

The Quest for Celiac-Safe Wheat with good baking properties

Hetty van den Broeck

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The Quest for Celiac-Safe Wheat with good baking properties

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Thesis

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Chapter 1

General Introduction

Wheat

Evolutionary history

Wheat is a very important crop for our daily intake of proteins, vitamins, minerals and fibers. After maize and rice, wheat is the third most produced cereal in the world (<http://faostat.fao.org/site/567/default.aspx#ancor>). Wheat is not only used for preparation of foods such as bread, noodles, and biscuits, but wheat constituents are increasingly used in many food products such as soups, sauces, fruit gums, ice-cream, flavored potato chips, and even in medicines and vitamin supplements [1-4].

Wheat can be divided in hard wheat and soft wheat. Hard wheat or durum wheat is commonly used for preparation of pasta, and soft wheat is used for preparation of bread. Soft wheat or bread wheat (*Triticum aestivum*) evolved from diploid grasses originating from the Southeastern part of Turkey about 0.5 million years ago [5-7]. First, durum wheat originated from hybridization of a wild diploid wheat *T. urartu* (AA) with a wild diploid B-genome donor from the species *Aegilops speltoides* (Fig. 1). Durum wheat possesses two sets of genomes (AABB) with seven pairs of chromosomes in each. Bread wheat (*T. aestivum*) resulted from spontaneous hybridization between tetraploid *T. turgidum dicoccum* and the D-genome from the wild diploid species *Aegilops tauschii* about 10,000 years ago [8, and references therein]. Bread wheat possesses three sets of genomes (AABBDD) with seven pairs of chromosomes in each.

Thousands of years of cultivation, gene mutations and cross-fertilization resulted in genetic variability of tetraploid and hexaploid wheats. The introduction of the D-genome improved the bread-making properties [9-12] and increased the ability of wheat to adjust to environmental changes. Allopoloidization (fusion of genomes from different species) was shown to result in changes like gene loss, gene silencing, gene activation and duplications [13].

More than a hundred years ago, breeders started to improve hexaploid bread wheat varieties for disease resistance, higher yield, and adaptation to climate changes. Only about 50 years ago, breeders started to breed for good bread-making characteristics and milling yield.

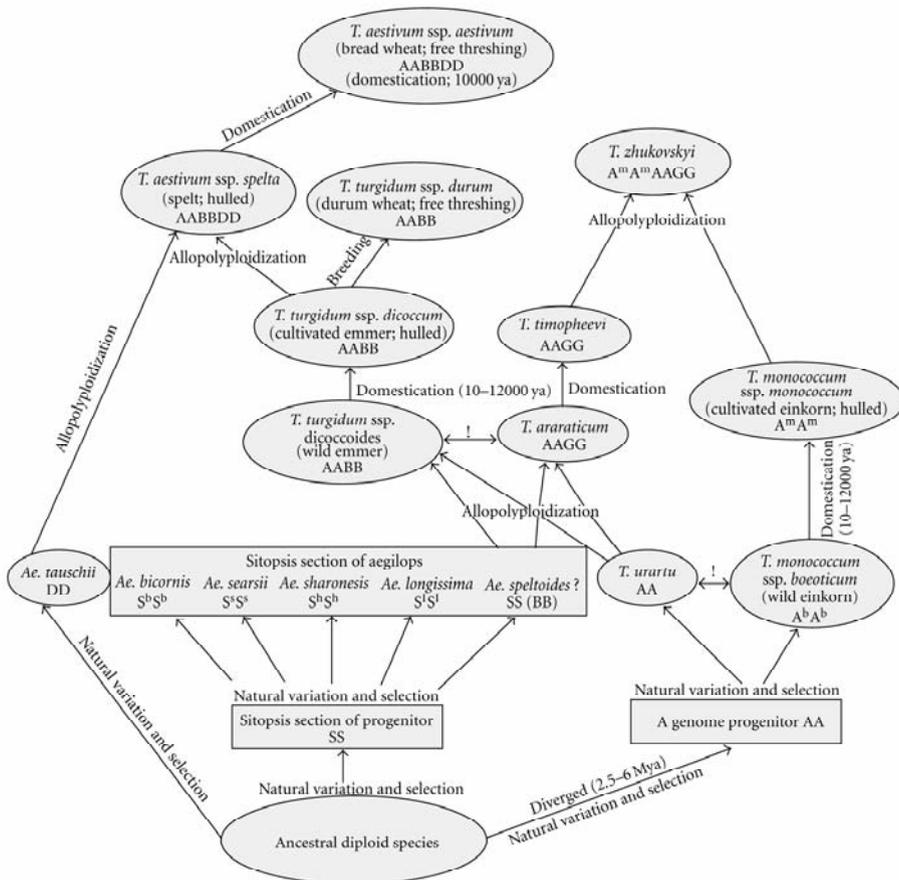


Figure 1. Schematic representation of the evolutionary history of wheat species. *T.* is *Triticum*; *Ae.* is *Aegilops*; ! is morphologically almost indistinguishable but not inter fertile. Reprinted from Gupta (2008) [8] with permission.

Wheat composition

The wheat kernel consists of the bran, the endosperm, and the germ. The bran is build up from several layers and comprises about 13.5% of the kernel (Fig. 2). The germ comprises about 3% of the wheat kernel and contains oil, unsaturated fats, enzymes, vitamins, and minerals. The largest part of the kernel is the endosperm, which comprises starch granules and storage proteins. Wheat flour is ground endosperm, which is separated from germ and bran.

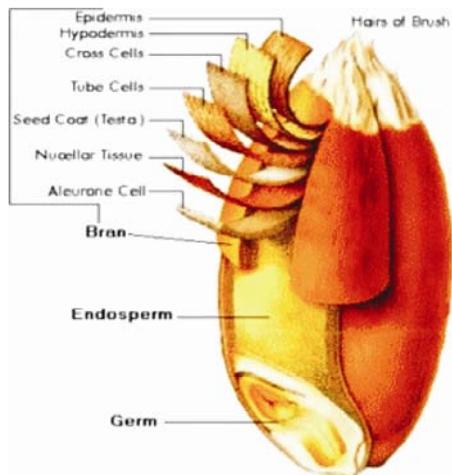


Figure 2. Composition of the wheat kernel. Adapted with permission. Copyright © 1999 by Noël Haegens.

Gluten proteins

Up to 50% of the total storage protein content is assigned to gluten proteins. In wheat, gluten proteins are comprised of gliadins and glutenins, which are present in approximately equal amounts and form 80% of the total storage protein content in the wheat kernel in combination with albumins (12%) and globulins (8%). These storage proteins have been classified by Osborne (1907) based on their solubility in water, salt, and alcohol solutions [14]. Albumins are soluble in water and diluted (neutral) buffers, globulins are salt-soluble, gliadins are alcohol-soluble (40–70%), and glutenins are soluble in alcohol under reducing conditions and in diluted acid or alkali. Gliadins and glutenins are in general extracted by alcohol solutions, containing a reducing agent for extracting glutenins [15]. The main purpose of the storage proteins in the wheat kernel is to provide amino acids during germination and seedling growth. Gluten proteins from wheat are classified in three groups: sulphur-rich (S-rich), sulphur-poor (S-poor) and high molecular weight (HMW) prolamins, with several subgroups within the S-rich group (Table 1).

Gliadins form a large protein family in which α -, β -, and γ -gliadins can be distinguished [16]. Glutenins can be subdivided into low-molecular weight glutenin subunits (LMW-GS) and high-molecular weight glutenin subunits (HMW-GS) [17]. LMW-GS are divided into B-, C- and D-type subunits [18] and the HMW-GS are divided in x-type and y-type subunits [19].

Table 1. Summary of the types and characteristics of wheat grain prolamins (gluten proteins). Reprinted from Shewry and Halford (2002) [20] with permission.

Components	M_r (% total)	Polymers or monomers	Partial amino acid composition (mol%)
HMW prolamins			
HMW subunits of glutenin	65–90 000 (6–10%)	Polymers	30–35% Gly, 10–16% Pro, 15–20% Gly, 0.5–1.5% Cys, 0.7–1.4% Lys
S-rich prolamins			
γ -gliadins	30–45 000 (70–80%)	Monomers	30–40% Gln, 15–20% Pro, 2–3% Cys, <1.0% Lys
α -gliadins		Monomers	
B- and C-type LMW subunits of glutenin ^a		Polymers	
S-poor prolamins			
ω -gliadins	30–75 000 (10–20%)	Monomers	40–50% Gln, 20–30% Pro, 8–9% Phe, 0–0.5% Lys, 0–<0.5% Cys ^b
D-type LMW subunits of glutenin ^a		Polymers	

^aC-type LMW subunits are essentially polymeric forms of α - and γ -gliadins and D-type LMW subunits polymeric ω -gliadins. The B-type LMW subunits constitute a discrete group of S-rich prolamins.

^bCys is present in D-type LMW subunits, but not ω -gliadins.

Genes encoding gliadin polypeptides are located in clusters on the short arms of chromosomes 1A, 1B, and 1D and on the short arm of chromosomes 6A, 6B, and 6D. The *Gli-1* loci on chromosomes 1 represent genes that are tightly linked and mainly encode γ - and α -gliadins. Copy numbers range from 17 to 39 for γ -gliadins with some silent genes [21], and for the α -gliadins it ranges from at least 15 to 18 copies [22]. The *Gli-2* loci on chromosomes 6 represent tightly clustered genes encoding the ω / α - and some γ -gliadins [10, 12]. Copy numbers range from 25–35 [23] to 100 [24] or even 150 [25] per haploid genome. About half of the genes are pseudo-genes [26]. LMW-GS are encoded by genes at the *Glu-3* locus present on the short arms of chromosome 1 [27, 28]. Copy numbers range from 30 to 40 [29]. HMW-GS are encoded by genes at the *Glu-1* locus present on the long arms of chromosome 1 [30]. Each locus encodes a ‘slow’ moving x-type and a ‘fast’ moving y-type HMW-GS indicating their mobility when analyzed by protein gel electrophoresis [19]. Because of silencing of some genes, only four to five HMW-GS encoding genes are expressed in different bread wheat varieties. All together, many gluten genes are present in the wheat genome and the presence of three genomes in bread wheat results in over a hundred gene copies encoding an equal amount of gluten proteins.

Gluten and celiac disease

Celiac disease has an ancient history and goes back to 100 AD. A Greek physician named Aretaeus of Cappadocia, identified patients suffering from their abdomen with ‘koiliakos’ (koilia means belly in Greek). In the 19th century, first Dr. Mathew Baillie and later Dr. Samuel Gee stated that chronic diarrheal disorder leading to malnutrition could be treated

by a restricted diet [31, 32]. In 1950, the Dutch pediatrician Dr. Willem Karel Dicke, recognized that the disease was caused by ingestion of wheat proteins [33, 34]. CD is a T-cell mediated gluten intolerance provoked by specific peptides (epitopes) from gluten proteins of wheat, rye and barley. Exposure to dietary gluten is essential to develop the disease together with genetic susceptibility of the individual. CD can occur at any age and about 0.5–2% of the population suffers from CD. At present probably 70–97% of the CD-population is still undiagnosed. Patients suffering from CD have a damaged intestinal mucosa resulting in villous atrophy and crypt hyperplasia [35, 36] (Fig. 4). This can cause many chronically symptoms such as malabsorption, malnutrition, diarrhea, growth retardation, osteoporosis, lymphoma, etc. For CD-patients the only treatment is restriction to a lifelong gluten-free diet.

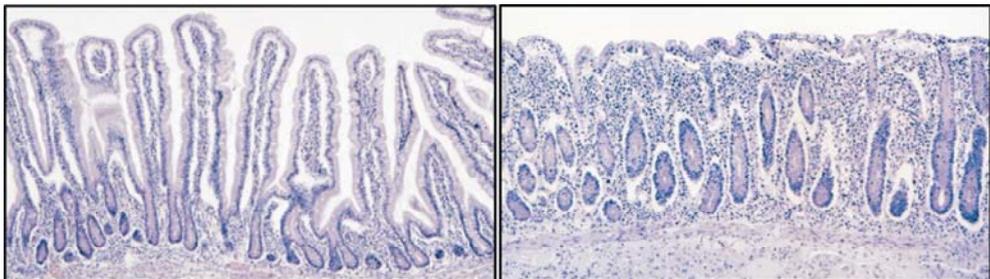


Figure 4. Villous atrophy and crypt hyperplasia of small intestine in CD (right) compared to small intestine in a healthy individual (left). Adapted with permission from Wahab et al. (2002) [36]. ©2002-2010 American Society for Clinical Pathology and ©2002-2010 American Journal of Clinical Pathology.

The adaptive immune response is mediated mainly by human leukocyte antigen (HLA-) DQ2 and DQ8 receptors expressed on the surface of antigen presenting cells (APCs) that recognize certain gluten peptides and present them to CD4+ T-cells [37-40] (Fig. 5). HLA-DQ2 is at least present in 90–95% of CD-patients and HLA-DQ8 is found in the remaining 5–10% [40-42]. However, few patients have different HLA-typings [43]. There is evidence that CD is sex-specific and that female patients more often than male patients carry the allele coding for DQ2 or DQ8 [44]. About 30–35% of the Western population is HLA-DQ2, but only 3% does develop CD of which two-third is woman [45]. The high proline and glutamine content makes gluten proteins resistant to complete proteolytic digestion by enzymes present in the human intestine, which might be specific for CD-patients [46-48]. It is still in debate how the undigested peptides cross the epithelial layer. It was shown that Zonulin, a protein released from the lamina propria, alters permeability and is increased in CD-patients [49, 50]. Alternatively, a transcellular transport pathway

The innate immune response is induced by an identified gliadin epitope (peptide 31–49 or 31–43) [59 and references therein] and is initiated in the intestinal epithelium. Overexpression of interleukin-15 by the enterocytes results in activation of intraepithelial CD8⁺ T-lymphocytes [60] expressing the activating natural-killer-cell marker, NK-G2D, which kill specific enterocytes [61] (Fig. 5).

Other autoimmune diseases, such as diabetes type 1, Crohn’s disease, and rheumatoid arthritis, are associated with CD [61] and occur 3–10 times more frequently than in the general population [62, 63]. Besides autoimmune diseases, neurological disorders such as Down’s and Turner’s syndrome, and many other diseases can be associated with CD [64].

Gluten peptides from wheat, rye, and barley resulting from partial gluten protein digestion may contain T-cell stimulatory epitopes triggering and maintaining celiac disease in patients [65] (Table 2). Especially the epitopes from the α - and γ -gliadins, and, to a lesser extent, the β -gliadins from wheat are considered to have by far the highest clinical relevance with regard to CD [58, 66-73].

Table 2. Overview of T-cell stimulatory peptides in wheat gluten and the hordeins, secalins, and avenins, the gluten-like molecules in barley, rye, and oats, respectively. Reprinted from Koning et al. (2005) [65] with permission.

Protein source	Cereal	Peptide sequence	HLA-DQ
α -Gliadin	Wheat	PQPQLPY P Q	DQ2
Hordein	Barley	PQPQP P F P Q	DQ2
Secalin	Rye	PQPQP P F P Q	DQ2
α -Gliadin	Wheat	PF P QPQL P Y	DQ2
Hordein	Barley	PF P QP Q Q P F	DQ2
Secalin	Rye	PF P QP Q Q P F	DQ2
Avenin	Oats	PY P EQ Q E P F	DQ2
Avenin	Oats	PY P EQ Q Q P F	DQ2
α -Gliadin	Wheat	FR P Q Q P P Y P Q	DQ2
Hordein	Barley	FR P Q Q P P F P Q	DQ2
α -Gliadin	Wheat	Q GS F Q P S Q Q	DQ8
γ -Gliadin	Wheat	FP Q Q P Q Q P F	DQ2
Avenin	Oats	FV Q Q Q Q Q P F	DQ2
γ -Gliadin	Wheat	P Q Q S F P Q Q Q	DQ2
Secalin	Rye	P Q Q S F P Q Q P	DQ2
γ -Gliadin	Wheat	II Q P Q Q P A Q	DQ2
LMW-glutenin	Wheat	FS Q Q Q Q S P F	DQ2
LMW-glutenin	Wheat	FS Q Q Q Q Q P L	DQ2
HMW-glutenin	Wheat	Q G Y Y P T S P Q	DQ8
Gluten	Wheat	QL P Q Q P Q Q F	DQ2

The Q-residues in bold are modified by tTG

By introduction of the D-genome in hexaploid wheat, the number of T-cell stimulatory epitopes increased highly as was determined by gene sequence analysis [74, 75] and by T-cell testing using different wheat species [76-78].

Gluten and baking quality

Wheat gluten proteins are, however, very important for the preparation of bread, pasta, noodles, and biscuits. They determine the elasticity and viscosity of dough. The first step in bread making is mixing flour with water to obtain elastic dough [79]. The quality of the dough is not only determined by the flour protein content but also by the quantity and type of gluten proteins present in the flour, especially the glutenin macropolymer (GMP) [80]. The amount of this highly aggregated glutenin fraction is strongly correlated to bread making quality [81]. During mixing, a three-dimensional structure is formed by the gluten proteins, which is capable of retaining gas bubbles produced by the yeast added during dough proofing (Fig. 6).

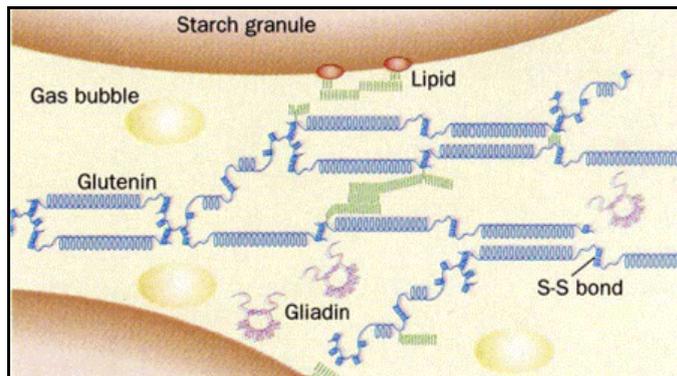


Figure 6. The disulfide-bonded polymeric structure of wheat glutenin, which forms the protein matrix between the starch granules and gas bubbles in dough. Reprinted from Wrigley (1996) [82] with permission.

Both glutenins and gliadins are determinants of bread-making quality [12, 83]. In principle, the network forming LMW-GS and HMW-GS determine gluten elasticity. The monomeric gliadins act as a plasticizer and determine gluten viscous properties. Dough strength is described by the balance between the elastic and viscous properties of a dough. High dough strength is characterized by long development time and high resistance to extension (high elasticity and low viscosity), whereas weak dough has shorter development time and low resistance to extension (high viscosity and low elasticity) [84]. Other components, besides gluten proteins, that also have an effect on quality are starch, salt, water, lipids, and yeast. Each bakery product such as bread, cake, biscuits, croissant, ciabatta, requires its own specific dough, which differs in viscoelastic properties and gas retention.

Why the need for celiac-safe wheat?

CD-patients are restricted to a lifelong gluten-free diet. Originally, Dr. Willem K. Dicke treated his patients by giving the gluten-free porridge. Because this was very difficult to stick to, the development of gluten-free bakery products was initiated. A gluten-free diet not only requires exclusion of gluten proteins containing CD-epitopes, but because CD-patients suffer from malabsorption, it should contain sufficient amounts of proteins, vitamins, minerals, and fibers. A lot of effort has already been put in the analysis of the nutritional quality of oat and pseudo-cereals such as buckwheat, amaranth, sorghum, rice, millet, corn, and quinoa and how to optimize the process of bread baking [85-96]. Cereals belong to the family of grasses and the majority of cereals is evolutionary related and contains prolamins as storage proteins in the seeds (Fig. 7).

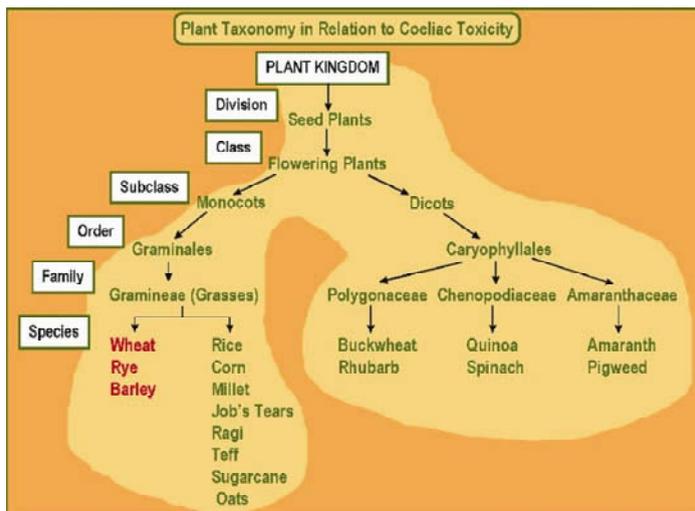


Figure 7. Plant taxonomy in relation to celiac disease. Printed from Kasarda D. (1999) at: wheat.pw.usda.gov/ggpages/topics/celiac.html

These proteins from wheat (gliadins and glutenins), rye (secalins), and barley (hordeins) are characterized by high contents of the amino acids proline and glutamine and are therefore called prolamins, but as a consequence contain CD-epitopes. Gluten proteins from maize (zeins), millet (pennisetins), and rice (oryzeins) are much lower in glutamine and proline content and oat avenins have a high glutamine content but a relatively low proline content. Oat gluten proteins account for 10–15% of the total protein content in contrast to gluten proteins in wheat that account for about 50% of the total

protein content [97]. Flour from oat, other cereal, or pseudo-cereals is not suitable for bread making due to the lack of polymeric gluten proteins in their protein profile. A part of wheat flour can be replaced by flours from other cereals or pseudo-cereals, or adding the right additives can be used for preparation of nice looking and tasty breads. Many Western CD-patients, however, were raised with consumption of wheat bread and many other wheat products such as cookies, cake and pie. Introduction of gluten-free products, prepared from different ingredients, are not very much appreciated because of texture and taste.

Outline of this thesis.

A significant increase has been observed in the prevalence of CD during the last decades. Increased consumption of wheat and gluten is considered a major cause. In developing countries, prevalence of CD is increasing because of the change to Western style diets. Celiac disease is a food-related disease caused by certain gluten peptides from wheat, rye, and barley containing CD-epitopes. Reduction of the presence of these CD-epitopes in wheat (and wheat products) could be a valuable approach to decrease the incidence of CD and to improve the quality of life of CD-patients and many still undiagnosed CD-patients having silent CD or latent CD, which is about 70–97% of the CD-patients as is illustrated by the so-called ‘celiac iceberg’ (Fig. 8) [98-100].

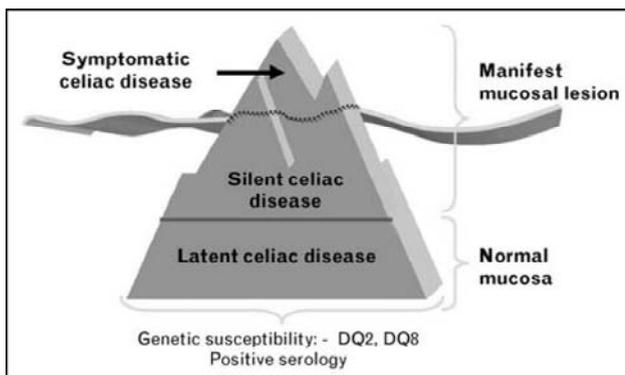


Figure 8. The celiac iceberg. The clinical outcome of the interplay between celiac disease genetic makeup and exposure to gluten, the environmental trigger of the disease, is typically represented by the iceberg model, with the symptomatic forms present at the visible part of the iceberg and the silent and potential forms being submerged below the water line. Reprinted from Fasano (2006) [98] with permission

Since CD is a food-related disease, it allows a prevention strategy, which focuses on elimination of the CD-epitopes directly from the food. Not all gluten proteins contain CD-epitopes, but many gluten proteins exist and minor changes in amino acid composition

can create an epitope and make them cause CD. Among wheat species, large variation exists in immune responses [76-78, 101-106]. This makes it possible to search and select varieties low in CD-stimulating epitopes.

During many years of breeding up till now no attention has been paid to wheat properties in relation to the presence of CD-epitopes in gluten proteins resulting in gluten intolerance causing celiac disease.

The selection and breeding strategies can be carried out at several wheat starting material levels as illustrated in Fig. 9 [107]:

1) short develop time would be required if varieties having a reduced level of CD-epitopes could be selected from the currently used pool of modern bread wheat varieties;

2) selection could also be performed using bread wheat varieties and landraces available from genebanks or using non-natural varieties such as deletion lines (http://www.k-state.edu/wgrc/Germplasm/Deletions/-del_index.html); this probably would need more develop time to commercialization because breeding steps would be necessary after selection;

3) the search for wheat low in CD-epitopes could also start using tetraploid wheat accessions; this will need a reconstruction of a bread wheat variety by hybridization of a selected tetraploid durum wheat varieties (AABB) low in CD-epitopes, with a selected *T. tauschii* species (DD) low in CD-epitopes, thus mimicking the original allopolyploidization that led to bread wheat and occurred ~10,000 years ago. Reconstructions with high variation in especially the D-genome are already available (http://www.cimmyt.org/english/docs/research_report/ResRep02MKazi.pdf);

4) selection for species low in CD-epitopes could also take place using diploid wheat accessions, such as *T. monococcum* or *T. urartu*. These species allow cultivation resulting in reasonable yield, but baking properties of these diploids may be a problem;

5) a total reconstruction of hexaploid bread wheat would be a final approach by building a new bread wheat starting from selected diploid lines low in CD-epitopes. The time to commercialization of a hexaploid bread wheat using this approach will take long, maybe even too long.

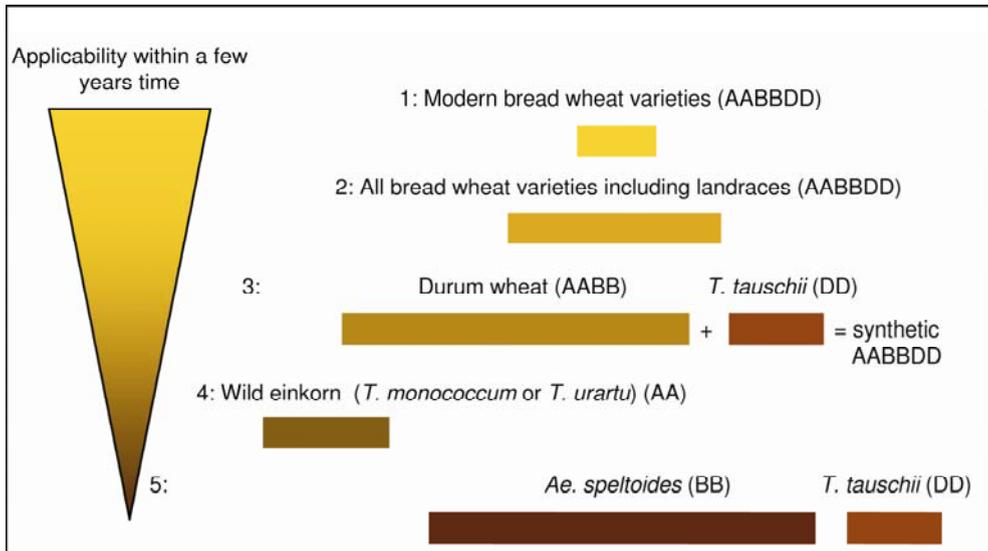


Figure 9. Hypothetical distribution of genetic variation within wheat (as indicated by the width of the bars underlining the different depicted wheat varieties or species), used to develop various strategies to search for wheat varieties safe for celiac disease patients. The triangle and colors represent the decline in food technological applicability and the agronomic value from 1 to 5, with 5 being the least applicable. *T.* is *Triticum*; *Ae.* is *Aegilops*.

To search for wheat low in CD-epitopes as described above, a high throughput method for analysis of the level of CD-epitopes is necessary. In contrast to large-scale testing using T-cell assays, large-scale testing with antibodies using immunoblotting is feasible in such an approach.

Following the general introduction in chapter 1, chapter 2 describes the development of an optimized extraction protocol for wheat gluten proteins. The importance of the extraction procedure used for extracting gluten proteins from wheat is shown. SDS-PAGE analysis and immunoblotting using mAbs against CD-epitopes show the incompleteness of extraction methods described in literature

Chapter 3 describes the extraction of wheat gluten proteins and the different staining methods to visualize them.

Chapter 4 describes the screening of two sets of hexaploid wheats for the presence of CD-epitopes GliA-9 and GliA-20 using immunoblotting. Most CD-patients are responding to α -gliadin epitopes and mAbs have been raised against two immunodominant α -gliadin epitopes. One of the screened sets of wheat consists of modern cultivated wheats grown in Europe and the second set consists of older wheat varieties and landraces obtained from places all over the world.

Chapter 5 describes the screening of a large set of tetraploid wheat landraces from all over the world. These landraces can be very heterogeneous and can be composed of several genotypes within one accession. It also shows that wheat identified as being tetraploid can be contaminated with hexaploid wheat. This contamination increases the number of CD-epitopes.

Chapter 6 describes the analysis of deletion lines of the bread wheat model 'Chinese Spring' that have specific deletions of gluten encoding loci present on the short arms of group 1 and 6 chromosomes. The effect of these deletions on the reduction of the number of CD-epitopes and the effect on dough properties of the lines with the largest deletions is studied. Deletion lines were selected and used in crossing experiments to obtain new deletion lines reduced in higher amounts of CD-epitopes.

Chapter 7 describes the analysis of the technological dough properties of a selection of 'Chinese Spring' deletion lines reduced in CD-epitopes. The selected lines have the smallest deletion possible resulting in the highest reduction in CD-epitopes possible. It is analyzed whether avenins from oat can compensate the loss of the deleted wheat gliadins in small scale mixing experiments.

To finalize, chapter 8 is the general discussion on the usefulness of the used methods and approaches, the obtained results, and how to continue with the search for wheat low in CD-epitopes to decrease the prevalence of CD.

Chapter 2

A modified extraction protocol enables detection and quantification of celiac disease-related gluten proteins from wheat

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Abstract

The detection, analysis, and quantification of individual celiac disease (CD) immune responsive gluten proteins in wheat and related cereals (barley, rye) require an adequate and reliable extraction protocol. Because different types of gluten proteins behave differently in terms of solubility, currently different extraction protocols exist. The performance of various documented gluten extraction protocols is evaluated for specificity and completeness by gel electrophoresis (SDS-PAGE), immunoblotting and RIDASCREEN[®] Gliadin competitive ELISA. Based on these results, an optimized two-step extraction protocol has been developed.

Introduction

Gluten proteins (prolamins) from wheat, rye, and barley are characterized by high proline and glutamine content. Due to the occurrence of especially these amino acids in specific motifs, these proteins can provoke celiac disease (CD) in susceptible individuals possessing T-cells with HLA-DQ2 or HLA-DQ8 receptors. HLA-DQ2 is present in 90–95% of CD patients [108] and HLA-DQ8 is found in the remaining 5–10% [41, 42, 109]. Exposure to gluten proteins leads to damage of the small intestine [40, 110], which causes a range of symptoms including altered bowel habits, malnutrition and weight loss. About 0.5–2% of the Western population suffers from CD, which makes it one of the largest food sensitivities worldwide. The only way to avoid symptoms is to maintain a life-long strict gluten-free diet. Currently, there is an increasing application of wheat gluten in various processed food products, ranging from meats to sweets, with several of these products unexpectedly containing gluten proteins. This increasing application in food products hinders CD-patients to maintain their gluten-free diet and makes them dependent on reliable testing and labeling by food producers. Such testings require optimal gluten detection and quantification protocols.

In wheat, gluten proteins are comprised of monomeric gliadins and polymeric glutenins, which are present in approximately equal amounts. Together they form 80% of the total storage protein content in the wheat kernel. The remaining proteins are albumins (12%) and globulins (8%). These storage proteins have been classified based on their solubility in water, salt, and alcohol solutions [14]. Albumins are soluble in water and diluted (neutral) buffers, globulins are salt-soluble, gliadins are alcohol-soluble (40–70%), and glutenins are soluble in alcohol under reducing conditions and in diluted acid or alkali. The gliadins form a large protein family in which α -, β -, and γ -gliadins can be distinguished [16], whereas the glutenins are subdivided into low-molecular weight glutenin subunits (LMW-GS) and high-molecular weight glutenin subunits (HMW-GS) [18]. Gliadins and glutenins are in general extracted by alcohol solutions, containing a reducing agent for extracting glutenins [15, 111-119]. In spite of the differences in solubility characteristics, co-extraction of glutenins in gliadin extracts and vice versa appears to be inevitable.

Gluten extracts can be tested for the presence of T-cell stimulatory proteins by immunoassays and immunoblotting using mAbs against T-cell stimulatory gluten peptides. Existing immunoassays differ in the antibodies used and in the test format [120-127]. A drawback of these immunoassays is their extraction protocol. It uses only 60% aqueous

ethanol, because the presence of reducing agents would interfere with the immunoassay [121, 128]. However, the use of reducing agents (2-mercaptoethanol, DTT) has been shown to improve the extraction efficiency of glutenins. Further studies have shown that the use of reducing agents (2-mercaptoethanol and guanidiniumchloride) in a 1:100 dilution does not affect immunoreactivity and is used in the R5 capture ELISA [126, 127, 129]. This ELISA is approved by the Codex Alimentarius Commission (2008) [130]. The monoclonal antibody in this assay, named R5, mainly recognizes QQPF, QQQF, LQPF, and QLPF sequences that occur in gliadins, secalins, and hordeins [127, 131]. The R5 assay thus focuses on gliadins and not on T-cell stimulatory epitopes. However, also T-cell stimulatory motifs are known from glutenins [58, 77], which will not be detected using the R5 antibody. It is not known to what extent the R5 assay yields different results from direct detection of T-cell stimulatory epitopes.

Here, we describe the development of a fast and efficient two-step extraction protocol for gluten proteins from wheat flour, based on a combination and optimization of documented extraction protocols for gliadins and glutenins. First, we analyzed the degree of (co-)extraction of individual extraction protocols by SDS-PAGE and gel staining, followed by comparative testing of the efficiency with our two-step protocol. Our two-step extraction protocol was compared to an existing 60% aqueous ethanol extraction protocol, first by immunoblotting using specific monoclonal antibodies against T-cell stimulatory epitopes Gli- 9 [126, 132] and LMW-GS [77, 132], and second in the RIDASCREEN® Gliadin competitive ELISA based on the R5 monoclonal antibody. We used the RIDASCREEN® Gliadin competitive ELISA instead of the RIDASCREEN® Gliadin, because that is a sandwich ELISA that needs at least two epitopes for detection. Our two-step protocol enabled a highly complete extraction of gliadins and glutenins from wheat, compatible with a reliable detection assay to test for CD-toxicity of foods using specific mAbs based on T-cell stimulatory peptides.

Materials and Methods

Flour

Wheat grains of four hexaploid wheat varieties, Bovictus, Combi, Rektor, and Bussard, were obtained from Limagrain, The Netherlands, and were ground in an analytical mill (A 11 Basic, IKA-Werke) and sieved through mesh (0.5 mm).

Different protocols for extraction of gluten proteins from wheat flour were tested as illustrated in Fig. 1 and are described below.

Extraction of gluten proteins from wheat flour

Separate 'gliadin' and 'glutenin' extracts

See Fig. 1A. According to Singh *et al.* (1991) [15] first, gliadins were extracted from 200 mg wheat flour by addition of 1 ml of 50% (v/v) aqueous iso-propanol with continuous mixing (MS1 Minishaker, IKA Works, Inc.) at 1,000 rpm for 30 min at room temperature, followed by centrifugation at 2,500 x g for 15 min at room temperature. The supernatant is referred to as '1st gliadin extract'. The residue was extracted twice with 50% (v/v) aqueous iso-propanol resulting in a '2nd and 3rd gliadin extract'. Second, the 'glutenin extract' was obtained by extraction of the residue with 1 ml of 50% (v/v) aqueous iso-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% (w/v) DTT for 30 min at 60°C with mixing every 5 to 10 min, followed by centrifugation at 10,000 x g for 10 min at room temperature. Extracts were analyzed by SDS-PAGE followed by silver staining and PageBlue™ staining.

Reducing agent extract

See Fig. 1B. For developing a method for combined extraction of both gliadins and glutenins, we tested whether the total gluten protein content could be extracted directly using 50% (v/v) aqueous iso-propanol containing 1% (w/v) DTT as reducing agent. Fifty milligrams of flour was extracted with 0.5 ml 50% (v/v) aqueous iso-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% (w/v) DTT for 30 min at 60°C followed by centrifugation at 10,000 x g for 10 min at room temperature. The supernatant is referred to as the 'DTT extract' and was analyzed by SDS-PAGE followed by silver staining and PageBlue™ staining.

60% ethanol extract

See Fig. 1C. To obtain 60% aqueous ethanol extracts, 50 mg of flour was extracted with 1.5 ml 60% (v/v) aqueous ethanol for 1 h with continuous mixing at room temperature followed by centrifugation at 10,000 x g for 10 min at room temperature. The supernatant is referred to as the '60% EtOH extract' and was analyzed by SDS-PAGE followed by silver staining, immunoblotting and RIDASCREEN® Gliadin competitive ELISA (see below). The residue was extracted once with 60% ethanol.

Two-step gluten extract

See Fig. 1D. Gluten protein extraction from wheat flour in our two-step protocol, as proposed and evaluated here, was carried out in 50% (v/v) aqueous iso-propanol (flour ratio of 1:10, w/v) and continuous mixing (MS1 Minishaker, IKA Works, Inc.) at 1,000 rpm for 30 min at room temperature, followed by centrifugation at 10,000 x g for 10 min at room temperature. The residue was extracted twice with 50% (v/v) aqueous iso-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% (w/v) DTT for 30 min at 60°C with mixing every 5–10 min, followed by centrifugation at 10,000 g for 10 min at room temperature. Upon addition of each sequential extraction solution the residue was resuspended by shaking in a Fastprep FP220A Instrument for 10 s at speed 6.5 m/s followed by sonication for 10 min in an ultrasonic bath (Branson 3510, Branson Ultrasonics Corporation). The supernatants are referred to as the '1st gluten extract', '2nd gluten extract' and '3rd gluten extract'. The extracts were analyzed separately, than combined and referred to as the 'two-step gluten extract'. Extracts were analyzed by SDS-PAGE, immunoblotting and RIDASCREEN® Gliadin competitive ELISA. The remaining proteins (mostly albumins and globulins) in the residue were extracted with 25 mM Tris-HCl (pH 8.0) containing 2% SDS (residue ratio of 1:20, w/v) and finally with 25 mM Tris-HCl (pH 8.0) containing 2% (w/v) SDS and 1% (w/v) DTT (residue ratio of 1:20, w/v) [133]. Both for 30 min at 60°C with mixing every 5–10 min, followed by centrifugation at 14,000 rpm for 10 min at room temperature. The supernatants, referred to as the 1st and 2nd residual extracts, were analyzed for protein content and analyzed by SDS-PAGE followed by silver staining and by immunoblotting to test for any remaining T-cell stimulatory gluten proteins.

SDS-PAGE and immunoblotting

The protein content of all extracts was quantified using Bio-Rad Protein Assay, based on the Bradford dye-binding procedure, according to manufacturer's instruction. Proteins were separated on SDS-PAGE gels (10%) [134] using a Hoefer SE 260 mighty small II system (GE Healthcare) followed by silver staining [135] or staining with PageBlue™ (Fermentas). For immunoblotting, proteins were blotted onto nitrocellulose (0.2 µm, Bio-Rad Laboratories), omitting methanol from the blotting buffer, using a Mini Trans-Blot Cell (Bio-Rad Laboratories) at 100 V for 1 h. Blots were incubated and visualized as described in [136] and using monoclonal antibodies (mAbs) specific for T-cell stimulatory epitope Gliadin 9 [126, 132], LMW-glutenin (LMW-2) [77, 132] and HMW-glutenin [126, 132] obtained from Dr. L. Dekking and Prof. dr. F. Koning, Leiden University Medical Centre, The Netherlands.

RIDASCREEN® Gliadin competitive ELISA

For detection of T-cell stimulatory epitopes by the peroxidase R5 conjugated antibody (R5-HRP), we used the RIDASCREEN® Gliadin competitive ELISA instead of the RIDASCREEN® Gliadin, which is a sandwich ELISA and needs at least two epitopes for detection. The RIDASCREEN® Gliadin competitive assay (R-Biopharm AG) was used according to manufacturer's instruction. The 60% ethanol extracts were compared to the two-step gluten extracts of the four hexaploid wheat varieties. Extracts were diluted to fit the standard curve. The same extracts were analyzed by immunoblotting using the R5-HRP antibody, visualized by chemiluminescence.

Database searching

The protein database of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) was searched on November 6th, 2008, for the presence of the sequences recognized by the mAbs R5 (QQFPF, QQQFP, LQPFP and QLPFP), Gli-9 (QFPFQPQ) and LMW-glutenin (QSPF). From the NCBI protein database, five different groups of gluten protein sequences from *Triticum aestivum* were extracted: / -gliadins, -gliadins, -gliadins/D-type LMW-GS, LMW-GS, and HMW-GS. Within the -gliadins, four sequences (AAA34286, P04729, P04730, and AAA34285) were more similar to LMW-GS and were transferred to the LMW-GS. The -gliadins/D-type LMW-GS group consisted three -gliadin sequences and three D-type LMW-GS sequences.

Results

Extraction of gluten proteins from wheat flour

Separate 'gliadin' and 'glutenin' extracts

To develop a quantitative gluten protein extraction protocol for wheat, several described extraction protocols were analyzed for their level of co-extraction of glutenins and gliadins (Fig. 1). Pre-extraction of gliadins from flour with 50% aqueous iso-propanol is in general performed prior to glutenin extraction to prevent cross-contamination of gliadins in the glutenin fraction. However, pre-extraction could also remove glutenins from the sample. To analyze the level of co-extraction of glutenins while pre-extracting gliadins with 50% aqueous iso-propanol, three 'gliadin' extracts were obtained by sequential extraction with 50% aqueous iso-propanol (Fig. 1A). Finally, a 'glutenin' extract was obtained by extraction

of the residue with 50% aqueous iso-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% (w/v) DTT (Fig. 1A).

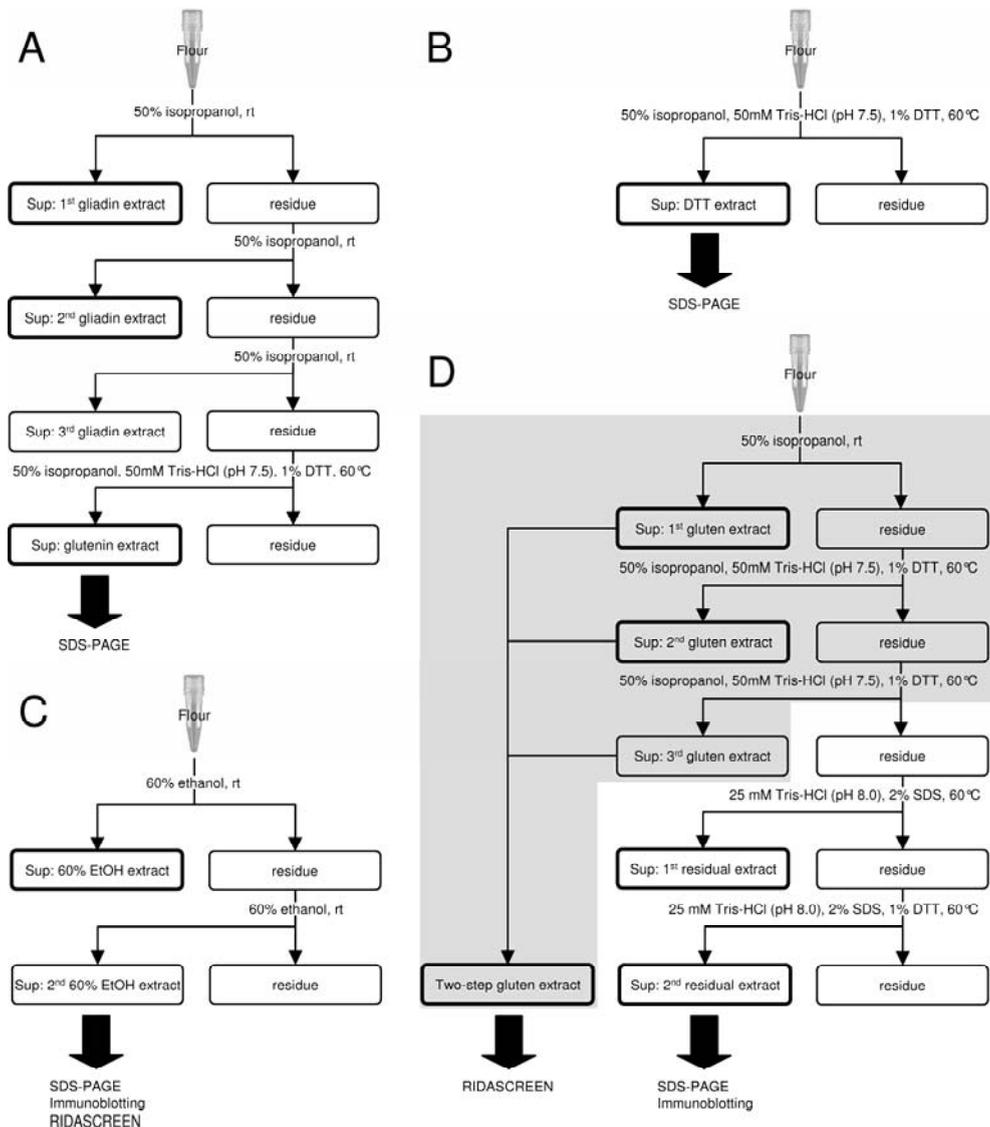


Figure 1. Protocols used for extraction of gluten proteins from wheat flour. (A) Separate gliadin and glutenin extraction; (B) Extraction using reducing agent; (C) 60% ethanol extraction; (D) Two-step gluten extraction (gray box). Extracts from boxes with bold lines were analyzed. 'rt' is room temperature

The average protein content in the '1st gliadin extract' of the four hexaploid wheat varieties was 1.21 (± 0.01) mg/ml. In the '2nd gliadin extract' the average protein content was 0.71 (± 0.03) mg/ml, while in the '3rd gliadin extract' it was only 0.02 (± 0.01) mg/ml, demonstrating that all 'gliadins' were extracted from the flour before the final glutenin extraction. The average protein content in the 'glutenin extracts' was 0.61 (± 0.01) mg/ml. The '1st and 2nd gliadin extracts' and the 'glutenin extracts' were compared to analyze the level of co-extraction of gliadins in glutenin extracts and vice versa. The '3rd gliadin extract' was not analyzed further because of the low protein content. These extracts were compared with 'DTT extracts'. Proteins were separated on SDS-PAGE gels followed by silver staining (Fig. 2A) or staining with PageBlue™ (Fig. 2B). PageBlue™ is an end-stain procedure that stains HMW-GS more efficiently than silver nitrate does [137]. Results in Fig. 2 show that large amounts of overlapping proteins are present in the 'gliadin' and 'glutenin' extracts.

In all four varieties the amount of HMW-GS is even higher in the '2nd gliadin extract' than in the '1st gliadin extract', showing that HMW-GS are easily extracted with 50% aqueous iso-propanol without reducing agent. Proteins from the -gliadins/D-type LMW-GS region are more abundantly present in the '1st and 2nd gliadin extracts' compared to the 'glutenin extracts' and 'DTT extracts' (Fig. 2, boxed proteins), especially for variety Bovictus.

Reducing agent extract

To analyze whether the total gluten protein content could be extracted by one single extraction using a reducing agent, wheat flour was extracted with 50% aqueous iso-propanol containing 1% (w/v) DTT. The average protein content in these extracts was 1.01 mg/ml (± 0.00). Proteins were separated on SDS-PAGE gels followed by silver staining (Fig. 2A) or staining with PageBlue™ (Fig. 2B). Results in Fig. 2 (boxed proteins) show that proteins from the -gliadins/D-type LMW-GS region are more abundantly present in the '1st and 2nd gliadin extracts' compared to the 'glutenin extracts' and 'DTT extracts', especially for variety 'Bovictus'. These results show that the gluten proteins cannot be extracted quantitatively by one single extraction including 1% DTT. In addition, these results show that to extract gluten proteins quantitatively from wheat flour, the protocol should start with a 50% iso-propanol extraction without DTT.

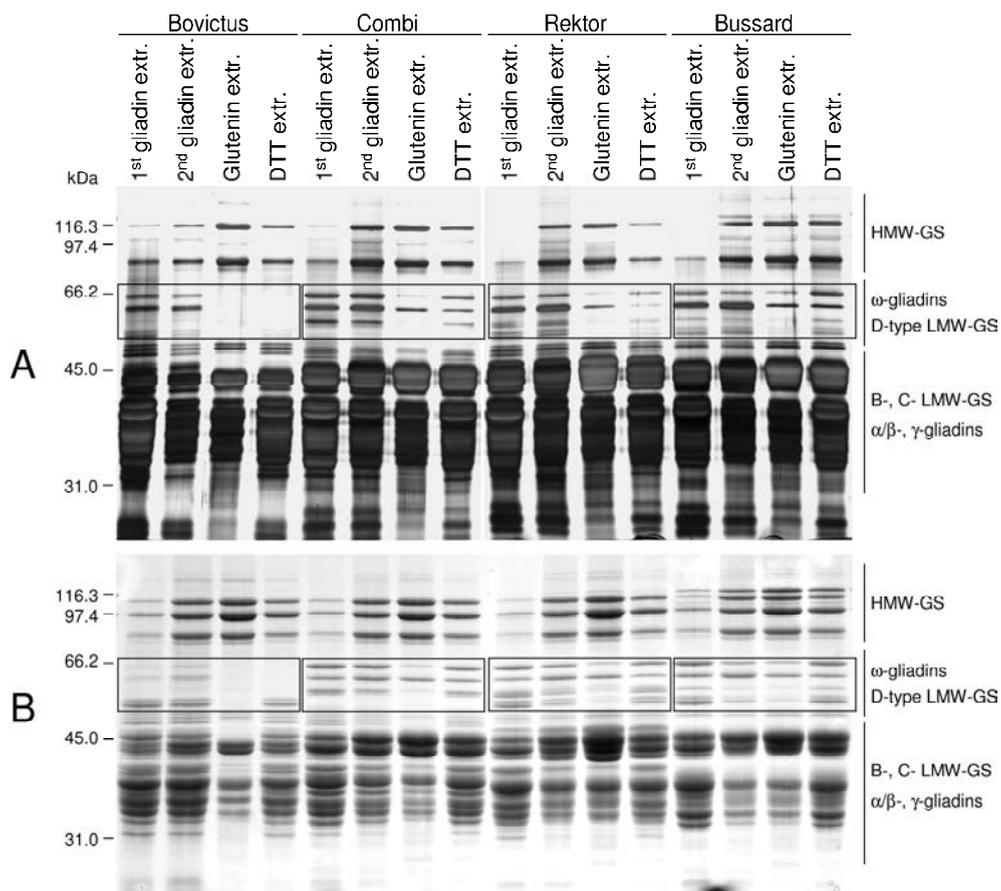


Figure 2. Gluten proteins (1 μ g) from wheat varieties Bovictus, Combi, Rektor, and Bussard separated on SDS-PAGE gels (10%) after various extraction protocols: 50% (v/v) iso-propanol (1st gliadin and 2nd gliadin extract); 50% (v/v) iso-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% DTT (glutenin extract); 50% (v/v) iso-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% DTT (DTT extract). (A) Silver staining; (B) PageBlue™ staining.

60% ethanol extract

To test gluten protein extracts in immunoassays, in general, extraction is performed with 60% aqueous ethanol. Extractions were performed on the flour of the four wheat varieties and the average protein content of the 1st 60% aqueous ethanol extracts was 0.33 mg/ml (\pm 0.03). The residue obtained after the 1st 60% aqueous ethanol extraction was extracted once more, but the average protein content of the supernatants was only 0.03 mg/ml (\pm 0.00). Proteins from the 1st 60% aqueous ethanol extract were separated on SDS-PAGE gels followed by silver staining (Fig. 3).

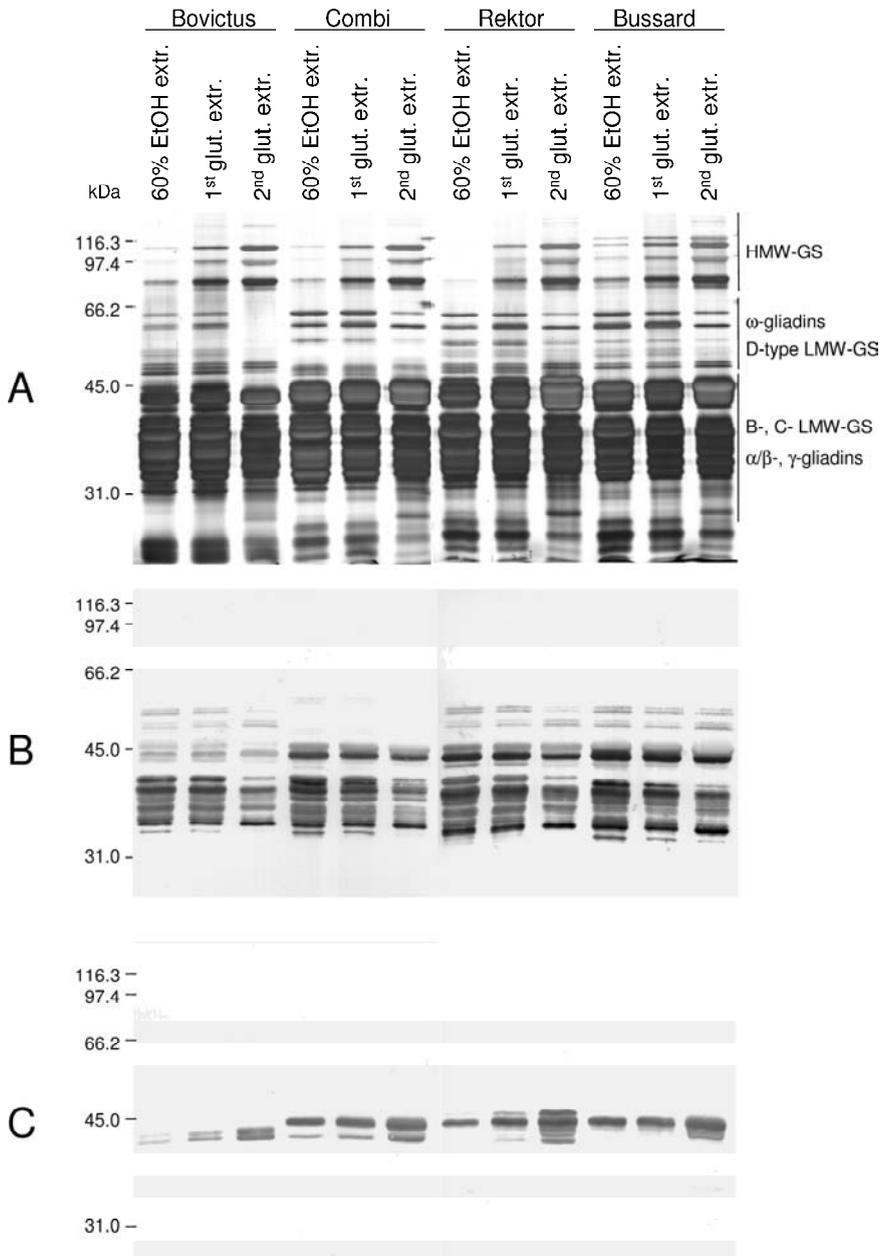


Figure 3. Gluten proteins (1 μ g) from wheat varieties Bovictus, Combi, Rektor, and Bussard separated on SDS-PAGE gels (10%) after various extraction protocols: 60% ethanol (60% EtOH); 50% iso-propanol (1st gluten extract); 50% iso-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% DTT (2nd gluten extract). (A) Silver staining; (B) Immunoblot using mAb against Gli-a-9; (C) Immunoblot using mAb against LMW-glutenin (LMW-2).

Two-step gluten protein extract

To determine whether we could completely extract gluten proteins in a two-step protocol, we performed sequential extractions starting first with 50% aqueous iso-propanol followed by extracting the residue twice with 50% aqueous iso-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% DTT (Fig. 1D). To optimize the extraction of the gluten proteins from the flour, a ratio flour to buffer of 1:10 (w/v) was used instead of 1:5 (w/v). The average protein content in the three gluten extractions obtained was 1.52 (± 0.14) mg/ml, 0.33 (± 0.03) mg/ml, and 0.06 (± 0.01) mg/ml, respectively. The 3rd extraction resulted in very low protein concentrations indicating that three extractions were sufficient to highly complete extraction of the gluten proteins. Proteins from the 1st and 2nd gluten extracts were separated on SDS-PAGE gels followed by silver staining (Fig. 3). Extraction of the residue with 25 mM Tris-HCl (pH 8.0) containing 2% SDS (1st residual extract) and with 25 mM Tris-HCl (pH 8.0) containing 2% SDS and 1% DTT (2nd residual extract) resulted in an average protein concentration of 0.84 (± 0.09) mg/ml and 0.08 (± 0.02) mg/ml, respectively. Proteins from the residual extracts were separated on SDS-PAGE gels followed by silver staining and immunoblotting. Silver stained gels showed that some HMW-GS and probably some α -gliadins/D-type LMW-GS were still extracted with 25 mM Tris-HCl (pH 8.0) containing 2% SDS (Fig. 4A).

Immunoblotting

The extracts obtained by 60% ethanol (Fig. 1C, 1st 60% EtOH extract) and by our two-step protocol (Fig. 1D, 1st and 2nd gluten extracts) were subsequently compared. They were analyzed for T-cell stimulatory gliadins and glutenins by immunoblotting using mAbs specific for T-cell stimulatory epitopes GliA- 9 (Fig. 3B), LMW-glutenin (Fig. 3C), and for the R5-HRP antibody (results not shown). Immunoblotting was followed by analysis using the RIDASCREEN[®] Gliadin competitive ELISA (Table 1) to determine the degree of completeness of both extraction protocols in order to relate the protocols to CD. Results showed that by extraction with 50% iso-propanol in our two-step protocol, higher concentrations of HMW-GS were obtained compared to extraction with 60% ethanol (Fig. 3A). This result suggests that 60% ethanol is less efficient to extract HMW-GS, maybe because 50% iso-propanol is more apolar. Immunoblotting using mAb GliA- 9 showed that 60% aqueous ethanol extracts and 50% aqueous iso-propanol extracts gave comparable results (Fig. 3B). Extracts obtained by extraction with 50% aqueous iso-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% DTT showed less proteins reacting with mAb GliA- 9, because most gliadins already had been extracted with 50% aqueous iso-propanol. Using

mAb against LMW-glutenin showed increased levels of proteins in both the 50% iso-propanol extracts and the extracts obtained by 50% aqueous iso-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% DTT, in comparison to the 60% aqueous ethanol extracts (Fig. 3C). Proteins from the residual extracts were separated on SDS-PAGE gels followed by immunoblotting using mAbs specific for T-cell stimulatory epitope Glia- 9, LMW-glutenin, and HMW-glutenin. Immunoblots did not show any reaction of proteins with the mAbs Glia- 9 (Fig. 4B) and LMW-glutenin (Fig. 4C). In contrast, some HMW-GS were indeed still present that reacted with the mAb against HMW-glutenin (Fig. 4D). Apparently, these HMW-GS are part of a complex that could not be extracted by 50% aqueous iso-propanol or by 50% aqueous iso-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% DTT. Peptides from HMW-GS were identified to bind to HLA-DQ8 receptors [65, 138], whereas purified HMW-GS were shown to stimulate T-cells of patients that were HLA-DQ2 and HLA-DQ8 [72, 139]. As the largest part of the HMW-GS was extracted in our two-step protocol, it was concluded that the main gluten protein content was extracted with our two-step protocol.

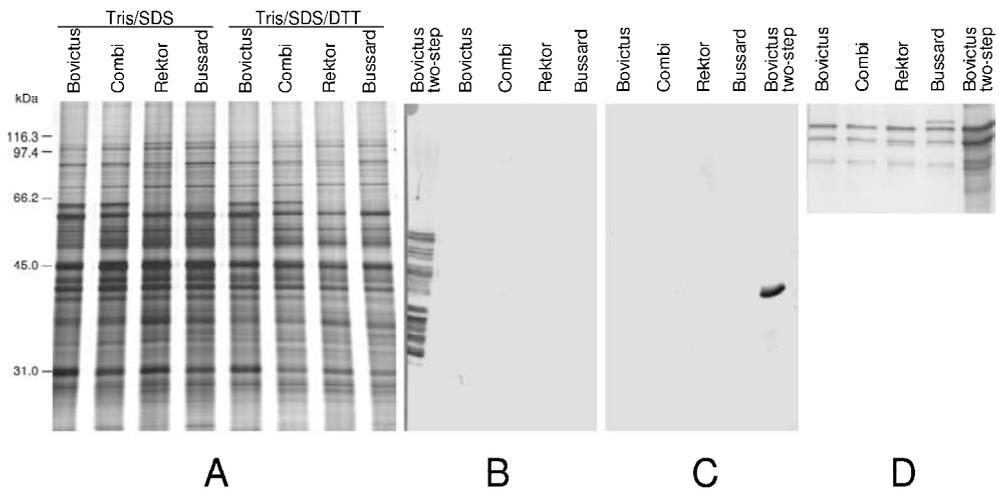


Figure 4. Proteins (1 μ g) after extraction with 25 mM Tris-HCl (pH 8.0) containing 2% SDS (Tris/SDS) and with 25 mM Tris-HCl (pH 8.0) containing 2% (w/v) SDS and 1% (w/v) DTT (Tris/SDS/DTT) from varieties Bovictus, Combi, Rektor, and Bussard separated on SDS-PAGE gels (10%); (A) Silver staining. Tris/SDS extracts were used in immunoblotting; (B) mAb against Glia- 9; (C) mAb against LMW-glutenin; (D) mAb against HMW-glutenin. The Bovictus two-step extract was used as a control in immunoblotting (B, C, and D).

RIDASCREEN® Gliadin competitive ELISA

The sequentially obtained extracts in our two-step protocol were combined to obtain two-step gluten extracts (Fig. 1D). The two-step gluten extracts and the 60% ethanol extracts were analyzed and compared using the RIDASCREEN® Gliadin competitive ELISA. The results (Table 1) showed no major differences between the two different extracts of each variety, only differences among the varieties were observed. The values measured for total gluten content (in ppm) were high, which is expected when pure wheat samples are analyzed. In accordance with the RIDASCREEN® Gliadin competitive ELISA, the immunoblot resulted in similar patterns for the 60% ethanol extracts and two-step gluten extracts from each variety (results not shown).

Table 1. RIDASCREEN® Gliadin competitive ELISA.

		ppm ($\times 10^6$)
60% ethanol extract		
	Bovictus	21.3 (± 3.5)
	Combi	13.9 (± 0.7)
	Rektor	8.3 (± 0.7)
	Bussard	13.9 (± 0.2)
Two-step gluten extract		
	Bovictus	21.2 (± 0.2)
	Combi	11.3 (± 0.9)
	Rektor	8.3 (± 0.2)
	Bussard	10.0 (± 0.4)

Comparison of 60% ethanol extracts and two-step gluten extracts of hexaploid wheat varieties. Errors are standard errors of the mean of duplicate measurements.

Database searching

A protein database search (<http://www.ncbi.nlm.nih.gov/>) of gluten protein sequences from *T. aestivum* was searched for the presence of the sequences recognized by the mAbs R5 (QQPFP, QQQFP, LQPFP and QLPFP), Gli- 9 (QPFPQPQ) and LMW-2 (QSPF). This search resulted in three α -gliadin/D-type LMW-GS sequences out of six that contained the QQPFP sequence (Table 2). Within two α -gliadin sequences, the QQPFP sequence appeared 13 and 14 times. Within β -gliadin sequences, QQPFP appeared with a maximum of eight times per sequence. Within γ -gliadin sequences, QQPFP appeared mostly once per sequence. Within LMW-GS sequences, 60 out of 236 contained the QQPFP sequence, while as many as 155 LMW-GS sequences contained the LMW-2 mAb sequence (QSPF). This explains the higher signals in immunoblotting in both the 1st gluten extracts (50%

aqueous iso-propanol) and 2nd gluten extracts (50% aqueous iso-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% (w/v) DTT) compared to 60% aqueous ethanol extracts, when using the LMW-glutenin mAb (Fig. 3C). No HMW-GS sequences were found that contained QQFPF, QQQFP, LQPFP, or QLPFP sequences.

Table 2. Database search result for epitope sequences present in gluten proteins from *T. aestivum*.

mAb		/ -gliadins	-gliadins	-gliadins/ D-type LMW-GS	LMW-GS	HMW-GS	total
R5 :	QQFPF	63 (65)	91 (354)	3 ¹ (47)	60 (79)	0	218 (545)
	QQQFP	42 (59)	78 (134)	5 ² (56)	17 (43)	0	142 (292)
	LQPFP	69 (77)	0	0	0	0	69 (77)
	QLPFP	0	36 (36)	1 ³ (1)	0	0	37 (37)
LMW-2:	QSPF	0	0	0	155 (155)	0	155 (155)
Glia-α9 :	QFPQPQ	68 (68)	67 (152)	3 ⁴ (10)	0	0	138 (230)
Total no. of protein sequences in database		84	93	6	236	68	419

Number of gluten proteins that contain the different sequences recognized by the mAbs R5, LMW-2, and Glia- 9. In brackets the total number of epitope sequences present in the proteins.

¹2 -gliadins, 1 D-type LMW-GS

²3 -gliadins, 2 D-type LMW-GS

³1 -gliadin

⁴2 -gliadins, 1 D-type LMW-GS

Discussion

Here we describe a two-step extraction protocol that is especially suitable for highly complete extraction of gluten proteins and is compatible with assays for detection and quantification of T-cell stimulatory epitopes with specific monoclonal antibodies. The protocol is based on the subsequent combination of extraction of gliadins and glutenins. Based on comparison with other existing gluten extraction protocols, we obtained more complete and higher overall gluten protein content and specifically higher concentrations of glutenins. The strength of our two-step extraction protocol is that it starts directly with the extraction of gluten proteins without pre-extraction to remove albumins and globulins. Starting the protocol with 50% iso-propanol enables the extraction of -gliadins/D-type LMW-GS that are not extracted in 50% iso-propanol containing DTT.

Bread and pasta wheat breeding has resulted in thousands of different wheat varieties. Recent research using crude extracts from a limited selection of wheat varieties showed large variation of epitope-specific T-cell responses and antibody binding between

these varieties [77]. It is relevant to analyze the variation in immune response among these existing varieties in detail in order to find varieties having low (or none) T-cell stimulatory epitopes for direct use or for further breeding. Multiple T-cell stimulatory gluten epitopes are especially found in α -gliadins, but also in γ -gliadins and glutenins [58, 77]. To analyze the immune response of many wheat varieties and species (including diploids, tetraploids, and hexaploids), our two-step extraction protocol proved to be highly useful.

Obtained gluten protein extracts were tested with the RIDASCREEN[®] Gliadin competitive ELISA. Competitive ELISA is preferred over the sandwich ELISA because the latter requires at least two epitope sequences if the same mAb is used for binding and detection. In that case, the sandwich ELISA may result in underestimation of T-cell stimulatory epitopes. On the other hand, competitive ELISA will only produce an accurate estimation of T-cell stimulatory epitopes if specific mAbs are used. Less specific antibodies may result in false negative or false positive results. The high signals generated by the antibody used in the RIDASCREEN[®] Gliadin competitive ELISA correlates with the known presence of multiple and diverse epitope sequences within the proteins. The low sensitivity of the R5 mAb for LMW-GS explains why the RIDASCREEN[®] Gliadin competitive ELISA did not detect obvious differences in LMW-GS presence across extraction protocols. Although no remarkable differences between the 60% ethanol extracts and the two-step gluten extracts were observed using the RIDASCREEN[®] Gliadin competitive ELISA, clear differences between the extracts were shown by immunoblotting using specific mAbs against other T-cell stimulatory epitopes. The R5 mAb recognizes different small peptide sequences (QQPFP, QQQFP, LQPFP, and QLPPF) which are present mainly in gliadin proteins. The sequence LQPFP, which is strongly recognized by the R5 mAb, is present in the T-cell stimulatory proteolysis-resistant 33-mer [47]. However, this sequence is only present in γ -gliadins. The RIDASCREEN[®] Gliadin competitive ELISA is used to detect 'gluten' in general in food products. Because of the presence of many recognition sites for the R5 mAb, this may lead to overestimation of T-cell stimulatory epitopes. In case of the recognition of the R5 mAb of LMW-GS epitopes, there is an underestimation of T-cell stimulatory epitopes. These facts make the R5 mAb not well suitable for quantification. Immunoblotting using specific mAbs against CD immune responsive epitopes as applied in our protocol is therefore more useful to characterize the T-cell stimulatory capacity of wheat varieties, as the epitopes are not distributed randomly across gliadins and glutenins [74-76]. The antibodies used in the present study, which are specifically generated against

specific T-cell stimulatory epitopes, in combination with our two-step extraction protocol, thus enable to quantify T-cell stimulatory epitopes in a certain food product.

Conclusion

The extraction protocol proposed here enables to highly complete extraction of the gluten proteins from wheat flour. For quantification of the 'epitope load' for CD-patients, the combination with a set of CD-related T-cell stimulatory epitope specific antibodies is essential.

Acknowledgments

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Chapter 3

Staining efficiency of specific proteins depends on the staining method: Wheat gluten proteins

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Abstract

To analyze gluten proteins involved in celiac disease (CD) by proteomic analysis, prolamins extracted from hexaploid wheat varieties were analyzed by SDS-PAGE and 2-dimensional gel electrophoresis. Differences between staining methods (CBB, silver nitrate, SYBRO Ruby, and CyDye) were analyzed in comparison to immunoblotting. Staining efficiency varied per protein across methods and complete staining of all gluten proteins could not be achieved by one of these methods. Care should be taken in the selection of staining method especially if one wants to relate the results to data obtained by immunoblotting.

Introduction

Hexaploid wheat (*Triticum aestivum*) is worldwide grown and used for preparation of baked products, such as bread, due to the presence of gluten proteins. Gluten proteins form an elastic matrix when wheat flour is mixed with water and give volume to bread during rising. Wheat gluten extracts are also increasingly used as an additive in many food products such as soups, sauces, candies, etc. Gluten proteins from wheat are composed of monomeric gliadins and polymeric glutenins. Gliadins are subdivided into α -, β -, and γ -gliadins. The individual polypeptides of glutenins are subdivided into high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS). These subunits are linked by intermolecular disulfide bonds. Gliadins and glutenins together are called prolamins and contain large amounts of the amino acids proline and glutamine and only small amounts of arginine, lysine, and histidine. A negative aspect of prolamins is that they can contain immune responsive peptides that can cause celiac disease (CD; gluten intolerance) in genetically susceptible individuals [40]. After consumption of gluten-containing foods, specific peptides from prolamins trigger an immune response that causes damage to the small intestine in these patients. This leads to a range of symptoms including altered bowel habits, malnutrition, and weight loss [140].

In wheat breeding programs a lot of effort has been put in analyzing allelic codes for gliadins and glutenins in order to come to high yield and disease/pest resistant wheat cultivars with good baking quality [141]. These allelic codes that are used in breeding to predict dough properties are not always suitable for the identification of prolamins containing CD immune responsive peptides because of the low abundance of some of these proteins. To study the presence and variance of these immune responsive prolamins peptides in different modern wheat varieties, a proteomics study was initiated.

As prolamins have an atypical amino acid composition, the staining behavior of these proteins was expected to deviate from standard. We therefore investigated the effect of different staining procedures after gelelectrophoretic separation of total prolamins extracts in comparison to immunoblotting using antibodies that visualize prolamins containing CD immune responsive peptides. Prolamins from bread wheat varieties were analyzed by SDS-PAGE and 2-dimensional gel electrophoresis (2-DE). Separate SDS-PAGE gels were stained with Coomassie Brilliant Blue (CBB) R-250 (traditional), G-250 (colloidal, PageBlue™), silver nitrate, and SYPRO Ruby. Because of similar molecular masses, many gliadins share a mobility region with LMW-GS when

analyzed by SDS-PAGE. 2-DE results in increased resolution of these proteins. One variety was selected for 2-DE, for which proteins of one gel were visualized with CBB R-250, SYPRO Ruby, and CyDye labeling. In the CyDye experiment, 2-DE was performed using Cy3, one of the CyDye DIGE fluor minimal dyes (Cy2, Cy3, and Cy5) used in DIGE [142]. In DIGE, CyDyes are used for labeling different protein samples prior to analyzing them on the same gel. The CyDyes have very similar molecular masses and are positively charged to match the lysine residues of the protein to avoid shifting from the unlabeled protein. Cy2 is frequently used to label a reference sample that can be included as an internal standard on each gel. DIGE makes it possible to detect and quantitate differences between experimental pairs of samples resolved on the same gel and between gels. This method is very sensitive, 0.25–0.95 ng can be detected [143], and by using an internal standard sample, comparison between gels is highly facilitated. For further downstream characterization methods, such as mass spectrometry, 2-DE gels can be post-stained by SYPRO Ruby or CBB.

Materials and Methods

Gluten protein extraction

The total prolamin content was extracted from six modern hexaploid wheat varieties: Bovictus, Combi, Sperber, Rektor, Toronto, and Ambras, as followed: Fifty mg of wheat flour was extracted with 50% iso-propanol with continuous mixing (MS1 Minishaker, IKA Works, Inc.) at 1,000 rpm for 30 min at room temperature, followed by centrifugation at 10,000 rpm for 10 min. The residue was re-extracted twice with 50% iso-propanol/1% DTT/50 mM Tris-HCl (pH 7.5) for 30 min at 60°C. After addition of each next extraction solution, the residue was resuspended by shaking in a Fastprep® FP220A Instrument for 10 s at speed 6.5 m/s followed by sonification for 10 min in an ultrasonic cleaner bath (Branson 3510, Branson Ultrasonics Corporation). Extracts were combined and proteins were analyzed on SDS-PAGE gels.

SDS-PAGE

Proteins were analyzed on SDS-PAGE gels (10%) using the Hoefer SE 260 mighty small II system (GE Healthcare). Separate SDS-PAGE gels were stained with traditional CBB R-250 (0.1% w/v in 45% methanol/10% acetic acid, for 1 h), with colloidal CBB G-250 (PageBlue™, Fermentas, for 16 h), silver nitrate [135], and SYPRO Ruby Protein gel stain

(1/20 dilution in 20% ethanol, Bio-Rad Laboratories, according to manufacturer's instructions). For CBB R-250 staining, 15 µg protein was loaded of each sample, and for CBB G-250 staining, 2 µg protein was loaded of each sample. For staining with silver nitrate and SYPRO Ruby, 1 µg protein was loaded. Gels stained with CBB R-250 were destained for 2 h or alternatively for 16 h in 10% ethanol/7.5% acetic acid.

Immunoblotting

From one gel, proteins were blotted onto nitrocellulose (0.2 µm, Bio-Rad Laboratories), omitting methanol from the blotting buffer, using a Mini Trans-Blot Cell (Bio-Rad Laboratories) at 100 V for 1 h. Blots were incubated and visualized as described in [136] using monoclonal antibodies (mAbs) specific for stimulatory T-cell epitope GliA- 20 [126].

2-DE

Prolamins from wheat variety Rektor were also analyzed by 2-DE and staining properties were compared by applying CBB R-250 staining, SYPRO Ruby staining, and Cy3 labeling. For 2-DE, proteins were separated in the first dimension on Immobiline Drystrip pH 3–10 of 24 cm (GE Healthcare). Protein (50 µg) was labeled with Cy3 (GE Healthcare) according to manufacturer's instructions. For overnight rehydration of IPG strips, Cy3 labeled protein was combined with unlabeled protein (100 µg) to a final volume of 450 µl in rehydration buffer (6 M urea, 2 M thio-urea, 2% CHAPS, 20 mM DTT) complemented with 0.5% IPG buffer pH 3–10 (GE Healthcare) according to manufacturer's instructions. The rehydrated strips were focused on an IPGphor (GE Healthcare) until 50,000 Vh. Prior to second dimension, strips were equilibrated for 15 min in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl, pH 8.8) containing 1% w/v DTT, followed by 15 min in equilibration buffer containing 2.5% w/v iodoacetamide. Separation in the second dimension was performed on SDS-PAGE gels (10%) using the Hoefer ISO-Dalt System (GE Healthcare). After electrophoresis, a gel image was obtained for Cy3 with an FX-scanner (Bio-Rad laboratories) using an excitation wavelength of 532 nm and an emission wavelength filter of 555 nm. Following the Cy3 scan, the gel was post-stained with Sypro Ruby or with CBB R-250.

Results and discussion

SDS-PAGE and 2-DE analysis of prolamins from wheat flour using the different staining procedures resulted in different staining intensities for individual proteins depending on the dye used (Fig. 1 and 2).

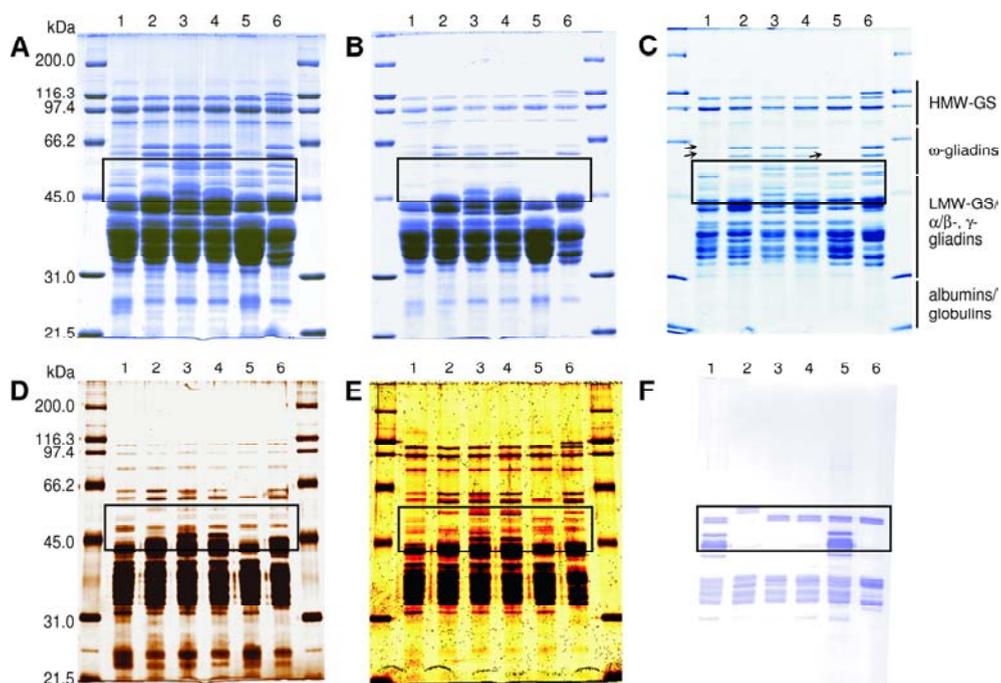


Figure 1. SDS-PAGE (10%) analyses of prolamins from hexaploid wheat varieties. 1. Bovictus; 2. Combi; 3. Sperber; 4. Rektor; 5. Toronto; 6. Ambras. (A) Protein (15 μ g) stained with CBB R-250 and 2 h destaining; (B) Protein (15 μ g) stained with CBB R-250 and 16 h destaining; (C) Protein (2 μ g) stained with PageBlue™. Arrows indicate poorly stained proteins; (D) Protein (1 μ g) stained with silver nitrate; (E) Protein (1 μ g) stained with SYPRO Ruby; (F) Western blot analysis with mAb against T-cell epitope Gli-a-20.

Each method resulted in a slightly different protein pattern. Traditional CBB R-250 staining has the limitation of a low detection sensitivity (8–16 ng) as compared to PageBlue™ (5 ng), silver nitrate (4–8 ng), and SYPRO Ruby staining (1–2 ng) [144]. This was compensated by loading more protein on these gels (Figs. 1A, 1B, and 2A). CBB R-250 interacts with lysine and arginine residues [145]. All CBB staining methods are easy and compatible with characterization methods such as mass spectrometry. However, it was observed that the length of destaining appeared to be very important for the resulting

protein pattern. All CBB R-250 stained prolamins destained to some extent. They were destained in water, as was already shown by Hurkman and Tanaka (2004) [112]. Proteins in the α -gliadin region (66.3–55.4 kDa) destained to a greater extent than other proteins and even diffused out of the gel. We determined that destaining also occurs in 10% ethanol/7.5% acetic acid.

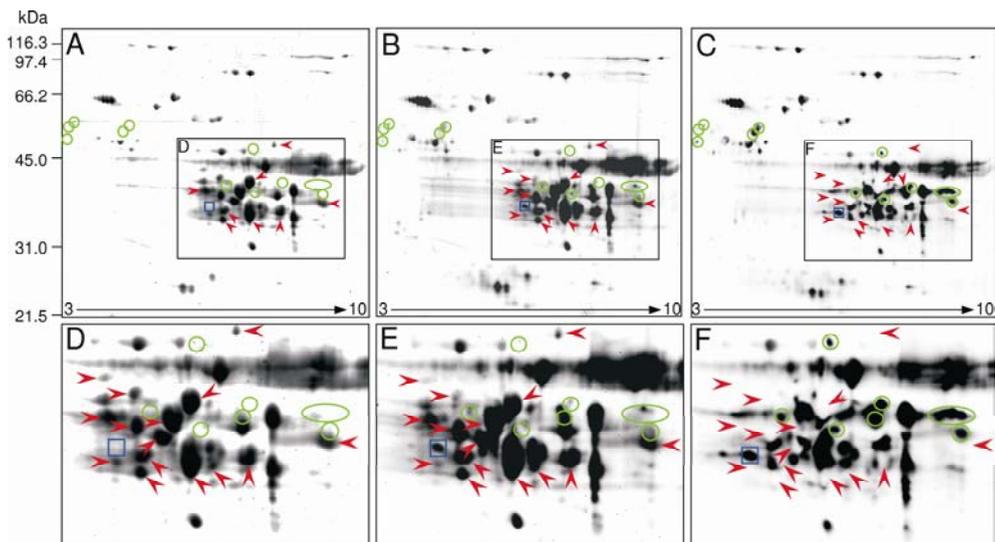


Figure 2. 2-DE of the prolamin extract from hexaploid wheat variety Rektor, 50 μ g protein labeled with Cy3 combined with 100 μ g unlabeled protein. IEF performed on 24 cm linear IPG strip pH 3–10, followed by SDS-PAGE (10%). (A) Stained with CBB R-250; (B) Stained with SYPRO Ruby; (C) Cy3 signal. Red arrowheads represent spots selectively visualized with CBB R-250 and SYPRO Ruby. Green circles represent spots only detected with Cy3. The spot indicated with the blue square is detected with SYPRO Ruby and Cy3, but not with CBB R-250. D, E, and F: Enlargements of boxes in A, B, and C, respectively.

Comparing Fig. 1A with 1B shows that not only proteins in the particular α -gliadin region diffused out of the gel, but also proteins with lower molecular masses disappeared from the gel (boxed proteins in Fig. 1). Some of these proteins were identified with immunoblotting to contain the specific T-cell epitope Gliab-20 (Fig. 1F) and are therefore important in the analysis of variation in prolamin protein patterns among different wheat cultivars in relation to CD.

Colloidal staining with PageBlue™ (CBB G-250) does not require a destaining step and is much more sensitive than traditional CBB R-250 staining (compare Fig. 1A with 1C). It is an endpoint staining and it interacts with lysine, arginine, histidine, and tyrosine residues [145]. The linear dynamic range extends over two orders of magnitude. Fig. 1C shows that

some HMW-GS could be visualized more clearly compared to silver staining (Fig. 1D) but some proteins were poorly stained (indicated with arrows in Fig. 1C). The boxed proteins shown in Fig. 1 also diffused out of the gel when kept in water (result not shown). Gels should be kept in 20% ammonium sulfate to prevent this loss [145].

Silver staining provides high sensitivity and interacts with lysine residues [145]. However, the procedure is quite complex and over-development can occur, as is seen with staining of prolamins (Fig. 1D). Because HMW-GS stained less with silver nitrate than other prolamins, the duration of development had to be increased, which led to over-development of gliadins and LMW-GS (Fig. 1D, 45–31 kDa range). As a result, the intensities of protein bands or gel spots do not reflect the true abundance of different proteins, which implies that silver staining cannot be used in quantification [142].

SYPRO Ruby is an end-point stain with a high sensitivity and is quantitative over three orders of magnitude. It is reported to detect lysine, arginine, as well as histidine [145]. It is an easy one-step staining of proteins without long destaining steps and is compatible with mass spectrometry. Fig. 1E shows that some HMW-GS (the same as with Page Blue™) could be visualized more clearly compared to silver staining. Gliadins and LMW-GS in the 45–31 kDa region were more discrete due to end-point staining. A disadvantage of SYPRO Ruby is the speckled background staining.

In DIGE, CyDye DIGE fluor minimal dyes (Cy2, Cy3, and Cy5) are used for labeling of protein samples prior to gel electrophoresis. Labeling only 1–2% of all the lysine residues with CyDye was found to be optimal [142]. However, prolamin proteins contain only small amounts of lysine, arginine, and histidine [146]. Therefore, the efficiency of CyDye labeling of prolamins may be affected as well. Indeed, some prolamins were not labeled (Fig. 2C and 2F, indicated with arrowheads). On the other hand, some proteins were labeled that were not visualized with SYPRO Ruby (compare Fig. 2B with 2C and Fig. 2E with 2F, encircled spots), or CBB R-250 (compare Fig. 2A with 2C and Fig. 2D with 2F, encircled spots).

Immunoblotting using specific antibodies against CD immune responsive peptides is also a very sensitive detection method, but only for the detection of specific gliadins and glutenins (Fig. 1F). Prolamins from different varieties appearing on the same height in a SDS-PAGE gel display differences in antibody binding capacity. The mAbs staining indicates that similarly abundant proteins of the same molecular weight, which may have been identified by the same allelic code for dough properties [141], can be very different in immune staining, and that some low abundant proteins have a very strong immune response. Our results show that in identification of proteins containing immune

responsive peptides in relation to CD it is imperative to detect also low abundant proteins that are not used in band identification for dough properties.

Conclusion

For visualizing gluten proteins all staining methods described here are applicable, though prominent selective staining is observed for each method. As a consequence, no single staining method provides complete staining of the prolamin profile on SDS-PAGE and 2-DE gels, but the methods show an inconsistent pattern for individual spots on 2-DE gels. For traditional CBB R-250 staining, a higher amount of protein has to be loaded on a gel and destaining for more than 2 h should be avoided because of the selective disappearance of proteins. Colloidal PageBlue™ (CBB G-250) staining is very sensitive compared to traditional CBB staining, but unfortunately, some proteins are poorly stained (Fig. 1C). Silver staining may result in over-development, but if one attempts to visualize lower abundant proteins this is a suitable method. SYPRO Ruby is a very sensitive stain and it displays better staining of HMW-GS compared to silver staining. Wheat varieties can be compared using SYPRO Ruby staining on 2-DE gels. However, small shifts in protein patterns (e.g. due to allelic differences) can be difficult to detect without the use of an internal reference. In this sense, the use of DIGE is preferable. CyDye labeling in DIGE analysis is suitable, although some proteins cannot be visualized, compared to CBB and SYPRO Ruby staining. On the other hand, some prolamins are labeled with CyDye that are not visualized by CBB and SYPRO Ruby. In terms of efficiency, the possibility of running three samples on the same gel, including a reference sample, reduces the number of gels and this makes DIGE a very useful method for the analysis and comparison of large numbers of wheat varieties.

Our study shows that especially for gluten proteins, due to their aberrant amino acid composition and their solubility, the staining method used to visualize the proteins in 1-D or 2-D gel electrophoresis affects the resulting protein pattern. In CD research the most important criterion in the selection of the staining method is that as many proteins as possible should be visualized, including proteins present in small amounts, as all of them may be recognized by specific antibodies against CD immune responsive peptides.

Acknowledgments

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Chapter 4

Presence of celiac disease epitopes in modern and old hexaploid wheat varieties: Wheat breeding may have contributed to increased prevalence of celiac disease

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Abstract

Gluten proteins from wheat can induce celiac disease (CD) in genetically susceptible individuals. Specific gluten peptides can be presented by antigen presenting cells to gluten-sensitive T-cell lymphocytes leading to CD. During the last decades, a significant increase has been observed in the prevalence of CD. This may partly be attributed to an increase in awareness and to improved diagnostic techniques, but increased wheat and gluten consumption is also considered a major cause.

To analyze whether wheat breeding contributed to the increase of the prevalence of CD, we have compared the genetic diversity of gluten proteins for the presence of two CD-epitopes (GliA- 9 and GliA- 20) in 36 modern European wheat varieties and in 50 landraces representing the wheat varieties grown up to around a century ago. GliA- 9 is a major (immunodominant) epitope that is recognized by the majority of CD-patients. The minor GliA- 20 was included as a technical reference. Overall, the presence of the GliA- 9 epitope was higher in the modern varieties, whereas the presence of the GliA- 20 epitope was lower, as compared to the landraces. This suggests that modern wheat breeding practices may have led to an increased exposure to CD-epitopes. On the other hand, some modern varieties and landraces have been identified that have relatively low contents of both epitopes. Such selected lines may serve as a start to breed wheat for the introduction of 'low CD-toxic' as a new breeding trait. Large-scale culture and consumption of such varieties would considerably aid in decreasing the prevalence of CD.

Introduction

Wheat is the third most produced cereal in the world after maize and rice (<http://faostat.fao.org/site/567/default.aspx#ancor>). It is a very important food crop for the daily intake of proteins, vitamins, minerals and fibers in a growing part of the world population [147]. Wheat consumption, and especially the intake of its gluten, is, however, also a major cause of the development of celiac disease (CD). CD is an inflammatory disorder of the small intestine resulting in a wide variety of chronically symptoms (diarrhea, bowel pain, headache, growth retardation, osteoporosis, infertility, lymphoma, etc.) in about 1% of the wheat consuming world population. The prevalence and the risk of death in undiagnosed CD have increased dramatically during the last 50 years in the United States [148]. Lohi *et al.* (2007) [149] described a doubling of the prevalence of CD in Finland in the last two decades, which definitely could not be ascribed to improved detection only. In Asia the prevalence of CD is increasing because of a change towards Western-style diets [147]. Changes in life style (e.g., the increasing exclusion of breast feeding) and the time and amount of the first introduction of wheat containing products in early life can be considered major environmental factors causing this increase [60, 98, 150]. Wheat consumption as wheat flour and wheat-based products per capita is high in Europe and the Middle East and increasing in Asia [148] and is again increasing in the United States (<http://www.ers.usda.gov/AmberWaves/september08/findings/wheatflour.htm>). In addition, wheat gluten is increasingly applied as an additive in a wide and growing variety of processed foods and in other products, including medicines [1-4].

Bread wheat (*Triticum aestivum*) is an allohexaploid species resulting from natural hybridization between a tetraploid *T. turgidum* (*dicoccum*) carrying the AB-genome and a wild diploid species *Aegilops tauschii* carrying the D-genome [8, and references therein]. Especially, the introduction of the D-genome improved the bread-making properties [9-12]. Over 100 years ago, breeders started to systematically cross and select bread wheat for higher yields, adaption to climate changes, better bread-making characteristics, and improved disease resistance. Little information is available about the breeding history of landraces on these aspects [151]. Breeding has resulted in many thousands of different wheat varieties that are stored in genetic resource centers and breeding company stocks. Modern wheat breeding, focusing on the increase of yield, initially narrowed down the genetic base of its germplasm [152]. However, genetic diversity has increased again in the set of varieties released since the 1990s because breeders started to use wild relatives and synthetic wheats for introgression of, among others, disease resistances [153]. The net

effect was neither a decrease nor an increase of genetic diversity, as measured by neutral genetic markers, over the last century. However, this does not exclude the possibility that diversity in some traits may have been reduced or increased. Notably for prolamins, landraces can contain many different chemotypes in a single population [154], but modern varieties have only a small number of different gene combinations for some of the prolamins [19]. This raises the important question about possible breeding-induced differences in the presence of T-cell stimulatory epitopes in modern varieties compared to landraces and older varieties.

In wheat, gluten proteins are comprised of gliadins and glutenins, which are present in approximately equal amounts and form 80% of the total storage protein content in the wheat kernel, next to albumins (12%) and globulins (8%). The gliadins form a large protein family in which α -, β -, and γ -gliadins can be distinguished [16], whereas the glutenins can be subdivided into low-molecular weight glutenin subunits (LMW-GS) and high-molecular weight glutenin subunits (HMW-GS) [17]. The high proline and glutamine content makes gluten proteins resistant to complete proteolytic digestion [46, 47, 155]. Gluten peptides resulting from partial digestion of all gluten protein groups (α -, β -, γ -gliadins, LMW-GS, and HMW-GS) may contain T-cell stimulatory epitopes [26, 27], but the epitopes from the α -gliadins are considered to have by far the highest clinical relevance with regard to both the adaptive immune response and the innate immune response that lead to the development of CD [66-73, 156-160].

In the present paper, we use two monoclonal antibodies (mAbs) that were raised against the GliA-9 and GliA-20 epitopes [77, 126, 132] for comparison of the presence of T-cell stimulatory epitopes in gluten protein extracts from different wheat landraces and modern varieties. The GliA-9 epitope is especially known as a major immunodominant epitope that can be recognized by the majority of CD-patients [69, 156]. The GliA-9 epitope sequence (L) is part of the proteolytic-resistant 33-mer in α -gliadins that has a strong T-cell stimulatory effect [47, 161]. The GliA-20 epitope, which is used in this study as a technical reference, is a minor epitope that is recognized by a minority of patients. For these epitopes proper mAbs are available. Unfortunately, not all epitopes can be studied properly with existing antibodies, and the consequence of the shorter epitope recognition site of the mAbs compared to the T cell recognition site might be over-staining, which would result in overestimation of the toxicity.

Recent research using protein extracts from a limited selection of wheat varieties demonstrated a large variation in immune responses, as measured as epitope-specific T-cell responses or in mAb binding studies [76, 77]. In the present study, we set out to

compare the occurrence of T-cell stimulatory gluten epitopes of modern European varieties with landraces to determine to what extent breeding might have changed the presence of T-cell stimulatory epitopes in wheat. The results will be discussed in view of the question to what extent wheat breeding can contribute to the prevalence of CD.

Materials and Methods

Search for the occurrence of sequences recognized by mAbs and T-cell epitopes

The frequency of known T-cell stimulatory epitope sequences in deduced α -gliadin proteins was analyzed using the expressed sequence tag (EST) sequences obtained from hexaploid varieties Lavett and Baldus as described by Salentijn *et al.* (2009) [75]. The deduced α -gliadin sequences were analyzed for the different minimal recognition sequences of mAbs and T-cells for GliA- 9 (QFPQPQ and PFPQPQLPY, respectively) and GliA- 20 (RPQQPYP and FRPQQPYPQ, respectively) [77, 126, 132, 162]. No mismatches were allowed.

Grain samples

A set of 36 modern hexaploid wheat varieties, available for the European market, were obtained from Limagrain, Lelystad, The Netherlands. The varieties were selected for maximum *Glu-3* and *Gli-1* diversity, in the genetic background of the most frequent composition of HMW-GS alleles (*Glu-A1*: 0 or 1; *Glu-B1*: 7+9; *Glu-D1*: 5+10; Table 1), based on allozyme patterns that are routinely produced for wheat varieties. The set of landraces (Table 2) was obtained from the Centre for Genetic Resources (CGN), the Netherlands (<http://www.cgn.wur.nl/uk/>), except for accession RICP01C0203330, which was obtained from the Research Institute of Crop Production (RICP), Czech Republic. The accessions were selected based on collection period and diversity of geographic origin.

Gluten protein extraction

For extraction of gluten proteins from wheat, we used the method as described by Van den Broeck *et al.* (2009) [163], which combines three sequentially obtained extracts to extract nearly all gliadins and glutenins. Extraction of the residue left after the third extract with 25mM Tris/HCl (pH 8.0) containing 2% SDS and with 25mM Tris/HCl (pH 8.0) containing 2% SDS and 1% DTT showed that only some HMW-GS and probably some

omega-gliadins/D-type LMW-GS were still present in the sample. The gluten extracts analyzed in this study therefore contained most of the gluten proteins present.

Table 1. Details of modern wheat varieties.

	Variety	Type	Quality group	<i>Glu-1</i>	<i>Glu-3</i>	<i>Gli-1</i>	Year of release	Country of origin
1	Kornett	W	A	0, 7 + 9, 5 + 10	f, g, c	f, f, b	1997	Germany
2	Eta	S	B	1, 7 + 9, 5 + 10	f, f, c	f, g, b	1986	Poland
3	Thasos	S	E	1, 7 + 9, 5 + 10	f, f, c	a, f, g	1994	Germany
4	Triso	S	E	1, 7 + 9, 5 + 10	f, c, c	f, f, b	1996	Germany
5	Star	S	A	0, 7 + 9, 5 + 10	f, c, c	a, f, g	1986	Germany
6	Ramiro	W	A	1, 7 + 9, 5 + 10	f, c, a	f, b, g	1969	Germany
7	Bovictus	W	B	0, 7 + 9, 5 + 10	e, j, c	b, l, b	1993	Germany
8	Combi	S	E	0, 7 + 9, 5 + 10	e, g, c	f, f, l	1990	Germany
9	Zentos	W	E	0, 7 + 9, 5 + 10	e, g, c	a, f, g	1989	Germany
10	Naxos	S	A	1, 7 + 9, 5 + 10	e, g, c	a, c, a	1992	Germany
11	Astron	W	A	0, 7 + 9, 5 + 10	d, g, c	o, f, b	1989	Germany
12	Belisar	W	A	0, 7 + 9, 5 + 10	d, g, c	o, e, a	1995	Germany
13	Glockner	W	E	0, 7 + 9, 5 + 10	d, g, c	l, b, b	1993	Germany
14	Sperber	W	A	0, 7 + 9, 5 + 10	d, g, c	a, h, d	1982	Germany
15	Rektor	W	E	1, 7 + 9, 5 + 10	d, g, c	a, f, a	1980	Germany
16	Tinos	S	E	1, 7 + 9, 5 + 10	d, f, c	a, f, g	1993	Germany
17	Trakos	W	B	0, 7 + 9, 5 + 10	d, c, c	b, f, b	1997	Germany
18	Toronto	W	A	0, 7 + 9, 5 + 10	a, j, c	b, l, b	1990	Germany
19	Tambor	W	A	0, 7 + 9, 5 + 10	a, h, c	b, f, b	1993	Germany
20	Aristos	W	A	0, 7 + 9, 5 + 10	a, g, c	f, f, d	1997	Germany
21	Pegassos	W	A	1, 7 + 9, 5 + 10	a, g, c	f, f, a	1996	Germany
22	Bussard	W	E	1, 7 + 9, 5 + 10	a, g, c	f, c, b	1990	Germany
23	Mikon	W	B	1, 7 + 9, 5 + 10	a, g, c	f, b, l	1988	Germany
24	Winni	W	B	1, 7 + 9, 5 + 10	a, g, c	b, f, d	1998	Germany
25	Klaros	S	A	1, 7 + 9, 5 + 10	a, g, c	a, f, b	1995	Germany
26	Quattro	S	A	1, 7 + 9, 5 + 10	a, g, c	a, f, a	1995	Germany
27	Batis	W	A	1, 7 + 9, 5 + 10	a, g, c	a, e, l	1994	Germany
28	Aron	W	E	0, 7 + 9, 5 + 10	a, g, c	a, e, i	1992	Germany
29	Munk	S	A	0, 7 + 9, 5 + 10	a, g, c	a, e, g	1993	Germany
30	Ambras	W	A	1, 7 + 9, 5 + 10	a, g, c	a, b, k	1990	Germany
31	Urban	W	E	0, 7 + 9, 5 + 10	a, g, c	a, b, i	1980	Germany
32	Ralle	S	E	1, 7 + 9, 5 + 10	a, c, c	a, f, b	1984	Germany
33	Bold	W	B	0, 6 + 8, 5 + 10	d, c, c	o, s, i	1996	Germany
34	Borenos	W	E	0, 7 + 9, 2 + 12	a, d, a	f, h, g	1987	Germany
35	Dakota	W	B	1, 7 + 8, 2 + 12	e, j, a	m, l, a	1996	Germany
36	Cadenza	S	B	0, 14 + 15, 5 + 10	a, h, c	a, d, b	1992	UK

Allele identification for *Glu-1*, *Glu-3*, and *Gli-1* loci is according to Jackson et al. (1996) [164]. W is winter wheat. S is spring wheat. Quality group according to the German classification (German Federal Office for Plant Varieties): E is Elite wheat, A is Quality wheat, and B is Bread wheat.

Table 2. Details of landrace accessions.

	Accession no.	Accession name	<i>T. aestivum</i> subspecies	Population type	Type	Collection date	Country of origin
1	RICP01C0203330	Arcade	<i>aestivum</i>	A	S		Belgium
2	CGN19307	Minaret	<i>aestivum</i>	A	S	1982	The Netherlands
3	CGN04210	Weissahr Rotkorn Binkel	<i>compactum</i>	L	S	1951	Italy
4	CGN08510	Hilgendorf	<i>aestivum</i>	A	S	1947	New Zealand
5	CGN08315	Rouge de la Gruyere	<i>compactum</i>	L	S		Switzerland
6	CGN12393	Sappo	<i>aestivum</i>	A	S	1969	Sweden
7	CGN19285	Baldus	<i>aestivum</i>	A	S	1990	The Netherlands
8	CGN08025	Sinde	<i>aestivum</i>	L	S	1972/1973	Ethiopia
9	CGN10640	–	<i>compactum</i>	L	S	1974	Pakistan
10	CGN05620	–	<i>aestivum</i>	L	W		Afghanistan
11	CGN05359	Alty Agac Mestnaja	<i>aestivum</i>	L	W		Armenia
12	CGN12271	Kamtschatka Mestnaja	<i>compactum</i>	L	S	1964	Armenia
13	CGN05414	Chyrda Bugda Mestnaja	<i>aestivum</i>	L	W		Azerbaijan
14	CGN06118	Gomborka	<i>aestivum</i>	L	S	1959	Azerbaijan
15	CGN08330	–	<i>compactum</i>	?	W		Azerbaijan
16	CGN08306	–	<i>spelta</i>	L	S		Azerbaijan
17	CGN08309	–	<i>spelta</i>	L	S		Azerbaijan
18	CGN05500	–	<i>aestivum</i>	L	W	1955	Iran
19	CGN06440	Tritaeg 1	<i>aestivum</i>	L	S	1955	Iran
20	CGN08274	Weisser Kolbenspelz	<i>spelta</i>	L	S		Germany
21	CGN12269	Iran 404c	<i>spelta</i>	L	S		Iran
22	CGN12270	Iran 416a	<i>spelta</i>	L	S		Iran
23	CGN04065	Bagdad	<i>aestivum</i>	L	S	1902	Iraq
24	CGN06150	Humera	<i>aestivum</i>	L	S		Iraq
25	CGN06162	Iraqua	<i>aestivum</i>	L	S	1969	Iraq
26	CGN08327	Irak	<i>compactum</i>	?	S	1964	Iraq
27	CGN06183	–	<i>aestivum</i>	L	S	1969	Israel
28	CGN04191	–	<i>compactum</i>	L	S	1967	Israel
29	CGN09728	–	<i>aestivum</i>	L	S	1981	Pakistan
30	CGN12071	–	<i>aestivum</i>	L	S	1976	Pakistan
31	CGN10639	–	<i>compactum</i>	L	S	1974	Pakistan
32	CGN11461	BraunerSpelzAusSchettlenz	<i>spelta</i>	L	W		Germany
33	CGN04041	Ak 702	<i>aestivum</i>	L	S	1963	Turkey
34	CGN04267	Ak Guzluk	<i>aestivum</i>	L	W	1948	Turkey
35	CGN05392	Bolu	<i>aestivum</i>	L	W	1948	Turkey
36	CGN10653	Turk 169	<i>aestivum</i>	L	I		Turkey
37	CGN04345	Galickaja	<i>aestivum</i>	L	W		USSR
38	CGN05541	Krymka	<i>aestivum</i>	L	W		USSR
39	CGN06033	Artemovka/Lutescens 1418	<i>aestivum</i>	A	S	1945	USSR
40	CGN04042	Akagomughi/Akakomugi	<i>aestivum</i>	L	S	1960	Japan
41	CGN04275	Alton, Ghirka winter	<i>aestivum</i>	L	W	1900	USSR
42	CGN05465	Gelderse Ris	<i>aestivum</i>	L	W	<1900	The Netherlands
43	CGN04381	Lammas Red	<i>aestivum</i>	L	W		UK
44	CGN04080	Chiddam	<i>aestivum</i>	L	S	1863	France
45	CGN04164	Noe	<i>aestivum</i>	L	S	1880	France
46	CGN04087	Chul Bidaj; Idaho Hard	<i>aestivum</i>	L	S	1902	USSR
47	CGN06361	Red Fife	<i>aestivum</i>	L	S	1900	USA
48	CGN04236	Pyrothrix 28	<i>aestivum</i>	A	S	1973	USSR
49	CGN05640	Rode Dikkop	<i>aestivum</i>	L	W	<1901	The Netherlands
50	CGN11465	Spelt van Hoosterhof	<i>spelta</i>	L	W		Belgium

Passport details are from CGN, Wageningen, The Netherlands. A is advanced cultivar; L is landrace/traditional cultivar; S is spring wheat; W is winter wheat; I is intermediate.

Wheat grains were ground in an analytical mill (A 11 Basic, IKA-Werke) and sieved through mesh (0.5 mm). Gluten proteins were extracted from 50 mg wheat flour by addition of 0.5 ml of 50% aqueous iso-propanol with continuous mixing (MS1 Minishaker, IKA Works, Inc.) at 1,000 rpm for 30 min at room temperature, followed by centrifugation at 10,000 rpm for 10 min at room temperature. The residue was re-extracted twice with 50% aqueous iso-propanol/1% DTT/50 mM Tris-HCl, pH 7.5, for 30 min at 60°C with mixing every 5–10 min followed by centrifugation at 10,000 rpm for 10 min at room temperature. After addition of each next extraction solution, the residue was resuspended by shaking in a Fastprep[®] FP220A Instrument for 10 s at speed 6.5 m/s followed by sonification for 10 min in an ultrasonic bath (Branson 3510, Branson Ultrasonics Corporation). The three obtained supernatants were combined and considered the gluten protein extract. The protein content was quantified using Bio-Rad Protein Assay (Bio-Rad Laboratories), based on the Bradford dye-binding procedure, according to manufacturer's instruction.

SDS-PAGE and immunoblotting

Equal amounts of gluten proteins were loaded and were separated on SDS-PAGE gels (10%) [134] using a Hoefer SE 260 mighty small II system (GE Healthcare). Proteins were blotted onto nitrocellulose (0.2 µm, Bio-Rad Laboratories), omitting methanol from the blotting buffer, using a Mini Trans-Blot Cell (Bio-Rad Laboratories) at 100 V for 1 h. Blots were stained using a MemCode[™] Reversible Protein Stain Kit for Nitrocellulose Membranes (Fisher Scientific) prior to incubation with mAbs. Blots were incubated as described [136] using monoclonal antibodies (mAbs) specific for T-cell stimulatory epitopes GliA- 9 [77, 132], GliA- 20 [132, 162], GliT 156 (LMW-1 and LMW-2) [77, 132], and HMW-gliT [126, 132]. Antibody binding to the blots was visualized by staining for alkaline phosphatase conjugated secondary antibody, using Nitro Blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma). The gluten protein extract of 'Toronto' was used on each separate immunoblot as an 'inter-gel' control.

Blots were scanned using a Bio-Rad GS-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories) and saved as TIFF images. Pixel intensities were calculated per lane using Quantity One software (Bio-Rad Laboratories). Relative intensities differed specifically per mAb used, but were normalized to values obtained for the 'inter-gel' control. Intensities per lane were categorized into three classes: low (+), medium (++), and high (+++) according to linear subdivision of the maximum intensities.

Two-dimensional gel electrophoresis (2-DE)

For 2-DE, gluten proteins were separated in the first dimension by IEF. Immobiline Drystrips pH 3–10 of 7 cm (GE Healthcare) were rehydrated overnight with 5 µg protein in rehydration buffer (6 M urea, 2 M thio-urea, 2% CHAPS, 20 mM DTT) complemented with 0.5% IPG buffer pH 3–10 (GE Healthcare) to reach a final volume of 125 µl, according to manufacturer's instructions. The rehydrated strips were focused on an IPGphor (GE Healthcare) using the following conditions: 300 V during 30 min, gradient to 1,000 V in 30 min, gradient to 5,000 V in 1h 20 min, step and hold at 5,000 V until 6,500 Vh. Prior to second dimension, strips were equilibrated for 15 min in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl, pH 8.8) containing 1% (w/v) DTT, followed by 15 min in 5 ml equilibration buffer containing 2.5% (w/v) iodoacetamide. Separation in the second dimension was performed using SDS-PAGE gels (10%) and the SE 260 mighty small II system (GE Healthcare). Gels were used for immunoblotting or stained with PageBlue™ (Fermentas).

Tandem mass spectrometric analysis and data analysis

Protein bands were excised from SDS-PAGE gel and digested with chymotrypsin (Boehringer Mannheim). Digested peptide mixtures were separated and analyzed by electrospray tandem mass spectrometry (LTQ-Orbitrap, Thermo Fisher Scientific) as described by Van Esse *et al.* (2008) [165]. Proteins were identified by automated database searching (OMSSA, <http://pubchem.ncbi.nlm.nih.gov/omssa/>) against the *T. aestivum* protein sequence database.

Results

Search for frequency of epitope sequences

Monoclonal Ab staining, which is much easier than T-cell testing, is currently the only way to perform a comparative study such as carried out here for a large number of varieties and landraces. It is currently also the only tool to quantify for CD-epitopes in breeding programs. One worry could be that, as a consequence of the shorter epitope recognition site of the mAb, staining with the mAbs will not reveal all T-cell epitopes present, while other sites may be stained that do not represent complete epitopes.

To evaluate if there is an under- or overestimation of CD-epitopes when using monoclonal antibodies, we analyzed the frequency of minimal recognition sequences of

mAbs and T-cells for GliA- 9 and GliA-20 in deduced α -gliadin proteins encoded by EST sequences obtained from hexaploid varieties Lavett and Baldus. The numbers of protein sequences that contain the various sequences recognized by GliA- 9 and GliA- 20 mAbs and T-cells are shown in Table 3. Sequences obtained from varieties Lavett and Baldus [75] contained the sequences recognized by either GliA- 9 (mAb and T-cell) or GliA- 20 (mAb and T-cell) or both. Five sequences obtained from the D-genome of 'Baldus' contained the T-cell epitope sequence for GliA- 9 but only three contained the GliA- 9 mAb sequence. On the other hand, a single sequence from 'Lavett' (B-genome) and two sequences from 'Baldus' (B-genome) were obtained that contained the GliA- 9 mAb sequence but not the corresponding T-cell sequence. The fact that α -gliadins encoded by the B-genome do not contain sequences recognized by the GliA- 9 and GliA- 20 T-cells was consistent with the results of Van Herpen *et al.* (2006) [74].

As we calculated, over 92% of the found mAb epitopes were recognized by the corresponding T-cells (Table 3), which means that there is a small over-estimation of the CD-epitopes in a cultivar using mAb staining compared to T-cell testing.

Table 3. Search results for GliA- 9 and GliA- 20 epitope sequences present in deduced α -gliadin sequences from hexaploid varieties Lavett and Baldus [75].

Variety	Genome	GliA- 9		GliA- 20		GliA- 9 + GliA- 20	
		mAb	T-cell	mAb	T-cell	mAb	T-cell
Lavett	A (n=4)	4	4	2	2	2	2
	B (n=1)	1	–	–	–	–	–
	D (n=7)	6	6	5	5	5	5
Baldus	A (n=2)	2	2	1	1	1	1
	B (n=7)	2	–	–	–	–	–
	D (n=5)	3	5	5	5	3	5
Total (n=26)		18	17	13	13	11	13

The number in each cell represents the presence of the recognized sequences by mAb or T-cell. The symbol '–' in a cell means that the sequence was not present.

To analyze whether α -gliadin epitopes are present in other protein than only α -gliadins, the NCBI protein database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein>) was screened for sequences that could be recognized by the GliA- 9 and GliA- 20 mAbs and T-cells. The search showed that α -gliadins and α -gliadins/D-type LMW-GS exist that contain the GliA- 9 mAb recognition sequence, although no corresponding T-cell recognition sequence was present in these α -gliadins and α -gliadins/D-type LMW-GS. For the wheat lines from which the NCBI protein database sequences have been derived, this

would lead to overestimation of GliA- 9 epitopes using antibody staining. No α -gliadins and β -gliadins/D-type LMW-GS were found that contain sequences recognized by the GliA- 20 mAb or T-cells.

SDS-PAGE and immunoblotting

The gluten protein content in the grains of 36 modern wheat varieties was on average 24.4 ± 3.6 g/mg flour. In grains from landrace accessions, the average gluten protein content was 30.9 ± 3.4 g/mg flour. The difference in protein content is most likely caused by the different environments in which the varieties were grown. A higher amount of starch in combination with a stable total amount of protein leads to a reduction of the gluten protein content per mg of flour.

The wheat varieties and landraces were analyzed by immunoblotting using mAbs against T-cell stimulatory epitopes GliA- 9 and GliA- 20 to detect the number of proteins present containing these epitope sequences. Equal amounts of protein were loaded on the gels.

Modern varieties

Most modern varieties showed a number of the same (or very comparable) staining patterns for both mAbs against GliA- 9 and GliA- 20 epitopes (Figs. 1A, B), but distinct patterns were obtained for 'Kornett' (Table 1, no. 1), 'Bovictus' (Table 1, no. 7), 'Trakos' (Table 2, no. 17), 'Toronto' (Table 1, no. 18), 'Tambor' (Table 1, no. 19), 'Bold' (Table 1, no. 33), 'Dakota' (Table 1, no. 35), and 'Cadenza' (Table 1, no. 36).

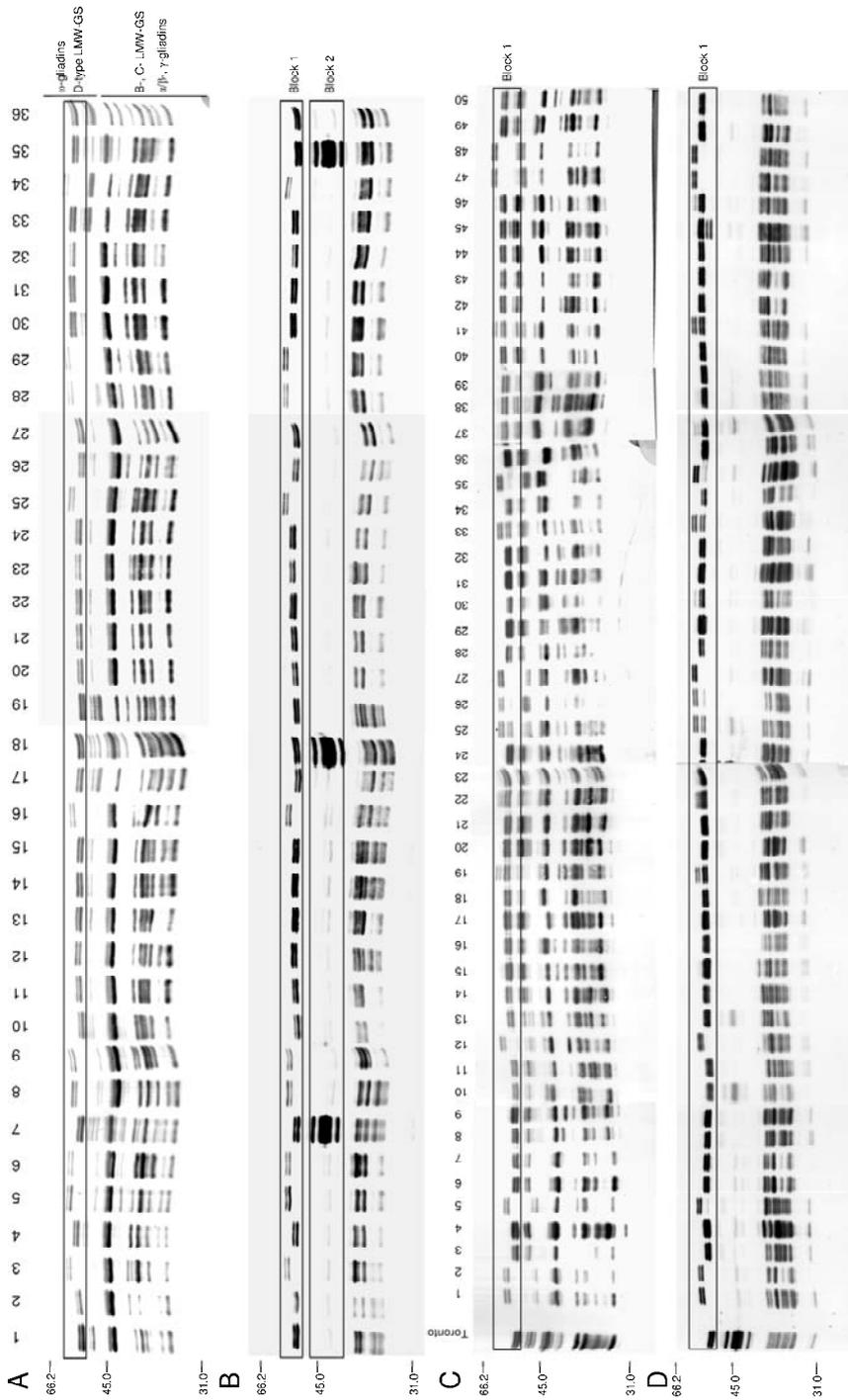
Some distinct blocks of bands were visible that are identical in several cultivars. Using the GliA- 20 mAb, two closely linked proteins assigned as β -gliadins/D-type LMW-GS were stained, which we will refer to as 'block-1' proteins (Fig. 1B). The molecular masses of these two linked proteins were ~ 55 kDa. This block occurred in 26 modern wheat varieties. Ten varieties had two proteins of ~ 60 kDa, probably linked α -gliadins/D-type LMW-GS, which can also be stained with the GliA- 9 mAb (Fig. 1A). The lower one of these two bands from variety Bovictus (Table 1, no. 7) was characterized by LC-MS/MS and showed 54.6% protein coverage with a D-type LMW-GS from *T. aestivum* (CAR82265) and 29.6% protein coverage with an α -gliadin from *T. aestivum* (AAG17702) (results not shown). Within those two sequences, no recognition sequence is present for the GliA- 20 mAb, with which the protein bands gave a clear signal. However, among the peptide sequences that were identified by LC-MS/MS, sequences were obtained that carry the GliA- 20 mAb and T-cell epitope sequence. Until now, only a few β -gliadin and D-type

LMW-GS proteins have been sequenced because they are difficult to clone due to the presence of large repetitive domains [166]. Molecular masses of 1B- and 1D-encoded ω -gliadins are often overestimated by SDS-PAGE compared to mass spectrometry [167]. These ω -gliadins are encoded by two closely linked genes on the *Gli-D1* locus [168]. From studies on 'Chinese Spring' deletion lines [169], we know that these proteins are absent in plants in which parts of the short arm of chromosome 1D were deleted. In contrast, no ω -gliadins reacting with mAb Gli- 9 and Gli- 20 were removed in lines having deletions of the short arms of chromosome 1A or 1B. Our epitope staining data support earlier results [69, 170, 171] showing that ω -gliadins may contain epitopes that are involved in gluten-sensitive response of CD-patients.

When stained by the Gli- 20 mAb, the three varieties Bovictus (Table 1, no. 7), Toronto (Table 1, no. 18), and Dakota (Table 1, no. 35) showed another block of tentatively linked gluten proteins between 42 and 50 kDa (Fig. 1B, referred to as 'block 2') that were also stained by the Gli- 9 mAb. This indicates that these proteins contained both epitope sequences. The 'block-2' proteins are also detected in immunoblot analyses of CIMMYT synthetic hexaploid wheat lines (results not shown). The proteins are not visible in the tetraploid parent lines and occur independently of the 'block-1' gluten proteins. Hence, they are most likely encoded by chromosome 1D.

The epitope sequence search revealed that deduced ω -gliadin sequences contained both sequences recognized by the Gli- 9 mAb and the Gli- 20 mAb (Table 3). That ω -gliadin sequences can contain sequences recognized by both mAbs is confirmed by the results in Fig. 2, which shows gluten proteins containing either the sequence recognized by the Gli- 9 mAb, the Gli- 20 mAb or both. Overall, most of the ω -gliadins contain the Gli- 9 epitope and only few, depending on the variety, contain just the Gli- 20 epitope.

Figure 1. Analyses of gluten protein extracts from 36 modern wheat varieties and hexaploid landrace accessions. Immunoblots modern varieties using (A) mAb Gli- 9; (B) mAb Gli- 20. Immunoblots landraces using (C) mAb Gli- 9; (D) mAb Gli- 20. Boxes indicate 'block-1' and 'block-2' proteins.



The epitope sequence search revealed that deduced α -gliadin sequences contained both sequences recognized by the GliA- 9 mAb and the GliA- 20 mAb (Table 3). The fact that α -gliadin sequences can contain sequences recognized by both mAbs is confirmed by the results in Fig. 2, which shows gluten proteins containing either the sequence recognized by the GliA- 9 mAb, the GliA- 20 mAb or both. Overall, most of the α -gliaidins contain the GliA- 9 epitope and only few, depending on the variety, contain just the GliA- 20 epitope.

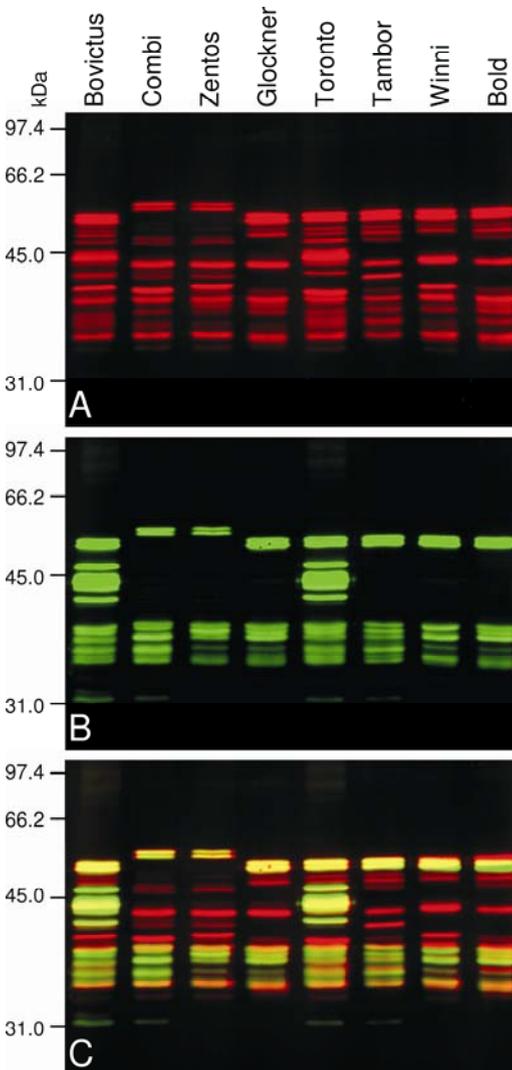


Figure 2. Images represent immunoblot results for modern wheat varieties Bovictus, Combi, Zentos, Glockner, Toronto, Tambor, Winni, and Bold. (A) Red channel shows the results with mAb GliA- 9; (B) Green channel for results with mAb GliA- 20; (C) Overlay of both images in (A) and (B). In yellow identical gluten protein bands are shown.

Other mAbs that were used in screening were raised against epitopes from HMW-glutenin and LMW-GS (Glt-156). Immunoblotting showed that all HMW-GS present in our modern varieties (a limited set: *Glu-A1*: 0 or 1; *Glu-B1*: 7+9; *Glu-D1*: 5+10; Table 1) stained with the mAb against the HMW-Glt epitope (not shown). This was confirmed by a NCBI database search in which all full-size HMW-GS were shown to contain the HMW epitope. The T-cell epitope from LMW-GS (Glt-156) is covered by two mAbs. Both mAbs were used in screening (data not shown). The mAb covering the N-terminal part as well as the antibody against the C-terminal part of the T-cell epitope resulted in one to three bands appearing on immunoblots of which mostly one, and sometimes two, were overlapping. Both epitopes are not considered major epitopes.

The existing mAbs against GliA- 1, unfortunately, only recognize two amino acids of the T-cell epitope [132] and were therefore not used for screening for the GliA- 1 epitope, which is the most important of the α -gliadin epitopes (Salentijn *et al.*, in prep).

2-DE

When gluten proteins from 'Bovictus' (Table 1, no. 7) and 'Sperber' (Table 1, no. 14) were separated in more detail by 2-DE, a single band on an immunoblot often yielded more than one protein spot (Fig. 3). Because of the complexity of protein bands, care has to be taken in assigning protein bands directly as alleles to varieties, based on one-dimensional SDS-PAGE protein patterns alone.

Landraces

The results for the landraces showed much more diverse protein patterns for proteins that bind GliA- 9 or GliA- 20 mAb (Figs. 4A, B). The two closely linked α -gliadins/D-type LMW-GS were also present in the landraces (boxed proteins in Figs. 1C, D). In some landraces, both the higher and lower molecular mass α -gliadins/D-type LMW-GS were present. This may be caused by genetic heterogeneity within the accessions that were obtained from a genebank since several kernels were combined for protein extraction. The 'block-2' gluten proteins were not found in any of the landraces we have tested.

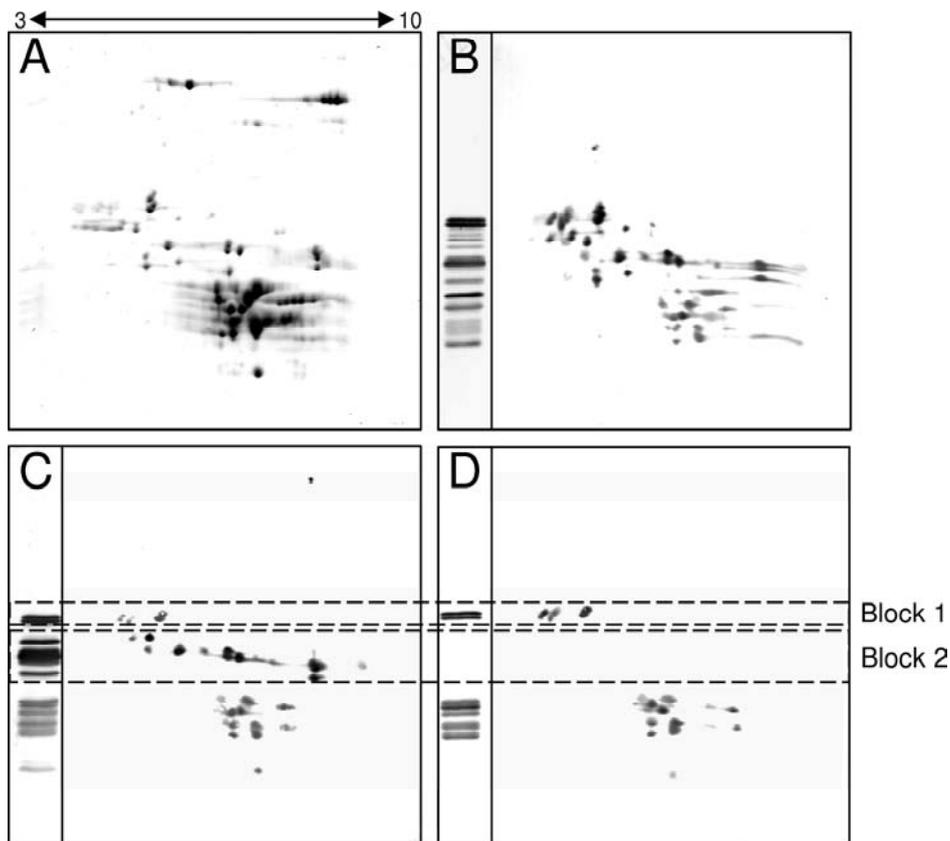


Figure 3. 2-DE analysis of the gluten protein extracts from modern wheat varieties *Bovictus* and *Sperber*. (A) 2-DE gel of '*Bovictus*' stained with PageBlue. (B) Immunoblot of '*Bovictus*' using mAb Glia- 9; (C) Immunoblot of '*Bovictus*' using mAb Glia- 20; (D) Immunoblot of '*Sperber*' using mAb Glia- 20. Lanes at the left in (B), (C), and (D) are one-dimensional immunoblots. Boxes indicate 'block-1' and 'block-2' proteins.

Immunoblot intensities

Immunoblots were scanned from Figs. 1A, B, C, and D and the pixel intensity of each lane was calculated. Our results indicate that α -gliadins/D-type LMW-GS may contain T-cell stimulatory epitopes Glia- 9 and Glia- 20. For this reason, these bands have been included in the pixel intensity measurements of the variety and landrace gel lanes to quantify the overall intensity as presented in Fig. 4. In addition, also signals caused by response of α -gliadins to the Glia- 9 mAb have been included in the intensity measurements. Although we did not find an intact epitope in our database analysis, the set of α -gliadin sequences present in the NCBI database might be too limited to exclude the possibility of the presence of the Glia- 9 T-cell epitope. We sorted the intensities for

both mAb stainings in three classes: low (+), medium (++), or high (+++) (Fig. 4). Clear differences in the immunoblot staining of GliA- 9 and GliA- 20 epitopes were found among and between varieties and landraces as groups. The calculation of relative intensities is used to measure the level of CD-epitopes in the different wheat samples. It is, at the moment, the most accurate way to compare the presence and levels of CD-epitopes in wheat, as it takes into account the fact that there are differences in relative expression levels and that some gluten proteins may contain multiple copies of an epitope. Furthermore, it does not rely on the daunting task of fully separating all gluten protein variants into individual spots on a 2-DE gel.

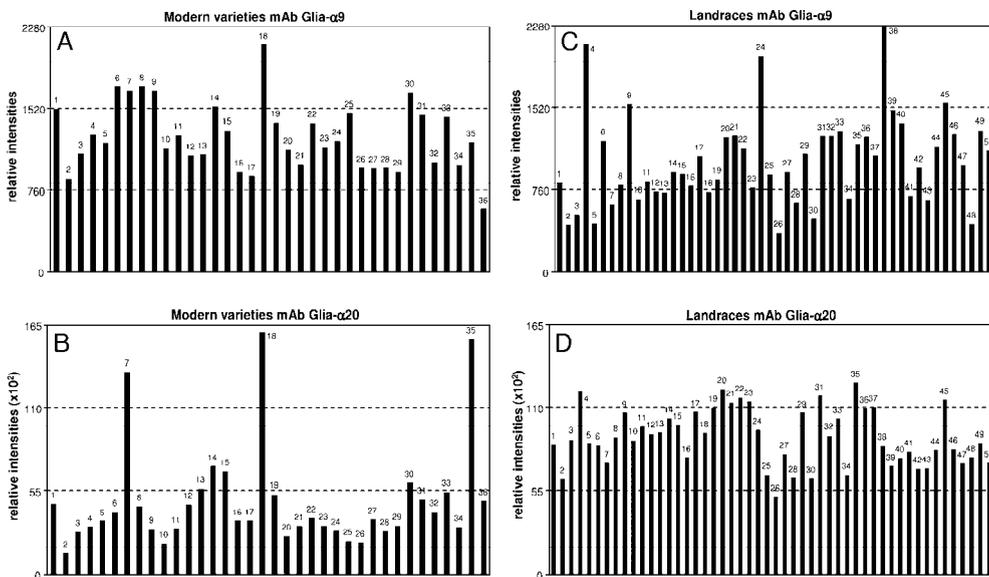


Figure 4. Immunoblots from Fig. 1 were scanned and the relative intensities are shown for mAbs GliA- 9 and GliA- 20. (A) GliA- 9 in modern wheat varieties; (B) GliA- 20 in modern wheat varieties; (C) GliA- 9 in wheat landraces; (D) GliA- 20 in wheat landraces.

From the modern varieties, Cadenza (Table 1, no. 36) showed the lowest response to both mAbs. Among the landraces, CGN08327 (Table 2, no. 26) showed the lowest response to both mAbs. Other landraces showing low response to the GliA- 9 mAb (but medium to the GliA- 20 mAb) are ‘Minaret’ (CGN19307, Table 2, no. 2, advanced cultivar), ‘Weissahr Rotkorn Binkel’ (CGN04210, Table 2, no. 3), ‘Rouge de la Gruyere’ (CGN08315, Table 2, no. 5), CGN12071 (Table 2, no. 30), and ‘Pyrothrix 28’ (CGN04236, Table 2, no. 48, advanced cultivar).

No systematic differences were observed between spring and winter wheat varieties based on the relative intensities for both mAbs. Among the landraces the accessions classified as subspecies *compactum* and *spelta* did not differ systematically from the others. In addition, landraces could not be grouped according to their country or region of origin. This may be partly due to because of the fact that the recorded country is the country of the first genebank collection, which often may not be the country where it originated from.

Discussion

Looking back over the last five decades, several trends are apparent in wheat consumption: an increase in wheat consumption per capita [148], (<http://www.ers.usda.gov/AmberWaves/september08/findings/wheatflour.htm>) an increase in CD-related T-cell stimulatory epitopes in wheat (as for the major epitope GliA- 9, this paper), an increase in the use of gluten in food processing [1, 2], and an increase in the consumption of processed foods. To some extent this can be attributed to an increase in awareness and improved diagnostic techniques. Given the relation between incidence of CD and exposure to cereals, it cannot be ruled out that an increased content of T-cell stimulatory epitopes has also contributed to this increased prevalence.

A diet based on wheat varieties reduced in T-cell stimulatory epitopes may help in the prevention of CD, as it has been observed that the amount and duration to gluten exposure are associated with the initiation of CD [60, 98, 150]. Wheat gluten proteins determine the elasticity and viscosity of the dough [12, 83] but they also showed to be highly useful in food processing. Gluten is widely applied in the production of soups, sauces, meat products, potato chips, candies, ice creams, and even in medicines, vitamin supplements, etc. Wheat varieties with very low amounts of T-cell stimulatory epitopes may be tolerated by many CD-patients [70, 172]. The reduction of the amount of major T-cell stimulatory epitopes in food will especially benefit children, in which the onset of CD may be delayed or even prevented, and in non-diagnosed CD-patients (the vast majority of all CD-patients) to strongly reduce their symptoms. This means that breeding for wheat with considerably reduced T-cell stimulatory epitopes is to be considered as a serious option.

This study explored differences in the presence of T-cell stimulatory gluten epitopes between modern wheat varieties and landraces, aiming at breeding strategies for the

reduction of T-cell stimulatory epitopes in gluten-containing food products. Immunoblotting was used to compare 36 modern wheat varieties and 50 landraces. Monoclonal Abs specific for the T-cell stimulatory epitopes GliA- 9 and GliA- 20 were used. We found that the diversity in banding patterns was lower in the modern varieties, clearly indicating a reduced genetic diversity. This implies that non-diagnosed CD-patients nowadays may encounter a less diverse set of gluten proteins than several decades ago. The set of landraces included accessions from all over the world and the modern varieties are all European varieties. This does not affect the comparison as the decrease in diversity of α -gliadins in varieties is the same when only landraces of European origin are considered.

So, can wheat breeding successfully be used to lower exposure to T-cell stimulatory epitopes? In the set of wheat varieties studied, we found a number of candidates that exhibit a low content of the epitopes measured. Among the 36 modern varieties, only one variety was identified with a low response against the GliA- 9 mAb (the immunodominant epitope), compared to 15 out of 50 landraces, whereas the frequencies of high responders to this antibody were almost the same. The opposite was found regarding the GliA- 20 mAb, which showed a significantly higher overall antibody response in the landraces. Considering the epitope impact on CD-patients of the major immunodominant GliA- 9 epitope, it is concluded from these data that in general the toxicity of modern wheat varieties has increased.

Landraces will require further breeding to increase their agronomical and food-technological value. On the other hand, with a worldwide occurrence of 0.5–2% of CD-patients and a high frequency (70–97%) of undiagnosed individuals, some agronomic drawbacks may be acceptable.

Monoclonal Ab staining is a less time-consuming and much easier method to screen a large number of varieties for the presence of T-cell stimulatory epitopes than dealing with T-cell clones, but requires specific mAbs. These are available for a subset of all epitopes that have been identified in wheat. As GliA- 9 is a major T-cell epitope from α -gliadins which is recognized by most patients [69, 156], we have used it here as a proxy for CD-toxicity of all α -gliadins. The α -gliadins also contain various T-cell epitopes that are recognized by groups of patients, although by fewer patients than the α -gliadins. Unfortunately, there are no specific mAbs against α -gliadin epitopes that recognize any of these T-cell stimulatory epitopes reliably. New methods are currently being developed, using high throughput sequencing of transcripts and proteomics of gluten proteins. Hopefully, it will become possible to qualitatively and quantitatively assess the presence

of all CD T-cell stimulatory epitopes in wheat varieties. To prove, however, that wheat varieties can be considered safe for consumption by CD-patients, we will need T-cell testing using T-cells from a large number of patients and, ultimately, a trial in which CD-patients will be challenged by hopefully non-CD-stimulatory wheat varieties.

Conclusion

It stands to reason that reduction of T-cell stimulatory epitopes in wheat may directly contribute to increasing the quality of life of many individuals. Further selection of varieties and landraces low in T-cell stimulatory α -gliadin epitopes and other major epitopes (e.g. from β -gliadins), can be considered a responsibility of wheat breeding companies together with research organizations and government. Starting from such selections of 'low in CD-epitopes' wheat, 'low in CD-epitopes gluten' may become an important new trait in wheat breeding. The inclusion in breeding programs of varieties from different origins will assure the maintenance of a broad genetic diversity. Such selection strategies should also include the use of tetraploid (durum) and diploid wheat species. Further application of advanced breeding technologies, including re-synthesizing of hexaploids and specific gene silencing, will additionally be helpful.

Acknowledgments

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Chapter 5

In search of tetraploid wheat accessions reduced in celiac disease-related gluten epitopes

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Abstract

Tetraploid wheat (durum wheat) is mainly used for the preparation of pasta. As a result, of breeding, thousands of tetraploid wheat varieties exist, but also tetraploid landraces that are still maintained and used for local food preparations. Gluten proteins present in wheat can induce celiac disease, a T-cell mediated auto-immune disorder, in genetically predisposed individuals after ingestion. Compared to hexaploid wheat, tetraploid wheat might be reduced in T-cell stimulatory epitopes that cause celiac disease because of the absence of the D-genome. We tested gluten protein extracts from 103 tetraploid wheat accessions (obtained from the Dutch CGN genebank and from the French INRA collection) including landraces, old, modern, and domesticated accessions of various tetraploid species and subspecies from many geographic origins. Those accessions were typed for their level of T-cell stimulatory epitopes by immunoblotting with monoclonal antibodies against the α -gliadin epitopes Gli-a-9 and Gli-a-20. In a first selection, we found 8 CGN and 6 INRA accessions with reduced epitope staining. Fourteen of the 57 CGN accessions turned out to be mixed with hexaploid wheat, and 5 out of the 8 selected CGN accessions were mixtures of two or more different gluten protein chemotypes. Based on single seed analysis, lines from two CGN accessions and one INRA accession were obtained with significantly reduced levels of Gli-a-9 and Gli-a-20 epitopes. These lines will be further tested for industrial quality and may contribute to the development of safer foods for celiac patients.

Introduction

Tetraploid wheat is a so-called hard wheat and is generally used for preparation of pasta, but in Mediterranean countries it is also used for preparation of various types of breads [173]. Tetraploid wheat has originated from a hybridization between *Triticum urartu* (an A-genome wheat species) and most likely *Aegilops speltoides* (a B-genome wheat species) resulting in a tetraploid species having the AB-genome [174-177]. Tetraploid wheat domestication occurred in Southeastern Turkey [5-7, 178, 179]. During thousands of years, cultivated tetraploid species spread throughout Europe and Asia because of human migration. Further cultivation and natural selection resulted in an enormous variation of tetraploid wheats, many of which have been collected in genebank collections as landraces. The features of the landraces depend on the local environmental conditions and on the local food traditions and food preparations. Many landraces are still maintained in some regions because the properties of their flour are considered important for traditional food applications. Landraces are adapted to local climatic conditions, cultural practices, and (partly) to disease and pests [179]. A landrace may be a mixture of genotypes, which evolved under the environmental conditions where they were grown because of natural selection and selection by the farmer. Tetraploid wheat can mix up with hexaploid bread wheat very easily under agricultural conditions and care should be taken if the tetraploid wheat should be maintained as a pure genotype [151]. As a result, many commercial lots, currently sold as durum wheat nearly always contain a part hexaploid bread wheat.

The typical food-technological characteristics of wheat are related to the presence of gluten proteins. These proteins are water-insoluble, making them especially useful in a wide and still rapidly increasing variety of food applications. Gluten proteins are comprised of gliadins and glutenins, which are present in approximately equal amounts and form 80% of the total storage protein content in the wheat kernel [180]. In tetraploid wheat, the genes encoding gliadins are located on the short arm of chromosomes 1A and 1B (*Gli-1* loci) and on the short arm of chromosomes 6A and 6B (*Gli-2* loci). The *Gli-1* loci represent genes that are tightly linked and mainly encode the α - and β -gliadins. The *Gli-2* loci represent tightly clustered genes encoding the γ / δ - and some ϵ -gliadins [10, 12].

Gluten proteins from wheat varieties used for preparation of pasta or bread (but also from rye and barley) can induce celiac disease (CD) in genetically susceptible individuals [40]. CD is characterized by an inflammation of the small intestine. The prevalence is about 1% in the Western (Caucasian) population. The vast majority (~95%) of this group is

unaware of having CD because of large-scale underdiagnosis and wrong diagnosis. The incidence of CD appears to be increasing [148, 149]. The only cure today is a strict life-long gluten-free diet. However, it is difficult to avoid wheat gluten, because of its rapidly increasing application in an increasing number of food products [1-4]. The starting point of our research is that any reduction in the total CD-stimulating gluten consumption will contribute to an overall reduction of the prevalence and symptom severity of CD.

Overall, tetraploid wheats contain less T-cell stimulatory α -gliadin epitopes than hexaploid bread wheat because of the absence of the D-genome, [74, 75]. The highly immunodominant T-cell stimulating 33-mer [47, 161] is exclusively present in α -gliadins encoded by the D-genome. In addition, the levels of T-cell stimulatory epitopes have been shown to vary among varieties [76, 77, 106, 181, 182]. This opens possibilities to select for wheat varieties with significantly reduced α -gliadin epitope levels, aiming at direct use or to apply in breeding programs directed towards large-scale reduction or even total elimination of CD-stimulating gluten-elements from wheat.

The aim of this study is to analyze, as a first step, the genetic diversity and heterogeneity regarding the presence of T-cell stimulatory epitopes of old and modern tetraploid wheat accessions collected from many geographic origins world-wide.

Materials and Methods

Grain samples

A set of 57 tetraploid wheats was obtained from the Centre for Genetic Resources (CGN), the Netherlands (<http://www.cgn.wur.nl/uk/>) (Table 1). The accessions were selected based on collection period and diversity of geographic origin. Some CGN accessions showed heterogeneous seed compositions. In this study, the taxonomy of *Triticum* of Morris & Sears (1967) [183] is followed. A second set of 46 tetraploid wheat accessions was obtained from INRA (Montpellier) that was selected on genetic diversity based on 10 microsatellites polymorphism and resulted in a core collection as described by David *et al.* (2003) [184] and Thuillet *et al.* (2005) [185] (Table 2). Accessions not present at INRA were provided by The United States Department of Agriculture (USDA) or the International Center for Agricultural Research in the Dry Areas (ICARDA). Seed compositions of INRA accessions were homogeneous. Both sets included *T. turgidum ssp dicoccoides* accessions from the Near East (wild ancestors), domesticated *T. turgidum ssp dicoccon* accessions (domesticated ancestors), domesticated *T. turgidum ssp polonicum* accessions, old *T.*

turgidum ssp *durum* varieties, and elite varieties from the French catalog. For comparison, three hexaploid bread wheat varieties (*T. aestivum* 'Bovictus', 'Toronto', and 'Minaret') have been included.

Accessions CGN06223, CGN08006, and CGN12037 were selected from a previous study on hexaploid wheat accessions [182]. Based on the banding pattern, these accessions seemed to be tetraploids instead of hexaploids. Their tetraploidy was confirmed in flowcytometric ploidy level determination of young leaf material.

Gluten protein extraction

Multiple (~10–30) wheat grains of 57 tetraploid landraces obtained from CGN, and single grains of selected landraces were ground in an analytical mill (A 11 Basic, IKA-Werke) and sieved through mesh (0.5 mm). From 46 tetraploid wheat accessions obtained from INRA (Montpellier) single seeds were used for gluten protein extraction. Gluten proteins were extracted according to Van den Broeck *et al.* (2009) [163], which describes extraction of most of the gluten proteins. Gluten proteins were extracted from 50 mg wheat flour or ground single seeds by addition of 0.5 ml of 50% aqueous iso-propanol with continuous mixing (MS1 Minishaker, IKA Works, Inc.) at 1,000 rpm for 30 min at room temperature, followed by centrifugation at 10,000 rpm for 10 min at room temperature. The residue was re-extracted twice with 50% aqueous iso-propanol/1% DTT/50 mM Tris-HCl, pH 7.5, for 30 min at 60°C with mixing every 5–10 min followed by centrifugation at 10,000 rpm for 10 min at room temperature. After addition of each next extraction solution, the residue was resuspended by shaking in a Fastprep® FP220A Instrument for 10 s at speed 6.5 m/s followed by sonification for 10 min in an ultrasonic bath (Branson 3510, Branson Ultrasonics Corporation). The three obtained supernatants were combined and considered the total gluten protein extract. The protein content was quantified using Bio-Rad Protein Assay (Bio-Rad Laboratories), based on the Bradford dye-binding procedure, according to manufacturer's instruction.

Chapter 5

Table 1. Details of tetraploid accessions obtained from CGN Wageningen, The Netherlands

	Acc. no.	Acc name	Species	Subspecies	Population type	Type	Collection date	Collection site
1	CGN06223	Lugaymi	<i>T. aestivum</i>	<i>aestivum</i>	L		1968	Saudi Arabia
2	CGN08006	Dibillik Sinde	<i>T. aestivum</i>	<i>aestivum</i>	L		1972	Ethiopia
3	CGN12037	Mate Sinde	<i>T. aestivum</i>	<i>aestivum</i>	L		1972	Ethiopia
4	CGN04221	Black Persian	<i>T. turgidum</i>	<i>carthlicum</i>	L	S	1962	Iran
5	CGN06550	Dniepopetrovk	<i>T. turgidum</i>	<i>durum</i>	B		1965	USSR
6	CGN06551	Gaza1	<i>T. turgidum</i>	<i>durum</i>		S		Egypt
7	CGN06552	Gaza 2; Gaya W 277	<i>T. turgidum</i>	<i>durum</i>		S	1937	Egypt
8	CGN06553	Gaza 8; Gaza W 277	<i>T. turgidum</i>	<i>durum</i>		S	1950	Egypt
9	CGN06555	Kaza kylcyk	<i>T. turgidum</i>	<i>durum</i>	L			Azerbaijan
10	CGN06567	Hansia Broach	<i>T. turgidum</i>	<i>durum</i>	L	S	1915	India
11	CGN06576	Beladi	<i>T. turgidum</i>	<i>durum</i>	L	S		Egypt
12	CGN06578	Beladi dakar	<i>T. turgidum</i>	<i>durum</i>			1948	Egypt
13	CGN06582	Gaurani	<i>T. turgidum</i>	<i>durum</i>	L	S	1949	India
14	CGN06590	Eretria	<i>T. turgidum</i>	<i>durum</i>	B	S	1955	Greece
15	CGN07975	Abessinischer Emmer	<i>T. turgidum</i>	<i>dicoccon</i>		S	1965	Ethiopia
16	CGN07989	Beladi	<i>T. turgidum</i>	<i>durum</i>	L	S	1939	Egypt
17	CGN07991	St. 472 Ethiopia	<i>T. turgidum</i>	<i>durum</i>	L	S	1950	Ethiopia
18	CGN08143	Hongrie 2883	<i>T. turgidum</i>	<i>durum</i>	B	S	1965	Portugal
19	CGN08145	Durazio Molar 918	<i>T. turgidum</i>	<i>durum</i>	B	S	1965	Portugal
20	CGN08146	Candial	<i>T. turgidum</i>	<i>durum</i>	B	S	1949	Portugal
21	CGN08154	Akmolinka 5	<i>T. turgidum</i>	<i>durum</i>	B	S	1959	USSR
22	CGN08173	Gharnovka	<i>T. turgidum</i>	<i>durum</i>			1900	USSR
23	CGN08190	Deves; Kamboyra	<i>T. turgidum</i>	<i>durum</i>	L	S	1916	Greece
24	CGN08202	Gandumi Andojird	<i>T. turgidum</i>	<i>durum</i>			1940	Iran
25	CGN08232	Psathas	<i>T. turgidum</i>	<i>durum</i>	L	S	1957	Cyprus
26	CGN08235	Hebda 3	<i>T. turgidum</i>	<i>durum</i>	B	S	1960	Algeria
27	CGN08239	Etrusco	<i>T. turgidum</i>	<i>durum</i>		S	1961	Italy
28	CGN08240	Capelti	<i>T. turgidum</i>	<i>durum</i>	B	S	1961	Italy
29	CGN08245	Mokhtar II	<i>T. turgidum</i>	<i>durum</i>	L	S		Egypt
30	CGN08262	Probstdorfer Pandur; Pandur	<i>T. turgidum</i>	<i>durum</i>	B	S	1972	Austria
31	CGN08339	Weisser Sommer	<i>T. turgidum</i>	<i>dicoccon</i>	L	S	<1945	Germany
32	CGN08341	Yaroslav Spring Emmer	<i>T. turgidum</i>	<i>dicoccon</i>	L	S		USSR
33	CGN08357	Diha	<i>T. turgidum</i>	<i>carthlicum</i>	L	S	1964	USSR
34	CGN08360	Diha Dzhavakhetskaja	<i>T. turgidum</i>	<i>carthlicum</i>	L	S	1959	USSR
35	CGN08361		<i>T. turgidum</i>	<i>carthlicum</i>	L	S	1958	USSR
36	CGN08367		<i>T. turgidum</i>	<i>turgidum</i>	L?	W		
37	CGN08379	Asa de Corvo 5279	<i>T. turgidum</i>	<i>turgidum</i>	L?	S		Portugal
38	CGN08390	LHTAXO 549/65	<i>T. turgidum</i>	<i>polonicum</i>	L?	S	1965	
39	CGN08396	Acme	<i>T. turgidum</i>	<i>durum</i>	B	S	1963	USA
40	CGN08397	Arnoutka	<i>T. turgidum</i>	<i>durum</i>	B	S	1954	USA
41	CGN08398	Golden Ball	<i>T. turgidum</i>	<i>durum</i>	B	S	1961	South Africa
42	CGN08407		<i>T. timopheevi</i>	<i>timopheevi</i>		S		USSR
43	CGN10468	Vernal	<i>T. turgidum</i>	<i>dicoccon</i>	B	S		USA
44	CGN10495		<i>T. timopheevi</i>	<i>timopheevi</i>	L?	S	1964	USSR
45	CGN11494	Brkulja	<i>T. turgidum</i>	<i>durum</i>	L	W	1949	Yugoslavia
46	CGN12275	Vernal Emmer	<i>T. turgidum</i>	<i>dicoccon</i>	L	S	1962	USA
47	CGN12282		<i>T. turgidum</i>	<i>dicoccon</i>			1952	Iran
48	CGN12283	Polba 3	<i>T. turgidum</i>	<i>dicoccon</i>	B	S		USSR
49	CGN12287	Tripshiro	<i>T. turgidum</i>	<i>durum</i>		S	1921	Libya
50	CGN12293	PI 294478	<i>T. turgidum</i>	<i>polonicum</i>	L?	S	1964	France
51	CGN12296	Biskri x Bruteille	<i>T. turgidum</i>	<i>durum</i>	B	S	1965	Morocco
52	CGN14995	Sari Bugday	<i>T. turgidum</i>	<i>durum</i>	B	W		Turkey
53	CGN16051		<i>T. turgidum</i>	<i>dicoccon</i>		S	1964	France
54	CGN16057	Gaza 6; Gaza 277	<i>T. turgidum</i>	<i>durum</i>		S	1941	Egypt
55	CGN16068	Saba 22738	<i>T. turgidum</i>	<i>durum</i>	B	S	1960	Algeria
56	CGN16069	Durum H-2	<i>T. turgidum</i>	<i>durum</i>		S	1939	Egypt
57	CGN16074	WTC 1229/65	<i>T. timopheevi</i>	<i>timopheevi</i>	L?	S	1964	USSR

Passport details (CGN, Wageningen, The Netherlands): B is breeder line; L is landrace/traditional cultivar; W is winter wheat; S is spring wheat; Empty cell means unknown information.

Table 2. Details of tetraploid wheat accessions obtained from INRA-Montpellier, France.

	Acc. no.	Subspecies	Collection site	Type		Acc. no.	Subspecies	Collection site	Type
1	45383	<i>dicoccon</i>	Bulgaria	D	24	84866	<i>durum</i>	Syria	O
2	45351	<i>dicoccon</i>	Iran	D	25	95920	<i>durum</i>	Syria	O
3	45239	<i>dicoccon</i>	Italy	D	26	82697	<i>durum</i>	Turkey	O
4	45354	<i>dicoccon</i>	Russia	D	27	82726	<i>durum</i>	Turkey	O
5	45280	<i>dicoccon</i>	Slovakia	D	28	82715	<i>durum</i>	Turkey	O
6	45309	<i>dicoccon</i>	Slovakia	D	29	Brumaire	<i>durum</i>	France	O
7	352365	<i>dicoccon</i>	Germany	D	30	Primadur	<i>durum</i>	France	E
8	355484	<i>dicoccon</i>	Spain	D	31	Neodur	<i>durum</i>	France	E
9	94635	<i>dicoccon</i>	Iran	D	32	IXOs9442	<i>durum</i>	France	E
10	415152	<i>dicoccon</i>	Israel	D	33	Villemur	<i>durum</i>	France	E
11	94648	<i>dicoccon</i>	Italy	D	34	Armet910	<i>durum</i>	France	E
12	113302	<i>dicoccoides</i>	Iran	W	35	Durental	<i>durum</i>	France	E
13	45963	<i>dicoccoides</i>	Jordan	W	36	Lloyd945	<i>durum</i>	France	E
14	46391	<i>dicoccoides</i>	Jordan	W	37	B6Rtchir	<i>durum</i>	Bulgaria	O
15	117887	<i>dicoccoides</i>	Syria	W	38	LRB6R595	<i>durum</i>	Bulgaria	O
16	46470	<i>dicoccoides</i>	Syria	W	39	68405	<i>polonicum</i>	–	D
17	46253	<i>dicoccoides</i>	Turkey	W	40	585015	<i>polonicum</i>	China	D
18	116172	<i>dicoccoides</i>	Turkey	W	41	384266	<i>polonicum</i>	Ethiopia	D
19	467014	<i>dicoccoides</i>	Israel	W	42	14140	<i>polonicum</i>	–	D
20	428133	<i>dicoccoides</i>	Lebanon	W	43	81285	<i>turgidum</i>	–	D
21	46516	<i>dicoccoides</i>	Syria	W	44	7786	<i>turgidum</i>	Ethiopia	D
22	487255	<i>dicoccoides</i>	Syria	W	45	387456	<i>turgidum</i>	Ethiopia	D
23	Kubanka	<i>durum</i>	USSR	O	46	134952	<i>turgidum</i>	Portugal	D

D is domesticated; *W* is wild; *O* is old; *E* is elite.

SDS-PAGE and immunoblotting

Equal amounts of gluten proteins were separated on SDS-PAGE gels (10%) [134] using a Hoefer SE 260 mighty small II system (GE Healthcare) followed by staining with PageBlue™ (Fermentas). Proteins were blotted onto nitrocellulose (0.2 µm, Bio-Rad Laboratories), omitting methanol from the blotting buffer, using a Mini Trans-Blot Cell (Bio-Rad Laboratories) at 100 V for 1 h. Blots were stained using a MemCode™ Reversible Protein Stain Kit for Nitrocellulose Membranes (Fisher Scientific) prior to incubation with mAbs. Blots were incubated as described [136] with monoclonal antibodies (mAbs) specific for T-cell stimulatory epitopes Gli- 9 [126, 132] and Gli- 20 [132, 162]. Antibody binding to the blots was visualized by staining for alkaline phosphatase conjugated secondary antibody, using Nitro Blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma). Blots were scanned using a Bio-Rad GS-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories) and saved as TIFF images. Pixel intensities were calculated per lane using Quantity One software (Bio-Rad Laboratories). The gluten protein extracts of either the modern hexaploid wheat varieties Bovictus or Toronto

(having high amounts of T-cell stimulatory epitopes, as analyzed before) and/or hexaploid wheat variety Minaret (with relatively low amounts of T-cell stimulatory epitopes) were used on each separate immunoblot as an 'inter-gel' control [182].

Results

The gluten protein extracts from all accessions were analyzed by immunoblotting using mAbs against the T-cell stimulatory epitopes GliA- 9 and GliA- 20 to identify the gluten proteins containing epitope sequences (Fig. 1). In 14 out of 57 CGN accessions, an additional set of bands at ~55–60 kDa was observed reacting with both mAbs GliA- 9 and GliA- 20 (Fig. 1, proteins indicated by arrows). These proteins are also present in the hexaploid wheat variety Bovictus (Fig. 1, lane B). It was shown before [169] that the genes encoding these two proteins, being γ -gliadin/D-type LMW-GS, are located on the short arm of chromosome 1D in 'Chinese Spring' deletion lines. These proteins are also detected in diploid *T. tauschii*, containing the D-genome, and in the immunoblot analysis of CIMMYT synthetic hexaploid wheat lines, but are not stained in their tetraploid parent lines (results not shown). Therefore, the accessions showing the two chromosome 1D encoded proteins are, at least, admixed with hexaploid wheats. Single seeds from accession CGN08006 were used in cDNA sequencing as described by Salentijn *et al.* (2009) [75] and confirmed its general tetraploidy by the absence of the D-genome sequences. Such admixtures were detected in wheat accessions originating from Ethiopia, Germany, USSR, Iran, France, and the USA (Fig. 1, lanes 18, 20, 23, 26, 28, 34, 35, 36, 45, 46, 51, 52, 55, and 56, Table 1). Two different seeds present in landrace accession CGN8360 (Fig. 1, Lane 34) were confirmed in flowcytometric ploidy level determination to be hexaploid.

Five accessions originating from Egypt shared the same gluten protein pattern for mAb GliA- 20 but differed in one protein band for mAb GliA- 9 (Fig. 1, lanes 6, 7, 8, 11, and 12). These accessions probably share the same progenitors, or they may be independently propagated accessions in which small differences have been evolved e.g. because of mixtures with (related) genotypes. Accessions CGN16069 from Algeria and CGN16069 from Egypt seemed very similar to each other, as they show the same patterns on immunoblots for both mAbs and are both mixed with hexaploid wheat (Fig. 1, lanes 55 and 56). For all other accessions, the gluten protein patterns on immunoblots were quite different, even if accessions originate from the same country.

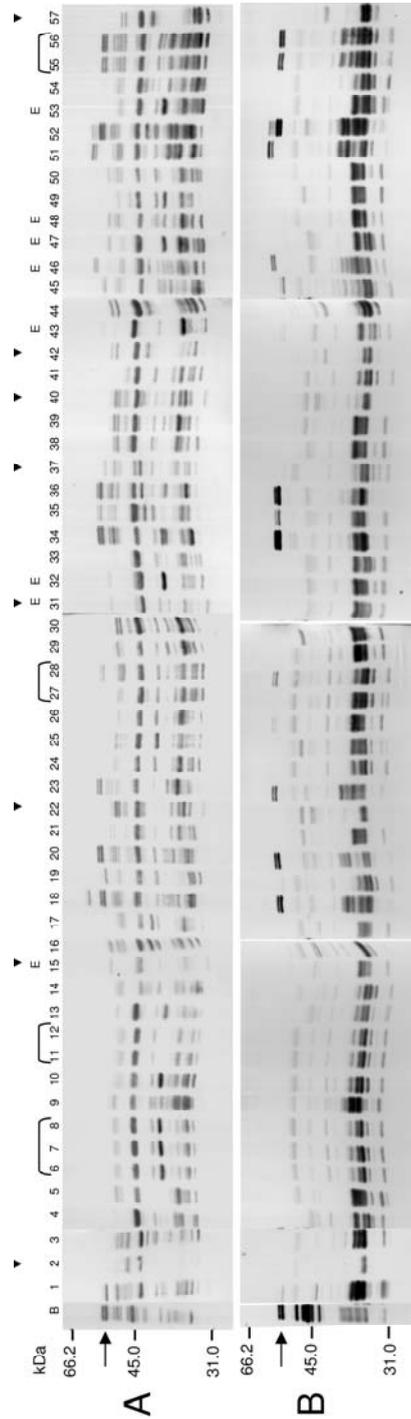


Figure 1. Immunoblot analysis of gluten protein extracts from 57 tetraploid wheat accessions compared to the gluten protein extract of modern hexaploid variety Bovictus. (A) mAb GliA- 9; (B) mAb GliA- 20. Brackets group accessions with similar protein patterns. E is emmer wheat. ▼ Indicates accessions selected for further analysis

To evaluate the presence and the level of T-cell stimulatory epitopes in tetraploid wheat accessions, eight accessions were selected that showed to contain a lower amount of T-cell stimulatory gluten proteins in the overall (seed mixture) protein pattern (Fig 1, indicated with ▼). The seeds of accessions CGN08006, CGN07975, CGN08173, CGN08339, CGN08379, CGN08397, CGN08407, and CGN16074 were subsequently examined for the presence of different seed phenotypes. The phenotypes of the seeds of accession CGN08006 were diverse in seed color and shape and when the gluten proteins from nine phenotypically different seeds (A to I) were analyzed by immunoblotting, six different gluten protein chemotypes could be detected (Fig. 2). Accession CGN08006 is a landrace collected from Ethiopia which was originally classified as a hexaploid wheat. Another ten landraces from Ethiopia have been analyzed by immunoblotting and six of these were mixed with hexaploid wheat. In contrast, seeds from accessions CGN07975, CGN08339, and CGN08407 showed only one phenotype. Seeds from accessions CGN08173, CGN08379, and CGN16074 showed two different phenotypes, but immunoblot protein patterns were the same using both mAbs. Seeds from accession CGN08397 showed four different phenotypes and two different immunoblot protein patterns were detected.

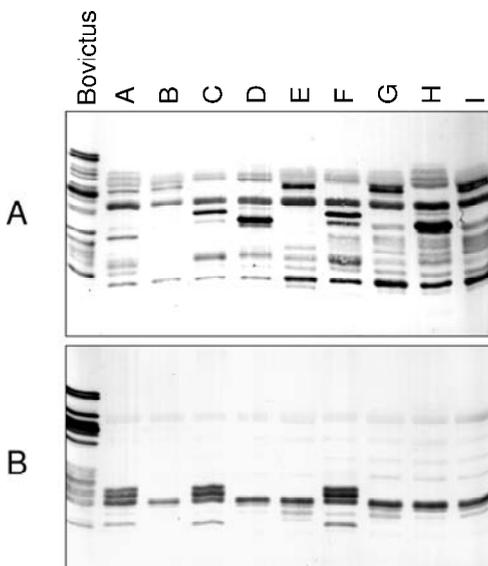


Figure 2. Immunoblot analysis of gluten protein extracts of nine single seeds (A to I) from accession CGN08006. (A) mAb Glia-9; (B) mAb Glia-20.

To compare the levels of T-cell stimulatory epitopes present in tetraploid accessions to hexaploid wheat varieties, relative intensities were calculated from the obtained immunoblots of the eight tetraploid CGN accessions (Fig. 3). The calculation of relative intensities is our method to measure the amount of epitopes in the different wheat samples and is the method we use to compare the presence and levels of epitopes in wheat, as it takes into account the fact that there are differences in relative expression levels of proteins and that some may contain multiple copies of an epitope.

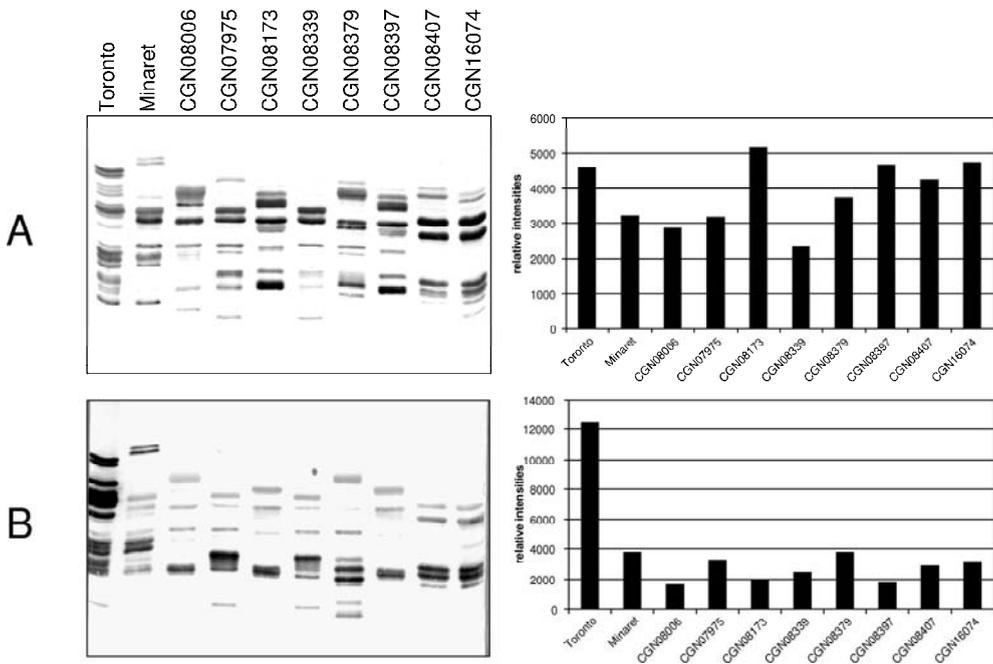


Figure 3. Immunoblot analysis and calculated intensities of single seed gluten protein extracts from accessions CGN08006, CGN07975, CGN08173, CGN08339, CGN08379, CGN08397, CGN08407, and CGN16074 compared to hexaploid varieties Toronto and Minaret. (A) mAb Glia- 9; (B) mAb Glia- 20.

Intensities of single seed gluten protein extracts were compared to gluten protein extracts of the modern bread wheat variety Toronto and old bread wheat variety Minaret. Results presented in Fig. 3 showed that when using the mAb against Glia- 9, five of the eight accessions contained lower amounts of T-cell stimulatory epitopes than the hexaploid variety Toronto. Two of these five accessions, CGN08006 and CGN08339, also contained a lower amount of T-cell stimulatory epitopes than the relatively low hexaploid variety Minaret. When using the mAb against Glia- 20, all eight accessions showed more

than three-fold reduced amounts of T-cell stimulatory epitopes than hexaploid variety Toronto. Seven accessions contained lower amounts of T-cell stimulatory epitopes than hexaploid variety Minaret. The lowest value was obtained for accession CGN08006, which was about two fold lower than variety Minaret.

Using the same methods as for the 57 CGN accessions, the 46 tetraploid wheats from INRA (Montpellier) were subsequently analyzed by immunoblotting using mAbs against the same T-cell stimulatory epitopes Glia- 9 and Glia- 20 to identify the number of proteins present containing these epitope sequences. Lines 45963, 117887, 46516, 84866, 95920, and Neodur contained the lowest amount of T-cell stimulatory epitopes and were therefore compared to CGN08006 and CGN08339 and compared to hexaploid variety Minaret (Fig 4). Although the gluten protein patterns on the immunoblot are different, these lines overall do not contain less T-cell stimulatory epitopes than the accessions selected from CGN. A calculation of the relative intensities on the immunoblots (Fig. 4) showed that accessions 84866 and Neodur are comparable to accessions CGN08006 and CGN08339 regarding Glia- 9 epitopes. Analysis of the Glia- 20 epitopes showed that Neodur together with CGN08006 are the lowest responders.

Discussion

In this study we analyzed the genetic diversity and heterogeneity regarding the presence of T-cell stimulatory epitopes of old and modern tetraploid wheat accessions collected from many geographic origins world-wide. The most striking result is that even true tetraploid wheat accessions have a level of CD-epitopes that is not orders of magnitude lower than observed for hexaploid wheat. This may seem surprising, as the D-genome contains many ω -gliadins with all epitopes present [74, 75]. On the other hand, the antibodies we used in this study (mAbs against Glia- 9 and Glia- 20) detect epitopes that are also present in the A-genome ω -gliadins, which may form a majority of the gliadins being expressed in tetraploid wheat varieties [75]. There are no antibodies that specifically detect D-genome ω -gliadin specific epitopes, such as the 33-mer (Salentijn, personal communication). As a result, the actual differences may be larger than what we detect in this study using immunoblotting with mAbs against T-cell stimulatory epitopes Glia- 9 and Glia- 20.

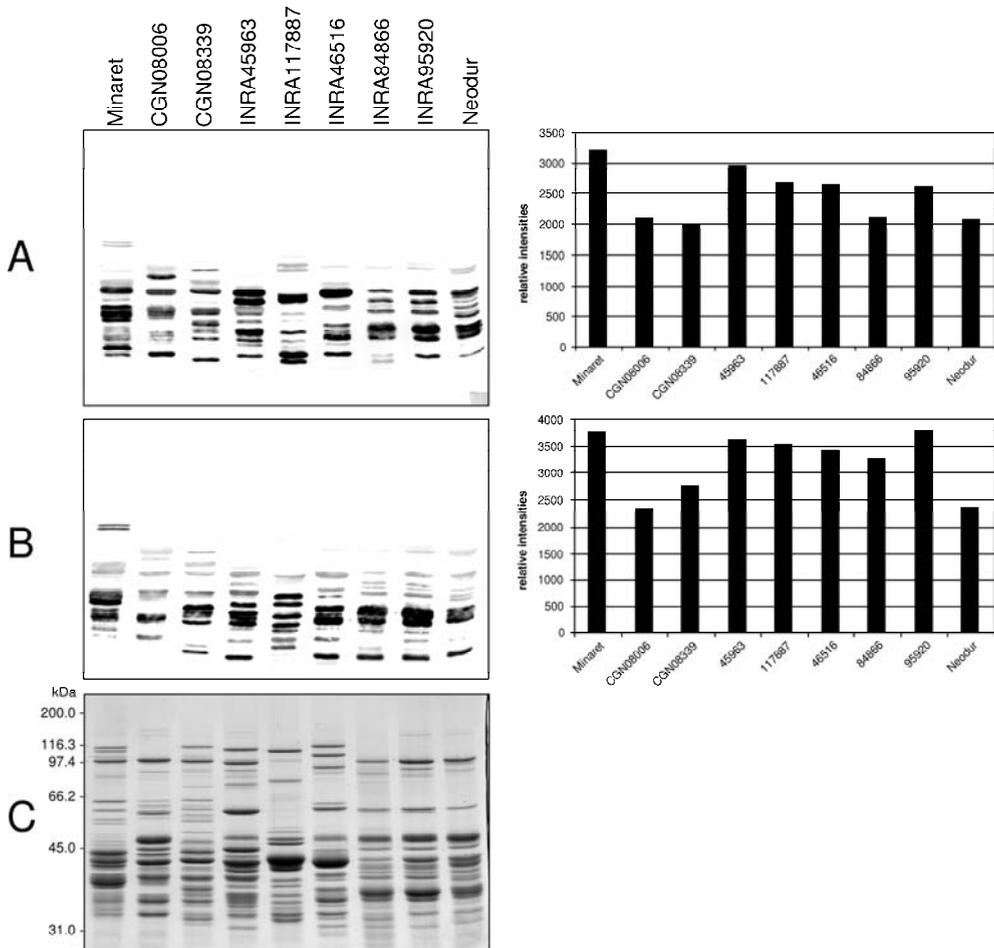


Figure 4. Immunoblot analysis and calculated intensities of single seed gluten protein extracts from accessions Minaret, CGN08006, CGN08339, INRA45963, INRA117887, INRA46516, INRA84866, INRA95920, and Neodur compared to hexaploid variety Minaret. (A) mAb GliA- 9; (B) mAb GliA- 20; (C) SDS-PAGE and PageBlue staining.

Differences among wheat varieties in gluten proteins occur because of allelic variation (genotype) that determines the gluten protein composition. The approach we used in this study, analyzes this genotypic variation by comparing the same amount of gluten protein per accession. Changes in gluten protein composition have been described, but are mainly expected if growth conditions are extreme (high or low temperature, dry or wet conditions) [186]. The varieties and accessions we have analyzed were grown under normal wheat growth conditions and therefore, influence on the gluten protein composition is not expected.

Accessions showing different gluten protein patterns with low CD-epitope content can be selected for making crosses to obtain varieties with different combinations of gluten proteins and reduced numbers of T-cell stimulatory epitopes. To improve dough and baking quality, tetraploid wheats low in T-cell stimulatory epitopes may be highly useful for the construction of synthetic hexaploid bread wheats by hybridization with D-genome species low in T-cell stimulatory epitopes. Baking quality and dough properties of tetraploid wheat can be improved by introduction of or substitution with chromosome 1D [173, 187-191] or by breeding. In addition, baking quality of tetraploid wheat can also be improved by sexual transfer of the HMW-GS from the D-genome (the *Glu-D1* locus) to chromosome 1A without adding the gliadins [192, 193] or by genetic transformation using both the 1Dx5 and 1Dy10 HMW-GS genes [194].

The occurrence of different genotypes and even different ploidy levels in a single genebank accession is a complicating phenomenon for genebank managers to accurately characterize landraces. Many landraces often result from maintenance and selection practices by local farmers directed towards optimizations to local agronomic and food applications. As a consequence, genebank passport data turned out to be poor predictors of the real genetic composition of landrace accessions that may be mixtures of genotypes of tetraploid and even hexaploid wheat species. Salentijn *et al.* (2009) [75] analyzed cDNA sequences of α -gliadins and observed that some accessions described as tetraploid contained *Gli-D2* transcripts and in one accession described as hexaploid no *Gli-D2* transcript could be found.

It is to be expected that the reduced levels of CD-epitopes as found among some of the accessions, will not directly result in safe foods to (all) CD-patients. However, we believe that an overall lowering of the 'CD-epitope load' of gluten that is daily consumed by many millions of people world-wide may contribute to delay or even prevent the onset of CD and its symptom development in that part of the population that is genetically susceptible, especially in children [182, 195]. The quantity of consumed CD-epitopes is a major factor that may influence the clinical representation of CD, along with some other recognized factors such as the type of cow's milk formulas, omission of breast feeding, and age at gluten introduction [196-199]. A reduction in overall 'CD-epitope load' might especially help to release the constraint of the still undiagnosed CD-patients (approximately 95% of all CD-patients) that are daily consuming CD-stimulating gluten proteins without realizing its effect on their health and well being. With the present results we show a first and relevant contribution into this direction.

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Chapter 6

Removing celiac disease-related gluten proteins from bread wheat while retaining technological properties: A study with Chinese Spring deletion lines

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Abstract

Gluten proteins can induce celiac disease (CD) in genetically susceptible individuals. In CD-patients gluten-derived peptides are presented to the immune system, which leads to a CD4⁺ T-cell mediated immune response and inflammation of the small intestine. However, not all gluten proteins contain T-cell stimulatory epitopes. Gluten proteins are encoded by multigene loci present on chromosomes 1 and 6 of the three different genomes of hexaploid bread wheat (*Triticum aestivum*) (AABBDD).

The effects of deleting individual gluten loci on both the level of T-cell stimulatory epitopes in the gluten proteome and the technological properties of the flour were analyzed using a set of deletion lines of *Triticum aestivum* cv. Chinese Spring. The reduction of T-cell stimulatory epitopes was analyzed using monoclonal antibodies that recognize T-cell epitopes present in gluten proteins. The deletion lines were technologically tested with respect to dough mixing properties and dough rheology. The results show that removing the α -gliadin locus from the short arm of chromosome 6 of the D-genome (6DS) resulted in a significant decrease in the presence of T-cell stimulatory epitopes but also in a significant loss of technological properties. However, removing the α -gliadin, β -gliadin, and LMW-GS loci from the short arm of chromosome 1 of the D-genome (1DS) removed T-cell stimulatory epitopes from the proteome while maintaining technological properties.

The consequences of these data are discussed with regard to reducing the load of T-cell stimulatory epitopes in wheat, and to contributing to the design of CD-safe wheat varieties.

Introduction

Celiac disease (CD) is a disorder that is characterized by a permanent intolerance to gluten proteins from wheat, rye, and barley. Over 0.5% of the Western population suffers from CD, which presents itself by chronic diarrhea, fatigue, osteoporosis, lymphoma, and several other clinical symptoms after prolonged gluten consumption. Until now, the only treatment is a complete and life long elimination of gluten from the daily diet [200]. In the small intestine, several native gluten peptides can bind directly to specific human leukocyte antigen (HLA)-DQ2 or DQ8 receptors on antigen presenting cells (APCs). However, after deamidation by tissue transglutaminase (tTG), the affinity of the peptides for these HLA-receptors is strongly increased. The gluten peptides can be presented by APCs to gluten-sensitive T-cell lymphocytes leading to the release of cytokines, which will cause inflammation reactions and result in damaged intestinal villi [58].

Gluten are major storage proteins and have many interesting characteristics for food industrial applications, e.g. in baking bread. Gluten proteins can be divided into three main groups: high molecular weight glutenin subunits (HMW-GS), low molecular weight glutenin subunits (LMW-GS), and gliadins. The HMW-GS are divided in x-type and y-type subunits [201]. The LMW-GS are divided into B-, C-, and D-type subunits [18]. Gliadins are divided into α -, β -, and γ -gliadins [16]. Multiple T-cell activating gluten peptides were mainly found in α -gliadins, but also in β -gliadins and both LMW-GS and HMW-GS [40, 58, 67, 200]. Especially peptides derived from α -gliadins are recognized by T-cells from most CD-patients, while T-cell responses to β -gliadins and glutenins are less frequently found [58, 67, 68, 202, 203].

Wheat varieties with very low amounts of T-cell stimulatory epitopes may be tolerated by many CD-patients [70, 203], while a diet based on wheat varieties reduced in T-cell stimulatory epitopes may help in the prevention of CD, as it has been observed that the amount and duration to gluten exposure is associated with the initiation of CD [60, 98, 150]. Breeding for bread wheat (*Triticum aestivum*) with less T-cell stimulatory gluten may result, however, in varieties with unwanted loss of technological properties, because glutenins and gliadins together contribute largely to dough quality. A correct mixture of both glutenins and gliadins is essential to obtain optimal viscoelastic dough [204], and the quantity and the size distribution of the gluten proteins are important factors for polymerization [205, 206].

Gluten-encoding genes are located on the three homoeologous genomes of bread wheat: A, B, and D. A few (for HMW-GS) to a hundred (for α -gliadins) gene copies are

present in hexaploid wheat. Sequences of individual gene copies within the same gluten family, such as the α -gliadins, are very similar and may contain multiple and different T-cell stimulatory epitopes [74]. Gluten proteins are encoded by 15 major loci. The HMW-GS are encoded by loci on the long arm of group 1 chromosomes (*Glu-A1*, *-B1*, and *-D1*) [207]. The LMW-GS are mainly encoded by the *Glu-3* loci on the short arms of group 1 chromosomes (*Glu-A3*, *-B3*, and *-D3*) [208] and are tightly linked to the loci encoding the α -gliadins and β -gliadins (*Gli-A1*, *-B1*, and *-D1* and *Gli-A3*, *-B3*, and *-D3*). Most γ -gliadins are encoded by loci on the short arms of group 6 chromosomes (*Gli-A2*, *B2*, and *D2*) [209].

In this study, deletion lines of *Triticum aestivum* cv. Chinese Spring (CS) were selected [210-212]. These deletion lines are generally lacking one locus containing gluten genes from one of the three homoeologous chromosomes. Here, we explore the feasibility to reduce T-cell stimulatory epitopes in hexaploid bread wheat by screening with epitope-specific monoclonal antibodies [77, 126, 132], while maintaining the technological properties. By crossing of existing deletion lines, new deletion lines can be created that have multiple deletions and show higher reduction of T-cell stimulatory epitopes.

Materials and Methods

Wheat materials

From the Wheat Genetic & Genomic Resources Center (WGGRC) Kansas State University, USA (http://www.k-state.edu/wgrc/Germplasm/Deletions/del_index.html), 26 *T. aestivum* Chinese Spring deletion lines were selected as described [210-212]. The deletion lines had partial deletions of the long and short arms of chromosomes 1 and 6, which was characterized by cytogenetics (Figs. 2C and 3B). One line contained deletions of both the short arm of chromosome 1 and chromosome 6 (1BS-19/6DS-4, Fig. 2C). All deletion lines were grown in containment glasshouses. No morphological differences were observed. Seeds were harvested from mature wheat plants.

Database search for the specificity of the sequences recognized by mAbs compared to T-cell epitopes

The frequency of occurrence of known T-cell epitopes involved in the onset of CD was analyzed by searching within the National Center for Biotechnology Information (NCBI) database. From the NCBI protein database (<http://www.ncbi.nlm.nih.gov/>) five different groups of gluten protein sequences were extracted and subsequently converted into

FASTA formats, using the following search queries: 'alpha gliadin', 'gamma gliadin', 'omega gliadin' 'D-type LMW-GS', 'LMW glutenin', and 'HMW glutenin'. All non-*Triticum*, non-*Aegilops* entries, and sequences containing less than 100 amino acids were removed. For the 'HMW glutenin' group only full size sequences were analyzed. The obtained protein sequences were aligned using ClustalW to validate if the correct groups were assigned to the sequences. Within the 'gamma gliadin' group, four sequences (AAA34286, P04729, P04730, and AAA34285) were more similar to LMW glutenins and were transferred to the 'LMW glutenin' group. In the 'omega gliadin/D-type LMW-GS' group, one sequence (ABI20696) was specific for the 'alpha gliadin' group and was transferred to the 'alpha gliadin' group. The sequences in the five established groups were analyzed for the different minimal recognition sequences of mAbs and T-cells [162]. No mismatches were allowed. Scores were expressed as the number of sequences of the sequences in the established group that contained one or more recognition sequences. The T-cell minimal recognition sequences used in the analyses were: Glia- 9 (PFPQPQLPY), Glia- 20 (FRPQQPYPQ), LMW-glt (PFSQQQSPF), HMW-glt (QGYPTSPQ) and mAb minimal recognition sequences used were: Glia- 9 (QFPQPQ), Glia- 20 (RPQQPYP), LMW-1 (PPFSQQ), LMW-2 (QSPF), HMW-glt (QGQQGYYP) [77, 126, 132, 162].

Extraction of gluten proteins

Gluten proteins were extracted from wheat grains according to Van den Broeck *et al.* (2009) [163]. Grains were ground in an analytical mill (A 11 Basic, IKA-Werke) and sieved through mesh (0.5 mm). Gluten proteins were extracted from 50 mg wheat flour by addition of 0.5 ml of 50% (v/v) aqueous iso-propanol with continuous mixing (MS1 Minishaker, IKA Works, Inc.) at 1,000 rpm for 30 min at room temperature, followed by centrifugation at 10,000 rpm for 10 min at room temperature. The residue was re-extracted twice with 50% (v/v) aqueous iso-propanol, 50 mM Tris-HCl, pH 7.5 containing 1% (w/v) DTT, for 30 min at 60°C with mixing every 5 to 10 min followed by centrifugation at 10,000 rpm for 10 min at room temperature. After addition of each next extraction solution, the residue was resuspended by shaking in a Fastprep[®] FP220A Instrument for 10 s at 6.5 m/s followed by sonication for 10 min in an ultrasonic bath (Branson 3510, Branson Ultrasonics Corporation). The three obtained supernatants were combined and considered the gluten protein extract. The protein content was quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories), based on the Bradford dye-binding procedure, according to manufacturer's instruction with BSA as a standard.

SDS-PAGE

Gluten proteins were separated on SDS-PAGE gels (10%) using a SE 260 mighty small II system (GE Healthcare, UK). SDS-PAGE was followed by immunoblotting or by silver staining [135] with some modifications. Gels were fixed in 50% (v/v) ethanol/10% (v/v) acetic acid in water for 30 min. Then, gels were washed in 5% (v/v) ethanol/1% (v/v) acetic acid in water for 10 min, followed by three times washing for 5 min in MilliQ water. Gels were sensitized in 0.02% (w/v) sodium thiosulfate for 1 min and again washed three times for 30 sec in MilliQ water. Gels were incubated in 0.1% (w/v) silver nitrate for at least 20 min. After this incubation, gels were rinsed 2 times for 5 sec in MilliQ water and developed in 6% (w/v) sodium carbonate containing 0.05% (v/v) formaldehyde (37%)/0.4% (w/v) sodium thiosulfate. Development of staining was stopped by addition of 5% HAc/water.

Immunoblotting

Proteins were blotted onto nitrocellulose (0.2 μ m, Bio-Rad Laboratories), in buffer omitting methanol, using a Mini Trans-Blot Cell (Bio-Rad Laboratories) at 100 V for 1 h. Blots were incubated and visualized as described [136] using mAbs specific for T-cell stimulatory epitopes against GliA- 9 [126, 132], GliA- 20 [132, 162], GLT-156 (LMW-1 and LMW-2) [77, 132], HMW-glt [126, 132]. Monoclonal Ab binding was visualized by staining for alkaline phosphatase, using Nitro Blue tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma).

Quadrumat milling

To obtain white wheat flour, wheat kernels (total weight ranging 7.6–36 g) were milled using a Quadrumat JR (Brabender, Germany). Kernel moisture was adjusted to 16.5%. Bran was separated from endosperm flour by sieving through mesh (150 μ m). After sieving the average yield was 50% (w/w), noting that samples 6AS-1 and 6DS-2 had a typically higher flour yield of 64% and 60%, the other samples ranged from 43% to 51%.

Total protein content in flour

Flour protein content was estimated by the Dumas method [213] using an NA2100 Nitrogen and Protein Analyzer (ThermoQuest-CE Instruments, Rodeno, Italy). The Dumas method is based on the measurement of total nitrogen in the sample ($N \times 5.7$). Methionine was used as a standard.

Isolation of glutenin macro polymer from flour and glutenin particle size analysis

Dough strength is correlated to the amount of the glutenin macro polymer (GMP) and to the size of glutenin particles. Glutenin macro polymer was isolated by dispersing wheat flour in 1.5% (w/v) SDS followed by ultracentrifugation as described [214]. Fresh GMP from flour was dispersed in 1.5% (w/v) SDS (10 ml) by rotating overnight at room temperature. Particle size distributions were measured using a Mastersizer 2000 (Malvern Instruments, UK). The laser diffraction pattern obtained with the instrument was correlated to the particle size distribution based on Fraunhofer theory, assuming a spherical particle shape. The range of the instrument was 0.02–2,000 μm . Dispersions of GMP were transferred to the water filled sample vessel at an obscuration of approximately 8%. The surface area mean ($D_{3,2}$) was used from the particle size distribution data for comparisons. Further details of this method are described by Don *et al.* (2005) [215] and Wang *et al.* (2005) [216].

Mixing experiments

Dough strength was determined using a micro-Mixograph. A 2 g Mixograph (National Manufacturing Co., USA) pin-mixer was used to analyze the mixing properties of the different flour samples. Mixing was performed at 20°C. Water was added as estimated by Approved Method 54-40A [217] using the calculated protein and moisture contents. Dough contained 2% (w/w) sodium chloride (Merck, Germany). Bandwidth at peak resistance (BWPR) in percentages and dough development time (DDT) in minutes were used from the midline analysis for comparison.

Flow-relaxation measurements

Relaxation tests were performed to study dough elasticity. Longer relaxation half times indicate more elastic dough behavior [218, 219]. Dough was mixed to peak in the 2 g Mixograph pin-mixer, carefully removed from the mixer and transferred to the Bohlin VOR rheometer (Bohlin Instruments, Sweden). Flow-relaxation measurements were performed using an aluminum grooved plate geometry with a cross-section of 30 mm and a gap of 1 mm [219]. Moisture loss from the dough piece was prevented using paraffin oil. The actual measurement was performed after an equilibration time of 30 min to allow appropriate release of dough stress. The measuring temperature was 20°C. During measurement, the sample was deformed to a strain of 100% at a shear rate of 0.0208 s^{-1} . The strain was kept constant and the subsequent decrease of stress of the dough was recorded as a function of time. The time necessary for the dough to relax to a stress of

50% of the initial stress, recorded directly after stopping deformation, was used as the flow-relaxation half time ($T_{1/2}$).

Crossing of CS deletion lines

Deletion lines 6DS-4/1BS-19 and 1DS-5 were crossed using 6DS-4/1BS-19 as a pollen recipient and as a pollen donor. The plants were grown in 10 L pots with seven plants per pot in a climatized greenhouse. Ears were emasculated two to three days before flowering and covered with glassine bags. Pollination took place three to four days after emasculation. The resulting F1 seeds were multiplied and harvested as separate plants. Of the F2 seeds 270 were analyzed of crossing 6DS-4/1BS-19 (♀) X 1DS-5 (♂) and 288 of the reciprocal crossing for the presence of the different deletions. Genomic DNA was extracted from seedlings using a DNA extraction kit (Qiagen), according to manufacturers' instructions. DNA was used in PCR with primers specific for gene markers on the chromosome arms where the deletions are present. These markers are gwm147 for deletion 1DS-5, cfd49 for deletion 6DS-4, and wmc798 for deletion 1BS-19. Plants with single deletion and multiple deletions were selected, self-pollinated and grown until seeds were mature.

Results

Protein database search

The NCBI protein database search was performed to analyze the number of proteins that contain the different sequences recognized by mAb and T-cells. This search provided insight in how many proteins were expected to contain the different sequences and which different sequences were present within the proteins. The numbers of protein sequences that contain the various sequences involved in the onset of CD that are recognized by T-cells and mAbs are shown in Table 1. It was observed that the mAb and T-cell minimal sequences were specific for the epitopes in each of the expected protein group, with the exception of the mAb recognizing GliA- 9, whose minimally recognized sequence was also present in a number of - and -gliadin proteins. The sequence recognized by the T-cells was not present within any other protein group except for the / -gliadins. The minimal sequences recognized by mAbs LMW-1 and LMW-2 were more frequently found in the LMW-GS group than the sequence recognized by the corresponding T-cells. The sequences

recognized by mAb and T-cells for HMW-glt was present in nearly all HMW-GS protein sequences.

Table 1. Results of database search for sequences recognized by mAbs and T-cells.

Epitope	Protein groups				
	/ -gliadins	-gliadins	-gliadins/ D-type LMW-GS	LMW-GS	HMW-GS
mAb GliA- 9 (QFPQPQ)	68	67	3	–	–
T-cell GliA- 9 (PFPQPQLPY)	44	–	–	–	–
mAb GliA- 20 (RPQQPYP)	48	–	–	–	–
T-cell GliA- 20 (FRPQQPYPQ)	48	–	–	–	–
mAb LMW-1 (PPFSQQ)	–	–	–	233	–
mAb LMW-2 (QSPF)	–	–	–	163	–
T-cell LMW-glt (PFSQQQSPF)	–	–	–	21	–
mAb HMW-glt (QGQQGYYP)	–	–	–	–	67
T-cell HMW-glt (QGYPTSPQ)	–	–	–	–	65
No. of sequences retrieved	84	93	6	263	67

The number in each cell represents the presence of the recognized sequence by mAb or T-cell within a protein group. ‘–’ in a cell means that the sequence was not present.

SDS-PAGE

To obtain the gluten protein patterns from the CS deletion lines, gluten proteins were extracted and analyzed by SDS-PAGE followed by silver staining. Major differences compared to CS wild type are indicated by boxes in Fig. 1. Differences in gluten protein content compared to CS wild type were mostly observed in the B-, C- type LMW-GS and / -, -gliadin region. Lines with deletions of the short arms of chromosomes 1D were missing several gluten protein bands in the -gliadin/D-type LMW-GS region. The double deletion line, 1BS-19/6DS-4 (Fig. 1), was missing the largest number of gluten protein bands because of two deletions in gluten encoding regions. Unexpected results were obtained for deletion line 1BS-18, which is the line with the smallest deletion of chromosome arm 1BS. This line is missing an extra band compared to the other 1BS deletion lines having larger deletions. This does not fit with reported results on deletion lines [210, 212]. Deletion line 6BS-4 (Fig. 1) missed a gluten protein band that is present in the other deletion lines of chromosome 6B, even though deletion line 6BS-1 has been reported (WGGRC; Fig. 2C) to contain a larger deletion than 6BS-4. Deletion line 6BS-4 also contains the 5BS-2 deletion, but, to our knowledge, no gluten protein locus has ever been identified onto the short arm of chromosome 5B. We do not have any explanation for these discrepancies

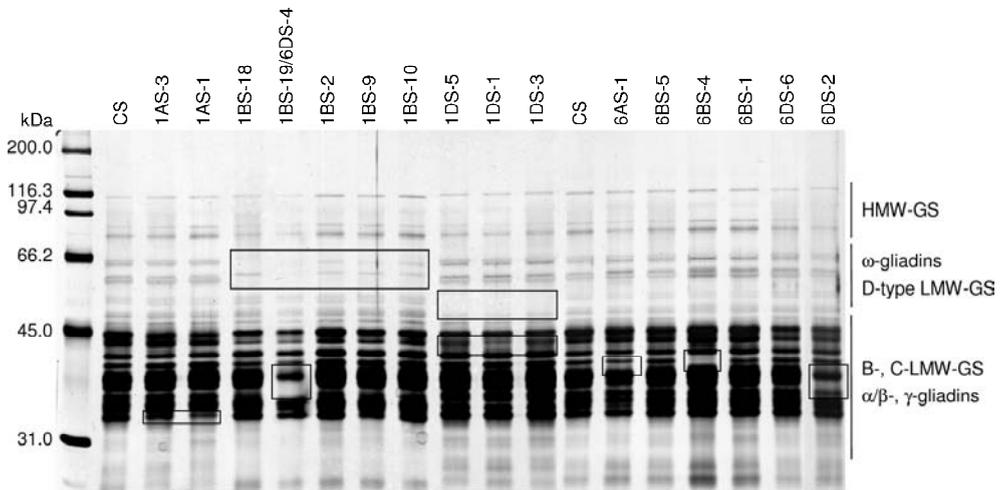


Figure 1. SDS-PAGE analysis of prolamins extracts from Chinese Spring deletion lines. CS: Chinese Spring wild type. Boxes indicate differences in protein bands.

Gli-1 deletions

CS deletion lines were analyzed for their contribution to T-cell stimulatory epitopes by using various mAbs recognizing different T-cell epitopes. In Fig. 2A, immunoblot results are presented using mAbs GliA- 9 and GliA- 20 for deletion lines of the short arm of chromosomes 1 (*Gli-1*) and 6 (*Gli-2*). Major differences, compared to CS wild type, are indicated with arrowheads. Deletion lines 1AS-3 and 1AS-1 were missing one gluten protein band by using mAb GliA- 9 and no gluten protein bands by using mAb GliA- α 20 (Figs. 2A, B). This suggests that this missing gluten protein only contains the epitope sequence recognized by mAb GliA- 9 and the loci encoding these gluten protein map to bin 1AS3-0.86-1.00 (the terminal 14% of chromosome arm 1AS) (Fig. 2C). All five deletion lines of the short arm of chromosome 1B (Fig. 2A) lacked one gluten protein band by using mAb GliA- 9 and no gluten protein band by using mAb GliA- 20. The double deletion line 1BS-19/6DS-4 (Fig. 2A) was missing two extra bands using mAb GliA- 9 and four by using mAb GliA- 20, which is caused by the 6DS-4 deletion. Two gluten protein bands were recognized by both mAbs GliA- 9 and GliA- 20. All 1BS deletion lines (Fig. 2A) lacked the same gluten protein band recognized by mAb GliA- 9 and because of that the loci encoding corresponding gluten protein map to bin 1BSsat18-0.50-1.00 (Fig. 2C). All three

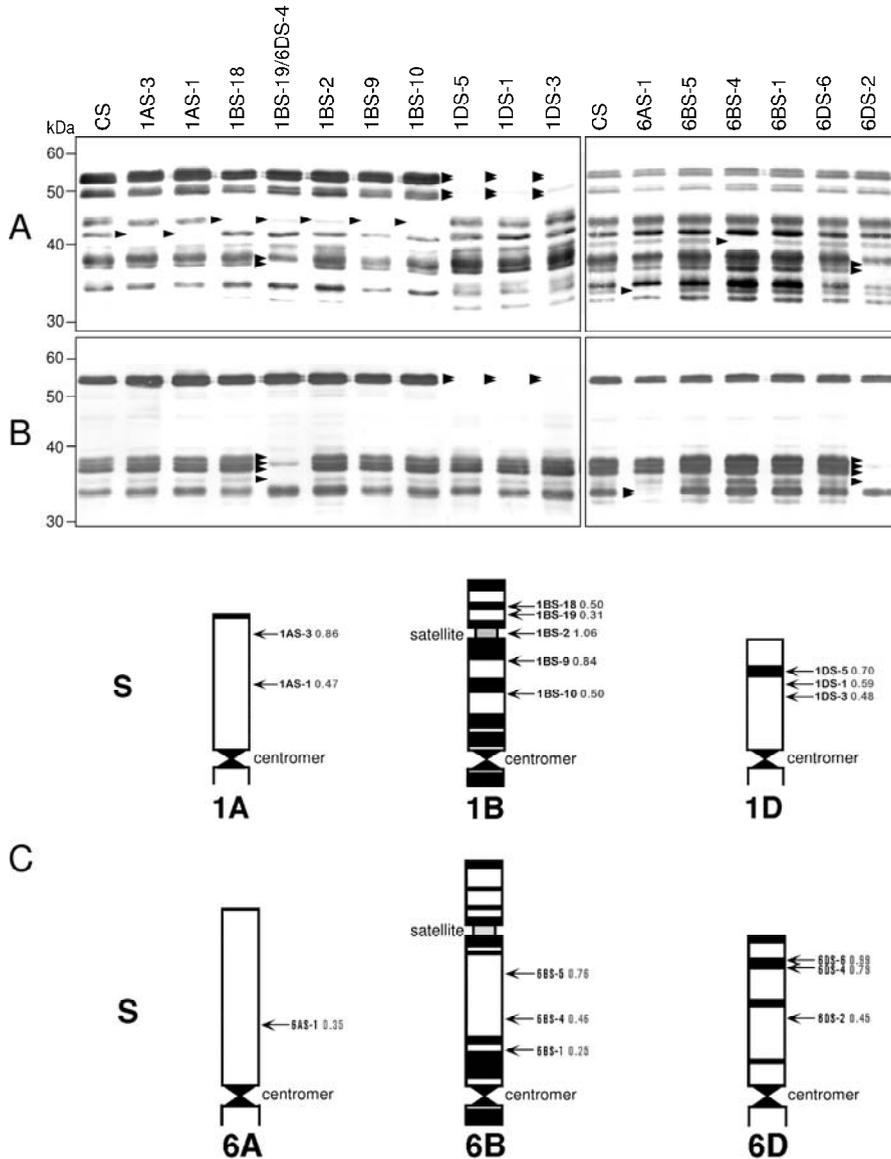


Figure 2. Immunoblot analysis of Chinese Spring deletion lines of the short arm of chromosome 1 and 6. (A) Using mAb GliA- 9; (B) Using mAb GliA- 20. CS: Chinese Spring wild type. Arrowheads indicate absent protein bands; (C) Physical maps of the short (S) arms of wheat chromosomes 1A, 1B, 1D, 6A, 6B, and 6D from centromer to telomeric ends (Wheat Genetic and Genomic Resources Centre, Kansas State University, USA). Arrows on the right of each chromosome indicate the deletion lines with their breakpoint (indicated as fraction length from the centromer). The banding patterns within the chromosomes are according to Gill et al. (1991) [211].

deletion lines of the short arm of chromosome 1D (Fig. 2A) lacked four gluten protein bands by using mAb Gli-a- 9 and two gluten protein bands by using mAb Gli-a- 20. These missing protein bands correspond to the boxed (missing) proteins in Fig. 1. One gluten protein band did not completely disappear by using mAb Gli-a- 9. This is probably because of the presence of gluten proteins from different loci but having the same molecular weights, therefore becoming visible only as one gluten protein band. The loci encoding the recognized gluten proteins map to bin 1DS5–0.7–1.0 (the terminal 30% of 1DS) (Fig. 2C). The two gluten protein bands recognized by mAb Gli-a- 20 were the same as recognized by mAb Gli-a- 9.

***Gli-2* deletions**

When analyzing CS deletion lines that are lacking parts of the short arm of chromosome 6, deletion line 6AS-1 (Fig. 2A) lacked one gluten protein band in immunoblotting using mAb Gli-a- 9 and two bands by using mAb Gli-a- 20. Deletion line 6BS-4 (Fig. 2A) lacked one gluten protein band by using mAb Gli-a- 9, but this was not the case for the other two 6BS deletion lines, 6BS-1 and 6BS-5 (Fig. 2A), which is not consistent with the reported sizes of the deletions. In the 6BS deletion lines, no changes were observed in gluten protein bands compared with CS wild type by using mAb Gli-a- 20 (Fig. 2B). These results suggest that the short arm of chromosome 6B encodes no gluten proteins containing T-cell stimulatory epitopes recognized by both mAbs Gli-a- 9 and Gli-a- 20, at least not mapping to bin 6BS–0.25–1.00 (terminal 75% of 6BS) (Fig. 2C). Deletion line 6DS-2, the line with the largest deletion (Figs 2A and B) lacked two gluten protein bands recognized by mAb Gli-a- α 9 and four bands by mAb Gli-a- α 20. One gluten protein band has not completely disappeared probably because of the presence of different gluten proteins having the same molecular weight within one gluten protein band. The same gluten protein bands are also absent in the double deletion line 1BS-19/6DS-4 (Fig. 2A). These missing protein bands correspond to the boxed (missing) proteins in Fig. 1. Hence, the loci encoding these gluten proteins map to bin 6DS4–0.79–0.99 (Fig. 2C).

***Glu-1* deletions**

Within the protein database, nearly all HMW-GS had epitope sequences recognized by mAb HMW-glt. The immunoblot results for the deletion lines of the long arm of chromosome 1 using the mAb recognizing HMW-glt are shown in Fig. 3A. In CS wild type, all four HMW glutenin subunits were detected. No contribution to HMW-GS was observed for the long arm of chromosome 1A, as expected for a transcriptional silent locus. Two

HMW-GS, 1Bx7 and 1By8, were absent in deletion lines 1BL-1 and 1BL-6. This suggests that the locus encoding HMW-GS 1Bx7 and 1By8 map to bin 1BL1–0.47–0.69 (Fig. 3B). The two HMW-GS, 1Dx2 and 1Dy12, were absent in deletion line 1DL-4. This suggests that the loci encoding HMW-GS 1Dx2 and 1Dy12 map to bin 1DL4–0.18–0.41 (Fig. 3B).

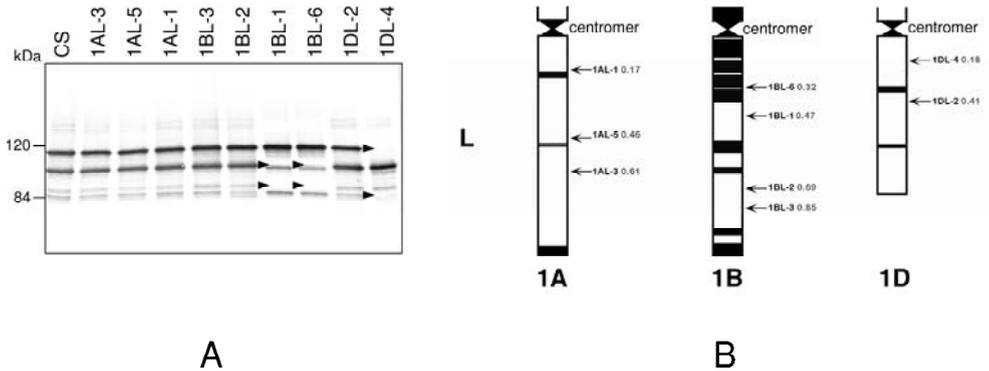


Figure 3. Immunoblot analysis of Chinese Spring deletion lines of the long arm of chromosome 1. (A) Using mAb HMW-glt. CS: Chinese Spring wild type; (B) Physical maps of the long (L) arms of wheat chromosomes 1A, 1B, and 1D from centromer to telomeric ends (Wheat Genetic and Genomic Resources Centre, Kansas State University, US). Arrows on the right of each chromosome indicate the deletion lines with their breakpoint (indicated as fraction length from the centromer). The banding patterns within the chromosomes are according to Gill et al. (1991) [211].

Glu-3 deletions

The immunoblot results using mAb LMW-2 for the deletion lines of the short arm of chromosome 1 are shown in Fig. 4. One band was observed in all the deletion lines and in CS wild type without significant differences. Immunoblot results using mAb LMW-1 showed similar patterns (results not shown).

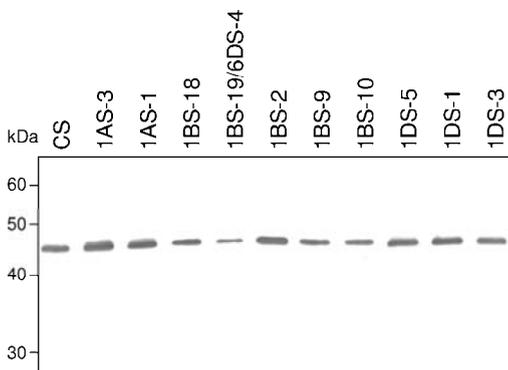


Figure 4. Immunoblot analysis of Chinese Spring deletion lines of the short arm of chromosome 1, using mAb LMW-2. CS: Chinese Spring wild type.

Rheological parameters of Chinese Spring deletion lines

The lines with the largest deletions from chromosomes 1 and 6, according to our results, were used for technological testing. Parameters among flours of different deletion lines are presented in Fig. 5.

Total protein content in flour (% w/w) of all deletion lines was higher compared to CS wild type flour. Especially protein content in flour of line 6AS-1 was high (20.5%), followed by protein content in flour of deletion line 1BS-19/6DS-4 (18.6%).

The glutenin macro polymer (GMP) content expressed as volume per mg protein was decreased in deletion line 1BL-1 and was nil in deletion line 1DL-4 (Fig. 5). GMP represents the highly aggregated glutenin protein network that is the prime determinant of dough elastic properties. A decrease in GMP is therefore expected to lead to a decrease in dough strength [80, 214, 220]. Because of the low amount of GMP present in flour of the deletion lines 1BL-1 and 1DL-4, it was impossible to estimate glutenin particle sizes for these lines. Flours of the two deletion lines, 1BS-10 and 6DS-2, showed a small decrease in GMP volume. For all other deletion lines, the GMP volume was increased.

Glutenin particle size is a predictor of dough mixing properties [215]. Average glutenin particle size was increased in flours of deletion line 1AL-1 and 6AS-1. In deletion lines 6DS-2, 6BS-1, 1BS-10, 1BS-19/6DS-4 and 1AS-1 the average particle size was decreased compared to CS wild type.

Dough made from flours of the two deletion lines 1BL-1 and 1DL-4, lacking HMW-GS, showed a significant decrease in dough development time (DDT) (Fig. 5). Dough made from all other deletion lines showed increase in DDT, especially the lines with deletions of the short arm of chromosome 6 (6AS-1, 1BS-19/6DS-4, 6DS-2, and 6BS-1) and 1AS-1. Deletions of the *Gli-2* loci seem to have a substantial effect on increasing DDT.

Bandwidth at peak resistance (BWPR) is a measure of dough stability. The BWPR was slightly decreased for deletion line 1DL-4 and was increased for all other deletion lines compared to CS wild type dough (Fig. 5). The BWPR was especially high for deletion lines 6AS-1 and 1BS-19/6DS-4. It is relevant to note that these are the same deletion lines having the highest protein content in flour.

Dough elasticity, indicated by relaxation half time ($T_{1/2}$), was decreased in flours of deletion lines 1BL-1 and 1DL-4, which lack HMW-GS, and in deletion lines 6BS-1 and 6DS-2 (Fig. 5). In contrast, deletion lines 1BS-19/6DS-4 and 1AS-1 showed an increase in $T_{1/2}$, indicating more elastic dough [218, 219].

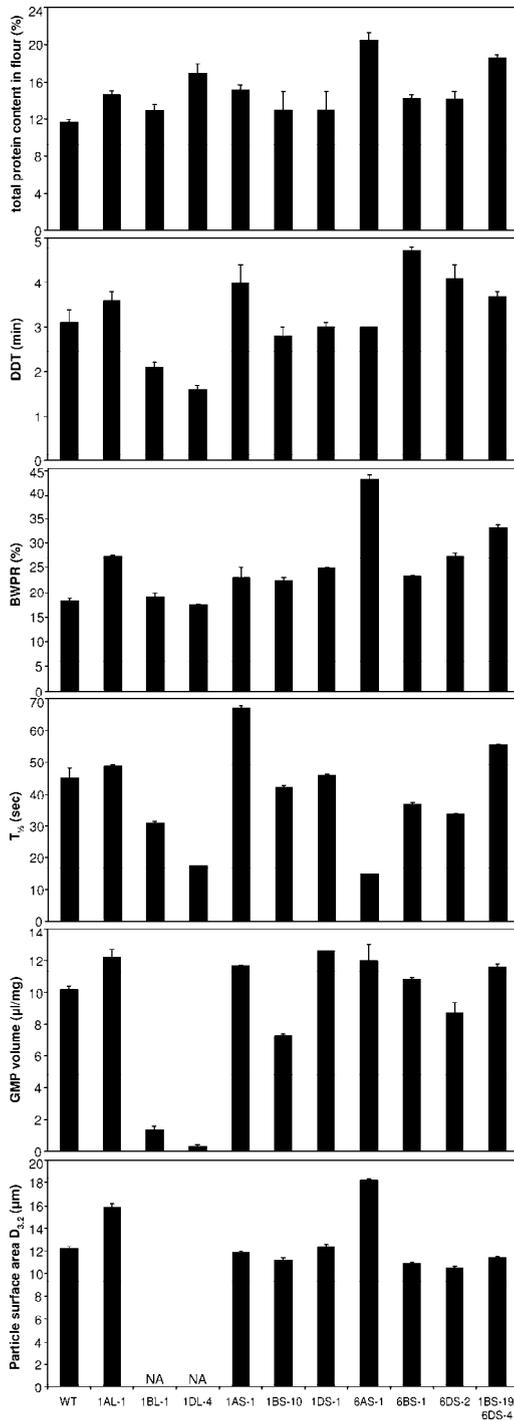


Figure 5. Rheological parameters tested for Chinese Spring deletion lines. All technological measurements were performed in duplicate, except the relaxation test ($T_{1/2}$) for deletion lines 1DL-4, 6AS-1, 6DS-2, and 6DS-4/1BS-19. Error bars represent the standard error. 'NA' means not analyzed for particle surface area ($D_{3,2}$) because the amount of GMP was too low.

Crossing of CS deletion lines

F2 seedlings from crossings of 6DS-4/1BS-19 X 1DS-5 were tested for the presence of single or multiple deletions. For both crossings, F2 seedling were obtained having the separate deletions 6DS-4, 1BS-19, and 1DS-5, but none of the analyzed seedlings contained all three deletions and none contained the combination of the deletions 1DS-5 and 1BS-19. However, from crossing 6DS-4/1BS-19 X 1DS-5, four seedlings were obtained and from the reciprocal crossing one seedling was obtained having both deletions 6DS-4 and 1DS-5. Mature kernels were harvested and analyzed for the presence of gluten proteins by SDS-PAGE and PageBlue staining, and for the presence of T-cell stimulatory epitopes by immunoblotting using mAbs against T-cell epitopes GliA- 9 and GliA- 20. SDS-PAGE analysis of the gluten protein extracts clearly showed the absence of all gluten proteins absent in both parents (Fig. 6A). In addition, immunoblotting showed the absence of all gluten proteins reacting with mAbs GliA- 9 and GliA- 20 absent in both parents (Figs. 6B and 6B). New deletion lines were obtained having the deletions 6DS-4, 1BS-19, and 6DS-4/1DS-5. Deletion line 6DS-4/1DS-5 is highly reduced in T-cell stimulatory epitopes GliA- 9 and GliA- 20

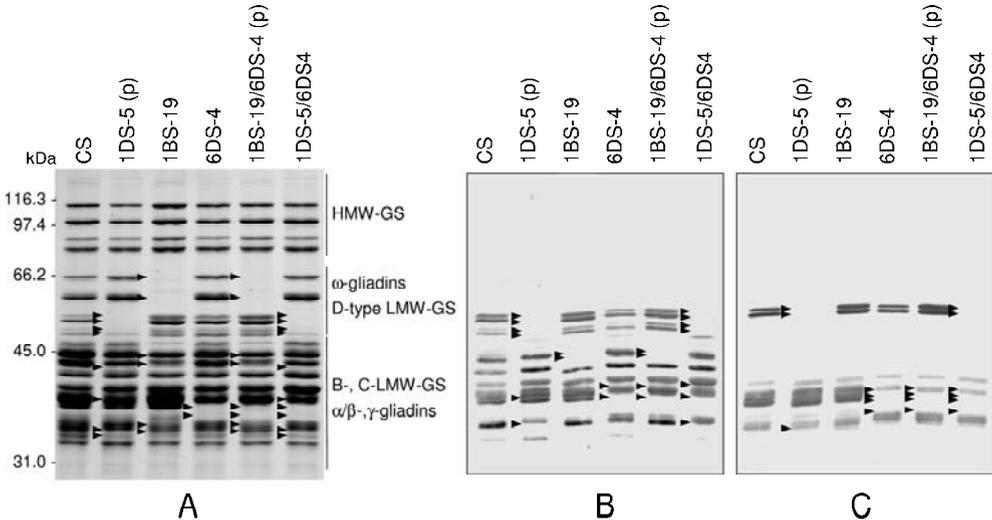


Figure 6. Analysis of 'Chinese Spring' deletion lines of the short arm of chromosome 1 and 6 including the new deletion line 1DS-5/6DS-4 obtained from crossing. (A) SDS-PAGE gel stained with PageBlue; (B) Immunoblot using mAb GliA- 9; (C) Immunoblot using mAb GliA- 20. CS: Chinese Spring wild type; (p): parental line used in crosses. Arrowheads indicate absent gluten protein bands.

Discussion

In this study, we examined the possibilities to develop a bread wheat variety with both reduced levels of T-cell stimulatory epitopes and good technological properties. We used a set of Chinese Spring deletion lines that lack different gluten protein-encoding loci from the group 1 and 6 chromosomes to determine whether reduction in T-cell stimulatory epitopes can be achieved by removal of certain gluten protein encoding genes with minimal effect on the technological properties of bread wheat. Many cytogenetic resources have been developed in *T. aestivum* cv Chinese Spring, which is considered as a model variety for hexaploid wheat. However, differences among varieties may exist.

CD immunogenic epitopes

On the short arm of the group 6 chromosomes, the gluten loci that encode ω -gliadins are located. The ω -gliadins are considered the most immunogenic concerning both the adaptive immune response and the innate immune response [40, 58, 68, 70, 202]. We observed that the locus on the short arm of chromosome 6D, mapped to bin 6DS-0.45-0.99, is responsible for most of the T-cell stimulatory ω -gliadin proteins. These results are in agreement with the results obtained by Molberg *et al.* (2005) [76] who showed no decrease in response of DQ2- β T-cells for deletion line 6DS-6 and a significant decrease in T-cell response for deletion lines 6DS-4 and 6DS-2. In addition, results are in agreement with results of Van Herpen *et al.* (2006) [74], based on relative presence of CD-epitopes in ω -gliadin ESTs from the three homoeologous loci, and with results of Salentijn *et al.* (2009) [75] on the presence in cDNAs from two hexaploid and two tetraploid cultivars. When using mAb GliA-20 in immunoblotting also two gluten protein bands were stained that were encoded by the short arm of chromosome 1D. We tentatively assign these as ω -gliadins/D-type LMW-GS containing the mAb GliA-20 sequence. Only a few ω -gliadin proteins have been sequenced so far because they are difficult to clone due to the presence of large repetitive domains [166]. It has been shown that ω -gliadins may have epitopes that are involved in gluten-sensitive response of CD-patients [170, 171]. The ω -gliadins encoded by chromosome 6 seem to be related to gliadins encoded by chromosome 1 from which they might have originated through gene duplication and/or translocation [180, 221]. Analysis of the minimal sequence recognized by mAb GliA-9 indicated that this sequence also occurs in some α - and β -gliadins. Indeed, mAb GliA-9 recognized gluten protein bands that disappeared in deletion lines of the short arm of chromosome 1A, 1B, and 1D (where α - and β -gliadin encoding genes are

located). We observed that genes mapped to bin 1DS-0.48-1.00 had the highest contribution to the number of T-cell stimulatory epitopes.

New deletion lines were obtained having the deletions 6DS-4, 1BS-19, and 6DS-4/1DS-5. Deletion line 6DS-4/1DS-5 is highly reduced in T-cell stimulatory epitopes GliA- 9 and GliA- 20 and lacks all gluten proteins that are absent in both deletion lines 6DS-4 and 1DS-5.

Technological properties

Studies have shown that the technological parameters of wheat flours are influenced by alleles encoding different HMW-GS [222-225], LMW-GS [12, 226], and gliadins [227]. Deleting parts of the short arm of chromosome 1A resulted in an increased dough development time (DDT) and volume of glutenin macro polymer (GMP). A decrease in LMW-GS or gliadins results in a relative increase of ratios for HMW-GS/LMW-GS or glutenins/gliadins. Such a change was suggested to increase dough strength [204, 206]. Indeed, we found that removal of the locus from the short arm of chromosome 1A resulted in increased dough elasticity. In the deletion lines 1AS-1 and 1DS-1, higher GMP volumes were observed, while in deletion line 1BS-10 a decreased GMP volume was found together with decreased DDT. On chromosome 1B, also a *Glu-B2* locus is located encoding a B-type LMW-GS [228, 229] and a *Glu-B3* locus is located encoding two tightly linked genes for an ω -gliadin and a B-type LMW-GS [230]. This suggests that LMW-GS encoded by these loci are important for the formation of the GMP [231, 232]. Removal of the loci could affect the ratios for HMW-GS/LMW-GS or glutenins/gliadins. Chromosome 1D encodes a D-type LMW-GS containing a single cysteine residue and therefore may act as a chain terminator [233, 234]. The absence of the protein could increase the GMP volume in deletion line 1DS-1. It would be expected that the GMP volume would decrease in deletion line 1AS-1 because of removal of the locus encoding major LMW-GS. We observed, however, that no T-cell stimulatory epitopes present in LMW-GS disappeared from the immunoblot using mAbs LMW-1 and LMW-2, which is possible if expression from the deleted locus is compensated for by the other two loci present on the homoeologous chromosomes, for example by a higher expression of *Glu-B3*. Compensation behavior of storage protein synthesis in wheat was observed by Wieser *et al.* (2006) [235] after inhibition of the expression of ω -gliadins by RNA interference (RNAi). Also Gil-Humanes *et al.* (2008) [236] recently observed while RNAi reduced the proportion of ω -gliadins by 55-80% and β -gliadins by 63%, this did not lead to similar reduction in proteins detected by the sandwich ELISA using the R5 monoclonal antibody. The R5 assay was, however,

developed for the detection of gluten proteins from different sources and not optimized to detect T-cell stimulatory gluten proteins [127]. Hence, although the R5 assay is currently considered the standard test for identification of gluten contaminants, we regarded this test unsuitable in the context of this study.

With respect to technological properties, deletion line 6AS-1 showed an increase in GMP volume and a strong increase in glutenin particle size. In contrast, deletion lines 6BS-1 and 6DS-2 showed a decrease in glutenin particle size and a decrease in GMP volume for deletion line 6DS-2. Gliadins of the γ - and δ -type have been identified to contain an extra cysteine residue that makes them act as chain terminators. We suggest that the short arm of chromosome 6A in CS is encoding a chain terminating γ -gliadin. The lower content of chain terminators could account for a larger size of glutenin particles as observed in deletion line 6AS-1. Because of compensation, deletions of the short arm of chromosome 6B and 6D could lead to an increased expression of chain terminating α -gliadins encoded by the short arm of chromosome 6A and result in observed smaller glutenin particle sizes. The deletions of the short arm of chromosome 6B and 6D resulted in stronger dough as shown by increased DDT. This effect on dough strength is expected because a decrease in γ -gliadins results in a relative increase of the glutenin/gliadin ratio. The GMP volume of flour from deletion line 6DS-2 was decreased, which indicates weaker dough, whereas the DDT was increased, which indicates stronger dough. Because of this effect, the decrease in GMP volume in deletion line 6DS-2 resulted in decreased elasticity rather than decreased dough strength.

We observed that technological properties of flour from deletion lines were strongly affected by the removal of the different HMW-GS with the strongest effect in deletion line 1DL-4. Dough strength (as expressed as DDT and GMP volume) and dough elasticity ($T_{\frac{1}{2}}$) were both strongly decreased, which is in agreement with published results [204, 237, 238]. Deletion of the locus on the long arm of chromosome 1A resulted in some increase in dough strength (DDT and GMP volume) and elasticity ($T_{\frac{1}{2}}$). In addition, glutenin particle sizes were significantly increased. Both the x-type and y-type encoding genes of CS at *Glu-A1* are silent [207]. In most studies, the silent locus at *Glu-A1* was not found to be important to determine dough strength compared to non-silent loci [12, 226], so the effect of deletion of the long arm of chromosome 1AL might be because of the absence of other gene products. Based on these results, the *Glu-1* loci of CS are considered inappropriate as a focus to breed for wheat with less T-cell stimulatory epitopes if technological properties are to be preserved.

Conclusions

A strategy to breed for bread wheat with less T-cell stimulatory gluten epitopes while retaining technological properties is feasible by focusing on eliminating genes present on the short arms of chromosome 1D and 6D. This will result in a wheat variety with highly decreased T-cell stimulatory epitopes. However, eliminating genes might decrease dough elasticity because of a changed ratio in glutenin and gliadin proteins. This ratio could be compensated for by the addition of monomeric proteins with no T-cell stimulatory epitopes to the flour, for example from safe sources like oats, or by the introduction through breeding or genetic modification of CD-safe gliadin genes. In addition, wheat varieties with limited but not complete reduced levels of T-cell stimulatory epitopes may still contribute to lower the gluten load for the entire population and it may reduce the development of CD in a number of potential patients.

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Chapter 7

Dough quality of bread wheat lacking -gliadins with celiac disease epitopes and addition of celiac-safe avenins to improve dough quality

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Abstract

Celiac disease is a T-cell mediated immune response in the small intestine of genetically susceptible individuals caused by ingested gluten proteins from wheat, rye, and barley. In the allohexaploid bread wheat (*Triticum aestivum*), gluten proteins are encoded by multigene loci present on the homoeologous chromosomes 1 and 6 of the three homoeologous genomes (A, B, and D). The effect of deleting individual gluten loci was analyzed in a set of deletion lines of *T. aestivum* cv. Chinese Spring with regard to the level of T-cell stimulatory epitopes (Glia- 9 and Glia- 20) and to technological properties of the dough including mixing, stress relaxation, and extensibility.

Deletion of loci encoding α -gliadins, β -gliadins, and LMW-glutenins located on the short arm of chromosome 1D, reduced the number of T-cell stimulatory epitopes and caused a minor deterioration of dough quality by increase of elasticity. Deletion of loci encoding γ -gliadins located on the short arm of chromosome 6D, resulted in a significant reduction in T-cell stimulatory epitopes and in parallel, the dough became more stiff and less elastic. We demonstrated here that γ -gliadins from wheat can largely be compensated by the addition of avenins from oat to the flour to meet technological requirements.

Introduction

Dough quality for bread making highly depends on the presence and composition of wheat gluten proteins. These gluten proteins are composed of monomeric gliadins and polymeric glutenins, which together determine the bread-making quality [12, 83]. Glutenins are responsible for the elastic properties of the dough, whereas the gliadins are responsible for the viscous properties. High molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) can form large polymers by inter- and intramolecular disulfide bonds. The LMW-GS can be divided in typical LMW-GS B-subunits that can act as chain extenders because of their ability to form intermolecular disulfide bonds and in gliadin-like LMW-GS C- and D-subunits that can act as chain terminators because they lack the ability of forming intermolecular disulfide bonds [239]. The amount of large glutenin macro polymers (GMP) is an important quality parameter and strongly relates to dough properties [80, 225, 240-242]. Gliadins can be divided into α -, β -, and γ -gliadins [16] which have specific water-retaining capacities important for dough viscosity.

In bread production, the dough mixing process (i.e. the controlled addition of water to the wheat flour) is a very important step [80, 243, 244]. During mixing, the gluten proteins are rehydrated again and homogeneously distributed throughout the dough. Upon resting, a three-dimensional gluten network structure is formed that will determine the viscoelastic and gas holding properties of the dough. Detailed mixing and rheological studies have revealed a direct relationship between gluten composition on structural properties and dough properties [for a review see 245]. For example, the ratio between glutenins and gliadins is especially relevant for the viscous vs. elastic properties of dough. A high ratio of monomeric vs. polymeric proteins will lead to a less stiff and more viscous dough [246, and references therein].

Apart from their role in dough quality, gluten proteins can affect health in genetically susceptible individuals. Many gluten proteins contain T-cell stimulatory epitopes that can cause celiac disease (CD; gluten intolerance) [40]. After consumption of gluten proteins from wheat, rye, or barley, the epitopes trigger an immune response that causes damage to the small intestine. CD-patients are therefore restricted to a lifelong gluten-free diet. About 0.5–2% of the Western population suffers from CD and the numbers are increasing because of better diagnosis but also because of increased intake and usage of wheat gluten proteins as food additive. Among the different gluten epitopes that have been identified, the α -gliadin epitopes are considered the most immunogenic [66-73, 156] of

which the Glia- 9 is a major immunodominant epitope. The Glia- 9 epitope sequence (I) is part of the proteolytic resistant 33-mer identified by [47, 161] that has a high T-cell stimulatory effect. The 33-mer sequence (LQLQFPQPQLPYQPQLPYQPQLPYQPQPF) is only present in D-genome protein sequences of / -gliadins (Salentijn, personal communication).

Gluten proteins are encoded by 15 major loci. The HMW-GS are encoded by *Glu-1* loci on the long arm of group 1 chromosomes (*Glu-A1*, *-B1*, and *-D1*) [207]. The LMW-GS are encoded by the *Glu-3* loci on the short arms of group 1 chromosomes (*Glu-A3*, *-B3*, and *-D3*) [208] and are tightly linked to the loci encoding the - and -gliadins (*Gli-A1*, *-B1*, and *-D1* and *Gli-A3*, *-B3*, and *-D3*). Most / -gliadins are encoded by loci on the short arms of group 6 chromosomes (*Gli-A2*, *B2*, and *D2*) [209].

Deletion lines of *T. aestivum* cv. Chinese Spring (CS) were selected having specific deletions on the short arms of group 1 and 6 chromosomes and generally lacking one locus of the three homoeologous chromosomes encoding gluten proteins [210, 212].

Removing gliadins will result in a loss in T-cell stimulatory epitopes but may also lead to a change in dough technological properties with the dough becoming more stiff. This former may impair the potential use of deletion lines in the development of CD-safe wheat. To compensate for dough technological properties, we searched for a CD-safe alternative of these monomeric proteins. Here, we investigate the potentials of CD-safe oat avenins, which are similar to wheat gliadins, to compensate in the technological properties of 'Chinese Spring' deletion lines, selected for their reduction in T-cell stimulatory epitopes Glia- 9 and Glia- 20 [77, 132], as well as for having the smallest chromosome deletions [169].

Material and Methods

Plant material

From the Wheat Genetic & Genomic Resources Center (WGGRC) Kansas State University, USA (http://www.k-state.edu/wgrc/Germplasm/Deletions/del_index.html) seeds were obtained from *T. aestivum* Chinese Spring (CS) deletion lines 1AS-3, 1DS-5, 6AS-1, 6DS-2(/5BS-1), and 1BS-19/6DS-4 as described [210-212]. The deletion lines had partial deletions of the short arms of chromosomes 1 and 6, which were characterized by cytogenetics (Fig. 1A). Deletion line 6AS-1 was marked as being probably heterozygous. Seeds of CS wild type (wt) (CGN04086) were obtained from the Centre for Genetic

Resources (CGN), the Netherlands (<http://www.cgn.wur.nl/uk/>). Plants of CS wild type (wt) (CGN04086) and deletion lines were grown in a climatized greenhouse and seeds were harvested at maturity. Ears of deletion lines 1AS-3 and 6AS-1 looked similar as the CS wt. Ears of deletion lines 1DS-5, 6DS-2(/5BS-1), and 1BS-19/6DS-4 were only half filled with grains at maturity. Analysis of these deletion lines is also described in Van den Broeck *et al.* (2009) [169], however, new rounds of seed multiplications have been performed to obtain sufficient amounts of seeds. This might have caused differences in flour properties and explain the difference in results obtained for mixing, extension testing and GMP-volume.

Milling

Grain samples were milled using a Quadrumat JR laboratory mill (Brabender, Germany) according to Approved Method 26-50 [217]. Prior to milling, kernel moisture was adjusted to 15% by incubation on a roller bank overnight at room temperature. Bran was separated from endosperm flour by sieving through mesh (150 μm). After sieving, the flour yield ranged from 34 to 47%. These flour samples were used for mixing experiments and rheology experiments. Commercial oat flakes (De Halm, The Netherland) were ground in an analytical mill (A 11 Basic, IKA-Werke) and sieved through mesh (315 μm).

Total protein content in flour

The total nitrogen content of the flour ($\text{N} \times 5.7$ for wheat and $\text{N} \times 6.26$ for oat [96]) was calculated using the Dumas method [213] using a Flash EA 1112 Nitrogen and Protein Analyzer (Thermo Scientific) according to Approved method 46-30 [217]. Moisture content was measured according to Approved Method 44-15A [217].

Gluten protein content in flour

Gluten proteins were extracted (according to Van den Broeck *et al.* (2009) [169]) from 50 mg wheat flour by addition of 0.5 ml of 50% (v/v) aqueous iso-propanol with continuous mixing (MS1 Minishaker, IKA Works, Inc.) at 1,000 rpm for 30 min at room temperature, followed by centrifugation at 10,000 rpm for 10 min at room temperature. The residue was re-extracted twice with 50% (v/v) aqueous iso-propanol, 50 mM Tris-HCl, pH 7.5 containing 1% (w/v) DTT, for 30 min at 60°C with mixing every 5–10 min, followed by centrifugation at 10,000 rpm for 10 min at room temperature. After addition of each next extraction solution, the residue was resuspended by shaking in a Fastprep[®] FP220A Instrument for 10 s at 6.5 m/s followed by sonication for 10 min in an ultrasonic bath (Branson 3510, Branson Ultrasonics Corporation). The three obtained supernatants were

combined and considered the gluten protein extract. The gluten protein content was quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories), based on the Bradford dye-binding procedure, according to manufacturer's instruction with BSA as a standard.

SDS-PAGE and immunoblotting

Wheat gluten proteins and oat avenins were separated on SDS-PAGE gels of 10% and 11%, respectively, as described [134] using a Hoefer SE 260 mighty small II system (GE Healthcare) followed by staining with PageBlue™ (Fermentas).

For immunoblotting, proteins were blotted onto nitrocellulose (0.2 µm, Bio-Rad Laboratories), omitting methanol from the blotting buffer, using a Mini Trans-Blot Cell (Bio-Rad Laboratories) at 100 V for 1 h. Blots were stained using a MemCode™ Reversible Protein Stain Kit for Nitrocellulose Membranes (Fisher Scientific) prior to incubation with mAbs. Incubation with mAbs specific for T-cell stimulatory epitopes Glia- 9, Glia- 20, and Glt-156 (LMW-glutenin) [77, 132] was performed as described [136]. Antibody binding to the blots was visualized by staining for alkaline phosphatase conjugated secondary antibody, using Nitro Blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma). Blots were scanned using a Bio-Rad GS-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories) and saved as TIFF images.

Two-dimensional gel electrophoresis (2-DE)

For 2-DE, gluten proteins were separated in the first dimension by isoelectric focusing (IEF). Immobiline Drystrips pH 3–10 of 7 cm (GE Healthcare) were rehydrated overnight with 10 µg protein in rehydration buffer (6 M urea, 2 M thio-urea, 2% CHAPS, 20 mM DTT) complemented with 0.5% IPG buffer pH 3–10 (GE Healthcare) to reach a final volume of 125 µl according to manufacturer's instructions. The rehydrated strips were focused on an IPGphor (GE Healthcare) using the following conditions: 300 V during 30 min, gradient to 1,000 V in 30 min, gradient to 5,000 V in 1h 20 min, step and hold at 5,000 V until 6,500 Vh. Prior to second dimension, strips were equilibrated for 15 min in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl, pH 8.8) containing 1% (w/v) DTT, followed by 15 min in 5 ml equilibration buffer containing 2.5% (w/v) iodoacetamide. Separation in the second dimension was performed using SDS-PAGE gels (10%) and the SE 260 mighty small II system (GE Healthcare). Gels were stained with PageBlue™ (Fermentas).

Mixing

Mixing studies were performed with a 2g-Mixograph (Pin mixer, TMCO, Lincoln, NE, USA) using water absorptions estimated by Approved Method 54-40A [217] using the calculated protein and moisture contents. The quantities of flour and water were chosen to provide a constant 3.5 g of dough. To all flours, 2% NaCl was added. Mixing was performed for 10 min at 20°C in duplicate. Mixing experiments with flour from deletion line 6DS-2 were in addition performed with replacing the flour with 10% or 25% of oat flour. Mixograms were evaluated using Mixsmart® software. Computer-analyzed mixograph parameters obtained included midline peak time (MPT), midline peak height (MPH), midline peak width (MPW), midline right slope (MRS), bandwidth at 8 min (MTxW), and energy to peak (ETP).

Extension testing

Dough for extension testing was mixed to peak in a 10-g mixograph (National Manufacturing, USA). A dough piece of 2.3 g was used for stress relaxation measurement, and the rest of the dough was pressed into a Teflon mould pre-warmed to 30°C and rested for 45 min at 30°C in a water-saturated atmosphere prior to testing. The dough strips (5–7) were removed from the Teflon block, mounted on the Kieffer dough and gluten extensibility rig, and immediately tested on a TA.XT2i texture analyzer (Stable Micro Systems, UK) at a hook speed of 3.3 mm/s and a trigger force of 2 g. From the extension graph, the maximum resistance (R_{\max} in g) and extensibility (Ext in mm) were calculated. R_{\max} is a measure of the stiffness of the dough; a higher R_{\max} reflects a stronger, more stiff dough. Ext is a measure of the elasticity of the dough and a high Ext-value reflects a more viscous and less elastic dough.

Stress relaxation

A dough piece of 2.3 g obtained from the 10 g mixograph was placed between two parallel plates (25 mm diameter) of an Advanced Rheometer AR2000 (TA Instruments, USA). The gap between the plates was adjusted to 3 mm and the dough was covered with paraffin oil to prevent drying out. The dough was rested for 45 min at 30°C to allow relaxation of stresses resulting from dough handling. Then a strain was applied from 0–0.2 s^{-1} in 50 s, after which the dough was allowed to relax for 5 min. The relaxation half-time ($T_{1/2}$) was calculated as the time required for the stress to decay to half the strain. A viscous dough is reflected by a short $T_{1/2}$ value, while a high $T_{1/2}$ value reflects a more elastic dough.

Extraction and addition of oat avenins

Commercial oat flakes from variety Gigant (De Halm, The Netherlands) were ground in an analytical mill (A 11 Basic, IKA-Werke) and 25 g of the resulting flour was defatted with petroleum ether (60:40) [247] by resuspending three times in 500 ml. The flour was collected on filter paper and left to dry overnight in a fume hood. Then, the dry flour (22.6g) was extracted once with 120 ml of 50% (v/v) aqueous iso-propanol by rotation for 30 min at room temperature, followed by centrifugation at 10,000 rpm for 10 min. The residue was re-extracted once with 50 ml 50% (v/v) aqueous iso-propanol. DTT appeared not necessary as a reductant for total avenin extraction. The supernatants were combined and dialyzed against 0.1 mM acetic acid which results in a pH close to that of dough (~pH 6) [244]. After dialysis, the avenin fraction was freeze-dried. Of this freeze-dried oat avenin fraction, 1, 2, 5, 10, or 20 mg was added to flour of deletion lines 1AS-3, 1DS-5, 6AS-1, 6DS-2, and 1BS-19/6DS-4. Two-gram mixing experiments were performed and parameters obtained included midline peak time (MPT), midline right slope (MRS), band width at 8 min (MTxW), and energy to peak (ETP).

Isolation of glutenin macro polymer (GMP)

GMP was isolated by suspending 0.5 g flour in 11 ml 1.5% (w/v) SDS. After ultracentrifugation (Beckmann L-80, 80,000 x g, 30 min, 20°C) [214] the supernatant was discarded and the thickness of the gel layer was measured. From this, the GMP volume was calculated.

Results

The reduction of CD-stimulating epitopes (Glia- 9 and Glia- 20) in the context of quality aspects of wheat dough (regarding mixing, stress relaxation, and extensibility) was analyzed in a set of deletion lines of *T. aestivum* cv. Chinese Spring having deletions from the short arms of chromosomes 1 and 6. Additionally, the capacity of oat avenins to compensate for the deleted gliadins was investigated.

Flour properties

Flour milling yield of CS wt and the deletion lines was calculated as percentage of flour obtained from the initial total kernel weight. Flour yields obtained after milling and sieving were remarkably lower for the deletion lines 6DS-2 and 1BS-19/6DS-4 compared to CS wt

(Table 1). Deletion line 6DS-2 produced a wide variety of seed dimensions, generally smaller than the CS wt seeds. Seeds of deletion line 1BS-19/6DS-4 were comparable to CS wt seeds. Seeds of both deletion lines had a somewhat darker appearance than the CS wt seeds.

Flour water content from CS wt and deletion lines was 12%. Total protein content in flour was ~18% for CS wt and for deletion lines 1AS-3, 1DS-5, and 6AS-1, but was lower for deletion line 6DS-2 and higher for deletion lines 6AS-1 and 1BS-19/6DS-4 (Table 1).

The gluten protein content as percentage of the total protein content in the flour (and as percentage of flour) was for CS wt 33% (5.8%), for 1AS-3 33% (6.1%), for 1DS-5 29% (5.2%), for 6AS-1 26% (5.0%), for 6DS-2 28% (4.2%), and for 1BS-19/6DS-4 29% (5.8%).

SDS-PAGE, immunoblotting and 2-DE

Gluten protein extracts from flour of CS wt and deletion lines were analyzed by SDS-PAGE and PageBlue staining, immunoblotting and 2-DE. Results are shown in Fig. 1, arrow heads indicate the major differences compared to CS wt. Differences to CS wt in gluten proteins were mostly observed in the B-, C- type LMW-GS and γ -gliadin region. Line 1DS-5 was missing several gluten protein bands in the γ -gliadin/D-type LMW-GS and in α -gliadins regions. The double deletion line, 1BS-19/6DS-4, was missing the most gluten protein bands because of two deletions in different gluten encoding loci.

Deletion line 6AS-1 turned out to be heterozygous as was shown by SDS-PAGE and immunoblotting (Figs. 1A, B, and C). To characterize the protein extracts in more detail, 2-DE analysis was performed. Single protein bands on the SDS-PAGE gel (Fig. 1A) were separated into individual protein spots (Fig. 1E). This 2-DE gels enabled to identify the absent gluten proteins in the deletion lines more clearly than by SDS-PAGE. If proteins are near identical, it is still possible that they appear in the same spot on the 2-DE gel.

Mixing

Dough mixing behavior and technological dough properties reflect the composition and structure of the gluten protein profile in the dough. A high glutenin/gliadin (glu/gli) ratio results in a more stiff and less extensible dough, whereas a high HMW-/LMW-GS ratio will increase dough elasticity [for a review see 245]. In addition, other compensation type of factors also influences dough mixing behavior and properties. For example, if one type of gliadins is deleted, it is possible that the synthesis of another type of gliadins is increased, effectively keeping the glutenin/gliadin ratio constant. In addition, deleting one type of

LMW-GS could lead to an increased synthesis of either glutenins or gliadins, leading to an overall change in glu/gli ratio.

Mixograms obtained from the 2g-mixograph were evaluated using Mixsmart[®] software. Optimum water addition for mixing was based on flour protein content and flour water content according to Approved Method 54-40A [217]. This procedure provided good curves for all flours tested. Parameters selected for further analysis included midline peak time (MPT), midline peak height (MPH), midline peak width (MPW), midline right slope (MRS), bandwidth at 8 min (MTxW), and energy to peak (ETP). The results are shown in Table 1. The CS wt dough was weak, with a very short mixing time (MPT) and required energy input compared to high quality wheat used for bread making. Deletion of gliadins (as in lines 6DS-2 and 1BS19/6DS4) resulted in higher levels of required energy to peak (ETP) and larger values of MtxW, indicating a relatively higher content of glutenins [237]. Alternatively, deleting LMW-GS (as with line 1DS-5) resulted in a lower MtxW and a lower ETP, indicating a relatively higher content of gliadins. These data are reflected by changes in GMP content (see p. 113). Parameters were correlated individually and relatively high correlation coefficients were obtained for all mixograph parameters except the correlation coefficients for values for MPH and MRS ($R^2=-0.32$). Values for FPC gave a high correlation coefficient with MPH ($R^2=0.77$) and a lower correlation coefficient with MPW ($R^2=0.41$).

Extension testing

Dough extension relates to its gas-holding properties during bread making [248]. Relevant extension parameters are extensibility (Ext in mm) and maximum resistance (R_{\max} in g) (Table 1). Values for R_{\max} were higher for deletion lines 6DS-2 and 1BS19/6DS-4 and values for Ext were lower compared to CS wt, assuming a more stiff and less extensible dough. For deletion lines 1AS-3 and 1DS-5, values for R_{\max} were lower and higher for Ext compared to CS wt, indicating a weaker and more extensible dough. High correlations coefficients were obtained for R_{\max} values and values for the other dough parameters. The correlation coefficient for R_{\max} and Ext was high ($R^2=-0.885$) and inverted.

Figure 1. Analysis of 'Chinese Spring' deletion lines of the short arm of chromosome 1 and 6. (A) Physical maps of the short arms of wheat chromosomes 1A, 1B, 1D, 6A, and 6D from centromer to telomeric ends (Wheat Genetic and Genomic Resources Centre, Kansas State University, USA). Arrows on the right of each chromosome indicate the deletion lines with their breakpoint (indicated as fraction length from the centromer). The banding patterns within the chromosomes are according to Gill et al. (1991) [211]. Gluten protein extracts from flour analyzed by: (B) SDS-PAGE (10%) stained with PageBlue; (C) Immunoblot using mAb GliA- 9; (D) Immunoblot using mAb GliA- 20; (E) 2-DE gels stained with PageBlue. CS: Chinese Spring wild type. Arrowheads indicate absent gluten protein bands.

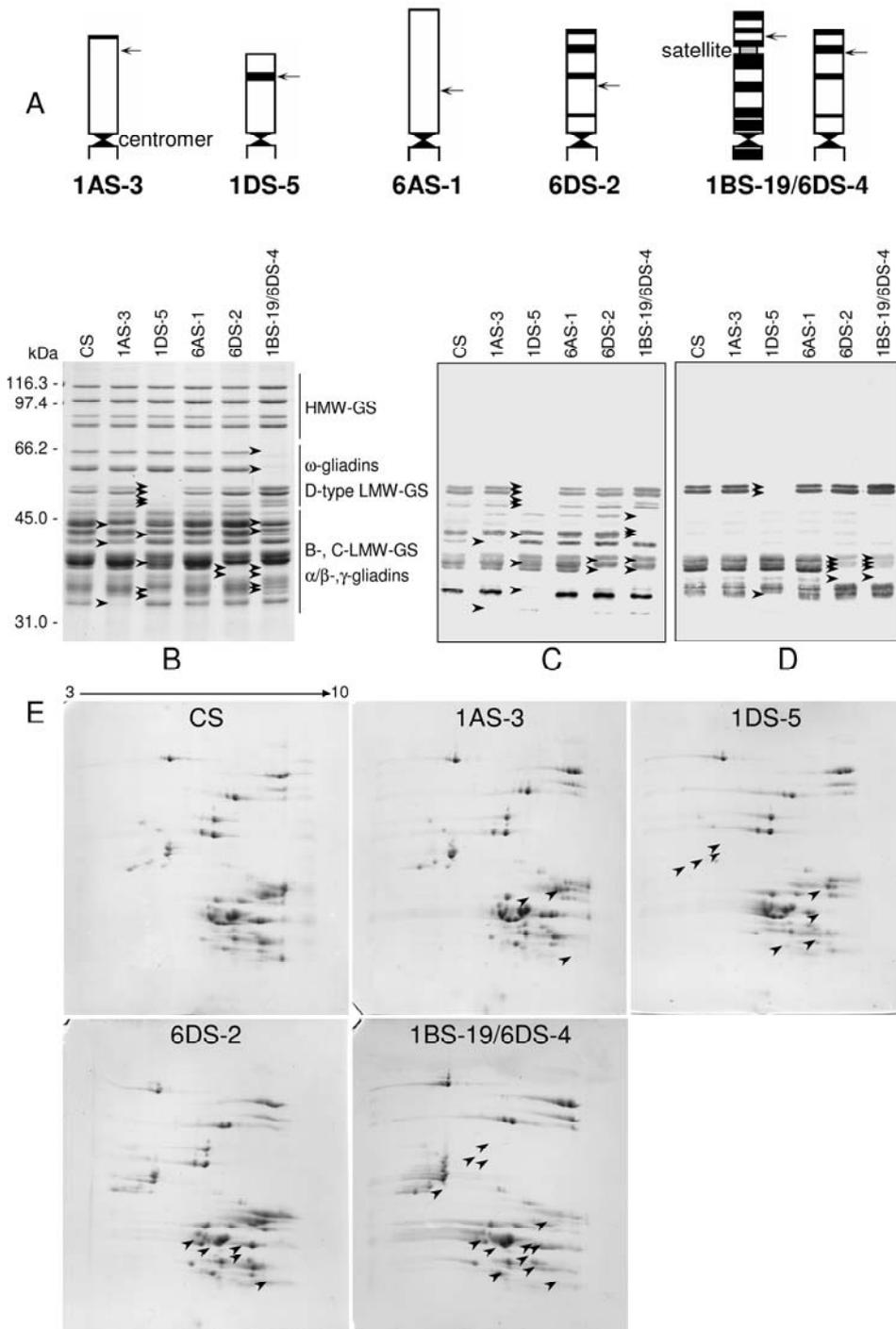


Table 1. Dough parameters obtained for 'Chinese Spring' wild type and deletion lines.

parameter	CS wt	1AS-3	1DS-5	6AS-1	6DS-2	1BS-19/6DS-4
FY	47	44	45	41	34	34
FPC	17.6±0.3 ^a	18.1±0.4 ^a	17.7±0.3 ^a	19.0±0.8 ^a	15.2±1.2 ^a	20.3±0.3 ^a
MPT	1.8±0.2 ^a	1.6±0.2 ^a	1.5±0.0 ^a	2.0±0.0 ^a	2.3±0.1 ^a	2.2±0.1 ^a
MPH	65.3±0.2 ^a	72.0±0.5 ^a	65.5±0.4 ^a	67.5±1.7 ^a	63.4±3.0 ^a	83.2±0.3 ^a
MPW	32.3±1.2 ^a	30.0±3.1 ^a	29.4±0.6 ^a	34.1±1.2 ^a	34.3±2.6 ^a	38.9±0.8 ^a
MRS	-7.2±0.9 ^a	-10.0±0.7 ^a	-9.9±0.0 ^a	-4.2±0.9 ^a	-4.0±1.3 ^a	-7.9±1.1 ^a
MTxW	15.4±0.4 ^a	16.0±0.1 ^a	13.7±0.1 ^a	16.8±0.4 ^a	19.0±1.3 ^a	18.7±0.3 ^a
ETP	83.5±11.7 ^a	89.3±13.4 ^a	70.4±3.2 ^a	89.8±3.5 ^a	97.1±10.8 ^a	123.4±6.4 ^a
R _{max}	5.9±0.9 ^d	4.8±0.6 ^d	3.9±0.4 ^b	7.2±0.7 ^c	9.8±0.9 ^c	11.9±0.8 ^c
Ext	115.7±10.3	130.4±27.4	136.2±31.2	129.9±14.1	102.3±17.1	101.7±14.5
T _½	21.3	20.5	19.3	22.3	24.1	23.1
GMP	15.3±0.0 ^a	14.8±0.2 ^a	13.6±1.9 ^a	18.7±0.7 ^a	17.3±1.2 ^a	17.5±1.6 ^a

FY: Flour yield (%); FPC: Flour protein content (%) (at 12% moisture content); MPT: Midline peak time (min); MPH: Midline peak height (%); MPW: Midline peak width (%); MRS: Midline right slope (%/min); MTxW: Bandwidth at 8 min (%); ETP: Energy to peak (%Tq/min); R_{max}: Maximum resistance (g); Ext: Extensibility (mm); T_½: Flow relaxation half time (s); GMP: GMP-volume (l/mg protein); ^astdev of two measurements; ^bstdev of four measurements; ^cstdev of five measurements; ^dstdev of seven measurements.

Stress relaxation

In stress relaxation experiments, a mechanical strain is applied to the dough to induce a stress. The time needed for relaxation of this stress after removing the strain provides information on the structure of the dough [249]. A fast relaxation may indicate the presence of small structures, whereas a slow relaxation may indicate the presence of larger structures. A piece of the same dough material as used for extension testing was used for stress relaxation testing. The relaxation halftime (T_½ in s) was calculated for the stress to decay to half the strain. Although the data were obtained from a single experiment because of limited flour starting material, a clear trend became visible. For deletion lines 6DS-2 and 1BS19/6DS-4 the T_½ was increased by 2.8 s and 1.8 s, respectively, compared to CS wt (T_½ = 21.3 s), which is an increase of 13.4% and 8.5%, respectively. An increased relaxation half time indicates a stronger gluten network. For deletion lines 1AS-3 and 1DS-5 the T_½ was decreased by 0.8 s and 1.0 s, respectively, compared to CS wt, which is a decrease of 3.8% and 4.7% respectively. Relaxation data correlated highly with the other obtained mixing parameters.

When deletions lead to a higher glu/gli ratio (as was the case in lines 6DS-2 and 1BS-19/6DS-4), the stiffness of the dough is increased, the extensibility is decreased, and the flow relaxation half time (T_½) is increased. When deletions lead to a lower glu/gli ratio (as

with lines 1AS-3 and 1DS-5), the reverse was observed. These observations are completely in line with current theory [245].

Flour and avenins from oat and addition to wheat flour

In order to investigate the potential of oat avenins to replace the absent gliadins and to restore dough quality in the 6DS deletion lines, oat flour and extracted oat avenins were added to flour of the wheat deletion lines 6DS-2. Total protein content in oat flour was $10.8\% \pm 0.1$ and water content in oat flour was 10%. The avenin content in the oat flour was 7.7 mg/g (0.8%). The oat flour used in this study was not suitable for dough preparation because oat gluten proteins do not possess the unique viscoelastic properties as wheat gluten proteins, especially by the lack of glutenins (Fig. 2 and Table 2). Oat flour was added to flour of deletion line 6DS-2 and tested by using the 2g-mixograph to see whether this would affect dough mixing properties. Replacing 10% of the 6DS-2 flour with oat flour did not significantly affect the dough mixing properties; however, replacing 25% of the 6DS-2 flour with oat flour resulted in a dough with decreased values for MPH, MPW, and MTxW and less mixing tolerance.

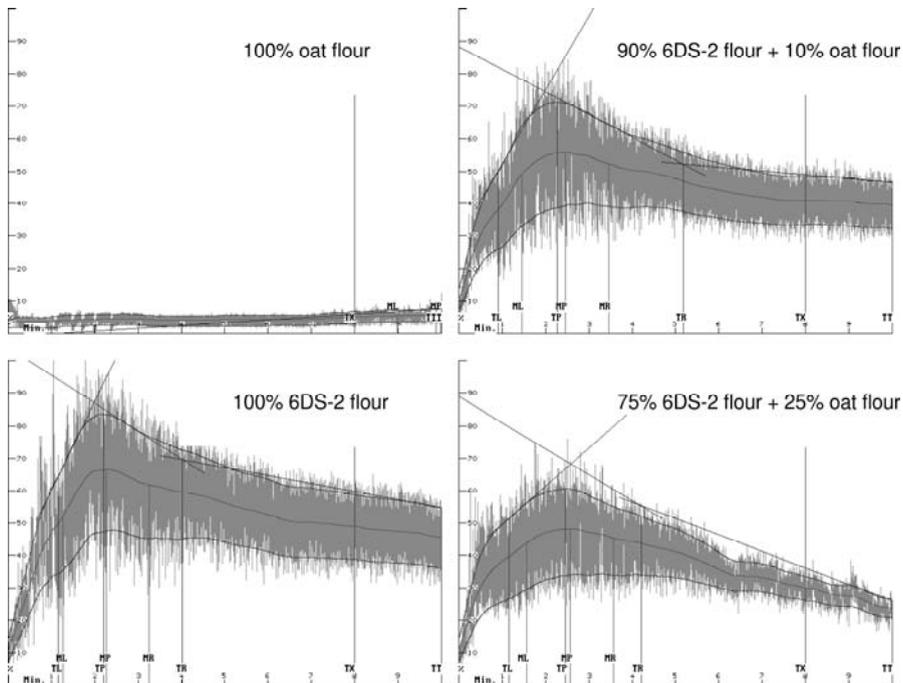


Figure 2. Mixograms of flour from deletion line 6DS-2, oat flour, and blends.

Table 2. Addition of oat flour to deletion line 6DS-2 flour. Comparison of values obtained for 2g-mixograph parameters.

	MPT	MPH	MPW	MRS	MTxW	ETP
oat	—	—	—	—	3.5	—
6DS-2	2.3	63.4	34.3	-4.0	19.0	97.1
6DS-2:oat 9:1	2.5	55.9	31.5	-4.3	14.9	102.0
6DS-2:oat 3:1	2.6	48.1	26.3	-3.6	7.3	98.0

MPT: Midline peak time (min); MPH: Midline peak height (%); MPW: Midline peak width (%); MRS: Midline right slope (%/min); MTxW: Band width at 8 min (%); ETP: Energy to peak (%Tq/min).

Because substitution with oat flour also added other components, which may influence baking quality and taste, we decided to use the avenin fraction from oat. This avenin preparation contained several protein bands (Fig. 3). Six protein bands were isolated from SDS-PAGE gel and identified by LC-MS/MS. The peptide sequences obtained could be identified as oat avenin sequences according to the Mascot protein database (results not shown). The avenins were analyzed by immunoblotting for the presence of the T-cell stimulatory epitopes GliA- 9, GliA- 20, and Gli-156 (LMW-1 and LMW-2) [77, 132] known from wheat to be T-cell stimulatory in celiac disease. No staining was observed for both epitopes when analyzing 10 g of avenins, as was expected (results not shown). Of the lyophilized oat avenin fraction, 1, 2, 5, 10, or 20 mg was added to flour of deletion lines 1AS-3, 1DS-5, 6AS-1, 6DS-2, and 1BS-19/6DS-4. Two-gram mixing experiments were performed and parameters measured included midline peak time (MPT), midline right slope (MRS), band width at 8 min (MTxW), and energy to peak (ETP).

Addition of 5 mg of avenins to CS wt flour resulted in a decreased MPT and ETP, whereas MRS was highly increased. Addition of 2 or 5 mg avenins to flour of deletion lines 1AS-3 and 1DS-5 resulted in decreased MPT, MRS, and ETP.

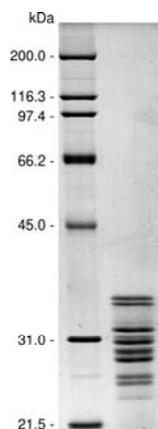


Figure 3. Avenins extracted from oat flakes ('Gigant') separated by SDS-PAGE (11%) and stained by PageBlue.

Table 3. Addition of purified oat avenins to flour of deletion line 6DS-2. Comparison of values obtained for 2g-mixograph parameters.

	MPT	MPH	MPW	MRS	MTxW	ETP	GMP
4086 (CS wt)	1.8	68.1	32.2	-9.5	15.4	85.3	15.3±0.0
4086 + 2 mg avenins	–	–	–	–	–	–	15.9±1.1
4086 + 5 mg avenins	1.4	63.9	31.8	0.7	14.8	65.7	–
6DS-2	2.1	70.7	34.2	-5.8	17.3	106.0	17.3±1.2
6DS-2 + 1 mg avenins	2.3	65.7	35.4	-4.9	19.1	105.2	–
6DS-2 + 2 mg avenins	2.6	67.1	36.3	-3.0	19.0	122.0	16.6±0.0
6DS-2 + 5 mg avenins	2.5	67.4	36.4	-1.0	18.4	123.4	–
6DS-2 + 20 mg avenins	1.8	67.9	29.7	-10.0	16.1	78.9	–

MPT: Midline peak time (min); MPH: Midline peak height (%); MPW: Midline peak width (%); MRS: Midline right slope (%/min); MTxW: Band width at 8 min (%); ETP: Energy to peak (%Tq/min); GMP: GMP-volume (l/mg)

The MTxW did not significantly change for addition of 5 mg to CS wt and both deletion lines 1AS-3 and 1DS-5. Addition of 2 or 5 mg avenins to flour of deletion line 6AS-1 only increased MRS. Addition of 5 mg to deletion line 1BS-19/6DS-4 did not significantly change MPT and ETP, but the MRS was highly decreased. Addition of 20 mg resulted in decrease in MPT and MRS. Results for addition of avenins to flour of deletion line 6DS-2 are shown in Table 3. The data show that dough strength of deletion line 6DS-2 increased up to addition of 5 mg avenins. Addition of 20 mg avenins, however, resulted in a weaker dough. Addition of 1, 2, 5, and 20 mg of avenins to flour of deletion line 6DS-2 corresponded to addition of 0.3%, 0.7%, 1.6%, and 6.6% protein, respectively.

GMP content

GMP was isolated from CS wt, 1AS-3, 1DS-5, 6AS-1, 6DS-2, 1BS-19/6DS-4, CS wt plus 1‰ oat avenins, and 6DS-2 plus 1‰ oat avenins. GMP was isolated from wheat flour by suspension in 1.5% (w/v) SDS. After ultracentrifugation the supernatant was discarded and the thickness of the gel layer was measured, from which the GMP volume in l/mg protein could be calculated. GMP isolated from CS wt and deletion lines showed different textural characteristics. GMP isolated from CS wt, deletion lines 1AS-3 and 1DS-5 appeared to be watery and flowed easily from the tube after removing the supernatant after ultracentrifugation. The GMP content from deletion lines 6DS-2 and 1BS-19/6DS-4 was higher and the GMP gel was clearly more stiff and did not flow at all. The GMP isolated from the flours with added oat avenins did not appear different compared to the GMP from the flours without addition of oat avenins.

Deletion line 6AS-1

Deletion line 6AS-1 turned out to be heterozygous as was indicated by the WGGRC and was confirmed by results from SDS-PAGE and immunoblotting (Figs. 1A, B, and C). Data obtained from mixing and extension testing did not reveal significant differences compared to CS wt. However, data from stress relaxation testing ($T_{1/2}$) and GMP extraction showed differences compared to CS wt. These values were increased indicating an increased dough strength. The flour of deletion line 6AS-1 may be a mixture of wt flour and flour from a true 6AS-1 deletion. This could, to some extent, influence dough properties because of absence of part of the gliadin proteins, which influences the glu/gli ratio.

Discussion

In this study, various deletion lines of bread wheat 'Chinese Spring' were analyzed for the effect that elimination of specific groups of gluten proteins (that contain specific CD-stimulating epitopes) might have on dough-making quality. The selected lines have deletions of gluten-encoding loci present on the short arms of chromosomes 1 and 6.

Storage proteins account for about 50% of the total protein in mature cereal grains [20] of which 80% comprises gluten proteins. This means that about 40% of the total protein in wheat consists of gluten protein. Gluten protein content obtained from CS wt and deletion lines was not as high as expected. It was ~30% from the total protein content instead of the expected 40%. This may be explained by the fact that most gluten proteins are located in the sub-aleuronic layer [250, 251], which partially remains attached to the bran after one round of milling and sieving.

Kernel texture is known to influence milling yield [252] It could be that seed development and, as a result, milling yield are influenced by the 6DS deletion. Although from literature the trait for milling yield is not mapped on chromosome 6D, it seems to be related with e.g. the *Pinb* locus on the short arm of chromosome 5A, B, and D [252-255]. Deletion line 6DS-2 also has a 5BS deletion, whereas deletion line 1BS-19/6DS-4 does not. In addition, a minor locus for kernel hardness is mapped to chromosome 6D [256, 257].

The overall gluten protein content is not changed in the analyzed deletion lines. This means that if gluten protein encoding loci are deleted, the amount of protein synthesized from the remaining gluten loci must have increased. It can be expected that removal of gluten protein encoding loci will affect dough properties because of a change in the glu/gli

ratio and in the HMW-/LMW-GS ratio. 'Chinese Spring' has null, 7 + 8, and 2 + 12 HMW-GS at the *Glu-1* loci, which results in poor baking quality [83, 258, 259] and in a sticky and weak dough [260]. The presence of gliadins can 'dilute' the gluten network and can make the dough less stiff and more extensible [247, 261-263]. Addition of gliadins, especially α - and β -gliadins, was shown to weaken the dough by decreasing the glu/gli ratio resulting in decreased mixing time and increased extensibility [84, 264, 265]. Therefore, it was assumed that removing gliadin encoding loci, as in deletion lines 6DS-2 and 1BS-19/6DS-4, would result in a less elastic and more stiffer dough when compared to CS wt, because of higher amounts of glutenins and an increase in glu/gli ratio. The latter is generally associated with a decrease in extensibility [265] and is known to increase mixing time [266]. Dough extensibility and resistance to extension can be related to gas-holding capacity of dough during bread making [248]. The ability of dough to retain gas is of key importance. Gianibelli *et al.* (1998) [267, 268] have shown that glu/gli ratios could be increased by deletion of gliadins encoded by the *Gli-2* loci, which increased MPT and polymeric protein. Deletion lines 1AS-3 and 1DS-5 indeed resulted in weaker doughs. Masoudi-Nejad *et al.* (2002) [269] showed that the deletion in line 1AS-3 is too small to delete β -gliadins, as was indicated by the presence of these proteins on A-PAGE. Our results also showed that all β -gliadins present in CS wt are still present in deletion line 1AS-3. The few proteins, most likely some LMW-GS that were removed from deletion line 1AS-3 apparently have sufficient impact to make the formation of a nice gluten network more difficult. From deletion line 1DS-5, two linked β -gliadins were removed that contain both GliA- 9 and GliA- 20 epitopes. The decrease in dough quality may be caused by the removal of these β -gliadins or by removal of LMW-GS, which will decrease the glu/gli ratio and the formation of a good gluten network. The two linked 1D β -gliadins are referred to as D-type LMW-GS [233, 234] and contain a small number of cysteine residues by which they can interact with the polymeric gluten fraction. There is an increasing interest in β -gliadins and D-type LMW-GS because of their relevance to baking quality [167, 270] and our results indicate that they may contain T-cell stimulatory β -gliadin epitopes. Several authors have shown that β -gliadins may contain epitopes involved in gluten-sensitive response of CD-patients [69, 170, 171].

In this study, oat flour and oat avenins were added separately to flour of deletion line 6DS-2 to analyze the effect on dough properties, and to determine whether the effect of the deletion could be reversed by addition of the gliadin-like proteins from oat. Oats have been proven to be well tolerated in CD-patients as part of a gluten-free diet [271 and references therein] and to contribute to the improvement of the nutritional value of a

gluten-free diet [272]. By addition of oat flour not only avenins are added but also lipids and β -glucans, which will have a negative effect on the dough properties [273]. High β -glucan content was shown to increase water absorption and also to decrease dough plasticity [274, 275]. Therefore, only limited amounts of oat flour can be included in wheat flour. Flander *et al.* (2007) [96] reported that a reasonable quality bread could be prepared with addition of 51 g oat flour per 100 g of wheat flour. This may be explained by the assumption that the monomeric oat avenins alter the ratio between monomeric and polymeric proteins in wheat.

Addition of purified oat avenins to the wheat flour might circumvent introduction of the negative effects by addition of oat flour. Our results showed that, to a certain level, the addition of oat avenins improves dough quality and probably strengthens the formation of the gluten network. Addition of oat avenins may be different from incorporation in which firstly the disulfide linkages are reduced before addition [276]. However, others have found that there is a similarity between the effects of 'addition' and 'incorporation' of glutenin subunits on dough properties [277]. Especially in deletion line 6DS-2 we observed an improvement of the dough, which may be because the avenins integrate in the wheat gluten network, although it is unknown whether avenins possess free SH-groups. The increase in values for mixing parameters suggests an increase of the HMW-/LMW-GS ratio [278] or an increased glu/gli ratio [268]. When the amount of added avenins is too high, as was seen when 20 mg avenins were added to 2 g 6DS-2 flour, the gluten network became diluted again, which resulted in a weaker dough. This result showed that reduction of CD-stimulating epitopes from gluten proteins in wheat to reduce the response in CD-patients influences the dough making quality and that this could be compensated by addition of purified avenins. Unfortunately, for industrial use the extraction of avenins from oats would be a costly and labor-intensive process. It has been demonstrated that heterologously expressed gluten reveal the same characteristic protein function as native gluten proteins [279]. To simplify the process of purification, avenins could be recombinantly expressed in bacteria or yeast, but this would have a high cost price as well. Another option to improve the dough protein network is the addition of additives such as enzymes and hydrocolloids as described by Hüttner and Arendt (2010) [280, and references therein].

Deletion lines 1DS-5 and 1BS-19/6DS-4 were missing a large amount of gluten proteins as was shown by SDS-PAGE and by immunoblotting using mAbs against T-cell epitopes GliA- 9 and GliA- 20. A comparison of immunoblotting with total gluten protein staining in the gel also showed that not all gluten proteins still present in the deletion lines contained the T-cell stimulatory epitopes GliA- 9 and GliA- 20. In CD-patients, T-cells

responding to the GliA- 20 epitope are less frequently present. This may be caused by the fact that the GliA- 20 epitope can be degraded better in CD-patients, or because deamidation occurs slower, which gives a slower response [57], or because the GliA- 20 epitope is present in much lower amounts in today's cultivated wheats compared to the presence of the GliA- 9 epitope [182]. Molberg *et al.* (2005) [76] showed by using deletion lines 1BS-19/6DS-4 and 6DS-2 in human T-cell testing a significant decrease in T-cell response in T-cells from a patient responding to the 33-mer to which 50% of the CD-patients respond [69, 156]. To be able to use the deletion of the *Gli-D2* locus in commercial wheat varieties, the locus may have to be deleted from wheat varieties with a high production and intrinsically good industrial properties. Preferably, the *Gli-D2* locus needs to be deleted without eliminating larger parts of the chromosomes, as this may affect the fitness of the crop.

Conclusion

Removing of gluten-encoding loci reduces T-cell stimulatory epitopes causing CD, but also affects dough quality. In 'Chinese Spring', deletion of the *Gli-D1/Glu-D3* loci decreased dough quality (i.e. dough strength and elasticity), but deletion of the *Gli-D2* locus improved dough strength by a decrease in elasticity. Oat avenins are a good alternative for wheat gliadins and could be added to the flour of certain wheat lines that lack CD-stimulating encoding gluten loci, to improve or restore dough quality.

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Chapter 8

General Discussion

Celiac disease

Celiac disease (CD) has become a worldwide public health problem with an occurrence of about 0.5%–2% of the Western population, of which about 70–97% is still undiagnosed. CD-patients are genetically susceptible and it has been hypothesized that they lack specific brush border enzyme activities to degrade gluten peptides rich in the amino acids proline and glutamine completely [48, 281, 282]. Undegraded gluten peptides can enter the lamina propria and certain peptides (epitopes) can be recognized by T-cells when presented by antigen presenting cells (APCs), leading to inflammation of the small intestines. Therefore, CD-patients are restricted to a lifelong gluten-free diet to prevent symptoms such as malnutrition, malabsorption, diarrhea, abdominal pain, and in the worst case lymphoma and cancer as can occur in refractory celiac disease (RCD) in which patients no longer respond to a gluten-free diet [197, 283-286]. At the same time, CD can occur in a silent form without any clear symptoms and with or without villous atrophy and histological changes [287-292]. In addition, other organs beyond the gastrointestinal system can be affected by gluten sensitivity including joints, the heart, thyroid, bone, brain cerebellum and the neuronal synapsins, which are involved in the regulation of the neurotransmitter release [293]. CD can develop at any age but exposure to gluten at a young age by breastfeeding delays the development of CD [60, 195, 294-297]. In children, CD can lead to growth retardation. Adherence to a gluten-free diet is of high importance for CD-patients because it allows healing of the intestinal mucosa, thus removing symptoms, and it improves the quality of life. Maintaining to a gluten-free diet, however, is very difficult because gluten proteins are increasingly applied as ingredient in many processed foods [1-4]. Wheat gluten and starch can also be present as a ‘hidden’ and not expected ingredient in food products and even in medication, which causes problems especially to the very sensitive CD-patients. This will also increase the exposure to gluten in the many, still undiagnosed, CD-patients and increases the development of symptoms in CD-patients that, so far, were symptom-free. Nowadays, food labeling of gluten-free products still lags behind. It is only recently that labeling of wheat constituents in foods is regulated and mandatory. This does not safeguard CD-patients, since producers are not always aware that their ingredients may contain gluten. In addition, the gluten-free market is a niche market, making gluten-free products limited available and expensive and thus only relevant for diagnosed CD-patients and not for prevention. In a common Western diet about 20 g of gluten is consumed per day [298]. For a food to be labeled as ‘gluten-free’, the maximum amount of gluten is 20 mg/kg (Commission Regulation (EC

No. 41/2009), which corresponds to about 7 wheat kernels per kg. Another category is 'low-in-gluten' labeled food, in which the maximum of gluten is 100 mg/kg.

In developing countries, such as China and India, consumption of wheat is increasing since rice is being replaced by wheat as a staple food. This means that an increase of CD can be expected in the near future. In India the prevalence of CD is already increasing and there is no legislation for gluten labeling. As a result patients are not able to stick to a gluten-free diet [299]. In the United States, although working on regulations, there are currently no regulations for 'gluten-free' labeling on food products [300]. To adhere to a gluten-free diet requires reading of food labels. Of the Dutch population, only 1.5% is illiterate, but most illiterate persons are found in Southeast Asia (46%) followed by Africa (39%) (<http://nl.wikipedia.org/wiki/Analfabetisme>), which may considerably hinder the purchase of gluten-free foods (if available) and cause an early ending of the gluten-free diet.

CD-patients show symptoms such as malabsorption because of a flat mucosa. Therefore, a gluten-free diet not only requires exclusion of all gluten proteins, but should contain sufficient amounts of proteins, vitamins, minerals, and fibers. Oats have been proven to be well tolerated by most of the CD-patients as part of a gluten-free diet and contributes to the improvement of the nutritional value of a gluten-free diet [271 and references therein, 272]. However, no long-term studies (10–30 years or more) of the effect of oat consumption by CD patients have been completed yet. Few CD-patients (1% of the diagnosed CD-patients) show an immune response and intestinal damage to oat avenins [203, 301, 302]. The most important issue of introduction of oat to the gluten-free diet is that there should be no contamination with wheat, rye or barley, which can easily occur if this is not taken into account. When 'pure oats' can be grown, it can contribute to decreased exposure to CD-epitopes.

The study performed in this thesis focuses on the identification and characterization of wheat varieties that are low in CD-epitopes to which the majority of CD-patients respond and that also have good commercial potential. A requirement in the development of wheat varieties low in CD-epitopes is to maintain the technological properties of wheat to produce a viscoelastic dough for preparation of food products, especially bread. Ultimately, when wheat low in CD-epitopes can be grown commercially, the development of symptoms in CD-patients will be reduced and the exposure to wheat gluten will be reduced for the entire population including the undiagnosed CD-patients.

This left us with the question: how to develop CD-safe wheat while retaining technological properties? This will be challenged and discussed into more detail in the

following paragraphs by addressing a set of questions on CD-epitopes, screening, and on elimination and modification with relevance to technological properties.

What are the epitopes to screen for in wheat?

For more than 20 years, many epitopes have been identified and reported [38, 42, 47, 58, 67, 68, 77, 138, 171, 203, 303-313]. Epitopes were tested *in vitro* and *in vivo* to support their CD-stimulatory response. The epitopes having the highest immune response are characterized by the fact that they cannot be degraded in the human gastrointestinal tract and by the presence of glutamine residues that can be deamidated by tTG. Important is the position of these glutamines, and the rate by which they become deamidated [57]. Gluten proteins are very complex and because of the presence of three homoeologous genomes in bread wheat, many gluten proteins exist. The α -gliadins harbor the most active epitopes, because they may contain the 33-mer (LQLQFPQPQLPYQPQLPYQPQLPYQPQPF) that can not be degraded further. This 33-mer contains the peptides PFPQPQLPY (Glia- 9; I), PQPQLPYPQ (Glia- 2; II), and PYPQPQLPY (Glia- 9; III) [47], which individually also stimulate T-cells and bind to HLA-DQ2 or DQ8. The complete sequence of the 33-mer is only found in proteins encoded by chromosome 6 of the D-genome (Salentijn, personal communication). The sequence PFPQPQLPY (Glia- 9; I) is found in proteins encoded by the 6A- and 6D chromosome, whereas the sequences PQPQLPYPQ (Glia- 2; II) and PYPQPQLPY (Glia- 9; III) are found in proteins only encoded by chromosome 6D [75]. The most immunogenic epitopes are therefore located on the short arm of chromosome 6D [74, 75]. About 50% of the children with CD respond to the 33-mer [58]. Cornell and Stelmasiak (2007) [282] identified another peptide, RPQQPYPQPQPQ, that could not be degraded further in CD-patients, but was degraded by duodenal mucosa from controls. This peptide is identified as the Glia- 20 epitope [58]. The optimal length of a peptide causing T-cell response is 10 to 15 amino acids [314, 315]. Binding studies showed which peptides could bind to the HLA-DQ molecules and which positions are relevant in binding [67, 311, 316-318]. Residues can become negatively charged by deamidation by tTG. A single deamidation is already sufficient for strong binding [67, 157]. Some peptides, however, stimulate T-cells in the natural state and require no deamidation. This seems to occur more often in children than in adult CD-patients [58]. These peptides are derived from glutenins, α -gliadins, and β -gliadins (Glia- 20 epitope) [314]. The binding and presenting of the peptide to the T-cell receptor (TCR)

is the main event in the adaptive immune response of CD. Next to initiation by peptides of the adaptive immune system, some gluten peptides can also trigger the innate immune system. The gluten peptide identified in the innate immune response, peptide 31–49 or 31–43, can be present in α -gliadins encoded by the short arm of chromosome 6A, B, and D [75]. Of the innate gluten peptide, deamidation is not required to stimulate immune response [59 and references therein]. Innate immune response is especially found in HLA-DQ2 CD-patients and may be necessary to initiate the adaptive immune response [71]. However, the exact mechanism of innate response is still under debate [319, 320].

In the study performed in this thesis, we used several criteria for analyzing the presence of relevant CD-epitopes. The focus was on HLA-DQ2 restricted CD-epitopes from α -gliadins because these are the most active, have proven immune response, and are not easily degraded in CD-patients. Therefore, we selected epitope Glia- 9, which is an immunodominant CD-epitope and is present within the 33-mer, and epitope Glia- 20, which is indicated as a minor CD-epitope, as observed in two cohort studies [58, 69]. The selected epitopes do not cover all epitopes but are of main importance because of the availability of reliable and specific monoclonal antibodies. Furthermore, they are present in gluten proteins encoded by the A- and D-genome. In durum wheat, the D-genome is absent and is therefore more ‘safe’ than bread wheat containing the D-genome.

How to screen for CD-epitopes in wheat?

Screening for wheat varieties low in CD-epitopes requires an accurate qualitative or quantitative detection method and knowledge about the safe gluten threshold for CD patients (which might be different among patients [321]). For CD-epitope screening, different methods can be used such as T-cell testing and antibody based assays. Testing of wheat species using T-cells relies on the availability of epitope specific T-cell clones obtained from patients. A T-cell clone is produced from a single T-cell from a single patient. Although hypothetically many T-cell clones with a typical epitope-specificity can be obtained from many patients, this detection system directly shows potential drawbacks such as the questionable representation of the entire CD-population and the technical laborious of the T-cell based screening tests.

Immunoassays

For detecting levels of gluten in food products, immunoassays can be performed based on mAbs. Monoclonal Ab staining is less laborious than T-cell testing and is the only way to perform comparative analysis of large numbers of wheat varieties. It is currently the only tool to quantify the level of CD-epitopes in wheat breeding programs. Assays using mAbs depend on the specificity of the antibody used and on the standards used as a reference. Monoclonal antibodies have been raised against gluten proteins, gluten extracts, and against CD-epitopes by many researchers [77, 121, 124-126, 131, 322-328]. Important is that mAbs used in detection kits do not only detect wheat gluten, but also gluten proteins from rye and barley to confirm the presence of all proteins containing CD-epitopes. Detection is quantitative and the extraction method should extract the complete gluten content from the sample for correct quantification. Detection also needs to be very sensitive (<20 ppm) to secure the labeling of 'gluten-free' on products. The ELISA based detection kits can be performed in any lab equipped with a plate-reader. ELISA assays were primarily developed for detection of gluten proteins and not necessarily for detection of CD-epitopes. At this moment, the R5-antibody ELISA as developed by Sorell *et al.* (1998) [125] is approved by the Codex Alimentarius as the method to detect whether a food product can be labeled gluten-free [130]. The R5 antibody detects gluten proteins in general and not only gluten proteins containing CD-epitopes. Recently, a new competitive ELISA has been developed by EuroProxima (<http://www.europroxima.com>) named Gluten-Tec[®] ELISA based on mAbs against the CD-epitopes GliA- 9 and GliA- 20 [126, 132, 329].

The sequence recognized by the mAb raised against the T-cell epitope is usually shorter than the sequence recognized by the T-cell. As a consequence, screening using mAbs might result in overestimation of the number of T-cell epitopes that are actually present. Overestimation is better to accept than underestimation, but needs to be taken into account when screening for CD-epitopes.

For the study performed in this thesis, a qualitative screening method was applied using immunoblotting and mAbs raised against non-deamidated CD-epitopes GliA- 9 and GliA- 20 as described by Spaenij-Dekking *et al.* (2004, 2005) and Mitea *et al.* (2008) [77, 126, 132]. A complete extraction method was developed for isolating intact gluten proteins from wheat samples. Obtained immunoblots were also used for quantitative densitometric analysis.

To be able to screen quickly a large number of wheat varieties for variation in the presence of CD-epitopes, a few technological hurdles had to be taken, which are described in Chapter 2 and 3. To screen for gluten proteins present in many wheat varieties, an extraction method should be used that extracts all of the gluten proteins present in the

wheat kernel. Chapter 2 reports on the analysis of different extraction protocols described in literature showing that gliadins and glutenins can not be separated completely, because of their overlapping solubility in aqueous alcohol solutions with or without a reducing agent. To meet this requirement, an extraction protocol was developed that combines extraction steps for gliadins and glutenins resulting in a single extract that contains both groups of gluten proteins. Chapter 2 also shows the screening of these gluten protein extracts by immunoblotting using monoclonal antibodies (mAbs) against the GliA-9 epitope and against the Glt-156 epitope present in LMW-GS. Clear differences are seen between different extraction protocols, and our two-step extraction protocol shows a highly complete extraction of all gluten proteins. The often used ELISA screening method for the presence of gluten proteins, as in the RIDASCREEN[®] Gliadin competitive ELISA, uses an antibody (R5) that is not specific for T-cell epitopes stimulating CD. This has important consequences and asks for consideration if these methods are to be used in testing for 'gluten-free' food labeling. Because the R5 antibody is not specific it will result in an over- or underestimation of the actual number of epitopes present. Limitations of gluten detection methods involve the used mAb that needs to be specific but also the used gluten standard that should be well characterized [330, 331].

To screen wheat varieties in this study, an ELISA based assay was not used, because at that moment extraction procedures were still incomplete and used mAbs did detect more than only CD-epitopes.

Chapter 3 reports on the visualization of gluten proteins using SDS-PAGE gels. Because of the structure of gluten proteins (high in glutamine and proline; low in lysine, arginine, and histidine) different staining methods result in different images. An end-staining method, such as PageBlue staining, appeared the best method for visualizing all gluten proteins.

Next generation sequencing

For high throughput generation of DNA sequences, new DNA sequencing techniques are being developed continuously and 'next-next' generation sequencing methods are under development. These sequencing techniques result in thousands of sequences and start with just single molecules derived from transcripts. In this way, expressed genes can be detected in various developmental stages of wheat kernels from which expression levels can be analyzed. The disadvantage of this method to screen for CD-epitope levels is that mRNA has to be extracted from a certain stage during kernel development. Another disadvantage is that mRNA levels do not always correlate to protein levels [332 and references therein]. Therefore, screening methods based on next generation sequencing

of mRNA (converted into cDNA) extracted from wheat kernels in developmental stages, may not reflect the actual presence and level of gluten proteins in the mature kernels.

Proteomics

An even more direct method to screen for presence and level of CD-epitopes in gluten proteins from a certain wheat variety is liquid chromatography (LC) coupled to mass spectrometry (MS) by which the mass of proteins or proteolytic degraded peptides can be analyzed and quantified [333-336]. Mass spectrometry can be used as a marker technique to identify CD-toxic and non-CD-toxic peptides. To identify small structural differences, electrospray ionization (ESI) MS in combination with high pressure liquid chromatography (HPLC) can be applied because of the high resolution. Commonly, gluten protein extracts are first separated by 2-DE and protein spots are excised from the gel followed by proteolytic digestion, generally with trypsin [337]. Trypsin cleaves proteins C-terminal to lysine and arginine, but not if followed by proline. However, gluten proteins from wheat have an atypical amino acid sequence and are low in both these amino acids. Therefore, an alternative for proteolytic digestion of gluten proteins is needed e.g. by using combinations of pepsin, trypsin, chymotrypsin and thermolysin [338, 339]. Unique peptides can be created and targeted peptides can be used for identification and quantification.

The sequences and structures of gluten proteins are very similar. The same holds for their mobility when separated by SDS-PAGE. For true identification of peptides by sequence or by mass, these peptide, protein or DNA sequences should be present in the database that is used for identity search [339]. Development of a large database containing many gluten gene sequences from different cultivars helps the proteomics analysis methods to a high extent.

Proteomic analysis can be performed using the mature wheat kernel in comparison to analysis by cDNA sequencing in next generation sequencing, which starts from mRNAs that are abundantly present in developmental wheat kernels but less in mature kernels.

What to screen, where to start?

Many thousands of wheat varieties exist, both modern and old hexaploid and tetraploid varieties, landraces, cultivated and wild species, and the ancestor diploid species. Also non-natural hexaploid wheats were developed including e.g., synthetic hexaploid wheats

(<http://www.k-state.edu/wgrc/Germplasm/synthetics.html>) and deletion lines in hexaploid wheat 'Chinese Spring' (http://www.k-state.edu/wgrc/Germplasm/Deletions/del_index.html). So far, the presence of CD-epitopes in all these wheat accessions has only been analyzed in limited amounts [76-78, 101-106]. Diploid and tetraploid wheats are commonly shown to contain less CD-epitopes compared with hexaploid wheats because of the absence of the D-genome. Screening wheat varieties in this study is performed by using immunoblotting with specific mAbs against CD-epitopes by the strategy that is described and shown in Fig. 7 in Chapter 1 (General Introduction). If screening of different hexaploid wheat varieties that are nowadays grown and used for breeding would result in the discovery of a variety that is reduced in CD-epitopes, this line would immediately be applicable for further breeding. However, when the genetic diversity among the modern hexaploid varieties would not be large enough and a variety low in CD-epitopes cannot be identified among modern hexaploid wheats, older hexaploid varieties, including landraces, can be analyzed. Therefore, we analyzed the occurrence and level of CD-epitopes in a set of modern wheat varieties from Europe and compared this with a set of older varieties and landraces obtained from all over the world. Results in Chapter 4 show that genetic diversity is indeed reduced in modern wheat varieties compared with the older varieties and landraces. Modern wheat varieties appear to contain an increased number of GliA- 9 epitopes and a reduced number of GliA- 20 epitopes. That may not only explain why currently patients respond more to the GliA- 9 epitope and not so many patients respond to the GliA- 20 epitope [58, 69], but also be the reason for a higher prevalence of CD, because the level of the immunodominant epitope GliA- 9 seems to have increased as a result from breeding during the last decades. Wheat varieties used for breeding are mainly selected for high yield and good baking properties. So far, breeders did not include selection for presence of CD-epitopes, which will probably influence baking properties. Older hexaploid varieties were identified that are reduced in CD-epitopes, compared with modern varieties. Further breeding can be started aiming at reduced levels of CD-epitopes as a new quality trait.

The next step would be the analysis of tetraploid wheats used for pasta preparation to search for an accession low in CD-epitopes. Such a tetraploid (durum) wheat can be used in the development of a hexaploid bread wheat low in CD-epitopes, by hybridization with a diploid D-genome species low in CD-epitopes. The resulting synthetic hexaploid may, however, show different gluten protein patterns because of epigenetic regulation of gene expression [340, 341] and still needs to be tested for presence of CD-epitopes. Chapter 5 reports on the screening of tetraploid accessions including landraces, old,

modern, and domesticated accessions of various tetraploid species and subspecies from many geographic origins. The resulting immunoblot banding patterns of these tetraploids are less complex compared with the immunoblot patterns of hexaploid accessions. This is most likely caused by the absence of the D-genome that contains many ω -gliadin epitopes. However, from the locally obtained tetraploid accession nearly 25% is contaminated with hexaploid wheat. Durum wheat may contain up to 3% of common wheat, legally [342], but for selection of accessions low in CD-epitopes this is not useful. Tetraploid accession should be selected that are reduced in CD-epitopes when compared with hexaploid wheats, and need therefore be uniform.

GMO approach

Although selection of wheat varieties and accessions may result in useful lines low in CD-epitopes further improvements will still be needed for enhanced reduction of the amount of CD-epitopes or with regard to the technological quality. Here, the application of genetic modification (GM) may become relevant. Several strategies are possible. RNA interference (RNAi) can be used for targeted down-regulation of any gene of interest. Constructs resulting in hairpin-shaped RNA molecules produce interfering RNAs that silence the expression of genes or even whole gene families. RNAi has shown to be an effective method to reduce the amount of CD-epitopes. Taking into account that wheat is hexaploid and that the many gluten genes are very similar, complete elimination seems unlikely [343]. Gil-Humanes *et al.* (2008) [236] showed that by using an RNAi construct containing a part of a ω -gliadin, most of the ω -gliadins were not expressed in progeny plants. The disadvantageous consequence by choosing ω -gliadin was that other gluten genes such as the γ -gliadins were higher expressed. This will increase the number of ω -gliadin epitopes and make the wheat not useful for consumption by most CD-patients responding to ω -gliadin epitopes and also not for decrease in prevalence of CD. Wieser *et al.* (2006) [235] and Becker *et al.* (2006) [344] described the use of an RNAi construct containing a part of an ω -gliadin gene that resulted in the loss of ω -gliadins (>63%) in progeny plants, which was compensated by increased levels of γ - and β -gliadins. These wheats might be of interest for most CD-patients responding to ω -gliadin epitopes, but not for CD-patients responding to γ -gliadin epitopes. Vader *et al.* (2002) [58] and Camarca *et al.* (2009) [69] demonstrate that many CD-patients may have a specific tendency in their sensitivity towards either ω -gliadin or γ -gliadin epitopes. The silencing of the ω -gliadins may now result in a wheat variety that might be tolerated by the ω -gliadin sensitive CD-patients, irrespective of the amount of ω -gliadin epitopes, and vice versa. In addition, the interesting

question now is what about the gliadin expression (and complementary compensation) in crossings between -gliadin- and -gliadin-silenced lines?

Tetraploid wheats reduced in CD-epitopes can also be improved for baking quality through a GMO-approach by transfer of HMW-GS from the D-genome, *Glu-D1* locus, to chromosome 1A without adding the gliadins [192, 193]. Or more specifically by transformation using genes encoding both the 1Dx5 and 1Dy10 HMW-GS as these are the glutenin proteins especially involved in the formation of a high quality dough network [191, 194, 345].

The use of transgenics for consumption is, however, still in debate. CD-patients will probably accept more easily the use of genetic modification because of their desire for CD-safe wheat and food products. If transgenic wheat would be used for all wheat products to prevent CD and to reduce the exposure to CD-epitopes for the entire population, it will probably not be acceptable for many non CD-patients. To convince all consumers to accept genetic modification, it probably first needs to be proven that there is improvement of health aspects for all. GM CD-safe wheat will for the present be limited to the 'gluten-free' niche market.

Zinc finger nuclease technology

Zinc finger technology can be applied to specifically eliminate targeted gluten loci containing CD-epitopes. For example, the *Gli-D2* locus on chromosome 6D could be targeted leaving all other loci intact. By introduction of zinc finger nuclease domains, the targeted locus will be knocked out [346, 347 and references therein]. By selection of the right wheat variety on baking properties, this locus, encoding the main CD-epitopes, can be removed without decreasing the baking quality. The technique is still under development regarding its application in plants, and especially in genetically complex traits like CD-epitopes. It may take several years before the technique will have proved to be successful in wheat and it is still considered to be a GMO approach.

What about technological properties?

With the development of CD-safe wheat the baking quality needs to be maintained for the applicability on commercial scale. To maintain technological properties of wheat certain gluten proteins cannot be removed, while other gluten proteins can be removed without affecting dough properties. Technological quality and its maintenance are the subjects of Chapters 6 and 7. These Chapters show the analysis of deletion lines of bread wheat

'Chinese Spring' that are missing the gluten encoding loci *Glu-1*, *Glu-3*, *Gli-1*, *Gli-2* and *Gli-3* located on chromosomes 1 and 6. Absence of these loci reduces the number of CD-epitopes significantly, but also has an effect on dough properties. Dough properties were analyzed by mixing experiments, stress relaxation testing, extensibility testing, and glutenin macropolymer (GMP) analysis, because these tests best reflect dough quality and bread making properties [248, 249, 348]. These tests allow small scale analyses to be performed on the limited amount of kernel material available. HMW-GS and LMW-GS are very important for the dough structure because of the formation of the gas retaining gluten network. Deletion of the loci on the long arms of chromosome 1B and 1D encoding HMW-GS (no HMW-GS are expressed by chromosome 1A in 'Chinese Spring') results in loss of dough structure, which proves that HMW-GS encoded by both loci are definitely needed in dough preparation. Similar results were obtained from studies on specific deletion lines having different compositions of HMW-GS [237]. Deletions of loci from the short arms of chromosomes 1A and especially of chromosome 1D remove many CD-epitopes. In case of 1AS and 1DS deletions, LMW-GS and α - and β -gliadins encoding genes are removed. Absence of LMW-GS prevents the formation of a strong gluten network with the HMW-GS and results in weaker, more elastic dough. Deletion of the locus from the short arm of chromosome 6 (6DS) removes γ -gliadins containing CD-epitopes, including the 33-mer to which most CD-patient respond [46, 76]. 'Chinese Spring' is used as model wheat for research purposes and has poor dough making quality (wet and sticky dough). Because gliadins act as plasticizers in the gluten network [247, 261-263], removal of gliadins from 'Chinese Spring' results in a more stiff dough. Gliadins are therefore necessary in high quality bread wheat with a strong gluten network. In addition, it is analyzed whether CD-safe gliadin-like proteins from oat, the avenins, can compensate wheat gliadins. Addition of purified avenins to flour of the 6DS deletion line shows to improve dough quality and results in a more stiff dough. Our studies show that clear limits exist in removing gluten proteins. However, removal of γ -gliadins, which also removes major CD-epitopes, is possible and can be compensated by addition of oat avenins.

Crosses were performed between two lines having deletions on the short arms of chromosome 1D and 6D, respectively. Progeny plants that carried both deletions show a reduction of a large amount of CD-epitopes as was shown by immunoblotting. This new deletion line has to be tested for dough quality. The final proof of principle is testing of all deletion lines for bread making properties on a regular scale, but this requires at least 1 kg flour. The 'Chinese Spring' deletion lines may be used in breeding as a receptor to introduce high quality HMW-GS to improve baking properties.

For breeding bread wheat with lower amounts of CD-epitopes and reduced numbers of gliadins containing these epitopes, lower quality HMW-GS may be selected for. Such wheat produces a less strong gluten network that doesn't need gliadins to dilute it. In general, the 'deletion line-approach' seems to be useful for other wheat varieties that have a higher baking quality and not necessarily need α -gliadins (and β -gliadins) in their gluten network.

Final conclusions

In case of CD, both prevention and treatment are of the utmost importance. The results presented in this thesis mainly contribute to prevention. Since the majority of the CD-patients is undiagnosed (70–97% of the CD-patients) and wheat and wheat constituents are increasingly applied in food products, reduction of CD-epitopes should become a major and general objective in wheat breeding. We have shown that expanding screening protocols used in wheat breeding with screening for CD-epitopes (e.g. GliA-9 and GliA-20) will be highly beneficial and lead to a decreased exposure. We feel that the availability and use of wheat low in CD-epitopes on a large scale will also benefit the large undiagnosed CD-population as well as the diagnosed CD-patients. Use of CD-safer wheats will also lead to less 'unintended' exposure and with that improve the quality of life for diagnosed and undiagnosed CD-patients. Companies that breed wheat for human consumption should start to analyze and quantify their wheat varieties for CD-epitopes and use these selection criteria in breeding. In time, we expect that this can lead to a significantly lower prevalence of CD. Using these screening tools, we were able to identify tetraploid wheats and hexaploid wheats with reduced amounts of CD-epitopes. In addition, we developed a hexaploid wheat low in CD-epitopes by crossing two hexaploid wheat deletion lines. We have not been able to find wheat varieties with no CD-epitopes, which might be explained by the use of mAbs in the screenings that recognize shorter sequences than the human T-cells, and will result in an overestimation of the presence of epitopes. Future studies, involving next generation sequencing and proteomics, next to mAb assays and T-cell testing, will lead to improved methods for accurate quantification of CD-epitopes. We are confident that the methods and materials developed and the strategies proposed in this thesis will contribute to further approaches directed to the production of CD-safe wheat and wheat containing food products.

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Summary

Gluten proteins from wheat have the unique property to interact with each other and form a network in dough preparation. In this gluten network gas bubbles can be retained that are produced by yeast, which is added during dough preparation. The result is a voluminous, viscoelastic dough for bread making. Besides these network forming properties, gluten proteins contain remarkably high amounts of the amino acids proline and glutamine. Therefore, these proteins can not be degraded completely in the human gastrointestinal tract and so called epitopes are generated in the small intestine after ingestion of gluten containing foods such as bread and cookies. Intolerance to gluten proteins can cause inflammation of the small intestine, which leads to villous atrophy (flatten mucosa) and malabsorption. This intolerance to gluten proteins is also called celiac disease. The with celiac disease occurring inflammation can lead to many symptoms such as malnutrition, weight loss, stomach pain, diarrhea, dermatitis and in the worst case lymphoma. In children, celiac disease can cause growth retardation. Gluten proteins from rye and barley, because of their similarity to gluten proteins from wheat and high proline and glutamine content, can also cause celiac disease. Celiac disease patients therefore have to avoid life-long all food products containing wheat, rye, and barley (gluten-free diet). Not only gluten protein intake but also genetic susceptibility is necessary to develop celiac disease. Therefore, relatives of celiac disease patients have a highly increased risk to develop celiac disease. About 0.5–2% of the Western population suffers from celiac disease of which 70–97% is undiagnosed, and the prevalence of celiac disease is still increasing. The reason for this may be the consumption of many bread, cookie and pasta products, but also because wheat constituents, such as gluten and starch, are increasingly used as food additive in soups, sauces, sausage, candy, ice-cream, and even in medicines. Because bread wheat originated thousands of years ago by fusion of three different grass species, many different gluten proteins exist. Pasta wheat originated from two of the three grass species and lacks the species that contains most of the celiac disease epitopes. Not all gluten proteins can be removed because baking properties might be lost. For analysis of the presence of celiac disease epitopes, it is important that all gluten proteins are extracted from the wheat kernels. Therefore, an extraction protocol was developed to extract as many gluten proteins as possible, which allows analysis of many samples at the same time. Because thousands of different wheats exist that have never been tested for celiac disease epitopes, this extraction and detection method was applied to analyze whether modern wheats, which are used by breeders, exist with lower amounts of celiac

disease epitopes. These modern wheats were subsequently compared to old wheats collected from all over the world. From these analyses it seemed that there was an increase in celiac disease epitopes in modern wheats. This might be the reason for the increase of prevalence of celiac disease. For future breeding of bread wheat it is important to select and breed not only for high yield, disease/pest resistance, and baking quality, but also for the (reduced) presence of relevant celiac disease epitopes. In older bread and pasta wheats, we observed that fewer celiac disease epitopes are present. Knowing this, a more celiac-safe bread wheat could be developed by performing crossings with a selection of wheats. In the analysis of wheat lines that miss specifically genetic parts of one of the three grass species from which bread wheat evolved, it appeared that if the part is removed that encodes celiac disease epitopes to which most patients respond, the dough properties improved compared to the control dough. By addition of similar proteins from oat (avenins), the dough properties improved even more. These gluten proteins from oat are tolerated by most celiac disease patients. The results show that it is feasible to develop wheat that contains less celiac disease epitopes without decreasing dough properties.

Celiac disease patients, however, respond differently to different celiac disease epitopes which complicates the development of wheat that is suitable for all celiac disease patients. The research performed in this thesis shows that selection of wheat for the presence of celiac disease epitopes is extremely relevant because it can reduce the number of celiac disease patients in the future. At the same time, we demonstrate that celiac-safe wheat can still maintain good baking properties. Diagnosed celiac disease patients will benefit as well from celiac-safe wheat because exposure to 'hidden' gluten proteins will decrease.

Samenvatting

Gluteneiwitten uit tarwe bezitten de unieke eigenschap dat ze met elkaar bindingen aan kunnen gaan en zo een netwerk kunnen vormen tijdens deegbereiding. In dit glutennetwerk worden gasballetjes vastgehouden die door de aanwezige gist worden geproduceerd gedurende de deegbereiding. Hierdoor ontstaat een luchtig geheel voor broodbereiding. Naast hun eigenschap dat ze een netwerk kunnen vormen, zijn gluteneiwitten ook bijzonder omdat ze extreem veel van de aminozuren proline en glutamine bevatten. Hierdoor kunnen ze niet goed worden afgebroken in het menselijk maag-darmstelsel en ontstaan er zogenaamde epitopen in de dunne darm na consumptie van glutenbevattende producten zoals brood en koekjes. Bij mensen met glutenintolerantie kunnen er problemen optreden die tot ontsteking van de dunne darm leiden waarbij de villi in de darmwand worden afgebroken dat vervolgens leidt tot malabsorptie. Deze intolerantie voor gluteneiwitten wordt ook wel coeliakie genoemd. De bij coeliakie optredende ontstekingen kunnen tal van lichamelijke klachten geven zoals ondervoeding, gewichtsverlies, pijn in de darmen, diarree, dermatitis en in het ergste geval darmkanker. Kinderen kunnen tevens een groeiachterstand oplopen. Gluteneiwitten uit rogge en gerst kunnen, omdat ze veel lijken op gluteneiwitten uit tarwe en veel prolines en glutamines bevatten, ook leiden tot dezelfde symptomen in coeliakiepatiënten. De enige remedie voor coeliakiepatiënten is op dit moment om geen gluteneiwitten uit tarwe, gerst en rogge te consumeren. Zij moeten daarom alle producten waar deze granen inzitten hun leven lang vermijden (glutenvrij dieet). Niet alleen de inname van gluteneiwitten, maar ook erfelijke aanleg is essentieel voor het ontwikkelen van de ziekte en daarom hebben familieleden van coeliakiepatiënten ook een grote kans om coeliakie te ontwikkelen. Circa 0,5–2% van de Westerse bevolking heeft coeliakie, waarvan ongeveer 70–97% nog niet gediagnosticeerd is. Er is ook nog steeds een toename te zien in het aantal patiënten. Dit komt mogelijk doordat we tegenwoordig veel meer gluten binnen krijgen via allerlei brood, koek- en pastaproducten, maar ook omdat tarwebestanddelen, zoals gluten en zetmeel, steeds vaker worden toegevoegd aan vóorbewerkte producten zoals soepen, sausen, worst, snoepjes, ijs en zelfs medicijnen. Doordat broodtarwe duizenden jaren geleden ontstaan is uit een samensmelting van drie verschillende grassoorten, komen er veel en verschillende gluteneiwitten voor. Pastatarwe is ontstaan uit twee van de drie grassoorten en mist de soort waarin de meeste coeliakie-epitopen aanwezig zijn. Niet alle gluteneiwitten kunnen worden verwijderd, omdat dan de broodbakeigenschappen verloren gaan. Voor het analyseren van de aanwezigheid van

coeliakie-epitopen is het belangrijk dat alle gluteneiwitten uit de tarwekorrels geïsoleerd worden. Daarvoor werd een extractieprotocol opgezet dat zoveel mogelijk gluteneiwitten extraheert en ook dusdanig efficiënt is dat veel tarwemonsters tegelijkertijd geanalyseerd kunnen worden. Doordat er duizenden verschillende tarwes bestaan die nooit getest zijn op de aanwezigheid van coeliakie-epitopen, werd deze extractie- en detectiemethode toegepast om te analyseren of er tussen moderne broodtarwes, die door veredelaars worden gebruikt, tarwerassen aanwezig zijn die minder coeliakie-epitopen bevatten. Deze moderne tarwes zijn vervolgens vergeleken met oude tarwes verzameld over de hele wereld. Daaruit bleek dat er een toename te zien was van het aantal coeliakie-epitopen in de moderne tarwes. Dit kan de reden zijn dat het voorkomen van coeliakie nog steeds toeneemt. Bij de toekomstige veredeling van broodtarwe zou er dus niet alleen naar hoge opbrengst, ziekteresistentie en bakkwaliteit gekeken moeten worden, maar ook naar de aanwezigheid van relevante coeliakie-epitopen. In oudere broodtarwe en pastatarwe zien we dat er minder coeliakie-epitopen voorkomen. Met deze kennis kan door middel van kruisen met een moderne tarwe een meer veilige broodtarwe worden ontwikkeld. Bij de analyse van broodtarwelijnen die bepaalde genetische stukjes missen van één van de drie grassen waaruit broodtarwe is ontstaan, blijkt dat, als het stukje dat codeert voor coeliakie-epitopen waarop de meeste patiënten reageren, is verwijderd, de deegeigenschappen zelfs beter werd in vergelijking met het controledoeg. Als daar vervolgens vergelijkbare eiwitten uit haver aan werden toegevoegd, werden de deegeigenschappen nog beter. Deze eiwitten uit haver zijn voor de meeste coeliakiepatiënten geen probleem. De resultaten laten dan ook zien dat er mogelijkheden zijn om een tarwe te ontwikkelen waar minder coeliakie-epitopen inzitten zonder dat de deegkwaliteit ten nadele wordt beïnvloed.

Patiënten kunnen echter allemaal verschillend reageren op verschillende coeliakie-epitopen wat het ingewikkeld maakt om tarwe te ontwikkelen dat voor alle coeliakiepatiënten geschikt is. Het onderzoek in dit proefschrift laat zien dat selecteren van tarwe op de aanwezigheid van coeliakie-epitopen buitengewoon relevant is omdat het kan leiden tot een verlaging van het aantal coeliakiepatiënten in de toekomst. Tevens laten we zien dat coeliakie veilige tarwe ook nog steeds goede bakeigenschappen kan hebben. Ook huidige coeliakiepatiënten zullen hier baat bij hebben doordat via dit soort tarwe de blootstelling aan zogenaamde 'verborgen' gluteneiwitten afneemt.

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To complete the content of my thesis, I finally can write a more easy part, which is the acknowledgments. It is of course a great pleasure to say thanks to everybody who contributed in some way to the accomplishment of this thesis. At the same time, it gives me the creeps because the date of the defense is rapidly coming closer now and after that this challenge is over.

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Curriculum vitae

Hetty van den Broeck was born on June 10th 1966 in Breda, The Netherlands. After completion of secondary school at the Newman College in Breda, she received her High school (HAVO) diploma in 1983. In the same year, she started a HLO study at the Hogeschool West-Brabant, Dr. Struycken institute in Etten-Leur, The Netherlands. In 1987 she graduated and obtained her bachelor degree in Medical Biochemistry. In the final part of her study she followed an internship at the Genetics department within the section Molecular Genetics of Industrial Microorganism (MGIM) of the former Agricultural High school in Wageningen, The Netherlands. In June 1987, she was assigned as molecular and biochemical research technician in the same research group. The major research topics included fundamental and applied research on cell wall degrading enzymes and glycolytic enzymes from fungi, mainly *Aspergillus*. The many projects she contributed to, resulted in 10 patent publications. The Agricultural High school changed in the mean time to Wageningen Agricultural University and is now known as Wageningen University, part of Wageningen University and Research Centre (WUR). In September 1998 she was assigned as senior research assistant at the department of Cell Biology (now Business unit Bioscience) at the Centre for Plant Breeding and Reproduction Research (CPRO-DLO), now known as Plant Research International (PRI) in Wageningen. She changed research topic from lower eukaryotic (fungi) to higher eukaryotic (plant) research, starting with a study on antioxidants and genes from fruits and vegetables. In 2000 she changed subject within the same department and used her knowledge on plant cell walls to study the cell wall building enzymes of fiber hemp, *Cannabis sativa*, by cDNA microarray analysis as part of the EU project HARMONIA. At the end of 2004 she commenced her career in gluten related research performed within the Dutch Celiac Disease Consortium (CDC). She started to study gluten proteins from wheat in relation to celiac disease (gluten intolerance). In 2006 she accepted the opportunity to start a PhD study on this topic, which resulted in the underlying thesis. The first CDC project ended in 2009 and from 2010 a follow-up project, CDC2, started in which she will continue to explore the quest for celiac-safe wheat and food products.



Curriculum vitae



Hetty van den Broeck is geboren op 10 juni 1966 in Breda. In 1983 behaalde ze haar HAVO diploma aan het Newman College te Breda. In datzelfde jaar begon ze aan een HLO-opleiding bij de Hogeschool West-Brabant, Het Dr. Struycken instituut te Etten-Leur. In 1987 voltooide zij deze opleiding en kreeg haar bachelor titel in de richting Medische Biochemie. Haar stage en afstudeerperiode ter afsluiting van die opleiding volgde zij bij het laboratorium voor Erfelijkheidsleer van de toenmalige Landbouw Hogeschool in Wageningen bij de sectie Moleculaire Genetica van Industriële Micro-organismen (MGIM). Daar heeft zij sinds 1987 gewerkt als moleculair biologisch en biochemisch analiste. De onderwerpen waar zij aan werkte betroffen zowel fundamenteel als toegepast onderzoek aan celwandafbrekende enzymen en glycolytische enzymen uit schimmels, voornamelijk *Aspergillus*. De vele projecten waar zij in heeft bijgedragen, resulteerde in 10 patentpublicaties. De naam van Landbouw Hogeschool veranderde in Landbouwwuniversiteit en is nu Wageningen University onderdeel van Wageningen University and Research Centre. In september 1998 veranderde Hetty van baan en werd werkzaam als senior onderzoeksassistente bij de afdeling Celbiologie (later Celcybernetica en nu Business unit Bioscience) van het toenmalige CPRO dat nu Plant Research International is. Hier werd het schimmelonderzoek ingeruild voor plantenonderzoek en is zij begonnen aan onderzoek naar antioxidanten en genen uit groente en fruit. In 2000 veranderde zij van onderwerp en keerde zij terug naar het celwandonderzoek en dit keer werd het onderzoek naar de celwandopbouwende enzymen uit vezelhennep (*Cannabis sativa*) m.b.v. cDNA microarray analyse als onderdeel van het EU-project HARMONIA. Eind 2004 begon zij aan onderzoek aan gluten gerelateerd onderzoek binnen het Celiac Disease Consortium (CDC) waarin een relatie werd gelegd tussen gluten eiwitten uit tarwe en de ziekte coeliakie (= gluten intolerantie). In 2006 accepteerde zij de mogelijkheid om promotieonderzoek te beginnen op dit onderwerp dat resulteerde in dit proefschrift. Eind 2009 eindigde het eerste CDC-project en vanaf 2010 is er een vervolgproject, CDC2, gestart waarin zij de zoektocht naar coeliakie-veilige tarwe en voedingsproducten voortzet.

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Overview of completed training activities

Discipline specific activities

- Xth Cell Wall Meeting, Sorrento (Italy), Aug. 29–Sept. 3, 2004, oral presentation.
- XIIth International Celiac Disease Symposium, New York (USA), Nov. 9–11, 2006, 2 posters.
- 10th International Symposium on Immunological, Chemical and Clinical Problems of Food Allergy, Parma (Italy), May 26–29, 2008, oral presentation.
- 13th International Coeliac Disease Symposium, Amsterdam (NL), April 6–8, 2009, 3 posters.
- 10th International Gluten Workshop, Clermont-Ferrand (France), Sept. 7–9, 2009, oral presentation.
- 2nd International Symposium on Gluten-Free Cereal Products and Beverages, Tampere (Finland), June 8–11, 2010, oral presentation.

General courses

- Techniques for Writing and Presenting Scientific Papers, graduate school SENSE, Jan. 14–16, 2004.
- Practical Statistics for Microarray Data, Centre for Biostatistics, Utrecht University (NL), March 4–5, 2004.
- Biomolecular Mass Spectrometry Course, Dept. of Pharmaceutical Sciences, Utrecht University (NL), Sept. 25–29, 2006.
- Proteomics, graduate schools VLAG/EPS, Oct. 20–23, 2008.
- Postdoc Career Development Initiative (PCDI) Postdoc Retreat, Heeze (NL), April 21–23, 2010.

Optionals

- Preparing PhD research proposal.
- Monthly or bimonthly Celiac Disease Consortium meetings, presentations in years 2005, 2006, 2007, 2008, and 2010.
- Weekly BU Bioscience Thematic Meetings, presentation in years 2005, 2006, 2007, 2008, 2009, and 2010.
- Weekly, BU Bioscience Cluster meetings, presentations in years 2005, 2006, 2007, and 2008.

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