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Characterization and quantification of low molecular weight glutenins in durum wheats

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Summary – Durum wheat proteins have been considered as a model because of the very clear-cut relationship previously evidenced between the electrophoretic type '42' or '45' of the components that are coded by the *Gli-B1* chromosome locus and the intrinsic quality (gluten viscoelasticity) of cultivars.

The proteins from 4 cultivars were subjected to sequential extraction and separated into five groups, respectively, in: NaCl, EtOH (gliadins-I), EtOH + mercaptoethanol (ME) (gliadins-II), AcOH + ME (glutenins-I) and SDS + ME (glutenins-II) and characterized using polyacrylamide gel electrophoresis (PAGE), SDS–PAGE and 2-dimensional (NEPHGE × SDS – PAGE) electrophoretic systems. EtOH-soluble fractions were also separated by ion–exchange chromatography, each fraction being characterized in PAGE and SDS–PAGE and its composition in major bands determined by densitometry. From the ratio of each chromatographic fraction and of each solubility group, an estimation of the major bands or electrophoretic zones was also made in respect to the whole proteins.

In 'type 45' cultivars, it was shown that only 67% of the EtOH-soluble fraction (although considered as classical gliadins) had a monomeric character, giving rise to discrete bands in PAGE systems. The remainder (33%) were aggregated fractions, essentially those referred to as low molecular weight glutenins (LMWG), that migrate, upon reduction only, in SDS–PAGE systems. LMWG make up 27% of total proteins and are revealed as a strong triplet in the 44 500–51 500 MW region, in gliadin-I and especially in gliadin-II groups. In type '42' cultivars, the LMWG ratio is reduced about by half (18% of EtOH soluble fraction, 14% of total proteins). This difference, coupled with their aggregative behavior, leads to their consideration as the major functional markers of gluten quality, gliadins 42/45 being genetic markers only. Without excluding possible physicochemical differences between different LMWG allelic types, it is hypothesized that quantitative differences could explain by themselves the quality differences between the two durum wheat genetic types. Concerning the other aggregative fractions, like high molecular weight glutenin (HMWG) subunits in glutenin-I and II groups, they do not show (unlike bread wheats) quantitative or qualitative differences large enough to play a major role in explaining genetic differences in durum wheat gluten characteristics.

It is recommended, especially for physicochemical studies of wheat quality, to rely on a protein classification based on monomeric or aggregative characteristics, instead of Osborne's scheme based only on fractionation by solubility. The ratio: LMWG/monomeric gliadins or total aggregative proteins/monomeric gliadins was also proposed in view of an efficient prediction of gluten quality in the selection of durum wheat varieties.

durum wheat / gluten / low molecular weight glutenin / gliadin / aggregation / electrophoresis / densitometry

Résumé – Caractérisation et quantification des gluténines de faible poids moléculaire des blés durs. *Les protéines du blé dur ont été retenues comme modèle en raison de la relation très étroite précédemment mise en évidence entre le type électrophorétique (« 42 » ou « 45 ») des composants codés au niveau du locus chromosomique* Gli-B1 *et la qualité intrinsèque (viscoélasticité du gluten) des variétés.*

Les protéines de 4 variétés ont été soumises à une solubilisation différentielle et séparées en 5 groupes, respectivement, dans NaCl, EtOH (gliadines-I), EtOH+ME (gliadines-II), AcOH+ME (gluténines-I) et SDS+ME (gluténines-II) et caractérisées par des électrophorèses de type PAGE, SDS–PAGE et bidimensionnelles (NEPHGE×SDS–PAGE). Les fractions EtOH-solubles ont été également séparées par chromatographie d'échanges d'ions, chaque fraction étant caractérisée en PAGE et SDS–PAGE et le % des composants majeurs estimé par densitométrie. Compte tenu du % de chaque fraction chromatographique et de chaque groupe de solubilité, une estimation des bandes ou des zones électrophorétiques principales a également été faite par rapport à l'ensemble des protéines.

Chez les variétés de type « 45 », il est montré que 67% seulement de la fraction EtOH-soluble (pourtant considérée comme la gliadine classique) correspondent à des monomères donnant lieu à des bandes en système PAGE, le reste (33%) s'apparentant à des fractions agrégées, essentiellement des gluténines de faible poids moléculaire (LMWG), ne migrant qu'après action d'un réducteur, en système SDS–PAGE. Ces LMWG constituent par ailleurs 27% des protéines totales, et apparaissent sous la forme d'un triplet très intense dans la région des PM 44 500–51 500 dans les groupes gliadines-I et surtout gliadine-II. Chez le type génétique « 42 », les LMWG ont des % environ 2 fois plus faibles (18% de la fraction EtOH-soluble, 14% des protéines totales). Cette différence quantitative, associée à leur nature agrégative, amène à les considérer comme les principaux marqueurs fonctionnels de la qualité du gluten, les gliadines 42/45 n'étant que des marqueurs génétiques. Sans exclure la possibilité de différences physico-chimiques entre les différents types alléliques LMWG, on émet l'hypothèse que les différences quantitatives pourraient expliquer, à elles seules, les différences de qualité des glutens des deux types génétiques de blés durs. Quant aux autres fractions agrégatives telles que les sous-unités HMWG des gluténines I et II, elles ne semblent pas présenter (contrairement au cas des blés tendres) de différences quantitatives ou qualitatives pour qu'elles puissent jouer un rôle majeur dans l'explication des différences génétiques de qualité des glutens de blé dur.

Il est recommandé, pour de telles études sur les bases physico-chimiques de la qualité des blés, de s'appuyer sur une classification des protéines fondée sur le caractère monomérique ou agrégatif, en remplacement du schéma d'Osborne de fractionnement des protéines par solubilité. Le rapport: LMWG/gliadines monomériques (ou encore: ensemble des protéines agrégatives/gliadines monomériques) est également proposé pour permettre une prédiction efficace de la qualité du gluten lors de la sélection des variétés de blé dur.

blé dur / gluten / gluténines de faible poids moléculaire / gliadines / agrégation / électrophorèse / densitométrie

Introduction

For a long time, gliadins have been defined as those storage proteins of wheat endosperm which dissolve in 70% (v/v) ethanol at room temperature while glutenins remain insoluble. Because both gliadins and glutenins contribute to rheological properties of dough (gliadins being responsable for extensibility and glutenins for elasticity), a considerable number of biochemical, genetic and technological investigations has been carried out on these fractions within this solubility scheme, as previously reviewed [1–4]. However, gliadin preparations (even extracted without a reducing agent and examined in different chromatographic media) contain aggregated fractions called either 'aggregated gliadins' [5], 'high molecular weight gliadin' [6, 7], or 'low molecular weight glutenin' [8]. By gel–filtration of gliadin on Sephadex G–100, Jackson *et al.* [9] identified in the void volume several subunits coded by genes different from those coding for other gliadin fractions. On the other hand, glutenin fractions have been shown to contain gliadin-like subunits [10], giving additional evidence of the limits of the solubility-based Osborne classification. Other classifications have been proposed based upon sulfur content [11, 12], aggregative properties [13, 14], biological functions [14], N-terminal amino acid sequences [15], and chromosome locations of genes coding for the proteins [16].

It has now been fully demonstrated that gluten proteins consist of 3 major storage protein families: 1) one monomeric family that corresponds to classical gliadins (apparent molecular weights: 25 000–70 000) and that includes $\alpha+\beta$ types (genes mostly located on the short arm of chromosome groups No. 6) and $\gamma+\omega$ types (genes mostly located on the short arm of chromosome groups No. 1); 2) one aggregative family, generally reported as 'high molecular weight glutenin' or 'HMWG', corresponding to native aggregates of apparent MW from 1–several million, which, upon the effect of reducing agents, yields subunits of apparent MW

65 000–130 000 (genes located on the long arm of chromosome groups No. 1); 3) one aggregative family that we shall refer to as 'low molecular weight glutenin' or 'LMWG', corresponding to large aggregates which, upon reduction, yield subunits with apparent MW of 12 000–60 000 only (the major types belonging to the 45 000–50 000 range), most of them having genes located on the short arm of chromosome groups No. 1, in the same complex locus as the $\gamma+\omega$ gliadin locus.

LMWG remain the least characterized group. They differ from HMWG by their subunit molecular weight, the chromosome location of the encoding genes and their amino acid composition (lower glycine content [12]). Their amount in gluten is controversial; their different allelic types and their contribution to the gluten functional properties are poorly known; the physicochemical basis of their aggregative behavior has not yet been explained.

Although our investigations have been carried out both on bread wheats and durum wheats, we chose for this paper to illustrate the results by restricting ourselves to the durum wheat proteins as a model. Durum wheat LMWG have never been thoroughly examined (Payne *et al.* [17]). Moreover, durum wheat proteins afford a unique example of a clear-cut relationship between a functional property which is essential in determining cooking quality of pasta (gluten viscoelasticity) and a genetic type, *i.e.*, the presence of a given allele at one locus coding for some γ-gliadins and LMWG [18–20].

The aim of this study was to characterize the durum wheat LMWG and to determine their quantitative importance in gluten proteins, more specifically, within ethanol-soluble fractions which have been considered for a long time as typical gliadins.

Based upon ion–exchange chromatography and densitometry from one- and two-dimensional electrophoreses, an explanation for the LMWG functional role in determining intrinsic quality differences among durum wheats is proposed.

Materials and methods

Plant material

The cultivars of durum wheat (*Triticum durum* Desf.) used were *Agathe* and *Mondur* (both having good pasta quality and high gluten strength), *Calvinor* (medium pasta quality and poor gluten strength) and *Tomclair* (poor pasta quality and gluten strength). They were grown in 1985 in the INRA experimental field in Montpellier.

Wheats were milled into semolina in a pilot mill (yield 76%) [21].

Gliadin preparation

100 g of semolina were extracted at room temperature (20°C) with 1000 ml of 70% (v/v) ethanol/water (without reducing agent). After centrifugation at 38 000 × *g*, proteins were precipitated from the supernatant by the addition of 3000 ml of 0.25 M sodium chloride. The mixture was allowed to stand overnight at 4°C and the precipitate was collected by centrifugation. Proteins were then dissolved in 200 ml of 0.01 M acetic acid, dialyzed thoroughly against distilled water at 4°C, shell-frozen and freeze-dried.

Sequential extraction

Salt-soluble proteins, gliadin-I, gliadin-II, glutenin-I and glutenin-II, were extracted at room temperature (approx. 20°C) from semolina (ratio of extractant to solid: 10:1) according to the sequential procedure developed by Landry [22] with respectively: 0.5 M sodium chloride, 60% ethanol (v/v), 60% ethanol + 0.6% 2-mercaptoethanol, 1% acetic acid (v/v), + 0.6% 2-mercaptoethanol, 1.5% SDS (w/v) + 0.6% 2-mercaptoethanol. Each step consisted of one extraction plus two washes of the residue. The ratio of each solubility group was obtained from protein determination in aliquots of the pooled supernatants. The remainder was dialyzed against distilled water and freeze-dried.

Column chromatography of gliadin-I

Gliadin-I was separated by ion–exchange chromatography on Whatman CM–52 as described by Kasarda *et al.* [23]. The column (2.5 × 25 cm) was equilibrated with a 5 mM sodium acetate/1 M dimethylformamide (DMF) buffer (pH 3.5). 1 g of protein was dissolved in the column buffer, clarified by centrifugation, and applied to the column. 5 ml fractions were eluted (flow rate: 15 ml/h) at room temperature with a 5–100 mM sodium acetate gradient in the column buffer, monitored at 254 nm, dialyzed against distilled water and freeze-dried.

Electrophoresis

Fractions from column chromatography and sequential extraction were analyzed by acid–polyacrylamide gel electrophoresis (A–PAGE) in aluminum lactate buffer, pH 3.2, according to Bushuk and Zillman [24] and identified according to Zillman and Bushuk's two-number nomenclature [25], but using the durum wheat γ-gliadin 51 as the reference band [18] and by polyacrylamide gel electrophoresis in Tris-glycine buffer containing sodium dodecyl sulfate (SDS) pH 8.4, (SDS–PAGE) as described by Payne and Corfield [26] and slightly modified [19], and named according to their mobility by reference to a specific 'subunit 1000' [27].

Two-dimensional characterizations of the basic fractions were carried out using a NEPHGE (non-equilibrium pH gradient electrophoresis) × SDS–PAGE system as

described by Holt *et al.* [28] with a pH range from 7 to 10.5

Densitometry
Black and white prints of the gels were scanned with a soft laser LKB Ultroscan densitometer. The densitometric curves were processed (baseline subtraction, peak identification, integration) with an LKB Gelscan software on Apple IIe microcomputer. Reproducibility of the densitometric analyses has been evaluated to ± 2% when scanning the same electrophoretic pattern and to only ± 10% when scanning different patterns of the same sample, which is consistent with previous reports [29, 30]. In all tables, estimated percentages correspond to the means of 4 determinations (for example, 2 chromatographies, 1 electrophoresis and 2 scannings). Trace amounts of components were not taken into account and were eliminated by baseline subtraction.

Other laboratory tests
Protein content (%N × 5.7) was determined using the Kjehldahl method. Glutens were extracted and subjected to Viscoelastograph measurements to determine firmness and elastic recovery as previously reported [20].

Results

Protein composition and gluten properties

Protein content and gluten viscoelasticity of the samples are given in Table I. In accordance with previous works [18, 20], cv. *Agathe* and *Mondur,* which belong to durum wheat genetic type 'γ-gliadin 45', show much higher gluten firmness and elastic recovery than cv. *Kidur* and *Calvinor,* which belong to type 'γ-gliadin 42'.

The samples differ with respect to the percentages of the 6 protein classes: the gliadin-I ethanol-soluble fraction (without reducing agent), which prevails in all samples (from 30.7 to 39.2%), corresponds to the classical 'gliadin' of most of the previous works. The percentage of gliadin-II (ethanol-soluble in the presence of reducing agent), which is lower, clearly differentiates between the two types of wheats (from 14.2 to 15.0% in types '42' and from 22.8 to 24.5% in types '45').

All these fractions have been further characterized by SDS–PAGE, as illustrated on Fig. 1 in the case of cv. *Agathe* (very similar results have been obtained from the other cultivars). In spite of their specific patterns, each fraction consists of subunits covering a wide range of apparent molecular weights with considerable overlaps with other fractions. For all cultivars, soluble proteins range from 15–62.5 kDa; gliadins-I from 22.5–68 kDa and gliadin II from 37.5–110 kDa. Glutenin (I and II) patterns contain 4 major regions: 1) one low mobility region (95–110 kDa) that corresponds to HMWG subunits, which, in the particular case of cv. *Agathe,* are likely to correspond to B genome bands referred to as the allelic block 6–8 (G. Branlard and J.C. Autran, unpublished results); 2) a strong single band, with a mobility comparable to that of one salt-soluble component; 3) one region with intermediate mobility subunits (44.5–51.5 kDa) that is likely to correspond to a

Table I. Protein composition and gluten characteristics.

	Cultivar (genetic type)			
	Agathe ('45')	*Mondur* ('45')	*Calvinor* ('42')	*Tomclair* ('42')
Protein content (% d.b.)	15.4	14.9	16.0	14.6
Gluten Firmness (mm)	2.37	2.19	1.50	1.63
Gluten elastic recovery (mm)	1.78	1.69	0.75	0.79
Protein composition (in% total proteins):				
Albumins + globulins	19.2	20.8	23.5	26.2
Gliadin-I	34.3	30.7	39.2	36.5
Gliadin-II	22.8	24.5	15.0	14.2
Glutenin-I	8.6	7.8	7.5	6.8
Glutenin-II	6.0	8.0	5.2	7.9
Residue	9.1	8.2	9.6	8.4

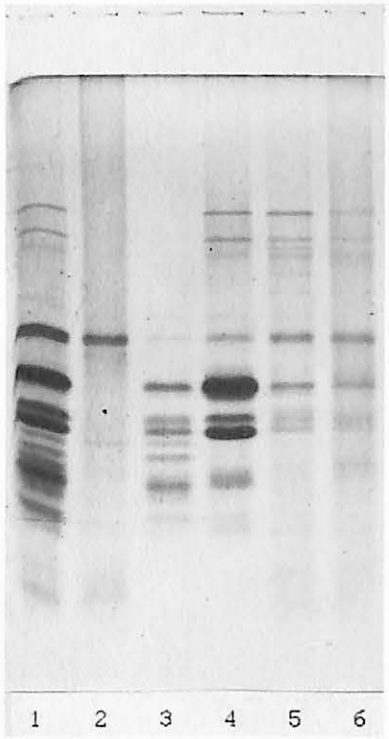

Fig. 1. Electrophoretic (SDS–PAGE) characterization of the different protein fractions from cv. *Agathe*: 1: total reduced proteins; 2: NaCl-soluble; 3: ethanol-soluble (gliadin-I); 4: ethanol + mercaptoethanol-soluble (gliadin-II); 5: acetic acid + mercaptoethanol-soluble (glutenin-I); 6: SDS + mercaptoethanol-soluble (glutenin-II).

low molecular weight fraction of glutenin subunits (LMWG); 4) one fast-moving region (15–40 kDa) with mobilities similar to some salt-soluble proteins and that we shall refer to as very low molecular weight glutenins (VLMWG).

Consequently, when considering a whole protein extract (lane 1, Fig. 1), it turns out that, with the exception of the HMWG subunits that can be easily located in the 95–110 kDa region, no subunit can be unambiguously assigned to a well-defined group or to a monomeric or an aggregative type.

Therefore, prior to one-dimensional electrophoresis and in order to achieve a better classification of the bands, an ion–exchange chromatographic step has been performed on the gliadin-I fraction.

Chromatography of ethanol-soluble proteins on CM-cellulose

A relatively high resolution and a satisfactory recovery of the proteins (85–90%) were obtained when using CM–52 with the acetic acid/sodium acetate/DMF buffer (pH 3.5).

A typical separation of the ethanol-soluble proteins is shown of Fig. 2 in the case of cv. *Agathe*.

14 fractions were collected and examined by A–PAGE without reduction (Fig. 3a) and by SDS–PAGE after reduction (Fig. 3b). The amounts of each fraction were evaluated: 1) by Kjehldahl determinations on each recovered product, and 2) by measuring the areas under the

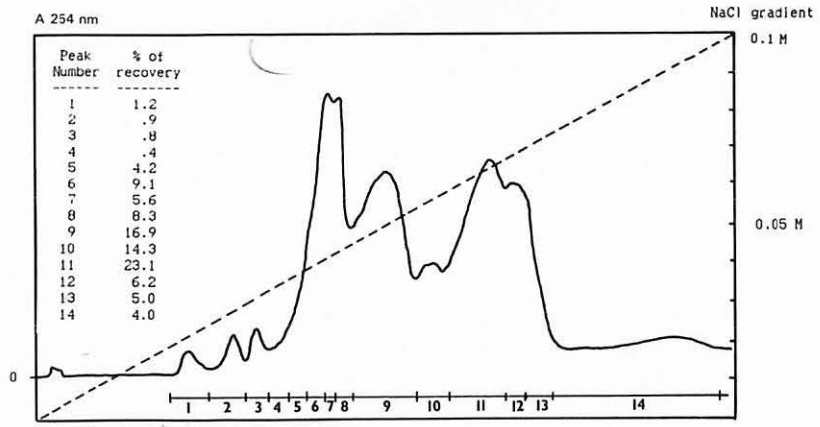

Fig. 2. Fractionation of whole ethanol-soluble proteins by ion–exchange chromatography on a CM–52 column (2.5 × 25 cm) equilibrated with 0.005 M sodium acetate, 1 M DMF, pH 3.5. – – : gradient of NaCl concentration. Fractions were pooled as indicated for analyses by A–PAGE and SDS–PAGE.

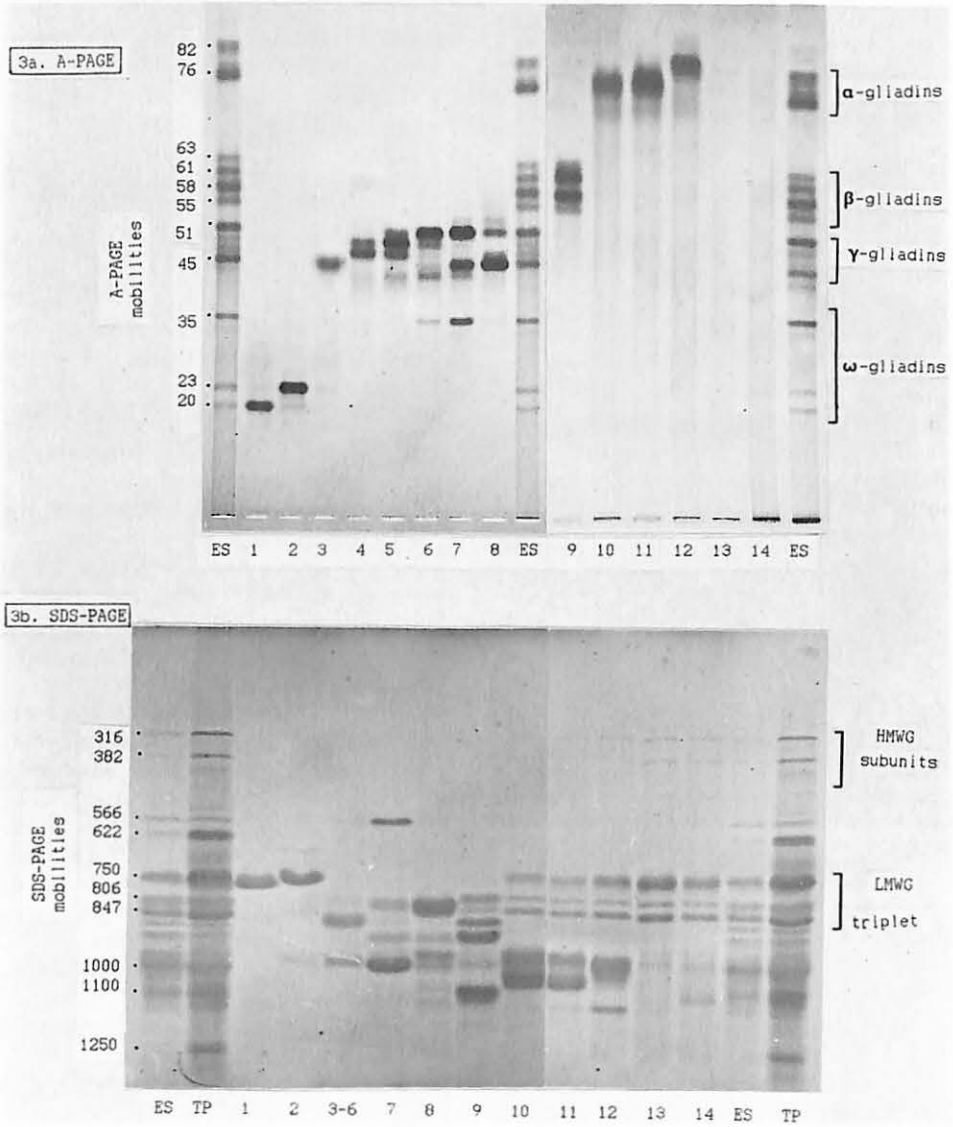

Fig. 3. Electrophoretic patterns of the fractions separated by ion–exchange chromatography on CM–52 of ethanol-soluble proteins (cv. *Agathe*). Lanes 1–14 correspond to chromatographic peaks on Fig. 2; TP: Total proteins; ES: ethanol-soluble proteins. **3a.** A–PAGE system. **3b.** SDS–PAGE system.

chromatographic curve using graph paper. Comparable results were obtained, except for ω-gliadins which gave higher values by densitometry. Mean values between the two techniques are reported on Fig.1.

In spite of overlaps between peaks and of the fact that most SDS–PAGE components correspond to several A–PAGE bands (for example, the reference subunit No. 1000 covers at least 3 major bands including one γ (51), one β and one α), several interesting trends in the fraction composition can be reported: 1) the first 2 peaks contain ω-gliadin bands (A–PAGE mobilities 20 and 23, respectively) in a relatively pure state, which correspond to 2 close SDS–PAGE subunits with mobilities around 750; 2) the 2 major γ-gliadin bands, 45 and 51, are mainly found in peaks 7 and 8 and are identified to subunits 806 and 1000, respectively;

3) ω-gliadin bands (A–PAGE mobilities: 20–35) yield SDS–PAGE subunits 566–750; major γ-gliadins (43–51), subunits 806–1000; β-gliadins (55–68), subunits 806–1105; α-gliadins (76–86), subunits 928–1105.

The most interesting result is that only the chromatographic peaks 1–12 yield bands in A–PAGE, peaks 13 and 14 only streaks, while peaks 10–12 yield streaks and α-gliadin bands; this is not the case in SDS–PAGE in which intense subunits are visible in all fractions. It is clearly apparent that all peaks yielding streaks (along with some slot material) in A–PAGE, show the strong triplet 750–806–847 (44.5–51.5 kDa), and some faint subunits 316–382 of higher apparent molecular weight: mobilities 316–382 (95–110 kDa), which agree with the above-mentioned HMWG subunits.

The strong triplet 750–806–847 is present in all fractions eluted with an ionic strength higher than 60 mM NaCl and cannot be confused with ω-gliadin 750 or γ-gliadin 847 subunits, which are eluted much earlier and without overlap in A–PAGE patterns. Therefore, it must be concluded that these intense triplet subunits, strongly retained on the column and probably having a very basic nature, do not fall into any classical monomeric gliadin category. These proteins must have an aggregative behavior, since they yield bands only in detergent media and after the use of a reducing agent. The streaks and slot materials observed in the corresponding A–PAGE patterns (and in whole gliadin) might evidence a large number of randomly combined subunits (excepted in some more apparent regions that could correspond to more probable associations) that give rise to continuous streaks instead of giving discrete bands.

When comparing the patterns of chromatographic peaks 10–14 with gliadin-II or glutenin-I patterns on Fig. 1, a striking similarity is noted. Gliadin-II consists essentially of the same strong triplet 750–806–847. The same proteins are visible in the patterns of glutenin-I, in which they represent the central part of the patterns, intermediate between HMWG and VLMWG regions. It must therefore be concluded that this triplet corresponds to an equivalent of what has been identified in bread wheats as 'low molecular weight glutenin' (LMWG). These proteins are present in durum wheat fractions extracted with a reducing solvent (gliadin-II or glutenin-I) and without reducing agent (gliadin-I); from simple visual examination, they seem to account for a major part of the whole gliadin pattern (see Fig. 1, lane 3, or Fig. 3a, lane ES).

Very similar elution curves were obtained with other cultivars (patterns not shown). Unlike A–PAGE patterns which show different gliadin compositions between cultivars, apparent molecular weight distributions in SDS–PAGE were nearly identical. The only difference that has been noticed in the case of type '42' cultivars (*Tomclair* and *Calvinor*) concerned the compositions of their LMWG groups: the 4 fainter subunit bands 750–776–806–839 (45–51.5 kDa) were present instead of the strong triplet that characterizes all 'type 45' cultivars ([31] and D. H. Du Cros and J.C. Autran, unpublished results). These 2 types of LMWG groups are very likely to correspond to the allelic types referred to as LMWG1 (linked to γ-gliadin 42 in type '42' cultivars) and LMWG2 (linked to γ-gliadin 45 in type '45' cultivars) by Payne *et al.* [17] and Autran and Berrier [19].

Quantitation of the major protein subunits in gliadin-I

All chromatographic fractions, as well as whole gliadin-I, were analyzed by laser densitometry. Considering that the capacity of protein molecules to bind to Coomassie blue in acid solution is roughly proportional to the basic amino acid contents of proteins, we assumed that most components of a given chromatographic peak would be similar in this respect so that optical density provided a valid measure of the relative amounts of protein. As in previous works using densitometric measurements of wheat proteins [29, 30], we thought this was a reasonable assumption, but recognized the possibility that bands within a given group could contain proteins with different amino acid compositions. When different kinds of proteins, such as salt-soluble gliadins, LMWG and HMWG, are compared, densitometric results must be interpreted with care. However, since salt-soluble fractions (rich in basic groups) contribute little to the total proteins that have been examined (gliadin-I), all the other groups being storage proteins with similar basic/acid amino acid ratios, we thought that reliable estimates could be made nevertheless.

Densitometric analyses were carried out (Fig. 4) in order to estimate, in gliadin-I and its chromatographic fractions, the percentages of the major α-, β-, γ- and ω-gliadins or of LMWG and HMWG (traces being neglected). For example, in the case of cv. *Agathe* (see Figs. 2 and 3), peaks 1 and 2 were found to be 100% ω-gliadins; peak 7 consisted of ω-gliadin 35 (20%), γ-gliadins 43 (14%), 45 (21%) and 51 (45%); peak 9 consisted of 100% β-gliadins; peak 11 consisted of the

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Densitometric analyses were carried out (Fig. 4) in order to estimate, in gliadin-I and its chromatographic fractions, the percentages of the major α-, β-, γ- and ω-gliadins or of LMWG and HMWG (traces being neglected). For example, in the case of cv. *Agathe* (see Figs. 2 and 3), peaks 1 and 2 were found to be 100% ω-gliadins; peak 7 consisted of ω-gliadin 35 (20%), γ-gliadins 43 (14%), 45 (21%) and 51 (45%); peak 9 consisted of 100% β-gliadins; peak 11 consisted of the

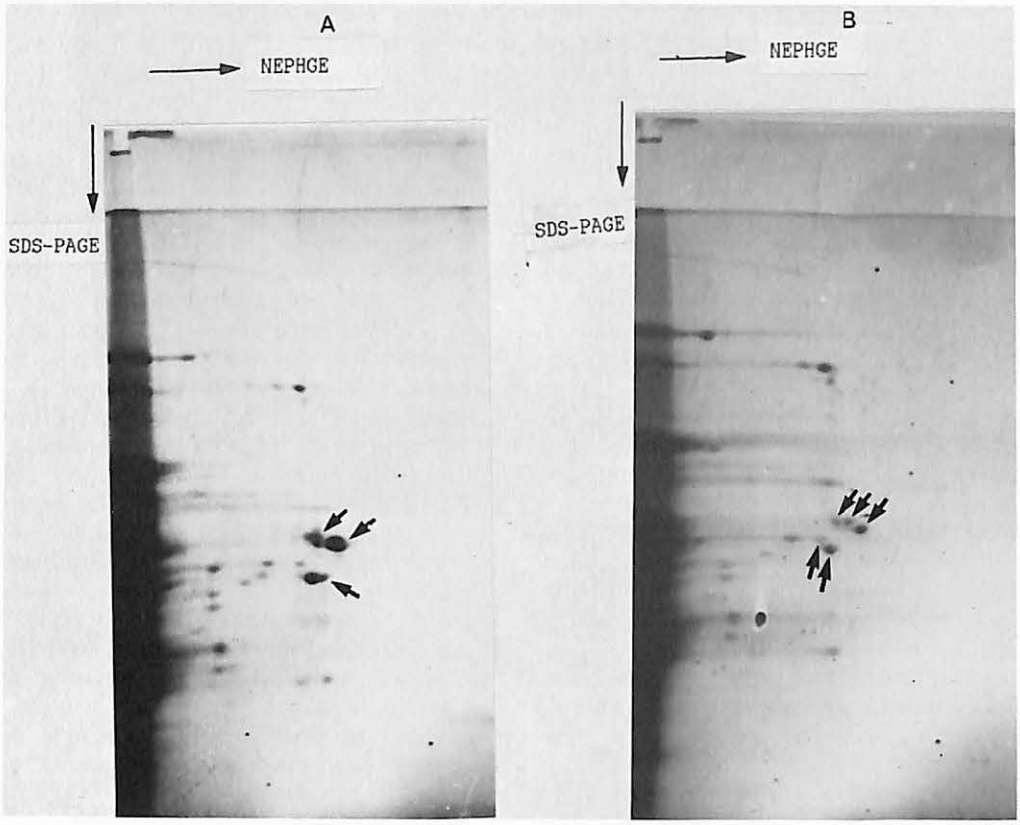

Fig. 4. Two-dimensional characterization (NEPHGE × SDS–PAGE) of the most basic subunits (arrows) from a total protein extract. **A.** cv. *Agathe*. **B.** cv. *Calvinor*.

LMWG triplet (83.7%), HMWG (5.9%), α-gliadins (8.5%) and traces (1.9%) of albumin-like fast-moving material.

From the relative amount of each fraction in gliadin-I (see Fig. 2), an estimation was made of the amount of each protein group in each major SDS–PAGE band or zone and the results were added up for total gliadin-I. These estimations are summarized on Table II for cv. *Calvinor* and *Agathe,* respectively. This estimation was not possible without a preliminary chromatographic step because of the impossibility of assigning an SDS–PAGE band to a well-defined group from a whole gliadin one-dimensional pattern.

The main trends that appear from these estimations are the following: 1) in both types of cultivars (*Calvinor* and *Agathe*), classical gliadin contains, as expected, high and similar percentages of α, β and γ fractions, much lower percentages (about 5 and 10%, respectively) of ω fractions, and some traces of fast-moving components (residual salt-soluble fractions or fast-moving gliadins referred to as F42 or F45 in a previous report [2]; 2) the major γ-gliadins (42, 45 and 51), previously purified [33], represent respectively 10.1, 7.6 and 8.7% of the total ethanol-soluble fraction. As compared to the total proteins, their respective ratios are: 3.9, 2.6 and 3.4%; 3) interestingly, both types contain small amounts of high molecular weight fractions (about 4 and 6%) and particularly large amounts of LMWG fractions for which they also strongly differ: about 14% in type 42 and 27% in type 45.

Densitometric analyses demonstrate the presence of a high percentage of LMWG fractions (along with small amounts of HMWG), in ethanol-extracted proteins, in addition to what can be called 'true gliadins' (*i.e.,* those that behave as monomers and give rise to bands in A–PAGE gels) (Table II).

These LMWG and HMWG contents are even likely to be underestimated, since the determinations have been based on ethanol-soluble fractions

Table II. Composition of the major SDS–PAGE bands or zones in the ethanol-soluble fraction (gliadin-I).

SDS–PAGE mobility	MW estimate (kDa)	% total gliadin	Composition (% of each band or zone)
cv. *Agathe*			
316–382	95–110	1.95	HMWG (100)
566	68	3.95	ω 35 (100)
622	62.5	3.95	HMWG (100)
750	51.5	18.65	LMWG (90); ω 20 (6); ω 23 (4)
806	47	6.60	LMWG (69); β (28); γ misc. (3)
847	44.5	14.30	LMWG (41); β (4); γ misc. (2); γ 45 (53)
872	43	5.10	β (75); γ misc. (25)
901	41.5	8.15	β (68); γ misc. (32)
928–1071	35–40.5	28.95	α (43); β (23); γ misc. (4); γ 51 (30)
1105	33	5.80	α (26); β (74)
>1250	<23	2.70	alb.? (76); Fγ 45 (24)
cv. *Calvinor*			
316–382	95–110	0.95	HMWG (100)
566	68	8.10	ω 33–38 (100)
622	62.5	3.35	HMWG (100)
750	51.5	4.70	LMWG (53); ω 20 (27); ω 23 (20)
776	49.5	3.80	LMWG (100)
806	47	10.15	LMWG (38); β (23); γ misc. (39)
839	45	15.30	LMWG (28); β (6); γ 42 (66)
872	43	6.80	β (83); γ misc. (17)
901	41.5	6.95	β (100)
928–1071	35–40.5	27.45	α (65); β (3); γ 51 (32)
1105	33	9.90	α (20); β (80)
>1250	<23	2.55	alb.? (81); Fγ 42 (19)

Table III. Global estimates of the different A–PAGE classes of gliadins and HMWG and LMWG in ethanol-soluble fractions (gliadin-I) (in % of the total ethanol-soluble fraction).

	cv. *Calvinor* (type '42')	cv. *Agathe* (type '45')
α-gliadins	19.9	14.0
β-gliadins	24.6	22.7
γ-gliadins	23.9[a]	21.9[b]
ω-gliadins	10.3	5.7
HMWG	4.3	5.9
LMWG	14.4	27.1
Misc. fast-moving material	2.6	2.7

[a] Including γ 42: 10.1%
[b] Including γ 45: 7.6%.

that redissolved in the chromatographic buffer and on recovered chromatographic peaks only (insoluble or not, eluted fractions usually contain more aggregated fractions than monomeric ones).

However, since this first estimation concerned the gliadins only, it was necessary to go further in estimating the percentages of LMWG and HMWG in the other fractions and in total proteins.

Quantitation of the major protein subunits in whole durum wheat proteins

SDS–PAGE patterns (Fig. 1) of the 5 fractions (salt-soluble, gliadin-I, gliadin-II, glutenin-I and glutenin-II) were scanned and the percentage of each major band or zone was estimated as for gliadin-I described above.

Salt-soluble fractions (as opposed to gliadins and glutenins) consist mainly of fast-moving com-

ponents (mobilities > 850) and a strong slow-moving band (mobility: 622), and do not exhibit any particular band in the LMWG region. Since soluble proteins and true (monomeric) gliadins are likely to have been removed prior to gliadin-II extraction, the patterns of gliadin-II and subsequent fractions consist of aggregative components only, that fall, respectively, into HMWG (mobilities: 316–622), LMWG (750–847) and VLMWG (>900) subunits. In the case of cv. *Agathe,* the distribution of these 3 classes was: 16, 58 and 26%, respectively, in gliadin-II; 36, 37 and 27% in glutenin-I; 30, 37 and 33% in glutenin-II.

Taking into account the percentage of each solubility fraction in total proteins (according to the data presented on Table I), the contribution of each major band can then be calculated, like the true percentages (with respect to total proteins) that, respectively, belong to salt-soluble fractions, α-, β-, γ- and ω-gliadins, LMWG, HMWG and residue. From the analysis of these data, presented on Tables IV and V, it appears that: 1) several fractions are present at similar ratios in both types of wheats: HMWG,VLMWG and residual proteins (about 10% each) and salt-soluble (about 20%, a little more in cv. *Calvinor*); 2) the major differences between the two types of cultivars essentially concern gliadins and LMWG components. True gliadin bands represent 32% in cv. *Calvinor,* but only 23% in cv. *Agathe.* Conversely, LMWG occur at almost double the ratio in cv. *Agathe* (28%) compared to cv. *Calvinor* (15% only); 3) a more accurate illustration of these differences can be shown using two-dimensional electrophoresis (NEPHGE × SDS–PAGE) of most basic proteins of cv. *Agathe* and *Calvinor* (Fig. 4). Compared to the total protein extract, the subunits of LMWG groups are among the few that migrate toward the cathode in the 7–10.5 pH gradient. In the case of cv. *Agathe,* they give rise to 3 major spots (and several minor ones) with isoelectric pHs estimated to be between 7.5 and 8.0, whereas in the case of cv. *Calvinor,* the 4 fainter bands of the LMWG1 group give rise to 5 spots in a similarly basic p*I* region; 4) LMWG

Table IV. Calculated contribution of each major SDS–PAGE subunit to the total wheat proteins (in % of total proteins, not including residue).

Subunit mobilities	Main components	cv. *Calvinor*		cv. *Agathe*	
281–316	HMWG	4.8		5.5	
566	ω-gliadin	3.0		1.1	
622	HMWG; alb.–glob.	16.3		13.4	
750	LMWG; ω-gliadin	4.8		16.3	
776	LMWG	5.0		–	
806	LMWG; γ-gliadin	7.2	-[a]	6.6	-[b]
839	LMWG; γ-gliadin	8.9		–	
847	LMWG; γ-gliadin	–		9.8	
872	β-gliadin; γ-gliadin alb.–glob.	4.2		2.9	
901	β-gliadin	3.9		4.1	
928–1071	α-gliadin; β-gliadin; VLMWG	15.1		16.1	
1105	α-gliadin; β-gliadin; alb.–glob.; VLMWG	8.2		6.3	
>1250	alb.–glob.; VLMWG	9.0		8.1	

[a] Total: 25.9% including 66.7% of LMWG.
[b] Total: 32.7% including 84.3% of LMWG.

Table V. Calculated contribution of salt-soluble proteins, gliadins, HMWG, LMWG, VLMWG and residue to total wheat proteins (in % of total proteins).

	cv. *Calvinor*		cv. *Agathe*	
Salt-soluble	23.5		19.2	
α-Gliadins	8.0		5.0	
β-Gliadins	10.0	31.9	8.0	22.7
γ-Gliadins	9.7[a]		7.7[b]	
ω-Gliadins	4.2		2.0	
HMWG	10.2		11.5	
LMWG	15.1	33.5	27.7	49.5
VLMWG	8.2		10.3	
Residue	9.6		9.1	
Aggregated monomeric ratio	1.35		2.58	

[a] Including 42: 3.9%.
[b] Including 45: 2.6%.

comprise a major fraction (and even one of the easiest fractions to identify from SDS–PAGE patterns) of both total reduced proteins and ethanol-soluble proteins. This result is illustrated on Fig. 5 by the densitometric tracings of these 2 samples, in comparison to chromatographic peak 13 in which the percentage of the LMWG triplet is 83%; 5) LMWG and HMWG are present in all gliadin and glutenin fractions. About 30–35% of LMWG are ethanol-soluble, 45–50% are ethanol + ME-soluble, 15–20% belong to the 2 glutenin groups. Concerning HMWG, 10–15% are ethanol-soluble, 35–45% are ethanol + ME-soluble, 20–50% belong to the glutenin groups; 6) in the two cultivars, the distribution of the aggregated groups (LMWG and HMWG) between the different solubility fractions is not the same: LMWG are less ethanol-soluble and behave more like 'glutenin-types' in *Agathe,* while the process is reversed for HMWG. In other words, the type '45' cultivar contains more LMWG proteins than the type '42' and these proteins are less ethanol-soluble in the former than in the latter, which explains the large predominance of the gliadin-II fraction in cv. *Agathe* as compared to cv. *Calvinor.*

A more detailed examination of the quantitated results shows that: 1) the triplet zone of a total protein pattern of cv. *Agathe* (subunits 750–849) is essentially constituted by LMWG subunits: 84% LMWG, 3% β-gliadins, 10% γ-gliadins and 3% ω-gliadins. The equivalent quadruplet in cv. *Calvinor* is made up of 67% LMWG, 5% β-gliadins, 24% γ-gliadins, 4% ω-gliadins; 2) when considering a total ethanol-extract (34.3% in *Calvinor*), the percentage of true gliadins (those that migrate in a regular A–PAGE gel) with respect to the extracted proteins, is largely overestimated: only 79% of these extracted proteins in cv. *Calvinor* and 65% in cv. *Agathe* are true monomeric gliadins, the remainder being assignable to aggregated protein materiel; 3) the ratio LMWG/gliadins affords an excellent discrimination of the two types of cultivars: respectively, 0.47 and 1.22 for *Calvinor* and *Agathe.* The same is true for the ratio: aggregated fractions (HMWG + LMWG + VLMWG + residue)/monomeric fractions (gliadins): respectively, 1.35 and 2.58. These ratios, more closely aligned with a physicochemical parameter of technological quality than the glutenin/gliadin ratios previously proposed, should be successful in predicting the gluten characteristics at the breeding stage.

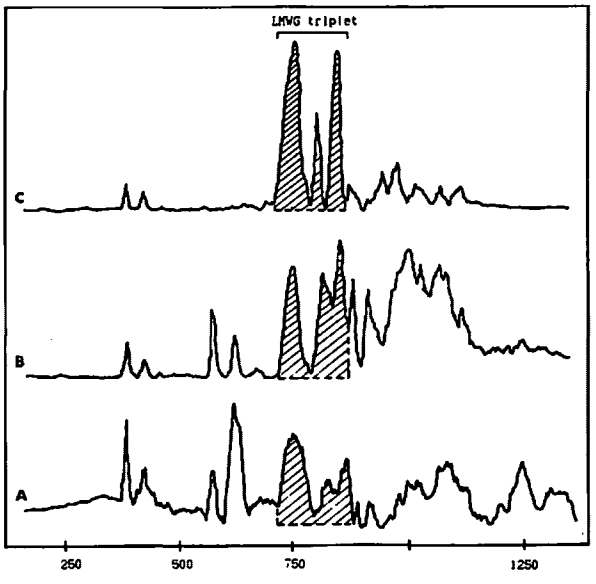

Fig. 5. Densitometric tracings of: **A.** Total reduced proteins from cv. *Agathe.* **B.** Whole ethanol-soluble fraction. **C.** Chromatographic peak No. 13.

Discussion

In durum wheats, gluten viscoelasticity, an important factor in pasta cooking quality, is strongly associated with the allelic type of proteins that are coded by locus *Gli-B1* [17, 19]: ω-gliadins, γ-gliadins and LMWG, and perhaps, to a lesser extent, those coded by locus *Glu-B1* [34]. Two major alleles have been discovered at locus *Gli-B1* in the world collection: allele 42 (γ-gliadin 42 and LMWG No. 1) and allele 45 (γ-gliadin 45 and LMWG No. 2) that correspond, respectively, to poor and good cultivars with respect to gluten viscoelasticity. In early studies, the quality difference between these two genetic types was attributed to gliadin components and a breeding strategy based on γ-gliadin type determination by A–PAGE was developed. However, purification [33] and physicochemical studies of γ-gliadins 42 and 45, in spite of some difference in surface hydrophobicity [35], did not evidence any strong difference capable of explaining such different gluten characteristics. This work has shown that the ratio of γ-gliadin 42 to total proteins is higher (3.9%) than the ratio of γ-gliadin 45 (2.6%). Since γ-gliadins are monomeric proteins (although we recognize that their behavior in dough, gluten or pasta could be different than in diluted ethanol or in A–PAGE buffers), it is difficult to explain how they could play a significant role in imparting gluten

viscoelasticity, this property being certainly based on the occurrence of large aggregates [13]. Conversely, it has been demonstrated that the LMWG proteins, that are major components within the *Gli-B1* locus, present both qualitative and quantitative differences and belong to an aggregative type. LMWG are therefore much more likely to be functional markers of gluten viscoelasticity in durum wheats, the other *Gli-B1*-coded proteins being only genetic markers. It is not known if LMWG1 and LMWG2 allelic types present any differences in their functional properties, but such a gap in their amounts (14% of the total proteins in type '42' and 27% in type '45') could explain the differences in durum wheat gluten characteristics.

In bread wheats *(Triticum aestivum)*, it has been shown [36, 37] that baking quality is more closely associated with *Glu-1*-coded proteins (HMWG subunits) than to *Gli-1* ones. In durum wheats, the opposite situation seems to prevail. In spite of some relationship between HMWG subunits and gluten or pasta quality [34], LMWG would be primarily involved in gluten viscoelasticity in durum wheat glutens. We can therefore hypothesize that all aggregative protein fractions could contribute to quality, that HMWG would act in bulk with little varietal specificity, and that LMWG would be the major functional markers of genetic differences in durum wheat glutens. The fact that the world durum wheat collection essentially consists of two allelic types differing at the *Gli-B1* locus may explain the occurrence of two clear-cut groups of cultivars with respect to gluten viscoelasticity. In bread wheats, by contrast, the occurrence of many allelic types at *Glu-A1*, *Glu-B1* and *Glu-D1* loci may explain the continuous evolution in gluten quality without two distinct classes of cultivars, as in durum wheats.

Conclusions

Using durum wheat proteins as the model, this work confirms that Osborne-type fractionation, which is still used in many studies, is unsatisfactory, at least when investigating the physicochemical basis of quality. The ethanol-soluble fraction, considered by many authors as well-defined gliadins, consists of different types of proteins: some that migrate in A–PAGE gels and that correspond to monomeric molecules and those that can be studied in SDS–PAGE gels only, upon reduction. The latter are far from being negligible in ethanol extracts, since minimum estimates of the only LMWG triplet are between 18 and 33% according to the durum wheat type. It must be emphasized that analyzing A–PAGE bands gives information only on a part of the extracted proteins, the remainder giving rise to streaks and slot material. It is even possible that some ethanol-soluble fractions that have been recently found to be correlated to quality [38] actually correspond to LMWG types.

We subscribe to a protein classification based on aggregation characteristics in A–PAGE buffers: true gliadins (from 23 to 32% of the total proteins in this work) correspond to monomeric proteins separated into discrete bands in A–PAGE, whereas glutenins (from 33 to 50%) are separated in SDS–PAGE gels only after a preliminary treatment by a reducing agent, even if some of them (particularly LMWG fractions) have been partly extracted by aqueous ethanol. Calculation of the ratio of aggregated/monomeric components in gluten proteins can be recommended to predict gluten viscoelasticity, rather than the classical glutenin/gliadin ratio.

The glutenin subfractions do not exhibit the same solubility characteristics. LMWG might be less aggregated than HMWG, since about one third of total LMWG has been found in ethanol extracts (less than 15% for HMWG). However, the differences, if any, in functional properties of these two fractions and their different allelic types among durum wheats are not known, but should be clarified soon, particularly from sequence determinations.

LMWG appear as a major fraction among gluten proteins, although they have certainly been underestimated in this study, since non-eluted and residual fractions (that are likely to consist mainly of aggregated proteins) have not been considered. However, LMWG do not contribute to the same ratio of total proteins in the different durum wheat genetic types. They can be estimated at 27% in type '45' but to 14% only in type '42'. Due to their aggregative behavior and without considering any difference in the functional properties between their allelic types, only the difference in their contributions to the protein pool could explain the opposing characteristics of '42' and '45' durum wheat types with respect to gluten strength and elasticity.

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