HIGH MOLECULAR WEIGHT GLUTENIN SUBUNITS OF WHEAT

QUALITATIVE AND QUANTITATIVE VARIATION IN RELATION TO BREAD-MAKING QUALITY

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HIGH MOLECULAR WEIGHT GLUTENIN SUBUNITS OF WHEAT:
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Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op woensdag 27 mei 1992
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

Foto op de omslag: PR Stad, centrale fotodienst DLO

Het in dit proefschrift beschreven onderzoek is tot stand gekomen met financiële ondersteuning van de Stichting Nederlands Graancentrum (NGC). Het proefschrift is gesubsidiëerd door de Stichting 'Fonds Landbouw Export Bureau 1916/1918' (LEB-Fonds).

STELLINGEN

1. De HMW glutenine subunit allelen verschillen in bijdrage tot de bakkwaliteit van tarwe op grond van type én hoeveelheid van de subunits. Aan het tweede aspect is voorheen nauwelijks aandacht besteed.
   Dit proefschrift.

2. De veredelaar moet er rekening mee houden dat het verband tussen het HMW glutenine subunit genotype en de bakkwaliteit van tarwe minder sterk is en ook gecompliceerder van aard dan veelal werd aangenomen.
   Dit proefschrift.

3. Met snelle kwaliteitsvoorspellende toetsen dient men in elk geval het aandeel van de HMW glutenine subunits in de totale hoeveelheid eiwit van de tarwekorrel te kunnen bepalen, gezien het belang van deze zeer variabele parameter voor de kwaliteit.
   Dit proefschrift.

4. De gebruikelijke techniek voor het identificeren van HMW glutenine allelen (SDS-PAGE) geeft geen waterdichte resultaten. Bij het relateren van de bakkwaliteit van een allel aan zijn genstructuur mag men daarom niet volstaan met één ras, maar dient men genen die schijnbaar voor eenzelfde subunit coderen uit verschillende rassen te isoleren.
   Flavell RB, Goldsbrough AP, Robert LS, Schnick D, Thompson RD (1989) Bio/technology 7: 1281-1285.
   Dit proefschrift.

5. De bewering van Goldsbrough et al. dat Pogna et al. aantonen dat de y-type subunits 10 en 12 verantwoordelijk zijn voor de verschillen in kwaliteit van de allelen voor de subunit combinaties 2+12 en 5+10 is onjuist.
   Goldsbrough AP, Bulleid NJ, Freedman RB, Flavell RB (1989) Biochem J 263: 837-842.
   Pogna NE, Mellini F, Dal Belin Peruffo A (1987) In: Agriculture. Hard wheat: agronomic, technological, biochemical and genetic aspects, pp 53-69.

6. De conclusie van Khan et al. dat de aanwezigheid van HMW glutenine subunit 8 of van subunit 9 een invloed heeft op het eiwitgehalte van de bloem is onterecht.
   Khan K, Tamminga G, Lukow O (1989) Cereal Chem 66: 391-396.

7. Het verbeteren van de kwaliteit van tarwe door veredeling en door teeltmaatregelen verdient de voorkeur boven het compenseren van een lage kwaliteit door middel van additieven.

8. Bij het automatisch koppelen van het predikaat "slechte kwaliteit" aan de in Nederland verbouwde tarwe wordt vergeten dat behalve in de broodindustrie er nog andere toepassingen bestaan en nog ontwikkeld worden. Daarbij kan het juist een voordeel zijn dat er duidelijke verschillen zijn met de tarwes uit concurrerende landen.

9. Voor een optimaal gebruik van de mogelijkheden van de gentechnologie in de veredeling is meer kennis vereist van het traject tussen een gen en het uiteindelijke produkt.

10. Voor het welslagen van de introductie van het "vierde gewas" is het landbouwkundig van groot belang het derde gewas in stand te houden.

11. De problemen met de landbouwoverschotten binnen de EG zouden voor de consument beter te verteren zijn indien deze overschotten gefinancierd zouden worden met een 'voedsel-garantie heffing'.

12. Voor de bosbouw in de EG was het beter geweest als de beschrijvende rassenlijst voor populiererassen uit 1967 als eerste nummer voor een reeks van rassenlijsten was opgevat.
    Kolster HW (1967) Populiererassen in de E.E.G.-landen en hun bruikbaarheid voor Nederland, 150 p.

13. Bij het grootschalig gebruik van "eetbaar" verpakkingsmateriaal geproduceerd uit landbouwprodukten dient de Nederlandse consument te weten dat deze materialen ontwikkeld zijn voor het terugdringen van milieuproblemen, en niet zozeer om als voedsel te dienen.

Proefschrift van P Kolster.
Titel: High Molecular Weight glutenin subunits of wheat: qualitative and quantitative variation in relation to bread-making quality.

Wageningen, 27 mei 1992.

VOORWOORD

Dit proefschrift is tot stand gekomen op twee instituten. Het grootste deel van het experimentele werk is uitgevoerd op de voormalige Stichting voor Plantenveredeling (SVP), nu onderdeel van het Centrum voor Plantenveredeling en Reproduktie Onderzoek (CPRO-DLO). Na bijna drie jaar daar gewerkt te hebben ben ik overgestapt naar het Instituut voor Agrotechnologisch Onderzoek (ATO-DLO), waar voor wat betreft het proefschrift de nadruk heeft gelegen op de verwerking van de resultaten met daarnaast afrondende experimenten. Iedereen die aan het tot stand komen van dit proefschrift een bijdrage heeft geleverd, zowel binnen deze instituten als daarbuiten, wil ik hierbij bedanken. De groep mensen is te groot om iedereen hier afzonderlijk te kunnen bedanken.

In het bijzonder gaat mijn dank uit naar mijn promotor, Prof.dr.ir. J.H. van der Veen, voor de stimulerende en kritische begeleiding van de interpretatie van het onderzoek. Ondanks (of juist dankzij?) het feit dat het onderzoek niet geheel binnen uw vakgebied valt ontstonden dikwijls boeiende discussies waarbij het onderzoek in een breed kader geplaatst werd. Een bijzonder woord van dank aan mijn co-promotor, Wim van Gelder. In de belangrijke beginfase op de SVP heb je het onderzoek op het juiste spoor gezet. Later in het onderzoek heeft jouw vertrouwen voor de broodnodige vrijheid en ruimte gezorgd. Jouw correcties in de manuscripten, van dikke rode strepen in het begin tot min of meer voorzichtige suggesties in een later stadium, zijn van groot belang geweest. Daarnaast heeft Jaap Visser van de vakgroep Erfelijkheidsleer (LUW) een inspirerende rol gespeeld, zowel in de doctoraalfase van mijn studie als tijdens het promotieonderzoek. Verder wil ik ook Albert Eenink voor de waardevolle hulp die hij, zowel als directeur van de SVP als van het ATO, op verschillende manieren op een doortastende wijze gegeven heeft.

Van de voormalige SVP wil ik met name Kees Krechting, Jans Schepers, Harry Jonker en Fred van Eeuwijk bedanken voor hun waardevolle hulp en voor de bijzondere werksfeer. Op het ATO bedank ik Johan Vereijken voor zijn rol als klankbord voor de eerste versies van de meeste hoofdstukken en Paul de Bie voor de assistentie bij het afronden van het experimentele gedeelte.

Als laatste wil ik Anja bedanken voor het vele typewerk, het corrigeren van de manuscripten en voor de vele andere bijdragen die je geleverd hebt. Hoewel deze technische assistentie zeer belangrijk is geweest, is voor mij jouw morele steun van nog grotere waarde geweest.

aan Anja,
aan mijn ouders.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Production and uses of bread-wheat

In the total agricultural world production, cereals -wheat, rice, barley, maize, rye, sorghum, oats and millet- are the most important crop with respect to the production volume. Among these, wheat takes the first place with an annual production volume of $508 \cdot 10^6$ tons in 1988, directly followed by rice [1]. Bread-wheat (*Triticum aestivum* L.) is the most common cultivated species of Triticum [2]. The production volume of durum or macaroni wheat (*T. durum*) wheat, which is primarily used for the manufacturing of pasta's (macaroni, spaghetti etc.), was about 5% of that of bread-wheat (1975-1984; [3]). Because durum wheat is not grown in The Netherlands, the name wheat will be used in this thesis exclusively for bread-wheat (*T. aestivum*).

The most important food application of wheat is the manufacturing of bread [4]. Other applications are e.g. in the pastry, the biscuit, the feed and the starch and gluten industry. Not all wheat fulfills the specifications outlined by the millers and bakers for the manufacture of bread. The criteria which determine the suitability of wheat for this application are: the milling properties, the dough properties and the bread properties. The milling quality is beyond the scope of this thesis.
Bread-making quality refers here to the processing quality of wheat flour, which includes the dough- and bread-properties.
Bread-making quality is a complex characteristic of wheat. Firstly, the consumers demands and the bread-making technology differs largely between countries [4-6]. Therefore, it is not possible to formulate universally applicable criteria for bread-making quality. In general, the volume of a loaf is considered as the most important criterion for it [4], although other characteristics of the loaf (e.g. shelf-life, crumb-structure, colour and appearance) and of the dough (e.g. strength, stickiness, mixing requirement, water absorbance) are of importance as well [4,7]. Secondly, it is not easy to determine the bread-making quality of a flour unambiguously, because baking tests are prone to variability depending on the baker

and on technological differences. The conditions of the baking test should be optimized for each flour [4].

Only a small proportion of the wheat grown in The Netherlands fulfills the requirements of the baking industry. Of the wheat milled for human consumption by the Dutch milling industry between 1985 and 1990, about 25% was produced in The Netherlands. This corresponds to 300,000 tons, which is only about 30% of the total production volume of the Dutch farmers [8]. The rest of the wheat used in Dutch milling industry originates from other EC countries. The low quality of the Dutch wheat results to a large extent from the low quality of the varieties and from the agronomic and climatologic conditions. In some years pre-harvest sprouting occurs, which has a deteriorating effect [9].

To improve the bread-making quality of flours, technological measures (e.g. improved technology and the use of additives) can be successful. The improvement of the intrinsic bread-making quality of wheat by agronomic measures or by plant breeding is important as this would enable the milling industry to use more domestic wheat without requiring technological compensation for decreased flour quality. As such, improved wheat quality is important to the Dutch farmers and the milling industry.

The subject of this thesis is studying genetic factors that influence the bread-making quality of wheat, but agronomic factors will be considered as well. The research aims primarily at developing a strategy for improving the bread-making quality by plant breeding.

Flour components

A flour contains a large number of components, which essentially originate from the endosperm of the kernel [10], but components from other kernel structures (e.g. aleurone layer, germ and bran) are present as well. Differences in the baking performance of flours are related to differences in the flour composition. The major classes of the flour components are [4]:

- carbohydrates (70-80% of dry flour)
- proteins (8-18% of dry flour)
- lipids (1.5-2.5% of dry flour)

Each of these classes is composed of a large number of individual components, which will be briefly discussed. Differences in characteristics of the components which affect the bread-making quality of flours will also be discussed, with special emphasis on characteristics which can be improved by plant breeding.

In the carbohydrate fraction, starch is the main component. The amount of water which must be added to a flour for an optimal baking result depends partly on the level of 'starch damage'[4,11] (i.e. the amount of sound starch granules which are damaged, or 'activated' by mechanical stress during milling), which in turn depends on the milling conditions and the kernel hardness [10]. The kernel hardness is to a large extent genetically determined [12]. As only a small number of genes controls kernel hardness, this quality-affecting wheat characteristic can be controlled by plant breeding [12,13]. Differences in the starch granule size may also affect bread-making quality [14]. According to Lelievre et al. [15] the optimal granule size depends on the protein content of the flours, which could be the cause of the different effects of variation in starch granule size on the bread-making quality reported in the literature [15].

The lipid fraction of a flour can be divided into two main fractions [16]: the polar (glycolipids and phospholipids) and non-polar lipids (mainly triglycerides). Part of the lipid fraction (about ⅓ of the total amount [17]) is bound to starch, the so-called starch lipids [18,19]. Another part of the lipids becomes bound to the flour proteins during mixing. It is generally accepted that these lipid-protein interactions are of importance for the bread-making quality [17,20,21]. The effects of lipids on the bread-making process are summarized by Pomeranz [16]. Shortening, or fat, is frequently added to flours to improve loaf volume, dough properties and shelf-life [22-24]. For a good bread-making quality of a flour, a high content of the native lipids and a high ratio of polar lipids : non-polar lipids is required [4]. Differences in lipid content and composition between varieties, and differences between samples of varieties grown at different locations, could be related to differences in bread-making quality [23,25,26], but in other studies, these relationships were less clear-cut [27-29].

In conclusion, differences in the composition of starch and lipids can affect the bread-making quality of flours. This knowledge has so far not been used for breeding for bread-making quality because the starch and lipid characteristics studied do normally not appear to control the quality. The protein fraction is more important in determining this characteristic of wheat.

Wheat flour proteins

The flour proteins can be classified according to their extractability and solubility in various solvents. Traditionally, the Osborne classification procedure is used, which has been developed in 1924 [see 30]. In this procedure, sequential extraction of

flour results in the following protein fractions: the albumin (water extraction), globulin (salt solution extraction), prolamines (aqueous alcohol extraction) and glutelins (dilute acid or alkali extraction). Later, the names gliadin and glutenin were adopted for the prolamins and glutelins, respectively [30]. These two fractions are known as gluten. In some studies, a protein fraction which is not extracted by any of the Osborne solvents was identified and called 'residue protein' [31]. The glutenin and gliadin fraction accumulate in the endosperm of the wheat kernel during kernel-filling and are considered to be true storage proteins (see [32]). Some of the proteins belonging to the albumin and globulin fractions have enzymatic functions; the functions of other proteins are unknown [33]. In general, the albumin and globulin fractions each account for about 10% of the total amount of flour proteins, the gliadin and glutenin fractions each for about 40% [33-35].

The amount of the flour proteins is positively correlated with the loaf volume. An increase in the protein content of flours from 10% to 14% results in an increase in loaf volume of about 50% [36-38]. Because only the amount of the gluten proteins increases with an increase in protein content [4], the improvement of the loaf volume wil be due to a higher amount of gluten. The slope of the regression line of loaf volume on protein content differs between genotypes, which reflects differences in protein quality.

Breeding for bread-making quality by increasing the protein content is hampered by a negative correlation between the yield and the protein content of the kernels (see [13]). Furthermore, the *amount* of protein present in a flour is largely determined by environmental conditions, which also hampers breeding for protein content [13,39]. In contrast, the protein *quality* is primarily genetically determined. Selection for protein quality enables breeding for improved bread-making quality without a yield-penalty.

In determining the protein quality, the albumin and globulin fractions are believed to be of no [4], or only of minor [33] importance. However, enzymes such as proteases and amylases, which are part of these fractions, do have an effect on the bread-making quality. By far the most important are the gluten proteins, which impart visco-elastic behavior to a dough. This property, which makes gluten unique among the plant proteins, is essential for the production of a leavened dough. During mixing and rising of the dough, a proper gluten allows the formation and expansion of gas-cells so that a high loaf volume and a good texture are formed. If the gluten is weak and highly extensile, a poor bread-texture will result; a gluten which is not sufficiently extensile will result in a poor loaf volume [33]. Therefore,

especially the characteristics of the gluten proteins are of importance when breeding for bread-making quality.

Small-scale tests for bread-making quality in breeding

Selection in the early stages of plant breeding by fast and small-scale tests is required for efficient programmes, also in the case of bread-making quality. Fast and small-scale tests can also be used in the milling and baking industry. It should be emphasized however that the aims of the industry are not in keeping with those of the breeders. The milling and baking industry is interested in a reliable estimate of the *phenotypic* quality, whereas the breeder is interested in the *genotypic* quality. The tests which have been developed aim at an as high as possible correlation with the phenotypic bread-making quality.

The requirements for a test applicable in early generation selection are:

1) small sample size, preferably a whole meal flour,
2) simple and fast methods,
3) good correlation with the genetically determined bread-making quality, and little influences of variation in environmental conditions.

The traditional baking and dough-tests [4,7] are too time- and labor-consuming and require far too much flour for application in early stages of a breeding programme. These tests are however of importance in later stages of breeding programmes.

Small-scale tests for bread-making quality are e.g. the Zeleny-, the Pelshenke-, the residue protein- and the SDS-sedimentation test [see 4,40,41]. The correlation coefficients between the results of these small-scale tests and the bread-making quality ranges between 0.35 and 0.86 [see 42-44]. In studies of Blackman and Gill [42] and Axford et al. [43], the correlation was superior for the SDS-sedimentation test. The results of the small-scale tests are not only determined by the protein quality, but are also affected by differences in the protein content of the flours [45]. As the protein content is mainly environmentally determined, this will decrease the selection response. The heritability of small-scale tests was medium to high, and varied between crosses [46] and between growing seasons [47]. The occurence of genotype * environment interactions limits the applicability of the tests [45,48,49].

Also Near-Infrared Reflectance (NIR) has been studied as a technique for the prediction of the bread-making quality. In some studies the quality prediction was poor [50-52]. However, Williams et al. [53] obtained accurate estimates for the

dough-characteristics and the loaf volume for hard spring wheat. In all, it has not been proven yet whether NIR has a potential for predicting bread-making quality in breeding programmes. In predicting other quality related characteristics of wheat, e.g. protein content and wheat moisture, NIR can be applied successfully [see 52].

In the small-scale tests for early generation selection flours have to be used, which implies that the tests are destructive. This is a drawback as in early generations only small amounts of kernels are available. Furthermore, the tests give no insight in the biochemical and the genetic basis of differences in protein quality.

Biochemical characterization of the gluten proteins

The poor solubility of the gluten proteins has limited the applicability of biochemical separation techniques. For experiments using gel-filtration, extraction procedures have been developed in which buffers were used containing urea and CTAB (hexadecyltrimethylammonium [54,55]), SDS (sodium dodecylsulphate [56]), or SDS in combination with sonification of the sample [57,58]. Because of these stringent extraction conditions, the results of these studies may not be representative for the situation *in-vivo* or in a dough.

Gel-filtration studies revealed that the gluten proteins are highly polydisperse in their molecular weight, with molecular weights up to several millions. Covalent bonds (S-S bonds) and non-covalent interactions (hydrogen bonds and hydrophobic interactions) are present between the gluten proteins. These cross-links play an important role in the bread-making quality [33,59,60]. The glutenin (solubility) fraction comprises large protein aggregates (up to 20,000 kD), composed of High Molecular Weight (HMW) glutenin subunits cross-linked by disulphide bonds. The gliadins are smaller, with elution positions ranging from 11 to 63 kD. Also a peak corresponding with a molecular weight of about 100 kD is present in the gliadin fraction [61]. This peak comprises Low Molecular Weight (LMW) glutenin subunits, which are linked by intermolecular disulphide bonds.

Electrophoretic techniques, such as PolyAcrylamide Gel Electrophoresis (PAGE) at a low pH (A-PAGE), PAGE in the presence of Sodium Dodecyl Sulphate (SDS-PAGE) and various two-dimensional techniques, were used to study the individual proteins which constitute the solubility fractions.

The gliadin fraction of bread-wheats is composed of about 50 monomeric proteins. Based on their electrophoretic behavior during A-PAGE, these proteins are divided in α-, β-, γ- and ω-gliadins showing decreasing electrophoretic mobilities, respectively. The ω-gliadins show molecular masses of about 70 kD, the other

groups of gliadins between 30 and 40 kD. Cysteine residues of the gliadins are involved in intra-molecular disulphide bonds [30].
Upon reduction of the disulphide bonds of the glutenin, the component proteins can be studied separately. Based on the molecular weight of the glutenin subunits, two classes can be distinguished: the HMW (97-136 kD) and the LMW (31-48 kD) glutenin subunits. A wheat variety contains 3-5 HMW glutenin subunits and about 15 LMW glutenin subunits [7,16,30,33,62,63]. Although the exact structure of the glutenin aggregates is unknown, it is believed that subunits are head-tail linked by disulphide bonds, and form linear aggregates [64,65, see also 59].
It is generally accepted that the gliadins contribute to the dough-extensibility, whereas the glutenins confer strength and elasticity to a dough [4,35,66]. Based on the sequence of HMW glutenin subunits and on the results of analysis with physical techniques, Tatham and coworkers [67] suggested that the repetitive β-turn conformation of the central region of the subunits form a β-spiral, which could be responsible for the elastic properties. Ewart [65] suggested a role of the gliadins as plasticizers between glutenin aggregates. The role of the LMW glutenin subunits is unknown.

In conclusion, the solubility fractions differ considerably in their biochemical characteristics and in their contribution to the physical gluten properties and hence to the bread-making quality. It is believed that the ratio between the amounts of gliadin and glutenin is of great importance for the bread-making quality [4], although according to Schofield and Booth [33] this has not yet been proven. Therefore, it is important to study the effect of variation in the amount of protein fractions in wheat on the quality of flours.

Variation in the amount of protein fractions in wheat
Variation in the amount of solubility fractions has been studied extensively in relation to the bread-making quality. Because different extraction procedures were used by research groups, an accurate comparison of the results of different groups is virtually impossible [33,68]. Moreover, the biochemical and genetic interpretation of these studies is hampered by the large experimental error in the isolation of these protein fractions, by the complexity of each fraction and by the overlap in the composition between fractions [30,31,68].

Differences between flours in the amount of solubility fractions - which probably have resemblance to the Osborne gliadins and glutenins - have been related to differences in bread-making quality [69-76]. Because different extraction procedures

were used, the results can not be interpretated in terms of functionality of the protein fractions. These studies revealed nevertheless several causes for the differences in amount of the fractions. Firstly, the amount of the fractions may differ between varieties and between locations [72,73]. Secondly, the ratio between these fractions is changing during kernel development [77,78] and during germination or sprouting of the kernel [79,80]. Thirdly, increase in the level of nitrogen-fertilization, which is known to increase the total amount of protein, results in an increase in the gliadin/glutenin ratio caused by an increase in the proportion of the gliadins [81].

Differences in the amount of groups of storage proteins have also been studied using biochemical separation techniques, viz. SDS-PAGE and Reversed Phase-High Performance Liquid Chromatography (RP-HPLC). Molecular weight regions of the storage proteins as determined by SDS-PAGE patterns correspond roughly to the various solubility classes [82]. Differences in the amount of proteins in these molecular weight classes were found between species related to bread wheat [82,83] and between varieties [84]. Sulphur deficiency during kernel development resulted in an increase in the relative amounts of the HMW glutenin subunits and of the ω-gliadins [85,86]. This is probably a consequence of the relatively low S-content of these proteins. An increase in the nitrogen fertilization resulted with three out of four varieties in an increase in the proportion of the gliadins, whereas the proportions of the albumins and globulins decreased. In contrast, the proportion of the glutenins seemed largely independent from differences in protein content [87]. In another study with different levels of nitrogen fertilization, it was shown by electrophoretic analysis of the gliadins that the proportion of the ω-gliadins relative to the total amount of gliadins increased as kernel protein content increased [88]. Variation in growing location may also affect the relative amount of gliadins [89] and glutenin subunits [90], as determined by RP-HPLC analysis. Using the same separation technique, changes were shown during kernel maturation in the proportion of groups of glutenins (including the proportion of the HMW glutenins), in the amount of gliadins and in the ratio between the individual HMW glutenin subunits [91,92]. Also the location of the kernel in the spike affects the proportion of gliadins [92]. Pests during kernel filling may also affect the amount of proteins [93,94].

Care should be taken however with the interpretation of the results of Seilmeier et al. [91] and Huebner et al. [92]. In these studies, the gliadins and glutenins were isolated as solubility fractions, before a detailed analysis by RP-HPLC of the

fractions. This could mean that the results reflect differences in the extractability of these proteins, rather than differences in the amounts produced by the plants. A study of Kruger and Marchylo [95] illustrates that the extractability of proteins may change. A part of the HMW glutenin subunits become soluble in 50% 1-propanol during germination, and may therefore be lost when isolating the glutenin as a solubility fraction.

The above results show that the quantitative composition of the kernel proteins of wheat, which is of prime importance for the bread-making quality, is subject to environmental variation. The results obtained so far give no information on the nature of the underlying genetic variation, consequently it is not known whether the variation in amount of the protein fractions is amenable to plant breeding.

Biochemical techniques as small-scale tests for predicting bread-making quality
Knowledge of the relations between biochemical characteristics of the proteins and bread-making quality can be exploited in the development of small-scale tests for the prediction of the bread-making quality. A biochemical separation technique as HPLC can be a usefull test because it is relatively simple and fast and allows the use of small amounts of sample.

The aggregation behavior of the kernel proteins has been related to bread-making quality. A high proportion of large aggregates, which probably corresponds with a high proportion of glutenin, has been related positively [55,96,97] or negatively [98] with bread-making quality. This contradiction can be caused by differences in extraction-efficiency; especially large aggregates appear to be poorly soluble [59]. Size Exclusion (SE)-HPLC for determining the aggregation behavior of the gluten proteins, has been suggested as a fast and relatively simple selection technique. A drawback of this technique is that the results of gel-filtration depend on the level of extraction. Furthermore, other flour components [99] may also affect the level of aggregation.

The amount of glutenin subunits separated by RP-HPLC which are present in two peaks has been related to the bread-making quality [100]. The constituent proteins of these peaks were not identified. Regression equations to predict the bread-making quality, using the amount of the protein in these peaks as variables, allowed discrimination between poor, mediocre and good bread-making quality. In a second year of testing, the predictive power of the equations was lower [101], but still enabled discrimination between poor and good bread-making quality.

The amount of gliadins too was related to bread-making quality. A specific

region in a RP-HPLC chromatogram, named the 'Baking Quality Gliadin Fraction (BQGF), was negatively correlated with quality [102]. Samples grown at different locations were used, with different sets of varieties at each location. Correlation coefficients between the proportion of the gliadins in the BQGF fraction and the bread-making quality of varieties grown at the same location differed between locations and ranged between -0.61 to -0.93 [102]. In another year, these correlation coefficients were comparable [103]. For samples grown at different locations, the correlation coefficients between BQGF and bread-making quality was less than -0.36 or were not significant [103]. Therefore, quantification of BQGF for predicting the quality can only be used to compare wheats grown at the same location.
Peak regions of the 70% ethanol soluble proteins separated by RP-HPLC have been related to dough-extensibility but there was no relation with other dough characteristics and loaf volume [104].
The amount of the HMW glutenin subunits extracted in 50% 1-propanol relative to the amount of these proteins extracted in 50% 1-propanol + dithiothreitol was inversely related with dough strength. The ratio between the HMW glutenin subunits and the LMW glutenin subunits, extracted in 50% 1-propanol + dithiothreitol, increased with increasing dough strength [105]. The proteins were quantified using RP-HPLC. In a subsequent study, only the first relationship could be confirmed [106].

In conclusion, the studies using biochemical separation techniques have shown relations between the amount of specific groups of proteins and bread-making quality. It is not known whether these techniques estimate the *genotypic* or *phenotypic* bread-making quality. It has not been proven either whether such tests are to be preferred to classical quality prediction tests. In general, the protein (peaks) are poorly characterized (with the exception of [105] and [106]); therefore the results can not be used to define specific components which contribute to the quantitative variation.

The *type* of the gluten proteins, as determined by electrophoresis, is a major determinant of protein quality. There is a considerable knowledge on the relation between the bread-making quality and differences in the type of the gliadins (see [107-109]) and the type of HMW glutenin subunits (see [110,111], see also Chapter 2 of this thesis). There is only limited information on this relation for the LMW glutenin subunits [112,113]. Because the type of the proteins is genetically determined, and not affected by variation in environmental conditions, these relations are of major importance for breeding for improved bread-making quality.

Genetics of the gluten proteins

Bread wheat (*T. aestivum*) is an allo-hexaploid species. Each genome (denoted by A, B and D) is composed of 7 pairs of chromosomes (numbered 1 to 7). Glutenins and gliadins are encoded by genes at the following loci [11,63,66,114]

- *Glu-1* loci, located on the short arm of chromosomes 1A, 1B and 1D. Two closely linked genes encoding the HMW glutenin subunits are located at these loci [115].
- *Gli-1* loci, located on the short arm of 1A, 1B and 1D, encoding the LMW glutenin subunits and the ω- and γ-gliadins.
- *Gli-2* loci, located on the short arm of chromosomes 6A, 6B and 6D; encoding the α- and β-gliadins.

In wheat varieties, an extensive allelic variation is present at these loci. For each of the *Gli*-loci, about 10 gliadin alleles were described [116], which are identified by differences in the mobility during A-PAGE of the gliadins encoded. For the LMW glutenin loci, 6 (A-genome), 9 (B-genome) and 5 (D-genome) alleles were described by Gupta and Shepherd [117]. Because the gliadin genes are linked to the LMW glutenin subunit genes at the *Gli-1* loci, the effects of allelic variation in gliadins and LMW glutenins cannot easily be distinguished.

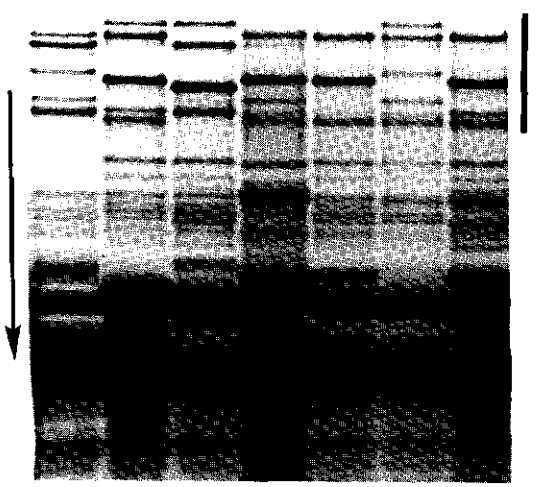

Figure 1.1 Separation of the storage proteins of seven wheat varieties by SDS-PAGE. The arrow indicates the direction of electrophoresis. The region of the gel which contains the HMW glutenin subunits is indicated by a bar.

The HMW glutenin subunit alleles are identified by the relative mobilities (R_m) during SDS-PAGE of the subunits encoded (Figure 1.1 and 1.2). For the three *Glu-1* loci, 3 (*Glu-A1*), 11 (*Glu-B1*) and 6 (*Glu-D1*) alleles have been described [118]. Landraces of wheat and wild relatives of *T. aestivum* contain a large number of alleles which are not present in established varieties. The number of possible permutations at the loci is therefore enormous. Because only the HMW glutenin subunits are studied in this thesis, literature concerning the gliadins and LMW glutenins will not be discussed hereafter.

Because of their specific contribution to the dough properties, imparting visco-elastic behavior to a dough, and because allelic variation has been related to differences in bread-making quality (see hereafter), the structure of the HMW glutenin subunit genes has been object of several studies. As the subunits are virtually insoluble in solvents traditionally used in biochemical studies, most of the knowledge of protein structure is derived from the nucleotide-sequences of the genes. The nucleotide sequence of a number of HMW glutenin subunits has been published [119-123]. These genes differ only slightly in their sequences and have a similar basic structure: the N-termini of the mature subunits are preceded by a 21 residues long signal peptide [120]. The mature subunit consists of three domains [see 124 for a review]; a central domain, composed of repetitive motifs and two non-repetitive domains at the N- and C-terminal part of the protein. The cysteine residues, which form inter-molecular disulphide bonds and as such being of importance for the bread-making quality, are primarily located in the N-termini. One cysteine residue is present in the C-terminal part and one cysteine residue is present in the central domain of some subunits. Sequences involved in the regulation of the gene expression, including the TATA and CAAT box, are present as well. A '-300 element', which is present in storage protein genes of wheat, barley and maize [121] may also be involved in the regulation of the expression of HMW glutenin subunit genes [120], although there is some controversy about its significance [125].The HMW glutenin subunits are synthesized only in the endosperm of the developing wheat kernel, and not in other tissues [see 126]. This tissue-specific expression is also observed when the genes are introduced in tobacco [127]. It has been established that a *cis*-acting 433 base-pair long element in the promoter region of the gene was essential for tissue specificity. In this segment, a 40 base-pair enhancer element is present, which is located 170 bp upstream of the transcription initiation site of the particular gene studied, which encodes subunit 12 in Chinese Spring. This enhancer is believed to be involved in the spatial and

temporal regulation of the gene expression [125].

Differences in quality of HMW glutenin alleles

A large number of research groups studied allelic variation at the *Glu-1* loci in relation to the protein quality (see Chapter 2 of this thesis). This variation appears to be important for the bread-making quality in countries in western Europe [110], probably because of the low protein content of wheat grown in this area. In other countries, e.g. Canada, the Soviet Union and the United States, the protein content is higher and as such the protein quality appears to be less important [see 128].

At each locus, alleles have been identified which differ in their contribution to the bread-making quality (see Figure 1.2).

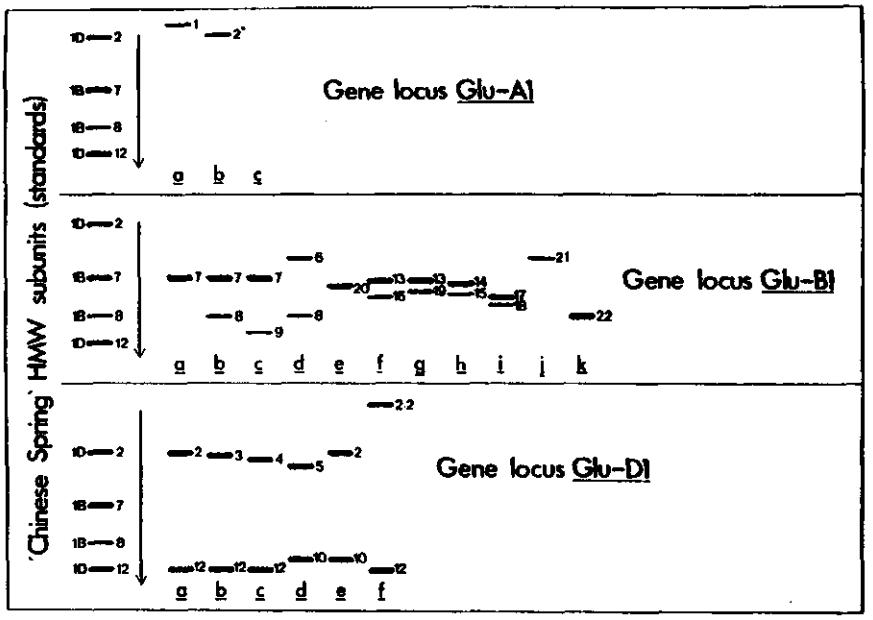

Figure 1.2 SDS-PAGE of the HMW glutenin subunit(s) encoded by alleles at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci of wheat, respectively. The arrow indicates the direction of electrophoresis. A variety normally contains at each locus one of these alleles, although other alleles can occur. On the left hand side of each group of alleles is the banding pattern of the subunits of Chinese Spring. Reprinted with permission from Payne and Lawrence [118].

The contribution of the HMW glutenin subunit alleles to the quality has been determined by relating the presence or absence of an allele in varieties or in segregating progenies to the bread-making quality. Only the 'quality' of alleles which are most frequent in established wheat varieties has been determined.

Methods which enable a less time-consuming and a less expensive prediction of the contribution to the quality of an allele are not known. Attempts have been made to relate differences in the structure of the subunits to the differences in quality of the alleles. Differences in the length of a continuous β-spiral of the central region of allelic subunits, which may contribute to the elastic properties of the gluten according to Tatham and coworkers [67], has been suggested as a cause of differences in quality [129]. Also the number of cysteine residues and the composition of the amino acids adjacent to the cysteines differs between subunits, but there is no evidence that this is causing the differences in quality [130,131].

A large amount (30%-79%) of the variation in bread-making quality between varieties has been ascribed to variation in the HMW glutenin subunit genotype (see Chapter 2). The HMW glutenin subunit alleles can therefore be considered as major genes in the quantitative trait bread-making quality. The 'high-quality' HMW glutenin subunit alleles which have been identified so far (Figure 1.2) can be used by plant breeders for improving bread-making quality. The question whether the HMW glutenin subunit alleles are also a useful selection criterion depends on their contribution to the variation in bread-making quality in breeding material. This contribution in breeding lines may differ from that in established varieties (see also Chapter 2).

Outline of the thesis

This thesis presents studies on the variation in the type and the amount of the HMW glutenin subunits in relation to the bread-making quality of wheat. In contrast to differences in the type of the HMW glutenin subunits, little is known about the variation in the amount of these proteins, especially about the mechanisms underlying this variation. This is remarkable because it is well known that differences in (relative) amounts of groups of gluten proteins (including the HMW glutenin subunits) have considerable effects on the bread-making quality. Therefore, in the research described here, genotypic and environmental variation in the amount of the HMW glutenin subunits has been studied. The results are discussed in relation to the possibilities to use variation in HMW glutenin subunit composition, qualitatively as well as quantitatively, in breeding for improved bread-making quality. The results can also be important for improving the quality of wheat for other applications, such as in the pasta-, pastry-, cookie- and starch-industry and for application of the gluten produced by the starch industry.

In Chapter 2, the effect of variation in HMW glutenin subunit genotype on the

loaf volume is studied. The alleles were ranked for quality, but also the contribution of variation at the *Glu-1* loci to variation in bread-making quality was analyzed. To exploit the differences in contribution to quality of alleles in plant breeding, it is essential that these alleles can be identified unambiguously. However, this identification can be hampered by the wide allelic variation. Therefore, variation in the type of the subunits is studied in Chapter 3, with special emphasis on the limitations of SDS-PAGE for allele identification. Chapter 4 to Chapter 7 deal with variation in the amount of individual HMW glutenin subunits. Because the methods for quantification of the subunits all have drawbacks for this particular application, a new method has been developed (Chapter 4). In Chapter 5 and 6, the effect of variation in environmental conditions and the effect of genetic variation on the amount of the individual HMW glutenin subunits is studied. The results are used to study genetic variation in the amount of the subunits in a set of varieties in Chapter 7. In Chapter 8, the level of expression of the HMW glutenin genes is investigated. It is shown that the level of gene expression can differ by a factor 2 within a variety. Furthermore, the relation between the contribution to the bread-making quality of an allele and the amounts of the subunits produced will be studied, a subject which has not been studied in the literature so far.

References

1 Anonymus (1989) The state of food and agriculture 1989 FAO agriculture series no. 22. Food and Agricultural Organization of the United Nations, Rome, pp 103

2 Orth RA, Shellenberger JA (1988) Origin, production, and utilization of wheat. In: Pomeranz Y (ed) Wheat: chemistry and technology, volume I. American Association of Cereal Chemists, St. Paul, pp 1-14

3 Dick JW, Matsuo RR (1988) Durum wheat and pasta products. In: Pomeranz Y (ed) Wheat: chemistry and technology, volume II. American Association of Cereal Chemists, St. Paul, pp 507-547

4 MacRitchie F (1984) Baking quality of wheat flours. Adv Food Res 29: 201-277

5 Mesdag J (1985) Developments in breeding winter wheat for bread-baking quality in some north-western European countries. Neth J Agric Sci 33: 215-234

6 Bolling H (1989) Qualitätsbewertung von Weizen in den EG-Ländern. Getreide Mehl Brot 43: 378-382

7 Finney KF, Yamazaki WT, Youngs VL, Rubenthaler GL (1987) Quality of hard, soft, and durum wheats. In: Wheat and wheat improvement, Agronomy Monograph 13. ASA-CSSA-SSSA, Madison, pp 677-748

8 Anonymus (1990) Jaarverslag 1990 Produktschap voor granen, zaden en peulvruchten, 's-Gravenhage, pp 60-72

9 Flintham JE, Gale MD (1988) Genetics of pre-harvest sprouting and associated traits in wheat: review. Plant Varieties and Seeds 1: 87-97

10 Bass EJ (1988) Wheat flour milling. In: Pomeranz Y (ed) Wheat: chemistry and technology, volume II. American Association of Cereal Chemists, St. Paul, pp 1-68
11 Payne PI, Holt LM, Jackson EA, Law CN (1984) Wheat storage proteins: their genetics and their potential for manipulation by plant breeding. Phil Trans R Soc London Ser B 304: 359-371
12 Bingham J (1962) The contribution of plant breeding to grain quality in wheat. In: Recent advances in processing cereals, monograph 16. Society Chemical Industry, London, pp 34-54
13 Law CN, Krattiger AF (1987) Genetics of grain quality in wheat. In: Morton ID (ed) Cereals in a European context. Weinheim, New York; VCH, Chichester, pp 33-47
14 Soulaka AB, Morrison WR (1985) The bread making quality of six wheat starches differing in composition and physical properties. J Sci Food Agric 36: 719-727
15 Lelievre J, Lorenz K, Meredith P, Baruch DW (1987) Effects of starch particle size and protein concentration on breadmaking performance. Starch/Stärke 39: 347-352
16 Pomeranz Y (1988) Composition and functionality of wheat flour components. In: Pomeranz Y (ed) Wheat. Chemistry and technology, volume II. American Association of Cereal Chemists, St. Paul, pp 219-370
17 Chung OK (1986) Lipid-protein interactions in wheat flour, dough, gluten, and protein fractions. Cereal F W 31: 242-256
18 Meredith P, Dengate HN, Morrison WR (1978) The lipids of various sizes of wheat starch granules. Starch/Stärke 30: 119-125
19 Morrison WR (1988) Lipids In: Pomeranz Y (ed) Wheat: chemistry and technology, volume I. American Association of Cereal Chemists, St. Paul, pp 373-439
20 Frazier PJ, Daniels NWR, Russell Eggitt PW (1981) Lipid-protein interactions during dough development. J Sci Food Agric 32: 877-897
21 Zawistowska U, Bekes F, Bushuk W (1985) Gluten proteins with high affinity to flour lipids. Cereal Chem 62: 284-289
22 Bell BM, Daniels DGH, Fisher N (1977) Physical aspects of the improvement of dough by fat. Food Chem 2: 57-70
23 Rogers DE, Zeleznak KJ, Lai CS, Hoseney RC (1988) Effect of native lipids, shortening, and bread moisture on bread firming. Cereal Chem 65: 398-401
24 Kulp K (1988) Bread industry and processes. In: Pomeranz Y (ed) Wheat: chemistry and technology, volume II. American Association of Cereal Chemists, St. Paul, pp 371-406
25 Panozzo JF, O'Brien L, MacRitchie F, Bekes F (1990) Baking quality of Australian wheat cultivars varying in their free lipid composition. J Cereal Chem 11: 51-57
26 Bekes F, Zawistowska U, Zillman RR, Bushuk W (1986) Relationship between lipid content and composition and loaf volume of twenty-six common spring wheats. Cereal Chem 63: 327-331
27 Larsen NG, Humphrey-Taylor VJ, Baruch DW (1989) Glycolipid content as a breadmaking quality determinant in flour from New Zealand wheat blends. J Cereal Sci 9: 149-157
28 Bell BM, Daniels DGH, Fearn T, Stewart BA (1987) Lipid compositions, baking qualities and other characteristics of wheat varieties grown in the U.K.. J Cereal Sci 5: 277-286
29 Morrison WR, Law CN, Wylie LJ, Coventry AM, Seekings J (1989) The effect of group 5 chromosomes on the free polar lipids and breadmaking quality of wheat. J Cereal Sci 9: 41-51
30 Law CN, Payne PI, Worland AJ, Miller TE, Harris PA, Snape JW, Reader SM (1984) Studies of genetical variation affecting grain protein type and amount in wheat. In: Cereal grain protein improvement, proceeding of meeting FAO/IAEA/GSF/SIDA. IAEA, Vienna, pp 279-300
31 Bietz JA, Wall JS (1975) The effect of various extractants on the subunit composition and associations of wheat glutenin. Cereal Chem 52: 145-155

32 Spencer D (1984) The physiological role of storage proteins in seeds. Phil Trans R Soc London Ser B 304: 275-285
33 Schofield JD, Booth MR (1983) Wheat proteins and their technological significance. In: Hudson BJF (ed) Developments in food proteins, volume 2. Applied Science Publishers, London, pp 1-65
34 Payne PI, Rhodes AP (1982) Cereal storage proteins: structure and role in agriculture and food technology. Encyc Plant Physiol 14: 346-369
35 Krull LH, Wall JS (1969) Relationship of amino acid composition and wheat protein properties. Baker's Dig 43: 30-39
36 Finney KF, Barmore MA (1948) Loaf volume and protein content of hard winter and spring wheats. Cereal Chem 25: 291-312
37 Finney KF (1985) Experimental breadmaking studies, functional (breadmaking) properties, and related gluten protein fractions. Cereal F W 30: 794-801
38 Tipples KH, Kilborn RH (1974) "Baking strength index" and the relation of protein content to loaf volume. Can J Plant Sci 54: 231-234
39 Johnson VA, Mattern PJ, Kuhr SL (1979) Genetic improvement of wheat protein. In: Seed protein improvement in cereals and grain legumes: proceedings of an international symposium, volume 2. IAEA, Vienna, pp 165-179
40 Zeleny L (1947) A simple sedimentation test for estimating the bread-baking and gluten qualities of wheat flour. Cereal Chem 24: 465-475
41 Orth RA, O'Brien L (1976) A new biochemical test of dough strength of wheat flour. J Aust Inst Agric Sci 42: 122-124
42 Blackman JA, Gill AA (1980) A comparison of some small-scale tests for bread-making quality used in wheat breeding. J Agric Sci 95: 29-34
43 Axford DWE, McDermott EE, Redman DG (1979) Note on the sodium dodecyl sulfate test of breadmaking quality: comparison with Pelshenke and Zeleny tests. Cereal Chem 56: 582-584
44 O'Brien L, Orth RA (1977) Effect of geographic location of growth on wheat milling yield, farinograph properties, flour protein and residue protein. Aust J Agric Res 28: 5-9
45 Preston KR, March PR, Tipples KH (1982) An assessment of the SDS-sedimentation test for the prediction of Canadian bread wheat quality. Can J Plant Sci 62: 545-553
46 O'Brien L, Ronalds JA (1987) Heritabilities of small-scale and standard measures of wheat quality for early generation selection. Aust J Agric Res 38: 801-808
47 Briggs KG, Shebeski LH (1971) Early generation selection for yield and breadmaking quality of hard red spring wheat (*Triticum eastivum* L. em Thell.). Euphytica 20: 453-463
48 Borghi B, Testoni A, Cattaneo M, Corbellini M (1975) Effects of genetic-environment interaction on different tests for bread making quality evaluation. Genet Agrar 29: 391-404
49 Bhatt GM, Derera NF (1975) Genotype x environment interactions for, heritabilities of, and correlations among quality traits in wheat. Euphytica 24: 597-604
50 Starr C, Morgan AG, Smith DB (1981) An evaluation of near infra-red reflectance analysis in some plant breeding programmes. J Agric Sci 97: 107-118
51 Devaux MF, Bertrand D, Martin G (1986) Discrimination of bread-baking quality of wheats according to their variety by near-infrared reflectance spectroscopy. Cereal Chem 63: 151-154
52 Rubenthaler GL, Pomeranz Y (1987) Near-infrared reflectance spectra of hard red winter wheats varying widely in protein content and breadmaking potential. Cereal Chem 64: 407-411
53 Williams PC, Jaby El-Haramein F, Ortiz-Fereira G, Srivastava JP (1988) Preliminary observations on the determination of wheat strength by near-infrared reflectance. Cereal Chem 65: 109-114
54 Meredith OB, Wren JJ (1966) Determination of molecular-weight distribution in wheat-flour

proteins by extraction and gel filtration in a dissociating medium. Cereal Chem 43: 169-186
55 Field JM, Shewry PR, Miflin BJ (1983) Solubilisation and characterisation of wheat gluten proteins: correlations between the amount of aggregated proteins and baking quality. J Sci Food Agric 34: 370-377
56 Bottomley RC, Kearns HF, Schofield JD (1982) Characterisation of wheat flour and gluten proteins using buffers containing sodium dodecyl sulphate. J Sci Food Agric 33: 481-491
57 Batey IL, Gupta RB, MacRitchie F (1991) Use of size-exclusion high-performance liquid chromatography in the study of wheat flour proteins: an improved chromatographic procedure. Cereal Chem 68: 207-209
58 Singh NK, Donovan GR, Batey IL, MacRitchie F (1990) Use of sonification and size-exclusion high-performance liquid chromatography in the study of wheat flour proteins. I. Dissolution of total proteins in the absence of reducing agents. Cereal Chem 67: 150-161
59 Schofield JD (1986) Flour proteins: structure and functionality in baked products. In: Blanshard JMV, Frazier PJ, Galliard T (eds) Chemistry and physics of baking. Royal Society of Chemistry, London, pp 14-29
60 Bloksma AH, Bushuk W (1988) Rheology and chemistry of dough. In: Pomeranz Y (ed) Wheat: chemistry and technology, volume II. American Association of Cereal Chemists, St. Paul, pp 131-217
61 Bietz JA (1985) High performance liquid chromatography: how proteins look in cereals. Cereal Chem 62: 201-212
62 Payne PI, Holt LM, Jackson EA (1984) The genetics of gliadin and glutenin, storage proteins of the wheat endosperm. Kulturpflanze 32: 63
63 Payne PI (1987) Genetics of wheat storage proteins and the effect of allelic variation on bread-making quality. Ann Rev Plant Physiol 38: 141-153
64 Graveland A, Bosveld P, Lichtendonk WJ, Marseille JP, Moonen JHE, Scheepstra A (1985) A model for the molecular structure of the glutenins from wheat flour. J Cereal Sci 3: 1-16
65 Ewart JAD (1979) Glutenin structure. J Sci Food Agric 30: 482-492
66 Payne PI (1986) Varietal improvement in the bread-making quality of wheat: contributions from biochemistry and genetics, and future prospects from molecular biology. In: Day PR (ed) Biotechnology and crop improvement and protection. BCPC monograph 34, Thornton Heath, pp 69-81
67 Tatham AS, Miflin BJ, Shewry PR (1985) The beta-turn conformation in wheat gluten proteins: relationship to gluten elasticity. Cereal Chem 62: 405-412
68 Byers M, Miflin BJ, Smith SJ (1983) A quantitative comparison of the extraction of protein fractions from wheat grain by different solvents, and of the polypeptide and amino acid composition of the alcohol-soluble proteins. J Sci Food Agric 34: 447-462
69 Graybosch RA, Peterson CJ, Hansen LE, Mattern PJ (1990) Relationships between protein solubility characteristics, 1BL/1RS, high molecular weight glutenin composition, and end-use quality in winter wheat germ plasm. Cereal Chem 67: 342-349
70 Khan K, Tamminga G, Lukow O (1989) The effect of wheat flour proteins on mixing and baking -correlations with protein fractions and high molecular weight glutenin subunit composition by gel electrophoresis. Cereal Chem 66: 391-396
71 MacRitchie F, Kasarda DD, Kuzmicky DD (1991) Characterization of wheat protein fractions differing in contributions to breadmaking quality. Cereal Chem 122-130
72 Orth RA, Bushuk W (1972) A comparative study of the proteins of wheats of diverse baking qualities. Cereal Chem 49: 268-275
73 Tanaka K, Bushuk W (1972) Effect of protein content and wheat variety on solubility and electrophoretic properties of flour proteins. Cereal Chem 49: 247-257
74 Chakraborty K, Khan K (1988) Biochemical and breadmaking properties of wheat protein components. II. Reconstitution baking studies of protein fractions from various isolation procedures. Cereal Chem 65: 340-344

75 MacRitchie F (1987) Evaluation of contributions from wheat protein fractions to dough mixing and breadmaking. J Cereal Sci 6: 259-268
76 Finney KF, Jones BL, Shogren MD (1982) Functional (bread-making) properties of wheat protein fractions obtained by ultracentrifugation. Cereal Chem 59: 449-453
77 Kapoor AC, Heiner RE (1982) Biochemical changes in developing wheat grains. Changes in nitrogen fractions, amino acids and nutritional quality. J Sci Food Agric 33: 35-40
78 Dell'Aguila A, Taranto G, Colaprico G (1982) Biochemical changes in developing hexa-, tetra and diploid wheat seeds. Genet Agrar 36: 353-364
79 Lukow OM, Bushuk W (1984) Influence of germination on wheat quality. II. Modification of endosperm protein. Cereal Chem 61: 340-344
80 Ariyama T, Khan K (1990) Effect of laboratory sprouting and storage on physicochemical and breadmaking properties of hard red spring wheat. Cereal Chem 67: 53-58
81 Doekes GJ, Wennekes LMJ (1982) Effect of nitrogen fertilization on quantity and composition of wheat flour protein. Cereal Chem 59: 276-278
82 Cole EW, Fullington JG, Kasarda DD (1981) Grain protein variability among species of *Triticum* and *Aegilops*: quantitative SDS-PAGE studies. Theor Appl Genet 60: 17-30
83 Mansur-Vergara L, Konzak CF, Gerechter-Amitai ZK, Grama A, Blum A (1986) Quantitative variation in the kernel proteins among 841 accessions of *Triticum dicoccoides* estimated by SDS-PAGE. Theor Appl Genet 72: 296-301
84 Fullington JG, Cole EW, Kasarda DD (1980) Quantitative SDS-PAGE of total protein from different wheat varieties. J Sci Food Agric 31: 43-53
85 Fullington JG, Miskelly DM, Wrigley CW, Kasarda DD (1987) Quality-related endosperm proteins in sulfur-deficient and normal wheat grain. J Cereal Sci 5: 233-245
86 Wrigley CW, Du Cros DL, Fullington JG, Kasarda DD (1984) Changes in polypeptide composition and grain quality due to sulfur deficiency in wheat. J Cereal Sci 2: 15-24
87 Fullington JG, Cole EW, Kasarda DD (1983) Quantitative sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total proteins extracted from different wheat varieties: effect of protein content. Cereal Chem 60: 65-71
88 Timms MF, Bottomley RC, Ellis JRS, Schofield JD (1981) The baking quality and protein characteristics of a winter wheat grown at different levels of nitrogen fertilisation. J Sci Food Agric 32: 684-698
89 Huebner FR, Bietz JA (1988) Quantitative variation among gliadins of wheats grown in different environments. Cereal Chem 65: 362-366
90 Marchylo BA, Kruger JE, Hatcher DW (1990) Effect of environment on wheat storage proteins as determined by quantitative reversed-phase high-performance liquid chromatography. Cereal Chem 67: 372-376
91 Seilmeier W, Wieser H, Belitz H-D (1990) Weizen während der Reifung. Analyse der Gliadine und Glutenine mittels RP-HPLC. Z Lebensm Unters Forsch 191: 99-103
92 Huebner FR, Kaczkowski J, Bietz JA (1990) Quantitative variation of wheat proteins from grain at different stages of maturity and from different spike locations. Cereal Chem 67: 464-470
93 Fullington JG, Nityagopal A (1986) Effect of rust infection on the protein components of wheat. Phytochem 25: 1289-1292
94 Bechtel DB, Kaleikau LA, Gaines RL, Seitz LM (1985) The effects of *Fusarium graminearum* infection on wheat kernels. Cereal Chem 62: 191-197
95 Kruger JE, Marchylo BA (1990) Analysis by reversed-phase high-performance liquid chromatography of changes in high molecular weight subunit composition of wheat storage proteins during germination. Cereal Chem 67: 141-147
96 Huebner FR, Wall JS (1976) Fractionation and quantitative differences of glutenin from wheat varieties varying in baking quality. Cereal Chem 53: 258-269
97 Singh NK, Donovan R, MacRitchie F (1990) Use of sonification and size-exclusion high-

performance liquid chromatography in the study of wheat flour proteins. II. Relative quantity of glutenin as a measure of breadmaking quality. Cereal Chem 67: 161-170
98 Dachkevitch T, Autran J-C (1989) Prediction of baking quality of bread wheats in breeding programs by size-exclusion high-performance liquid chromatography. Cereal Chem 66: 448-456
99 Zawistowska U, Bekes F, Bushuk W (1985) Involvement of carbohydrates and lipids in aggregation of glutenin proteins. Cereal Chem 62: 340-345
100 Sutton KH, Hay RL, Griffin WB (1989) Assessment of the potential bread baking quality of New Zealand wheats by RP-HPLC of glutenins. J Cereal Sci 10: 113-121
101 Sutton KH, Hay RL, Mouat CH, Griffin WB (1990) The influence of environment, milling and blending on assessment of the potential breadbaking quality of wheat by RP-HPLC of glutenin subunits. J Cereal Sci 12: 145-153
102 Huebner FR, Bietz JA (1986) Assessment of the potential breadmaking quality of hard wheats by reversed-phase high-performance liquid chromatography of gliadins. J Cereal Sci 4: 379-388
103 Huebner FR (1989) Assessment of potential breadmaking quality of hard spring wheats by high-performance liquid chromatography of gliadins - year two. Cereal Chem 66: 333-337
104 Scanlon MG, Ng PKW, Lawless DE, Bushuk W (1990) Suitability of reversed-phase high-performance liquid chromatographic separation of wheat proteins for long-term statistical assessment of breadmaking quality. Cereal Chem 395-399
105 Kruger JE, Marchylo BA, Hatcher D (1988) Preliminary assessment of a sequential extraction scheme for evaluating quality by reversed-phase high-performance liquid chromatography and electrophoretic analysis of gliadins and glutenins. Cereal Chem 65: 208-214
106 Marchylo BA, Kruger JE, Hatcher DW (1989) Quantitative reversed-phase high-performance liquid chromatographic analysis of wheat storage proteins as a potential quality prediction tool. J Cereal Sci 9: 113-130
107 Branlard G, Dardevet M (1985) Diversity of grain proteins and bread wheat quality. I. Correlation between gliadin bands and flour quality characteristics. J Cereal Sci 3: 329-343
108 Payne PI, Seekings JA, Worland AJ, Jarvis MG, Holt LM (1987) Allelic variation of glutenin subunits and gliadins and its effect on breadmaking quality of wheat: analysis of F5 progeny from Chinese Spring x Chinese Spring (Hope 1A). J Cereal Sci 6: 103-118
109 Mosleth E, Uhlen AK (1990) Identification of quality-related gliadins and prediction of bread-making quality of wheat from the electrophoretic patterns of gliadins and high molecular weight subunits of glutenin. Norw J Agric Sci 4: 27-45
110 Payne PI, Nightingale MA, Krattiger AF, Holt LM (1987) The relationship between HMW glutenin subunit composition and the bread-making quality of Britsh-grown wheat varieties. J Sci Food Agric 40: 51-65
111 Branlard G, Dardevet M (1985) Diversity of grain protein and bread wheat quality. II. Correlation between high molecular weight subunits of glutenin and flour quality characteristics. J Cereal Sci 3: 345-354
112 Gupta RB, Singh NK, Shepherd KW (1989) The cumulative effect of allelic variation in LMW and HMW glutenin subunits on dough properties in the progeny of two bread wheats. Theor Appl Genet 77: 57-64
113 Gupta RB, Bekes F, Wrigley CW (1991) Predicting values of LMW glutenin alleles for dough quality of bread wheat. In: Bushuk W, Tkachuk R (eds) Gluten proteins 1990. American Association of Cereal Chemists, St Paul, Minnesota, pp 615-621
114 Shepherd KW (1988) Genetics of wheat endosperm proteins - in retrospect and prospect. In: Miller TE, Koebner RMD (eds) Proceedings of the Seventh International Wheat Genetics Symposium, volume 2. Institute of Plant Science Research, Cambridge, pp 919-931
115 Harberd NP, Bartels D, Thompson RD (1986) DNA restriction-fragment variation in the

gene family encoding high molecular weight (HMW) glutenin subunits of wheat. Biochem Genet 24: 579-596

116 Metakovsky EV, Novoselskaya AYu, Kopus MM, Sobko TA, Sozinov AA (1984) Blocks of gliadin components in winter wheat detected by one-dimensional polyacrylamide gel electrophoresis. Theor Appl Genet 67: 559-568

117 Gupta RB, Shepherd KW (1990) Two-step one-dimensional SDS-PAGE analysis of LMW subunits of glutelin. 1. Variation and genetic control of the subunits in hexaploid wheats. Theor Appl Genet 80: 65-74

118 Payne PI, Lawrence GJ (1983) Catalogue of alleles for the complex gene loci, Glu-A1, Glu-B1, and Glu-D1 which code for high-molecular-weight subunits of glutenin in hexaploid wheat. Cereal Res Commun 11: 29-35

119 Thompson RD, Bartels D, Harberd NP (1985) Nucleotide sequence of a gene from chromosome 1D of wheat encoding a HMW-glutenin subunit. Nucleic Acids Res 13: 6833-6846

120 Halford NG, Forde J, Anderson OD, Greene FC, Shewry PR (1987) The nucleotide and deduced amino acid sequence of an HMW glutenin subunit gene from chromosome 1B of bread wheat (*Triticum aestivum* L.) and comparison with those of genes from chromosomes 1A and 1D. Theor Appl Genet 75: 117-126

121 Forde J, Malpica J-M, Halford NG, Shewry PR, Anderson OD, Greene FC, Miflin BJ (1985) The nucleotide sequence of a HMW glutenin subunit gene located on chromosome 1A of wheat (*Triticum aestivum* L.). Nucleic Acids Res 13: 6817-6831

122 Anderson OD, Greene FC, Yip RE, Halford NG, Shewry PR, Malpica-Romero J-M (1989) Nucleotide sequences of the two high-molecular-weight glutenin genes from the D-genome of a hexaploid bread wheat, *Triticum aestivum* L. cv Cheyenne. Nucleic Acids Res 17: 461-462

123 Sugiyama T, Rafalski A, Peterson D, Söll D (1985) A wheat HMW glutenin subunit gene reveals a highly repeated structure. Nucleic Acids Res 13: 8729-8737

124 Shewry PR, Tatham AS (1989) New light on an old technology: the structure of wheat gluten and its role in breadmaking. Outlook Agric 18: 65-71

125 Thomas MS, Flavell RB (1990) Identification of an enhancer element for the endosperm-specific expression of high molecular weight glutenin. Plant Cell 2: 1171-1180

126 Colot V, Robert LS, Kavanagh TA, Bevan MW, Thompson RD (1987) Localization of sequences in wheat endosperm protein genes which confer tissue-specific expression in tobacco. EMBO J 6: 3559-3564

127 Robert LS, Thompson RD, Flavell RB (1989) Tissue-specific expression of a wheat high molecular weight glutenin gene in transgenic tobacco. Plant Cell 1: 569-578

128 Finney KF, Heyne EG, Shogren MD, Bolte LC, Pomeranz Y (1985) Functional properties of some European wheats grown in Europe and Kansas. Cereal Chem 62: 83-88

129 Goldsbrough AP, Bulleid NJ, Freedman RB, Flavell RB (1989) Conformational differences between two wheat (*Triticum aestivum*) 'high-molecular-weight' glutenin subunits are due to a short region containing six amino acid differences. Biochem J 263: 837-842

130 Anderson OD, Greene FC (1989) The characterization and comparative analysis of high-molecular-weight glutenin genes from genomes A and B of a heaxploid bread wheat. Theor Appl Genet 77: 689-700

131 Goldsbrough AP, Robert L, Schnick D, Flavell RB (1988) Molecular comparisons between bread-making - quality-determining high molecular weight glutenin subunits of wheat gluten - evidence for the nature of good and poor quality at the protein level. In: Miller TE, Koebner RMD (eds) Proceedings of the Seventh International Wheat Genetics Symposium, volume 1. Institute of Plant Science Research, Cambridge, pp 727-733

CHAPTER 2

ADDITIVE AND EPISTATIC EFFECTS OF ALLELIC VARIATION AT THE HIGH MOLECULAR WEIGHT GLUTENIN SUBUNIT LOCI IN DETERMINING THE BREAD-MAKING QUALITY OF BREEDING LINES OF WHEAT[1]

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Summary

The relation has been studied between the High Molecular Weight glutenin (HMWg) subunit alleles and the bread-making quality of 226 lines of winter wheat (*Triticum aestivum* L.), grown in The Netherlands. The lines represented a wide range of genetic backgrounds, and had not been selected for quality, in contrast to the established varieties used by other authors.
The variation in HMWg subunit genotypes accounted for about 20% of the total variation in loaf volume among the lines. Most important was the allelic variation at the *Glu-D1* locus. The *Glu-D1* allele encoding the subunit 5+10 was superior to its allelic counterpart, encoding 2+12. The difference in average loaf volume between groups of lines containing 5+10 or 2+12 was positively related with protein content of the flours. When protein content *Glu-1* loci also below 9.2%, no effect of allelic variation at the *Glu-D1* locus was present. Epistatic effects between the also contributed to the variation in loaf volume of the lines: i.e. the effect of allelic variation at *Glu-A1* and *Glu-B1* depended on the allele present at the *Glu-D1*. The contribution of the epistatic effects was about half the contribution of the additive effects, and should therefore be included in predictive models for bread-making quality.

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Introduction

The variation in amount and composition of the storage proteins of wheat (*Triticum aestivum* L.) contributes to the variation in bread-making quality between genotypes. The bread-making quality of wheat, here defined as the volume of the loaf, is positively correlated with the protein content of the flour. Moreover, the regression of loaf volume on protein content is genotype-specific, which reflects differences in protein quality (Finney and Barmore 1948). Protein quality depends on the genetically determined composition of the storage proteins. These proteins consist of a number of fractions, viz. the High Molecular Weight glutenin (HMWg) subunits, the Low Molecular Weight glutenin (LMWg) subunits and the gliadins (Schofield and Booth 1983; Shewry et al. 1986). Each of these fractions may contribute to the bread-making quality of wheat. Other kernel characteristics, such as hardness, lipid composition and starch properties are also important in this respect.

The relationship between the allelic variation in composition of the HMWg subunits and the bread-making quality has been studied, using varieties (Wrigley et al. 1982; Moonen et al. 1983; Branlard and Dardevet 1985; Campbell et al. 1987; Lawrence et al. 1987; Odenbach and Mahgoub 1987; Payne et al. 1987b; Ng and Bushuk 1988), breeding lines (Payne et al. 1979, 1981; Moonen et al. 1982; Cressey et al. 1987; Lorenzo et al. 1987; Van Gelder et al. 1987) and near-isogenic lines, inbred backcross lines or recombinant inbred lines (Moonen and Zeven 1985; Payne et al. 1987a; Odenbach and Mahgoub 1988; Carrillo et al. 1990). The HMWg subunits are encoded by three complex homeoallelic HMWg subunit loci, the *Glu-A1*, *Glu-B1* and *Glu-D1* loci. On each HMWg subunit locus, alleles are present which show different relationships with the bread-making quality. Payne and coworkers (1979, 1987b) ranked the alleles of the three *Glu-1* loci for their contribution to bread-making quality, after determination of the quality of the lines of segregating progenies by the SDS-sedimentation test according to Axford et al. (1979). In general, these observations were confirmed by others (Moonen et al. 1982; Branlard and Dardevet 1985; Campbell et al. 1987; Cressey et al. 1987), but in these studies, there was no, or just a weak, contribution of allelic variation at *Glu-A1* and *Glu-B1* to the variation in bread-making quality. In other studies, the ranking of the alleles of *Glu-A1* (Moonen et al. 1983; Van Gelder et al. 1987; Pogna et al. 1989) or *Glu-B1* (Odenbach and Mahgoub 1988) differed from the results obtained by Payne and coworkers. All authors agreed upon a considerable

effect of the *Glu-D1* locus.

To enable a prediction of the bread-making quality of lines in a breeding programme, Payne et al. (1987b) assigned a quality score to the alleles of the *Glu-1* loci - the *Glu-1* quality score - which is based on the ranking of the alleles for bread-making quality. Assuming the effect of the alleles to be additive, the bread-making quality was predicted by adding the scores of the alleles present in the particular line. Pogna et al. (1989) developed a scoring system based on a ranking of the HMWg alleles for the alveograph W value. A significant relationship between *Glu-1* quality score and bread-making quality was reported for varieties grown in Britain (Payne et al. 1987b), Spain (Payne et al. 1988), Canada (Lukow et al. 1989) and Germany (Rogers et al. 1989). About 30% to 79% of the variation in bread-making quality of these varieties was ascribed to the variation in *Glu-1* score. Ng and Bushuk (1988), who analyzed the HMWg subunit composition instead of a score, estimated 67.5% of the variation in bread-making quality of Canadian-grown wheat varieties to be accounted for by the variation in the HMWg genotypes.

In view of these findings, the quality of individual alleles of the HMWg loci has generally been suggested as a selection criterion in breeding programmes for bread-making quality. However, it is questionable whether the studies quoted, in which varieties are used, are representative of the breeding situation. In varieties, the amount of variation in bread-making quality which can be ascribed to allelic variation at the *Glu-1* loci, might be affected by common ancestry (pedigree effects) (Wrigley et al. 1982), and the selection for bread-making quality during the plant-breeding process (Campbell et al. 1987). Furthermore, it has been shown that score systems based on the HMWg alleles are not always adequate in predicting the bread-making quality of breeding lines (Van Gelder et al. 1987; Brunori et al. 1989; Graybosch et al. 1990). Finally, Van Gelder et al. (1987) showed that the relation between the presence of an HMWg allele and the bread-making quality depended on the HMWg allele composition.

The aim of this study was to investigate whether the HMWg subunit allele composition can be used as a means of predicting bread-making quality of breeding lines. For this purpose, it was decided to use lines which a) were randomly chosen, i.e. had not been selected for bread-making quality, and which b) originated from crosses between genotypes differing widely in genetic background. A preliminary analysis carried out on a limited number of the lines was presented elsewhere (Van Gelder et al. 1987).

Material and methods

Plant material

In this study, lines of winter wheat were used, which were developed in the breeding programme for bread-making quality of the former Foundation for Agricultural Plant Breeding (SVP) (Mesdag 1985). The lines had not been selected for bread-making quality, but for agronomic performance only. Therefore, they are considered at random with respect to the property studied here. In the crossing programme, SVP breeding lines and varieties were used. In total, 226 lines, originating from 156 crosses, were analyzed. Twenty crosses were between two varieties, 58 between a variety and a breeding line, and 78 between two breeding lines. About 74% of the crosses resulted in one line, 18% in two lines, and 8% in more than two lines. The varieties introduced in the crossing programme had diverse origins (Table 2.1).

Table 2.1 Country of origin of varieties used in the breeding programme, and the number of times that a cross with a variety resulted in a line after selection.

Country	Number of varieties	Number of times a variety was used as a parent					
		1	2	3	4	5	6
Netherlands	4	1	1	1			1
United States	11	7	2		1	1	
France	6	2		2	1	1	
Germany	11	4	3	1	3		
Belgium	1		1				
USSR	2	1				1	
Rumania	1	1					
Switzerland	4	1	3				
Austria	2	1	1				
United Kingdom	1	1					
Italy	2	2					
Bulgaria	1	1					
Unknown	1	1					

The breeding lines used in the programme originated from 92 crosses, and were likewise derived from a large number of varieties. Therefore, the lines used in the baking tests had a wide range of genetic backgrounds. Lines grown from 1980 to 1988, except 1984, were used. In 1984, only baking tests without the addition of

ascorbic acid were performed. Each year, a unique set of lines was grown in duplicate, under standard agronomic conditions on plots of at least 6 m^2.

Bread-making tests

The bread-making quality of flour from kernels milled on a Brabender Quadrumat-Junior mill, was determined using the micro-baking test described in detail by Meppelink (1981). Baking tests were carried out using unfortified flour and using the flour after addition of 25 ppm $KBrO_3$ (from 1980 to 1982) or 25 ppm ascorbic acid (in 1983 and from 1985 to 1988). All baking tests were in duplicate. Samples showing a falling number (ICC standard 107) lower than 170 were discarded. Only the results of baking tests with the addition of oxidizing agents are reported here. The protein content was determined as N x 5.7, after Kjeldahl digestion and using an auto-analyzer system, and was expressed on a 100% dry weight basis.

Electrophoresis

The HMWg subunit composition of flour samples was determined by SDS-PAGE using electrophoretic conditions described elsewhere (Kolster et al. 1988, Chapter 3 of this thesis) and 8.3% acrylamide gels (Lawrence and Shepherd 1980). If necessary, the samples were additionally analyzed using a 7.5% or 10% gel, to separate subunits 2 and 2* or 9 and 10, respectively (Lawrence 1986). HMWg subunits were identified according to Payne and Lawrence (1983).

Table 2.2 Occurrence of HMWg alleles at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci in a population of 226 random breeding lines of winter wheat.

Glu-A1 locus			*Glu-B1* locus			*Glu-D1* locus		
subunits	number	%	subunits	number	%	subunits	number	%
0	94	42	6+8	80	35	2+12	143	63
1	57	25	7	85	38	5+10	83	37
2*	75	33	7+8	44	19			
			7+9	17	8			

A small number of lines contained subunits 13+16, 20 and 21 (*Glu-B1* locus) and subunits 3+12 (*Glu-D1*). These lines were not included in the set of lines used in the statistical analyses. Heterogenous lines containing different HMWg subunit genotypes were also excluded from the set of lines. In the remaining 226 lines,

three *Glu-A1* alleles, seven *Glu-B1* alleles and three *Glu-D1* alleles were identified. Table 2.2 shows the frequencies of the HMWg subunits occurring in these lines. The presence of γ-secalines was determined in the lines, using a procedure derived from Payne et al. (1987b). Only five lines contained these proteins, therefore their possible effect on the bread-making quality was not included in the analyses.

Statistical analyses

The relationship between the alleles of the *Glu-A1*, *Glu-B1* and *Glu-D1* loci and loaf volume, which is a quantitative variable, can be analyzed in a familiar analysis of variance set-up with a few modifications to account for idiosyncracies of the data. A variation in water absorption and protein content of the flour and in kernel hardness can affect the loaf volume. Preliminary analyses showed that, besides the qualitative factors representing the *Glu-1* alleles, only the protein content merited incorporation in the statistical model. Years was treated as a block factor.

The combinations of the alleles at the three *Glu-1* loci did appear in unequal frequencies in the data. To compensate for this, weights were assigned to the individual observations, so as to make the total weight 1 for each of the possible *Glu-A1* * *Glu-B1* * *Glu-D1* allele combinations. It seems not superfluous to note that weighting may lead to biased estimates. The weighting resulted in what is, probably counter-intuitively, called an unweighted means analysis (Searle 1971; pp 365-369).

Although the model was balanced with respect to the allele combinations, it was not balanced when years and protein content were considered. As a consequence, treatment effects are not orthogonal, and the size and significance of terms depends on the order in which the terms are added to the model (Searle 1971; Mead 1988). A reasonable strategy for assessing the relevance of a term is to estimate its sums of squares when fitted as the first or the last in a row of comparable terms (the main effects for the loci and protein content on the one hand, and the interaction terms between two loci, or a locus and protein content on the other hand). This results in estimates of the maximum or minimum impacts of a term on the loaf volume respectively. Significance of the terms can be deduced from the variance ratios. Genstat (Genstat 5 committee 1987) was used for the statistical analyses.

Results

The results of the analysis of variance for the loaf volume of the lines are presented in Table 2.3. The sums of squares are given as percentages of the total sum of squares and can be interpreted as indications of the relevance of the various terms. Due to nonorthogonality of the terms, the percentages of the total sums of squares do not add up to 100%. The fitting order of the terms (see Material and methods) was found to be not relevant to the overall conclusions.

Table 2.3 Analyses of variance of loaf volume. Minimum and maximum estimates for the sums of squares (see Material and methods), expressed as percentages of the total sum of squares, and the corresponding variance ratios. Variance ratios are approximately F distributed. $\sigma^2 = 141$. Year was treated as a block term.

		Sums of squares		Variance ratios	
Source	df[1]	minimum	maximum	minimum	maximum
year	6	24	24		
protein content(pc)	1	15	21	93.1 ***	136.3
Glu-A1	2	1	2	1.8 n.s.	6.4
Glu-B1	3	2	4	3.9 **	8.3
Glu-D1	1	8	9	50.3 ***	55.4
Glu-A1 * *Glu-B1*	6	1	1	1.1 n.s.	1.2
Glu-A1 * *Glu-D1*	2	2	3	7.2 ***	10.0
Glu-B1 * *Glu-D1*	3	3	3	5.5 ***	7.3
Glu-A1 * pc	2	1	2	1.8 n.s.	6.8
Glu-B1 * pc	3	1	1	1.4 n.s.	1.2
Glu-D1 * pc	1	3	7	20.5 ***	46.4
error	195	31	31		
total	225				

[1] df = degrees of freedom
n.s. = not significant; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

The year in which the lines were grown accounted for 24% of the total variation in loaf volume. In this study, the year effect is caused by the variation in the growing conditions and the use of a different set of lines each year. The variation in protein content of the flours also had an important contribution to the variation in loaf volume. Because of a highly significant ($P \leq 0.001$) interaction between protein content and the *Glu-D1* locus in determining loaf volume, this effect is studied in more detail hereafter.

For loafs baked from flour of lines containing *Glu-D1* subunits 2+12, an increase in protein content of 1% resulted in an increase in loaf volume of 10.8 ml, whereas

flour of lines containing its allelic counterpart 5+10 showed an increase of 25.3 ml (Figure 2.1).

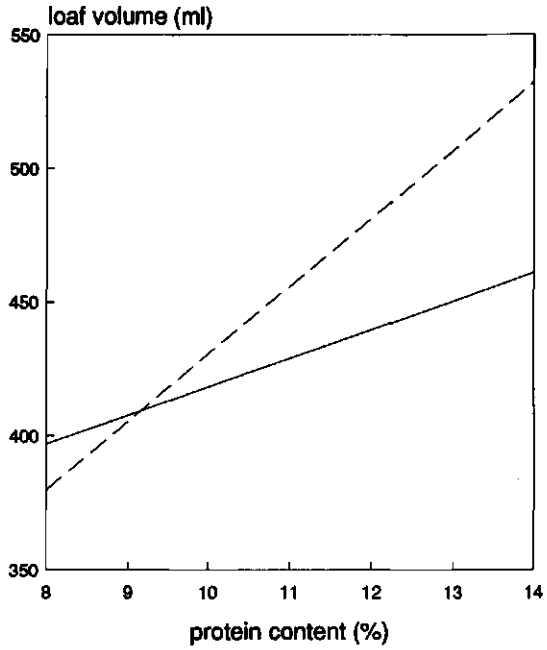

Figure 2.1 The fitted regression lines of loaf volume on protein content of flour of breeding lines containing *Glu-D1* allele 2+12 (———) and 5+10 (------).

The protein content accounted for 15-21% of the variation in loaf volume, the *Glu-D1* * protein content interaction accounted for an additional 3-7% of the variation. When the protein content was low, such as in 1983 when the average protein content was 9.2%, groups of lines containing *Glu-D1* subunits 2+12 or 5+10 did not differ in loaf volume (Figure 2.1). As the protein content increases to above 9.2%, in the present study up to 14%, the difference between these groups of lines increases. Branlard (1987), who studied genotypes with a protein content varying from 10 to 19%, found effects of HMWg alleles on the bread-making quality between 10% and 15% protein, and no effects when the protein content exceeded 15%. Therefore, the effect of variation in HMWg allele composition depends on the protein content of the flour used in the baking tests.

The allelic variation at the *Glu-D1* locus contributed importantly (Table 2.3) to the variation in loaf volume between the breeding lines. *Glu-D1* subunits 5+10 were superior to its allelic counterpart 2+12 (Figure 2.1 and Table 2.4), which agrees

with the results of others (Payne et al. 1987b; Pogna et al. 1989; Branlard and Dardevet 1985; Moonen et al. 1983; Van Gelder et al. 1987).

The effect of the variation in HMWg allele composition on the variation in loaf volume not only depended on additive effects between the loci; interactions between alleles of the *Glu-A1* and *Glu-D1* loci, and between alleles of the *Glu-B1* and *Glu-D1* loci, contributed significantly ($P \leq 0.001$, Table 2.3) to the variation in loaf volume. The epistatic effects of the *Glu-1* loci in determining the loaf volume are shown in Table 2.4. *Glu-A1* subunits 1 and 2* are superior to *Glu-A1* null (0) only in combination with *Glu-D1* subunits 5+10. This *Glu-A1* effect in the presence of *Glu-D1* subunits 5+10 is in agreement with the effects of the *Glu-A1* alleles irrespective of the allele composition of the other loci as found by Payne et al. (1987b). When *Glu-D1* subunits 5+10 are present, *Glu-B1* subunits 7+8 and 7+9 are superior to the other *Glu-B1* subunits. This effect is in general agreement with the results of Payne et al. (1987b), who ranked the subunits coded for by the *Glu-B1* locus as follows: 6+8 = 7 < 7+9 < 7+8. However, when the allele composition of the *Glu-D1* locus is taken into consideration, it appears that the *Glu-B1* allele encoding the subunits 7+8 are significantly inferior to its allelic counterparts only in the presence of *Glu-D1* subunits 2+12.

Table 2.4 The loaf volume of groups of lines, differing in their HMWg allele composition, according to the fitted statistical model. Protein content in the model was 11.6%, being the average for the data used. LSD (0.05) for *Glu-A1* * *Glu-D1* = 12, LSD for *Glu-B1* * *Glu-D1* = 14.

	Glu-D1			Glu-D1	
Glu-A1	2+12	5+10	*Glu-B1*	2+12	5+10
0	435	457	6+8	436	455
1	428	488	7	442	456
2*	445	471	7+8	423	483
			7+9	443	494

Another factor that interacted with the allele composition is the addition of oxidizing agents, as it appeared that when loafs were produced without addition, only the *Glu-B1* subunits 7+9 were superior to the other *Glu-B1* alleles in combination with *Glu-D1* subunits 5+10 (results not shown).

The interaction effect between the *Glu-A1* locus and protein content, when the term was fitted as the first in the row of interaction effects (see maximum columns in Table 2.3), is due to the *Glu-A1* * *Glu-D1* and *Glu-D1* * protein content

interactions.

The epistatic effects between the *Glu-1* loci accounted for 6-7% of the variation in bread-making quality between the lines, whereas the additive effects accounted for 11-15%. The interaction effects were slightly less pronounced when analyzing loaf volume without the addition of oxidizing agents (results not shown).

Discussion

The choice to use the HMWg allele composition in the statistical analyses, and not analyses of scores assigned beforehand (Payne et al. 1987b, 1988; Lukow et al. 1989; Rogers et al. 1989), was motivated by the fact that scores imply that the *Glu-1* alleles exert the same influence under all circumstances. As shown in this study, this is not the case. The variation in loaf volume between the breeding lines is, after correction for the nuisance factor years, primarily determined by the variation in protein content of the flour produced from the kernels of the lines. Allelic variation at the *Glu-D1* locus has an important effect upon the bread-making quality. In addition, epistatic effects of the *Glu-1* loci contributed to the variation in bread-making quality.

Interactions between the HMWg alleles in determining the bread-making quality have been described before. Lorenzo et al. (1987) reported in genotypes heterozygous on the *Glu-1* loci intra-allelic interactions (dominance). Payne et al. (1987a) showed that, in near-isogenic substitution lines, the reduction in bread-making quality (determined by the SDS- sedimentation test) as a result of the substitution of HMWg alleles by a null-allele depended on the composition of the remaining subunits. Odenbach and Mahgoub (1988) reported interactions in inbred-backcross lines between *Glu-B1* subunits 7+8 and 7+9 and *Glu-D1* subunits 5+10 and 2+12 in determining the bread-making quality, as determined by the Zeleny sedimentation test. This interaction is essentially the same as the *Glu-B1* * *Glu-D1* interaction reported in this study. Furthermore, epistatic effects between HMWg alleles in determining the SDS-sedimentation volume are also described by Carrillo et al. (1990), who used F_2-derived F_8 lines originating from one cross. The interactions reported in the studies quoted may only be present in the specific genetic backgrounds of the lines used, or the contribution of these interactions to the variation in bread-making quality can be affected by the genetic background. Therefore, it cannot be determined from these studies whether these epistatic effects

are of importance for the breeding for bread-making quality. The results presented in the present study do show that epistatic effects have an important contribution to the variation in loaf volume between Dutch breeding lines differing widely in genetic background, and should therefore be taken into consideration in breeding for bread-making quality. Furthermore, when establishing the contribution to the bread-making quality of a new HMWg allele, the interactions between the alleles on the other *Glu-1* loci might affect the phenotypic expression of the quality of the new allele. The cause of the epistatic effects between alleles on homeologous loci is not known. Causes can be at the biochemical (protein-protein interactions) or at the genetic level (gene-expression depending on the type of allele present at homeologous loci).

The degree of variation in bread-making quality which can be ascribed to variation in HMWg genotypes, 17-22%, is low compared with other studies (Payne et al. 1987b, 1988; Lukow et al. 1989; Rogers et al. 1989; Ng and Bushuk 1988). The unbiased experimental material - the wide genetic origin of the lines used in the present study, and the absence of selection for bread-making quality during the breeding process - may have contributed to this discrepancy. However, an accurate comparison is hampered by the use of the *Glu-1* score in the statistical analysis in the studies quoted, instead of the actual allele make-up of the HMWg genotypes, with the exception of Ng and Bushuk (1988). Comparison is furthermore hampered by the use of different criteria for bread-making quality, such as the Zeleny sedimentation test and the alveograph W value (Payne et al. 1988), the loaf volume (Lukow et al. 1989; Ng and Bushuk 1988), or the bread-making quality as determined by the classification of varieties after variety testing (Payne et al. 1987b; Rogers et al. 1989).

Differences in quality of HMWg alleles have generally been suggested as an important tool for the breeding for bread-making quality. Because of the low degree of variation in loaf volume, which can be attributed to the variation in HMWg genotypes in the set of lines used in the present study, its importance should not be overestimated. The response to selection obtained by selection techniques estimating the quality of the total flour proteins, such as the SDS-sedimentation test, equals the response obtained by selection for HMWg alleles (Kolster, unpublished results). However, the introduction into a breeding programme of high quality HMWg alleles is an important first step to increase the bread-making quality.

In studying the relation between the composition of the HMWg subunits and the bread-making quality, the possible effect of variation in amount of individual

HMWg subunits has hardly been studied so far. A study on this subject is in progress to see whether it is relevant and possible to optimize the composition of the HMWg subunits qualitatively as well as quantitatively.

References

Axford DWE, McDermott EE, Redman DG (1979) Note on the sodium dodecyl sulphate test of breadmaking quality: comparison with Pelshenke and Zeleny tests. Cereal Chem 56: 582-584

Branlard G (1987) Prediction of the bread wheat quality from HMW glutenins and gliadins. In: Lasztity L, Bekes F (eds) Gluten proteins. Proceedings of the IIIrd Gluten Protein Workshop. Budapest, May 9-12, pp 604-612

Branlard G, Dardevet M (1985) Diversity of grain protein and bread wheat quality. II. Correlation between high molecular weight subunits of glutenin and flour quality characteristics. J Cereal Sci 3: 345-354

Brunori A, Galterio G, Zannettino C, Pogna NE (1989) Bread-making quality indices in *Triticum aestivum* progenies. Implications in breeding for better bread wheat. Plant Breed 102: 222-231

Campbell WP, Wrigley CW, Cressey PJ, Slack CR (1987) Statistical correlations between quality attributes and grain-protein composition for 71 hexaploid wheats used as breeding parents. Cereal Chem 64: 293-299

Carrillo JM, Rousset M, Qualset CO, Kasarda DD (1990) Use of recombinant inbred lines of wheat for study of associations of high-molecular-weight glutenin subunit alleles to quantitative traits. 1. Grain yield and quality prediction tests. Theor Appl Genet 79: 321-330

Cressey PJ, Campbell WP, Wrigley CW, Griffin WB (1987) Statistical correlations between quality attributes and grain-protein composition for 60 advanced lines of crossbred wheat. Cereal Chem 64: 299-301

Finney KF, Barmore MA (1948) Loaf volume and protein content of hard winter and spring wheats. Cereal Chem XXV: 291-312

Genstat 5 committee (1987) Genstat 5 reference manual. Clarendon Press, Oxford

Graybosch RA, Peterson CJ, Hansen LE, Mattern PJ (1990) Relationships between protein solubility characteristics, 1BL/1RS, high molecular weight glutenin composition, and end-use quality in winter wheat germ plasm. Cereal Chem 67: 342-349

Kolster P, Krechting CF, Van Gelder WMJ (1988) Variation in high molecular weight glutenin subunits of *Triticum aestivum* and *T. turgidum* ssp. *dicoccoides*. Euphytica S:141-145

Lawrence GJ (1986) The high-molecular-weight glutenin subunit composition of Australian wheat cultivars. Aust J Agric Res 37: 125-133

Lawrence GJ, Shepherd KW (1980) Variation in glutenin protein subunits of wheat. Aust J Biol Sci 33: 221-233

Lawrence GJ, Moss HJ, Shepherd KW, Wrigley CW (1987) Dough quality of biotypes of eleven Australian wheat cultivars that differ in high-molecular-weight glutenin subunit composition. J Cereal Sci 6: 99-101

Lorenzo A, Kronstad WE, Vieira LGE (1987) Relationship between high molecular weight glutenin subunits and loaf volume in wheat as measured by the sodium dodecyl sulphate sedimentation test. Crop Sci 27: 253-257

Lukow OM, Payne PI, Tkachuk R (1989) The HMW glutenin subunit composition of Canadian wheat cultivars and their association with bread-making quality. J Sci Food Agric 46: 451-460

Mead R (1988) The design of experiments. Cambridge University Press, Cambridge

Meppelink EK (1981) Einsatzmöglichkeit des Mikrobackversuches in der Weizenzüchtung.

Getreide Mehl Brot 35: 107-109
Mesdag J (1985) Developments in breeding winter wheat for bread-baking quality in some north-western European countries. Neth J Agric Sci 33: 215-234
Moonen JHE, Zeven AC (1985) Association between high molecular weight subunits of glutenin and bread-making quality in wheat lines derived from backcrosses between *Triticum aestivum* and *Triticum speltoides*. J Cereal Sci 3: 97-101
Moonen JHE, Scheepstra A, Graveland A (1982) Use of the SDS-sedimentation test and SDS-polyacrylamidegel electrophoresis for screening breeder's samples of wheat for bread-making quality. Euphytica 31: 677-690
Moonen JHE, Scheepstra A, Graveland A (1983) The positive effects of the high molecular weight subunits 3+10 and 2* of glutenin on the bread-making quality of wheat cultivars. Euphytica 32: 735-742
Ng PKW, Bushuk W (1988) Statistical relationships between high molecular weight subunits of glutenin and breadmaking quality of Canadian-grown wheats. Cereal Chem 65:408-413
Odenbach W, Mahgoub E (1987) Beziehungen zwischen der Backqualität und dem Vorkommen der Gene für die hochmolekularen Untereinheiten des Glutenins bei den deutchen Winter- und Sommerweizensorten. Getreide Mehl Brot 41: 195-198
Odenbach W, Mahgoub E (1988) Relationships between HMW glutenin subunit composition and the sedimentation value in reciprocal sets of inbred backcross lines derived from two winter wheat crosses. In: Miller TE, Koebner RMD (eds) Proceedings of the seventh international wheat genetics symposium, pp 987-991
Payne PI, Lawrence GJ (1983) Catalogue of alleles for the complex gene loci, Glu-A1, Glu-B1, and Glu-D1 which code for high-molecular-weight subunits of glutenin in hexaploid wheat. Cereal Res Commun 11: 29-35
Payne PI, Corfield KG, Blackman JA (1979) Identification of a high-molecular-weight subunit of glutenin whose presence correlates with bread-making quality in wheats of related pedigree. Theor Appl Genet 55: 153-159
Payne PI, Corfield KG, Holt LM, Blackman JA (1981) Correlations between the inheritance of certain high-molecular weight subunits of glutenin and bread-making quality in progenies of six crosses of bread wheat. J Sci Food Agric 32: 51-60
Payne PI, Holt LM, Krattiger AF, Carrillo JM (1988) Relationships between seed quality and characteristics and HMW glutenin subunit composition determined using wheats grown in Spain. J Cereal Sci 7: 229-235
Payne PI, Nightingale MA, Krattiger AF, Holt LM (1987a) The relationship between HMW glutenin subunit composition and the bread-making quality of British-grown wheat varieties. J Sci Food Agric 40: 51-65
Payne PI, Holt LM, Harinder K, MaCartney DP, Lawrence GJ (1987b) The use of near-isogenic lines with different HMW glutenin subunits in studying bread-making quality and glutenin structure. In: Lasztity L, Bekes F (eds) Gluten proteins. Proceedings of the IIIrd Gluten Protein Workshop. Budapest, May 9-12, pp 216-226
Pogna NE, Mellini F, Beretta A, Dal Belin Peruffo A (1989) The high-molecular-weight glutenin subunits of common wheat cultivars grown in Italy. J Genet & Breed 43: 17-24
Rogers WJ, Payne PI, Harinder K (1989) The HMW glutenin subunit and gliadin compositions of German-grown wheat varieties and their relationship with bread-making quality. Plant Breeding 103: 89-100
Searle SR (1971) Linear Models. Wiley, New York
Schofield JD, Booth MR (1983) Wheat proteins and their technological significance. In: Hudson BJF (ed) Developments in food proteins, vol 2. Applied Science Publishers, London, pp 1-65
Shewry PR, Tatham AS, Forde J, Kreis M, Miflin BJ (1986) The classification and nomenclature of wheat gluten proteins: a reassessment. J Cereal Sci 4: 97-106
Van Gelder WMJ, Kolster P, Mesdag J, Van Eeuwijk FA (1987) The relationship between high-

molecular-weight glutenin subunits, bread-making quality and yield of winter wheat. In: Borghi B (ed) Agriculture. Hard wheat: agronomic, technological, biochemical and genetic aspects. Commission of the European Communities, Luxembourg, pp 159-172

Wrigley CW, Lawrence GJ, Shepherd KW (1982) Association of glutenin subunits with gliadin composition and grain quality in wheat. Aust J Plant Physiol 9: 15-30

CHAPTER 3

VARIATION IN HIGH MOLECULAR WEIGHT GLUTENIN SUBUNITS OF *TRITICUM AESTIVUM* AND *T. TURGIDUM* SSP. *DICOCCOIDES*[1]

P Kolster, CF Krechting[2], WMJ van Gelder

Summary

Two-dimensional electrophoresis revealed biochemical variation for *Glu-B1* High Molecular Weight glutenin (HMWg) subunit 7 present in Dutch wheat (*Triticum aestivum* L.) varieties. This variation was not recognized by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). In *T. turgidum* ssp. *dicoccoides*, HMWg subunits were detected which do not occur in varieties. Some of these subunits show similar relative mobilities during SDS-PAGE as subunits present in varieties and as such unambiguous identification is not possible. Before species related to *T. aestivum* are used in a breeding programme, their HMWg subunits should be identified by two-dimensional electrophoresis, in order to avoid the introduction of HMWg subunits, of which the influence on bread-making quality is not known.

[1] Euphytica S (1988): 141-145

[2] DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO)

Introduction

The content and the composition of the endosperm proteins are important characteristics with respect to the bread-making quality of wheat (*Triticum aestivum* L.). A particular group of endosperm proteins, the High Molecular Weight (HMW) glutenins, largely contribute to the bread-making quality. These HMW glutenins are composed of subunits showing molecular masses of 80 to 140 kD (Lawrence and Shepherd 1980) which form protein aggregates of 120 to over 10 million kD (Graveland et al. 1985). The HMW glutenin (HMWg) subunits are encoded by complex loci on the long arms of the chromosomes 1A, 1B and 1D of hexaploid wheat, the *Glu-A1*, *Glu-B1* and *Glu-D1* locus, respectively (Payne et al. 1984). Until now, more then 20 alleles of these loci have been found in cultivars (Branlard and Le Blanc 1985; Payne et al. 1987) and in addition, numerous other alleles have been found in landraces and in species related to bread wheat (Vallega and Waines 1987; Waines and Payne 1987). Variation in the composition of the HMWg subunits has been shown to be responsible for differences in bread-making quality of genotypes (Payne et al. 1979). Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) of these subunits has been used to identify the alleles and to study their effects on the bread-making quality of genotypes (Payne et al. 1979; Moonen et al. 1983; Branlard and Dardevet 1985; Payne et al. 1987). The identification of the HMWg subunits, and as such of the alleles, is based on their relative mobilities (R_m) during SDS-PAGE. As the R_m of some HMWg subunits differ only slightly a correct identification of the alleles is sometimes difficult (Lawrence 1986; Van Gelder et al. 1987).

Careful separation by SDS-PAGE of the HMWg subunits present in Dutch wheat varieties, revealed very small but reproducible differences in R_m between two subunits (Van Gelder et al. 1987) which both had formerly been identified as subunit 7 (Moonen et al. 1982). The research reported in this article shows that these subunits can be clearly separated by two-dimensional (2-D) electrophoresis, using IsoElectric Focussing (IEF) and SDS-PAGE and that their differences in R_m correspond to differences in IsoElectric Point (IEP). It further shows that introduction into a breeding programme of species related to bread wheat may introduce HMWg alleles which cannot be distinguished from alleles already present in bread wheat, and the consequences thereof are discussed.

Material and methods

Plant material

Seed of reference wheat varieties for identification of the HMWg subunits (Payne and Lawrence 1983) was obtained from Dr. P.I. Payne (PBI, UK). The material of *T. turgidum* ssp. *dicoccoides* was provided by Dr. W. Lange (Foundation for Agricultural Plant Breeding (SVP)) and originated from collections made in Israel by Dr. Z.K. Gerechter-Amitai, within the framework of a co-operative programme between the Volcani Center (Bet Dagan, Israel) and the Research Institute for Plant Protection (Wageningen, The Netherlands).

One-dimensional SDS-PAGE

Extraction of the proteins was of Moonen et al. (1982). SDS-PAGE was a modification of the procedure described by Lawrence and Shepherd (1980). Gels (180 * 140 * 1.5 mm) of 8.3% acrylamide were electrophoresed at a constant current during 30 minutes at 15 mA and during 8 hours at 35 mA per gel, using a Pharmacia GE-2/4 LS vertical slab gel apparatus. The electrophoresis buffer was cooled by recirculating water of 4°C. Gels were stained overnight at room temperature in a freshly prepared solution consisting of 0.2% (w/v) Coomassie Brilliant Blue R250, 45% (v/v) methanol, 10% (v/v) acetic acid and 45% (v/v) water and were destained using a solution consisting of 25% (v/v) ethanol (96%), 8% (v/v) acetic acid and 67% (v/v) water.

Two-dimensional gel electrophoresis

Two-dimensional electrophoresis (IEF * SDS-PAGE) was according to the procedure of Brown et al. (1979) using the following modifications. Two crushed kernels were extracted in 0.7 ml freshly prepared extraction medium. Twelve glass tubes (120 mm * 1.7 mm internal diameter) were simultaneously filled with the gel solution in order to obtain reproducible IEF gels. The Pharmalyte 5-8 concentration was 5.2% (v/v) and the 2-D Pharmalyte 3-10 concentration was 1.22% (v/v). After the pre-run, 25 µl extract was applied onto the gel and the gels were run for 15 hours at 400 V for isoelectric focussing. After separation in the first dimension, the glass tubes were placed at 4°C for 15 minutes in order to have them slip out of the glass tube more easily. After the gels were incubated in incubation buffer (Brown et al. 1979) for 30 minutes at 35°C, they were laid directly on top of an SDS-PAGE gel for separation in the second dimension. Running, staining and destaining were

as described for one-dimensional electrophoresis.

Results and discussion

Figure 3.1 shows the HMWg subunits of Chinese Spring and of the Dutch wheat varieties Arminda and Adonis, separated by carefully optimized SDS-PAGE. Subunit 7 of Adonis showed a slightly higher R_m than subunit 7 of Arminda and Chinese Spring. These differences were reproducible but became less pronounced when high amounts of proteins were brought onto the gel, which resulted in broad bands. The alleles of the *Glu-A1*, *Glu-B1* and *Glu-D1* loci of Arminda and Adonis had been identified by Moonen et al. (1982) as 0, 7, 2+12 and 1, 7, 2+12, respectively, using SDS-PAGE routinely applied for this purpose. Under such conditions the small differences as shown in Figure 3.1 are usually not noticed.

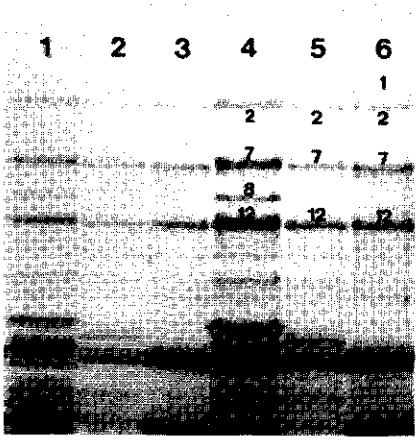

Figure 3.1 SDS-PAGE of the High Molecular Weight (HMWg) subunits of Chinese Spring (lane 1 + 4), Arminda (lane 2 + 5) and Adonis (lane 3 + 6). Volume of extract applied: lane 1-3, 20 µl; lane 4-6, 30 µl.

Figure 3.2 shows the separation of the HMWg subunits of Adonis and Arminda by 2-D electrophoresis. Subunit 7 from Adonis migrated to a more basic position compared to subunit 7 from Arminda, whilst the position of the subunits 2 and 12 agreed very well. 2-D electrophoresis of a mixture of Arminda and Adonis

confirmed the differences in IEP between the subunits 7. As subunit 7 of Arminda showed the same R_m and IEP as that of Chinese Spring, the subunit of Adonis is from now on referred to as subunit 7'.

The presence of the *Glu-A1* and *Glu-D1* encoded HMWg subunits suggests that subunit 7' is encoded by the *Glu-B1* locus. Moreover the position of this subunit on the 2-D gel is in agreement with the characteristic position of the *Glu-B1* subunits (Holt et al. 1981).

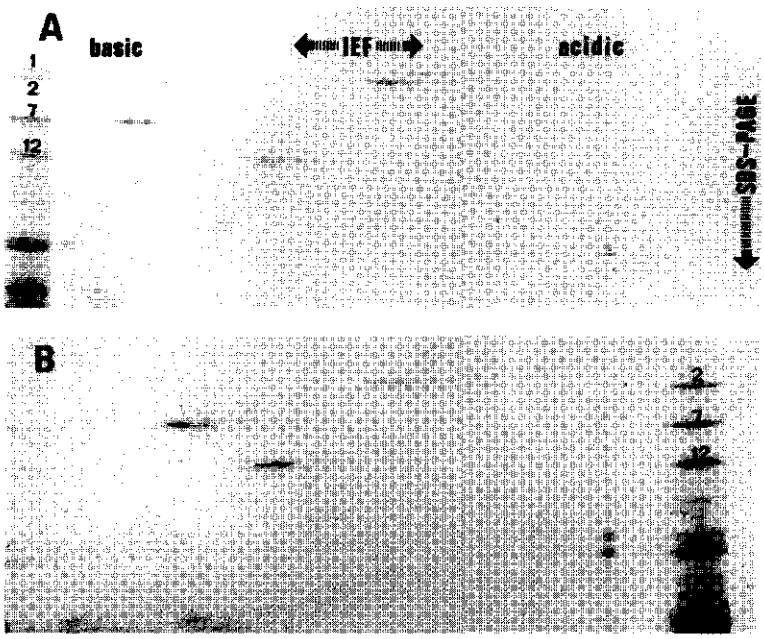

Figure 3.2 Separation of the High Molecular Weight (HMWg) subunits of Adonis (A) and Arminda (B) by two-dimensional electrophoresis (IEF * SDS-PAGE). As a reference, separation by SDS-PAGE (second dimension) is shown.

The differences in R_m and IEP between the subunits 7 and 7' strongly suggests that these proteins are allelic variants. Theoretically, the differences between the subunits could also result from post-translational modification of the same primary gene product. For some legume storage proteins post-translational modification has been demonstrated (Spencer 1984). Anderson and Hickman (1979) showed that chemical modification *in-vitro* of a certain protein which altered the charge of that protein, changed its SDS-binding characteristics and consequently its apparent

molecular mass determined by SDS-PAGE. If post-translational modification of a primary gene product of the *Glu-B1* allele could have caused such a modification *in-vivo*, the differences between the subunits 7 and 7' could be the result of this process.

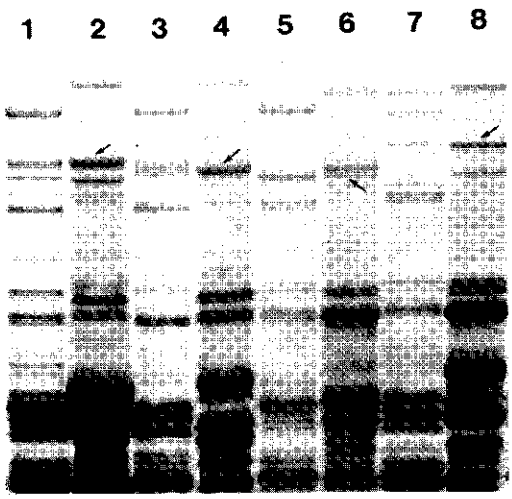

Figure 3.3 SDS-PAGE of the High Molecular Weight (HMWg) subunits of *T. turgidum* ssp. *dicoccoides* (lane 2, 4, 6 and 8) and *T. aestivum* (lane 1, 3, 5 and 7). Lane 1: Lancota (2*, 13+16, 2+12), lane 2: G315*-1M, lane 3: Sappo (2*, 14+15, 2+12), lane 4: G168-1-2-4 BM, lane 5: Gabo (2*, 17+18, 2+12), lane 6: G25-4M, lane 7: Hope (1, 6+8, 5+10) and lane 8: G4M-1M.

Baking tests using 168 wheat lines from Dutch wheat breeders revealed that the lines containing HMWg subunit 7 could be divided in two classes of baking quality and the results suggested that two types of subunit 7 occurred in these lines (Hamer and Marseille 1986). Our results support this hypothesis. A study is planned to investigate whether the subunits 7 and 7' show a different effect on bread-making quality, using the progeny from a cross between Arminda and Adonis. The inheritance of subunits 7 and 7' may provide information on the genetic basis of the differences between these subunits.

In *T. turgidum* ssp. *dicoccoides*, which has the genome constitution AB, HMWg subunits were found which have not been shown to occur in *T. aestivum* (Figure 3.3). In accordance with the position of *Glu-A1* and *Glu-B1* encoded HMWg subunits of *T. aestivum*, the subunit of *T. turgidum* ssp. *dicoccoides* showing the

lowest R_m is assumed to be *Glu-A1* encoded, the other subunit(s) *Glu-B1*. Some of the *Glu-B1* encoded HMWg subunits of *T. turgidum* ssp. *dicoccoides* (marked by <- in Figure 3.3) showed the same R_m as *Glu-B1* encoded subunits of *T. aestivum*. In spite of this, the alleles encoding for these subunits could be differentiated from their *T. aestivum* counterparts, due to the presence of a second subunit encoded by the alleles. This second subunit was present either in *T. turgidum* ssp. *dicoccoides* or *T. aestivum* or in both. In the latter case the R_m's of these accompanying subunits differed. In *T. tauschii*, the D-genome donor of *T. aestivum*, a HMWg allele was present which could not be distinguished by SDS-PAGE from its *T. aestivum* counterpart (Lagudah and Halloran 1988). It is not known whether biochemically different subunits, such as 7 and 7' and those found in species related to bread wheat, may differently influence bread-making quality. It might therefore be necessary to apply 2-D electrophoresis for unambiguously identifying the HMWg subunits, when alien germplasm is to be introduced into a breeding programme, or when making an inventory of the subunits present in breeding material. For subsequent screening of lines, SDS-PAGE will be useful for recognizing the identified subunits.

References

Anderson NL, Hickman BJ (1979) Analytical techniques for cell fractions. XXIV. Isoelectric point standards for two-dimensional electrophoresis. Anal Biochem 93: 312-320

Branlard G, Dardevet M (1985) Diversity of grain protein and bread wheat quality. II. Correlation between high molecular weight subunits of glutenin and flour quality characteristics. J Cereal Sci 3: 345-354

Branlard G, Le Blanc A (1985) Les sous-unités gluténines de haut poids moléculaire des blés tendres et des blés durs cultivé en France. Agronomie 5(6): 467-477

Brown JWS, Kemble RJ, Law CN, Flavell RB (1979) Control of endosperm proteins in *Triticum aestivum* (var. Chinese Spring) and *Aegilops umbellulata* by homeologous group 1 chromosomes. Genetics 93: 189-200

Graveland A, Bosveld P, Lichtendonk WJ, Marseille JP, Moonen JHE, Scheepstra A (1985) A model for the molecular structure of the glutenins from wheat flour. J Cereal Sci 3: 1-16

Hamer RJ, Marseille JP (1986) Electrophoresis as a tool for breeding of wheat for bread-making quality (in Dutch). IGMB rapport nr 86-130, pp 7

Holt LM, Austin R, Payne PI (1981) Structural and genetical studies on the high-molecular-weight subunits of wheat glutenin. Part 2: Relative isoelectric points determined by two-dimensional fractionation in polyacrylamide gels. Theor Appl Genet 60: 237-243

Lagudah ES, Halloran GM (1988) Phylogenetic relationships of *Triticum tauschii* the D genome donor to hexaploid wheat. 1. Variation in HMW subunits of glutenin and gliadins. Theor Appl Genet 75: 592-598

Lawrence GJ (1986) The high-molecular-weight glutenin subunit composition of Australian wheat

cultivars. Aust J Agric Res 37: 125-133
Lawrence GJ, Shepherd KW (1980) Variation in glutenin protein subunits of wheat. Aust J Biol Sci 33: 221-233
Moonen JHE, Scheepstra A, Graveland A (1982) Use of the SDS-sedimentation test and SDS-polyacrylamidegel electrophoresis for screening breeder's samples of wheat for bread-making quality. Euphytica 31: 677-690
Moonen JHE, Scheepstra A, Graveland A (1983) The positive effects of the high molecular weight subunits 3+10 and 2* of glutenin on the bread-making quality of wheat cultivars. Euphytica 32: 735-742
Payne PI, Lawrence GJ (1983) Catalogue of alleles for the complex gene loci, Glu-A1, Glu-B1, and Glu-D1 which code for high-molecular-weight subunits of glutenin in hexaploid wheat. Cereal Res Commun 11: 29-35
Payne PI, Corfield KG, Blackman JA (1979) Identification of a high-molecular-weight subunit of glutenin whose presence correlates with bread-making quality in wheats of related pedigree. Theor Appl Genet 55: 153-159
Payne PI, Holt LM, Jackson EA, Law CN (1984) Wheat storage proteins: their genetics and their potential for manipulation by plant breeding. Phil Trans R Soc London Ser B 304: 359-371
Payne PI, Nightingale MA, Krattiger AF, Holt LM (1987) The relationship between HMW glutenin subunit composition and the bread-making quality of British-grown wheat varieties. J Sci Food Agric 40: 51-56
Spencer D (1984) The physiological role of storage proteins in seeds. Phil Trans R Soc London Ser B 304: 275-285
Vallega V, Waines JG (1987) High molecular weight glutenin subunit variation in *Triticum turgidum* var. *dicoccum*. Theor Appl Genet 74: 706-710
Van Gelder WMJ, Kolster P, Mesdag J, Van Eeuwijk FA (1987) The relationship between high-molecular-weight glutenin subunits breadmaking quality and yield of winter wheat. In: Borghi B (ed) Agriculture. Hard wheat: agronomic, technological, biochemical and genetic aspects. Commission of the European Communities, Luxembourg, pp 159-172
Waines JG, Payne PI (1987) Electrophoretic analysis of the high-molecular-weight glutenin subunits of *Triticum monococcum*, *T. urartu* and the A genome of bread wheat (*T. aestivum*). Theor Appl Genet 74: 71-76

CHAPTER 4

QUANTIFICATION OF INDIVIDUAL HIGH MOLECULAR WEIGHT GLUTENIN SUBUNITS OF WHEAT USING SDS-PAGE AND SCANNING DENSITOMETRY[1]

P Kolster, CF Krechting[2], WMJ van Gelder

Summary

A method was developed for quantifying the individual High Molecular Weight glutenin (HMWg) subunits present in a wholemeal flour of wheat (*Triticum aestivum* L.). Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) was used to separate the subunits from each other, as well as from the other proteins present in an extract of flour. The extraction and separation of the HMWg subunits were optimized, which was necessary for accurate quantification of the proteins by scanning densitometry, as was the use of a sensitive method for staining proteins in the polyacrylamide gels, using a colloidal suspension of Coomassie Brilliant Blue. The stained bands were quantified by scanning densitometry. The coefficient of variation (CV) of the staining intensity of individual HMWg subunits was 12.4% for lanes on the same gel; variability between gels used on the same or different days introduced an additional CV of 7% each. The method developed has several important advantages over the quantification of the subunits by Reversed Phase-High Performance Liquid Chromatography (RP-HPLC). First, in contrast to RP-HPLC, a crude extract containing all the storage proteins can be used. Second, the proteins can be quantified and identified in the same analysis.

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[1] This chapter is based on a paper published in Journal of Cereal Science 15 (1992): 49-61

[2] DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO)

Introduction

The glutenin protein of wheat (*Triticum aestivum* L.) is of prime importance with regard to the bread-making quality of flour. Glutenin (traditionally defined according to the Osborne classification as the kernel proteins unextractable in 70% ethanol but extractable in dilute acid or alkali) (Byers et al. 1983) is a complex mixture of proteins in which two classes of subunits can be distinguished; the high and the low molecular weight glutenin subunits, respectively (Shewry et al. 1984). Bread wheat varieties possess 3-5 High Molecular Weight glutenin (HMWg) subunits, and approximately 15 Low Molecular Weight glutenin (LMWg) subunits (Payne et al. 1984). Genetic variation in the composition of the glutenin subunits, especially the HMWg subunits (Moonen et al. 1982; Branlard and Dardevet 1985; Payne et al. 1987; Van Gelder et al. 1987), has been related to the variation in bread-making quality between genotypes.

The variation in the total amount of the glutenin, isolated as described above, has been related to the bread-making quality also (Schofield and Booth 1983; MacRitchie 1984). Furthermore, variation in the ratio between the HMWg and the LMWg subunits (Huebner and Bietz 1985), and in the amount of groups of subunits (Sutton et al. 1989; Wieser et al. 1989) present in the glutenin fraction, also has been related to variation in bread-making quality.

The biochemical and genetic interpretation of these results is complicated by the complex subunit composition of glutenin and by the poor reproducibility of the isolation of the proteins on the basis of their extractabilities (Byers et al. 1983; Schofield and Booth 1983; Chakraborty and Khan 1988). Furthermore, extraction of flour with 70% ethanol (no reducing agents) to remove the gliadin fraction also leads to losses of a proportion of the LMWg subunits (Payne and Corfield 1979) and of the HMWg subunits from the glutenin fraction (Graybosch and Morris 1990). The use of extraction techniques to fractionate wheat endosperm storage proteins may therefore create difficulties in quantitative studies of glutenin subunits. Furthermore, varieties may possess subunits that are related to a poor bread-making quality in combination with subunits related to high quality. Allelic HMWg subunits may also differ in their contribution to bread-making quality. To discriminate between effects of variation in the amount of HMWg subunits and effects of differences in the type of subunits, it is necessary to determine the amount of individual and genetically well-defined HMWg subunits. The quantification of individual subunits also enables studies of the relationship between the contribution

to the bread-making quality of an allele and the amount of subunits encoded.

To separate the HMWg subunits from other storage proteins, two separation techniques are used routinely: Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) (Fullington et al. 1980; Cole et al. 1981; Mansur-Vergara et al. 1986) or reversed phase-high performance liquid chromatography (RP-HPLC) (Burnouf and Bietz 1984; Seilmeier et al. 1987; Kruger et al. 1988; Marchylo et al. 1989). In these studies (with the exception of Marchylo et al. 1989) the amounts of individual HMWg subunits present were not reported. In studies where the amounts of individual HMWg subunits separated by SDS-PAGE were determined (Branlard and Dardevet 1985; Payne et al. 1981; Uhlen 1990), methods were used that were developed for optimal resolution of the HMWg subunits rather than accurate quantification.

The aim of this investigation was to develop a method for the accurate quantification of all individual HMWg subunits. Because (genetic) identification of the HMWg subunits is based on their relative mobilities during SDS-PAGE (Payne and Lawrence 1983), this separation technique was used. Therefore, it was necessary to optimize the extraction and separation of the HMWg subunits, and to study the influence of variation in staining and electrophoretic conditions upon the quantification.

Material and methods

Seed of Dutch wheat varieties were milled using a Cyclotec-Udy mill equipped with a 1-mm sieve. Because of the possible existence of a protein gradient in wheat kernels (Lawrence 1986), wholemeal flour was used. Flour was stored at room temperature in airtight containers. The protein contents of the flours was determined as N * 5.7 after Kjeldahl digestion and using an auto-analyzer system. The protein contents of the samples used in the experiments ranged from 9% to 14% (dry-matter basis).

Extraction of flour

Proteins were extracted from flour with SDS-PAGE sample buffer (Moonen et al. 1982). The extraction procedure developed for complete extraction of the HMWg subunits was as follows. Wholemeal flour (50 mg) was vortexed with sample buffer (0.6 ml) for 10 s in a 2.5 ml Eppendorf tube. After 1 and 2 h, the sample was

vortexed again for 10 s and finally centrifuged in an Eppendorf centrifuge for 5 min at 16,000 g. An aliquot (0.3 ml) of the supernatant was retained and the rest was discarded. For the second extraction of the flour, the pellet was resuspended in sample buffer (0.3 ml) and re-extracted as before. After centrifugation, an aliquot (0.15 ml) of the second supernatant was added to the first. The extracts were stored at 4°C for electrophoretic analysis the day following the extraction.

SDS-PAGE

Preceding the SDS-PAGE analysis, the extracts were placed in a boiling water bath for 2 min, after which they were cooled using tap water, and centrifuged at 16,000 g for 5 min. The extracts (0.030 ml) were applied to SDS-PAGE gels containing 5% (Payne et al. 1981), 7.5% or 10% (Lawrence 1986), 8.3% (Lawrence and Shepherd 1980) or 12.5% (Hames 1981) acrylamide. The dimensions of the gels were 180 * 140 * 1.5 mm, except that, for 5% gels, they were 180 * 200 * 1.5 mm. A constant current of 15 mA was applied for 30 min, followed by 30 mA (5%, 7.5% or 8.3%) or 45 mA (10% or 12.5% acrylamide) for 8 h. A Pharmacia GE-2/4 LS vertical slab gel apparatus was used, and the tank buffer was kept at a temperature of 4°C. The gels were stained according Neuhoff et al. (1988), using Coomassie Brilliant Blue R250 at room temperature and in closed plastic boxes; 300 ml staining solution were used per gel per box, except that, for the 5% gels, 450 ml were used. During staining, the gels were agitated. After 40 h staining, protein bands were stained throughout the entire cross-section of the gel. Prolonged staining did not result in a significant increase in staining intensity. Therefore, the time of staining was standardized at 40 h. Because of the absence of background staining, no destaining was required. Stained gels were rinsed with 0.3% (w/v) Brij 35 (300 ml) and stored at 4°C in plastic bags containing 20% (w/v) ammonium sulphate (10 ml).

The HMWg subunits were identified according to Payne and Lawrence (1983).

Densitometric analysis

The amount of dye adsorbed by the proteins was determined using a Pharmacia-LKB Ultroscan XL laser densitometer (absorption at 633 nm). The densitometer traces were analyzed using the LKB 2400 Gelscan XL software package. The dye absorbance is determined as the area under the peak (absorbance units * mm peak width). For integration of the peaks, the horizontal, common, baseline option was selected, using the signal integration method.

Results and discussion

Gel staining

In preliminary experiments, the method routinely applied for protein staining in polyacrylamide gels was used. In that method, Coomassie Brilliant Blue (CBB) was dissolved in a mixture of methanol/water/acetic acid (Kolster et al. 1988, Chapter 3 of this thesis). Accurate quantification of HMWg subunits by scanning densitometry was hampered by the low staining intensity of the protein bands, however. It was not possible to increase the amount of protein applied to the gel either by decreasing the amount of extraction buffer used (because this decreased the extraction efficiency) or by increasing the amount of extract applied to the gel (because this overloaded the gel and led to a deterioration in the separation efficiency). Therefore, a more sensitive method for staining the proteins in polyacrylamide gels with CBB was evaluated for application to wheat storage proteins. By this method the proteins are stained with a colloidal suspension

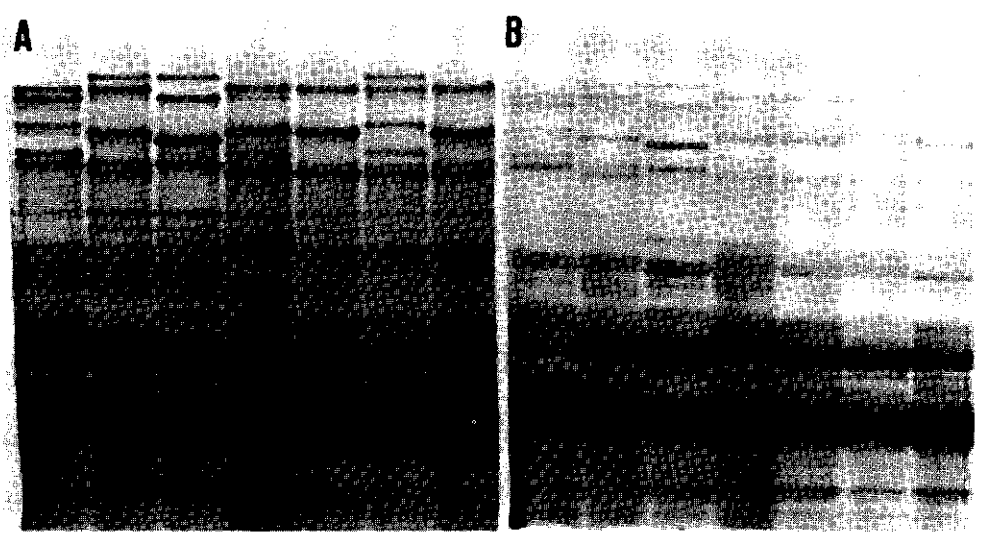

Figure 4.1 Comparison of gelstaining using (A) colloidal CBB R250 following Neuhoff et al. 1988 and (B) non-colloidal CBB R250. Two halves of the same gel were stained using these staining methods. Identical volumes of extracts of the same set of varieties were applied to each half.

of CBB (Neuhoff et al. 1988). The absence of destaining steps in this method should improve the reproducibility of the quantitative measurements. The higher staining intensity of proteins stained with colloidal Coomassie Brilliant Blue compared with non-colloidal CBB is shown in Figures 4.1A and 1B. The amount of dye adsorbed by the individual subunits depends on the staining method used (Kolster et al. 1988, Chapter 3 of this thesis).

A linear relationship between the staining intensity and the amount of protein applied to a gel is required for the quantification of proteins in gels. This linear relationship was shown over an absorption range of 0.10-1.5 absorbance units, which exceeded the range required for quantifying the HMWg subunits present in extracts of different varieties (results not shown).

Extraction of the HMWg subunits

A detailed study was made to evaluate the extraction procedure for the HMWg subunits from flour. The amount of HMWg subunits extracted from flour was defined here as the staining intensity after separation by SDS-PAGE. Although these proteins are difficult to extract, buffers containing a detergent and a reducing agent, such as SDS-PAGE sample buffer, are capable of extracting these proteins from flour quantitatively (Fullington et al. 1980).

Extraction of 50 mg of flour with 0.6 ml sample buffer for 10, 20, 30, 45 or 60 min showed that after 20 min the amount of HMWg subunits solubilized did not increase anymore. The intensity of shaking during the extraction did not affect the amount of HMWg subunits present in the supernatant. For practical reasons, it was decided to extract for 2 h, with vortexing after 0, 1 and 2 h.

The amounts of HMWg subunits extracted with two successive extractions of flour from two varieties are shown in Table 4.1. About 80% of the HMWg subunits extracted after the two successive extraction steps was present in the first extract. After the second step, the amount of N remaining in the pellet was determined after rinsing the pellet exhaustively with water to remove N containing buffer components. This revealed that 90-95% of the N-containing kernel components had been removed from the flour by the two extraction steps. This agrees with earlier findings that no more than 5% of the total amount of HMWg subunits remained in the residue after two extraction steps (Fishbein 1972). A third extraction was therefore omitted since this would merely result in a dilution of the extract.

Table 4.1 Absorbances of CBB stained HMWg subunits, present in two successive extracts of flours from the varieties Arminda and Citadel.

HMWg subunit	first extract[1]	second extract
Arminda		
2	44.6 ± 4.1	9.8 ± 1.3
7	52.9 ± 4.1	13.2 ± 2.3
12	46.5 ± 5.3	10.9 ± 2.4
total	144.0	33.8
Citadel		
2	37.4 ± 2.7	8.5 ± 2.1
6	24.1 ± 2.7	6.2 ± 0.8
8	23.1 ± 2.5	5.8 ± 1.5
12	50.5 ± 5.2	11.8 ± 2.9
total	135.1	32.3

[1] Average absorbances (100 * absorbance units * mm) ± standard error of the subunits present in successive extracts. Eight samples of each variety were extracted as described in Material and methods. The successive extracts (0.030 ml of the first en 0.015 ml of the second extract) were applied to adjacent lanes of an 8.3% SDS-PAGE gel.

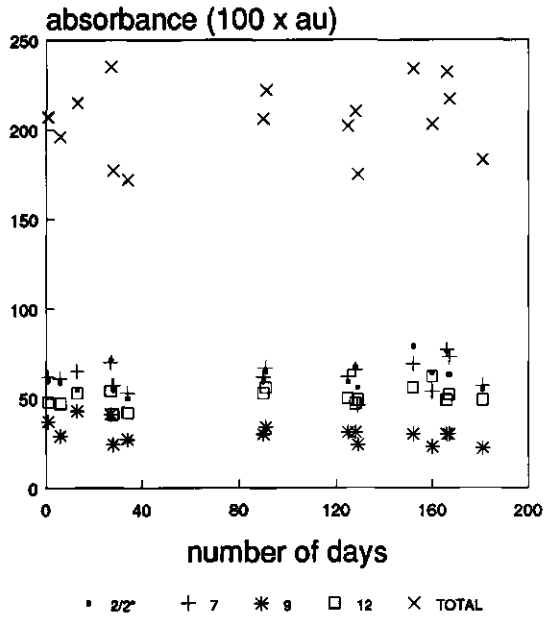

Figure 4.2 The stabilities of HMWg subunits of the variety Granada over a 5-month period after milling the kernels. Each value is the average absorbance of the CBB-stained subunits present in an extract, applied to four SDS-PAGE gels (8.3%). HMWg subunits 2 and 2* co-migrate on this type of gel.

To determine the stability of HMWg subunits in a wholemeal wheat flour, proteins were extracted from a flour that had been stored for different periods of time in an airtight container. As shown in Figure 4.2, the amount of the various HMWg subunits did not change during a storage period of five months. The effect of storage of the extracts at 4°C was determined also. Extracts of three varieties were applied to an SDS-PAGE gel, either directly following protein extraction or after storage of the (unboiled) extracts for 24 h at 4°C. The staining intensities of the HMWg subunits in the directly analyzed extracts did not differ from those in the stored extracts.

Variation in the duration of heating the extracts at 100°C (1, 2, 3, 5 or 10 min) also had no effect on the amount of HMWg subunits (results not shown).

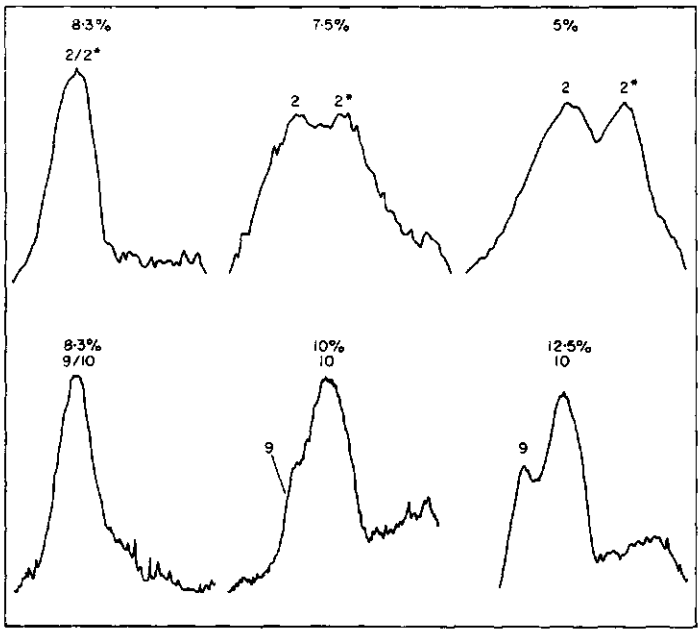

Figure 4.3 Densitometer traces of HMWg subunits 2 and 2*, and of 9 and 10 on SDS-PAGE gels differing in acrylamide concentration, showing differences in the separation of these subunits.

Electrophoretic conditions

The reproducibility of the measurement of the amount of dye adsorbed by a protein band depends not only on the reproducibility of the conditions during gel staining and destaining but on the electrophoretic conditions as well. The extent of diffusion of the proteins during electrophoresis, hence the bandwidth, affects the dye

adsorption of a protein band (Fishbein 1972; Neuhoff et al. 1990). Therefore, electrophoretic conditions determining the bandwidth, such as the temperature of the electrophoresis system, the volume of the sample applied to a gel, and the migration distance (Hames 1981), were standardized.

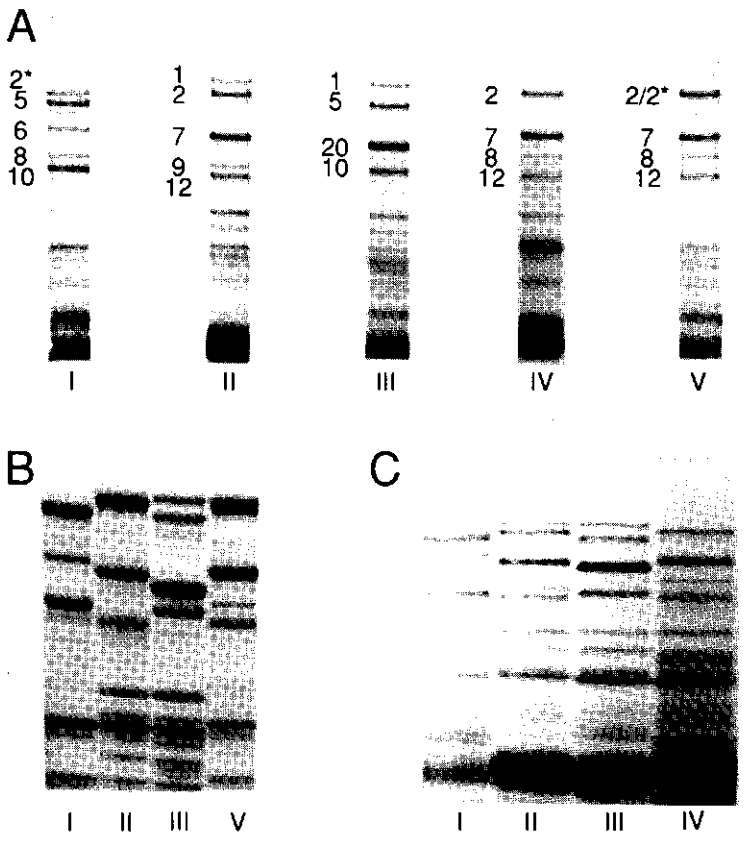

Figure 4.4 SDS-PAGE separation of the storage proteins present in varieties, using gels differing in acrylamide concentration. (a) 8.3%, (b) 5% and (c) 12.5% gel. The varieties were: (I) Granada, (II) Bastion, (III) Tenor, (IV) Chinese Spring, (V) Donjon. The HMWg subunits are designated according to the nomenclature of Payne and Lawrence 1983.

Another experimental variable affecting the bandwidth is the acrylamide concentration of the separation gel. Most combinations of HMWg subunits can be separated on an 8.3% or 10% SDS-PAGE gel (Lawrence 1986; Lawrence and Shepherd 1980). The most commonly occurring subunits co-migrating on 8.3 and

10% gels are the subunits 2 and 2*, and, on an 8.3% gel, 9 and 10. Consequently, the separation of some HMWg subunits requires the use of gels whose acrylamide concentrations are adjusted to the specific HMWg subunit under investigation.

Although subunits 9 and 10 could be recognized visually as two bands after separation on a 10% gel, the densitometer peaks for these subunits overlapped. The resolution achieved by a 12.5% acrylamide gel enabled a more precise determination of the boundary between the subunits (Figure 4.3). On a 12.5% gel, however, the separation of slow-moving subunits, such as 2* and 5, was inferior to that achieved on an 8.3% gel (Figure 4.4).

Likewise, the separation of subunits 2 and 2* increased with decreasing acrylamide concentration, and a gel of 5% acrylamide was required for accurate quantification (Figure 4.3). A consequence of the application of a 5% gel was the co-migration of the fast-moving subunits 8 and 10 and of subunits 9 and 12 (Figure 4.4), which were separated on an 8.3% gel.

Table 4.2 Absorbances of the HMWg subunits of two wheat varieties, separated on SDS-PAGE gels differing in acrylamide concentration.

HMWg subunit	acrylamide concentration		
	5%	8.3%	12.5%
Carimulti			
2	126.4 ± 7.4[1]	73.0 ± 6.7	27.0 ± 3.2
7	179.4 ± 8.2	100.8 ± 5.5	38.5 ± 3.2
12	124.8 ± 7.6	84.6 ± 6.7	25.5 ± 3.3
Granta			
2	128.6 ± 7.7	76.6 ± 7.0	27.5 ± 2.4
6	83.1 ± 6.0	53.8 ± 6.2	17.1 ± 2.0
8	96.8 ± 6.2	57.6 ± 5.8	11.4 ± 1.1
12	142.6 ± 10.0	109.0 ± 6.8	29.4 ± 3.6

[1] The average absorbances ± standard error (100 * absorbance units * mm) of eight scans is reported. For each gel type the extracts were separated on two gels, each gel containing four replicates of an extract. The same amount of extract was applied to the gels.

To study the effect of variation in acrylamide concentration on the amount of dye adsorbed by the HMWg subunits, extracts of two varieties were applied to 5%, 8.3% and 12.5% gels (Table 4.2). The relative proportions of the various subunits present in an extract differed only slightly between gels with different acrylamide concentrations. The dye adsorption of individual subunits on these gels was related inversely to the acrylamide concentration, in agreement with the results of Neuhoff

et al. (1990). Consequently, it was not feasible to compare quantitative measurements, when the HMWg subunits were separated on gels differing in acrylamide concentrations.

Because more HMWg subunits were separated on an 8.3% gel than on a 5% or a 12.5% gel, 8.3% gels were used routinely. The relative proportions of the most commonly occurring combinations of co-migrating subunits, subunits 2 and 2* and subunits 9 and 10 were determined after separation of these subunits on a 5% or 12.5% gel, respectively. The ratios of subunit 2 to subunit 2* and subunit 9 to subunit 10 obtained in this way were used to calculate the proportions of the absorbance of each individual subunit on an 8.3% gel.

Differences in staining intensity between gels

It is often necessary to compare results of different experiments. Therefore, a study has been made of the differences in the absorbance values of individual HMWg subunits arising from: (a) differences between lanes of the same gel; (b) differences between lanes of separate gels, which were cast and used simultaneously and; and (c) differences between lanes of gels which were cast and used on different days. The extracts were always produced the day preceding the separation, using the same flour, and were applied to four randomly chosen lanes of a gel. Table 4.3 gives an example of the results for two varieties. The staining intensity of the HMWg subunits present in an extract may differ significantly ($P \leq 0.05$) between the gels. Analyses of variance using the results of extracts of three varieties showed that the average coefficient of variation (CV) of the absorption of the HMWg subunits between lanes on the same gel was 12.4%, and that between lanes of separate gels produced and used on the same or different days was 7% in both cases. Thus, for optimal comparison of the amount of HMWg subunits present in samples, extracts should be applied in replicates on the same gel, thereby avoiding the variation between gels. The number of extracts that can be separated on one gel is small however. Consequently, in most studies, several gels will have to be used, which will lead to an increase in the experimental error.

The relatively high CV between lanes on the same gel will be caused to a large extent by the fact that only one arbitrarily chosen area of an HMWg subunit band is scanned. Because a band will never be totally homogeneous, determination of the amount of dye absorbed by the protein band by two-dimensional scanning should reduce this experimental error (Neuhoff et al. 1990). Differences between the gels were probably caused by differences in the pouring of the gels and in the staining

conditions.

For the quantification of proteins, separation by RP-HPLC is considered generally to give more reproducible results than SDS-PAGE in combination with scanning densitometry (Marchylo et al. 1989; Bietz 1986). Marchylo et al. (1988) reported a CV of 1-3% for pooled areas of the peaks present in segments of a chromatogram of the total storage proteins of wheat. An average CV of 25% has been reported for gliadin peaks of RP-HPLC chromatograms, produced over an eight-week period (Scanlon et al. 1989). The authors are not aware of any reports of the CV of peak areas of individual HMWg subunits separated by RP-HPLC. Consequently, for this specific application, it is not possible to compare the reproducibility of both methods of quantification on the basis of published data.

Table 4.3 Absorbances[1] of CBB stained HMWg subunits of two varieties on different gels.

	Experiment I[2]		Experiment II		
Subunits	gel 1	gel 2	gel 3	gel 4	LSD[3]
Tenor					
1	22	16	23	24	3.9
5	31	33	35	33	7.3
7	79	68	84	79	9.3
10	45	44	52	51	6.8
Urban					
1	8	9	8	9	1.7
5	37	44	37	34	6.7
7	56	61	63	52	14.2
9/10	61	68	67	56	9.4

[1] Average absorbances (100 * absorbance units * mm) of individual HMWg subunits present in extracts which are applied on four lanes of a gel.

[2] The gels within an experiment (gel 1, 2 and 3, 4 respectively) were produced and used simultaneously, the gels of experiment II were cast, used and stained four days after the gels of experiment I.

[3] Least Significant Difference at $P \leq 0.05$, n=4.

Conclusions

This study has shown that SDS-PAGE can be used in combination with scanning densitometry for the quantification of individual HMWg subunits. Disadvantages of the SDS-PAGE method developed are that the method is more laborious and

time-consuming than the RP-HPLC method and not automated easily. Furthermore, as the RP-HPLC method is probably more accurate in determining the amount of HMWg subunits in an extract, the SDS-PAGE method requires more replicates. For application in quality-prediction tests, the CV of the SDS-PAGE method is probably too high. For studying the causes of the variation in the amount of *individual* HMWg subunits and for biochemical studies, the SDS-PAGE method offers important advantages however. The identification of the HMWg subunits is based on their apparent molecular weight as determined by SDS-PAGE (Payne and Lawrence 1983; Bushuk 1991). Therefore, SDS-PAGE enables the quantification and the (genetic) identification of the HMWg subunits in one analysis. Subunits separated by RP-HPLC are identified by SDS-PAGE analysis of the peak fractions. SDS-PAGE also separates the HMWg subunits from the other proteins present in the wheat kernel (Payne et al. 1985). Consequently, this separation technique allows the use of a crude extract for the quantification of the HMWg subunits present in the kernel. In contrast, because some HMWg subunits co-elute with some of the other kernel proteins (Burnouf and Bietz 1989), selective extraction or precipitation of the HMWg subunits is required when RP-HPLC is to be applied. This is a potential source of error.

References

Bietz JA (1986) High-performance liquid chromatography of cereal proteins. In: Pomeranz Y (ed) Advances in Cereal Science and Technology, vol VIII. American Association of Cereal Chemists, St. Paul, MN, pp 105-170

Burnouf T, Bietz JA (1984) Reversed-phase high-performance liquid chromatography of reduced glutenin, a disulfide-bonded protein of wheat endosperm. J Chromatogr 299: 185-199

Burnouf T, Bietz JA (1989) Rapid purification of wheat glutenin for reversed-phase high-performance liquid chromatography: comparison of dimethyl sulfoxide with traditional solvents. Cereal Chem 66: 121-127

Branlard G, Dardevet M (1985) Diversity of grain protein and bread wheat quality. II. Correlation between high molecular weight subunits of glutenin and flour quality characteristics. J Cereal Sci 3: 345-354

Bushuk (1991) Nomenclature. In: Bushuk W, Tkachuk R (eds) Gluten proteins 1990. American Association of Cereal Chemists, St. Paul, pp 781-783

Byers M, Miflin BJ, Smith SJ (1983) A quantitative comparison of the extraction of protein fractions from wheat grain by different solvents, and of the polypeptide and amino acid composition of the alcohol-soluble proteins. J Sci Food Agric 34: 447-462

Chakraborty K, Khan K (1988) Biochemical and breadmaking properties of wheat protein components. I.Compositional differences revealed through quantification and polyacrylamide gel electrophoresis of protein fractions from various isolation procedures. Cereal Chem 65: 333-340

Cole EW, Fullington JG, Kasarda DD (1981) Grain protein variability among species of *Triticum*

and *Aegilops*: quantitative SDS-PAGE studies. Theor Appl Genet 60: 17-30
Fishbein WN (1972) Quantitative densitometry of 1-50 µg protein in acrylamide gel slabs with Coomassie Blue. Anal Biochem 46: 388-401
Fullington JG, Cole EW, Kasarda DD (1980) Quantitative SDS-PAGE of total protein from different wheat varieties. J Sci Food Agric 31: 43-53
Graybosch RA, Morris R (1990) An improved SDS-PAGE method for the analysis of wheat endosperm storage proteins. J Cereal Sci 11: 201-212
Hames BD (1981) An introduction in polyacrylamide gel electrophoresis. In: Hames BD, Rickwood D (eds) Gel electrophoresis of proteins. A practical approach. IRL Press Ltd, Oxford, pp 1-91
Huebner FR, Bietz JA (1985) Detection of quality differences among wheats by high-performance liquid chromatography. J Chromatogr 327: 333-342
Kolster P, Van Gelder WMJ (1991) A method for the quantification of individual high molecular weight glutenin subunits using SDS-PAGE and laser scanning densitometry. In: Bushuk W, Tkachuk R (eds) Gluten proteins 1990. American Association of Cereal Chemists, St. Paul, pp 349-362
Kolster P, Krechting CF, Van Gelder WMJ (1988) Variation in high molecular weight glutenin subunits of *Triticum aestivum* and *T. turgidum* ssp. *dicoccoides*. Euphytica S: 141-145
Kruger JE, Marchylo BA, Hatcher D (1988) Preliminary assessment of a sequential extraction scheme for evaluating quality by reversed-phase high-performance liquid chromatography and electrophoretic analysis of gliadins and glutenins. Cereal Chem 65: 208-214
Lawrence GJ (1986) The high-molecular-weight glutenin subunit composition of Australian wheat cultivars. Aust J Agric Res 37: 125-133
Lawrence GJ, Shepherd KW (1980) Variation in glutenin protein subunits of wheat. Aust J Biol Sci 33: 221-233
MacRitchie F (1984) Baking quality of wheat flours. Adv Food Res 29: 201-277
Mansur-Vergara L, Konzak CF, Gerechter-Amitai ZK, Grama A, Blum A (1986) Quantitative variation in the kernel proteins among 841 accessions of *Triticum dicoccoides* estimated by SDS-PAGE. Theor Appl Genet 72: 296-301
Marchylo BA, Hatcher DW, Kruger JE (1988) Identification of wheat cultivars by reversed-phase high-performance liquid chromatography of storage proteins. Cereal Chem 65: 28-40
Marchylo BA, Kruger JE, Hatcher DW (1989) Quantitative reversed-phase high-performance liquid chromatographic analysis of wheat storage proteins as a potential quality prediction tool. J Cereal Sci 9: 113-130
Moonen JHE, Scheepstra A, Graveland A (1982) Use of the SDS-sedimentation test and SDS-polyacrylamidegel electrophoresis for screening breeder's samples of wheat for bread-making quality. Euphytica 31: 677-690
Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9: 255-262
Neuhoff V, Stamm R, Pardowitz I, Arold N, Ehrhardt W, Taube D (1990) Essential problems in quantification of proteins following colloidal staining with Coomassie Brilliant Blue dyes in polyacrylamide gels, and their solution. Electrophoresis 11: 101-117
Payne PI, Corfield KG (1979) Subunit composition of wheat glutenin proteins, isolated by gel filtration in a dissociating medium. Planta 145: 83-88
Payne PI, Lawrence GJ (1983) Catalogue of alleles for the complex gene loci, Glu-A1, Glu-B1, and Glu-D1 which code for high-molecular-weight subunits of glutenin in hexaploid wheat. Cereal Res Commun 11: 29-35
Payne PI, Holt LM, Jackson EA (1984) The genetics of gliadin and glutenin, storage proteins of the wheat endosperm. Kulturpflanze 32: 63
Payne PI, Holt LM, Law CN (1981) Structural and genetical on the high-molecular-weight subunits

of wheat glutenin. Part 1: Allelic variation in subunits amongst varieties of wheat (*Triticum aestivum*). Theor Appl Genet 60: 229-236
Payne PI, Holt LM, Jarvis MG, Jackson EA (1985) Two-dimensional fractionation of the endosperm proteins of bread wheat (*Triticum aestivum*): biochemical and genetic studies. Cereal Chem 62: 319-326
Payne PI, Nightingale MA, Krattiger AF, Holt LM (1987) The relationship between HMW glutenin subunit composition and the bread-making quality of British-grown wheat varieties. J Sci Food Agric 40: 51-65
Scanlon MG, Sapirstein HD, Bushuk W (1989) Evaluation of the precision of high-performance liquid chromatography for wheat cultivar identification. Cereal Chem 66: 112-116
Schofield JD, Booth MR (1983) Wheat proteins and their technological significance. In: Hudson BJF (ed) Developments in food proteins, volume 2. Applied Science Publishers, London, pp 1-65
Seilmeier W, Wieser H, Belitz H-D (1987) High-performance liquid chromatography of reduced glutenin: amino acid composition of fractions and components. Z Lebensm Unters Forsch 185: 487-489
Shewry PR, Miflin BJ, Kasarda DD (1984) The structural and evolutionary relationships of the prolamin storage proteins of barley, rye and wheat. Phil Trans R Soc London Ser B 304: 297-308
Sutton KH, Hay RL, Griffin WB (1989) Assessment of the potential bread baking quality of New Zealand wheats by RP-HPLC of glutenins. J Cereal Sci 10: 113-121
Svensson G (1981) Varietal and environmental effects on wheat milling quality. Agri Hort Genet band XXXIX: 103 pp
Van Gelder WMJ, Kolster P, Mesdag J, Van Eeuwijk FA (1987) The relationship between high-molecular-weight glutenin subunits breadmaking quality and yield of winter wheat. In: Borghi B (ed) Agriculture. Hard wheat: agronomic, technological, biochemical and genetic aspects. Commission of the European Communities, Luxembourg, pp 159-172
Uhlen AK (1990) Quantitative analysis of high molecular weight glutenin subunits present in Norwegian wheats. Norw J Agric Sci 4: 19-26
Wieser H, Seilmeier W, Belitz H-D (1989) Reversed-phase high-performance liquid chromatography of ethanol-soluble and ethanol-insoluble reduced glutenin fractions. Cereal Chem 66: 38-41

CHAPTER 5

QUANTITATIVE VARIATION OF TOTAL AND INDIVIDUAL HIGH MOLECULAR WEIGHT GLUTENIN SUBUNITS OF WHEAT IN RELATION TO VARIATION IN ENVIRONMENTAL CONDITIONS[1]

P Kolster, CF Krechting[2], WMJ van Gelder

Summary

The total amount of the High Molecular Weight glutenin (HMWg) subunits, and the amount of the individual subunits, present in kernels of wheat (*Triticum aestivum* L.) were studied in relation to differences in growing conditions. The amounts of individual HMWg subunits in four varieties, each grown at six sites in The Netherlands, differed considerably between the sites. Only to a limited extent (0-66%, depending on variety) could this variation be ascribed to differences in protein content of the samples. The cause of the rest of the variation is unknown. Accordingly, the total amount of the HMWg subunits, relative to the total amount of proteins present in a wholemeal flour of a variety, differs between the samples produced at different sites.Thus the preference for synthesis of a certain protein fraction may vary depending on the environment. A second experiment showed that variation in protein contents of samples of two varieties grown on the same trial field accounted for about 90% of the variation in the total amount of HMWg subunits. The samples of each variety differed in protein content as a result of differences in the amount of N-fertilizer applied. Within each variety, the ratio between the individual HMWg subunits was less dependent on variations in environmental conditions than on the actual amount, indicating that the levels of expression of the individual HMWg subunit genes are regulated by a common mechanism.

The implications of the results for the selection possibilities of wheat with desired bread-making quality and for studies concerning the genetic variation in the amount of HMWg subunits are discussed.

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[1] Journal of the Science of Food and Agriculture 57 (1991): 405-415

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Introduction

The glutenins, a group of storage proteins of wheat (*Triticum aestivum* L.), have been studied extensively in relation to bread-making quality. Glutenin is composed of subunits which are connected by intermolecular disulphide bonds. According to their molecular weights these subunits can be subdivided into two classes: the High Molecular Weight glutenin (HMWg, 95-136 kD) and the Low Molecular Weight glutenin (LMWg, 42-51 kD) subunits (Payne and Corfield 1979). Allelic variation on the loci encoding the glutenin subunits, in particular the HMWg subunits (Moonen et al. 1982; Payne et al. 1979, 1987; Branlard and Dardevet 1985; Van Gelder et al. 1987), has been related to differences in bread-making quality between varieties. Differences in the amount of the glutenin (Schofield and Booth 1983) and in the amount of (groups of) glutenin subunits present in flour (Branlard and Dardevet 1985; Huebner and Bietz 1985; Kruger et al. 1988; Sutton et al. 1989; Wieser et al. 1989) have also been related to differences in quality as well. These authors did not study whether the variation in the amount of proteins had genetic causes or was the result of differences in environmental conditions during the growing of the wheat.

The agronomic and climatological conditions under which a variety is grown are known to contribute largely to the bread-making quality of a flour produced from the kernels. Accordingly, several studies have been published which are concerned with the relationship between differences in the environmental conditions and the amount of storage proteins produced by a variety. Sulphur deficiency during kernel development, which negatively influences the quality of flour produced from these kernels, has been associated with alterations in the ratio between groups of storage proteins, including the group of the HMWg subunits (Wrigley et al. 1984; Castle and Randall 1987; Fullington et al. 1987). The application of N-fertilizer in general results in improved bread-making quality by increasing the protein content of the flour. Densitometric analysis of the stained electrophoresis gels in which the endosperm proteins were separated showed that the amount of HMWg subunit group relative to the other storage proteins did not depend on the amount of fertilizer applied (Fullington et al. 1983; Levy et al. 1985). Nor did the proportion of glutenin, which is isolated on basis of solubility, depend on the amount of N-fertilizer (Doekes and Wennekes 1982). Differences in the year of growing and the location at which the varieties were grown resulted in small differences in the amount of groups of the glutenin subunits and groups of gliadins, according to

Marchylo et al. (1990).

The amount of individual HMWg subunits in relation to the differences in environmental conditions has not been reported in the studies quoted. However, because the HMWg subunits differ in their contribution to the bread-making quality, information concerning variations in the amount of well defined individual HMWg subunits due to differences in the environmental conditions is of importance. Therefore, the aim of this study was to investigate the relationship between the amount of individual HMWg subunits produced by a variety and the effect of different growing sites and the level of N-fertilization. The implications of environmental variation in the amount of the HMWg subunits for studies concerning the genetic variation in expression of the HMWg subunit genes are also discussed.

Material and methods

Wheat samples

The first set of samples (I) included Dutch winter wheat varieties Granada, Okapi, Citadel and Kraka, which were obtained from the former Governmental Institute for Research on Varieties (RIVRO, Wageningen). The varieties were grown in 1986 using standardized agronomic conditions, in trial fields of at least 36 m^2 at six sites: Nieuw Beerta (A), Kloosterburen (B), Wieringerwerf (C), Rijsenhout (D), 2Biddinghuizen (E) and Dronten (F).

A second set of samples (II) comprised kernels of two varieties, Urban and Obelisk, originating from an N-fertilizer experiment (Darwinkel 1987) on a trial field of the Research Station for Arable Farming and Field Production of Vegetables (PAGV, Lelystad). The total amounts of N applied on plots of 48 m^2 of these varieties were 80, 140, 180, 220 or 260 kg $N \cdot ha^{-1}$.

Electrophoresis

Because of the considerable variation in the amount of individual HMWg subunits between kernels of a sample (Kolster and Van Gelder 1991; Chapter 4 of this thesis), at least 30 gram of kernels (approximately 500 kernels) were milled to produce a wholemeal flour. Of each flour, 30 µl of duplicate extracts was applied on 8.3% SDS-PAGE (Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis) gels. The extraction, separation and staining of the proteins, using a

modified Coomassie Brilliant Blue (CBB) method for the quantification of individual HMWg subunits, was as described previously (Kolster and Van Gelder 1991; Chapter 4 of this thesis). To facilitate comparison between gels, an extract from a reference flour (variety Sunnan) was applied to the first and the last lane of each gel. For each variety of sample set I, a set of four simultaneously cast gels was used. Each gel contained duplicate extracts from the samples of a variety from all six sites.

Duplicate extracts from the samples of Obelisk and Urban (sample set II) were also applied to four simultaneously cast gels. In this experiment, an extract from flour of the complete set of samples of both varieties was applied to each gel. The duplicate extracts were divided over the four gels. The ratio between subunits 9 and 10 in Urban was determined using 12.5% SDS-PAGE gels (Kolster and Van Gelder 1991; Chapter 4 of this thesis). Of each lane two tracks were scanned using a Pharmacia-LKB laser densitometer. The absorbance of the stained protein bands at 633 nm was expressed as 100 * absorbance units * mm (100 * AU).

Statistical analysis

The results of the quantification of the CBB-stained HMWg subunits were analysed using Genstat (Genstat 5 committee 1987). The block structure of the data from sample set I used was gel/location/extract. The relation between the amount of HMWg subunits and protein content was analysed using regression analysis. The components of variance in the experiments concerning the absorbance of the stained HMWg subunits present in the samples of sample set I were estimated according to Searle (1971). Coefficients of variation were calculated using analysis of variance of the logarithm of the absorbance values.

Results and discussion

As quantitative densitometric measurements can be subject to large errors (see e.g. Pardowitz et al. 1990), the method for the quantification of the HMWg subunits *via* SDS-PAGE and densitometry has been optimized (Kolster and Van Gelder 1991; Chapter 4). From a practical point of view the extent of the experimental error in the quantification of the HMWg subunits must be compared with the differences in the staining intensity of the bands due to variation in the amount of the HMWg subunits in the samples. Therefore, the components of the variance in the

absorbance of the HMWg subunits present in the kernels of sample set I were estimated (see Materials and methods). In this experiment 65% of the variation in the staining intensity, i.e. the amount of individual subunits in a sample, can be ascribed to differences in the growing sites. The remaining variation is a result of the analytical procedure (Figure 5.1). In this experimental error, the contribution of differences between duplicate extracts and between scans of the same lane is limited (4% in each case). In particular, differences in the staining intensity between gels (21%) should be taken into consideration when determining the amount of the HMWg subunits. Table 5.1 shows the average absorption of the individual CBB-stained HMWg subunits present in the samples of the varieties grown at the six sites. The LSD values (Least Significant Difference, $P \leq 0.05$) show that differences of about 10% of the average absorption of a subunit are statistically significant. The extent of the variation in the absorbance of the subunits between the samples of different sites is clearly higher, which underlines the applicability of the quantification method used.

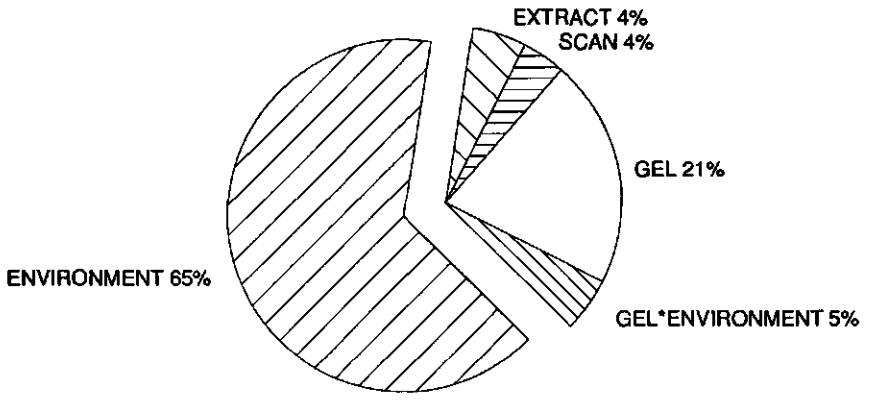

Figure 5.1 Pie diagram of the relative contribution of components of variance on the amount of individual HMWg subunits, as determined on gel, of the samples of sample set I. Environment = differences between samples of a variety grown at different sites; gel = differences in staining intensity between gels; scan = differences between densitometric recordings of the same lane; extract = differences between extracts of the same sample. Because of the experimental set-up, the contribution of variation between lanes could not be separately estimated. The gel * environment interaction is best interpreted as differences between lanes within a gel.

For each variety, the amount of individual HMWg subunits differs significantly ($P \leq 0.05$) between most of the samples which are produced at different sites (Table

5.1). The variation in the protein content between the samples of Okapi and Citadel accounts for 66% and 40% respectively of the variation in the total amount of HMWg subunits, as shown by regression analysis. However, no significant correlation between protein content and the total amount of the HMWg subunits was identified Kraka or Granada. Consequently, for wheat grown at different sites, the protein content of the kernels cannot be used to correct for differences in the amount of HMWg subunits.

Table 5.1 Amount[1] of individual HMWg subunits present in samples of four varieties, each grown at six sites denoted A to F.

HMWg subunit[2]	Site						Average	LSD[3]
	A	B	C	D	E	F		
Okapi								
2	37	25	25	41	46	30	34	3.7
7	64	38	42	58	78	51	55	4.9
12	45	30	30	42	54	39	40	4.9
pc[4]	11.7	9.7	11.1	11.7	12.1	11.1		
Granada								
2*	28	18	20	32	41	25	27	2.9
5	58	42	41	58	62	53	52	4.4
6	43	34	34	44	50	37	40	3.6
8	43	33	37	45	51	35	41	4.5
10	77	54	59	77	92	57	69	3.8
pc	11.7	10.2	11.8	12.0	11.4	11.3		
Kraka								
2	49	40	47	53	58	52	50	5.1
6	37	27	32	35	46	42	37	4.0
8	35	25	28	32	47	38	34	4.5
12	56	38	45	50	71	57	53	7.0
pc	11.1	8.7	10.0	11.5	10.5	10.7		
Citadel								
2	35	21	30	39	47	35	34	2.8
6	28	15	20	28	32	26	25	2.7
8	28	14	20	27	35	25	25	3.4
12	44	21	29	39	56	42	38	3.8
pc	11.3	9.3	10.5	11.4	10.7	10.8		

[1] Determined as absorbance (100 * AU) of CBB-stained subunits
[2] HMWg subunits encoded according to Payne and Lawrence (1983)
3 Least Significant Difference ($P \leq 0.05$, $n = 8$)
[4] Protein content

Figure 5.2 shows the total amount of HMWg subunits relative to the total kernel protein content of the samples of each variety. A considerable effect of the environmental conditions is revealed. The kernel proteins of the varieties grown at site E contain a higher proportion of the HMWg subunits group than the proteins of kernels produced at the other sites. The varieties, especially Okapi and Granada, produce a relatively low amount of the HMWg subunits at site C. The cause of the substantial variation in ratio between the amount of the HMWg subunits and the protein content is not known. It has been shown that the proportion of the groups of storage proteins - including the HMWg subunit group - is affected by sprouting (Ariyama and Khan 1990; Kruger and Marchylo 1990) and by pests during kernel development, such as *Fusarium graminearum* (Bechtel et al. 1985), *Puccinia striiformis* and *P. recondita* (Fullington and Nityagopal 1986) and the wheat-bug (Every et al. 1990). Furthermore, the ratio between groups of the storage proteins depends on the developmental stage of the kernel (Seilmeier et al. 1990).

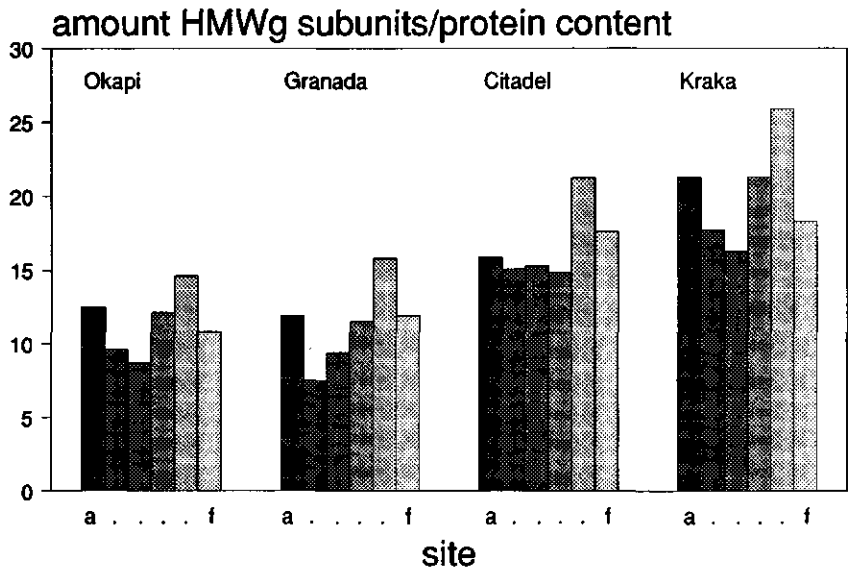

Figure 5.2 Ratio between the total amounts of CBB-stained HMWg subunits (100 * AU) and the protein contents of the kernels of the four varieties of sample set I. Samples of each variety were produced at six sites, see Material and methods.

There were no indications, in this set of samples, that the relationship between the

total amount of the HMWg subunits and the growing site differed between varieties (genotype * environment interactions absent). The same was true for the relationship between the total amount of the HMWg subunits relative to the total kernel protein content.

Table 5.2 shows the ratio between the individual HMWg subunits present in the samples. Within a variety, there are small but statistically significant differences in the relative amount of individual subunits between samples from different sites. The amount of the individual HMWg subunits produced by a variety therefore shows a proportional response to variations in environmental conditions. The coefficient of variation (CV) due to the differences at the growing site is five times smaller for the relative amount of the subunits than for the staining intensity itself (4.7% and 23.6%, respectively). Thus, compared with the total amount of the HMWg subunits, the relative amount is only to a limited extent influenced by the environmental conditions.

Within a site, varieties differed in the staining intensity of the HMWg subunits (Table 5.1). This probably reflects differences in the amount of subunits present in the kernels, but also differences in the molar absorption of CBB by the subunits (Van Kley and Hale 1977). Therefore, only for the varieties which contain the same combination of subunits, Kraka and Citadel, the total amount of HMWg subunits produced can be compared. Statistical analysis showed that at each site the kernels of Kraka had a significantly ($P \leq 0.001$) higher concentration of the four HMWg subunits than the kernels of Citadel. The differences in absorbance of the subunits of these varieties do not result from differences in the staining between the gels, as the average staining intensity of the HMWg subunits present in extracts from the reference flour (see Material and methods) differed only slightly between the gels to which the samples of these varieties were applied (1.31 AU for Kraka and 1.41 AU for Citadel). The dough and bread qualities of Kraka are superior to those of Citadel (Anonymous 1990), in spite of both varieties having the same HMWg subunit combination. It is therefore very probable that the quantitative aspect, the variation in the amount of the HMWg subunits, is also very important.

Comparison of the genetically determined HMWg subunit producing capacity of wheat genotypes is hampered by the considerable effect that differences between growing sites have on the amount of the HMWg subunits. Ideally, because genotype * environment interactions for the amount of the HMWg subunits cannot be completely excluded, the genotypes should be grown under different environmental conditions, as in sample set I. However, in most cases only a limited

number of sites, or replicates on the same trial field, will be used. Therefore, the

Table 5.2 Ratio[1] between the individual HMWg subunits present in the kernels of varieties, each grown at six different sites.

HMWg subunit[2]	Site						LSD[3]
	A	B	C	D	E	F	
Okapi							
2	0.25	0.27	0.26	0.29	0.26	0.25	0.026
7	0.44	0.41	0.43	0.41	0.44	0.43	0.026
12	0.31	0.32	0.31	0.30	0.30	0.32	0.024
Granada							
2*	0.11	0.10	0.11	0.13	0.14	0.12	0.011
5	0.23	0.23	0.22	0.23	0.21	0.25	0.012
6	0.17	0.19	0.17	0.17	0.17	0.18	0.009
8	0.17	0.18	0.19	0.17	0.17	0.17	0.014
10	0.31	0.30	0.31	0.30	0.31	0.28	0.014
Kraka							
2	0.28	0.31	0.31	0.31	0.26	0.28	0.016
6	0.21	0.21	0.21	0.21	0.21	0.22	0.012
8	0.19	0.19	0.18	0.19	0.21	0.20	0.011
12	0.32	0.29	0.30	0.30	0.32	0.30	0.017
Citadel							
2	0.26	0.29	0.30	0.29	0.28	0.27	0.017
6	0.20	0.21	0.20	0.21	0.19	0.21	0.015
8	0.21	0.20	0.20	0.20	0.21	0.19	0.018
12	0.33	0.30	0.30	0.29	0.33	0.33	0.022

[1] Ratio between absorbances of CBB-stained subunits, expressed as proportion of the total absorbance of the HMWg subunits in a lane.
[2] HMWg subunits encoded according to Payne and Lawrence (1983)
[3] Least Significant Difference ($P \leq 0.05$, $n = 8$)

differences in the amount of the HMWg subunitsbetween plots of the same site have been studied. The samples of two varieties, Urban and Obelisk (sample set II), differed in protein content as a result of differences in the amount of N-fertilizer applied. The ratio between the individual HMWg subunits differed only slightly between the samples of each variety (results not shown), in accordance with the results obtained with sample set I. However, the total amount of the HMWg subunits differed considerably between the samples. An increase in the protein content resulted in an increase in the total amount of HMWg subunits (Figure 5.3). Regression analysis showed that the latter increase could to a large extent - 87% and 88% in case of Urban and Obelisk, respectively - be explained by the increase

in the protein content. The ratio between the amount of HMWg subunits and protein content is relatively constant when compared to the differences in ratio in the samples of a variety of sample set I. This agrees with the results of Fullington et al. (1983) and Levy et al. (1985).

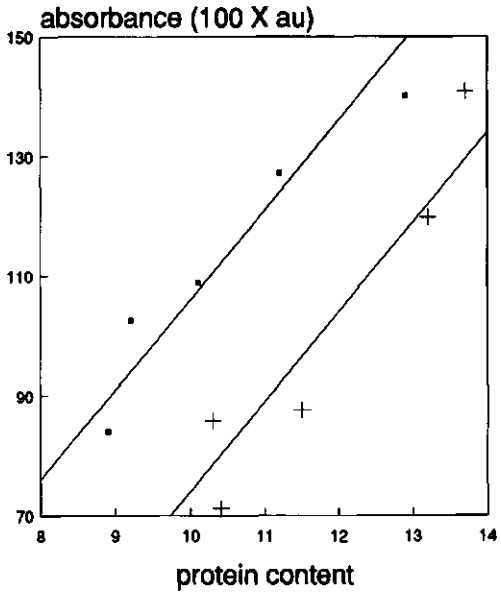

Figure 5.3 Relationship between protein content and total amount of HMWg subunits (100 * AU) - determined as the sum of absorption of the individual HMWg subunits - present in two varieties. Each point denotes the amount of HMWg subunits present in the kernels produced on one plot. (▪) = Obelisk (HMWg subunits 2, 7, 20, 9, 12), (+) = Urban (HMWg subunits 1, 5, 7, 9, 10). The regression equations are: absorption = -0.44 + 0.15 protein content and absorption = -0.76 + 0.15 protein content for Obelisk and Urban, respectively.

The degree of unexplained variation in the amount of the HMWg subunits between replicate samples of a variety was much smaller within a site than between sites. This indicates that differences in climatological conditions, such as rainfall and temperature, have an important effect upon the HMWg subunit production of a genotype. In contrast, the agronomic condition N-application, which affects the protein content, does not affect the ratio between the total amount of the HMWg subunits and the total amount of kernel proteins.

The slope of the regression lines of the total amount of the HMWg subunits on the protein content of the kernels of Obelisk and Urban did not differ significantly between the varieties (Figure 5.3). Differences in the dye adsorption of the subunits

of genotypes affect the slope of the regression lines. Therefore this slope cannot be used as an index to correct for differences in the staining intensity of subunits of varieties grown at the same location.

Conclusions

Variation in the amount of glutenin is of importance for the bread-making quality of wheat. This study shows that the amount of the HMWg subunits present in the kernels depends on:

a) Environmental conditions. The total amount of HMWg subunits, relative to the other endosperm proteins, differs strongly between the sites within each variety. This indicates that, at identical protein levels, the functional properties of total kernel proteins of a variety may differ between sites. This variation is of importance for the selection of wheat for bread-making quality. Interactions between the genotype and the environment in determining the amount of the HMWg subunits have not been found.
b) Genetic variation. The present study shows that varieties differ in their storage protein producing capacity which they invest in the production of HMWg subunits.

The environmental variation in the amount of the HMWg subunits interferes with studies concerning genetic variation in the amount of these proteins. In studies comparing the total amount of HMWg subunits produced by genotypes, it is advisable to grow these genotypes in the same trial field and to limit differences in protein content. The use of kernels affected by pests or sprouting should be avoided as there are indications that this affects the amount of the HMWg subunits. The ratio between individual HMWg subunits can be used to detect genetic variation in expression of genes of individual HMWg subunits because this ratio does not depend upon variation within a site nor between sites.

Because variation in the amount of HMWg subunits is of importance for bread-making quality, research is in progress to increase both our understanding of the agronomic conditions that affect the amount of HMWg subunits, and the prospects for exploitation of genetic variation in the amount of (individual) HMWg subunits in plant breeding.

References

Anonymous (1990) The Dutch descriptive list of varieties of field crops. Leiter-Nypels, Maastricht

Ariyama T, Khan K (1990) Effect oflaboratory sprouting and storage on physicochemical and breadmaking properties of hard red spring wheat. Cereal Chem 67: 53-58

Bechtel DB, Kaleikau LA, Gaines RL, Seitz LM (1985) The effects of *Fusarium graminearum* infection on wheat kernels. Cereal Chem 62: 191-197

Branlard G, Dardevet M (1985) Diversity of grain protein and bread wheat quality. II. Correlation between high molecular weight subunits of glutenin and flour quality characteristics. J Cereal Sci 3: 345-354

Castle SL, Randall PJ (1987) Effects of sulfur deficiency on the synthesis and accumulation of proteins in the developing wheat seed. Aust J Plant Physiol 14: 503-516

Darwinkel A (1987) Improvement of grain quality of winter wheat in arable farming in the Netherlands. In: Borghi B (ed) Agriculture. Hard wheat: agronomic, technological, biochemical and genetic aspects. Commission of the European communities, Luxembourg, pp 73-84

Doekes GJ, Wennekes LMJ (1982) Effects of nitrogen fertilization on quantity and composition of wheat flour protein. Cereal Chem 59: 276-278

Every D, Farrell JAK, Stufkens MW (1990) Wheat-bug damage in New Zealand wheats: the feeding mechanism of *Nysius huttoni* and its effect on the morphological and physiological development of wheat. J Sci Food Agric 50: 297-309

Fullington JG, Nityagopal A (1986) Effect of rust infection on the protein components of wheat. Phytochem 25: 1289-1292

Fullington JG, Cole EW, Kasarda DD (1983) Quantitative sodium dodecyl sulphate-polyacrylamide electrophoresis of total proteins extracted from different wheat varieties: effect of protein content. Cereal Chem 60: 65-71

Fullington JG, Miskelly DM, Wrigley CW, Kasarda DD (1987) Quality-related endosperm proteins in sulfur-deficient and normal wheat grain. J Cereal Sci 5: 233-245

Genstat 5 committee (1987) Genstat 5 reference manual. Clarendon Press, Oxford

Huebner FR, Bietz JA (1985) Detection of quality differences among wheats by high-performance liquid chromatography. J Chromatogr 327: 333-342

Kolster P, Van Gelder WMJ (1991) A method for the quantification of individual high molecular weight glutenin subunits using SDS-PAGE and laser scanning densitometry. In: Bushuk W, Tkachuk R (eds) Gluten proteins 1990. American Association of Cereal Chemists, St. Paul, pp 349-362

Kruger JE, Marchylo BA, Hatcher D (1988) Preliminary assessment of a sequential extraction scheme for evaluating quality by reversed-phase high-performance liquid chromatography and electrophoretic analysis of gliadins and glutenins. Cereal Chem 65: 208-214

Kruger JE, Marchylo BA (1990) Analysis by reversed-phase high-performance liquid chromatography of changes in high molecular weight subunit composition of wheat storage proteins during germination. Cereal Chem 67: 141-147

Levy AA, Galili G, Feldman M (1985) The effect of additions of *Aegilops longissima* chromosomes on grain protein in common wheat. Theor Appl Genet 69: 429-435

Marchylo BA, Kruger JE, Hatcher DW (1990) Effect of environment on wheat storage proteins as determined by quantitative reversed-phase high-performance liquid chromatography. Cereal Chem 67: 372-376

Moonen JHE, Scheepstra A, Graveland A (1982) Use of the SDS-sedimentation test and SDS-polyacrylamidegel electrophoresis for screening breeder's samples of wheat for bread-making quality. Euphytica 31: 677-690

Pardowitz I, Ehrhardt W, Neuhoff V (1990) Quantitative densitometry from the point of view of

information theory. Electrophoresis 11: 400-406
Payne PI, Corfield KG (1979) Subunit composition of wheat glutenin proteins, isolated by gel filtration in a dissociating medium. Planta 145: 83-88
Payne PI, Corfield KG, Blackman JA (1979) Identification of a high-molecular-weight subunit of glutenin whose presence correlates with bread-making quality in wheats of related pedigree. Theor Appl Genet 55: 153-159
Payne PI, Lawrence GJ (1983) Catalogue of alleles for the complex gene loci, Glu-A1, Glu-B1 and Glu-D1 which code for high-molecular-weight subunits of glutenin in hexaploid wheat. Cereal Res Commun 11: 29-35
Payne PI, Nightingale MA, Krattiger AF, Holt LM (1987) The relationship between HMW glutenin subunit composition and the bread-making quality of British-grown wheat varieties. J Sci Food Agric 40: 51-65
Searle SR (1971) Linear models. Wiley, New York
Seilmeier W, Wieser H, Belitz H-D (1990) Weizen während der Reifung. Analyse der Gliadine und Glutenine mittels RP-HPLC. Z Lebensm Unters Forsch 191: 99-103
Schofield JD, Booth MR (1983) Wheat proteins and their technological significance. In: Hudson BJF (ed) Developments in food proteins, volume 2. Applied Science Publishers, London, pp 1-65
Sutton KH, Hay RL, Griffin WB (1989) Assessment of the potential bread baking quality of New Zealand wheats by RP-HPLC of glutenins. J Cereal Sci 10: 113-121
Van Gelder WMJ, Kolster P, Mesdag J, Van Eeuwijk FA (1987) The relationship between high-molecular-weight glutenin subunits, bread-making quality and yield of winter wheat. In: Borghi B (ed) Agriculture. Hard wheat: agronomic, technological, biochemical and genetic aspects. Commission of the European Communities, Luxembourg, pp 159-172
Van Kley H, Hale S M (1977) Assay for protein by dye binding. Anal Biochem 81 485-487
Wieser H, Seilmeier W, Belitz H-D (1989) Reversed-phase high-performance liquid chromatography of ethanol-soluble and ethanol-insoluble reduced glutenin fractions. Cereal Chem 66 38-41
Wrigley C W, Du Cros D L, Fullington J G, Kasarda D D (1984) Changes in polypeptide composition and grain quality due to sulfur deficiency in wheat. J Cereal Sci 2 15-24

CHAPTER 6

THE EXPRESSION OF INDIVIDUAL HIGH MOLECULAR WEIGHT GLUTENIN SUBUNIT GENES OF WHEAT IN RELATION TO DIFFERENCES IN THE NUMBER AND TYPE OF HOMEOLOGOUS SUBUNITS AND DIFFERENCES IN GENETIC BACKGROUND

P Kolster, CF Krechting[1], WMJ van Gelder

Summary

The amount of the individual High Molecular Weight glutenin (HMWg) subunits of bread-wheat (*Triticum aestivum* L.) has been studied in relation to allelic variation at homeologous loci and variation in the general genetic background. The relations between *Glu-1* loci have been studied using near-isogenic lines (NILs) of the variety Sicco, in which the HMWg subunit alleles are substituted by allelic variants. The substitution of the *Glu-D1* allele by a null-allele resulted in a higher amount of the subunits encoded by genes at homeologous loci. The presence of a *Glu-A1* null-allele did not have a noticeable effect on the amount of the homeologous subunits. In three out of four NILs, and in the sister-lines of two crosses, the amount of the HMWg subunits did not depend on the allele make-up at the homeologous loci. Only in the NIL which contains the rare *Glu-D1* allele 2.2+12, the amount of the *Glu-A1* and *Glu-B1* encoded subunits was considerably lower than the amount of these subunits in Sicco. This study suggests a relation between the amount of the HMWg subunits encoded by an allele and its contribution to bread-making quality. The effect of the genetic background has been studied using F4 and F5 lines of two crosses. The total amount of the subunits, relative to the total amount of kernel proteins, showed a considerable variation between the lines. The ratio between the individual subunits did not differ between genetic backgrounds. Because the ratio between individual subunits is also largely independent from differences in environmental conditions, as shown by a previous study, it is concluded that the relative amount of a subunit is a valuable measure for the detection of genetically determined differences in expression of HMWg subunit genes. The results are discussed in relation to possible levels of genetic regulation of gene expression.

[1] DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO)

Introduction

The High Molecular Weight glutenin (HMWg) subunits, a group of storage proteins of wheat, have been extensively studied in relation to bread-making quality. In the allohexaploid bread-wheat (*Triticum aestivum* L.), these proteins are encoded by the genes at three complex loci (*Glu-A1*, *Glu-B1* and *Glu-D1*) at the homeologous chromosomes 1A, 1B and 1D respectively. At each locus two types of single copy genes are present (Harberd et al. 1986). These genes encode subunits which are classified as x-type or y-type HMWg subunits according to their higher or lower molecular weight, respectively (Payne et al. 1981). A large number of alleles has been found in wheat varieties and in species related to bread-wheat (see Kolster et al. 1988, Chapter 3 of this thesis). In wheat varieties, the *Glu-A1* alleles do not encode a y-type subunit, while one of the *Glu-A1* alleles does not result in an x-type subunit either. Alleles which do not encode HMWg subunits are referred to as null-alleles. Some *Glu-B1* alleles do not encode a y-type subunit. The *Glu-D1* alleles which occur in varieties all encode both types of subunits. *Glu-B1* and *Glu-D1* null-alleles are very rare.

Variation in the bread-making quality of genotypes has been associated with variation in HMWg subunit genotype (see Payne et al. 1979, 1987a; Moonen et al. 1982; Branlard and Dardevet 1985; Kolster et al. 1991a, Chapter 2 of this thesis). Also quantitative variation of the HMWg subunits (the total amount as well as the amounts of the individual subunits) is of importance for the bread-making quality (Schofield and Booth, 1983; Huebner and Bietz 1985; Kruger et al. 1988; Wieser et al. 1989). In wheat lines, the presence of null-alleles at each of the *Glu-1* loci, resulted in a decreased the total amount of the HMWg subunits and was negatively correlated with the bread-making quality (Payne et al. 1987b; Lawrence et al. 1988).

In general, phenotypic variation in the amount of the HMWg subunits was studied. In Chapter 5 of this thesis, a considerable effect of variation in environmental conditions on the amount of HMWg subunits os shown. There is a lack of knowledge about the genetic variation in the expression of HMWg genes. There is also limited information about the relation between the expression of HMWg subunit genes and the variation at the homeologous HMWg subunit loci and variation in genetic background.

In inter-varietal substitution lines, the amount of the HMWg subunits encoded by a substituted chromosome did not differ from the amount produced in the donor-variety (Galili and Feldman, 1985). The expression of HMWg subunit genes

at the *Glu-D1* locus was not affected by the deletion of a chromosome-arm carrying the *Glu-B1* locus in aneuploid wheat-lines (Galili et al. 1986). These results suggested that the expression of the HMWg subunit genes did not depend on the genetic background. In contrast, the results of other studies showed that the expression of the HMWg subunit genes may depend on the genetic background. Namely, an increase in the dosage of the HMWg loci in compensating nullisomic-tetrasomic lines was related to a decrease in the amount of homeologous HMWg subunits and in the amount of the gliadins (Galili et al. 1986), and other groups of gluten proteins of importance for the bread-making quality. Furthermore, Galili and Feldman (1986) reported an increase in the amount of HMWg subunits as a result of the deletion of a chromosome-arm encoding the so-called HMW gliadins. Aneuploid wheat lines were used to study the relations between the amount of storage proteins and the genetic background in most of the studies here referred to. These lines may not be representative for the relations in euploid wheat. Furthermore, allelic variation at the *Glu-1* loci may influence the expression of *individual* HMWg subunit genes, but this topic has not been studied so far.

The aim of the present investigation was to study the expression of HMWg subunit genes in euploid wheat genotypes in relation to:

a) differences in the number of genes resulting in a HMWg subunit, which in general ranges from 3 to 5 in bread-wheat,
b) differences in the type of alleles present at the homeologous *Glu-1* loci,
c) differences in the genetic background (i.e. in genes other than the HMWg subunit genes).

Near-isogenic lines (NILs) of Sicco, in which the HMWg subunit alleles are replaced by allelic variants, have been used to study in the Sicco genetic background the relations between the genes of the *Glu-1* loci in determining the gene expression (a and b). To study the expression of HMWg subunit genes in different genetic backgrounds (c), F4 and F5 lines of two crosses have been used. These lines gave also information on the relations studied under a) and b).

Material and methods

Plant material

Kernels of two subsets of near isogenic lines (NILs) of the Dutch spring-wheat

variety Sicco were kindly provided by from Dr PI Payne, PBI Cambridge, England (Payne et al. 1987b). Subset A was composed of NILs in which the *Glu-A1* and/or *Glu-D1* alleles of Sicco are replaced by null-alleles, subset B was composed of NILs in which the Sicco alleles are replaced by expressed allelic variants. Table 6.1 shows the HMWg subunit genotype of the NILs and the donor varieties. Each NIL had been produced by 5 consecutive backcrosses to Sicco. In the backcrossing procedure, kernels were selected for HMWg subunit genotype by electrophoretic analysis of embryo-less half kernels, while the embryo containing half was used for propagation. BC-5 plants from heterozygous kernels containing the Sicco alleles and the target allele were selfed. The resulting kernels were selected for HMWg subunit genotype; kernels homozygous for the donor allele resulted in the NIL-substituted (NIL-sub), kernels with the Sicco HMWg genotype served as NIL-control (NIL-con). Each NIL-sub and its specific control were derived from the same BC-5

Table 6.1 The HMW glutenin alleles of the *Glu-A1*, *Glu-B1* and *Glu-D1* locus of the near-isogenic lines of Sicco and the donor varieties.

		HMWg genotype			
	Lines	*Glu-A1*	*Glu-B1*	*Glu-D1*	Donor genotype
subset A					
	NIL-1A null	0+0[1]	7+9	5+10	(Nap Hal x Gabo[2])
	NIL-1D null	1+0	7+9	0+0	-
	NIL-1A/1D null	0+0	7+9	0+0	-
	control	1+0	7+9	5+10	-
subset B					
	NIL-2*	2*+0	7+9	5+10	Glenlea
	control	1+0	7+9	5+10	-
	NIL-7+8	1+0	7+8	5+10	Chinese Spring[3]
	control	1+0	7+9	5+10	-
	NIL-2+12	1+0	7+9	2+12	Chinese Spring[3]
	control	1+0	7+9	5+10	-
	NIL-2.2+12	1+0	7+9	2.2+12	Danchi
	control	1+0	7+9	5+10	-

[1] The subunits encoded by the x and the y type gene, respectively. A silent gene is represented by an 0.

[2] Represents a Gabo isogenic line in which the Nap Hal HMWg subunit genotype (0, 17+18, 0) was introduced by 4 backcross generations. During the backcrossing with Sicco, genotypes heterozygous for 1 and 5+10 were selected; after selfing of the BC-5 plants the different types of isogenic lines were selected.

[3] For the NIL 7+8 and NIL 2+12, the same donor variety was used. The lines were separated at BC-1 (see Material and methods).

plant. Consequently, if donor genes other than the target gene are unintentionally introduced in the NILs, these will be present in the NIL-sub as well as in the NIL-con. The plants resulting from 3-4 of these selfed kernels were selfed again, the resulting kernels were spring sown at trial fields of the PBI. The kernels produced upon selfing of the resulting plants were used in the present study.

The NILs are considered to be to a large extent isogenic for the other loci than the *Glu-1* loci, and therefore valuable material for studying the HMWg subunit gene expression in relation to differences in HMWg subunit genotype. It should be noted that a considerable proportion of the donor genome linked to the marker gene is introduced in the NIL, even after 5 backcrosses and selfing at BC-5, as shown by a theoretical study of Stam and Zeven (1981). It is therefore likely that proximally located *cis* or *trans* acting regulatory sequences - or other linked genes which interact with the HMWg subunit genes - are introduced together with the target gene. The enhancer element involved in the temporal and spatial regulation of the HMWg genes (Thomas and Flavell 1990) is an example of a proximal *cis* acting regulatory sequence.

A second set consisted of kernels of 42 F4 and 45 F5 lines resulting from two crosses (between brackets, the parents and their HMWg alleles at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci, respectively), 84024 [SVP-76025111 * Saiga; (2*, 7+8, 2+12) * (0, 7+9, 2+12)] and 83009 [SVP-75016453 * SVP-73003242; (1, 6+8, 5+10) * (0, 6+8, 2+12)]. The progenies of these crosses were bulk-propagated untill the F3. The F4 generation started with randomly chosen single kernels. The kernels used in this study were produced at autumn sown trial fields of the former Foundation for Agricultural Plant Breeding (now part of the Centre for Plant Breeding and Reproduction Research).

When studying the amount of the individual HMWg subunits in material with a wide genetic origin, the variation in genetic background will be higher than when using segregating progenies from crosses between 2 genotypes. However, subunits with an identical relative mobility during SDS-PAGE, which is the criterion used for the genetic identification of subunits, are encoded by identical genes, except for some rare recombinations. This is not the case for subunits in unrelated genotypes because 1) a higher level of expression of genes encoding identical subunits but differing in sequences involved in gene regulation cannot be distinguished from a higher level of expression as a result of differences in the genetic background 2) allelic subunits may have a similar R_m during SDS-PAGE (Kolster et al. 1988, Chapter 3 of this thesis).

Electrophoresis and quantification

The proteins present in extracts of wholemeal flours were separated on 8.3% SDS-PAGE electrophoresis gels. The extraction procedure, electrophoretic conditions, gelstaining and the densitometric analysis are as described previously (Kolster et al. 1992, Chapter 4 of this thesis). In this report, the amount of Coomassie Brilliant Blue (CBB) (expressed as 100 * absorbance unit times mm) adsorbed by the HMWg subunits will be referred to as the amount of the subunits.

Extracts of each genotype were applied on 4 different gels. The two subsets of NILs were analyzed separately, each gel contained a complete subset. In the statistical analysis of the quantitative measurements of the NILs (Genstat 5 committe 1987), gel was treated as a block factor.

Because of the large number of lines originating from the two crosses, different sets of 4 gels were required for the quantification of the subunits. For the statistical analyses, differences in staining-intensity between gels of different sets were assumed to be absent. To verify this assumption, an extract of a reference flour was applied in the first and last slot each gel.

A wholemeal flour is used for electrophoretic analysis of the HMWg subunits. Consequently, genotypes homozygous for the *Glu-A1* allele 1 cannot be distinguished from heterozygous genotypes containing *Glu-A1* alleles 1 and 0 as the flour of both genotypes contained subunit 1. This will have contributed to the variation in the amount of the *Glu-A1* allele 1. Consequenty, the ratio between the subunits will also be affected. However, when the relative amount of the *Glu-B1* and *Glu-D1* subunits was calculated without taking the amount of subunit 1 into consideration, the conclusions from these experiments remained the same.

Results and discussion

Differences in the number of the HMWg subunits

The relation between the number of HMWg subunits produced by a genotype and the amount of the individual subunits ('a' in Introduction) was studied using near-isogenic lines of Sicco, in which the alleles of the *Glu-A1* and *Glu-D1* locus have been replaced by null-alleles (subset A, Table 6.1). Table 6.2 shows the amount of the HMWg subunits present in these NILs. Comparison of the amount of the subunits present in Sicco, the NIL-con and the NIL-1A null shows that replacing the *Glu-A1* allele 1 for the null-allele did not result in a significant change in the amount of *Glu-B1* and *Glu-D1* subunits. In the NIL-1D null and the NIL-1A/1D null, the amount of the remaining subunits was significantly ($P \leq 0.05$) higher than the amount of these subunits in Sicco or the NIL-con

(Table 6.2). The higher protein content of the flour may have contributed to the higher amount of the subunits in the NIL-1D null (Kolster et al. 1991b) but this cannot be the cause of the higher amount of the HMWg subunits in the NIL-1A/1D null.

Table 6.2 Amounts of the individual CBB stained HMWg subunits[1] of near-isogenic lines (NILs) of Sicco, in which the alleles of the *Glu-A1* and/or *Glu-D1* locus are replaced by null-alleles.

		NILs				
HMWg subunit	Sicco	control	1A-null	1D-null	1A/1D-null	LSD[2]
1 (*Glu-A1x*)	20	18		25		3.4
7 (*Glu-B1x*)	44	42	47	60	50	5.9
9 (*Glu-B1y*)	18	18	18	28	26	3.3
5 (*Glu-D1x*)	27	25	27			4.2
10 (*Glu-D1y*)	32	35	33			5.8
protein content (%)	11.1	11.1	10.3	12.3	11.2	

[1] 100 * absorbance units·mm

[2] LSD = least significant difference of the absorbance values at P = 0.05, based on the densitometric analysis of 8 lanes (two independently produced extracts of each sample, each applied on four gels)

The same relation was also studied in the progeny of the two crosses. Both populations contain *Glu-A1* allele 0 and a *Glu-A1* allele encoding a HMWg subunit (1 in cross 83009 and 2* in cross 84024). By calculating the average absorbance of randomly chosen lines with the same HMWg genotype (Table 6.3), differences in the genetic background ('c' in Introduction) are largely removed. The amounts of the individual subunits in sister lines which differ only in their *Glu-A1* allele (compare for 83009 group A with B and group C with D in Table 6.3) were only slightly affected differences in the type of *Glu-A1* allele. The differences observed for the amount of the corresponding subunits in different groups could well be the result of the differences in the average protein content of the flours of the lines, and in the average staining-intensity of the gels. The latter is represented by the average absorbance of the HMWg subunits of a control sample, applied on each gel (Table 6.3). The analysis of 84024 is in full agreement (results not shown).

In conclusion, our experiments with NILs show in euploid wheat that a decrease in the number of subunits by substitution of an active *Glu-D1* allele by a *Glu-D1* null-allele results in an increase in the expression of the remaining HMWg subunit genes. The presence of a *Glu-A1* null-allele in the NILs did not have a noticeable effect on the amount of the homeologous subunits. The absence of an effect of the

presence of a *Glu-A1* null-allele is also found in the experiments with the sister-lines. The presence of the *Glu-A1* null-allele is common in wheat varieties, in contrast to the *Glu-D1* null-allele.

The absence of a significant effect of the presence of the *Glu-A1* null-allele may be caused by the low amount of subunit encoded, which is considerably lower than the amount produced by the *Glu-B1* and *Glu-D1* alleles (compare Table 6.2, amounts of subunits 1 and 5+10).

Table 6.3 Amounts of the individual CBB stained HMWg subunits[1] in 4 genotypic groups of sister lines (cross 83009), denoted A to D. HMWg subunit alleles present are for the *Glu-D1* locus 2+12 or 5+10 and for the *Glu-A1* locus 0 or 1.

HMWg subunit	A	B	C	D
0 (*Glu-A1x*)[2]	-		-	
1 (*Glu-A1x*)		21		22
6 (*Glu-B1x*)	32	37	34	38
8 (*Glu-B1y*)	31	33	34	38
2 (*Glu-D1x*)			45	48
12 (*Glu-D1y*)			61	65
5 (*Glu-D1x*)	45	50		
10 (*Glu-D1y*)	71	75		
protein content(%)[3]	13.0	13.1	13.3	13.6
control samples[4]	128	135	126	129
number of lines	7	15	7	16

- = no subunit present

[1] 100 * absorbance units·mm

[2] For the quantification of the HMWg subunits of a line, four replicate densitometric analysis were used. Due to the large differences in the staining intensity between the lines, none of the differences between a subunit present in different groups were significant

[3] The average protein content of the samples belonging to the same genotypic group

[4] Total absorbance of the HMWg subunits present in an extract, used as a control for different gels (see Material and methods)

Another cause of the differences in effect of the *Glu-A1* and *Glu-D1* null-alleles may be the differences in the causes responsible for the lack of subunits of the genes. The genes of the *Glu-D1* null-allele are deleted (Payne, pers comm). In contrast, the HMWg subunit genes of the *Glu-A1* locus (Harberd et al. 1986) are probably silent by mutations, as shown by sequence analysis of two *Glu-A1y* subunits genes (Forde et al. 1985; Harberd et al. 1987). No HMWg subunit transcription products of the silent *Glu-A1x* and *Glu-A1y* genes of Chinese Spring (Thompson et al. 1983) and of the *Glu-A1y* gene of Cheyenne (Forde et al. 1985) could be detected. The results of the present study suggest that the number of genes present, is determining the level of expression, and not the number of transcribed genes. The number of flancking sequences of genes which can form

complexes with proteins, and therefore can be involved in gene-regulation (see Waugh and Brown 1991) might be rate limiting for gene expression.

Differences in the type of the HMWg subunits

The relation between the expression of the HMWg genes and the variation in type of alleles present at homeologous loci ('b' in Introduction) has been studied by means of four near-isogenic lines, in which the HMWg subunit alleles of Sicco were replaced by allelic variants (subset B in Table 6.1). Because the introgressed alleles encoded the same number of subunits as the Sicco alleles, each NIL-sub contained the same number of subunits as Sicco. Table 6.4 shows that the protein content and the amount of the HMWg subunits differed between the NILs. Apart from the different *Glu-1* allele substitution, differences between pairs of NILs can be caused by the introduction of other genes than the target genes. Also environmental influences could cause the differences between NILs (Kolster et al. 1991b, Chapter 5), so further studies should be carried out to separate these causes.

The amounts of the individual HMWg subunits present in the NIL-sub should be compared with the amount of the corresponding subunits present in its specific control (Table 6.4). In the first, second and third pair, the amounts of the Sicco-type HMWg subunits was equal or only slightly higher in the NIL-sub than in the NIL-con. In the NIL 2.2+12, the amount of the Sicco-type HMWg subunits was significantly lower than the amount in the control. The difference cannot be explained by the difference in protein content between the NIL-sub and its control. Therefore, the probable cause is the substitution of the *Glu-D1* allele encoding the subunits 5 and 10 by an allele encoding 2.2 and 12 (including the genes linked to it, see material and methods, plant material). The latter allele originates from a Japanese variety (Payne et al. 1983) and is very rare in wheat varieties.

The effect of the substitution of an allele, without changing the number of subunits, can also be studied using the progeny of the two crosses. In groups of lines which differ only in the type of *Glu-D1* allele (encoding 2+12 or 5+10; compare A with C and B with D in Table 6.3), the amount of the *Glu-B1* subunits 6 and 8 was comparable. The small differences observed were probably a result of differences in the average protein content of the groups of lines and in the staining intensity of the gels (see Table 6.3). The results of cross 84024 agree (results not presented).

In conclusion, the *Glu-1* loci did not influence the HMWg subunit gene expression except in the case of substitution of 5+10 by the rare allele 2.2+12. Payne and coworkers (Payne, pers comm) found this *Glu-D1* allele to be of a "low quality"

with respect to bread-making. This low quality may thus result from the overall reduction in the amounts of the HMWg subunits.

In passing it may be noted that the absorbance of some of the subunits encoded by the alleles introgressed in Sicco differs from the absorbance of the corresponding Sicco-type subunits (Table 6.4). Assuming that subunits do not differ in binding capacity of the dye, these differences are considered as differences in the amount of subunit. As far as these limited data go, there is a coincidence between the combined amounts of the subunits and the contribution to the bread-making quality of the *Glu-B1* and *Glu-D1* alleles, as reported by Payne et al. (1987a). The same holds for the *Glu-D1* encoded subunits in the lines of cross 83009 (Table 6.3); the amount of the high-quality alleles encoding the subunits 5 and 10 is higher than the amount of subunits encoded by the low-quality allele 2+12. The present study therefore suggests a relation between the amount of HMWg subunits and their contribution to bread-making quality. Further studies on this subject are in progress.

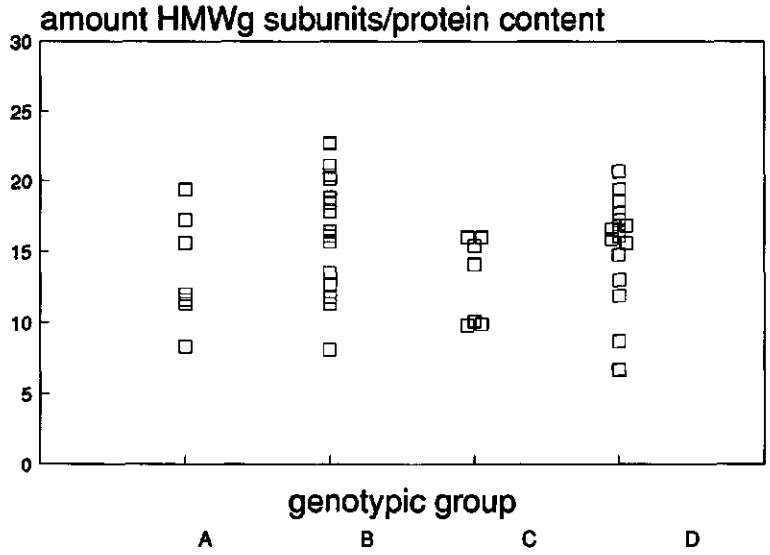

Figure 6.1 Variation in the ratio between the absorbance values of the CBB-stained HMWg subunits and the protein content of the sister-lines of cross 83009. The lines are grouped for HMWg subunit genotype, denoted A-D. See Table 6.3 for the HMWg subunit genotype of the groups. The protein content of the kernels of the lines ranges from 9.1 to 14.2%.

Differences in genetic background

The influence of variation in the genetic background on the expression of a HMWg

Table 6.4 Amounts of the individual CBB stained HMWg subunits[1] in near-isogenic lines of Sicco, in which alleles of the *Glu-A1*, *Glu-B1* or *Glu-D1* locus are replaced by allelic variants (given in parenthesis).

subunit	*Glu-A1* 2*			*Glu-B1* 7+8			*Glu-D1* 2+12			*Glu-D1* 2.2+12		
	control		substituted[2]	control		substituted	control		substituted	control		substituted
1 (*Glu-A1x*)	18	***	13(2*)	27	ns	30	27	ns	27	20	**	17
7 (*Glu-B1x*)	49	ns	51	67	ns	68 (7)	71	ns	79	67	***	47
9 (*Glu-B1y*)	16	ns	16	22	***	32 (8)	21	*	28	24	***	16
5 (*Glu-D1x*)	32	ns	30	43	ns	46	41	*	33 (2)	39	***	14 (2.2)
10 (*Glu-D1y*)	40	ns	40	65	*	70	66	ns	69(12)	59	***	43 (12)
protein content(%)	11.0		11.1	14.8		15.2	13.4		14.0	15.3		14.8

[1] 100 * absorbance units·mm

[2] the amount of subunits should be compared with the amount present in its specific control

*** : P < 0.001, ** : P = 0.01, * : P = 0.05, ns = not significant

gene ('c' in Introduction) has been studied in the F4 and F5 lines of the two crosses. Since the amount of the HMWg subunits of a genotype is related to the protein content of the lines, the absorbance is not the most optimal measure for this specific purpose.

Figure 6.1 shows the total amount of HMWg subunits - relative to the total amount of kernel proteins - for the different lines, grouped for HMWg subunit genotype. The total amount of the HMWg subunits, encoded by identical alleles, differed considerably between lines. This variation in proportion exceeds that observed for replicate plots of a variety and for plots of a variety grown at different locations (Kolster et al. 1991b, Chapter 5). In the latter study, also genetically determined differences in the proportion of the HMWg subunits between varieties with the same HMWg subunit make-up were shown. Therefore, it is likely that variation in genetic background considerably contributes to the variation in amount of the HMWg subunits. A quantitative analysis of this genetic contribution is in progress.

A second question is whether differences in the genetic background differentially affect the expression of individual HMWg subunit genes. The amount of individual HMWg subunits, relative to the total amount of HMWg subunits, is largely independent from the differences in protein content (Kolster et al. 1991b, Chapter 5). Although, there are some restrictions. Firstly, variation in relative amount of a subunit may result from variation in the amount of the subunit itself, but may also result from variation in the amount of (one of the) other subunits. Secondly, alleles may differ in number and amount of the subunits they encode. Consequently, comparison is only feasible for lines with the same HMWg subunit genotype. Table 6.5 therefore shows for lines from cross 83009 with the same HMWg subunit genotype the maximum and minimum relative amount of the individual subunits observed. The variation in the relative amount of a subunit within a group of genotypes is slightly higher than the observed variation as a result of differences in growing conditions (Kolster et al. 1991b, Chapter 5). The same holds for cross 84024 (results not shown).

These experiments give therefore no clear-cut evidence that individual HMWg genes are differentially affected by differences in genetic background. The total amount of the HMWg subunits however is clearly affected by differences in the genetic background. The effects of variation in storage protein genes on the expression of other storage protein genes as described by Galili (see Introduction) is another example of a relation between the genetic background and the expression of HMWg subunit genes.

Concluding remarks

The ratio between the HMWg subunits seems largely independent from differences in the gentic background. This suggests that a common regulation mechanism coordinates the expression of the genes at the *Glu-1* loci. Such a coordination between the HMWg subunit genes is also shown for wheat grown under different environmental conditions (Kolster et al. 1991b, Chapter 5). Only in the relation between the individual HMWg subunit genes, effects of the gene-dosage and the type of alleles on the gene-expression may occur, which effects remain unexplained. Bartels and Thompson (1986) showed for a genotype grown under standardized conditions that during kernel development, the expression of the genes of storage proteins is coordinated, probably at the level of transcription. The present study shows however that the total amount of the HMWg subunits relative to the total amount of protein depends on the genetic background. Therefore, other regulation mechanisms, superimposed on the mechanism regulating the coordinated expression of the HMWg subunit genes, are probably involved as well. Finally, environmental conditions are affecting the total amount of the HMWg subunits of a genotype (Kolster et al. 1991b, Chapter 5).

Table 6.5 Relative amounts of the individual HMWg subunits (absorbance of the HMWg subunits relative to the total absorbance of the subunits) in groups of sister-lines of cross 83009. The minimal and maximal relative amount present in the lines is reported[1].

HMWg subunit	A	B	C	D
1 (*Glu-A1x*)		0.02-0.15		0.06-0.16
6 (*Glu-B1x*)	0.17-0.18	0.15-0.19	0.19-0.20	0.16-0.19
8 (*Glu-B1y*)	0.16-0.18	0.15-0.19	0.19-0.20	0.17-0.20
2 (*Glu-D1x*)			0.26-0.29	0.19-0.25
12 (*Glu-D1y*)			0.31-0.37	0.27-0.35
5 (*Glu-D1x*)	0.24-0.28	0.22-0.26		
10 (*Glu-D1y*)	0.39-0.42	0.32-0.40		

[1] Average LSD ($P \leq 0.05$) for the relative absorbance of a line, based on 4 replicates: 0.025

The present study has significance for investigations on the genetic variation in the expression of HMWg subunit genes. Firstly, for the most commonly occurring HMWg subunit alleles, no interactions were found between the *Glu-1* loci in determining the expression of the genes. Therefore, it is possible to use the amount of a subunit as a measure of the gene expression, without taking the HMWg subunit genotype into consideration. Secondly, the relative amount of a subunit is only to a

limited extent influenced by differences in genetic background. The same holds for differences in environmental conditions (Kolster et al. 1991b, Chapter 5). Therefore, when in a genotype a subunit is found which differs strongly in proportion compared with the amount in other genotypes, it is probably caused by characteristics of the gene itself. A genetic analysis is required for determining the exact nature of the aberrant expression.

The results of the present study are used to assess the genetic variability of the expression of the HMWg subunit genes in Dutch wheat varieties and to study the relation between the quality of an HMWg subunit allele with respect to bread-making and the amount of protein produced.

References

Bartels D, Thompson RD (1986) Synthesis of mRNAs coding for abundant endosperm proteins during wheat grain development. Plant Sci 46: 117-125

Branlard G, Dardevet M (1985) Diversity of grain protein and bread wheat quality. II. Correlation between high molecular weight subunits of glutenin and flour quality characteristics. J Cereal Sci 3: 345-354

Forde J, Malpica JM Halford NG, Shewry PR, Anderson OD, Greene FC, Miflin BJ (1985) The nucleotide sequence of a HMW glutenin subunit gene located on chromosome 1A of wheat (*Triticum aestivum* L.). Nucleic Acids Res 13: 6817-6831

Galili G, Feldman M (1985) Genetic control of endosperm proteins in wheat. 3. Allocation to chromosomes and differential expression of high molecular weight glutenin and gliadin genes in intervarietal substitution lines of common wheat. Theor Appl Genet 69: 583-589

Galili G, Levy AA, Feldman M (1986) Gene-dosage compensation of endosperm proteins in hexaploid wheat *Triticum aestivum*. Proc Natl Acad Sci USA 83: 6524-6528

Genstat 5 committee (1987) Genstat 5 reference manual. Clarendon Press, Oxford

Harberd NP, Bartels D, Thompson RD (1986) DNA restriction-fragment variation in the gene family encoding high molecular weight (HMW) glutenin subunits of wheat. Biochem Genet 24: 579-596

Harberd NP, Flavell RB, Thompson RD (1987) Identification of a transposon-like insertion in a *Glu-1* allele of wheat. Mol Gen Genet 209: 326-332

Huebner FR, Bietz JA (1985) Detection of quality differences among wheats by high-performance liquid chromatography. J Chromatogr 327: 333-342

Kolster P, Krechting CF, Van Gelder WMJ (1988) Variation in high molecular weight glutenin subunits of *Triticum aestivum* and *T. turgidum* spp. *dicoccoides*. Euphytica S:141-145

Kolster P, Van Eeuwijk FA, Van Gelder WMJ (1991a) Additive and epistatic effects of allelic variation at the high molecular weight glutenin subunit loci in determining the bread-making quality of breeding lines of wheat. Euphytica 55: 277-285

Kolster P, Krechting CF, Van Gelder WMJ (1991b) Quantitative variation in individual high molecular weight glutenin subunits of wheat in relation to variation in environmental conditions. J Sci Food Agric 57: 405-415

Kolster P, Krechting CF, Van Gelder WMJ (1992) Quantification of individual high molecular weight glutenin subunits of wheat using SDS-PAGE and scanning densitometry. J Cereal Sci 15:

49-61
Kruger JE, Marchylo BA, Hatcher D (1988) Preliminary assessment of a sequential extraction scheme for evaluating quality by reversed-phase high-performance liquid chromatography and electrophoretic analysis of gliadins and glutenins. Cereal Chem 65: 208-214
Lawrence GJ, MacRitchie F, Wrighley CW (1988) Dough and baking quality of wheat lines deficient in glutenin subunits controlled by the Glu-A1, Glu-B1 and Glu-D1 loci. J Cereal Sci 7: 109-112
Moonen JHE, Scheepstra A, Graveland A (1982) Use of the SDS-sedimentation test and SDS-polyacrylamidegel electrophoresis for screening breeder's samples of wheat for bread-making quality. Euphytica 31: 677-690
Payne PI, Corfield KG, Blackman JA (1979) Identification of a high-molecular-weight subunit of glutenin whose presence correlates with bread-making quality in wheats of related pedigree. Theor Appl Genet 55: 153-159
Payne PI, Holt LM, Law CN (1981) Structural and genetical studies on the high-molecular-weight subunits of wheat glutenin. Part 1: Allelic variation in subunits amongst varieties of wheat (*Triticum aestivum*). Theor Appl Genet 60: 229-236
Payne PI, Holt LM, Lawrence GJ (1983) Detection of a novel high molecular weight subunit of glutenin in some Japanese hexaploid wheats. J Cereal Sci 1: 3-8
Payne PI, Nightingale MA, Krattiger AF, Holt LM (1987a) The relationship between the HMW glutenin subunit composition and the bread-making quality of British-grown wheat varieties. J Sci Food Agric 40: 51-65
Payne PI, Holt LM, Hardinger K, McCartney DP, Lawrence GJ (1987b) The use of near-isogenic lines with different HMW glutenin subunits in studying bread- making quality and glutenin structure. In: Lasztity L, Bekes F (eds) Gluten proteins. Proceedings of the IIIrd Gluten Protein Workshop. Budapest, May 9/12
Schofield JD, Booth MR (1983) Wheat proteins and their technological significance. In: Hudson BJF (ed) Developments in food proteins, vol 2. Applied Science Publishers, London, pp 1-65
Stam P, Zeven AC (1981) The theoretical proportion of the donor genome in near-isogenic lines of self-fertilizer bred by backcrossing. Euphytica 30: 227-238
Thomas MS, Flavell RB (1990) Identification of an enhancer element for the endosperm-specific expression of high molecular weight glutenin. Plant Cell 2: 1171-1180
Thompson RD, Bartels D, Harberd NP, Flavell RB (1983) Characterization of the multigene family coding for HMW glutenin subunits in wheat using cDNA clones. Theor Appl Genet 67: 87-96
Waugh R, Brown JWG (1991) Plant gene structure and expression. In: Grierson D (ed)
Wieser H, Seilmeier W, Belitz H-D (1989) Reversed-phase high-performance liquid chromatography of ethanol-soluble and ethanol-insoluble reduced glutenin fractions. Cereal Chem 62: 38-41

CHAPTER 7

VARIATION IN TYPE AND RELATIVE AMOUNTS OF THE HIGH MOLECULAR WEIGHT GLUTENIN SUBUNITS IN DUTCH WHEAT VARIETIES

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Summary

The High Molecular Weight glutenin (HMWg) subunit genotypes and the amount of the individual HMWg subunits were determined in 38 Dutch wheat varieties. In the winter wheat varieties, HMWg subunit alleles that in general have been shown to be related to a poor bread-making quality predominated. In the spring-wheat varieties, 'high quality' alleles were most frequent. When examining the published HMWg subunit genotypes of British and German varieties we found a similar difference in HMWg subunit genotype between spring- and winter wheats. Because the bread-making quality of spring wheat varieties is in general superior to that of winter wheats, probably also because of the lower protein content of the latter, the two groups should be taken separately when studying the relation between the HMWg subunit genotype and the bread-making quality.

There was a considerable variation in the relative amounts of allelic HMWg subunits and of subunits encoded by different genes (at the same or homeologous loci). However, between varieties with the same HMWg subunit genotype, the relative amounts of identical subunits differed only slightly. Some of these small differences may reflect differences in gene-expression, but it is not known to what extent differences in the genetic background or differences in the gene promoter are involved.

There were differences in the relative amounts of the HMWg subunits encoded by the alleles at the *Glu-B1* locus. Here, an increase in the relative amounts coincided with an increase in the bread-making quality of an allele.

Introduction

The high molecular weight glutenin (HMWg) subunits, a group of storage proteins of wheat (*Triticum aestivum* L.), are encoded by genes of the complex *Glu-1* loci at the A, B and D genome, the *Glu-A1*, *Glu-B1* and *Glu-D1* locus, respectively. At each locus, single copies of two types of HMWg genes are present (Harberd et al. 1986), which may encode an x- and an y-type subunit, respectively. The y-type subunits have a lower molecular weight than the x-type subunits. In wheat varieties, an extensive allelic variation is present at each complex locus (Payne et al. 1981). Some of the alleles of the *Glu-B1* locus contain y-type genes which do not result in a subunit. None of the *Glu-A1* alleles encode an y-type subunit; one of the *Glu-A1* alleles does not result in an x-type subunit either. All *Glu-D1* alleles encode both types of subunits. Accordingly, wheat varieties contain from 3, 4 or 5 subunits.

Allelic variation at the *Glu-1* loci has been related to differences in the bread-making quality (see Payne et al. 1979, 1987; Moonen et al. 1982; Branlard and Dardevet 1985). In a collection of 226 breeding lines of winter wheat, derived from 47 varieties of different countries (only with four old Dutch varieties), we found a ranking of the alleles which was in general agreement with the ranking found in the other studies (Kolster et al. 1991c, Chapter 2 of this thesis). At the *Glu-D1* locus, the allele encoding the combination of HMWg subunits 5+10 was related to a higher bread-making quality than the allele encoding 2+12. The *Glu-A1* null-allele (an allele which does not encode a HMWg subunit) was inferior to its allelic variants 1 and 2*, respectively. The *Glu-B1* alleles encoding subunits 6+8 and 7, were inferior to the alleles encoding the subunit combinations 7+8 and 7+9. In our study, about 20% of the variation in bread-making quality was ascribed to the variation in the HMWg genotype of the lines. Especially allelic variation at the *Glu-D1* locus contributed to the variation in quality (Kolster et al. 1991c, Chapter 2 of this thesis).

Also differences in the total amount of the HMWg subunits have been related to differences in the bread-making quality (Seilmeier et al. 1991; MacRitchie et al. 1991; see further Kolster et al. 1991c, Chapter 2 of this thesis). The total amount of the subunits of a variety was shown to be strongly affected by differences in the growing conditions (Kolster et al. 1991a, Chapter 5). Seilmeier et al. (1991) showed a considerable *phenotypic* variation in the amounts of individual subunits in 24 varieties. However, there are no literature reports on the extent of the *genetic* variation in the amount of individual subunits between wheat genotypes.

The molecular basis of the differences between HMWg subunit alleles in their contribution to bread-making quality is not known. It has been suggested that conformational differences between the HMWg subunits are involved (see Flavell et al. 1989). Differences in the amount of the subunits produced may be another cause of the differences in 'quality' between alleles, but until now, no studies on this possibility have been published.

In this report, the type and the amount of individual HMWg subunits present in the kernels of 38 Dutch wheat varieties is studied. The type of the HMWg alleles present is discussed in relation to the generally poor bread-making quality of Dutch wheat varieties. The results of the quantification of the individual HMWg subunits are used to study the differences in expression of the HMWg subunit genes, and to relate the amounts of the subunits produced by an allele to the contribution to the bread-making quality as established in other studies.

Material and methods

Plant material

The HMWg subunits were studied of 38 wheat varieties which are, or have been registered in the 'Dutch descriptive list of varieties of field crops' (Anonymous, 1978-1990). In addition, two old Dutch varieties (Mado and Orca) and 5 foreign varieties (Capelle Desprez, Chinese Spring, Norda, Kormoran and Tenor) were studied. Seeds of most of the varieties were grown at trial fields of the former Foundation for Agricultural Plant Breeding (SVP), Wageningen. The other seeds were obtained from the Governmental Institute for Research on Varieties (RIVRO), Wageningen. Of each variety, one sample was taken for analysis. The samples were collected in different years.

Electrophoretic analysis and quantification of the HMWg subunits

The HMWg subunits were extracted from wholemeal flours as described previously (Kolster and Van Gelder 1991). The set of varieties was randomly divided into 4 subsets. The proteins extracted from the flours of these subsets were separated on four replicate SDS-PAGE gels; each gel contained a complete subset. The identification and the numbering of the HMWg subunits, and consequently of the alleles, was according to Payne and Lawrence (1983). The amount of Coomassie Brilliant Blue (CBB) adsorbed by the HMWg subunits, which is correlated with the

amount of protein, was determined by laser scanning densitometry. The method used, and the reliability of the approach, was as described previously (Kolster et al. 1992, Chapter 4). The results of the quantitative measurements were analyzed using Genstat (Genstat 5 committee 1987).

Results and discussion

Variation in the type of HMWg subunits

Table 7.1 shows the frequencies of the HMWg subunit alleles at the *Glu-1* loci in 27 Dutch winter and 11 Dutch spring wheat varieties. These frequencies are derived from Table 7.3, which shows the HMWg subunit genotype of the varieties, together with the results of the quantification of the individual subunits.

Table 7.1 Frequencies of the *Glu-A1*, *Glu-B1* and *Glu-D1* HMWg subunit alleles in 26 Dutch winter- and 11 Dutch spring- wheat varieties[1].

Locus	Allele[2]	Winter wheat %	Spring wheat %
Glu-A1	0 (a)	73	36
	1 (b)	19	55
	2* (c)	8	9
Glu-B1	6+8 (d)	54	9
	7 (a)	30	18
	7+8 (b)	8	0
	7+9 (c)	8	73
Glu-D1	2+12 (a)	92	36
	5+10 (b)	8	55
	3+12 (c)	0	9

[1] These varieties are, or have been registered in the 'Dutch descriptive list of varieties of fieldcrops' (Anonymous, 1978-1990). Their HMWg subunit genotypes are given in Table 7.3
[2] Between brackets, the allele designation according to Payne and Lawrence (1983)

HMWg subunit alleles that had been related to a poor bread-making quality, according to Chapter 2 and the literature (see Introduction), predominated at the *Glu-1* loci in the Dutch winter wheat varieties (Table 7.1); viz. the *Glu-A1* null allele, the *Glu-B1* alleles which encode the subunits 6+8 and 7, and the *Glu-D1* allele encoding 2+12. In Dutch spring wheat varieties, the most commonly occurring HMWg subunit alleles were those generally related to a high bread-

making quality; viz. the alleles encoding subunit 1 (*Glu-A1* locus), 7+9 (*Glu-B1* locus) and 5+10 (*Glu-D1* locus). The HMWg subunit genotype of the varieties used could not be related to their bread-making quality as data on the bread-making quality of these samples are not available. Moreover, as stated before (Kolster et al. 1991c, Chapter 2), these relations should not be studied with varieties but preferably with lines which have a wide genetic background and which have not been selected for bread-making quality.

In spring wheat varieties from other countries too (Table 7.2) the good alleles are far more frequent than the poor alleles, except at the *Glu-D1* locus in the Soviet Union. With the winter wheat varieties a striking contrast is found. Whilst in Great Britain and Germany, like in The Netherlands, poor quality alleles predominate, high quality alleles are highly frequent in Canada, Finland and the Soviet Union, at least at the *Glu-A1* and *Glu-B1* locus. At the *Glu-D1* locus the high quality alleles are in the latter three countries not predominant, low frequent and highly frequent, respectively. This shows that the winter wheat varieties constitute separate (contrasting) gene pools.

It is generally accepted that the average bread-making quality of spring wheat is higher than that of winter wheat. This may result from a higher frequency of 'high quality' HMWg subunit alleles in the spring wheat varieties, but also from the higher average protein content of spring wheat varieties (see Jenkins et al. 1987; Shellenberger 1978). The difference in protein content of spring and winter wheats is associated with the lower yield of spring wheat, which is negatively related to the protein content. Spring and winter wheat varieties sown in winter had a higher yield and a lower protein content than the same varieties sown in spring (Jenkins et al. 1987). As agronomic differences contribute to the differences in the bread-making quality of spring and winter wheats, spring- and winter-sown wheat genotypes should be treated as separate groups when relating the presence of HMWg alleles to the bread-making quality. The high amount of variation in bread-making quality which has been ascribed to variation in the HMWg subunit genotype of British (47-60%; Payne et al. 1987) and Canadian (59-69%; Lukow et al. 1989) varieties is probably overestimated because the authors pooled the two classes of wheat. In studies where only winter wheat (German, Rogers et al. 1989; Dutch, Kolster et al. 1991c, Chapter 2) or spring wheat (Norwegian, Uhlen 1990) varieties were used, the contribution to bread-making quality of the variation in HMWg subunit genotype was considerably lower, but still substantial (approximately 30%, 20% and 27%, respectively).

Table 7.2 Frequencies (%) of the *Glu-A1*, *Glu-B1* and *Glu-D1* HMWg subunit alleles in spring (S) and winter (W) wheat varieties of 5 countries.

	Great Britain[1]		Canada[2]		Germany[3]		Finland[4]		Soviet Union[5]	
Locus	W	S	W	S	W	S	W	S	W	S
Glu-A1										
0	83	33	5	4	79	11	0	25	8	6
1	17	33	75	46	20	75	33	31	58	12
2*	0	33	20	46	2	14	67	44	33	82
other	0	0	0	4	0	0	0	0	0	0
Glu-B1										
6+8	53	11	5	8	47	0	0	6	3	2
7	35	0	0	2	23	18	20	0	0	0
7+8	9	22	20	24	11	0	0	44	17	20
7+9	2	33	75	56	20	71	73	50	80	76
other	2	33	0	10	0	11	7	0	0	2
Glu-D1										
2+12	73	28	45	10	64	18	67	25	11	49
5+10	8	61	55	90	36	82	33	75	89	51
other	20	11	0	0	0	0	0	0	0	0
Number of varieties	66	18	20	48	66	28	15	16	36	49

[1] Payne et al. 1987
[2] Lukow et al. 1989
[3] Sontag et al. 1986
[4] Odenbach and Mahgoub 1987
[5] Morgunov et al. 1990

In varieties of bread wheat, chromosome 1B (or its short arm) can be replaced by chromosome 1R (or its short arm) of rye. The presence of this rye chromosome (-arm) is known for its deteriorating effect on the bread-making quality (Zeller et al. 1982; Dhaliwal et al. 1987). The presence of the 1RS chromosome arm was established in 4 of the Dutch varieties (Clement, Donjon, Granada and Nautica), using an the electrophoresis system essentially according to Payne et al. (1987).

In conclusion, the poor bread-making quality of Dutch winter wheat varieties must partly result from the poor quality of their HMWg alleles. Therefore, the introduction of 'high quality' HMWg subunit alleles in breeding programs, such as *Glu-D1* allele encoding 5+10, is an important tool to enhance the bread-making quality.

Variation in the amount of HMWg subunits

The absolute amount of the individual subunits differed considerably between varieties (results not shown). This variation may result from environmental as well as genetic variation (and interactions between genotype and environment). In the present report, genetic differences in the expression of the HMWg subunit genes are studied. The interpretation of the variation in absolute amount of the subunits was hampered by the fact that the expression of HMWg subunit genes depends on the growing conditions of a variety (agronomic as well as climatologic) (Kolster et al. 1991a, Chapter 5) and may also depend on the general genetic background of a HMWg subunit gene (Kolster et al. 1991a, Chapter 5; 1991b). These studies also showed that the amount of an individual subunit, relative to the total amount of HMWg subunits, is largely independent from differences in the environmental conditions and in the genetic background. Accordingly, in the present study the relative amount of the individual HMWg subunits is used as a measure of gene-expression (Table 7.3).

Three classes of subunit specific differences in gene expression were distinguished:

a) differences in the expression of HMWg subunit genes, which are considered identical because of the indistinguishable relative mobility during SDS-PAGE of the subunits encoded and which are moreover encoded by genes in identical alleles. Thus, 'expression alleles' encoding the same *type* of subunits. These are differences between varieties (Table 7.3) with the same HMWg subunit genotype. Because allelic subunits may differ in amount, comparing varieties for relative amount of a subunit is only valid for varieties with the same HMWg subunit genotype.
b) differences in the expression between the genes for the x- and the y-type subunits at a locus. These are the differences in subunit expression within a variety.
c) differences in the amount of allelic HMWg subunits. These are the between HMWg genotype differences in Table 7.3.

A prerequisite for studying subject c) is that the expression of a HMWg subunit is not affected by the HMWg subunit genotype of the varieties. This demand was met for the HMWg subunit alleles which occur in the set of varieties used in this study (Kolster et al. 1991b, Chapter 6).

ad a) Table 7.3 shows for some varieties with identical HMWg subunit genotypes significant ($P \leq 0.05$) differences in the relative amounts of the individual HMWg subunits. However these differences are relatively small. Galili and

Table 7.3 The qualitative and quantitative HMWg subunit allele make-up of Dutch and 5 foreign wheat varieties varieties, grouped for HMWg subunit genotype.

variety	type and relative amounts[1] of the individual HMWg subunits													wheat
	Glu-A1x			*Glu-B1x*			*Glu-B1y*		*Glu-D1x*			*Glu-D1y*		
	0	1	2*	6	7	20	8	9	2	3	5	12	10	type[2]
Anouska	-			0.18			0.19		0.24			0.40		w
Citadel*	-			0.20			0.21		0.30			0.30		w
Clement	-			0.18			0.25		0.24			0.33		w
Durin	-			0.16			0.21		0.32			0.32		w
Granta*	-			0.20			0.20		0.26			0.35		w
Kraka*	-			0.20			0.21		0.28			0.32		w
Marksman	-			0.17			0.21		0.26			0.37		w
Sarno	-			0.15			0.20		0.25			0.40		w
Stratos*	-			0.18			0.19		0.27			0.37		s
Swifta	-			0.16			0.16		0.34			0.35		w
Tundra	-			0.19			0.21		0.27			0.33		w
Arminda*	-				0.38				0.33			0.29		w
Cappelle Desprez	-				0.40				0.28			0.32		-
Caribo	-				0.41				0.27			0.33		w
Carimulti	-				0.40				0.28			0.32		w
Miller*	-				0.40				0.25			0.35		w
Okapi*	-				0.37				0.28			0.36		w
Orca	-				0.41				0.25			0.34		-
Taurus*	-				0.40				0.28			0.32		w
Tombola*	-				0.41				0.30			0.29		w
Chinese Spring	-				0.33		0.14		0.22			0.31		-
Kaspar*	-				0.33			0.16	0.23			0.27		s
Obelisk*[3]	-				0.33			0.14	0.23			0.29		w
Pagode	-				0.33			0.15	0.23			0.30		w
Saiga	-				0.34			0.15	0.24			0.27		w
Obelisk*[3]	-					0.41			0.28			0.31		w
Manella		0.16			0.31				0.28			0.26		w

Table 7.3 continued

variety	*Glu-A1x*			*Glu-B1x*			*Glu-B1y*		*Glu-D1x*			*Glu-D1y*		wheat
	0	1	2*	6	7	20	8	9	2	3	5	12	10	type[2]
Adamant		0.15		0.16			0.17		0.21			0.32		w
Lely		0.16		0.15			0.17		0.24			0.29		w
Nautica		0.16		0.17			0.18		0.25			0.25		w
Bastion		0.12			0.29			0.15	0.20			0.24		s
Mado		0.16				0.33			0.23			0.28		-
Norda			0.09		0.20		0.24		0.22			0.25		-
Donjon			0.08		0.29		0.13		0.20			0.30		w
Sunnan*			0.08		0.30			0.14	0.23			0.25		s
Melchior	-				0.34			0.16		0.23		0.27		s
Kormoran	-				0.36			0.12			0.24		0.28	-
Selpek	-				0.36			0.12			0.24		0.28	s
Arkas		0.14			0.31						0.26		0.30	s
Toro		0.18			0.32						0.24		0.27	s
Cyrano		0.09			0.28		0.12				0.21		0.29	w
Minaret*		0.09			0.33			0.12			0.22		0.24	s
Ralle*		0.09			0.32			0.14			0.21		0.25	s
Sicco		0.14			0.29			0.11			0.23		0.24	s
Tenor		0.14				0.38					0.21		0.28	-
Granada			0.16	0.17			0.15				0.23		0.30	w

[1] The ratio between the subunits 2 and 2* and between subunit 9 and 10 were determined using a 5% and 12.5% SDS-PAGE gel, respectively (Kolster and Van Gelder 1991). For the relative amount of a subunit present in varieties with the same HMWg genotype the average LSD ($P \leq 0.05$, 4 replicate gels) is 0.02

[2] Spring (s) or winter (w) wheat variety. Old Dutch and foreign varieties (see Methods) are indicated by an '-' and are not included in Table 7.1. Varieties still listed in 1990 in the 'Dutch descriptive list of varieties of fieldcrops' (Anonymous, 1978-1990) are marked with an asterisk*

[3] The variety Obelisk is a mixture of two *Glu-B1* genotypes, which have been isolated at the Foundation for Agricultural Plant Breeding. These genotypes have been studied separately. Obelisk is not included in Table 7.1

Feldman (1983) observed a larger difference in relative amount of the *Glu-B1* encoded subunit 7 in two genotypes with identical HMWg genotype (1, 7+8, 2+12). Unfortunately, this combination of HMWg alleles did not occur in our set of varieties. The range of the relative amount of a subunit within groups with the same HMWg genotype is still larger than the variation in relative amount within a variety due to variation in the environmental conditions (Kolster et al. 1991a, Chapter 5), but of the same magnitude as the differences due to differences in the genetic background (Kolster et al. 1991b, Chapter 6). There was no apparent relation between the year of growing (not presented) and the relative amounts of the subunits of the different varieties. The differences between varieties observed in this study probably had environmental as well as genetic causes. A genetic analysis is required to separate effects of the genetic background from differences due to variation in the regulation of the expression of the *Glu-1* genes. Because the differences in the relative amount of a subunit within genotypic groups are small in general, quantitative measurements can be used as an indication of the presence of comigrating subunits. For instance, when the *Glu-D1* allele encoding 5+10 is present, the *Glu-B1* alleles encoding 7+9 can not be distinguished from the allele encoding 7 because the subunits 9 and 10 comigrate on a 8.3% SDS-PAGE gel. However, they can be distinguished quantitatively because the relative amount of the composite band (9 and 10) is clearly higher than the relative amount of subunit 10 separately.

ad b) The relative amount of the x- and the y-type HMWg subunits encoded by genes of the same allele differed considerably (Table 7.3). The *Glu-B1x* subunits 7 of the alleles encoding 7+8 and 7+9 are in all but one case (Norda), more intensely stained than the *Glu-B1y* subunits 8 and 9, respectively; the y-type subunit 8 encoded by *Glu-B1* allele 6+8 tends to be more intensely stained than subunit 6. The *Glu-D1y* subunits 10 and 12 are in the majority of varieties more intensely stained than the *Glu-D1x* subunits 5 and 2, respectively. The relative amount of the *Glu-A1x* subunits 1 is comparable to that of the *Glu-B1y* subunits.

These results show that the three to five HMWg subunit genes which can be expressed in wheat varieties may differ considerably in the amount of protein produced. Because subunits can differ considerably in molecular weight, the amount of protein should preferably be expressed on a molar basis when discussing these results in terms of differences in gene-expression. This will

be discussed in Chapter 8.

ad c) The relative amount of allelic HMWg subunits at a given *Glu-1* locus was compared between groups of varieties which do not differ in their allele make-up at the other *Glu-1* loci. For the *Glu-A1* locus, this was only the case for two varieties (Sunnan and Bastion). Therefore, the relative amounts of the *Glu-A1* encoded subunits could not be compared accurately. In general, the relative amount of 2* appears to be lower than the relative amount of subunit 1. Only in Granada, the amount of 2* is comparable to the relative amount of subunit 1 found in other varieties. The superiority of the *Glu-A1* alleles encoding the subunits 1 and 2*, respectively, to the *Glu-A1* null-allele is probably also a result of the lower total amount of the HMWg subunits in combinations with the latter allele (see Chapter 6).
Comparison of the relative amounts of the *Glu-B1* and *Glu-D1* encoded subunits was hampered by the low number of varieties within some of the *Glu-A1* x *Glu-B1* x *Glu-D1* allele combinations. As a previous study (Kolster et al. 1991b, Chapter 6) showed that the presence or absence of a *Glu-A1* encoded subunit does not affect the expression of genes at the *Glu-B1* and *Glu-D1* loci, the number of varieties within the groups with identical HMWg genotypes was increased by calculating the relative amount of the subunits encoded by the alleles of the B and D genome excluding the amount of the *Glu-A1* encoded subunit (Table 7.4). In the varieties containing the *Glu-D1* subunits 2+12 (Table 7.4), the *Glu-B1* alleles are ranked according to the relative amount of the subunits encoded as follows: 6+8 = 7 < 7+8 = 7+9. In combination with *Glu-D1* subunits 5+10, the ranking of *Glu-B1* alleles is identical. The *Glu-B1* alleles which encode subunit 7 (alleles encoding 7, 7+8 and 7+9) differed only in total amount of subunit encoded because of the differences in amount of y-type subunits.

In this study, the amounts of the subunits encoded by the *Glu-D1* allele 2+12 were equal to the relative amounts of the subunits encoded by its allelic counterpart encoding 5+10. In a previous study, using only a limited number of genotypes, the relative amounts of subunits 5+10 were higher than the relative amounts of 2+12.

The ranking of the relative amounts of the subunits encoded by the *Glu-B1* alleles found in this study was in general agreement with the ranking for bread-making quality of these alleles found under Dutch agronomic conditions (see Chapter 1) and the ranking found in other studies. Only the relation to bread-

making quality of the *Glu-B1* alleles encoding 7+8 and 7+9 is not consistent. In British breeding lines, the allele encoding 7+8 was superior to 7+9 (Payne et al. 1987), whereas in German genotypes (Odenbach and Mahgoub 1988) the allele encoding 7+9 was superior also. In Dutch genotypes, 7+9 tends to be superior to 7+8, although the differences in bread-making quality were not significant (Kolster et al. 1991c, Chapter 2). The amount of 7+8 was higher than the amount of 7+9 in near-isogenic lines of Sicco in which different *Glu-1* alleles were introduced, and which were grown in Britain (Kolster et al. 1991b, Chapter 6). This is in contrast to the results of the present study. This suggest that for these two *Glu-B1* alleles different types occur, which differ in their level of gene-expression.

Table 7.4 The relative amounts of the individual HMWg subunits encoded by *Glu-B1* alleles in varieties which contained the *Glu-D1* allele encoding 2+12 or 5+10[1] (collected from Table 7.3)

Glu-B1 allele[2]	*Glu-B1x*	*Glu-B1y*	*total Glu-B1*	*Glu-D1x*	*Glu-D1y*	n[3]
				Glu-D1 2+12		
6+8 (d)	0.18	0.20	0.38	0.27	0.35	14
7 (a)	0.39		0.39	0.28	0.32	10
7+8 (b)	0.32	0.14	0.46	0.25	0.29	3
7+9 (c)	0.33	0.16	0.49	0.24	0.28	6
				Glu-D1 5+10		
6+8 (d)	0.20	0.18	0.38	0.27	0.36	1
7 (a)	0.37		0.37	0.30	0.34	2
7+8 (b)	0.31	0.13	0.44	0.23	0.32	1
7+9 (c)	0.36	0.13	0.49	0.24	0.28	5

[1] If present, the amount of the *Glu-A1* encoded subunits was corrected for
[2] LSD ($P \leq 0.05$) for the relative amount of the individual *Glu-B1* encoded subunits = 0.013
[3] n = number of varieties

This could also be the case for the alleles at the *Glu-A1* locus. The ranking for quality of the Glu-A1 alleles 1 and 2* is not unambiguous (Chapter 2) and so is the ranking for the relative amounts of these subunits in the present study. For the *Glu-D1* locus, the allele encoding the subunits 5+10 is clearly superior to the allele encoding subunits 2+12 with repect to quality. However, in the present study, the relative amounts of the subunits encoded by these alleles is identical. This would indicate that differences in the amounts of the subunits do not cause the differences between these *Glu-D1* alleles. In a previous study (Chapter 6 of this thesis) the relative amounts of 5+10 were higher than the relative amounts of 2+12.

Conclusions

Because of the high frequency of 'poor-quality' HMWg subunit alleles in Dutch (winter) wheat varieties, it is of importance to introduce 'high-quality' alleles in breeding programmes, especially the *Glu-D1* allele encoding the subunits 5+10. Also genetic variation in expression of the HMWg subunit genes would be of importance for breeding for improved bread-making quality. In this study, the expression of identical HMWg subunit genes belonging to the same allele differed only slightly between the Dutch varieties. Although the range of these differences indicated that genetic variation contributed to this variation, alleles with an strongly aberrant gene-expression were not detected. More important for breeding is therefore the genetic variation in the total amount of the HMWg subunits, relative to the total amount of the storage proteins (Kolster et al. 1991b, Chapter 6). Furthermore, this study suggests that differences in the contribution to the bread-making quality between *Glu-B1* alleles are caused by differences in the amount of the subunits encoded. This relationship may enable the prediction of the contribution to the bread-making quality of a new allele by determining the relative amount of the subunits encoded. The relation between the contribution to the bread-making quality of the *Glu-A1* and *Glu-D1* alleles and the amount of subunits produced is inconclusive.

References

Anonymous (1978-1990) Dutch Descriptive list of varieties of fieldcrops. Leiter-Nypels, Maastricht, vol 53 (1978) till vol 65 (1990), The Netherlands (in Dutch)

Branlard G, Dardevet M (1985) Diversity of grain protein and bread wheat quality. II. Correlation between high molecular weight subunits of glutenin and flour quality characteristics. J Cereal Sci 3: 345-354

Dhaliwal AS, Mares DJ, Marshall DR (1987) Effect of 1B/1R chromosome translocation on milling and quality characteristics of bread wheats. Cereal Chem 64: 72-76

Flavell RB, Goldsbrough AP, Robert LS, Schnick D, Thompson RD (1989) Genetic variation in wheat HMW glutenin subunits and the molecular basis of bread-making quality. Biotechnology 7: 1281-1285

Galili G, Feldman M (1983) Genetic control of endosperm proteins in wheat. 2. Variation in high molecular weight glutenin and gliadin subunits of *Triticum aestivum*. Theor Appl Genet 66: 77-86

Genstat 5 committee (1987) Genstat 5 reference manual. Clarendon Press, Oxford.

Harberd NP, Bartels D, Thompson RD (1986) DNA Restriction-fragment variation in the gene family encoding high molecular weight (HMW) glutenin subunits of wheat. Biochem Genet 24: 579-596

Jenkins G, Payne PI, Sharp P (1987) In: Borghi B (ed) Agriculture. Hard wheat: agronomic,

technological, biochemical and genetic aspects. Commission of the European Communities, Luxembourg, pp 135-143

Kolster P, Van Gelder WMJ (1991) A method for the quantification of individual high molecular weight glutenin subunits using SDS-PAGE and laser scanning densitometry. In: Bushuk W, Tkachuk R (eds) Gluten proteins 1990. American Association of Cereal Chemists, St. Paul, pp 349-361

Kolster P, Krechting CF, Van Gelder WMJ (1991a) Quantitative variation in individual high molecular weight glutenin subunits of wheat in relation to variation in environmental conditions. J Sci Food Agric 57: 405-415

Kolster P, Krechting CF, Van Gelder WMJ (1991b) The expression of individual high molecular weight glutenin genes of wheat in relation to differences in the number and type of homeologous subunits and differences in the genetic background

Kolster P, Van Eeuwijk FA, Van Gelder WMJ (1991c) Additive and epistatic effects of allelic variation at the high molecular weight glutenin subunit loci in determining the bread-making quality of breeding lines. Euphytica 55: 277-285

Kolster P, Krechting CF, Van Gelder WMJ (1992) Quantification of individual high molecular weight glutenin subunits of wheat using SDS-PAGE and scanning densitometry. J Cereal Sci 15: 49-61

Lukow OM, Payne PI, Tkachuk R (1989) The HMW glutenin subunit composition of Canadian wheat cultivars and their association with bread-making quality. J Sci Food Agric 46: 451-460

MacRitchie F, Kasarda DD, Kuzmicky DD (1991) Characterization of wheat protein differing in contributions to breadmaking quality. Cereal Chem 68: 122-130

Moonen JHE, Scheepstra A, Graveland A (1982) Use of the SDS-sedimentation test and SDS-polyacrylamidegel electrophoresis for screening breeder's samples of wheat for bread-making quality. Euphytica 31: 677-690

Morgunov AI, Rogers WJ, Sayers EJ, Metakovsky EV (1990) The high-molecular-weight glutenin subunit composition of Soviet wheat varieties. Euphytica 51: 41-52

Odenbach W, Mahgoub E (1987) Beziehungen zwischen der Backqualität und dem Vorkommen der Gene für die hochmolekularen Untereinheiten des Glutenins bei den deutchen Winter- und Sommerweizensorten. Getreide Mehl Brot 41: 195-198

Odenbach W, Mahgoub E (1988) Relationships between HMW glutenin subunit composition and the sedimentation value in reciprocal sets of inbred backcross lines derived from two winter wheat crosses. In: Miller TE, Koebner RMD (eds) Proceedings of the seventh international wheat genetics symposium, pp 987-991

Payne PI, Lawrence GJ (1983) Catalogue of alleles for the complex gene loci, Glu-A1, Glu-B1, and Glu-D1 which code for high-molecular-weight subunits of glutenin in hexaploid wheat. Cereal Res Commun 11: 29-35

Payne PI, Corfield KG, Blackman JA (1979) Identification of a high-molecular-weight subunit of glutenin whose presence correlates with bread-making quality in wheats of related pedigree. Theor Appl Genet 55: 153-159

Payne PI, Holt LM, Law CN (1981) Structural and genetical on the high-molecular-weight subunits of wheat glutenin. Part 1: Allelic variation in subunits amongst varieties of wheat (*Triticum aestivum*). Theor Appl Genet 60: 229-236

Payne PI, Nightingale MA, Krattiger AF, Holt LM (1987) The relationship between the HMW glutenin subunit composition and the bread-making quality of British-grown wheat varieties. J Sci Food Agric 40: 51-65

Rogers WJ, Payne PI, Harinder K (1989) The HMW glutenin subunit and gliadin compositions of German-grown wheat varieties and their relationship with bread-making quality. Plant Breeding 103: 89-100

Seilmeier W, Belitz H-D, Wieser H (1991) Separation and quantitative determination of high-molecular-weight subunits of glutenin from different wheat varieties and genetic variants of the

variety Sicco. Z Lebensm Unters Forsch 192: 124-129
Shellenberger JA (1978) Production and utilization of wheat. In: Pomeranz Y (ed) Wheat. Chemistry and technology. American Association of Cereal Chemists, St. Paul, pp 1-18
Sontag T, Salovaara H, Payne PI (1986) The high-molecular-weight glutenin subunit compositions of wheat varieties bred in Finland. J Agric Sci Fin 58: 151-156
Uhlen AK (1990) The composition of high molecular weight glutenin subunits in Norwegian wheats and their relation to bread-making quality. Norw J Agric Sci 4: 1-17
Zeller FJ, Gunzel G, Fischbeck G, Gerstenkron P, Weipert D (1982) Veranderungen der Backeigenschaften des Weizens durch die Weizen-Roggen-Chromosomentranslokation 1B/1R. Getreide Mehl Brot 36: 141-143

CHAPTER 8

CONVERSION OF THE CBB ABSORBANCE VALUES OF HIGH MOLECULAR WEIGHT GLUTENIN SUBUNITS TO THE ABSOLUTE AND MOLAR AMOUNTS

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Summary

To enable the interpretation of the absorbance values of individual HMWg subunits in terms of amount of protein, the specific Coomassie Brilliant Blue (CBB) adsorbance of HMWg subunits (absorbance units/µg protein) has been determined. The specific adsorbance of the subunits 8 and 9 was lower than that of the subunits 1, 2, 2*, 5, 6, 7, 10 and 12. Differences in adsorbance of the subunits of *Glu-B1* alleles, shown in chapter 7, are not caused by differences in the CBB binding of these proteins. In flour of 5 varieties, the total amount of the HMWg subunits, relative to the total amount of flour proteins, ranged from 3 to 8%. This is lower than the generally assumed proportion of 10%.

To compare the levels of expression of individual genes, the amount of the various subunits were calculated on a molar basis. It was shown that the absorbance values can be misleading when comparing the level of expression of different HMWg subunit genes.

The *Glu-D1y* genes of the alleles encoding 2+12 and 5+10 have a considerably higher level of expression than the x-type genes. In contrast, the x-type genes of the *Glu-B1* alleles 7+9 have a higher expression level than the y-type genes. There are indications that the levels of expression of the x- and y-type genes of the *Glu-B1* allele 6+8 are similar. These differences between *Glu-B1* alleles could well be caused by differences in the promoter region of the gene encoding subunit 7.

Introduction

In the preceding chapters, the amounts of individual HMWg subunits were indirectly determined by measuring the amount of Coomassie Brilliant Blue (CBB) adsorbed by these proteins after their electrophoretic separation. The absorbance values of the stained protein bands enabled comparative studies of the amount of a subunit in relation to environmental- or genetic variation (see Chapter 5, 6 and 7 of this thesis). However, there are some restrictions to the use of the CBB adsorbance. The *specific* CBB adsorbance (absorbance units *per* unit of weight) differs between proteins (Van Kley and Hale 1977; Fullington et al. 1980), but it is not known whether HMW subunits differ in this respect. Differences in specific CBB adsorbance between subunits would hamper the comparison of the amount of *different* subunits on this basis. Furthermore, the *absolute* amount of the subunits on a weight basis is unknown. It is generally assumed that the HMWg subunits account for approximately 10% of the total amount of the flour proteins (Anderson et al. 1989; Payne et al. 1984; Pogna et al. 1989). However, firm experimental data to support this assumption are lacking. The absolute amount of a subunit on a weight basis cannot be used directly to compare the level of gene-expression when the subunits differ strongly in their molecular weight (e.g. x-type subunits versus y-type subunits). For this purpose, the *molar* amounts are required.

The aim of this investigation is to determine the *specific* CBB adsorbance of the HMWg subunits which are common in wheat in The Netherlands. For the purification of the subunits, traditional biochemical separation techniques (e.g. gel filtration, ion-exchange chromatography) can be used (see Shewry et al. 1984). However, the use of these techniques is laborious and complex due to the poor solubility of these proteins. Electroendosmotic preparative electrophoresis (EPE) enables a relatively simple and one-step purification of the subunits (Curioni et al. 1989). In this report, the CBB adsorption of the purified subunits is determined, using the separation and staining conditions described previously (Kolster et al. 1992, Chapter 4 of this thesis; Kolster and Van Gelder 1991). Amino acid analysis is used to determine the amount of the subunits.

Material and methods

Flour material

For the purification of the subunits, flours of varieties grown in The Netherlands were used. Flour of the following varieties (between brackets the protein contents on a 100% dry matter basis) were used in this Chapter to demonstrate the variation in the amount of the individual HMWg subunits: Katepwa (14.2%), Obelisk (11.1%), Okapi (10.1%), Toro (14.2%) and Urban (12.6%). The same samples of Okapi and Toro were used as a reference samples in the experiments in which the CBB adsorbance of the purified subunits was determined.

Purification of HMWg subunits

Proteins were extracted from wheat flour (0.3 g) for 30 min with sample buffer (2 ml) used in sodium dodecyl sulphate-polyacrylamide gelelectrophoresis (SDS-PAGE) (Kolster et al. 1988, Chapter 3 of this thesis). After heating the extract in a boiling water bath for 2 min, 1.5 ml of this extract was applied to the rod-gel (Ø 2 cm) for electroendosmotic preparative electrophoresis (EPE), which was performed essentially according to Curioni et al. (1989), with some minor modifications. Fractions (0.7 ml) of the electroendosmotic flow were monitored for protein composition with the Pharmacia phast-system (7.5% homogenous SDS-PAGE gels). Fractions containing the same single HMWg subunits were pooled and concentrated with Centriprep 30™ concentrators (MW cutoff of 30,000 D) to a final volume of approximately 1 ml. After filtration using Millipore filters (pore size 0.22 µm), an aliquot (0.3 ml) of each sample was taken for determining the CBB adsorption of the subunit after electrophoretic separation, the rest (approximately 0.7 ml) was used for amino acid analysis.

Amino acid analysis

The concentrated and filtered samples were dialyzed in vials closed with dialysis membrane for two days against water to remove the bulk of the glycine, which originated from the buffer compartment of the EPE apparatus. The water was refreshed three times. After freeze-drying of the remainder in the vials, the subunits were hydrolyzed in 6 N HCl (0.1-0.2 ml) for 24 h at 110°C. To minimize losses during the manipulation of the sample, for each sample the same vial was used for dialyses, freeze-drying and hydrolysis. Phenylthiocarbamyl (PTC) derivatives of the amino acids were analyzed with a Waters pico-tag column.

It is not necessary to use the amounts of all amino acids to determine the amount of subunit present in the concentrated and filtered samples. The amount of protein can be calculated from the amount of the amino acids that were accurately quantified, using the ratio between the molecular weight (M_r) and the number of these amino acid residues *per* molecule, the conversion factor. The following amino acids were selected for this purpose: glutamate/glutamine, proline, alanine, valine, leucine, histidine, lysine and arginine (marked with an asterisk in Table 8.1). Some other amino acids (i.e. tryptophan, threonine, serine, tyrosine, methionine and cysteine) may suffer from hydrolytic losses (Hare 1975). Because glycine was present in the buffer in which the subunits eluted from the EPE gel, and may not be completely removed from the subunits by the dialysis, this amino acid was also not selected. The amino acids which were present in the subunits in low amounts (aspartate and asparagine, isoleucine and phenylalanine) were not selected because the amounts of these amino acids appeared to be overestimated.

Instead of using replicate amino acid analyses of only one sample, for most subunits isolates from different varieties were used (see Table 8.3).

SDS-PAGE analysis

The buffer composition of the aliquots of the concentrated and filtered samples of the isolated subunits was made comparable to that of SDS-PAGE sample buffer by the addition of concentrated buffer. After heating the samples for 2 min in a boiling water bath, aliquots of 5, 10, 15, 25 and 30 µl of each sample were applied to an 8.3% SDS-PAGE gel. Because the volume applied to a slot affects the band width, which in turn influences the CBB adsorbance of a protein (Neuhoff et al. 1990), the total volume in a slot was adjusted to 30µl by adding sample buffer. The electrophoresis, staining and scanning conditions were identical to those described previously (Kolster et al. 1992, Chapter 4 of this thesis).

A linear relationship was found between the amount of subunit applied and the absorbance values. The absorbance value per µl sample was calculated as the slope of the regression line of the absorbance value on the amount of sample (Genstat 5 committee 1987). The intercept of the regression line of each of the samples did not differ significantly from 0. Several gels were used to analyze the CBB adsorbance of the subunits. To facilitate comparison between gels, extracts of two varieties (Okapi and Toro) were always applied as standards to each gel.

Results and discussion

Table 8.1 shows the results of the amino acid analysis of two aliquots of a sample of subunit 10. Equal volumes of this sample were pipetted in 2 vials, which were dialyzed, freeze dried and hydrolyzed separately. Generally, the mol% of the amino acids is in good agreement with that of identical subunits isolated from other varieties (Galili and Feldman 1985; Shewry et al. 1984; Wieser et al. 1990), and with the amino acid composition deduced from the coding sequence of a gene encoding this subunit (see Table 8.1).

Table 8.1 Amino acid composition of two independently processed subsamples of HMWg subunit 10, isolated from the same flour sample of the variety Toro, and the amino acid composition deduced from the nucleotide sequence of a gene of Cheyenne[1].

amino acid	sample A		sample B		gene sequence
	pmol/µl	mol%	pmol/µl	mol%	mol%
asx[2]	7	1.1	7	1.1	0.6
thr	26	3.8	25	4.2	3.8
ser	42	6.3	38	6.4	6.7
glx[3]*	226	33.7	208	34.0	35.6
pro*	91	13.6	81	13.1	11.0
gly	139	20.8	126	21.2	18.0
ala*	25	3.8	23	3.9	3.7
cys	1	0.3	2	0.6	1.1
val*	17	2.6	16	2.7	2.6
met	2	0.4	2	0.3	0.5
ile	10	1.4	8	1.3	0.6
leu*	29	4.3	26	4.4	3.8
tyr	13	2.0	10	1.6	5.4
phe	4	0.7	6	0.9	0.3
his*	12	1.8	12	2.1	2.1
lys*	10	1.5	9	1.5	1.1
arg*	14	2.1	13	2.2	2.1

[1] amino acid composition of mature subunit 10 (i.e. without signal peptide) derived from the coding sequence of subunit 10 of Cheyenne (Anderson and Greene 1989)
[2] aspartate and asparagine
[3] glutamate and glutamine
* amino acids used as a measure for the absolute amount of the subunits

The molecular weight (M_r) of the subunits is required to calculate the amount of the subunits from the amounts of the selected amino acids (see Material and methods). The M_r (and also the amino acid composition) was derived from the putative nucleotide sequence of the genes because the M_r cannot be accurately

determined by SDS-PAGE analysis of the HMWg subunits (see Bunce et al. 1985). As shown in Table 8.2 (compare column 4 and 5), there are considerable differences between the apparent molecular weight as determined by SDS-PAGE and the M_r derived from the nucleotide sequence of the gene. Because the M_r determined by viscometric analysis of a subunit agreed well with the deduced M_r (Field et al. 1987), it is assumed that extensive post-translational modifications do not occur. This assumption is further supported by the amino acid composition data of isolated subunits (Galili and Feldman 1985; Shewry et al. 1984; Wieser et al. 1990; this study), which differs only slightly from the composition derived from the putative amino acid sequences. Large deletions would alter the amino acid composition because the amino acid residues are not randomly distributed within the polypeptides. Unfortunately, the complete coding sequence is known only for a limited number of subunits.

Table 8.2 Characteristics of individual HMWg subunits derived from the putative amino acid sequence of the genes

subunit[1]	variety	size[2]	mol weight[3] (kD)	apparent mol weight (kD)[4]	conversion factor[5]
2* (*Glu-A1x*)	Cheyenne	794	86.3	116.5	0.175
7 (*Glu-B1x*)	Cheyenne	770	82.8	104.6	0.184
9 (*Glu-B1y*)	Cheyenne	684	73.5	93.9	0.176
2 (*Glu-D1x*)	Yamhill	817	87.0	116.5	0.171
5 (*Glu-D1x*)	Cheyenne	827	88.1	114.9	0.170
10 (*Glu-D1y*)	Cheyenne	627	67.5	92.5	0.174
12 (*Glu-D1y*)	Chin. Spr.	639	68.7	91.8	0.175

[1] Between brackets, the gene designation is given
[2] Size (number of amino acids) (Anderson et al. 1988)
[3] Molecular weight of the mature protein (i.e. without signal peptide) (Anderson et al. 1988)
[4] Estimated by 12% SDS-PAGE (Greene et al. 1988)
[5] The amount of subunit (µg) = amount of the stable amino acids (pmol) * conversion factor. This factor is based on the number of the selected amino acids per molecule (see Table 8.1) and the M_r of the subunits. These data were derived from the coding sequences of the genes, obtained from Genbank or directly from the publication (Halford et al. 1987; subunit 9)

The conversion factor of subunits differs only slightly (Table 8.2). The average value is 0.175. Because the amino acid composition of the subunits for which the amino acid sequence is not yet clarified, is found to be only slightly different from the compositions of subunits with a known sequence (Shewry et al. 1984), we assume that this conversion factor is also valid for other subunits.

Table 8.3 shows the specific CBB adsorbances of each of the purified HMWg

subunits, which are calculated from the absorbance value/µl of sample and the amount of the selected amino acids/µl found per sample after hydrolysis. Correction of the absorbance values of the purified subunits based on the absorbance values of the subunits in the reference varieties at the different gels does not change the conclusions (results not shown). The total experimental error in determining the specific adsorbance is estimated to be about 20%. This error is composed of (a) inaccuracy in determining the absorbance of CBB-stained subunits, which is estimated to be about 10%, and (b) experimental error in determining the total amount of the selected amino acids. This includes all steps from the dialysis of the samples to the final amino acid analysis. Based on the differences in the amount of the selected amino acids in Table 8.1 (414 and 381 pmol/µl for sample A and

Table 8.3 Specific Coomassie Brilliant Blue adsorbance of the HMWg subunits.

subunit	allele[1]	variety	absorbance units/ µg subunit [2]
Glu-A1 locus			
1	a	Sicco	0.23
2*	b	Granada	0.25
Glu-B1 locus			
6	d	Granada	0.23
7	a	Toro	0.23
7	c	Sicco	0.26
8	d	Citadel	0.18
8	b	Chinese Spring	0.19
8	b	Chinese Spring	0.18
8	d	Kraka	0.18
9	c	Sicco	0.17
Glu-D1 locus			
10	d	Tenor	0.25
10	d	Granada	0.25
10[3]	d	Toro	0.22
12	a	Chinese Spring	0.22
12	a	Kraka	0.24
2	a	Citadel	0.22
2	a	Chinese Spring	0.27
5	d	Sicco	0.26
5	d	Granada	0.25

[1] The HMWg subunit allele designations according to Payne and Lawrence (1983)
[2] The average conversion factor from Table 8.2 is used to calculate (see text) the amount of subunit from the amount of the selected amino acids
[3] Sample of subunit 10 used in Table 8.1

sample B, respectively), this error is estimated to be about 10%. Differences in the

specific adsorbance of identical subunits isolated from different varieties are not larger than the experimental error except for somewhat larger differences in the specific adsorbance of the subunits 2 which was probably due to the very low amount of subunit 2, isolated from flour of Citadel. The *Glu-B1y* subunits 8 and 9 have a lower specific adsorbance than the other subunits. The reason for these differences is unknown. There are no large differences in the specific adsorbance between the other subunits. Therefore, in this study a specific adsorbance of 0.18 absorbance units/μg protein is used for the subunits 8 and 9, and 0.24 absorbance units/μg for the other subunits.

In previous chapters, it is shown that allelic HMWg subunits stained using CBB may differ in their absorbance values. These differences can be interpreted as differences in the amount of protein because this chapter does not give any indications of differences in the *specific* CBB binding of these subunits.

The specific adsorbance of HMWg subunits has been used to calculate from the adsorbance values of the individual subunits the absolute amounts of these subunits in flours of five wheat varieties. The samples of Okapi and Toro were the same as those used as reference samples in determining the CBB-adsorbance of the isolated subunits (see Experimental). Correction of the adsorbance values of the subunits in this experiment, based on the adsorbance values of the subunits in Okapi and Toro, was found to have only a minor effect on the results and was therefore omitted. Table 8.4 shows that there are considerable differences in absolute amount of the individual subunits and in their proportion relative to the total amount of kernel proteins. As shown previously, these differences could be caused by:

a) variation in the type of HMWg subunits which is genetically determined (Chapter 6).
b) environmental variation (see Kolster et al. 1991, Chapter 5).
c) variation in genetic background of HMWg subunit genes (Kolster et al. 1991, Chapter 5 and Chapter 6).

Moreover, the total amount of the subunits present in a flour is affected by the number of subunits (see Chapter 6).

In this study, the total amount of HMWg subunits, relative to the total amount of protein, ranged from 2.8% to 8.3%. This is clearly below the estimate of 10% which has been commonly used until now, but which was not supported by experimental evidence. The differences in the new proportions presented here agree with the results of Chapter 5 which showed that the proportion of HMWg subunits is not constant but highly variable between flours.

Table 8.4 Amounts of the individual HMWg subunits of 5 varieties.

Variety	HMWg subunit	absorbance (AU.mm)[1]	amount (μg)[2]	molar amount (nmol)[2]	proportion of subunits to total protein (%)[3]
Katepwa	2* (Glu-A1x)	0.66	83	0.96	1.2
	7 (Glu-B1x)	1.36	170	2.05	2.4
	9 (Glu-B1y)	0.50	84	1.14	1.2
	5 (Glu-D1x)	0.77	97	1.10	1.4
	10 (Glu-D1y)	1.19	148	2.20	2.1
Obelisk	7 (Glu-B1x)	0.52	65	0.79	1.1
	20 (Glu-B1x)	0.54	68	-	1.1
	9 (Glu-B1y)	0.20	34	0.46	0.6
	2 (Glu-D1x)	0.52	65	0.74	1.1
	12 (Glu-D1y)	0.64	80	1.16	1.3
Okapi	7 (Glu-B1x)	0.43	54	0.65	1.1
	2 (Glu-D1x)	0.33	41	0.47	0.8
	12 (Glu-D1y)	0.37	46	0.67	0.9
Toro	1 (Glu-A1x)	0.33	41	-	0.6
	7 (Glu-B1x)	0.70	90	1.09	1.3
	5 (Glu-D1x)	0.43	54	0.61	0.8
	10 (Glu-B1y)	0.55	69	1.02	1.0
Urban	1 (Glu-A1x)	0.19	24	-	0.4
	7 (Glu-B1x)	0.80	100	1.21	1.7
	9 (Glu-B1y)	0.27	45	0.61	0.8
	5 (Glu-D1x)	0.54	68	0.77	1.2
	10 (Glu-D1y)	0.63	79	1.17	1.3

[1] The CBB adsorbance of the protein band in the gel. Extracts of the flours were applied to 2 SDS-PAGE gels; each extract was applied to two lanes of each gel. Average coefficient of variation of the absorbance of the subunits (four replicates) = 11%

[2] The amount (μg or nmol) of subunit present in 0.05g of flour. To each lane, 1/30 th of the total amount of protein extracted from these samples were applied. The specific adsorbance used was 0.18 AU/μg for the subunits 8 and 9 and 0.24 AU/μg for the other subunits (see Table 8.3)

[3] The proportion of the HMWg subunits relative to the total amount of flour proteins.

- The M_r of these subunits is not known

The molar amounts of the various subunits were then calculated to compare the levels of gene-expression. It should be noted that Obelisk is a mixture of two HMWg subunit genotypes (see Chapter 7), which differ in their *Glu-B1* allele (subunits 7+9 or subunit 20). The exact frequencies of these genotypes are unknown. Consequently, the levels of gene-expression of the *Glu-B1* genes of Obelisk cannot be compared with that of the other varieties. The ratio between the absorbance values of the subunits of the varieties used in this chapter is

representative for the ratios found in other varieties (see Chapter 7).
The level of expression of genes encoding the *Glu-D1y* subunits 10 and 12 is somewhat higher than expression of the genes encoding the *Glu-D1x* subunits 5 and 2, respectively. These differences in expression of these x- and y-type subunit genes are more pronounced than could be expected from the absorbance values. In contrast, the *Glu-B1x* genes encoding subunit 7 have a clearly higher level of expression than the *Glu-B1y* genes encoding subunit 9. Other *Glu-B1* alleles did not occur in this set of varieties. Based on the absorbance values reported in Chapter 7, it seems reasonable to assume that the same holds for the x- and y-type genes of the allele encoding 7+8. However, the x-type gene of the *Glu-B1* allele encoding 6+8 has probably a level of gene expression comparable to that of the y-type gene. Therefore, there are differences between alleles of the same locus in the type of gene (x- or y-type) which shows the highest level of expression.

A sequence involved in the regulation of the expression of the HMWg subunit genes, the -300 sequence element, occurs in two copies in the gene encoding this subunit from variety Cheyenne (Anderson and Greene 1989). In that study, subunit 7 appears to be the most intensely stained subunit band in Cheyenne. It is suggested that this is due to a high level of expression of the gene encoding subunit 7. Also in the homogenous varieties used in the present study, and in most of the varieties used in Chapter 7, the subunits 7 are the most intensely stained ones. However, the *Glu-D1y* genes and the genes encoding subunit 7 have a comparable level of expression (thus, *molar* amounts, not absolute amounts; see Table 8.4). The high staining intensity of subunits 7 compared to the *Glu-D1y* subunits is therefore due to the higher M_r of this subunit. The fact that the expression of the genes encoding subunits 7 is about twice as high as that of the *Glu-B1y* genes could however well be caused by the higher copy number of the -300 sequence element.

At this point, it should be remembered that the HMWg subunits are identified by their mobility during SDS-PAGE. Allelic variants which differ only slightly in the type of subunit may not always be recognized by this technique (see Chapter 3). The same holds for variants which differ only in their level of gene expression. It is for example not known whether the -300 element occurs always in two copies in the genes encoding subunit 7.

When comparing the level of expression of genes at homeologous loci, differences between allelic genes in their level of expression should also be considered. In the previous chapters, the number of alleles was limited however. Further studies, using the absorbance values of the subunits of a larger number of

alleles and more genotypes, are therefore required.

References

Anderson OD, Greene FC (1989) The characterization and comparative analysis of high-molecular-weight glutenin genes from genomes A and B of a hexaploid bread wheat. Theor Appl Genet 77: 689-700

Anderson OD, Halford NG, Forde J, Yip RE, Shewry PR, Greene FC (1988) Structure and analysis of the high molecular weight glutenin genes from *Triticum aestivum* L. cv Cheyenne. In: !!!(eds) Proceedings of the seventh International Wheat Genetics Symposium volume 1: 699-704

Anderson OD, Greene FC, Yip RE, Halford NG, Shewry PR, Malpica-Romero J-M (1989) Nucleotide sequences of the two high-molecular-weight glutenin genes from the D-genome of a hexaploid wheat, *Triticum aestivum* L. cv Cheyenne. Nucleic Acid Res 17: 461-462

Bunce NAC, White RP, Shewry PR (1985) Variation in estimates of molecular weights of cereal prolamins by SDS-PAGE. J Cereal Sci 3: 131-142

Curioni A, Dal Belin Peruffo A, Pogna NE (1989) Electroendosmotic preparative electrophoresis as a one-step method for purification of high molecular weight subunits of wheat glutenin. Cereal Chem 66: 133-135

Field JM, Tatham AS, Shewry PR (1987) The structure of a high-M_r subunit of durum-wheat (*Triticum durum*) gluten. Biochem J 247: 215-221

Fullington JG, Cole EW, Kasarda DD (1980) Quantitative SDS-PAGE of total protein from different wheat varieties. J Sci Food Agric 31: 43-53

Galili G, Feldman M (1985) Structural homology of endosperm high molecular weight glutenin subunits of common wheat (*Triticum aestivum* L.). Theor Appl Genet 70: 634-642

Genstat 5 committee (1987) Genstat 5 reference manual. Clarendon Press, Oxford

Greene FC, Anderson OD, Yip RE, Halford NG, Malpica Romero J-M, Shewry PR (1988) Analysis of possible quality-related sequence variations in the 1D glutenin high molecular weight subunit genes of wheat. In: Proceedings of the seventh International Wheat Genetics Symposium, volume 1: 735-740

Halford NG, Forde J, Anderson OD, Greene FC, Shewry PR (1987) The nucleotide and deduced amino acid sequences of an HMW glutenin subunit gene from chromosome 1B of bread wheat (*Triticum aestivum* L.) and comparison with those of genes from chromosomes 1A and 1D. Theor Appl Genet 75: 117-126

Hare PE (1975) Amino acid composition by column chromatography. In: Needleman SB (ed) Protein sequence determination. Springer-Verlag Berlin-Heidelberg-New York, pp 204-231

Kolster P, Van Gelder WMJ (1991) A method for the quantification of individual high molecular weight glutenin subunits using SDS-PAGE and laser scanning densitometry. In : Bushuk W, Tkachuk R (eds) Gluten proteins 1990. American Association of Cereal Chemists, St. Paul, pp 349-361

Kolster P, Krechting CF, Van Gelder WMJ (1988) Variation in high molecular weight glutenin subunits of *Triticum aestivum* and *T. turgidum* ssp. *dicoccoides*. Euphytica S: 141-145

Kolster P, Krechting CF, Van Gelder WMJ (1991) Quantitative variation in individual high molecular weight glutenin subunits of wheat in relation to variation in environmental conditions. J Sci Food Agric 57: 405-415

Kolster P, Krechting CF, Van Gelder WMJ (1992) Quantification of individual high molecular weight glutenin subunits of wheat using SDS-PAGE and scanning densitometry. J Cereal Sci 15: 49-61

Neuhoff V, Stamm R, Pardowitz I, Arold N, Ehrhardt W, Taube D (1990) Essential problems in

quantification of proteins following colloidal staining with Coomassie Brilliant Blue dyes in polyacrylamide gels, and their solution. Electrophoresis 11: 101-117
Payne PI, Lawrence GJ (1983) Catalogue of alleles for the complex gene loci, Glu-A1, Glu-B1, and Glu-D1 which code for high-molecular-weight subunits of glutenin in hexaploid wheat. Cereal Res Comm 11: 29-35
Payne PI, Holt LM, Jackson EA, Law CN (1984) Wheat storage proteins: their genetics and their potential for manipulation by plant breeding. Phil Trans R Soc London Ser B 304: 359-371
Pogna NE, Mellini F, Beretta A, Dal Belin Peruffo A (1989) The high-molecular-weight glutenin subunits of common wheat cultivars grown in Italy. J Genet & Breed 43: 17-24
Shewry PR, Field JM, Faulks AJ, Parmar S, Miflin BJ, Dietler MD, Lew EJ-L Kasarda DD (1984) The purification and N-terminal amino acid sequence analysis of the high molecular weight gluten polypeptides of wheat. Biochem Biophys Acta 788: 23-34
Van Kley H, Hale SM (1977) Assay for protein by dye binding. Anal Biochem 81: 485-487
Wieser H, Seilmeier W, Belitz H-D (1990) Characterization of high molecular weight subunits of glutenin separated by reversed-phase high-performance liquid chromatography J Cereal Sci 12: 223-227

CHAPTER 9

SUMMARY AND GENERAL DISCUSSION

In view of the poor bread-making quality of the wheat grown in The Netherlands, only a small part of production is used for baking of bread. Therefore quality improvement is a major aim of plant breeding. Unfortunately, breeding for bread-making quality is hampered by its complexity. The suitability of wheat flour for the manufacture of bread depends on the composition of a large number of kernel components, such as lipids, starch and proteins, and this kernel composition in turn depends on agronomic measures, climate and on the genetic make-up of the varieties. Moreover the suitability of wheat flour for the baking of bread depends on technological conditions, such as those in milling and in the baking process. Another drawback in breeding for improved bread-making quality is the lack of simple and fast techniques to assess bread-making quality. Dough and baking-tests, which take much labour and time are required to determine such characteristics as dough strength and elasticity, loaf volume and crumb structure.

Plant breeders in various countries have been aware that the kernel proteins play a major role in determining the bread-making quality. The total *amount* of kernel proteins, which is largely determined by environment and as such difficult to change by breeding, is positively correlated with the bread-making quality. More useful characteristics for breeding are the genetically determined differences in protein *quality*, which result in differences in bread-making quality at a given amount of protein, i.e. in differences in the ratio between loaf volume and protein content. Small-scale tests to estimate protein quality (e.g. the SDS sedimentation, the Pelshenke dough-ball or the residue-protein tests) have been used in breeding programmes to predict bread-making quality. However the results of these tests not only depend on the genotype but also reflect environmental conditions. After the development of biochemical techniques for separation of proteins, bread-making quality has been related to the composition of kernel proteins. Because protein composition is genetically determined, it has been proposed as a tool in breeding for bread-making quality.

In this thesis the bread-making quality of wheat was studied in relation to differences in type and amount of the various high molecular weight glutenin

(HMWg) subunits. These storage proteins are encoded by genes at three homoeologous loci (*Glu-1* loci: *Glu-A1*, *Glu-B1* and *Glu-D1*); at each locus several alleles occur that differ in type or number (0-2) of subunits encoded. At the start of this study, the relation between variation in the HMWg subunit genotype and bread-making quality has been studied extensively by various research groups. These studies revealed the occurrence of alleles that differ in their contribution to bread-making quality. In fact, a large part of the variation in bread-making quality was ascribed to variation in the HMWg genotype. Though it was known that variation in type of the HMWg subunits also affects the quality of wheat grown in The Netherlands, it was not known to what extent differences in bread-making quality can be ascribed to differences in the HMWg subunit genotype.

Two general aspects for breeding for bread-making quality required further research:

1) the difficulties which may occur in the identification of the HMWg subunit alleles
2) the variation in amount of the HMWg subunits on which aspect very little information was available.

Variation in the amounts of groups of kernel proteins, such as the gliadins and the glutenins, is of prime importance for bread-making quality (**Chapter 1**). Therefore, it was worthwhile studying whether genetic variation in the *amounts* of the HMWg subunits could also be used in breeding for bread-making quality. Variation in the amounts of the subunits is probably also important for other applications of wheat in which gluten characteristics are important, such as the manufacture of pastas and pastry. Another reason for studying the HMWg subunits quantitatively is that the cause of the 'differences' in quality of HMWg alleles is not known. Differences in the structure of the subunits (the intrinsic *quality* of the subunits) have been suggested, but also differences in the level of gene expression (the *quantity* of the subunits) could be responsible for the differences in quality between the alleles. Therefore a major aim was to study the variation in amount of individual HMWg subunits and to elucidate the mechanisms underlying this variation.

Chapter 2 shows that variation in the HMWg genotype does have a large effect on bread-making quality of wheat lines grown in The Netherlands. These lines represent a wide genetic background and can be considered to be random lines with respect to bread-making quality. A baking test developed in The Netherlands was used to determine quality. In general, the ranking of the alleles for quality was in agreement with the ranking found in studies in other countries. Because different

baking tests and different criteria for quality were used in the various studies, the relationship found can be considered as generally valid. Most important is the variation at the *Glu-D1* locus. The allele encoding the subunits 5+10 is related to a higher bread-making quality than its allelic counterpart encoding 2+12. In the literature, it is generally assumed that the effect of allelic variation at the 3 homoeologous *Glu-1* loci on quality is additive. The so-called scoring systems to screen lines for bread-making quality are based on this assumption. However this study shows that interactions between homoeologous loci do occur: an effect of allelic variation at the *Glu-A1* and *Glu-B1* loci was present only in combination with the 'high-quality' *Glu-D1* allele encoding the subunits 5+10. The mechanisms underlying these interactions are not known. At this point, it should be noted that in Dutch wheat varieties, 'poor quality' alleles predominate at each of the three *Glu-1* loci (Chapter 7). Therefore it is important to introduce 'high-quality alleles' into Dutch breeding programmes, in particular the *Glu-D1* allele encoding 5+10. About 20% of the variation in the breeding lines was ascribed to differences in HMWg genotype, which is much lower than in some of the other published studies. The remainder of the variation in bread-making quality was ascribed to variation in environmental conditions and to genetic variation in the composition of other kernel components, such as lipids, starch, gliadins and low molecular weight glutenin subunits. Therefore, genetic variation in other kernel components should also be taken into consideration when selecting for bread-making quality, although the HMWg subunit genes can be considered as major genes in determining that quality. Which of these other components is most important in this respect is not known.

A large number of HMWg alleles are present in varieties and in species related to bread-wheat. Because the contribution to quality is known for only a small number of these alleles, there could be alleles with an unknown 'quality' in breeding programmes. Unintended introduction of 'poor-quality' alleles can be avoided as far as these alleles can be distinguished from 'high-quality' ones. The HMWg alleles are routinely identified by determining the relative mobility (R_m) with one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the subunits encoded. As shown in **Chapter 3**, the resolution of this technique may be insufficient for an unambiguous identification because alleles may be present that differ only slightly in the R_m of the subunits encoded. Unambiguous identification by two-dimensional electrophoresis is especially necessary before introducing species related to bread-wheat in breeding programmes.

To study variation in the *amount* of the HMWg subunits effectively, one must develop a method for a reliable quantification of the subunits present in the kernel. Since one of the objectives was to investigate the amounts of the subunits encoded by different alleles and because a genotype may possess 'poor-quality' alleles in combination with 'high-quality' alleles, one must use a method that allows the quantification *and* identification of each subunit. In most methods described in the literature, reversed-phase high-performance liquid chromatography (RP-HPLC) is used to separate the HMWg subunits. However the sample preparation required before RP-HPLC is a potential source of error and may even result in systematic losses of the HMWg subunits, as discussed in **Chapter 4**. In addition, these methods require electrophoretic analysis to identify the HMWg subunits present in the RP-HPLC peaks. As an alternative, an efficient method was developed and is described in Chapter 4. The HMWg subunits are separated from other kernel proteins - and from each other - by SDS-PAGE. Scanning densitometry is used to determine the amount of Coomassie brilliant blue (CBB) adsorbed by the protein after gel staining, which is a measure for the amount of protein. The routine method for gel staining resulted in subunit bands with a low staining intensity. So it was essential to introduce a new and more sensitive staining method. Since sample preparation is not required in the method, systematic losses of the HMWg subunits can be avoided. Because the identification of the subunits is based on their R_m during SDS-PAGE, the method allows quantification and identification of the subunits in one analysis.

As shown in **Chapter 5**, the amount of individual HMWg subunits is subject to environmental variation. An increase in nitrogen fertilizer applied to two varieties (Obelisk and Urban), grown at the same trial field, resulted in an increase in both the total amount of kernel proteins and the amounts of all HMWg subunits. Variation in protein content accounted for 88% and 87% of the variation, respectively, in amount of the HMWg subunits. For wheat varieties grown at different sites, there was a weaker correlation between protein content and the total amount of HMWg subunits 'within varieties/between sites'. The proportions of variation in amount of the subunits accounted for by protein content were 40, 0, 66 and 0% for the varieties used, Citadel, Granada, Okapi and Kraka, respectively. This means that the proportion of these subunits relative to the total amount of kernel proteins, is highly variable as a result of differences in growing conditions (Figure 5.2). The mechanism responsible for this variation is not known. As discussed in Chapter 1, this proportion of the HMWg subunits largely determines

bread-making quality of a flour. The environmental effect on the proportion of the HMWg subunits may therefore be useful as an additional criterion for prediction of bread-making quality of flours by millers and bakers, besides traditional criteria such as protein content and variety. Genetic variation in the total amount of the HMWg subunits also plays a role (Chapter 5). This will be discussed later.

The environmental effects on the variation in the amount of the HMWg subunits and on the proportion of the subunits relative to the total content of protein hampers detection of genetic variation in amount. However the ratio between the individual subunits of a genotype is only slightly affected by differences in environment. Therefore one can use the amount of a subunit relative to the total amount of the subunits as a measure for the level of gene-expression.

The proportion of the HMWg subunits relative to the total amount of proteins can be changed by several breeding strategies. Firstly, it is possible to select for number of subunits because alleles occur at each *Glu-1* locus that differ in the number of subunits encoded. The number of subunits affects the total amount of the HMWg subunits. Secondly, it is shown in **Chapters 5** and **6** that the level of expression of the HMWg subunit genes is affected by variation in their genetic background. For instance, the proportion of the HMWg subunits of two varieties (Citadel and Kraka) with the same HMWg genotype, averaged over six growing sites, differed by about 30% (Chapter 5). This variation offers ways of changing the proportion of the HMWg subunits by breeding. However to achieve this, more information about the nature of this effect of genetic background is required, and techniques for determination of the proportion of the HMWg subunits in breeding programmes must be developed. Thirdly, one can use HMWg alleles that differ only in their level of gene expression, and not in the R_m or the number of subunits encoded (*expression alleles*). Because the introduction and selection for these alleles is relatively simple, this approach seems more practical than using the effect of variation in genetic background. Expression alleles can also be used to increase the amount of the HMWg subunits if the maximum number of subunits is present, or to improve the ratio between the individual HMWg subunits. Selection for expression alleles allows simultaneous selection for type of subunits. It is not yet possible to increase the amount of the HMWg subunits by gene technology (e.g. the introduction of extra copies of the genes).

Chapters 6 and 7 study genetic variation in expression of individual HMWg subunit genes. Chapter 6 describes experiments to determine the effect of variation at *Glu-1* loci on the expression of HMWg genes at homoeologous loci. In these

experiments, near-isogenic lines that differ in their HMWg genotype, were used and also the progeny of two crosses. The expression of the individual HMWg subunit genes is sometimes, but not always, affected by the number of subunits. Introduction of a *Glu-D1* null-allele resulted in an increase in expression, whereas introduction of a null-allele at the *Glu-A1* locus had no effect. Variation in the *type* of the HMWg subunits had, with one exception, no significant effect on the amount of the subunits encoded by homoeologous genes. Although variation in the genetic background strongly affected expression of HMWg genes, the ratio between the individual HMWg subunits of lines with the same HMWg genotype is only slightly affected by differences in genetic background.
An enhancer element, localized proximate to the transcription initiation site of the genes, is thought to be involved in the developmental regulation of the individual HMWg genes. Since variation in the genetic background and in environmental conditions affects the total amount of the HMWg subunits but does not change the ratio between the subunits, expression of the individual HMWg homoeologous genes is probably coordinated by a common regulation mechanism. This mechanism may differ between genetic backgrounds. Coordination of gene expression may involve regulatory proteins, but it may also only be the strength of the gene promoters that is of importance. The effect of the genetic background could then be caused by a non-specific mechanism, such as competition for transcriptional factors between the HMWg genes and other genes in the genetic background.

The relative amounts of the subunits can be used as a measure of the genetically determined level of gene expression because variation in environmental conditions, in genetic background and in HMWg genotype has only small effects on the ratio between the subunits. In a set of 38, mostly Dutch, wheat varieties the variation in relative amounts of the HMWg subunits was studied (**Chapter 7**). The subunits were identified according to their R_m during SDS-PAGE. There were only small differences in the relative amounts of identical subunits. So expression alleles were not detected in this set of varieties. This could be due to a common ancestry of the varieties studied, so one should study the expression of HMWg genes in a set of genotypes of wider genetic origin. However the occurrence of expression alleles may turn out to be generally rare.

Non-identical subunit alleles at the *Glu-B1* locus of the Dutch varieties differed considerably in the relative amounts of the subunits encoded, as indicated by the relative absorbance of the CBB stained bands. Chapter 8 shows that these differences in CBB adsorption reflect differences in the level of gene expression

(i.e. *molar* amounts of the subunits), and not differences between the subunits in their affinity for CBB nor in their molecular weight. The contribution to bread-making quality of these *Glu-B1* alleles, as established in Chapter 2 and in the literature, is positively related to differences in the amounts of the subunits. For the *Glu-A1* and *Glu-D1* loci, there was no clear evidence for such a relation. As discussed in Chapter 7, it is still possible that expression alleles occur at the *Glu-A1* locus, which could explain the conflicting ranking of the alleles found in the literature for quality of the alleles encoding the subunits 1 and 2*. Because only a few varieties used in Chapter 7 contained these alleles, this could not be investigated for Dutch varieties. The results of this thesis do not unambiguously show differences in the amounts of the allelic *Glu-D1* pairs of subunits 2+12 and 5+10. In Chapter 6, the total amount of the 'high-quality' subunits 5+10 is somewhat higher than the total amount of the 'low-quality' subunits 2+12; in Chapter 7, there is, however, no difference in the total amounts of these subunits. The *Glu-D1* alleles present in the genetic stock used (Dutch varieties) probably do not differ in their level of gene expression.
Consequently, the question whether differences in 'quality' between HMWg alleles are caused by differences in the amounts of the subunits encoded or by differences in the intrinsic quality of the proteins remains to be answered. Therefore further research on the amounts of the subunits is required, especially for those encoded by alleles at the *Glu-A1* and *Glu-D1* loci. If the relationship found between the amount of the *Glu-B1* subunits and bread-making quality can be confirmed for other *Glu-B1* alleles and for the alleles at the other loci, this relationship could be used for prediction of the 'quality' of novel alleles.

The following example, based on **Chapter 8**, illustrates that the HMWg subunits that are of crucial importance for the bread-making quality only constitute a minor fraction of the flour proteins (on a weight basis). An estimate of the proportion of the *Glu-B1* subunits (relative to the total amount of kernel proteins) of a genotype containing the 'high-quality' allele encoding 7+9 is 3% (Table 8.4). Accordingly, 1 kg of a flour (protein content 12%) of this genotype contains 3.6 g of these 'high-quality' subunits. The amount of the 'low-quality' subunits 6+8 (*Glu-B1* allele) is about 80% of the amount of 7+9 (Table 7.4). So 1 kg of a flour milled from a genotype possessing this allele contains 2.9 g of these subunits. In the research presented in Chapter 2, the differences in loaf volume of lines that possess these *Glu-B1* alleles is about 20%. Whether or not the differences in the amount of the subunits are responsible for the differences in quality of these alleles, this example

illustrates that differences in a minor flour fraction can have large effects on the bread-making quality.

In conclusion, not only qualitative variation but notably also quantitative variation in the composition of the HMWg subunits is important for bread-making quality. This quantitative variation in the HMWg composition probably also affects the suitability of wheat for other purposes, e.g. in the pasta, pastry, biscuit and starch-gluten industry.

Because genetic variation in the amount of the HMWg subunits occurs, it is possible to improve the proportion of the HMWg subunits by breeding. For example, if the average gluten strength is too low for good quality, a breeder can increase the amount of the subunits. Improvement by breeding of the quantitative HMWg composition can go together with breeding for type of the subunits. The results also show that the proportion of the HMWg subunits relative to the total amount of flour proteins is subject to environmental variation, which is important for improvement of the quality of wheat for the various applications, but it cannot be used fully because simple and fast tests are lacking for estimation of the HMWg subunits quantitatively.

Before the qualitative and quantitative variation can be exploited optimally in improving bread-making quality of wheat, additional research will be required. The proportion of the HMWg subunits required for good quality will depend on the amount and the composition of other kernel proteins, such as gliadins and low molecular weight glutenin subunits and probably also on the type of HMWg subunit. The optimum ratio between groups of kernel proteins probably also depends on the bread-making processes used. Therefore, it is necessary to study both genetic and environmental variation in the amount of these other groups of proteins in relation to wheat quality. Because both plant breeders and millers aim at the production of flour suited for as many bread-making processes as possible, the protein composition should preferably be related to several bread-making processes. Another subject requiring additional research is development of small-scale tests, which can be used in breeding programmes to determine the amounts of the HMWg subunits and other proteins affecting wheat quality. These tests could also be valuable for the milling and baking industry, but the requirements of the industry for such a test are not fully congruent with those of the breeders (phenotypic quality in contrast to genotypic quality).

The major new results presented are as follows:

- The relationship between the type of HMWg subunit and bread-making quality is

more complex than generally suggested in the literature.

- An efficient method for simultaneous identification and quantification of individual HMWg subunits has been developed.
- The amount of the individual HMWg subunits and the proportion relative to the other proteins are subject to environmental and genetic variation. Within genetic variation, effects of HMWg genotype and genetic background can be distinguished.
- The ratio between the individual subunits of a genotype is only slightly affected by environmental differences. On this basis, genetic variation in the level of expression of individual genes can be distinguished from environmental variation.
- Indications were found that there is a mechanism that coordinates the expression of individual HMWg genes and determines the total amount of the subunits.
- The amount of the *Glu-B1* subunits is positively correlated with the contribution to quality of the alleles encoding these subunits.
- The HMWg subunits, i.e. proteins of major importance for the bread-making quality, constitute only a minor fraction of the wheat flour proteins.

SAMENVATTING

Door de lage bakkwaliteit van de in Nederland verbouwde tarwe wordt slechts een gering deel van de produktie (ongeveer 30% in het seizoen 1990/1991) gebruikt voor het bakken van brood. Daarom is het voor de landbouw van belang om de bakkwaliteit van Nederlandse tarwe te verhogen, onder andere door het veredelen op deze eigenschap. Echter, de complexiteit van de bakkwaliteit bemoeilijkt veredeling. Zo zijn er naast de invloed van het genotype sterke invloeden van de milieu-omstandigheden, zoals de teeltmaatregelen en het klimaat. Tevens hebben technologische omstandigheden, zoals de condities tijdens het malen van de korrel en het bakproces, een grote invloed. Voorts wordt veredeling gehinderd door het ontbreken van snelle en eenvoudige testen waarmee de bakkwaliteit aan de hand van een kleine hoeveelheid korrels geschat kan worden. Deze testen zijn onmisbaar, vooral bij selectie in een vroeg stadium van de veredeling. De bakkwaliteit als zodanig kan alleen bepaald worden met arbeids- en kapitaals-intensieve methoden (deegonderzoek en bakproeven).

In de veredeling kan in principe gebruik gemaakt worden van het feit dat hoeveelheid en kwaliteit van de opslageiwitten in de korrel een grote invloed hebben op de bakkwaliteit. Het eiwitgehalte van de tarwekorrel is positief gecorreleerd met de bakkwaliteit, maar gezien de grote door milieuverschillen geïnduceerde variatie is het eiwitgehalte slechts moeizaam met veredeling te verhogen. Van meer belang zijn de genetisch bepaalde verschillen in de *kwaliteit* van het eiwit, die tot uiting komen als verschillen in bakkwaliteit per procent eiwit. De eiwitkwaliteit wordt bepaald door de samenstelling van het eiwit, die genetisch bepaald is en dus voor veredeling toegankelijk.

In dit proefschrift wordt de samenstelling van een specifieke groep opslageiwitten van tarwe, de hoog molecule gewicht-glutenine subunits (in het vervolg afgekort als HMWg subunits, 'High Molecular Weight glutenin subunits') bestudeerd in relatie tot de bakkwaliteit. Genen op drie homeologe *Glu-1* loci coderen voor deze eiwitten: de *Glu-A1*, de *Glu-B1* en de *Glu-D1* locus. Per locus wordt gecodeerd voor maximaal twee subunits. Ook komen nul-allelen voor, dat wil zeggen allelen die voor geen HMWg subunit coderen. Bij aanvang van dit onderzoek was al bekend dat op deze loci allelen voorkomen die sterk verschillen in bijdrage aan de bakkwaliteit. Gesuggereerd is zelfs dat een belangrijk deel van de genetische variatie in bakkwaliteit een gevolg is van verschillen in HMWg subunit genotype.

Het was echter niet bekend of dit ook zo is onder Nederlandse omstandigheden. Daarnaast was er slechts weinig kennis beschikbaar over twee onderwerpen die van belang zijn voor de veredeling op bakkwaliteit:

1) de beperkingen bij het identificeren van de HMWg subunit allelen,
2) de (genetische) variatie in de hoeveelheid van de HMWg subunits.

Uit de literatuur was bekend dat ook de hoeveelheid van bepaalde groepen korreleiwitten (inclusief de HMWg subunits) een belangrijk effect heeft op de bakkwaliteit (zie **hoofdstuk 1**). In dit proefschrift is daarom genetische variatie in zowel het type als in de hoeveelheid bestudeerd.

Het type HMWg subunit heeft een duidelijk effect op de bakkwaliteit van in Nederland geteelde tarwelijnen (**hoofdstuk 2**). Ongeveer 20% van de variatie in de bakkwaliteit van de lijnen is door de variatie in HMWg subunit genotype te verklaren. De rest van de variatie is te wijten aan variatie in milieu-omstandigheden en aan al dan niet genetische variatie in andere korrelbestanddelen. Van de drie *Glu-1* loci heeft allel variatie op het *Glu-D1* locus het grootste effect. Het allel dat codeert voor de HMWg subunits 5+10 gaat samen met een betere bakkwaliteit dan het allel dat codeert voor 2+12. Vermeldenswaard is dat in Nederlandse rassen vooral het allel coderend voor 2+12 voorkomt, en ook op de andere loci komen 'slechte allelen' duidelijk vaker voor dan 'goede allelen' (zie Hoofdstuk 7). De bijdrage tot de bakkwaliteit van de drie *Glu-1* loci blijkt niet additief te zijn, in tegenstelling tot wat in het algemeen in de literatuur vermeld wordt. Alleen bij aanwezigheid van het *Glu-D1* allel 5+10 is een effect aanwezig van variatie op de andere loci. Het mechanisme achter deze interacties is niet bekend.
Concluderend kan gesteld worden dat, hoewel de relaties complexer zijn dan voorheen gedacht, het HMWg subunit genotype een belangrijk effect heeft op de bakkwaliteit. Het is daarom van belang om in Nederlands veredelingsmateriaal 'kwaliteits-allelen' te introduceren, waarbij het *Glu-D1* allel 5+10 prioriteit verdient. Naast de genetische variatie in HMWg subunit samenstelling is het aannemelijk dat genetische variatie in andere korrelbestanddelen ook een rol kan spelen bij de veredeling.

In **hoofdstuk 3** wordt ingegaan op de problematiek van het identificeren van HMWg subunit allelen. In rassen en verwanten van broodtarwe is een groot aantal allelen aanwezig. De bijdrage tot de bakkwaliteit is slechts van een gering aantal hiervan bekend. Om introductie of selectie van allelen met een negatief effect op de bakkwaliteit te voorkomen is een betrouwbare allel identificatie essentieel. Deze identificatie is gebaseerd op de relatieve mobiliteit (R_m) tijdens 'sodium dodecyl

sulfaat-polyacrylamide gel electrophorese' (SDS-PAGE) van de door het allel gecodeerd subunits. Echter, de verschillen in R_m van allele subunits kunnen zeer gering zijn. In deze situatie is voor een betrouwbare identificatie twee-dimensionale electroforese (isoelectrische focussering gevolgd door SDS-PAGE) gewenst.

Voor het bestuderen van de variatie in hoeveelheid subunit diende de kwantificatie methode bij voorkeur zowel geschikt te zijn voor het individueel kwantificeren van de subunits als voor het identificeren van de subunits, en dus van de allelen. Dit is belangrijk omdat een genotype zowel 'goede' als 'slechte' allelen kan bevatten. Daarnaast is het interessant om de hoeveelheid subunit gecodeerd door allelen die verschillen in bijdrage tot de bakkwaliteit te bestuderen. Het bleek noodzakelijk om zelf een kwantificatie methode te ontwikkelen omdat de in de literatuur beschreven methoden niet aan de hierboven gestelde eisen voldoen. Deze methode wordt beschreven in **hoofdstuk 4**. De HMWg subunits worden hierbij met SDS-PAGE van de andere korreleiwitten en ook onderling gescheiden. De hoeveelheid Coomassie Brilliant Blauw (CBB) gebonden door de subunits na kleuring van de gel, hetgeen proportioneel is met de hoeveelheid subunit, wordt bepaald met scanning densitometrie. Omdat de identificatie van de subunits gebaseerd is op de R_m tijdens SDS-PAGE is het mogelijk om de subunits in één analyse zowel te identificeren als te kwantificeren.

In **hoofdstuk 5** wordt de invloed van variatie in milieu omstandigheden op de *hoeveelheid* HMWg subunits beschreven. Bij monsters van twee rassen, geteeld op dezelfde lokatie, bestonden grote N-gift invloeden op de hoeveelheid HMWg subunit. Deze verschillen konden voor een groot deel (meer dan 87%) uit verschillen in eiwitgehalte van de korrels verklaard worden. Bij monsters van een viertal andere rassen, geteeld op zes lokaties, bestonden grote lokatie-verschillen in hoeveelheid subunit. Hierbij speelden de verschillen in eiwitgehalte duidelijk minder een rol. Deze experimenten geven aan dat de hoeveelheid van de HMWg subunits ten opzichte van de andere korreleiwitten sterk afhankelijk is van milieufactoren. Omdat variatie in deze verhouding de bakkwaliteit beïnvloedt (zie Hoofdstuk 1) kan een test voor het bepalen hiervan voor de maal- en broodindustrie bruikbaar zijn als een extra parameter voor het schatten van de bakkwaliteit van tarwe(meel), naast de nu gebruikte parameters als het eiwitgehalte en de rasidentiteit. Tevens blijkt uit hoofdstuk 5 dat er voor de hoeveelheid van de subunits ook genetische variatie bestaat. Hierop zal nog terug gekomen worden.

Met veredeling kan de hoeveelheid HMWg subunits op verschillende manieren beïnvloed worden. De genetische achtergrond waarin HMWg subunit allelen

aanwezig zijn heeft een belangrijke invloed op de totale hoeveelheid subunit, zoals blijkt uit onderzoek gepresenteerd in de **hoofdstukken 5 en 6**. Dit biedt weliswaar perspectieven voor veredeling op hoeveelheid subunit, maar voor exploitatie is meer kennis vereist van de genetische basis van deze variatie en zijn snelle testen nodig voor het bepalen van de hoeveelheid subunits. Daarnaast kan gebruik worden gemaakt van variatie op de *Glu-1* loci. Het aantal subunits van een genotype, dat positief gecorreleerd is met de totale hoeveelheid subunits, is eenvoudig te sturen met veredeling door gebruik te maken van HMWg subunit allelen die verschillen in aantal subunits. Een andere benadering is het gebruik van 'expressie-allelen'. Dit zijn allelen die verschillen in de hoeveelheid van de subunits, maar niet in type of aantal subunits. Hiermee is het mogelijk om de totale hoeveelheid HMWg subunits te verhogen als het maximale aantal subunits reeds aanwezig is, en om de verhouding tussen de individuele subunits te veranderen.

Hoofdstuk 6 beschrijft onderzoek naar genetische invloeden op het niveau van expressie van individuele HMWg subunit genen. Als het totale aantal HMWg subunits afneemt door de introductie van een 'nul-allel' (een allel dat geen subunits codeert) van het *Glu-D1* locus neemt de expressie van de overige genen toe. De aanwezigheid van een nul-allel op het *Glu-A1* locus had dit effect niet. Variatie in het type allel had, met één uitzondering, geen significant effect op het niveau van expressie van genen op homeologe loci. De genetische achtergrond heeft weliswaar een effect op de totale hoeveelheid subunit, maar niet op de verhouding tussen de individuele subunits. Deze resultaten duiden er op dat de expressie van de genen op de drie *Glu-1* loci door een gemeenschappelijk mechanisme gecoördineerd wordt. Tussen genotypen bestaan verschillen in dit mechanisme, zoals blijkt uit het effect van variatie in de genetische achtergrond op de totale hoeveelheid subunit. Kennis hierover kan mogelijkheden bieden voor het doelgericht veranderen van de hoeveelheid HMWg subunit.

In **hoofdstuk 7** wordt het niveau van expressie van de HMWg subunit genen in 38 bijna uitsluitend Nederlandse rassen bestudeerd. De verhouding tussen de HMWg subunits - de relatieve hoeveelheid - blijkt een bruikbare maat te zijn voor het genetisch bepaalde niveau van expressie van de HMWg subunit genen omdat deze verhouding nauwelijks beïnvloed wordt door verschillen in de genetische achtergrond of door milieufactoren, zoals aangetoond in hoofdstuk 5. Er is slechts een beperkte variatie in het expressie niveau van *identieke* allelen (subunits hebben dezelfde R_m), zodat expressie-allelen in deze rassen niet gevonden zijn. Dit kan een gevolg zijn van onderlinge verwantschap van de Nederlandse rassen, maar het is

ook mogelijk dat expressie-allelen als zodanig zeldzaam zijn. Het is daarom van belang om in vervolgonderzoek een groep genotypen met een meer diverse genetische achtergrond te gebruiken.
Niet-identieke HMWg allelen op het *Glu-B1* locus van de Nederlandse rassen verschillen daarentegen aanzienlijk in het niveau van gen-expressie. De verschillen in de hoeveelheid van de subunits gecodeerd door de *Glu-B1* allelen zijn positief gecorreleerd met de 'kwaliteit' van de allelen, zoals deze is vastgesteld in het onderzoek gepresenteerd in hoofdstuk 2 en in onderzoek van anderen. Deze relatie kan echter niet aangetoond worden voor de allelen van de andere loci.
Voor de hoeveelheid van de HMWg subunits gecodeerd door de *Glu-D1* allelen 2+12 en 5+10 zijn in dit proefschrift tegenstrijdige resultaten verkregen. In de Nederlandse rassen (hoofdstuk 7) is de hoeveelheid subunit gecodeerd door deze allelen gelijk. In hoofdstuk 6 daarentegen, waar isogene lijnen van Sicco en een kruisingspopulatie gebruikt zijn, is de totale hoeveelheid 5+10 hoger dan de totale hoeveelheid 2+12. Dit kan er op duiden dat op dit locus toch expressie-allelen voorkomen. Dus, hoewel er een relatie aangetoond is tussen de hoeveelheid subunit en de bijdrage tot de kwaliteit van het allel dat deze subunits codeert geeft dit proefschrift geen uitsluitsel of de verschillen in 'kwaliteit' van de allelen veroorzaakt zijn door verschillen in de hoeveelheid of in de structuur van het eiwit. Hiervoor is extra onderzoek vereist, met name naar de hoeveelheid van de *Glu-A1* en *Glu-D1* gecodeerde subunits.

In **hoofdstuk 8** is in meel van vijf rassen de hoeveelheid van de HMWg subunits op gewichtsbasis bepaald. De hoeveelheid varieerde tussen de rassen van 3 tot 8 gram per 100 gram eiwit. Dit toont aan dat, hoewel de HMWg subunits van groot belang zijn voor de bakkwaliteit van tarwemeel, zij slechts een klein deel van de totale hoeveelheid eiwit uitmaken.

Samenvattend kan worden gesteld dat dit proefschrift aantoont dat zowel kwalitatieve als kwantitatieve variatie in de samenstelling van de HMWg subunits van belang is voor de bakkwaliteit van tarwe. Opgemerkt moet worden dat deze variatie ook van belang is voor andere toepassing van tarwe, zoals in de pasta-, de banket- en de gluten/zetmeel industrie. Bij de veredeling op kwaliteit kan naast variatie in het type subunit de aangetoonde genetische variatie in de hoeveelheid van de HMWg subunits gebruikt worden. Voor de tarweverwerkende industrie is de milieuvariatie in de hoeveelheid subunits van belang.
Voor een optimaal gebruik van de HMWg subunits in de veredeling en de

tarweverwerkende industrie is op een aantal gebieden meer onderzoek gewenst.

1) de optimale hoeveelheid HMWg subunits is onbekend. Deze zal afhankelijk zijn van de hoeveelheid van andere groepen eiwitten, zoals de α-, β-, γ of ω-gliadinen en de LMW glutenine subunits, en is hoogstwaarschijnlijk eveneens afhankelijk van het brood bereidingsproces dat gebruikt wordt. Het is daarom van belang om eveneens genetische en milieuvariatie in de hoeveelheid en het type van deze eiwitten te bestuderen.

2) simpele en snelle testen ontbreken voor het bepalen van de hoeveelheid van groepen eiwitten, inclusief HMWg subunits. Deze testen zijn van belang voor de veredelaars en voor maalderijen en bakkerijen. Echter, zoals gesteld in hoofdstuk 1, vallen de vereisten van de tarweverwerkende industrie niet samen met die van de veredelaar. Deze is geïnteresseerd in de *genotypische* kwaliteit, terwijl de tarwe-verwerkende industrie belang heeft bij de *fenotypische* kwaliteit.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 28 augustus 1962 te Wageningen. Na het behalen van het VWO-B diploma aan het Wagenings Lyceum, werd in 1981 begonnen met de studie plantenveredeling aan de Landbouwuniversiteit (voorheen Landbouw Hogeschool) te Wageningen. Het doctoraalexamen (met lof) met als hoofdvakken plantenveredeling (Prof.dr.ir. J.E. Parlevliet en Dr.ir. B.A. Uijtewaal) en erfelijkheidsleer (Prof.dr.ir. J.H. van der Veen en Dr.ir. J. Visser) en als bijvak fytopathologie (Prof.dr.ir. J. Dekker en Dr.ir. L.C. Davidse) werd in september 1987 behaald. Hij bracht zijn praktijktijd door bij de Z.P.C. te St. Annaparochie en bij 'Plant Genetic Systems' te Gent, België. Vanaf 1 januari 1987 tot 1 november 1989 heeft hij gewerkt aan dit promotieonderzoek bij de Stichting voor Plantenveredeling (SVP) te Wageningen in dienst van de Stichting Nederlands Graan Centrum (NGC). Per 1 november 1989 is hij werkzaam als onderzoeker industriële eiwitten bij de hoofdafdeling Agrificatie van het DLO-Agrotechnologisch Onderzoeksinstituut (ATO-DLO) te Wageningen.