-type proteins (Kasarda et al. 1983).

All currently available ω-gliadin gene sequences encode mature proteins with sequences that begin with the ω-2 motif of ARX and contain QEL or KEL motifs at amino acids 9–11. The data in this paper suggest that the ω-1-type protein, beginning KEL, is formed from the ω-2 type by the action of an asparaginyl endoprotease at an NK site (Fig 6). The molecular masses and sequences of the ARQ/KEL pairs from *T. urartu* provide the most striking evidence for this hypothesis. Also, the ω-1-type ω-gliadins, beginning KEL, were only observed for wheat varieties that had ω-2-type gliadins with an NK site. There was no evidence for cleavage between the N and P residues found at amino acids 4–5 or 5–6 of the 1A and 1D ω-gliadins, probably because cleavage by proteases tends to be inhibited by P.

We suggest that the ω-gliadins are partially cleaved by a legumain-like asparaginyl endoprotease. Legumains are involved in post-translational processing of seed storage proteins (Gruis et al. 2002; Muntz and Shutov 2002; Rotari et al. 2001; Sheldon et al. 1996) and are also found in animal cells (Dando et al. 1999). Related sequences are found in wheat EST databases.

An asparaginyl endoprotease may also be involved in processing LMW-GS. Mature LMW-GS begin with the sequences METSHIP, METSCIP or SHIP (Lew et al. 1992). The gene sequence for a SHIP-type LMW-GS encoded a signal peptide followed by the sequence MENSHIP (Fig. 6) (GenBank Y17845) (Masci et al. 1998). Thus, there is an N prior to the start of the SHIP sequence. If that sequence is compared with an example of a gene coding for the METSHIP-type, such as GenBank U86028, it can be seen that a single base mutation in the codon AAT for asparagine to ACT for

threonine or, vice versa, is sufficient to account for the difference between the presence or absence of the putative asparaginyl endoprotease cleavage site. Such a change may have followed an ancient gene duplication. All LMW-GS sequences currently in the databases have one to three additional asparagines in the C-terminal halves of the proteins, which must be protected from proteolysis for this theory to be correct.

Genes for wheat storage proteins tend to be present in multiple copies and this seems to be the case for the 1A ω-gliadins, since at least ten distinct ω-gliadin proteins were observed for *T. urartu,* three 1A ω-gliadins for CS, and six for Butte86. Fewer genes are required to account for the number of proteins observed if some differences in size and N-terminal sequence are accounted for by post-translational proteolysis at the N termini. It is not known whether there is also processing at the C-termini. Theoretically, it is now possible to determine the exact number and sequences of the ω-gliadin genes by sequencing the tightly linked ω-gliadin loci on the short arm of chromosome 1. If this is done, it would be informative to analyze all the ω-gliadin proteins for the same wheat variety.

The wheat storage proteins have unique protein sequences that give rise to structures that are of interest in terms of protein chemistry, models of protein structure, and structure-function relationships that are important to dough rheology and other aspects of food technology. Although the 1A ω-gliadins represent only one subclass of ω-gliadins and constitute a minor proportion of the total gliadin fraction, even this one subclass represents a complex protein family. This complexity appears to be due to a combination of gene duplication and post-translational modification. This paper presents the first evidence that suggests there is post-translation cleavage of the ω-gliadins by an asparaginyl endoprotease. The data in this paper demonstrate the relationship between the *T. aestivum* 1A ω-gliadins and the *T. urartu* ω-gliadins and confirm that the 1A and 1D ω-gliadins are closely related to each other and to the ω-secalins and C-hordeins and differ from the exceptionally glutamine-rich 1B ω-gliadins.

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