Factors affecting the hyper-aggregation of glutenin particles

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Proefschrift

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GENERAL INTRODUCTION

1.1 Wheat

Cereals are a vital part of our daily diet, on average about 53% of our daily caloric intake is obtained from cereal-based products. Wheat, rice and corn are the cereals that contribute the most to the world's food supply chain. About 50% of the protein consumed worldwide is of cereal origin. The role of wheat is the most prominent, 31% of all cereals produced is wheat. It was one of the first of the grains domesticated by humans. Historical evidence points at the cultivation of wheat in the Nile valley around 5000 B.C, the Indus and Euphrates valleys by 4000 B.C., China by 2500 B.C., and England by 2000 B.C. Apparently, wheat cultivation expanded from Mediterranean centers of leading agriculture, towards Asia and Europe.

In the beginning, the main concern of wheat farmers and wheat breeders, was a good harvest yield. Climatic conditions and plant pests and deseases depress yields and can ruin the technological quality of the crop. Selecting and breeding for resistance against these types of stresses are still primary objectives of most breeding programs around the world. Furthermore, every variety has a certain life-span. Therefore, production of new varieties is a constant activity for breeders.

These efforts have been quite successful already: of the use of better wheat varieties, and improved growing conditions (for example by improving the condition of the soil), has enormously increased wheat yield per acre and hence wheat production. In Europe this has even led to overproduction of wheat. It stands to reason that if availability is secured, other more use-quality related factors come into play: with wheat this has impelled the trend to focus breeding programs and growing studies on the end-use quality of wheat.

Producing leavened products like bread, requires wheat flour of suitable bread-making quality. Variation in the flour produced from wheat, even within pure varieties, is large. Therefore, a good prediction of wheat flour quality is of value for the flour milling and baking industry. The flour quality required depends on the processes in which it is used (bread, biscuit, pasta). Millers try to keep these qualities constant by careful selection and blending of wheat varieties.

Wheat technological quality derives mainly from two interrelated characteristics: grain hardness and protein composition and content. Grain protein composition and hardness are heritable traits. Protein content is weakly heritable and strongly dependent on environmental factors. The milling properties of wheat are determined by wheat kernel properties. Extraction rate: kg flour obtained per kg wheat, is affected by it. Kernel moisture is adjusted to kernel hardness; hard kernels require more moisture for milling than soft ones. In general flour from hard wheat contains more protein, despite adjusted moisture, damaged starch is often higher for flour from hard wheat. Quality of wheat flour is determined by the molecular structure of the major proteins present, which in turn,

control the interactions of the proteins during the bread-making or other food processes in which the flour is used.

1.2   Wheat flour quality

When removing the wheat kernel bran layer and/or when breaking a wheat kernel open, one will find a whitish powdery substance. This is the kernel's starchy endosperm (fig. 1.1). Wheat flour mainly consists of this starchy endosperm. Based on dry matter, wheat flour contains: ca. 70 % starch, 9 - 18% proteins, ca 2 % lipids and ca 2,5% non-starch polysaccharides.

There are two populations of starch granules: 1) the large, lenticular A-type granules with diameters between 14-40μm, 2) the spherical B-type granules with diameters between 1 – 10μm. Starch granules consist of amylose and amylopectin.
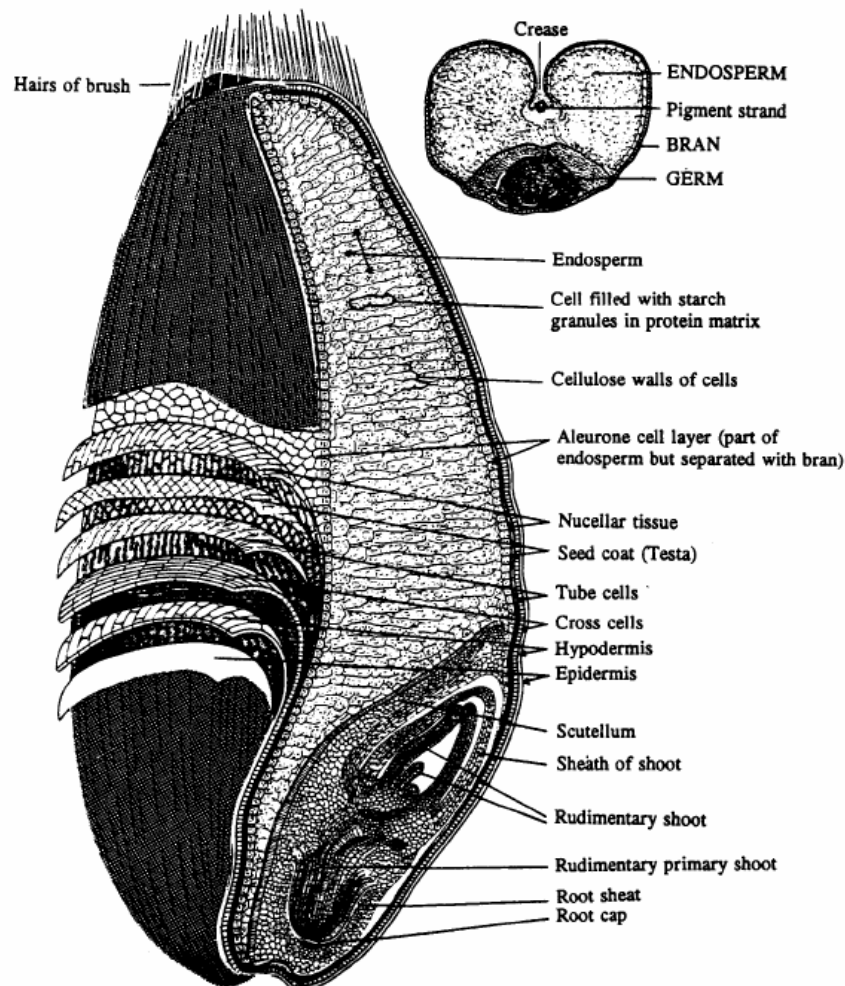

Figure 1.1   Structure of the wheat kernel (from Hoseney, 1986)

Flour protein content and composition, especially of the gluten part of the protein; have been shown to be the most important parameters determining the suitability of flour in the bread-making process. In this introduction we will concentrate on this aspect and not further discuss aspects related to wheat starch. Wheat gluten content related parameters give a high correlation with the variation in process requirements, dough rheological properties and bread volume(Hamer et al., 1999). Technological measurements like the Farinograph, Mixograph, Extensigraph and rheological measurements at both small (e.g. oscillation) and large deformations (extensograph) are used in combination with compositional data in order to give an explanation for the unique properties of wheat flour dough (Bloksma and Bushuk, 1988).

1.3   **Wheat flour proteins**

Protein is an important component for assessing wheat flour quality. In 1902 Osborne stated:

"*Of the protein substances used as food, none is of more importance than those contained in the seeds of wheat*"

Osborne (1893, 1907) was the first to systematically separate wheat protein into four fractions. He defined four wheat flour protein fractions based on differences in their solubility: albumins that are soluble in water; globulins that are soluble in water + NaCl; gliadins that are soluble in 70% ethanol and glutenins, partly soluble in dilute acid or alkali.

Finney and Barrymore (1948) revealed relations between flour protein content and loaf volume. However, it was soon learned that besides protein quantity there are also variations in protein quality (MacRitchie 1999). In the 1960s and 1970s a focus on the gluten complex, a heterogeneous mix of proteins was thought necessary to identify these differences in wheat flour protein quality.

1.3.1   Gluten

From Osborne's extractions it was learned that gluten consists of glutenins and gliadins. This visco-elastic gluten protein fraction has since long been recognized as an important part of the wheat proteins. It essentially started before Osborne's fractionation, with the experiment of Beccari, where he showed that gluten could be obtained by washing dough with water. Extensively rinsing dough in tap water removes albumins, globulins and starch, leaving a sticky elastic proteinaceous mass: gluten. In Beccari's time (early 18th century) further fractionation was not done yet, gluten was

viewed as a single protein then (Bailey 1941). Originally, in 1833, De Saussure called the proteinaceous mass not gluten, but 'Glutin'. Gluten represents the major (appr. 70-80%) part of the flour proteins. Even today, the Osborne method and the perhaps Beccari derived so-called hand wash method (AACC Method 38-10) are part of the cereal chemist's toolbox to assess wheat flour quality.

The glutenins and gliadins in gluten have different properties. The glutenins are the network proteins. Therefore glutenins contribute to dough strength. Gliadins do not have this network forming ability; they kind of adhere to the glutenin structures in the network. Glutenin affects the elastic properties of dough; gliadins the viscous properties of dough. The ratio between glutenin and gliadin plays a major role in dough rheological properties. Of the two, the variation in glutenin is most important. Today, glutenin is regarded as the key structuring protein in gluten and dough.

1.3.2   The insoluble glutenins: GMP, dough properties and baking performance

In an interesting pioneering paper by Mecham et al. (1965) it was shown that the mixing stability of gluten and gluten visco-elasticity decreased when gluten had been dispersed in acetic acid. This effect was even more dramatic when large protein aggregates were removed from dispersed gluten. In hindsight, Mecham's aggregates may well have been what are later called large insoluble 'glutenin polymers'. Chen and Bushuk (1970) revealed that part of the glutenin is soluble in acetic acid, thus making the distinction between an insoluble and a soluble fraction. The importance of this distinction became clear when Orth and Bushuk (1972) demonstrated a positive correlation between the amount of acetic acid insoluble glutenin and bread loaf volume. From then on insoluble glutenin became widely recognised as the key protein fraction that can explain differences in dough strength and bread-making quality (Khan and Bushuk 1978). The use of detergents (SDS, Graveland (1980)) and organic solvents (propanol) allowed an even better separation and led to the conclusion that insolubility was due to size and a very high degree of polymerization. Other groups developed methodology with propanol to further separate soluble proteins parts from the insoluble glutenin. Currently, two main methods are in use to quantify and characterize this fraction: the so-called UPP method: using propanol an Unextractable Polymeric Protein (UPP) fraction is obtained. Upon sonication, this fraction becomes soluble in SDS and can be analysed using Size Exclusion Chromatography (Gupta 1993). The other method is the SDS method as put forward by Graveland et al. (1980) resulting in the SDS insoluble gel protein fraction. This fraction was renamed Glutenin Macro Polymer (GMP) to reflect its highly aggregated nature (Weegels et al. 1996). Moonen et al.

(1986) found that the SDS insoluble glutenin-gel protein fraction highly correlated with SDS sedimentation values and loaf volume. Weegels et al. (1996, 1997) studied this fraction in great detail and presented firm evidence that GMP quantity correlates with bread loaf volume. Pritchard (1993) suggested that GMP-gel stiffness (G'), could be used as a wheat flour quality indicator. Using 16 varieties, Kelfkens and Lichtendonk (2000) confirmed that the stiffness of the GMP-gel, G', correlates with bread loaf volume. Kelfkens and Lichtendonk (2000) have also shown that GMP-quantity correlates with dough extensional properties. Bekkers et al. (2000) showed that the stiffness of the GMP-gel (G') from flour correlates with dough development time. Clearly, this GMP fraction is pivotal in understanding glutenin functionality. Cornec et al. (1994) and Lefebvre et al. (2000) showed a correlation between insoluble glutenin aggregate size and dough elasticity. Graveland (1980) and Weegels et al. (1997) showed that GMP is not a static entity: mixing renders GMP soluble in 1.5% SDS and during dough rest there is reassembly / repolymerization of GMP. Hamer et al. (1994) showed that dough mixing breaks down the GMP fraction differently than with chemical reduction. According to Weegels et al. (1996, 1997) GMP from flour is different than GMP from mixed and rested dough. Clearly, the decrease of GMP with mixing-time and its reassembly during dough rest is a complex physico-chemical process. The factors underlying this process, and how they are governed by initial properties are still poorly understood.

1.3.3 Glutenin composition: the glutenin subunits

Clearly, the glutenin fraction presents an important key to understanding and controlling flour end-use quality. When combining SDS and reducing agent (mercaptoethanol, DTT), the glutenin fraction fall apart into subunits that can be analyzed further. Bietz and Wall (1972) separated these glutenin subunits using SDS-PAGE. These subunits can be divided into two groups: 1) high molecular weight glutenin subunits (HMWGS) and 2) low molecular weight glutenin subunits (LMWGS), (Shewry et al. 1992). Payne and Lawrence (1983) extensively catalogued the genes coding for HMW subunits.

At present, the genetic location of all known HMW-GS has been reported and most HMW-GS have been cloned and sequenced. This has revealed strong structural similarities, but also characteristic differences. One of these differences, that is related to polymerisation behaviour and therefore regarded important, is related to the number of free SH-groups. Based on this distinction, HMW-GS are classified into two subgroups: X-type and Y-type subunits. The N-termini of the X-type subunits contain 4 cysteines (4 –SH) and the N-termini of Y-type subunits contain 5 cysteines (5 – SH). Also, the X-type HMW-GS have a lower electrophoretic mobility than Y-type subunits.

Payne et al. (1987) numbered the HMW-GS using electrophoretic mobility. The short hand notation for HMW-GS coded on loci 1A, 1B, 1D of wheat cultivar Soissons is: 2*, 7 + 8, 5 + 10. In figure 1.2, an example is given of four different HMW glutenin subunit patterns. The subgroups (X,Y) can be noted , like in: 'Dx5 + Dy10'.

Figure 1.2   HMW bands for Olympic Gabo lines. From left to right: wild-type, double deletion line with only HMW 5+10, double deletion with only HMW 1, double deletion with only HMW 17+18.

1.3.4   *Relevance of HMW-GS to end-use quality*

HMW-GS have been of very special interest in understanding wheat flour quality. This is because Payne revealed that HMW-GS 5+10 are associated with good bread-making quality flours and HMW-GS 2+12 are associated with poor bread-making quality (Payne et al,. 1987). This observation compelled Payne to set up a scoring system for bread-making quality based on HMW-GS. Although the initial observations of Payne for the difference between 5+10 and 2 +12 still hold (Lafiandra et al. 1993), the overall the correctness of this type of quality scoring is questionable (Hamer et al., 1999; Nicolas et al., 1997). Nevertheless, it provided an important tool to breeders, because it allowed them to better target wheat flour quality traits.

Uthayakumaran et al. (1999) show a relation between glutenin to gliadin ratio and wheat flour dough properties. Glutenin content clearly affects dough strength and this is linked with the HMW-GS composition. For an example flour from Glenlea with a double set of Bx7 is well known for its dough strength. In their review Dupont and Altenbach (2003) point out that HMW-GS quantity and glutenin quantity are genetically determined. Less is known about the effect of glutenin HMW/LMW ratio.

1.3.5   The role of LMW-GS

The HMW-GS have received a lot of attention, however Graveland et al. (1985), Keck et al. (1995) demonstrated that glutenin consists of HMW-GS *and* LMW-GS. The HMW-GS are considered as the main determinants of glutenin polymer size. Like HMW-GS there are A, B and D LMW-GS. The LMW-GS are not extensively studied in this thesis, the focus is on HMW-GS. It is interesting however to note a few ideas around LMW-GS. Kasarda (1999) points at D-LMW-GS, with only 1 free –SH. These LMW-GS may have a function as terminators of the glutenin polymerization processes. Hence, some LMW-GS may be determinants glutenin size as well. The LMW-GS have long been underestimated, only recently their role has been thoroughly reviewed by D'Ovidio and Masci (2004). Also, Hamer et al. (2004) showed that the D-LMW-GS affect dough mixing properties. This indicates that LMW-GS surely deserved more attention soon, befitting their importance in glutenin functionality.

1.4   The structure of glutenin

The discovery of Payne led to many publications that were based on a paradigm that baking quality was related to the presence or absence of individual HMWGS. It is now generally accepted that the situation is more complex and that it is the structure of the glutenin protein network that is important.

Revealing that glutenin consists of HMW-GS and LMW-GS motivated researchers to unravel the specific molecular structure. When more is learned about glutenin structure, perhaps more could be understood about the unique physico-chemical properties in dough. By reduction of glutenin (Kasarda 1999) and identification of important disulphide bonds, oligomers of HMW-GS and LMW-GS could be identified. Fractionation of glutenins and its subunits gave way to propose various chemical models for glutenin (Ewart (1968), Graveland et al. (1985), Gao et al. (1992), Belton (1999)). However, the complete glutenin structure is very large and insoluble, hampering a biochemical approach to unravel glutenin structure. The best description still is that glutenins are HMW-GS and LMW-GS that form disulfide linked 'polymers' (Shewry et al. 1989, 1992; Keck et al. 1995). There is general agreement on the importance of glutenin 'polymers' in determining dough properties; there is no consensus on the structure of glutenin 'polymers'. The more it is tried to separate glutenin into its HMWGS and LMWGS, the higher the distance with the original glutenin 'polymeric' structures in flour, dough and thereby perhaps the lower the relevance of experimental findings.

1.5   The importance of very highly aggregated glutenin networks

Breeding, growing and selecting wheat for quality requires a model linking composition to technological properties. Information on how composition affects glutenin network structure in terms of amount and properties, and how this in turn links to technological properties could lead to such a model. However, despite the considerable research done to further elucidate glutenin structure, the structural basis of gluten properties, is still an open question (Lefebvre and van Vliet 2003). This question summarizes the main aim of this thesis: What is the structural basis that controls gluten network properties and how is this structural basis related to the glutenins?

The method developed by Graveland et al. (1980) isolates a glutenin-gel by extraction and centrifugation with SDS. GMP consists of HMWGS and LMWGS. In this thesis an approach, based on the glutenin macro-polymer is used, to get better understanding of the factors affecting glutenin functionality in dough. The approach is described in detail in the following sections and paragraphs

1.5.1   The glutenin network: a combination of chemical and physical interactions.

The gluten network structure essentially rests upon glutenins. Highly insoluble glutenin fractions like GMP can be viewed as protein fractions that contain the main building blocks of the glutenin network in dough. With some exceptions, in most of the literature glutenin network formation and strength is related to disulphide bond formation. However, Lefebvre (2000) and Bloksma (1972) both have stated that just a small portion of possible disulphide bonds in the glutenin network is relevant for its rheological properties. Also, Tkachuk and Hlynka (1968) demonstrated that H-bridges play an important role. More recently, Belton (1999) proposed that gluten elasticity is based on H-bridges, via a 'loop-and-train' model. Although heavily debated, it was also proposed that it is not the disulphide bonds, but dityrosine cross-links that determine dough rheology (Tilley et al. (2001). Clearly, in addition to disulphide bonds, other protein interactions must be taken into account. In this respect, it is also important to define the length scale relevant for visco-elasticity. Again, this is debated. Belton (2005) states that it is at the molecular level; interactions between sections of glutenin molecules are relevant. According to Lefebvre and van Vliet (2003) the relevant structures affecting dough properties are at the meso-scopic length scale. As water is a bad solvent for gluten and glutenins, the glutenin network can be best considered to be a colloidal particle network held together by physical interactions. The hyper aggregation model of Hamer and Van Vliet (2000) takes covalent and non-covalent interactions into account to explain gluten(in) structure at various length scales. It uses a meso-scopic (ca 10 – 100μ) glutenin particle as the essential element in building-up the network.

1.5.2   The hyper-aggregation model

The hyper-aggregation model (Hamer and Van Vliet, 2000) presents a framework that views the glutenin network as a particle network in which both covalent and non-covalent processes are included.
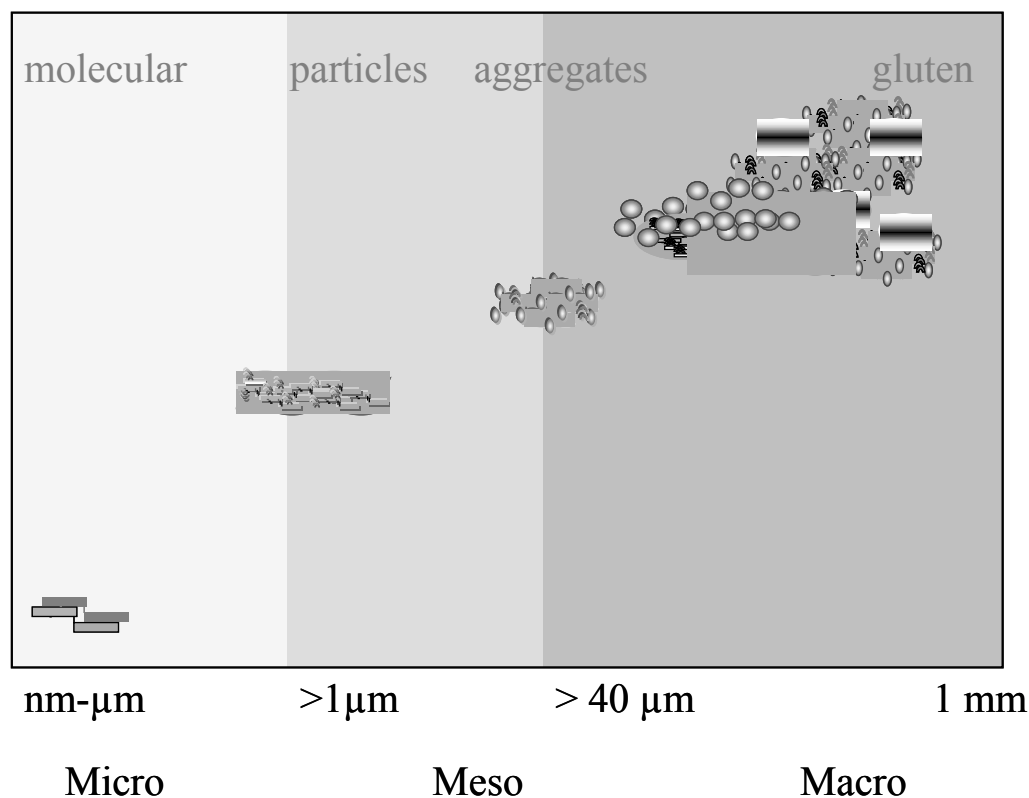

Figure 1.3   Schematic representation of the hyper-aggregation model

This model provides a basis to study the combined effect of different parameters on glutenin aggregation and can be briefly described as follows:

At level one, HMW-GS and LMW-GS from covalently linked polymers. Only covalent bonds are encountered at his level. The position of the disulphide bonds is determined by protein conformation, position of thiol groups and stabilizing effects like hydrogen bonds and other bonds. This step is determined by the presence of individual glutenin subunits, and their ability to propagate or terminate the network. At this level, genetically determined glutenin subunit composition will be important. At the second level the covalently stabilized glutenin polymers form larger aggregates by physical interactions: hydrogen bonds, electrostatic, hydrophobic interactions. This aggregation leads to close associations between protein chains allowing additional disulphide

bonds to be formed thus stabilizing the aggregate. The glutenin subunit composition of the particles formed by level I aggregation will largely determine the size of the level II aggregates. It is expected that level II aggregates play a role in affecting dough properties during certain stages of mixing and resting. At the third level, further aggregation occurs by physical interactions only. Here interactions with non-protein constituents come into play as well, since the aggregates have entered a size range of for example starch or fibre particles. The final size of the level III aggregates, their interaction properties and glutenin network quality, is predominantly affected by process conditions used.

1.6   Aim and objectives of this thesis

The general aim of this thesis is:

"What is the structural basis that controls gluten network properties and how is this structural basis related to the glutenins?"

We used the hyper-aggregation model as a guide to approach this research aim. This requires the thorough examination of GMP properties at various levels. Therefore, the following objectives were set out:

- to identify GMP protein network characteristics;
- to understand if and to what extent this network structure can be used to explain differences in dough mixing requirements between varieties of different technological quality;
- to understand how and to what extent GMP is involved in dough rheological properties after resting;
- to further clarify the physical and chemical changes of the glutenin properties during mixing
- to study how HMWGS composition effects GMP and its properties and how this in turn affects dough mixing and rheological properties.
- to study if the results of these studies can be integrated into one model linking composition to structure to functionality.

1.7   Outline of this thesis

In order to show the general setup of this thesis and the various connections between chapters, a schematic outline of this thesis is given in figure 1.4.
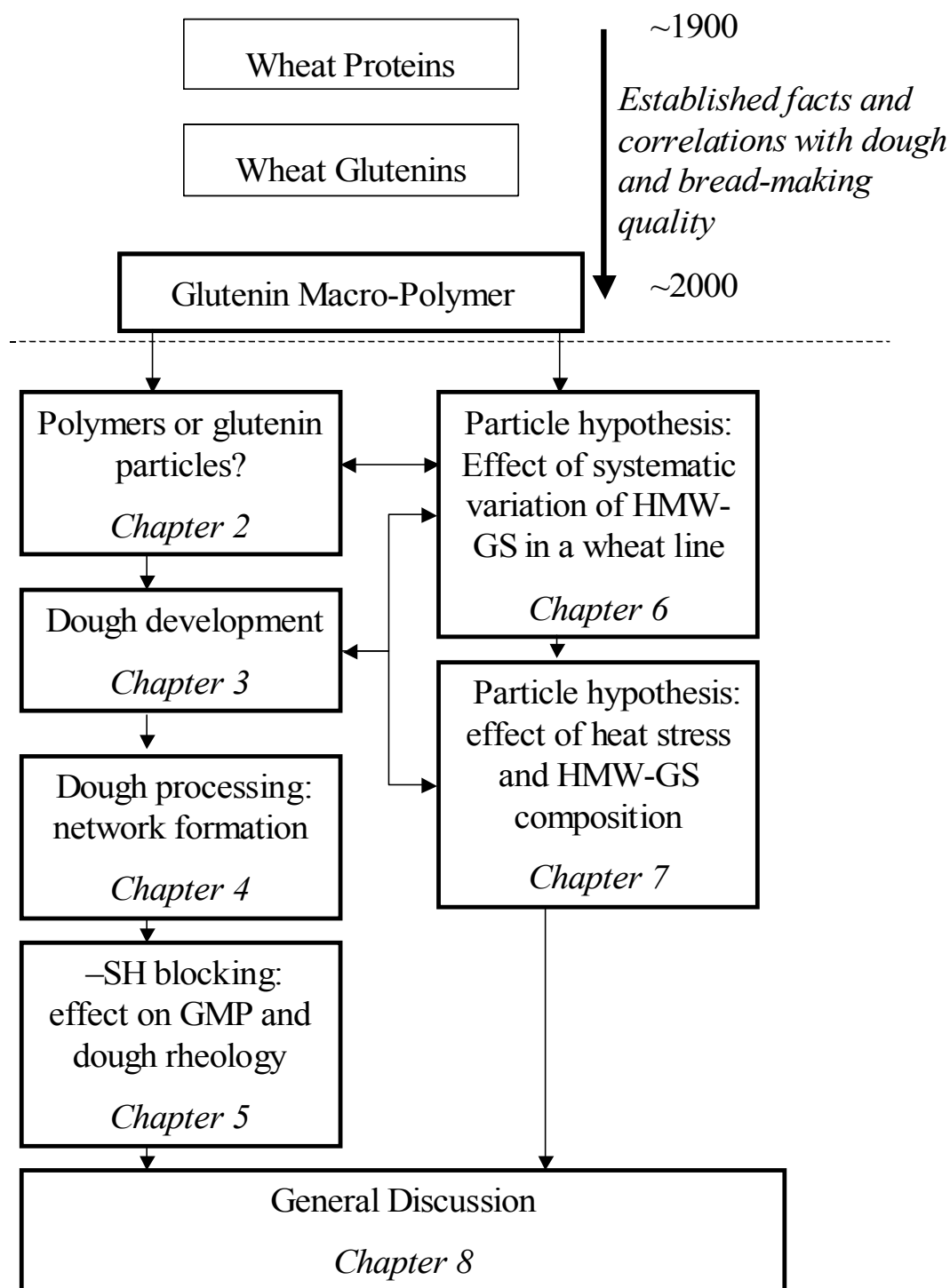

Figure 1.4   A schematic outline of the thesis

Chapter 2 deals with the GMP-fraction. It addresses the physical chemical nature of the GMP-fraction. In chapter 3 the relation between this fraction and dough mixing behaviour is studied.. In chapter 4, various dough mixing regimes and resting times are used to study the how changes in the glutenin network relate to changes in dough rheological properties, and to what extent this can be explained using the Hyper-aggregation model. Since both physical and chemical processes play a role in the formation of glutenin networks, chapter 5 investigates if and how chemical reactions related to the equilibrium between –SH ⇔ S-S effect dough processing and final dough properties. In chapters 2 – 5 commercial flour varieties are used (Classic, Baldus, Soissons, Estica, Amazone, Roblin, AC Phil). In chapters 6 and 7 another approach is followed, using specific wheat lines from breeding and growing programmes. These chapters focus on the generation of glutenin particles and how this is controlled by either genetic or environmental factors. Chapter 6 studies the role of HMW-GS composition in affecting glutenin size and functionality in dough. In chapter 7 special wheat lines, grown under different conditions of heat stress are used, to study how this effects glutenin network formation and resulting flour technological properties Chapter 8 provides a general discussion, questioning the validity of the approaches followed, the results obtained and how these fit with the hyper-aggregation model. Chapter 8 finishes with the possible impact of our findings for the cereals industry.

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2

GLUTENIN MACROPOLYMER: A GEL FORMED BY GLUTENIN PARTICLES

Don C., Lichtendonk W.J., Plijter J.J. , Hamer R.J., 2003. Glutenin Macropolymer: a gel formed by particles. Journal of Cereal Science 37, 1-7.

Abstract

The quality of wheat-based foods and the processing properties of wheat flour dough are strongly related to the presence and properties of very large glutenin protein aggregates. These very large aggregates are insoluble in 1.5 % (w/v) SDS and can be recovered after ultracentrifugation as a gel, the so-called Glutenin Macro Polymer (GMP) fraction. GMP quantity and gel properties strongly correlate with flour technological quality, but details on factors governing GMP quantity and properties is still missing. We therefore studied factors governing GMP formation. Flour from four wheat varieties: Galahad-7, Caprimus, Soissons and Classic were used in this study. GMP was isolated from each of the flour samples and characterised. Plateau values of G' paralleled differences in wheat quality. Further detail was obtained by studying dispersions of GMP in 1.5 % SDS. Re-aggregation, viscometry and Confocal Scanning Laser Microscopy confirmed that GMP consists of very large particles, able to form a gel. Clear differences in average particle size and re-aggregation (gel-forming) properties could be measured. The size and shape of the particles point to a possible origin from the protein bodies in immature wheat endosperm.

Key word index: SDS unextractable glutenins, GMP, particle network

Abbreviations: SDS = Sodium Dodecyl Sulphate, GMP = Glutenin Macro Polymer, HMWGS = High Molecular Weight Glutenin Subunits, LMWGS = Low Molecular Weight Glutenin Subunits, CSLM = Confocal Scanning Laser Microscopy, FITC = Fluorescent Protein Label, PE = Petroleum-ether

2.1   Introduction

The main fraction (85%) of wheat flour protein is gluten. Native gluten consists of gliadins and glutenins[1]. It has been generally accepted that the bread-making quality of wheat flour is related to the presence and properties of gluten proteins. The gliadin fraction has been reported to contribute to the viscous properties of wheat flour dough[1]. Whereas the glutenin fraction of wheat gluten has long been considered to have a prominent role in the strengthening of dough[2]. Part of the glutenin polymers can be isolated as an SDS insoluble gel-layer[3], named Glutenin Macro Polymer (GMP). GMP quantity correlates strongly with the elastic properties of dough and bread loaf volume[4]. Independent from the studies on GMP as the key to wheat quality prediction, the quantity of glutenin solubilized by controlled sonication has also been reported to strongly correlate with bread-making quality[5]. Pritchard et al.[6] have demonstrated that GMP dynamic rheological properties (G'), are also very relevant, due to their strong correlation with bread loaf volume and dough extensibility. Other studies have indicated and confirmed these results[7-9].

GMP consists of very large structures of both high molecular and low molecular weight glutenin subunits (HMWGS and LMWGS respectively). Several models, mainly based on the ability of the subunits to form inter chain disulfide bonds, have been proposed for the structure of the glutenin network[10-12], but no consensus has been reached. A link is suspected between HMWGS subunit composition and glutenin network properties. Payne et al[13] have demonstrated a positive correlation with loaf volume if HMWGS 5+10 are present. HMWGS are without doubt important in determining dough properties. Varieties lacking HMWGS (triple null varieties) are unable to form a visco-elastic gluten. Dough from a variety like Galahad-7, only having HMWGS-7, is extremely extensible and hardly exhibits elastic properties. In contrast, a variety like Glenlea (having two copies of HMWGS-7) is over-strong. Both Lafiandra et al[14] and Popineau et al[15] have demonstrated that the presence of HMWGS 5+10 is associated with a visco-elastic gluten and that gluten visco-elasticity substantially decreases when HMWGS 5+10 are substituted with HMWGS 2+12. It is clear that there must be a link between HMWGS composition, GMP and gluten properties. In the present study we set out to better understand the factors determining the formation and properties of GMP. Hamer and van Vliet have recently proposed[16] that GMP is a gel formed by both physical and chemical interactions. In doing so, they have pointed to the plausibility of the fact that particle-particle interactions largely determine GMP formation. When GMP is hypothesised to be a particle network or aggregate gel, it is clear that particle properties must be investigated. In order to unravel the particle nature of GMP, we have studied GMP formation and underlying

properties using flour from four technologically different varieties. In this way we expect to provide a more in-depth understanding of the particle nature of GMP and close in on factors determining wheat quality.

2.2   Experimental

2.2.1   Wheat flour

Flour was obtained from the following varieties: Galahad-7, Caprimus, Soissons and Classic. Protein content, HMWGS composition and an indication of quality are presented in table 2.1.

Table 2.1   Flour protein content, glutenin HMW-GS composition and wheat flour quality of flour from Galahad-7, Caprimus, Soissons and Classic

Wheat variety	Protein content of flour (%)	HMW subunit composition	Flour technological classification
Galahad 7	11.5	7	Very weak
Caprimus	10.3	6+8, 2+12	Weak
Soissons	10.6	2*, 7+8, 5+10	Strong
Classic	13.7	1, 7, 5+10	Very strong

2.2.2   Isolation of GMP

1.4g of petroleum ether defatted (percolation method) flour was suspended in 28 mL 1.5% SDS (SDS analytical grade from Merck™) and centrifuged at 80.000 g for 30 minutes at 20°C in a Kontron Ultracentrifuge[3]. The supernatant was decanted and the gel-layer collected as GMP. This procedure results in the removal of most, but not all of the soluble gluten proteins. Typically, the GMP gel contains ca 15-30 % soluble proteins that can be further removed by repeated washing. The GMP gel itself mainly consists of HMWGS and LMWGS[17].

2.2.3   Rheology of GMP gels

Material (1g) was carefully taken from the top of the gel and transferred into the measuring cell of a Bohlin VOR rheometer (Bohlin, Sweden). The cell consisted of two parallel plates (d=30 mm) with a gap of 1 mm. Measurements were performed at 20°C in a strain sweep mode at amplitudes ranging from 1% to 100%. Data were expressed as G' vs strain or delta (δ) vs strain.

2.2.4   GMP dispersion

GMP dispersions were prepared by transferring 1g of GMP gel to a tube containing 10 mL 1.5% (w/v) SDS solution. The gel was mixed with the solvent by briefly stirring with a spatula. Then the tube was sealed and placed on a roller-bank for 3h at ambient temperature. This produced a visually homogenous, opalescent dispersion, indicating that part of the protein polymers are not dissolved, which was used for further characterisation.

2.2.5   Protein analysis

Flour protein content was measured by the Kjeldahl method. Protein content of GMP and GMP dispersions were measured using an UV absorption method[18]. The UV method was successfully calibrated with a set of Kjeldahl protein values of GMP.

2.2.6   Starch analysis

Starch content of GMP was determined with a test-kit provided by Boehringer Mannheim – UV-method for the determination of native starch- Cat. No. 207748. The method was calibrated with a set of reference starch dispersions.

2.2.7   Re-aggregation / dis-aggregation experiments

Dispersions of GMP were diluted with 1.5 % (w/v) SDS solution to a concentration of 0.5, 1 or 2 mg/mL respectively. Each dispersion was then centrifuged for 30 min at 80000 g at 20°C. Each supernatant or newly formed gel was weighed and analysed for protein content. Results were expressed as % protein recovered in the gel phase.

2.2.8   The rheological characterisation of GMP dispersions

The viscosity of GMP dispersions could be measured using a Ubbelohde capillary viscometer. The pass through time was in the order of 300-400 seconds to optimise accuracy. Measurments were performed in duplicate. Between measurements the Ubbelohde was extensively rinsed with 1.5% SDS solution. Cleaning and measurements must be done cautiously in order to prevent air bubbles in the solution. Data were expressed as reduced viscosity L/g.

2.2.9   Confocal Scanning Laser Microscopy

Freshly prepared samples of GMP dispersions were stained for protein with FITC and observed using a Leica TCS NT Confocal Laser Scanning Microscope. In order to optimize contrast, GMP

dispersions with protein concentrations varying from 0.1 mg/mL protein to 5.0 mg/mL protein were observed with CSLM.

2.3   Results and discussion

GMP was isolated from each of the flour samples. Data on GMP wet weight, protein concentration, protein quantity and starch concentration of each GMP isolate are presented in table 2.2.

Table 2.2   The GMP wet weight and protein content of GMP gels isolated from Galahad-7, Caprimus, Soissons and Classic wheat flour

Wheat variety	GMP wet weight (g)	GMP protein concentration (mg/g)	Total GMP protein quantity (mg)	Starch concentration (mg/g)
Galahad 7	0.7 ± 0.1	14.2 ± 0.6	9.9 ± 0.7	2.9 ± 0.4
Caprimus	3.1 ± 0.2	8.0 ± 0.7	24.8 ± 1.2	2.9 ± 0.4
Soissons	2.9 ± 0.3	10.6 ± 0.7	30.7 ± 2.1	10.2 ± 1.2
Classic	3.9 ± 0.2	12.1 ± 0.8	47.2 ± 3.1	4.7 ± 0.9

n = 5

Table 2.2 shows that the amount of GMP obtained varies widely for the four varieties. Especially GMP protein quantity increases from: Galahad-7 < Caprimus < Soissons < Classic. In relation to total flour protein the percentage of glutenin proteins, recovered in the GMP fraction is 6%, 17%, 21% and 25% for Galahad-7, Caprimus, Soissons and Classic respectively. As reported earlier[5] the glutenin quantity parallels the variation in technological quality. GMP typically contains some starch. The variations in starch content are not completely clear, but it appears that GMP from strong varieties contain more starch.

2.3.1   Rheological characterisation of GMP

In figure 2.1 the typical strain vs G' plots are presented for each of the four varieties tested. Without exception each GMP demonstrates typical gel like behaviour.

The following plateau values of G' in the strain sweep were observed: 6, 13, 38 and 100 Pa for Galahad-7, Caprimus, Soissons and Classic respectively. Although there is starch in GMP, Bekkers et al. demonstrated that gel rigidity (G') is largely determined by the size and interactions of HMW glutenins[17].
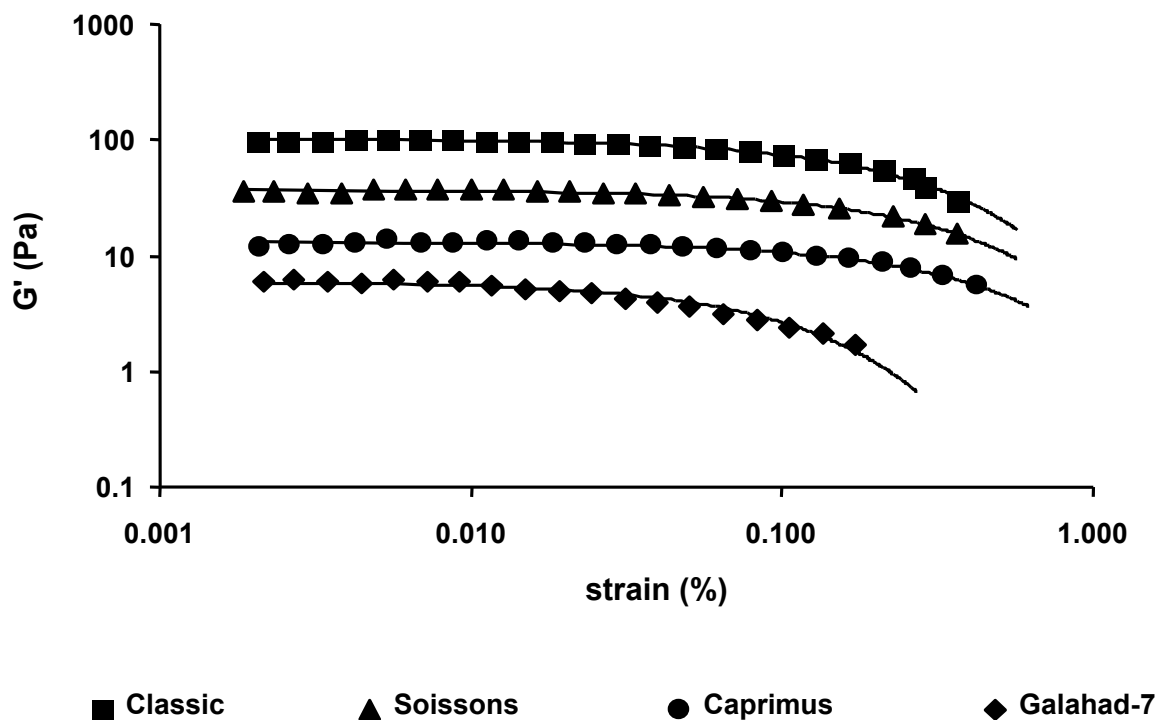
Figure 2.1   *The G' versus strain for GMP from Classic (squares), Soissons (triangles), Caprimus (circles) and Galahad-7 (diamonds).*

With increasing strain, the gel starts to deteriorate as evident from a decrease in G'. Especially GMP from Galahad-7 was observed to deteriorate at comparatively low strain, an indication of a weak gel (fig. 2.1). The ratio of viscous to elastic behaviour is reflected in δ. At a δ lower than 45°, elastic behaviour predominates in the structure, whereas a delta higher than 45° is representative of more viscous behaviour. The δ values are presented in figure 2.2.
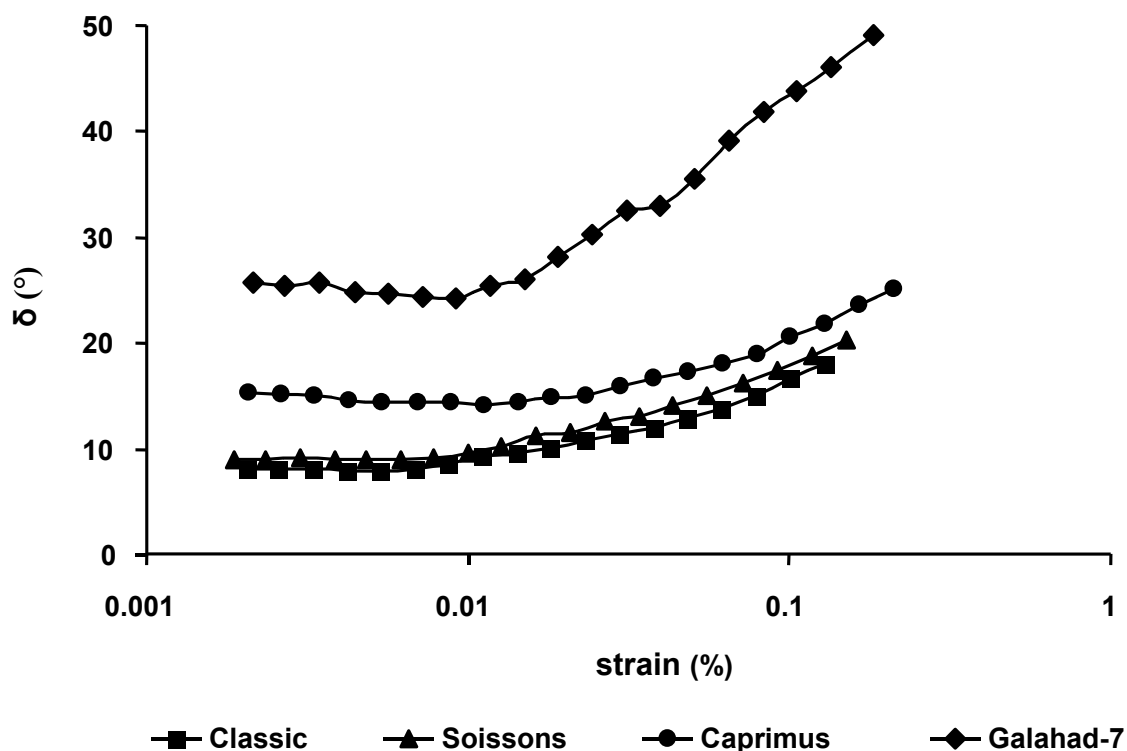
Figure 2.2   The δ versus strain for GMP from Classic (squares), Soissons (triangles), Caprimus (circles) and Galahad-7 (diamonds).

Values of delta in the plateau region for Galahad -7, Caprimus, Soissons and Classic are 25°, 14°C, 9° and 8° respectively. Again, Galahad-7 has the weakest GMP. With Galahad-7 GMP, the δ is increased the most (>45°) when the strain was increased and passed the plateau region. This is indicative of a very weakly stabilised network. It is the G' in the plateau region that parallels best the variation in technological classification.

2.3.2   Re-aggregation / dis-aggregation experiments

If the assumption is followed that GMP is a particle network stabilised by a combination of covalent and non-covalent interactions, classic physical theory on dilute suspensions and macromolecular solutions point to the importance of both concentration and properties of particles[19]. We investigated this by performing re-aggregation studies at different concentrations of protein in the suspension. The results of the re-aggregation experiments are shown in figure 2.3.
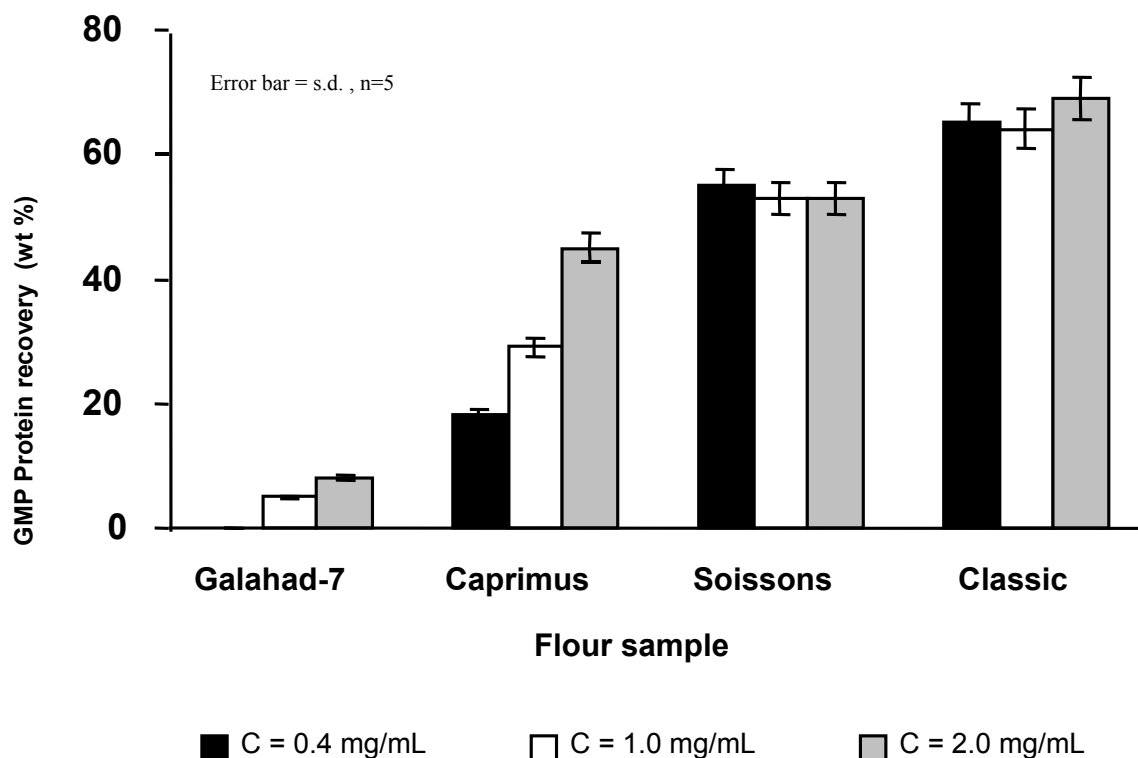
Figure 2.3   The recovered GMP weight in dis-aggregation / re-aggregation experiments; see squares for the used concentrations

With Galahad-7 and Caprimus lowering the protein concentration leads to a decrease in the amount of gel recovered. In contrast, with Soissons and Classic the recovery is not affected by protein concentration. The fact that the maximal yield < 100 % is due to the dilution of soluble proteins present in the first GMP fraction. It is clear from the experiment reported here that differences in GMP cannot be fully explained by differences in concentration. The recoveries obtained are also affected by differences in particle size and associative properties. The poor recovery at lower protein concentration for Galahad-7 and Caprimus is likely due to an increased glutenin solubilization at lower protein concentration. The firmer and more elastic GMP from '5+10' varieties Classic and Soissons appear to consist of comparatively stable aggregates. G' and δ (fig. 2.1 and 2.2) are related to the extent and strength of the interactions that are considered to stabilise GMP particle structure. The results in figure 2.3 indicate that the physical properties that fortify GMP-gel are reflected in a high recovery of GMP in a re-aggregation experiment.

2.3.3 Viscometric characterisation of GMP dispersions

Viscometric characterisation of the dispersions was carried out to further reveal differences in particle properties. Figure 2.4 shows the typical concentration vs reduced viscosity plots for the four varieties.
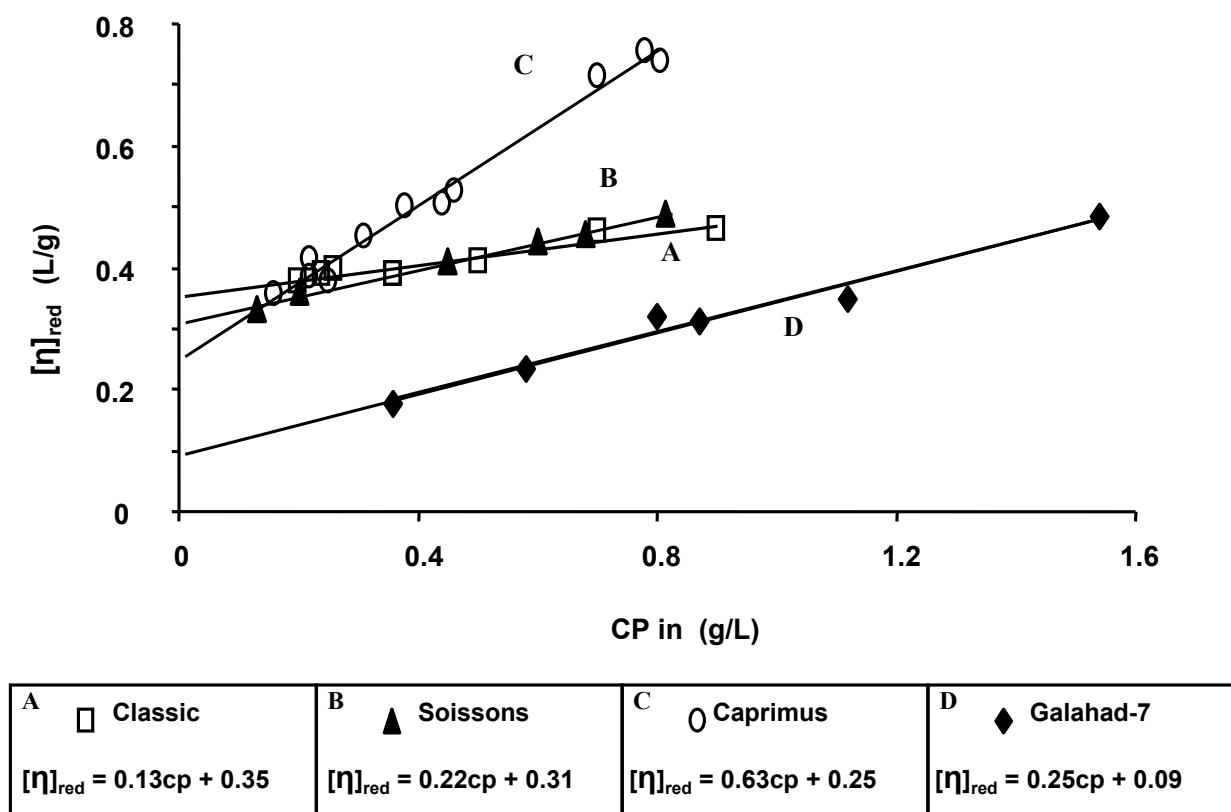

A	☐ Classic	B	▲ Soissons	C	○ Caprimus	D	◆ Galahad-7
$[\eta]_{red} = 0.13cp + 0.35$		$[\eta]_{red} = 0.22cp + 0.31$		$[\eta]_{red} = 0.63cp + 0.25$		$[\eta]_{red} = 0.25cp + 0.09$	

Figure 2.4   The reduced viscosity versus protein concentration (CP) for GMP dispersions (see legend for explanation of symbols and flour samples).
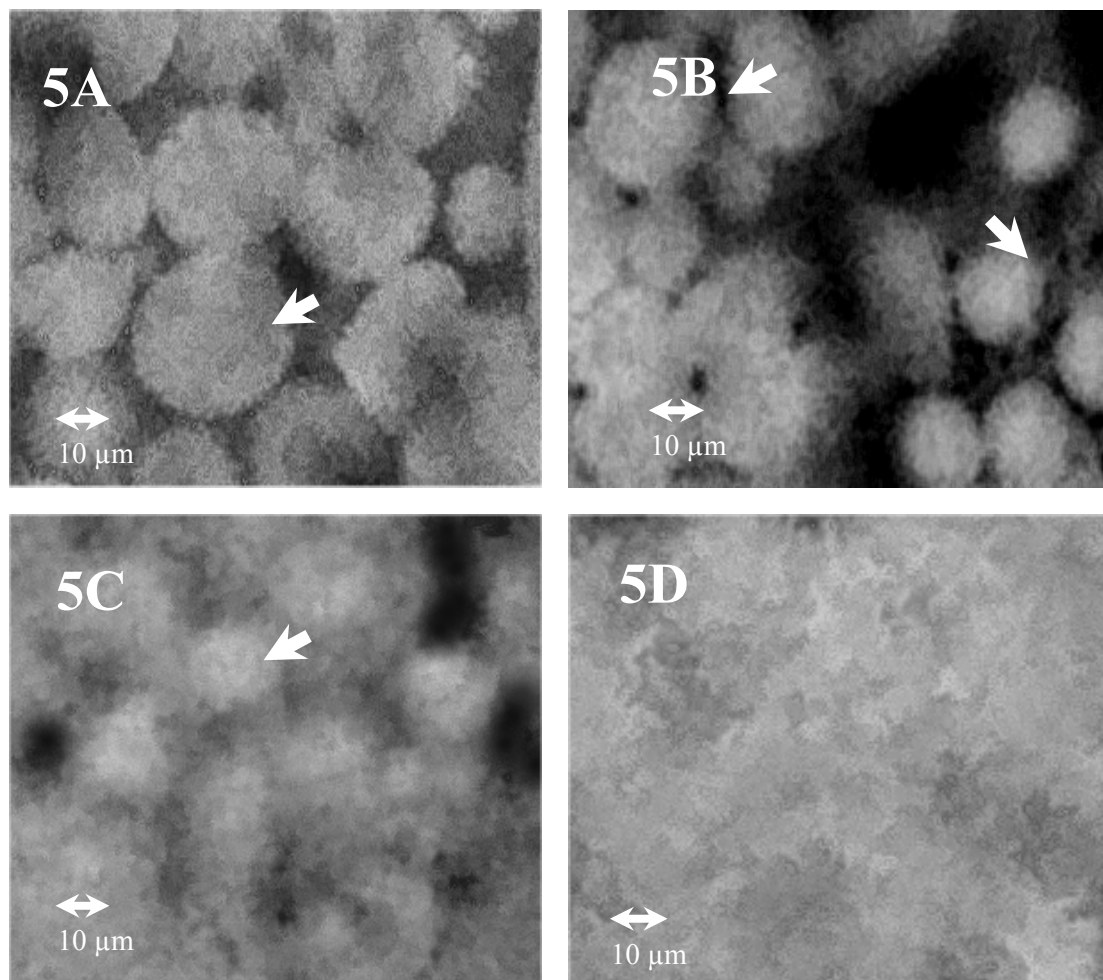
The intercept represents the voluminosity, that is expected to be related to the average size of the suspended particles. The voluminosity is increased from Galahad-7 to Classic. Assuming a spherical shape the relative average particle sizes for Galahad-7, Caprimus, Soissons and Classic can be estimated to be about 1: 8: 12: 15 respectively. We must stress the voluminosity is only related to the average size of the particles present in the GMP dispersion and does not give information on maximal or minimal sizes. Nevertheless, the ratios observed point to considerable differences between the four varieties.

Physical theory states that the rheological properties of a gel are related to the average size and concentration of the contained particles[16]. Clearly the results presented here indicate that the observed differences in average particle size are reflected in observed G' plateau values. On average, the GMP-gels isolated contain about 10 mg/mL of protein. Typical plateau values of G' observed are between 6 - 100 Pa. These are comparatively weak gels. Although it is expected that higher protein concentrations (ca 50-100mg/mL) will lead to a stronger gel, such concentrations cannot be obtained with the present isolation methods. The slope of the lines shown in figure 2.4 indicates differences in particle properties, that are paralleled by the results found with the re-aggregation experiments (fig. 2.3). This suggests that the viscosity vs concentration plots are influenced by GMP particles becoming soluble at lower protein concentration. When this is not the case, the plot for reduced viscosity vs. concentration has a relatively small slope, as can be seen for Soissons and Classic. The recentrifugation experiments have shown that Caprimus GMP is sensitive to concentration. Hence, the slope observed with this variety. With Galahad-7 the low intrinsic viscosity observed already points to solubilisation of particles over the concentration range tested.

2.3.4   Confocal Scanning Laser Microscopy

In general, the results on dispersion viscometry and re-aggregation point to the presence of large particles. We used CSLM to confirm this finding. GMP dispersions were analysed with CSLM at various protein concentrations. Both SDS soluble and insoluble proteins are stained by the method used. This affected contrast. Particles became discernible at a concentration of about 0.5 mg/mL protein. Especially with the two '5+10' varieties, Classic and Soissons, CSLM clearly revealed large (10-30 μm) sphere shaped particles (fig. 2.5A and 2.5B).

With Caprimus (fig. 2.5C) particles are less discernible, due to a lower contrast between soluble and insoluble protein. As observed earlier with Caprimus and especially Galahad-7 (fig. 2.5D), more GMP is solubilized at lower protein concentrations. With Galahad-7 contrast was too low to allow particles to be observed.

Figures 2.5 A-D   CSLM images of GMP dispersions of GMP isolated from: Classic (A), Soissons (B), Caprimus (C) and Galahad-7 (D).

2.3.5   Particles in GMP and their origin

In our aim to better understand GMP formation we found our experimental observations to agree with the earlier proposed hypothesis of a particle network[16]. The average size of the particles as deduced from viscometry and re-aggregation experiments prompted examination of GMP dispersions by CSLM. With this technique we were able to visualise the particles in dispersed GMP. Both the size (ca 5-30 μm) and spherical shape of the GMP particles in figures 2.5A-C reminded us of earlier TEM photographs of glutenin particles by Graveland and -more importantly- of protein bodies in the immature wheat endosperm[20, 21]. Endosperm protein is deposited in protein particles. This is well documented by Evers and Bechtel[21]. The latter are ca 3-5 μm diameter. The larger sizes found in this study (ca 5-30 μm) can be explained by swelling of the insoluble GMP particles in the solvent (1.5 % SDS). It has been confirmed that HMW-GS can be deposited in

dense protein bodies and are assembled into insoluble polymers[22]. Our data makes it plausible that the structural organisation we observed for GMP particles somehow reflects this structure as it was originally formed in the immature wheat endosperm. Soluble proteins present in the endosperm protein particles are lost during our extraction procedure leaving only the SDS insoluble GMP 'skeleton' particles.

2.4   Conclusions

In this chapter we have re-confirmed the importance of GMP and GMP rheological parameters in explaining the variation in technological quality of wheat. The hypothesis that GMP is a particle network was confirmed by GMP dispersion viscometry, re-aggregation and CSLM experiments. Clear differences between GMP particles from different varieties could be observed. These differences in particle properties are expected to have a relation with HMWGS composition and help explain differences in G' plateau values for GMP. Spherical protein particles are present in GMP. The resemblance of such particles with protein deposits in the endosperm[21] suggests that they are related.

Acknowledgements

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3

UNDERSTANDING THE LINK BETWEEN GMP AND DOUGH: FROM GLUTENIN PARTICLES IN FLOUR TOWARDS DEVELOPED DOUGH

Abstract

Clear correlations exist for GMP quantity and rheological properties vs. wheat quality and dough rheological properties, but real insight in understanding these links is still missing. The observation that GMP consists of glutenin particles opens up new possibilities to reveal the underlying mechanism linking glutenin network properties with dough preparation. GMP was isolated from flour of three wheat varieties: Estica, Soissons and Baldus, strongly varying in their mixing requirements (expressed as time-to-peak, TTP). Decrease of GMP quantity and G' vs. mixing energy was confirmed. More detail was obtained by studying the changes in GMP particles when mixing flour into dough. Mixing leads to a decrease in the average size of the particles. Interestingly, the TTP coincided with the work-input at which all particles just became soluble in SDS. At TTP, the average size of the GMP particles was the same for each variety. During mixing particles lost their globule shapes and appeared ruptured. Particle size analysis confirmed that particles were still present near TTP. Analysis of the change in particle size vs. energy input using physical principles revealed the following: 1) mixing energy is the predominant actuator in decreasing GMP particle size, 2) the initial GMP particle size in flour strongly determines the practical mixing requirements 3) the derived mixing energy vs.GMP particle size relationship was shown to be applicable for both Mixograph and Farinograph mixing. Our results demonstrate that, for the flour samples used, glutenin particle size determines TTP and GMP rheology, showing that glutenin particle properties could be a new key to understand the link between GMP and dough.

Key word index: GMP, GMP-particle size, mixing, dough development.

Abbreviations: SDS = Sodium Dodecyl Sulphate, GMP = Glutenin MacroPolymer, HMWGS = High Molecular Weight Glutenin Subunits, LMWGS = Low Molecular Weight Glutenin Subunits, CSLM = Confocal Scanning Laser Microscopy, FITC = Fluorescent Protein Label, PE = Petroleum-ether, SUP = supernatant fraction of GMP isolation, TTP = Time to Peak

3.1   Introduction

Undoubtedly, without the presence and unique properties of wheat gluten it would not be possible to prepare the visco-elastic dough from wheat flour, suitable for bread making[1-3]. Native gluten consists of gliadins and glutenins[4]. The gliadin fraction has been reported to contribute to the viscous properties of wheat flour dough; the glutenin fraction of wheat gluten has been shown to have a prominent role in the strengthening of dough[5]. The glutenin fraction consists of high molecular weight glutenin subunits (HMWGS) and low molecular weight glutenin subunits (LMWGS). Various authors have shown that both the quantity and composition of HMWGS correlates with dough properties and bread-making quality[6-9]. An important fraction of the glutenins can be isolated from flour as a SDS insoluble gel-layer[10], named Glutenin Macro Polymer (GMP). It has been shown that GMP quantity is an indicator of wheat flour quality[11] and that the rigidity of the GMP gel-layer, expressed as the G' (Pa) value obtained from a dynamic rheological measurement, correlates with bread-making quality[12]. Several studies have confirmed the importance of GMP in assessing wheat quality and predicting dough properties[13-15].

Nevertheless, GMP from flour is not the same as GMP from dough, as has already been pointed out by Weegels et al.[16]. Mixing is the key step that transforms wheat flour into dough[17]. When flour, salt, water and additives are combined in order to prepare dough, complex cascades of physical and chemical events have been reported to take place during mixing[18-20]. Glutenin and especially the GMP fraction, is considered to play a pivotal role in this. Uthayakumaran et al[22] have confirmed both effects of protein content as glutenin/gliadin ratio. It was confirmed that a relation between chemical composition and dough rheology exists and that this should be investigated further. However, various glutenin network structures have been proposed on the basis of fractionation and chemical analysis, but no consensus on this subject has been reached[23-25]. More importantly, it still remains difficult to explain dough rheological observations solely on the basis of gluten or glutenin composition data and hypothesised molecular structures. Belitz et al[27] have demonstrated that physical interactions can contribute considerably to gluten-strength. Amend et al[26] revealed the presence of gluten aggregates and strands during mixing. Hamer and Van Vliet[28] have proposed that GMP is a gel formed by both physical and chemical interactions. Recently, Don et al[29] showed that GMP gel isolated from flour contains large glutenin particles (~5 – 50 μm). Independently, Lefebvre et al[30] suggested that the gluten-network is a particle network, hence implying that physical interactions at the meso-scopic (0.1 – 100 μm) level affect dough properties.

The observation of large glutenin particles in GMP opens up new possibilities to reveal the mechanism that links the properties of GMP with dough properties. In the present study we set out to better understand how the GMP particles from flour are affected by mixing. In order to do this we have studied the effect of mixing on GMP from three flour varieties, strongly varying in their mixing requirements (TTP). GMP fractions were isolated from dough at various stages of mixing. We studied changes in physical properties using GMP-gel rheology, dispersion viscometry, particle size analysis and CSLM. In this way we expect to provide new insights on how mixing affects the GMP particles on the meso-scopic level and provide a better understanding of the link between GMP and dough.

3.2 Experimental

3.2.1 Wheat flour samples

Flour was obtained from the following varieties: Estica, Soissons and Baldus, by milling on a Buhler mill, extraction rates were 77.7%, 78.4% and 78.5% respectively. Protein content (based on dry matter), HMWGS composition and TTP are presented in table 3.1.

Table 3.1   The protein content, HMW-GS composition and time-to-peak of wheat flour from varieties Estica, Soissons and Baldus

Wheat variety	Flour protein content (%)	HMW subunit composition	TTP (min)
Estica	12.4	6+8, 2+12	5
Soissons	10.6	2*, 7+8, 5+10	10
Baldus	12.5	1, 14+15, 5+10	27

This set of three was used in a thorough analysis of GMP properties. For the mixing studies, 9 different flour varieties (also Buhler milled, extraction 76-79%) that vary in mixing requirements were added to this first set of three. Protein content for the set of 9 was within a range of $12.0 \pm 1.5\%$.

3.2.2 Protein analysis

Flour protein content was measured by the Kjeldahl method. Protein content of GMP and GMP dispersions were measured using an UV absorption method[31]. The UV method was successfully calibrated with a set of Kjeldahl protein values of GMP.

3.2.3 Isolation of GMP from flour

Flour defatted with petroleum ether was suspended in 1.5% SDS (1.4 g flour in 28 mL, SDS analytical grade from Merck™) and centrifuged at 80.000 g for 30 minutes at 20°C in a Kontron Ultracentrifuge[10]. The supernatant was decanted and the gel-layer collected as GMP.

3.2.4 Isolation of GMP from mixed dough sample

The dough sample was freeze-dried after mixing. The freeze-dried dough was powdered on a Retsch hammer-mill using a sieve of 0.25mm. Isolation of GMP was done as described for isolation from flour.

3.2.5 GMP dispersion

GMP dispersions were prepared by transferring 1g of GMP gel to a tube containing 10 mL 1.5% (w/v) SDS solution. The gel was mixed with the solvent by briefly stirring with a spatula. Then the tube was sealed and placed on a roller-bank for 3h at ambient temperature. This produced a visually homogenous, opalescent dispersion, indicating that part of the protein polymers are not dissolved, which was used for further characterisation.

3.2.6 Rheology of GMP gels

1 gram of material was carefully taken from the top of the gel and transferred into the measuring cell of a Bohlin VOR rheometer (Bohlin Instruments). The cell consisted of two parallel plates (d=30 mm) with a gap of 1 mm. Measurements were performed at 20°C in a strain sweep mode at amplitudes ranging from 1% to 100% at 0.15 Hz. Data were expressed as G' vs strain, G' plateau value or delta (δ) vs strain.

3.2.7 Voluminosity of GMP particles

The viscosity of GMP dispersions was measured using an Ubbelohde capillary viscometer. The pass through time was in the order of 300-400 seconds to optimise accuracy. Data were expressed as reduced viscosity L/g.

3.2.8 Coulter Laser particle size analysis

Particle size distributions of diluted GMP dispersions from '5+10' variety Baldus, Soissons and '2+12' variety Estica were determined by laser diffraction using a Coulter LS 130. In this instrument laser light is scattered by the suspended particles and the generated diffraction pattern, which is a composite of the diffraction patterns for all the particles, is measured. For GMP from flour[29] a spherical shape for GMP particles was observed. When particles are assumed spherical, the instrument can convert the composite diffraction pattern into a particle size distribution with 72 classes ranging from 0.3 to 900µm using Fraunhofer theory.

3.2.9 Confocal Scanning Laser Microscopy

Freshly prepared samples of GMP dispersions were stained for protein with FITC (a fluorescent label, specific for proteins) and observed using a Leica TCS NT Confocal Laser Scanning Microscope. GMP dispersions with protein concentration of ~1 mg/mL were observed with CSLM.

3.2.10 Mixing experiments with a Z-blade mixer type

Mixing of defatted flour was performed on a Brabender Plastograph equipped with the 10g Farinograph bowl, a Z-blade mixer type. In the preparation of the dough 2% (w/w) NaCl and water were added and the mixing speed was 63 rpm. The required amount of water was determined according to ICC procedure 115/1[40]. TTP was determined from the recorded Torque vs. time plot. Energy to peak was determined using the Energy vs. time integrator.

3.2.11 Mixing experiments with a pin-mixer

A 2g Mixograph (National Manufacturing Co.) pin-mixer was used to mix 9 flour types that strongly vary in TTP and Estica, Soissons and Baldus were included in this set (defatted). A water bath was used to keep the Mixograph mixing compartment at 30°C. Water addition was according to the Farinograph method (ICC115/1[40]) and also 2% (w/w) salt was used. The integrated value at peak from the midline analysis (Tq x min.) was taken to compare with the energy to peak obtained from the Z-blade mixer.

3.3   Results

GMP was isolated from each of the flour samples. Data reflecting the characteristics of the GMP gel isolated from flour and the GMP dispersions are presented in table 3.2.

Table 3.2   The characteristics of GMP gels and dispersions from flour samples Estica, Soissons and Baldus

Wheat variety	GMP wet weight* (g)	GMP protein concentration* (mg/g)	GMP protein quantity* (mg)	G' plateau value* (Pa)	K'‡	[η]‡ (L/g)
Estica	2.6 ± 0.2	11.2 ± 0.5	29.1 ± 2.2	26 ± 2	1.5	0.26
Soissons	2.9 ± 0.3	10.6 ± 0.7	30.7 ± 2.6	43 ± 3	0.71	0.31
Baldus	3.3 ± 0.4	12.9 ± 0.8	42.6 ± 3.4	158 ± 11	0.31	0.36

* n = 5, ± s.d.
‡ K' and [η] from linear regression of reduced viscosity vs. concentration plot with $R^2 > 0.95$

It is clear that samples not only differ in technological quality but also in GMP wet weight, the protein concentration of the GMP-gel (mg protein per gram of wet gel), total GMP protein quantity, G' plateau values and intrinsic viscosity of GMP dispersions. The region where the gel rigidity does not decrease vs. increased deformation is called the G' plateau value. Here the G' plateau values were resp. 26, 43 and 158 Pa for Estica, Soissons and Baldus. The differences in G' plateau values cannot be explained on the basis of differences in GMP-gel protein concentration (~11-13 mg/mL). Table 3.2 also shows the intrinsic viscosities of dispersed GMP isolated from flour: 0.36, 0.31, 0.26 L/g for Baldus, Soissons and Estica respectively. The intrinsic viscosity of the GMP dispersion is a measure of the average GMP particle voluminosity. GMP mainly consists of HMW and LMW glutenins, both Weegels[16] and Skerrit[21] have presented an analysis of GMP composition. From reduced viscosity vs. concentration data a value known as K' (Huggins constant) can be calculated[32]. The value of K' is a measure of glutenin particle-particle interactions[33]. Apparently K' values decrease from Estica > Soissons > Baldus. This result suggests that also interactions between dispersed GMP particles are different.

It is already known since 1984 that mixing leads to a decrease in GMP[34]. Figure 3.1 confirms this for Estica, Soissons and Baldus.   Although the wet weight of GMP is decreased, protein

concentration in the GMP gel does not decrease as much as the wet weight. This result shows that quantity is affected, but not concentration.

For Estica, Soissons and Baldus after mixing times of respectively: ~5, 10 and 27 minutes the GMP wet weights are < 0.1g. This illustrates that when TTP is reached, almost no GMP fraction can be isolated anymore.
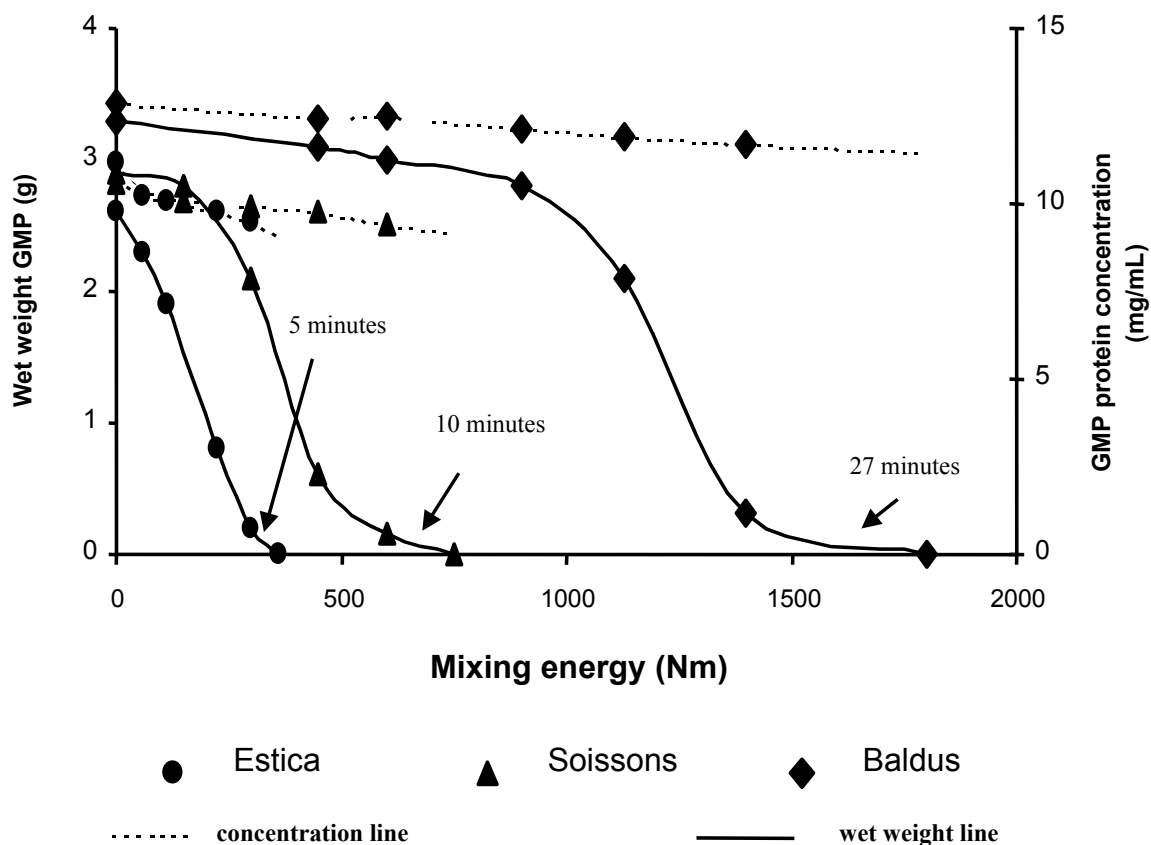

Figure 3.1   Decrease of the GMP-gel wet weight (y-axis left) versus mixing energy for varieties Baldus, Soissons and Estica and the respective protein content of the GMP-gels (y-axis right).

The G' plateau values of GMP gel proteins isolated from mixed flour dough versus mixing energy were measured. A typical decrease of G' versus mixing energy is represented in figure 3.2.
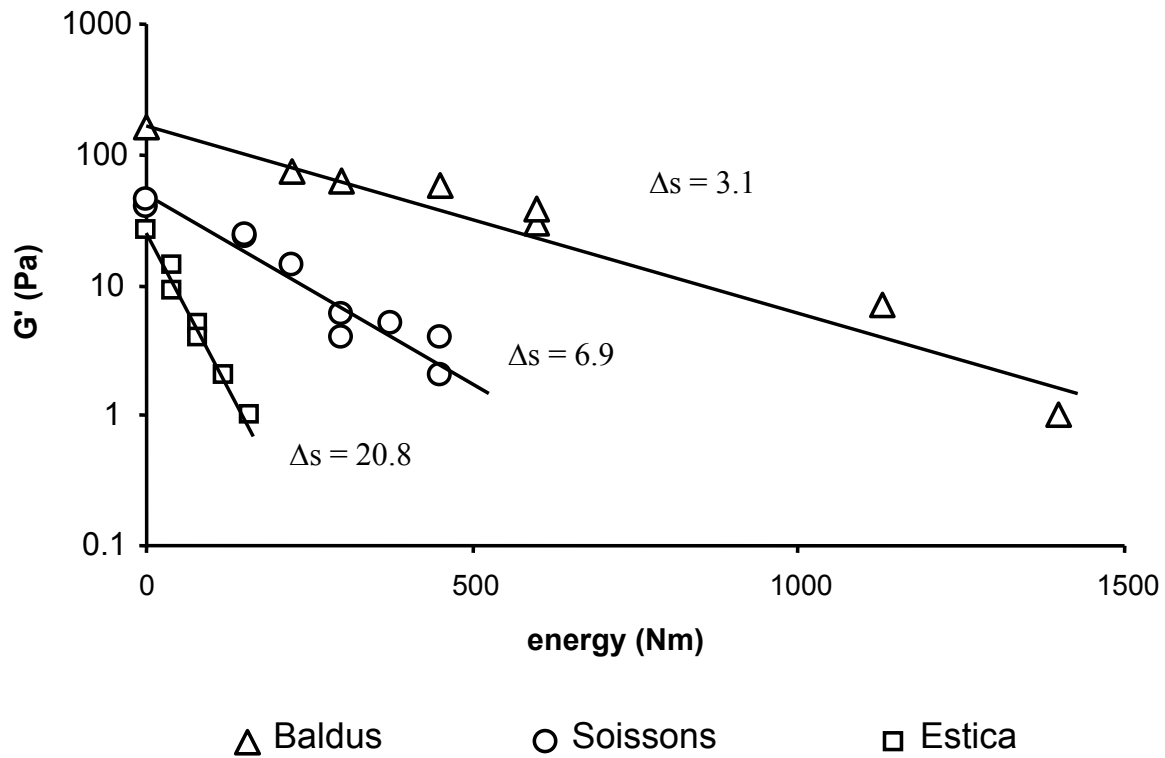
Figure 3.2   The decrease of GMP-gel strength expressed as G' vs. mixing energy applied to the respective dough by a Z-blade mixer (see legend for explanation of symbols and variety).

The semi-log plot shows a straight line for G' versus energy, indicating that GMP stiffness decreases with increasing energy input. Slopes are indicated by Δs, giving the rate of G' decrease. Given the fact that GMP gel protein concentration does not decrease much (fig. 3.1) the decrease in G' must be due to changes in GMP particle properties. GMP particle sizes can be decreased by cleavage of di-sulphide bonds on the one hand, but on the other hand it is also possible that GMP particles are disrupted by a physical mechanism.

GMP dispersion viscosities were measured to demonstrate how the glutenin particles are affected by mixing. Figure 3.3 shows the intrinsic viscosities versus energy plots for GMP isolated from flour and dough samples.
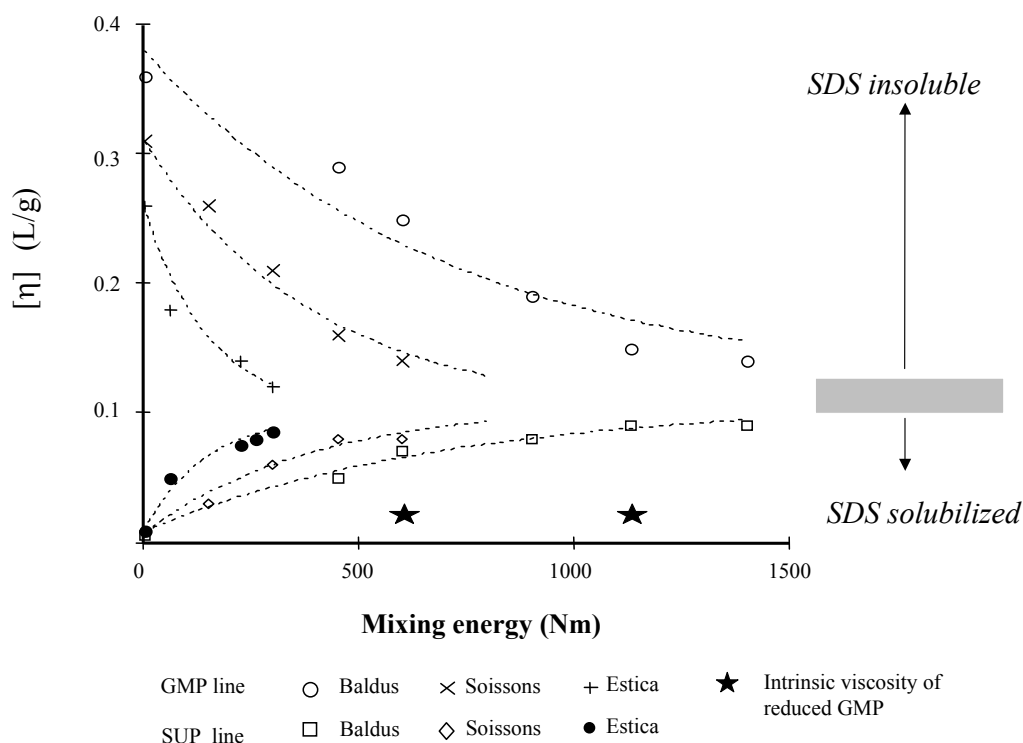
Figure 3.3   Voluminosity, expressed as the intrinsic viscosity, of SDS insoluble glutenin particles (GMP) and SDS soluble glutenin particles vs. mixing energy (SUP). See legend for a further explanation of symbols used.

The upper part of the graph shows how mixing decreases the average voluminosity of the GMP particles until a 'particle solubilization criterion' is reached. With this criterion is meant that the particles have reached a size that makes them soluble in 1.5% SDS. Interestingly, if we regard the TTP of the respective flour varieties, it becomes clear that the average voluminosities of the GMP particles are approximately the same near TTP. Completely in parallel the average voluminosity of the SDS soluble fraction (SUP) increases. The presented dotted lines in graph 3 do not 'touch' because the SUP-fraction also contains some water-soluble proteins, like wheat albumin, this slightly decreases the average voluminosity of the SUP-fraction. When –as a control- a reducing agent like DTT was added to the GMP dispersion of Baldus a dramatic decrease in viscosity was observed ('stars' shown fig. 3.3). This confirms that disulphide stabilised glutenin particles are still present in developed dough.

GMP isolated at various mixing times could be analyzed with Coulter laser. The results for variety Baldus are presented in figure 3.4.
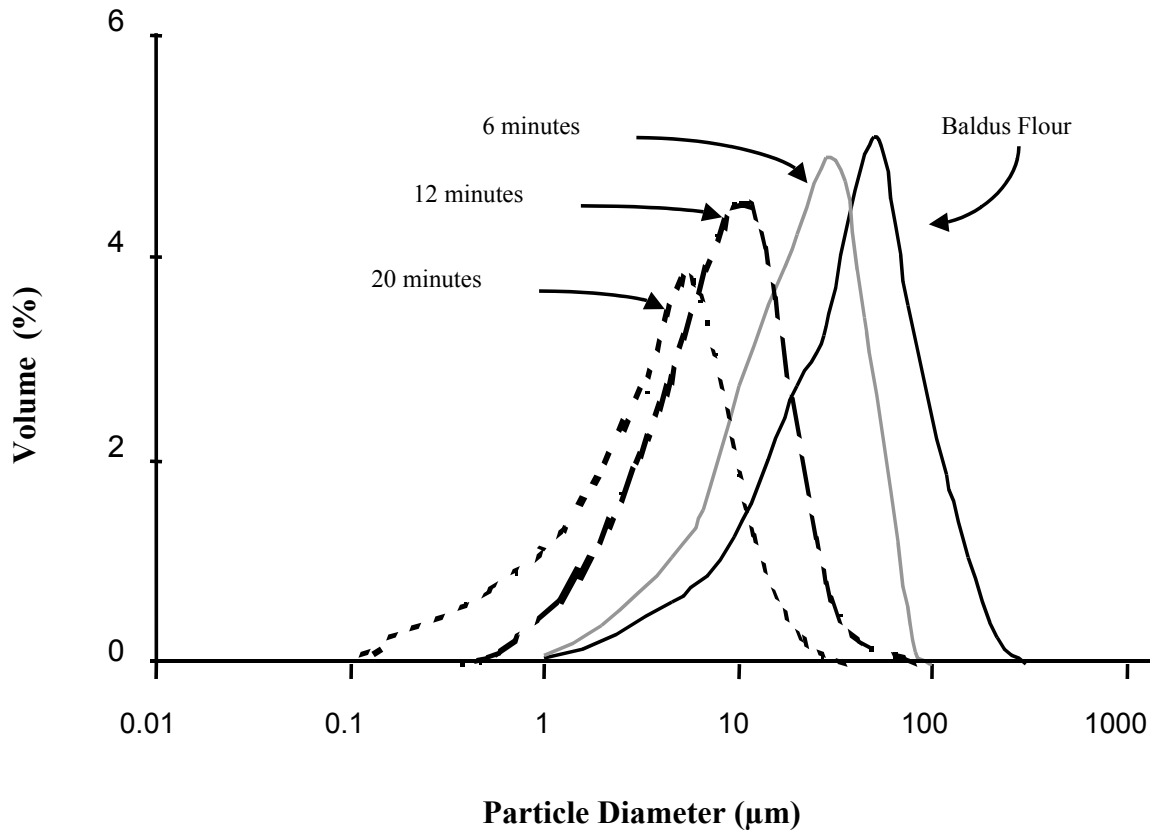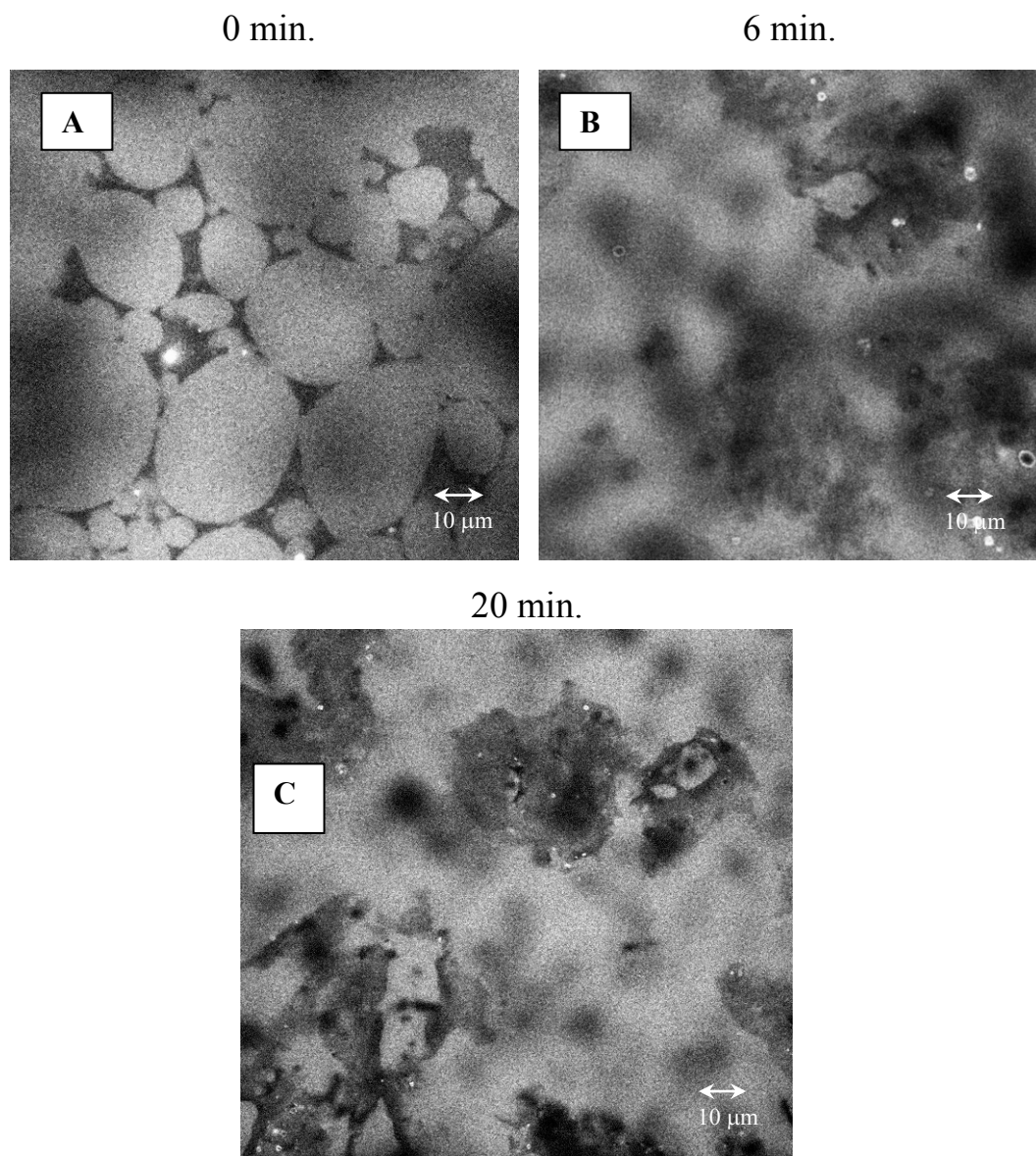

Figure 3.4   Size distributions of SDS insoluble glutenin particles isolated from Baldus flour dough vs. mixing energy

A similar pattern for size reduction was obtained for Estica and Soisons (not shown), only there starting sizes were smaller and mixing times were shorter. The results are confirmative for the decrease in GMP particle size vs. mixing energy. This decrease in size parallels the decrease in intrinsic viscosity presented in figure 3.3, hence showing that voluminosity decrease is closely related to a decrease in particle size measured with the Coulter Laser. The results in figures 3.3 and 3.4 confirm that GMP particles are still present at TTP, hence in developed dough. The presented size analyses demonstrate that at TTP, GMP particles have reached an average size that is very similar for each flour variety, as all reach a voluminosity of ~0.11 L/g.

Figures 3.5A-C show how the large particles observable with CSLM for Baldus (fig. 3.5A) have already been vigorously disrupted before TTP (fig. 3.5B-C).

0 min.   6 min.

20 min.

Figures 3.5A-C   CSLM images of GMP dispersions vs. various mixing times indicated above the respective image

The CSLM study on the GMP dispersions obtained from Baldus vs. mixing shows that particles are already affected in the very early stage of mixing. This confirms earlier findings[36], where it was concluded that development starts at the moment the mixer is turned on. Although mixing decreased average particle sizes, our observations indicate that GMP-particles are still present in

mixed samples (fig. 3.3 and 3.4), the contrast in figure 3.5B-C decreased. The limited attainable contrast with CSLM for mixed samples is thought to be due to an increase of particle-particle interaction (aggregation) and particle heterogeneity (fig 3.4). As CSLM is a static measurement, particles can stick together whereas Viscometry and Coulter laser are dynamic; here the particles are in a flow that disperses the particles.

Intrinsic viscosity represents average particle voluminosity and has a parallel with average particle size (fig. 3.3 and 3.4). G' plateau values of GMP gel are important in predicting dough properties and bread-making quality. In figure 3.6 it is shown that particle voluminosity and concentration govern G' plateau values of GMP from flour and dough.
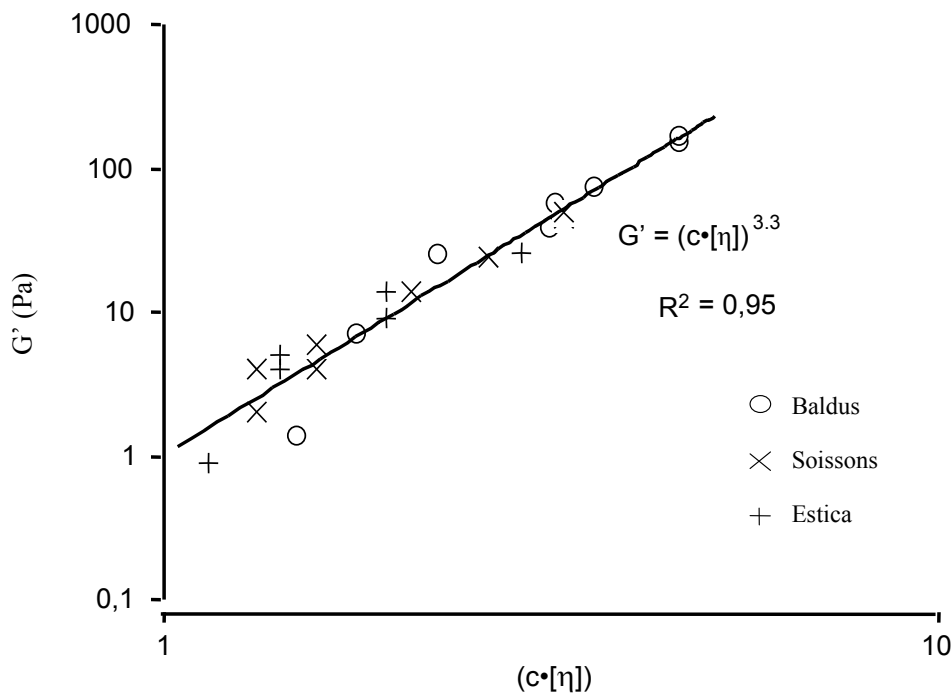

The plot shows G' (Pa) on the y-axis (logarithmic scale from 0,1 to 1000) versus (c•[η]) on the x-axis (logarithmic scale from 1 to 10). Data points for Baldus (○), Soissons (×), and Estica (+) follow the relationship:

$$G' = (c \cdot [\eta])^{3.3}$$

$$R^2 = 0,95$$

Figure 3.6   *GMP gel strength expressed as G' versus particle volume-concentration expressed as (c•[η]) isolated from flour and dough samples.*

Voluminosity of GMP particles from the three flour types and GMP particles from mixing experiments show a typical power-law correlation with G'. This is analogous to the equation proposed by Baker[37] for relating suspension viscosity to particle voluminosity and concentration. A power n between 3-5 is not unusual for a suspension of particles. The n value depends on particle

properties and concentration. Although it was observed that K' values were different for the three varieties (table 3.2), particle voluminosity appears to dominate observed G' plateau values in the range used here. For now it is not exactly clear why K' does not affect the power n in such a manner that n varies among varieties used, but for one, concentration variations are limited by GMP-gel isolation and gel deformations remain relatively small when G' plateau values are determined. This could indicate that the effects of K' do not come into play in this rheological analysis. Nevertheless, the fact that G' of GMP, a flour quality indicator, is determined by particle size clearly indicates that particle properties are the key to better understand the link between GMP and dough.

3.4   Discussion

Bekkers et al[38] have shown a strong positive correlation between dough development time and G' plateau values of GMP isolated from flour. It was therefore concluded that the mixing behaviour of flour under the conditions applied is related to the nature of the glutenin proteins as present in GMP, rather than the total amount of gel protein. Skerritt et al[21] recently reported changes in GMP composition induced by mixing, but on average the major changes in GMP composition were observed after peak resistance. As these compositional changes of GMP are clearly triggered after TTP, it is very likely that this represents a chemical breakdown of glutenin structure and is not within the scope of optimal development of gluten structure in dough. Furthermore in a study of Hamer et al.[35] it was demonstrated that physical breakdown of GMP induced by sonication resembled the mechanical breakdown found in mixing rather than the chemically induced breakdown by reduction. It was concluded that mechanical force is the predominant actuator in GMP breakdown during mixing. In this paper we have reported our efforts to better understand dough development and its relation with GMP particles. The results presented in figures 3.3 and 3.4 indicate that the decrease of glutenin particle voluminosity and size is a fundamental step in dough development. In engineering *Kick's law*[39] (eq. 1) is generally accepted to relate particle size reduction to energy-input.

$$\frac{[x]}{[x]_0} = \exp(-E/C) \qquad \text{Eq. 1}$$

where E is the energy input in Nm, [x] average particle size vs. E, $[x]_0$ the initial particle size (at E=0) and C is the rate constant. Assuming that this principle is applicable here, the decrease in glutenin particle voluminosity vs. energy is expected to follow *Kick's law*. From Δs values in fig. 3.2 it can also be deduced that the rate of particle voluminosity decrease, C, is linked with the initial

glutenin particle voluminosity. As a result we propose the following proportionality to describe glutenin particle size vs. energy (Eq. 2):

$$\frac{[\eta]}{[\eta]_0} \propto \exp\frac{-E}{[\eta]_0} \qquad \text{Eq. 2}$$

With $[\eta]_0$ as initial particle voluminosity at E=0 and $[\eta]$ the average voluminosity vs. E. When results observed for Estica, Soissons and Baldus agree with the proportionality in equation 2, the experimental data is super imposable. Derivation of shift factor F_E from equation 2, leads to equation 3:

$$\ln(F_E) = \frac{1}{[\eta]_0} - \frac{1}{[\eta]_{ref}} \qquad \text{Eq. 3}$$

where $[\eta]_0$ is the initial voluminosity of the sample and $[\eta]_{ref}$ the initial voluminosity of a reference sample. With Eq. 3 the shift factors F_E can be estimated, relative to Soissons ($[\eta]_{ref}$ = 0.31 L/g). Hence, for Soissons F_E = 1, for Estica F_E becomes 1.9 and for Baldus F_E = 0.6.

The superposition shown in fig. 3.7 indicates that the mechanism of particle size reduction vs. mixing energy is the same for the three varieties.
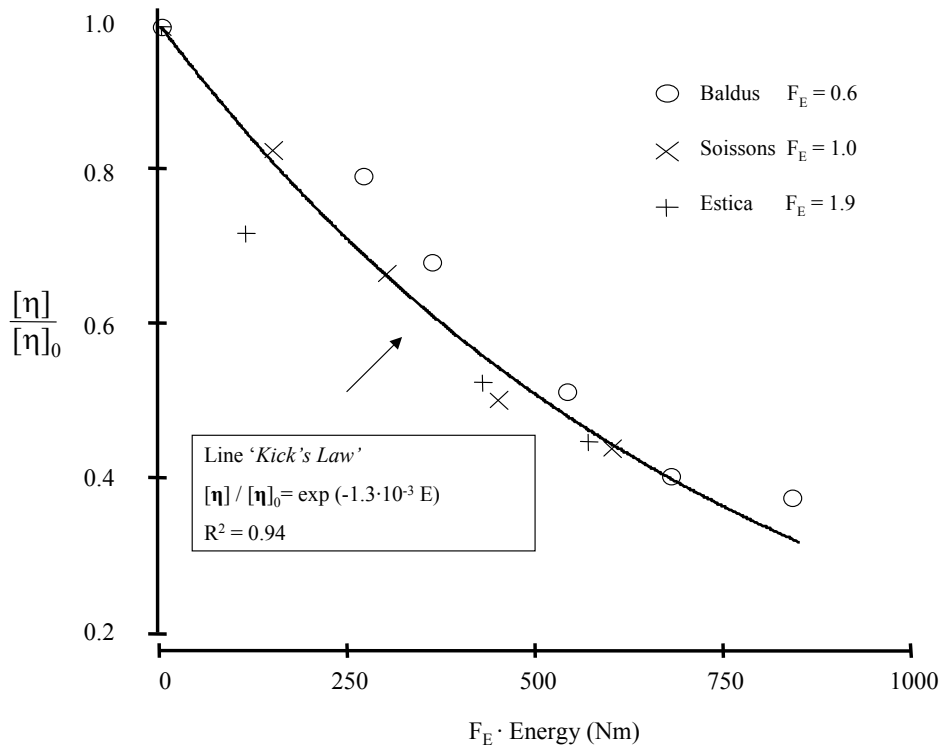

Figure 3.7   The glutenin particle voluminosity vs. energy superposition for particle voluminosity decrease vs. mixing energy applied by a Z-blade mixer

Also the agreement with eq. 2 and 3 confirms that mechanical force is the predominant actuator in GMP breakdown[35], during mixing towards TTP. It is the initial glutenin particle voluminosity that largely determines the required mixing energy. Although we do realise that only three varieties are used here, the fact that the widely differing mixing requirements for development could all be fitted with a R^2 of 0.94, is quite compelling.

3.4.1 *Energy to Peak in a pin-mixer vs. a Z-blade mixer*

The energy-voluminosity superposition shows that the required energy to peak resistance is closely related to the presence and size of GMP particles. The required TTP increases as glutenin particle size increases when mixing in a Z-blade mixer. Bloksma and Bushuk[18] reviewed some studies concerning dough development times determined with various recording mixers and concluded that, in general significant correlations were reported. However, a clear exception of this generalisation was reported in the original paper by Miller et al.[41], showing a poor correlation of R=0.52 between their farinograph (a Z-blade mixer) mixing times and their mixograph (a pin-mixer) mixing times. Even lower correlation of R=0.27 was found for their so-called bakery mixing time vs. Z-blade mixing time. Later on, Wilkins[42] reports some improvement of the earlier reported correlation of R=0.52 and claims a correlation of R=0.81 for pin-mixer peak time vs. Z-blade dough development time. About forty years later Zounis and Quail[43] confirmed the results found by Miller et al.[41], they demonstrated that farinograph Z-blade mixing times at 60 rpm had a poor correlation with the bakery mixing times. Both these papers[41,43] report fair to good correlation between the pin-mixer mixing times vs. bakery mixing times and – more importantly- poor correlations for Z-blade mixing times vs. pin-mixer mixing times. Clearly, the fact whether it is possible to obtain better correlations between pin-mixers and Z-blade mixers is still an important issue. The energy to peak – voluminosity relations presented in this paper could be extended to an apparently different type of mixer. For this the energy to peak of various flour samples mixed in the Z-blade mixer was compared with the energy to peak on the pin-mixer. The results are shown in figure 3.8.
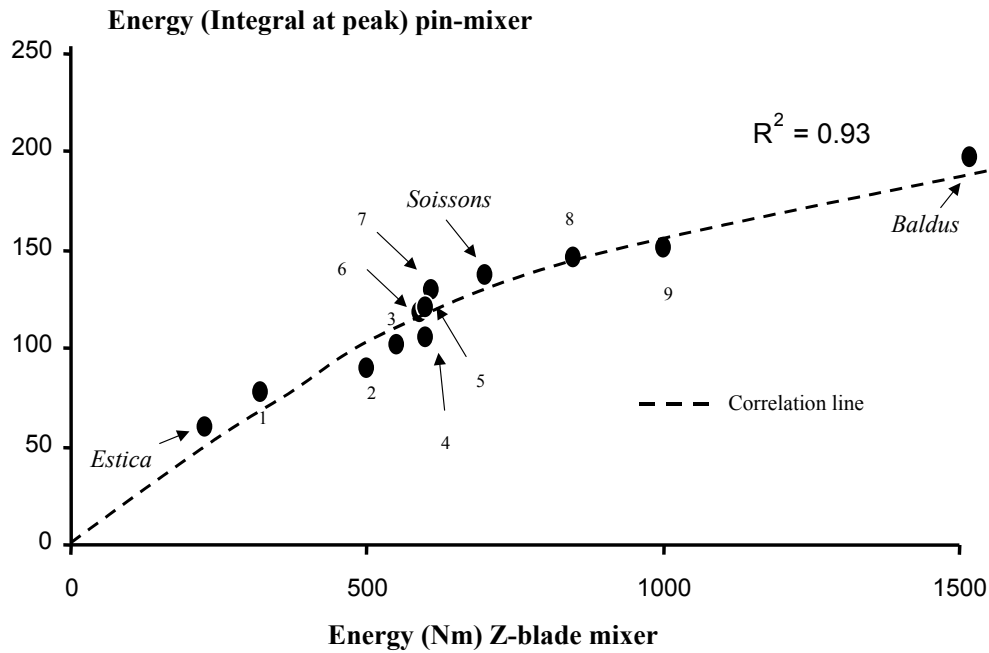
Figure 3.8   Correlating of the energy required for optimal dough development in a pin-mixer versus the energy required for developing dough in a Z-blade mixer.

Intuitively we suspect that a proper model that links the two types of mixers used should intercept at 0. Although representation of energy differs, because the mixing systems compared are quite different, a line could be fitted with $R^2 = 0.93$ (R=0.96). This demonstrates that when energy to peak is used instead of time, a better correlation between the pin-mixer and the Z-blade mixer is found. The relevant improvement in correlating these different mixer types also indicates that the glutenin particle voluminosity vs. mixing energy relations presented here is applicable to various mixer types. The demonstrated principles on how energy-input affects the insoluble glutenin particles could point the way in improving our understanding of various mixing processes and how they affect dough development.

3.4.2   Initial GMP Particle size and dough development

To further validate the importance of initial particle size it was investigated whether results found by Bekkers et al[38] also apply to initial particle voluminosity. The results presented in fig. 3.9 confirm that required energy to peak is strongly determined by initial glutenin particle size.
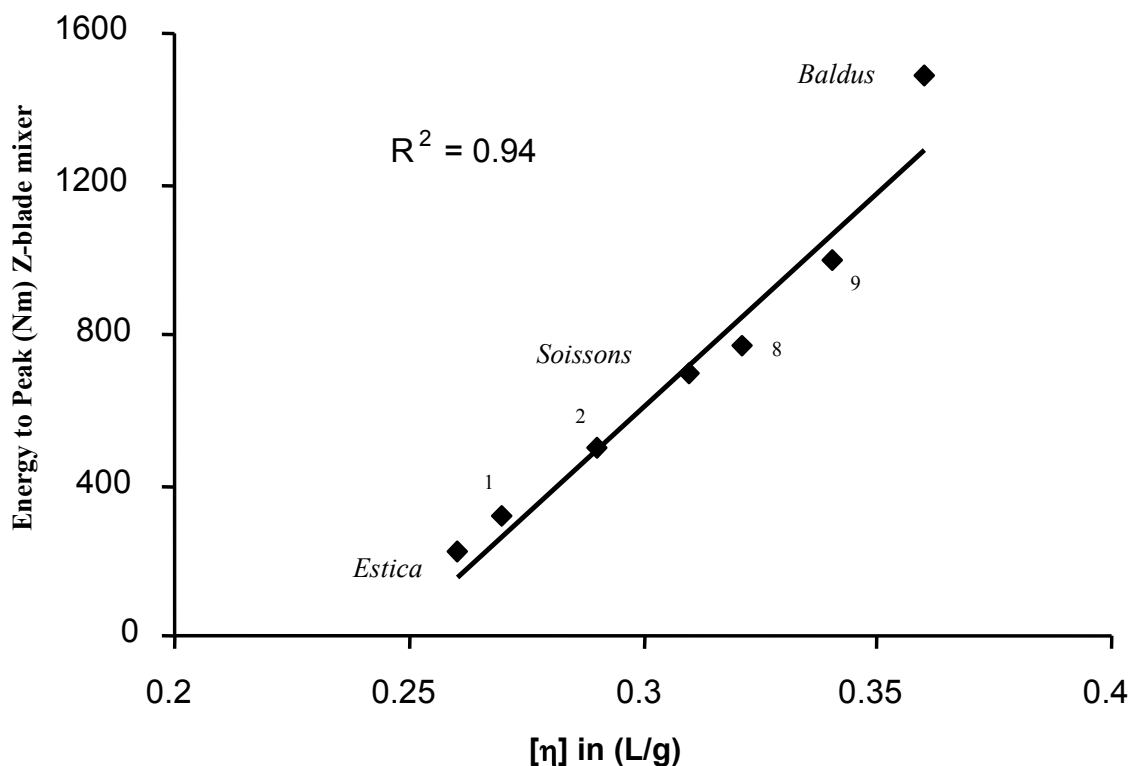
Figure 3.9   Total energy (Nm) required to optimally develop dough to peak resistance versus the initial glutenin particle size in flour, expressed as [η].

Clearly, the energy to peak is governed by initial glutenin particle size. Don et al[29] suggested a link between glutenin particles and protein particles in wheat endosperm. The results presented here strengthen our conviction that the glutenin particle size provides an important clue to understand glutenin structure-functionality relations.

Acknowledgements:

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4

THE EFFECT OF MIXING ON GLUTENIN PARTICLE PROPERTIES: AGGREGATION FACTORS THAT AFFECT GLUTEN FUNCTION IN DOUGH

Abstract

In previous chapters we have reported that the SDS insoluble gel-layer: the Glutenin Macro Polymer (GMP) can be considered a gel consisting of protein particles. These glutenin particles have a size of about $10^{-1} - 10^{2}$ µm and consist of HMW-GS and LMW-GS only. In GMP isolates from flour, the particles are spherical. In isolates from dough, glutenin particles have lost this shape. This seems relevant, since mixing disrupts the particles and the mixing energy required for dough development correlated with the glutenin particle size in flour. The question studied in this paper is how changes at a glutenin particle level affected the subsequent process of gluten network formation during dough rest and if this could be used to explain resulting dough reological properties. To this end, we studied how various mixing regimes affected the dough properties during and after resting (elasticity). We cannot fully explain the differences in the final dough properties observed using parameters such as the quantity of GMP in flour, the quantity of re-assembled GMP in dough and the size of re-assembled glutenin particles. However, other parameters were found to be important: 1) the Huggins constant K' reflecting the tendency of glutenin particles to interact at level II of the Hyper-aggregation model; 2) the composition of glutenin particles affecting the potential to form smaller or larger particles and 3) for over-mixed dough, covalent re-polymerisation at the so-called level I of hyper aggregation. Using these parameters we can better explain dough viscoelasticity after resting.

Running Headline: The effect of mixing on GMP particles

Key words: Glutenin Macro Polymer, Glutenin-particles, Mixing, Dough elasticity, Hyper-aggregation

Abbreviations: CSLM, Confocal Scanning Laser Microscopy; DTT, dithithreitol; FITC, Fluoro Iso-thiocyanate; GMP, Glutenin MacroPolymer; HMWGS, High Molecular Weight Glutenin Subunit; K'; Huggins constant; [η], particle voluminosity; LMWGS, Low Molecular Weight Glutenin Subunit; PE, Petroleum-ether; SDS, Sodium Dodecyl Sulphate; SUP, supernatant fraction from GMP isolation; TTP, Time to Peak; UPP, Unextractable Polymeric Protein.

4.1   Introduction

The presence of gluten proteins in wheat flour makes it uniquely suitable for the preparation of leavened bakery products (Bushuk 1998). The so-called wheat gluten contains glutenins and gliadins (Schofield 1994). The glutenin fractions are especially important for bread-making quality. The glutenin fraction that is insoluble in 1.5% SDS, the so-called Glutenin Macropolymer (GMP), consists of HMW-GS and LMW-GS (Graveland et al. 1982). The quantity of GMP that can be extracted from flour parallels differences in flour bread-making quality (Graveland 1984, Weegels et al. 1996). Similar parallels have been shown between the quantity of UPP (Unextractable Polymeric Protein), bread-making quality and wheat flour dough strength (Lafiandra and MacRitchie 1997). Both GMP and UPP fractions represent 'polymeric' glutenins (Lafiandra and MacRitchie 1997; Weegels et al 1996) and their respective quantities in flour were shown to correlate: $R^2 = 0.86$ with n=12 by Don et al. (2003d). Recently, it was shown that GMP isolated from flour contains spherical glutenin particles with sizes in the range $10^{-1} - 10^2$ µm (Don et al. 2003a). We stress that until recently it has not been possible to demonstrate directly the presence of glutenin particles in dough or flour, but only in the GMP fraction. At present no staining or labelling technique is available to discern the glutenin aggregates of different sizes from other wheat proteins and starch. The GMP isolation methodology is therefore useful in order to obtain glutenins without the other flour components that complicate staining or labelling. The average voluminosity of glutenin particles in flour has been shown to parallel the G', the GMP-gel rigidity (Don et al. 2003b). G' is an indicator of wheat flour quality that correlates with bread loaf volume (Kelfkens and Lichtendonk 2000, Pritchard 1993).

The fact that GMP represents the largest of the glutenin network proteins and the statistical relationship observed between the sizes of glutenin particles in GMP isolated from flour, the G'of the GMP-gel phase and bread loaf volume, prompted to look for a relationship between the occurrence of glutenin particles and final dough properties.

Mixing is a prerequisite for generating dough that can be leavened and baked into a loaf. Mixing has been shown to decrease the quantity of GMP-gel. However, more importantly, by extracting GMP from dough samples, Don et al. (2003b) showed that mixing disrupts the initially spherical particles extractable from flour. During the dough rest that follows mixing, glutenin polymers are reported to re-assemble, leading to a recovery of GMP-gel (Graveland 1984, Weegels et al. 1996, 1997). It is also clear from Weegels et al. (1997) that mixing changes the physico-chemical properties of GMP. Complex cascades of physical and chemical interactions have been suggested to take place during dough preparation. However, there is no clear consensus on how mixing induces

the formation of a viscoelastic network in dough (Bloksma and Bushuk, 1988; Hlynka, 1962; Hoseney and Rogers, 1990; Kaufman et al., 1986; Preston, 1989). Recently, Lefebvre et al (2000) suggested that the protein network in dough may be considered as a network of particles in the mesoscopic size range ($\sim10^{-1} - 10^2$ μm). More recently, Van Vliet and Lefebvre (2003) pointed out that the gluten network may be viewed as an aggregated network of insoluble colloidal particles. Our recent observations of spherical glutenin particles in GMP isolated from flour (insoluble in 1.5% SDS) (Don et al. 2003a) and disrupted, non-spherical glutenin particles isolated from mixed doughs (Don et al. 2003b) is in full agreement with this view and may clarify how mixing affects the glutenin fraction. Although at time to peak (TTP) the glutenin particles have a very similar size for three different flour varieties (Don et al, 2003b), other factors such as glutenin particle interactions and (re-)aggregation are expected to affect the macroscopic dough rheology after mixing. Combining the suggestions of Lefebvre and van Vliet (2003) with our observations lead us to believe that the glutenin particles may have properties that can be linked to the properties of the colloidal gluten particle network that determine final dough rheological properties. According to this hypothesis we would expect the properties of glutenin particles would also play a role during dough rest and in the final rheological properties of the dough. Therefore we studied factors underlying optimal glutenin functionality in dough, including both SDS soluble, glutenin particles and the insoluble GMP-fraction for three dough mixing regimes: under-mixed, optimally mixed and over-mixed.

4.2   Experimental

4.2.1   Wheat flour samples

Flour was obtained from the following varieties: Estica, Soissons and Baldus, by using a Bühler mill, extraction rates were 77.7%, 78.4% and 78.5% respectively. This is the same set of three used in a previous mixing study (Don et al., 2003b). Their protein content (based on dry matter), HMWGS composition and TTP are presented in table 4.1.

Table 4.1   Protein content, HMW-GS composition and time-to-peak.

Wheat variety	Flour protein content (%)	HMW subunit composition	TTP (min)
Estica	12.4	6+8, 2+12	5
Soissons	10.6	2*, 7+8, 5+10	10
Baldus	12.5	1, 14+15, 5+10	27

4.2.2   Protein analysis

Flour protein content was measured by the Kjeldahl method. Protein content of GMP and GMP dispersions were measured using an UV absorption method (Hall, 1996). The UV method was calibrated using a set of GMP Kjeldahl protein values.

4.2.3   Isolation of GMP from flour

Flour defatted with petroleum ether was suspended in 1.5% SDS (1.4g flour in 28 ml, SDS of analytical grade from Merck™) and centrifuged at 80,000 g for 30 min at 20°C in a Kontron Ultracentrifuge (Graveland, 1982). The supernatant (SUP) was decanted and the gel-layer collected as GMP.

4.2.4   Isolation of GMP from mixed dough sample

The dough sample was freeze-dried after mixing. The freeze-dried dough was powdered on a Retsch mill using a 0.25mm sieve. GMP was isolated as described for flour.

4.2.5   GMP dispersion

GMP dispersions were prepared by transferring 1g of GMP gel to a tube containing 10 ml 1.5% (w/v) SDS solution. The gel was mixed with the solvent by briefly stirring with a spatula. The tube was then sealed and placed on a roller-bank for 3h at ambient temperature. A visually homogenous, opalescent dispersion was obtained indicating that part of the protein polymers are not dissolved and was used for further characterisation.

4.2.6   Rheology of GMP gels

GMP (1 g) was carefully removed from the top of the gel and transferred to the measuring cell of a Bohlin VOR rheometer (Malvern Instruments, United Kingdom). The cell consists of two parallel plates (d=30 mm) with a gap of 1 mm. Measurements were performed at 20°C in a strain sweep mode at amplitudes ranging from 1% to 100% at 0.15 Hz. Data were expressed as G' vs strain, G' plateau value or delta (δ) vs strain.

4.2.7   Viscometry of GMP particles

The viscosity of GMP dispersions was measured using an Ubbelohde capillary viscometer. The flow through time was ~300-400 s to optimise accuracy. Rinsing with 1.5% SDS solution was frequently done between measurements. Ubbelohde measurements must be done cautiously as air bubbles are easily formed. The reduced viscosity at c-> 0 is represented by [η] and the Huggins constant K' (Huggins, 1942) was obtained by plotting reduced viscosity vs. concentration according to the Martin equation (Wang et al., 2003). We are aware that in polymer chemistry [η] is called 'the intrinsic viscosity of the polymer'. Intrinsic viscosities are often used to calculate molar masses of molecularly 'well-described' polymer-solvent combinations. We do not think this use is allowed for glutenin aggregates, since they are insoluble in the solvent used**.** Also, in contrast to 'simple' polymer-solvent combinations, with glutenin we lack a well-supported assumption of molecular structure. Viscometry can therefore only be used to provide data of the average volume of the glutenin particles in suspension. In order to avoid confusion with the more familiar term *intrinsic viscosity –*we have named the measured [η] in this context: the glutenin particle *voluminosity.* Again, this *voluminosity* was estimated from the intersect point for GMP concentration → 0. K', the Huggins-constant, reflecting particle interactions, was calculated from the slope of the plot.

4.2.8   Particle size analysis

Particle size measurements of diluted GMP dispersions from varieties Baldus, Soissons and Estica mixed to peak and rested for 45 and 90 min were performed using laser diffraction in a Coulter LS 130 (Beckman Coulter Inc, USA)**.** In this instrument, laser light is scattered by the suspended particles to generate diffraction patterns that are composites of the diffraction patterns for all the particles. For GMP from flour (Don et al., 2003a, b) the spherical shape of the GMP particles was confirmed. If the particles are approximately spherical, the instrument using Fraunhofer theory can convert the composite diffraction patterns into 72 classes particle size distributions ranging from 0.3 to 900μm. From the derived size pattern, the particle volume-surface ratio $D_{3,2}$ can be obtained.

4.2.9 Mixing experiments

Defatted flour was mixed using a Brabender Plastograph equipped with a 10g Farinograph bowl. Dough was prepared both in 2% (w/w) NaCl and water at a mixing speed was 63 rpm. The required amount of water was determined according to ICC procedure 115/1 (Standard Methods of the ICC, 1992). Energy input (Nm) and TTP can be calculated from the recorded torque vs. time plot with Plastograph software. Energy input is an important parameter in practical dough development. In general the time to dough peak resistance (TTP) is a reference point. It is agreed that by defenition: 1) dough is 'under-developed' or 'under-mixed' before TTP, 2) dough is 'developed' or 'optimally mixed' at TTP and 3) when mixing is continued beyond peak resistance the dough is 'broken down' or 'over-mixed'. The designation of 'under', 'optimal' or 'over-mixed' is inevitably somewhat arbitrary, since it varies with, for example, the wheat variety and mixing conditions used. For this reason, we have indicated in table 4.2 the energy input values used for each of the flour types and mixing ranges. Energy is expressed for 10g of flour mixed in the 10g Farinograph bowl.

Table 4.2   *The mixing regimes used for dough rheological study with Estica, Soissons and Baldus.*

Wheat variety	Under-mixed regime* (J)	Optimally mixed regime – TTP* (J)	Over-mixed regime* (J)
Estica	190-210	250-280	610-650
Soissons	305-335	595-635	945-995
Baldus	870-950	1570-1640	1930-1970

* Energy-input J/10 g flour

4.2.10 Flow-relaxation measurements

Flow-relaxation measurements were carried out using a Bohlin VOR rheometer using serrated plate geometry with a cross section of 30mm. As described (Lichtendonk et al., 2000), for flow relaxation measurements a dough piece was placed in the rheometer geometry and the gap set at 3 mm. Moisture loss from the dough piece was minimized by a plastic cover that was placed over the measuring geometry. Since the insertion of the dough piece may cause some stress in the dough, the actual measurement was made after an equilibration time of 10 min. The measuring temperature was 30°C. During the measurement the sample was deformed to a strain of 100% at a shear rate of

0.0208 s^{-1}. Afterwards the strain was kept constant and the decrease of stress of the dough recorded as a function of time. The time necessary for the dough to relax to a stress of 50% of initial stress, recorded directly after stopping deformation, was taken as the flow-relaxation halftime ($t_{1/2}$).

4.2.11 RP-HPLC

The HMW-LMW ratio of GMP from flour and dough samples was assessed with reversed phase HPLC (RP-HPLC) using a Waters HPLC with a C8 bonded phase column. GMP (1g) was extracted with 1 ml 50 (v/v) % 1-propanol, 0.02% (w/v) dithiothreitol, followed by extraction with 1ml solvent A. These two extracts were combined for HPLC analysis. Solvent A contained 6M urea, 20 % (v/v), 0.1 %(v/v) trifluoroacetic acid and 0.02% (w/v). Solvent B contained 5.5M urea, 50% (v/v) acetonitrile, 0.1 %(v/v) TFA and 0.02% (w/v) dithiothreitol. The column was loaded with 10μL of GMP extract and the temperature maintained at 50°C. Eluted was at a flow rate of 1ml/min, with a multi-step gradient starting at 20% B to 80% B. Typical elution times were 30-45 min for the HMW glutenins and 70-85 min for LMW glutenins.

4.2.12 Confocal Scanning Laser Microscopy

Freshly prepared samples of GMP dispersions were stained for protein with protein specific FITC (a fluorescent label) and observed at a protein concentration of ~1 mg/ml using a Leica TCS NT Confocal Laser Scanning Microscope. Nicolas et al. (2003) recently described a micro-rheology system that allows variation of shear rate rheology in a cone-plate cell. The glass plate allows observation of dispersed GMP particles under continuous shear using CSLM. The average shear rate applied ranges approximately from $10^{-1} – 10^2$ s^{-1}.

4.3   Results

4.3.1   Flour varieties and characteristics of GMP

Table 4.3 shows typical GMP data, for GMP wet weight, protein content and the G' plateau value, a measure of gel stiffness. Estica and Soissons have similar amounts of GMP protein (~30 mg), but Baldus has more (~40mg).

Table 4.3   *The characteristics of the GMP gels and dispersions from flour samples Estica, Soissons and Baldus.*

Wheat variety	GMP wet weight* (g)	GMP protein concentration* (mg/g)	GMP protein quantity* (mg)	G' plateau value* (Pa)	K'[‡]	[η][‡] (L/g)
Estica	2.6 ± 0.2	11.2 ± 0.5	29.1 ± 2.2	26 ± 2	1.5	0.26
Soissons	2.9 ± 0.3	10.6 ± 0.7	30.7 ± 2.6	43 ± 3	0.71	0.31
Baldus	3.3 ± 0.4	12.9 ± 0.8	42.6 ± 3.4	158 ± 11	0.31	0.36

* n = 5, ± s.d.

‡ K' and [η] from linear regression of reduced viscosity vs. concentration plot with $R^2 > 0.95$

Table 4.3 also provides data from GMP dispersion viscometry, reflecting GMP-particle properties. The intrinsic viscosity parallels particle size differences observed with CSLM, size measured with the laser and the G' plateau values (Don et al., 2003a, b). The parameter K', the Huggins constant, reflects the tendency of particles or polymers to interact (Huggins, 1942). With CSLM we have observed differences between flour particles from '5+10' and '2+12' varieties. A very strong '5+10'variety e.g. Baldus has clearly visible spherical glutenin particles. GMP- particles from '2+12' flour varieties e.g. Estica were shown to be less distinct, probably because they start to dissociate further into the solvent (Don et al., 2003a). These differences between '2+12' and '5+10'varieties observable with CSLM may indicate a stronger internal structure in '5+10'particles than '2+12' varieties. The voluminosity of glutenin particles extracted from flour is a predictor of the mixing time required to reach 'time to peaK'. It is not yet known exactly which factors (genetic, phenotypic) determine glutenin particle size and K'. However, viscometry data for GMP dispersions for six varieties (Don et al., 2003c) suggest a relationship with HMW-GS composition.

4.3.2   The effect of mixing on the voluminosity of SDS soluble glutenins

Mixing leads to a loss of GMP, which is explained by the dissociation of glutenin particles to sizes that dissolve in 1.5 % SDS (Don et al., 2003b; Weegels et al, 1996, 1997). Recently (Don et al., 2003b) showed how the mixing process affects both GMP and SDS solubilized glutenin particles up to TTP. The decrease in GMP in dough usually observed during mixing is schematically depicted in figure 4.1a.
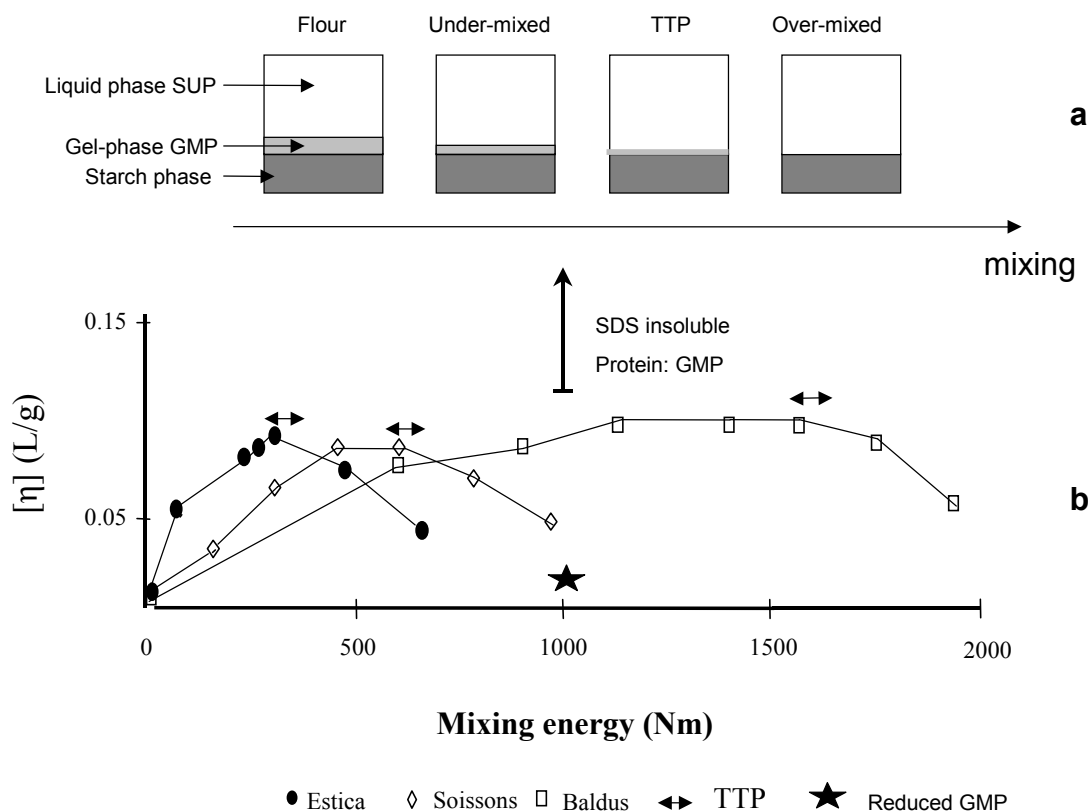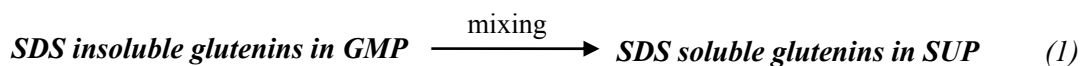
Figure 4.1a-b A schematic view of GMP loss versus mixing (a) and glutenin voluminosity in supernatant phase vs. mixing energy for Estica, Soissons and Baldus flour (b).

The SDS insoluble glutenin particles are converted so-called SDS soluble glutenin particles so that after TTP, nearly all glutenin particles have become soluble in SDS i.e. dissociated glutenin particles. Equation 1 schematically describes this process:

$$\textit{SDS insoluble glutenins in GMP} \xrightarrow{\text{mixing}} \textit{SDS soluble glutenins in SUP} \qquad (1)$$

The $[\eta]_{gmp}$ of GMP particles is typically: $0.10 < [\eta]_{gmp} < 0.40$ l/g. With mixing both GMP particle size (measured with Coulter LS 130) and $[\eta]_{gmp}$ decreases (Don et al., 2003b).When $[\eta]_{gmp} < \sim 0.11$ l/g very little GMP (<0.1g) can be isolated from 16g of dough (Don et al., 2003b). Earlier and more recent papers have focussed on the SDS insoluble GMP (Don et al., 2003a; Graveland et al., 1984; Weegels et al., 1996). To better understand the behaviour of glutenin particles dissociated in the SDS soluble phase (eq 1), we analysed the viscosity changes in this phase as a function of mixing.

Figure 4.1b shows that the average intrinsic viscosity of the 1.5% SDS soluble protein fraction increases with mixing. This is due to solubilisation of the glutenin particles during mixing and coincides with the mixing-driven decrease of GMP particle voluminosity ($[\eta]_{gmp}$) (Don et al., 2003b). Figure 4.1b further illustrates that the intrinsic viscosity of the SDS soluble part ($[\eta]_{sup}$) can be followed in the under-mixed regime, optimal mixing regime (TTP) and, more importantly, also for the over-mixed regime. These results show that when the glutenin particles have been sufficiently dissociated into SDS soluble glutenin particles, $[\eta]_{sup}$ is maximal and closely parallels dough TTP (fig. 4.1b). Thus TTP in the mixer is close to the maximal $[\eta]_{sup}$ observed for the SDS soluble fractions. When dough is over-mixed (i.e. beyond TTP), a decrease in $[\eta]_{sup}$ is observed. Presumably the result of further disruption of the SDS soluble glutenin aggregates. For comparison, the typical minimal intrinsic viscosity measured for dithitreitol-reduced SUP-fractions is just ~0.02 l/g (indicated by the star in figure 4.1b). With under-mixing, GMP particles are disrupted to a limited extent. Optimal mixing renders almost all GMP soluble, and over-mixing disrupts the SDS soluble glutenin aggregates so that the $[\eta]_{sup}$ approaches the $[\eta]$ of reduced SUP fractions.

4.3.3   The effect of mixing on the viscoelastic properties of dough

The viscometric characterisation of both SDS solubilised glutenins and GMP show that mixing has profound effects on both $[\eta]_{gmp}$ and $[\eta]_{sup}$. To examine how mixing affects dough rheology, the flow relaxation half-time ($t_{1/2}$) vs. rest was measured for under-mixed dough, dough mixed to TTP and over-mixed dough. Figures 4.2a-c show the relaxation half times vs. dough rest for mixed dough samples from Baldus, Soissons and Estica.
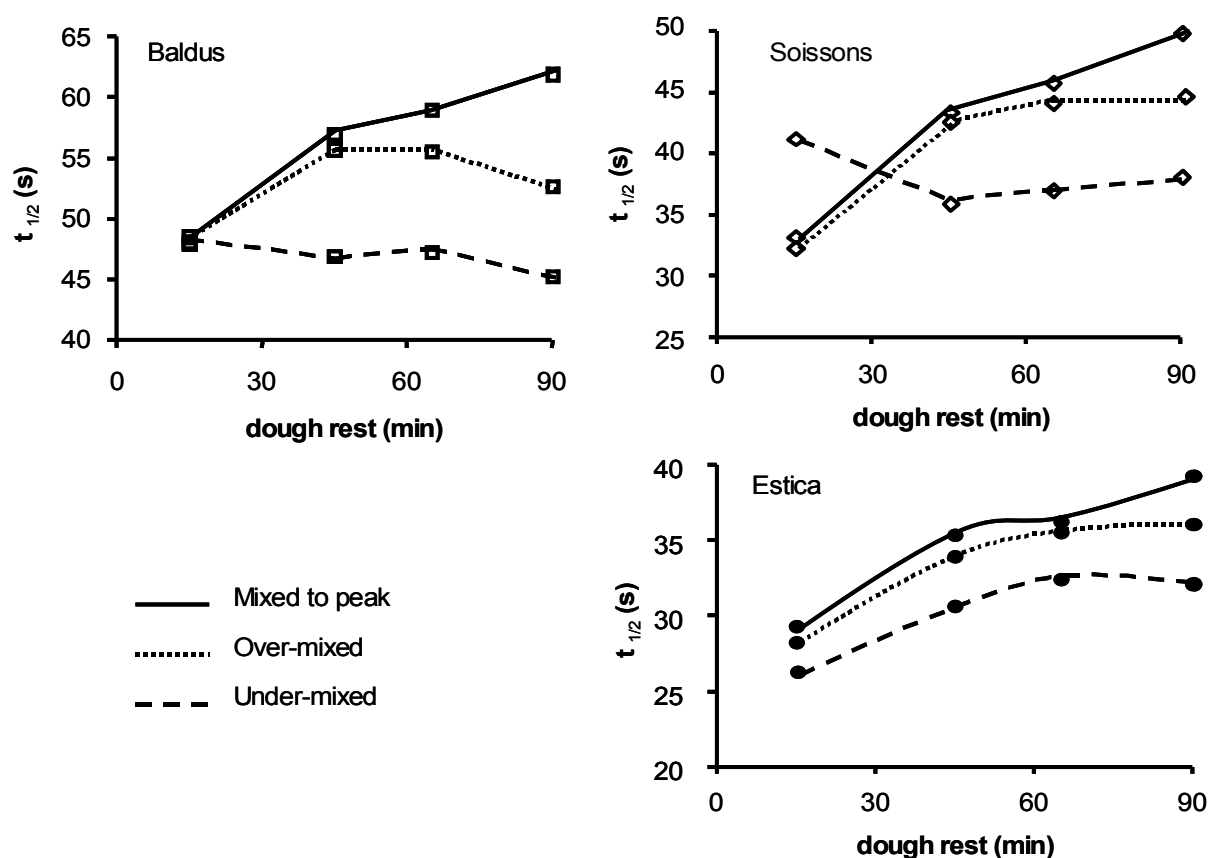
Figure 4.2a-c Flow relaxation half-time for under mixed, optimal mixed and over mixed dough prepared from Baldus (top left), Soisson (top right) and Estica (bottom left) flour.

Longer relaxation half times indicates a relatively more elastic dough behaviour. More specifically this implies that, on average, longer lasting (attractive) interactions between the structural elements in the dough are responsible for the rheological properties under the conditions used. Both Lasztity et al. (1996) and Lichtendonk et al.(2000) have successfully used relaxation half times to identify significant differences in the visco-elastic properties of dough samples. In our experiments doughs of all three varieties when mixed to TTP show an increase in $t_{1/2}$ against dough rest time. For under-mixing most $t_{1/2}$ values are lower than for optimally mixed dough. Furthermore, for Estica $t_{1/2}$ increase vs. rest but not for Baldus and Soissons. Over-mixing of dough results in $t_{1/2}$ values between under-mixed and optimally mixed dough. Over-mixing also results in a somewhat lower increase of $t_{1/2}$ for all three varieties. Clearly, mixing affects the ability to form a more elastic dough. Mixing to TTP results in doughs with the highest viscoelasticity. There are also differences in the values of $t_{1/2}$ between the three varieties. For example, figure 4.2 shows that $t_{1/2}$ of Estica

dough < Soissons dough. This cannot be explained by differences in the amount of GMP; Estica and Soissons flours have similar GMP contents (table 4.3). Both quantity and composition of glutenins or GMP and UPP glutenin fractions affect rheological properties of optimally mixed doughs (Lafiandra and MacRitchie, 1997; Weegels et al., 1996, 1997). Adding to this we studied if such a relation would hold for different mixing regimes. Figures 4.2a-c show clearly that each mixing regime has a distinct effect on subsequent dough rheology. To better explain these GMP-dough rheology relationships we measured the amount of GMP that re-assembles during dough rest-time.

4.3.4   *The effect of mixing on the balance between GMP and SDS soluble glutenin*

Figure 4.3 shows the recovery of SDS insoluble GMP-gel vs. dough rest for Estica, Soissons and Baldus doughs that were under-mixed, mixed TTP and over-mixed. The results for mixing to TTP confirm the findings of Graveland et al. (1984) and Weegels et al. (1996, 1997).
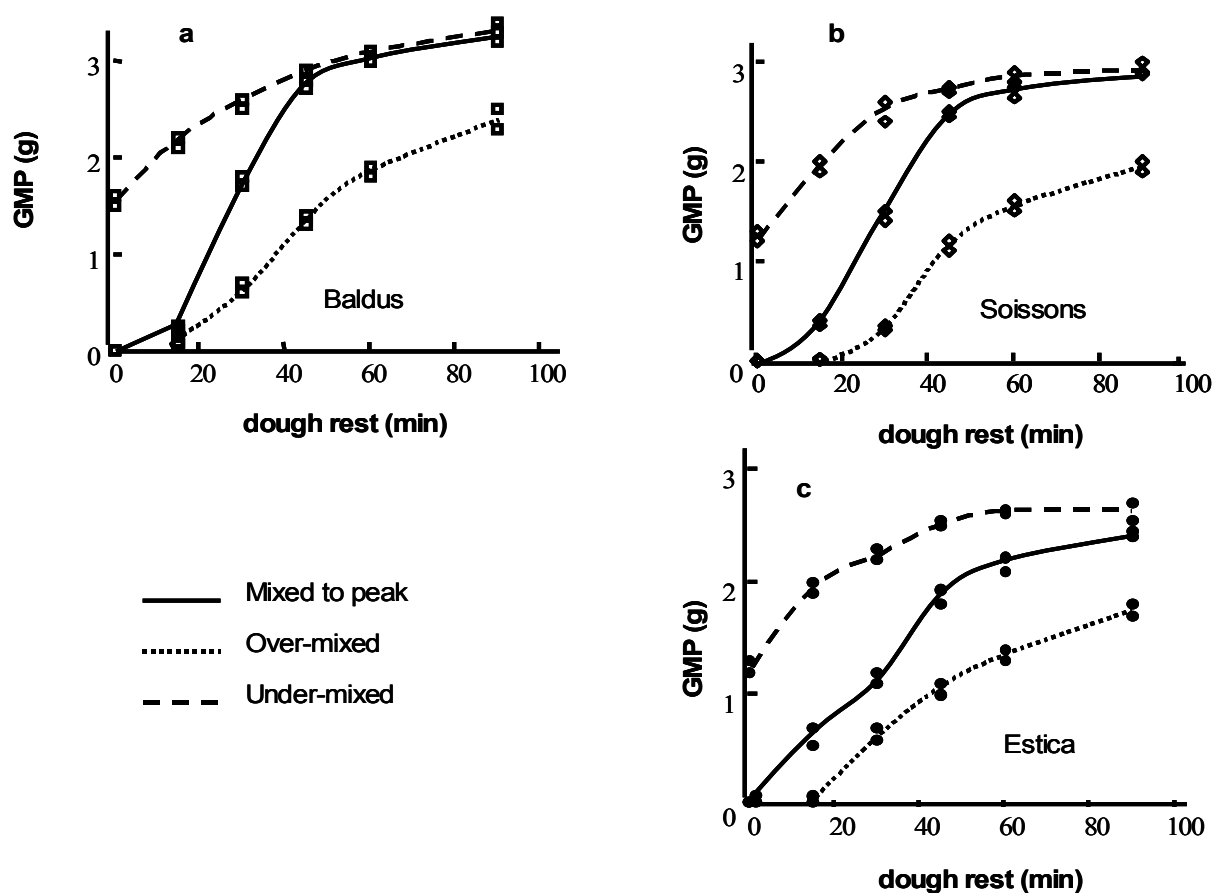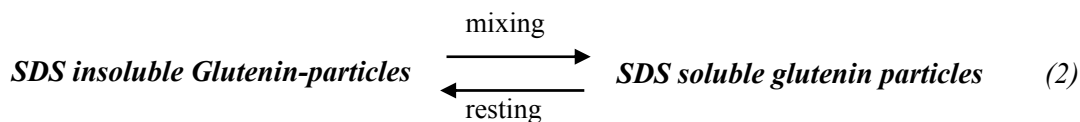
Figure 4.3a-c Recovery of GMP vs. rest-time for under mixed, optimal mixed and over mixed dough prepared from Baldus (a), Soisson (b) and Estica (c) flour.

The ability of the glutenins rendered soluble on mixing (fig. 4.1a and Eq. 1) to re-assemble into GMP-gel during dough rest (fig. 4.3a-c) is clearly shown. Equation 1 should therefore be modified to include a balance between SDS insoluble glutenin particles and SDS soluble glutenin particles:

$$\textbf{\textit{SDS insoluble Glutenin-particles}} \quad \xrightleftharpoons[\text{resting}]{\text{mixing}} \quad \textbf{\textit{SDS soluble glutenin particles}} \qquad (2)$$
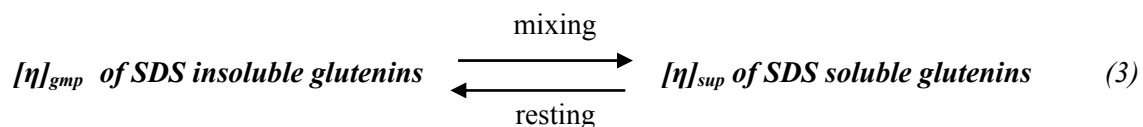
For all three varieties glutenin re-assembles during dough rest. However, differences exist between varieties and between mixing regimes in terms of the rate of reformation and the final quantity of GMP recovered at the end of dough rest. It should also be noted that the shown balance (Eq 2) does not imply that reformed glutenin particles are the same as the original glutenin particles in flour. When mixed to TTP, the re-assembly of soluble glutenins in SUP into insoluble GMP appears to occur more rapidly in the over-mixed than the under-mixed situation. When under-mixed, only part

of the GMP is rendered soluble. The SDS soluble glutenins could be recovered 60 min after dough rest. Over-mixing leads to a much slower GMP formation: a lag-time of ca 15 min was observed and the recovery of GMP was not complete after 90 min rest. The balance of GMP recovery (Eq 2) is clearly affected by the applied mixing energy. In turn, this may have affected the respective dough $t_{1/2}$ relaxation times.

Figures 4.3a-c shows the relationship between mixing-induced variation in dough rheology and the amount of recovered GMP during rest; after 45 min rest over-mixed doughs have less GMP than under-mixed doughs. However, the $t_{1/2}$ after 45 min rest is higher for over-mixed dough samples. When GMP in dough vs. $t_{1/2}$ is compared after 90 min rest, it can also be seen that over-mixed doughs have less GMP but higher $t_{1/2}$ than the under-mixed doughs. So, despite lower amounts of GMP in over-mixed dough at 15' and 45' rest, their $t_{1/2}$ values are quite close to the $t_{1/2}$ of optimally mixed dough. These data demonstrate that the link between amounts of dough GMP and dough rheological properties is not a simple one. The results indicate only that mixing alters the rate and extent of GMP re-assembly. Links between glutenins and dough properties have been demonstrated but in addition to the amount of GMP, aggregate size has been regarded as important (Lafiandra and MacRitchie 1997). To reveal any relationship between GMP particle voluminosity and dough rheology we investigated how GMP-particle voluminosity changes with dough rest.

4.3.5 Voluminosity of GMP re-assembling from the SUP-phase during dough rest

The amount of GMP recovered from doughs during dough rest suggests a re-aggregation of the SDS soluble glutenin, but the amount of re-assembled GMP in dough does not help explain the differing dough rheological properties although the voluminosity [η] which parallel particle size may do so. Only particles with an average [η] > 0.10 ± 0.02 l/g are able to form an SDS insoluble GMP phase gel. Particles with [η]< 0.10 ± 0.02 remain in the SDS soluble phase. Previous reports (Cornec et al., 1994; Lefebvre et al., 2000) and have shown the importance of aggregate size in dough rheology. When large GMP reaggregates are formed GMP in quantity they are likely to affect dough rheology. Glutenin aggregate voluminosity for both the SDS soluble ($[η]_{sup}$) and re-aggregated GMP ($[η]_{gmp}$) was followed during dough resting. As for the amount of GMP (Eq 2), a balance between $[η]_{sup}$ and $[η]_{gmp}$ exists (Eq 3):

$$\textit{[η]}_{gmp} \textit{ of SDS insoluble glutenins} \xrightleftharpoons[\text{resting}]{\text{mixing}} \textit{[η]}_{sup} \textit{ of SDS soluble glutenins} \qquad (3)$$

The results for $[η]_{sup}$ and $[η]_{gmp}$ are shown graphically in figure 4.4a-c.

4.3.6 The optimally mixed regime

In figure 4.4 the y-axis represents the average voluminosity of the proteins in the supernatant phase ($[\eta]_{sup}$) that is above the layer of GMP-gel (see fig. 4.1a) **.** The y-axis (att. For reasons of clarity the axis is reversed), shows the decrease of the $[\eta]_{sup}$ vs. dough rest time.
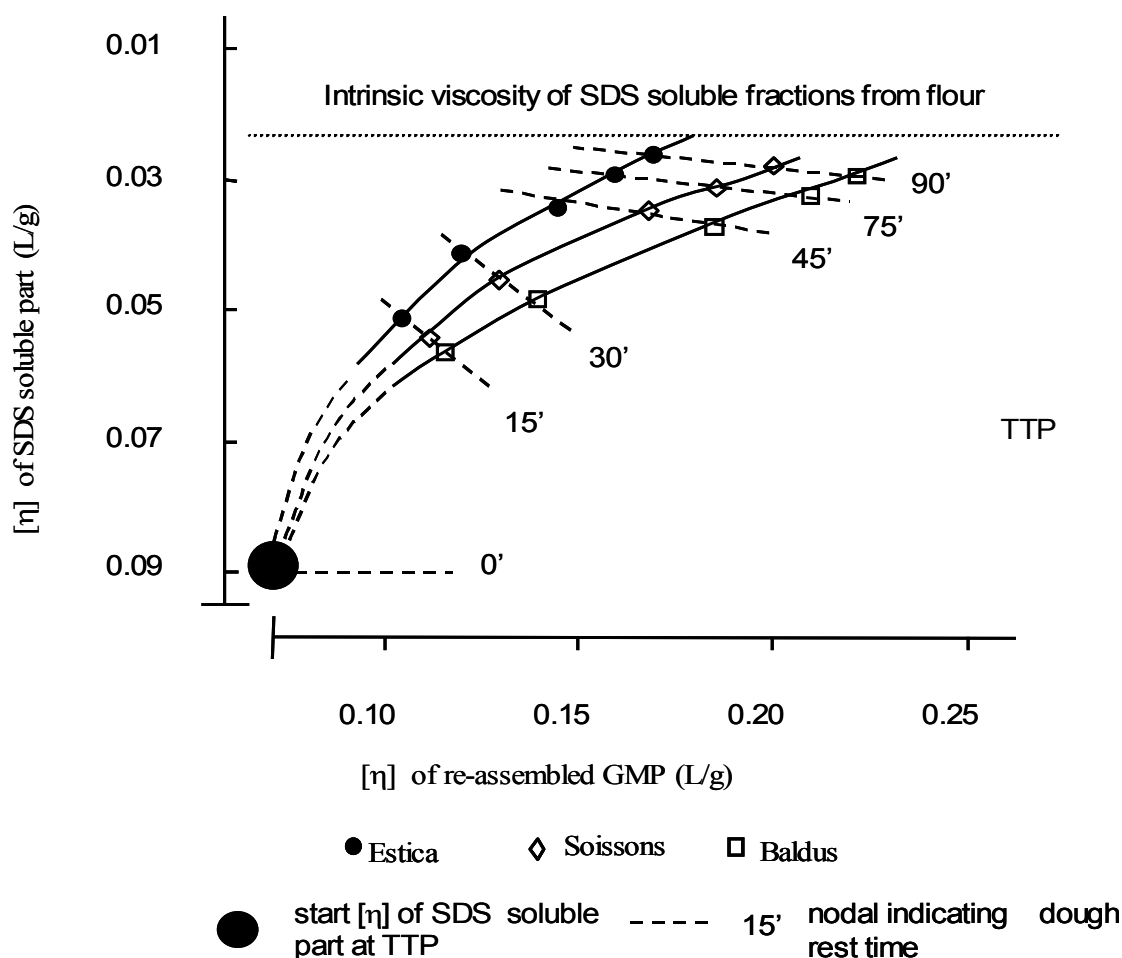

Figure 4.4   *Re-aggregation process of SDS soluble and insoluble glutenin at various rest times. The x-axis shows the increase of insoluble glutenin aggregates. The y-axis shows the decrease of glutenin size in the supernatant phase (TTP mixed).*

The x-axis represents the voluminosity of re-assembled glutenin particles ($[\eta]_{gmp}$) during dough rest. In figures 4.4 and 4.5 rest-times are represented by the dotted nodal lines. When these data are presented in this way some interesting features of GMP formation are revealed. Directly after mixing the $[\eta]_{sup}$ is approximately 0.09 l/g. This is in accordance with figure 4.1, where a peak $[\eta]_{sup}$ of ~0.09 l/g was observed. After 15 min dough rest, small amounts of re-formed GMP can be isolated. As $[\eta]_{sup}$ decreases, glutenin particles re-aggregate and form larger SDS insoluble

glutenin-particles, as shown by the increase in $[\eta]_{gmp}$. The viscometric analysis confirms that a GMP-gel can be recovered when glutenin aggregates have $[\eta] > 0.10$ l/g. The $[\eta]_{gmp}$ increases until $[\eta]_{sup}$ reaches a minimal value. This value is typically near that observed for SDS soluble fractions (e.g. flour albumins and globulins), $0.01 - 0.03$ l/g. Figure 4.4 clearly demonstrates how during dough rest the SDS soluble glutenin particles re-assemble into GMP aggregates of a mesoscopic size. Wang (2003b) reported a relationship between particle size (μm) and particle voluminosity $[\eta]_{gmp}$ with an R^2 between $[\eta]_{gmp}$ and particle $D_{3,2}$ (Laser) of 0.87. In this study, $D_{3,2}$ of GMP aggregates from Estica, Soissons and Baldus are 3.5, 4.4 and 5.2 μm, respectively at 45' rest; at 90 min $D_{3,2}$ increases to 4.3, 6.0 and 7.4 μm. Together with the $[\eta]_{gmp}$ shown in figure 4.4 these data confirm the relationship between GMP-particle $D_{3,2}$ measured with the Laser and $[\eta]_{gmp}$. Figure 4.4 also distinguishes between the re-assembly processes of the three varieties. With Estica the decrease of the $[\eta]_{sup}$ is faster (points closer together) than with Baldus. For the increase of $[\eta]_{gmp}$ the opposite is observed; in this case $[\eta]_{gmp}$ of Baldus increases faster than that for Estica. GMP re-assembly for Soissons in terms of $[\eta]_{sup}$ and $[\eta]_{gmp}$ is lies between Baldus and Estica. Thus, although at TTP particle voluminosities are of the same order of magnitude, for the three varieties the patterns of re-assembly of SDS soluble glutenins into large SDS insoluble GMP-aggregates is different.

4.3.7   *The under-mixed regime*

The re-aggregation of GMP from under-mixed dough was similarly followed. Figure 4.5 shows that GMP-aggregation starts at slightly different sizes but the pattern of increase compared to figure 4.4 is quite different. Although $[\eta]_{sup}$ decreases it is not accompanied by a gradual increase of $[\eta]_{gmp}$ as observed for doughs mixed to TTP (fig. 4.4). This result parallels the results in figure 4.2a-b where also no gradual increase of $t_{1/2}$ vs. rest is observed for under-mixed doughs of Baldus and Soissons. On the other hand Estica shows an increase in $t_{1/2}$ and $[\eta]_{gmp}$ also shows some increase. Size differences between GMP-particles after 90 min rest are $D_{3,2}$: 3.5, 5.0 and 6.7 μm for Estica, Soissons and Baldus, respectively. Clearly, the re-assembly process in the under-mixed dough is different from that in the TTP mixed dough.
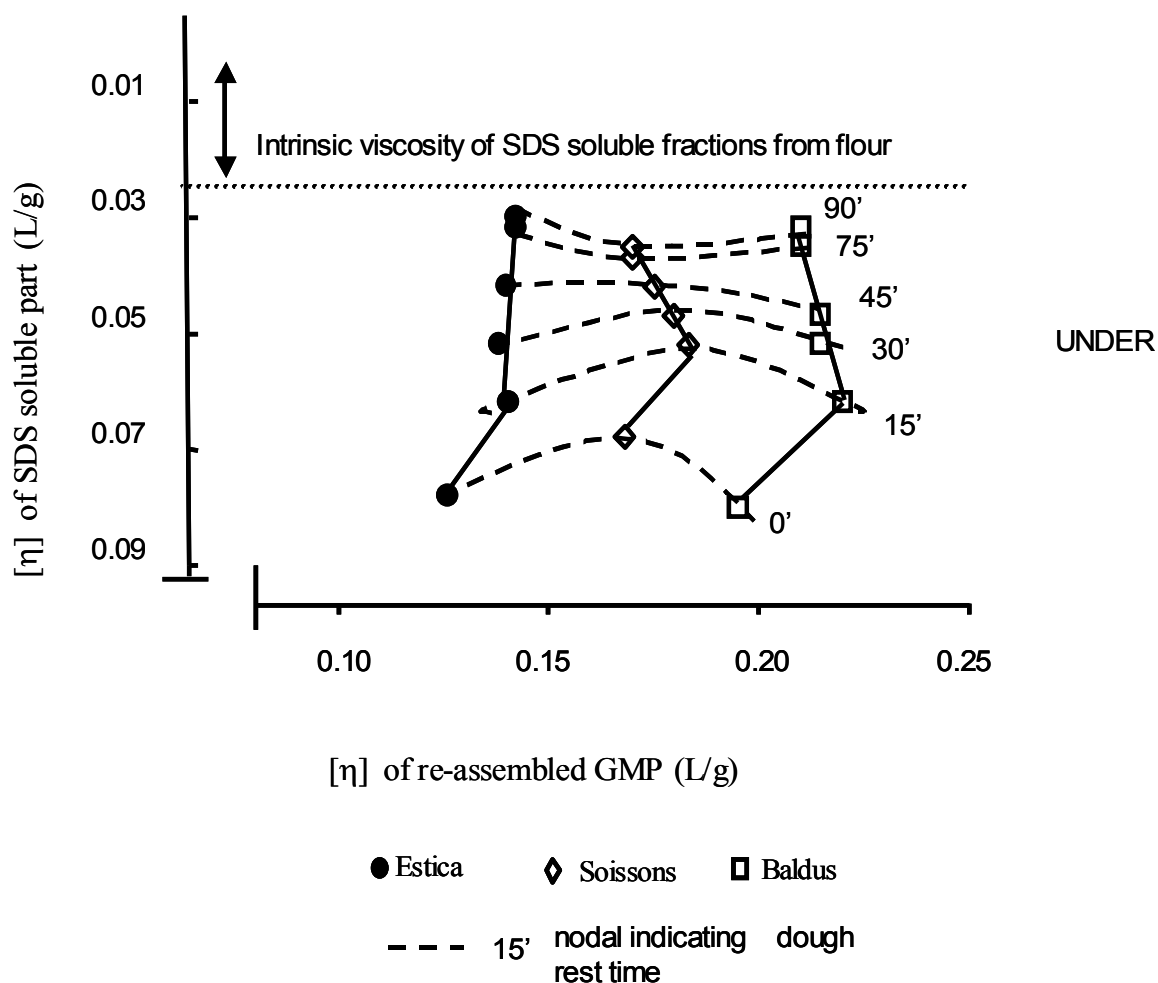
Figure 4.5   Re-aggregation process of SDS soluble and insoluble glutenin at various rest times. The x-axis shows the increase of insoluble glutenin aggregates. The y-axis shows the decrease of glutenin size in the supernatant phase (under-mixed).

4.3.8   The over-mixed regime

The results for $[\eta]_{sup}$ and $[\eta]_{gmp}$ are shown in figure 4.6 for over-mixed doughs. Compared to optimally mixed dough the first difference that is apparent is the initial $[\eta]_{sup}$ values As also shown in figure 4.1 the $[\eta]_{sup}$ decreases further after TTP. This also results in a start-point with a lower $[\eta]_{sup}$. In over-mixed doughs SDS soluble glutenin aggregates are further dissociated to sizes approaching those in dithiotheritol reduced SUP fractions (fig. 4.1).
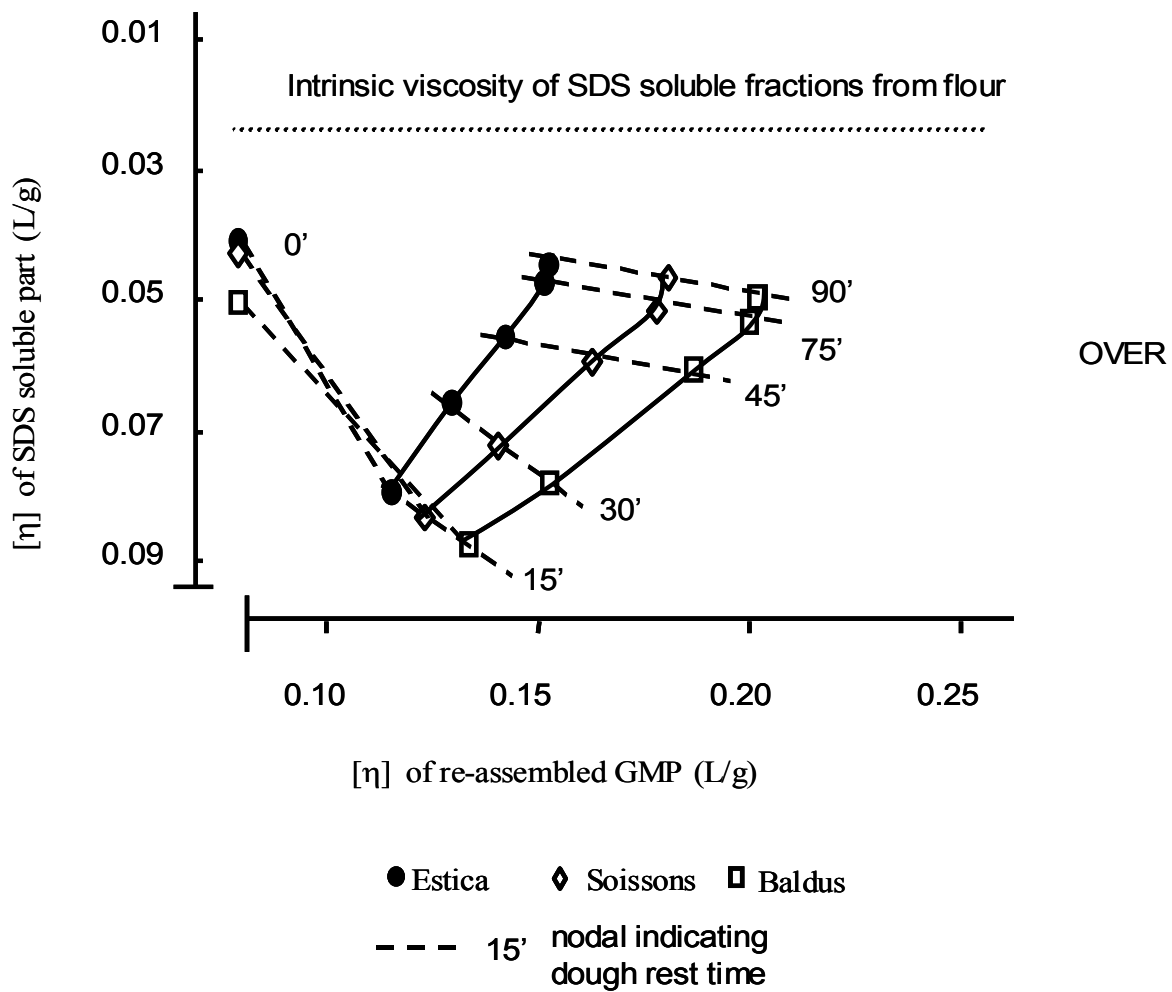
Figure 4.6   Re-aggregation process of SDS soluble and insoluble glutenin at various rest times. The x-axis shows the increase of insoluble glutenin aggregates. The y-axis shows the decrease of glutenin size in the supernatant phase (over-mixed).

During the first 15 min of rest, the $[\eta]_{sup}$ of over-mixed doughs increases. This may indicate that covalent associations the SDS soluble glutenin aggregates that have been disrupted by over-mixing (Fig. 4.1) first oligomerize and then form glutenin aggregates that are able to re-assemble into GMP-aggregates. After the increase in $[\eta]_{sup}$ the re-aggregation of glutenin particles and the decrease of $[\eta]_{sup}$ follows a pattern that shows some similarity to optimally mixed dough. Also, after 90 min $[\eta]_{gmp}$ is less than observed and the $[\eta]_{sup}$ remains higher than for optimally mixed doughs. This indicates that not all disrupted glutenin aggregates in SUP are able to re-assemble into GMP, and also that the overall voluminosities of the re-aggregated glutenin particles are smaller in the over-mixed doughs. The $D_{3,2}$ confirm the lower sizes of GMP aggregates at 90min compared to TTP mixed situation, as after 90min rest the values are: 3.7, 5.2 and 7.0 µm for Estica, Soissons

and Baldus, respectively. Extending rest time beyond 90 min (not shown) did not bring amount or the size of GMP closer to that for optimally mixed doughs.

4.3.9   Effect of re-assembled GMP aggregate size on dough rheological properties

As previously observed the results amounts of GMP in flours and in doughs cannot fully explain differences in dough rheology. Following the example of Cornec et al. (1994) and Lefebvre et al. (2000), Don et al. (2003b) we plotted the rheological parameter, $t_{1/2}$, against the glutenin particle size $[\eta]_{gmp}$ as shown in figure 4.7a-c.

Figure 4.7a-c Flow relaxation half-time against the voluminosity of glutenin particles in rested dough samples (a optimal mixed, b over-mixed, c under-mixed).

In general, positive correlations between $[\eta]_{gmp}$ and $t_{1/2}$ are observed and confirm that dough $t_{1/2}$ is related to the size of the re-assembled GMP-aggregates. This relationship is the same for both optimally mixed and over-mixed dough but the relationship is different in the under-mixed. The lines run parallel, but for each size, $t_{1/2}$ is lower than expected (fig. 4.5c). We believe this is due to

incomplete distribution of the gluten phase through the dough volume and that this prevents the formation of a continuous network. This is supported by micrographs of doughs (Lee et al., 2001; Peghambardoust et al., 2004), which show a non-continuous protein matrix in under-mixed doughs as opposed to a continuous matrix in optimally mixed and over-mixed doughs.

It is clear that the size of the re-assembled glutenin particle aggregates is a more relevant to the understanding of the dough rheology parameter $t_{1/2}$ than the amount of GMP. It is not yet clear why the mixing regimes have such a profound effect on glutenin re-aggregation. We therefore need to consider meso-scopic glutenin particle properties that may help explain the macro-scopic viscoelasticity of a dough. This is discussed in Section 4.4.

4.4   Discussion

4.4.1   *The role of mixing in dough rheological properties.*

The results obtained with the three mixing regimes, agree with earlier observations of Frazier et al (1975). Also Kilborn and Tipples (1975) have shown that variations in mixing time and speed, affect dough rheology and bread loaf volume. At the time these variations were thought to be related to proper dispersing of the gluten phase. In this paper we found that the size of glutenin aggregates insoluble in SDS, plays an important role in dough visco-elasticity as well. A combination of proper dispersion of gluten and aggregate size are likely candidates for explaining why dough visco-elasticity is so strongly dependent on its mixing time / energy-input. Various microscopy surveys on dough systems point at a physical shaping and changing of the gluten morphology (Lee et al., 2001, Amend et al., 1991 and Hoseney et al., 1990). It is clear that that the glutenin fraction plays a key role in the elastic properties of dough. For the glutenin fraction we see disruption of glutenin particles during mixing, and re-aggregation during rest. However, the elastic properties of the dough cannot only be explained by glutenin aggregate size alone. There seems to be interplay of mixing history and glutenin aggregate size.

4.4.2   *Deformability and disruption of glutenin particles: a model experiment*

The results of Amend and Belitz (1991) and Lee et al. (2001) point at a mechanism of distributing of the gluten protein phase. In these papers the gluten network is described as a network of gluten aggregates. However, in these studies microscopy can only show the whole gluten phase, and how it is distributed in the dough. Here we prefer to focus specifically on the glutenin fraction to better understand its role in affecting dough properties. By isolating GMP from freeze dried dough

samples Don et al. (2003b) showed with both CSLM and Coulter Laser that mixing disrupts and downsizes the initially spherical GMP particles. We do realise that links between particle deformation and mixing as yet are based on indirect observation. Samples were prepared, after which the SDS insoluble GMP gel was isolated and a sample for CSLM observation needed to be made. Now, using a newly developed rheometer cell (Nicolas et al., 2003), we were able to directly observe the behaviour of isolated particles subjected to a continuous shear field. Although the conditions are far from the actual conditions in a dough mixer, we believe the experiment helps to support our point that mixing can indeed change the shape and hence network formation properties of glutenin aggregates.
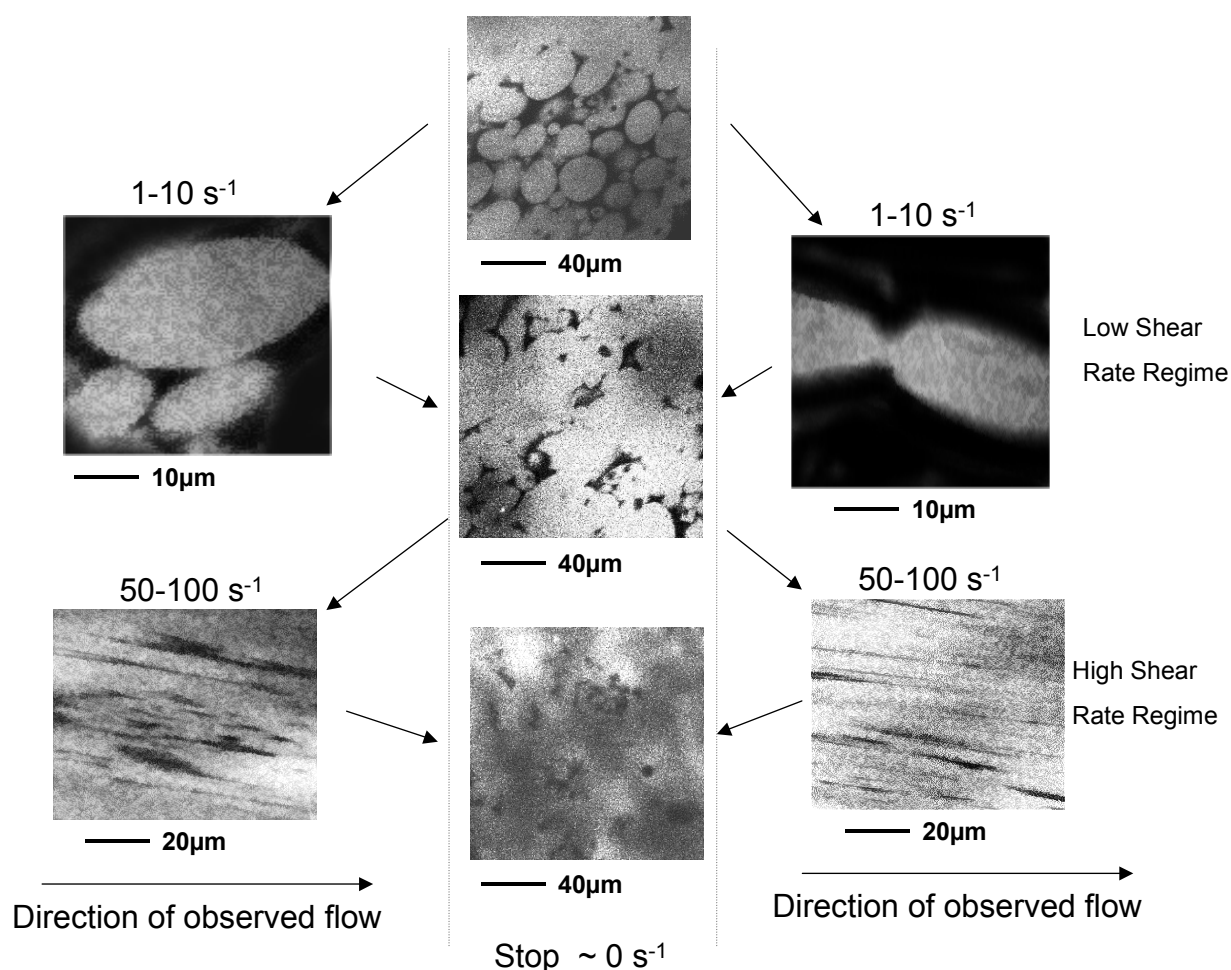

Figure 4.8   Observation of glutenin particles under various flow regimes.

Under the initial static condition the spherical glutenin particles are clearly visible (light areas in figure 4.8). In a so-called low shear rate ($10^{-1} - 10^{0}$ s^{-1}) regime the particles change from spherical to elliptic, some particle-particle adhesion is observed (right panel in figure 4.8). The particles are clearly deformed. When the shear is changed to zero, the system does not return to its initial state, contours of particles are still visible, but some aggregation has occurred. At a higher shear rate ($\sim 10^{2}$ s^{-1}) deformation is larger, leading to a seemingly continuous GMP phase. When the shear rate is stopped and the system relaxes, no glutenin particles are visible and a more continuous network seems to be formed. We suggest that the phenomena observed here are likely to have a link with mixing a dough. These phenomena clearly affect: the voluminosity, size and morphology of the glutenin particles.

4.4.3   The relation between mixing and the Huggins constant of the glutenin particle

The Huggins constant, K', reflects the tendency of the glutenin particles to interact. Interestingly, Wang et al. (2003) revealed that K' of glutenin particles isolated from gluten is correlated with gluten extensibility. Here, in order to see how mixing affects glutenin particle interactions we determined the Huggins constant of glutenin particles extracted from the mixed dough samples (freeze dried), the K'$_{gmp-dough}$. Figure 4.9 depicts the K'$_{gmp-dough}$ vs. mixing energy.
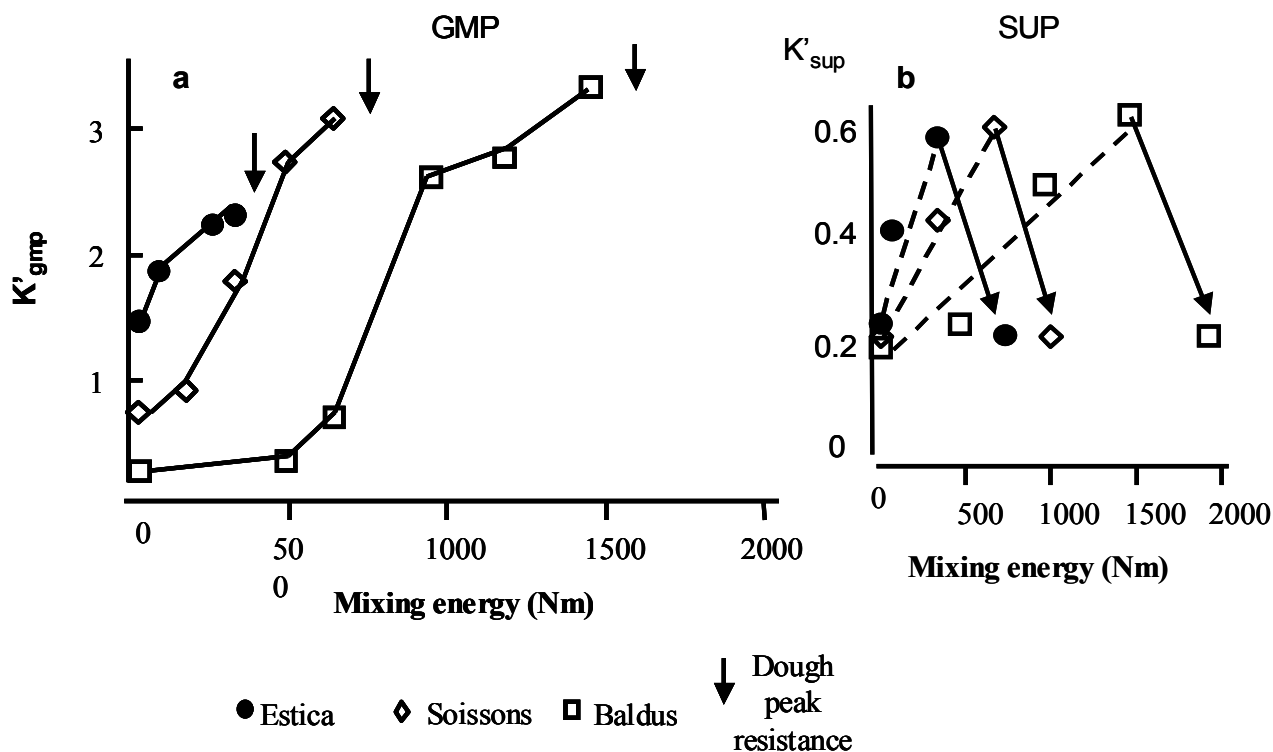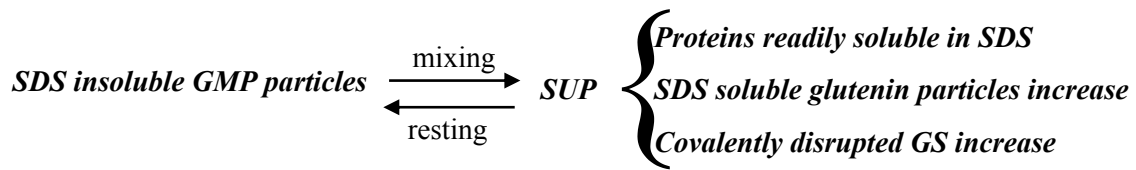
Figure 4.9   The change in K' of SDS insoluble glutenin (left) and SDS soluble glutenin (right) against mixing energy for Baldus (squares), Soissons (diamonds) and Estica (dot).

The $K'_{gmp\text{-}flour}$ (particles extracted from flour) is taken to reflect initial GMP particle interactions. The Huggins constant of GMP from dough, $K'_{gmp\text{-}dough}$, shows a strong increase vs. mixing for all three varieties. Usually a K' of ~0.3 is reported (Niezette et al., 1984) for flexible polymers in a good solvent. Particle systems that do show interactions have a K'>0.5. A K' larger than unity is indicative of strong particle-particle interactions (Russel et al., 1989). Our experiments show that $K'_{gmp\text{-}dough}$ reaches values greater than unity, indicating that mixing strongly promotes glutenin particle interactions. The increase of $K'_{gmp\text{-}dough}$ follows Baldus > Soissons > Estica. With resting, glutenin particle aggregates are re-assembled from smaller, SDS soluble glutenin particles or oligomers, that are present in the supernatant (SUP) fraction of the SDS extraction. Equation 4 describes this relation:

$$SDS\ insoluble\ GMP\ particles \underset{resting}{\overset{mixing}{\rightleftharpoons}} SUP \begin{cases} Proteins\ readily\ soluble\ in\ SDS \\ SDS\ soluble\ glutenin\ particles\ increase \\ Covalently\ disrupted\ GS\ increase \end{cases}$$

The changes in K'$_{sup}$ vs. mixing are shown in figure 4.9 (right panel). The SUP from flour has a K'$_{sup\text{-}flour}$ ~ 0.2 for all three varieties. The K' of a DTT reduced SUP fraction (star symbol in figure 4.1) also has a K' of 0.2. Such K' values indicate behaviour like soluble polymers (Niezette et al., 1984). Due to the predominance of readily SDS-soluble proteins in the SUP the K'$_{sup}$ vs. mixing is typically lower than the K'$_{gmp}$ vs. mixing. For all three varieties the K'$_{sup}$ showed an increase, from K'$_{sup}$ ~0.2 for flour, to a K'$_{sup}$ ~0.6 at TTP. This can only result from an increase of SDS soluble glutenin particles in the figure 4.1. This is paralleled by a decrease of K'$_{sup}$ from ca 0.6 to ~0.2. This is important, since such a decrease indicates a transition from more physical particle-particle interactions to more soluble polymer behaviour. In this respect, the increase in K'$_{gmp}$ during mixing can be explained by a change from: first large spherical particles representing the initial status of the GMP fraction (flour), into smaller irregular aggregates representing the status of the GMP in a mixed and rested situation.

4.4.4   The possible role of glutenin particle K' in glutenin aggregation

From the micro-rheology experiment, it is clear that after deformation or disruption of spherical particles the very irregularly shaped glutenin aggregates re-assemble in a different way. Figures 4.4, 4.5 and 4.6 show how glutenin particles are re-assembled. Figure 4.10-4.12 show the change in K' of these particles against [η] with resting for the three mixing regimes used. For reasons of comparison, figures 4.10-4.12 show both the pattern of K' of mixed samples as well as of K'of rested dough samples.

*Figure 4.10   The K' vs. the glutenin particle voluminsosity for mixing of Baldus flour; and K' vs.
the glutenin particle voluminosity for resting after respectively: TTP mixed, Over-
mixed and Under-mixed situation.*

Figure 4.11   The K' vs. the glutenin particle voluminsosity for mixing of Soissons flour; and K'
vs. the glutenin particle voluminosity for resting after respectively: TTP mixed,
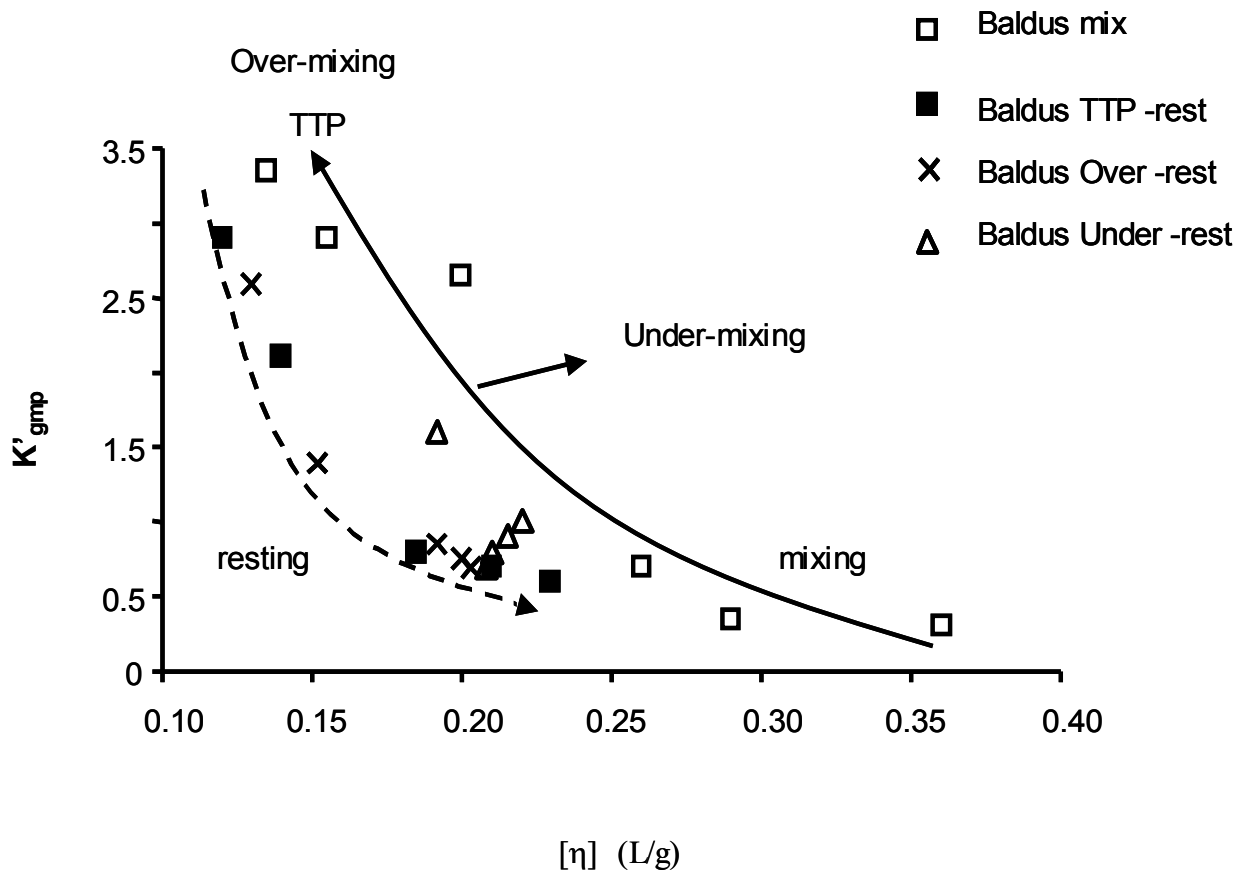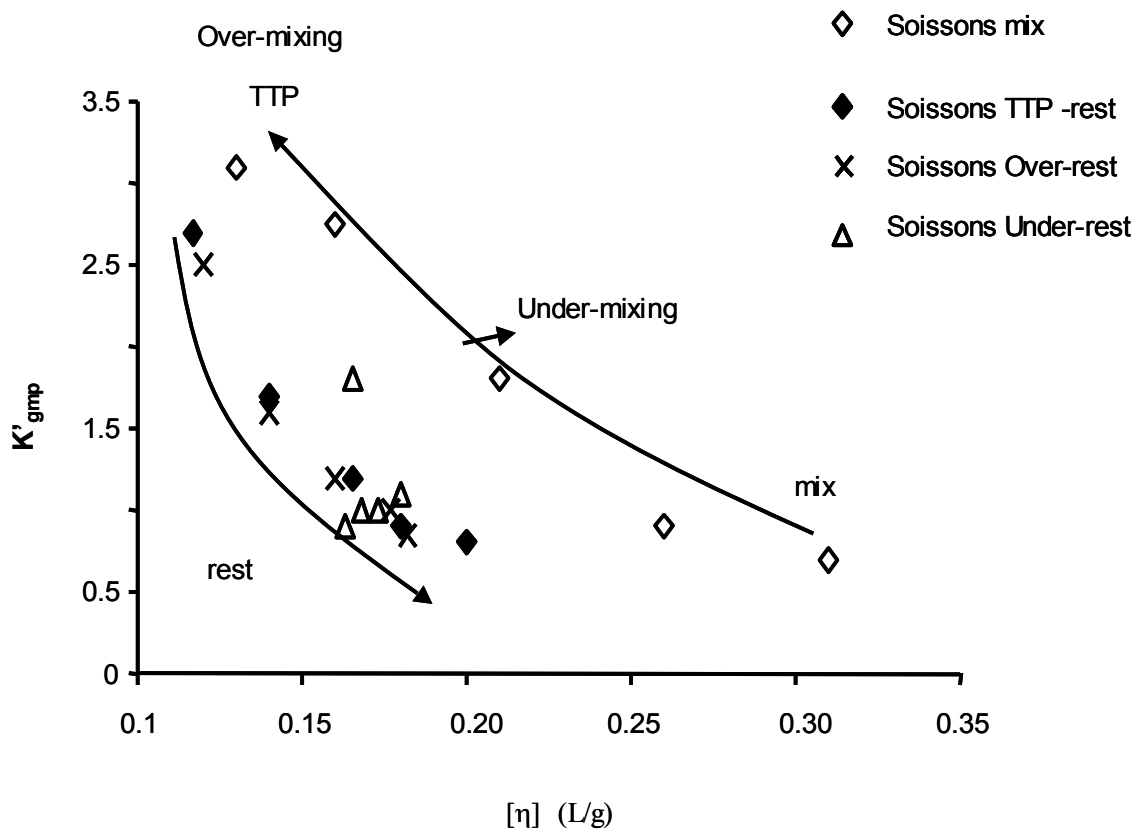*Over-mixed and Under-mixed situation.*

Figure 4.12   The K' vs. the glutenin particle voluminsosity for mixing of Soissons flour; and K' vs. the glutenin particle voluminosity for resting after respectively: TTP mixed, Over-mixed and Under-mixed situation.

Where K'increases during mixing, it decreases during resting. The changes in K' show that the increase in $[\eta]_{gmp}$ is accompanied by a decrease in K'. We stress that we do not consider that the glutenin aggregates in dough are spherical; it is more likely that mixing has irreversibly changed the particle morphology and size. Hence, during resting the more irregularly shaped glutenin aggregates form a network of very different properties and their size does remain lower than the spherical glutenin particles in GMP isolated from flour. This behaviour is observed for all three varieties. It is striking that, although K' and size directly after mixing are similar, large differences exist after resting. This could be due to differences in the internal composition (HMW/LMW ratio) of the glutenin particles.

4.4.5   The relationship between changes in glutenin particle properties during mixing and resting to glutenin network formation and dough rheology.

Our experiments have shown that the physical properties of the initially spherical glutenin particles present in GMP extracted from flour are changed by mixing to smaller, irregularly shaped particles. The two measurable parameters: size and K' in combination with observable changes in the glutenin aggregates could help understand how dough rheological properties are linked with glutenin particle properties. In the hyperaggregation model proposed by Hamer and Van Vliet (2000) to describe the different stages in glutenin protein network by aggregation from individual glutenin subunits to the macroscopic network three levels of organization are distinguished:

- the molecular level (Level I) where only covalent bonds are encountered, interactions are between the individual HMW and LMW glutenins and the formation of (SDS soluble) glutenin particles occurs

- the meso-scopic level (Level II) where physical aggregation is more important than covalent aggregation. At this level the interactions are between glutenin particles, affecting the formation of SDS insoluble GMP particles.

- the macro-scopic level (Level III) where aggregate formation is influenced by processing conditions.   Here glutenin particles participate in network formation affecting the subsequent macroscopic dough properties.

At level III, we assume the network is composed of soft interacting particles or particle aggregates. These soft particles form a network by interaction with one another and are known to exhibit the same viscoelastic behaviour during small and large deformation as that are characteristic of polymeric networks (Flickinger et al., 1999). This is similar to the proposal of Lefebvre and Van Vliet (2003) who suggested that a network of soft and deformable colloidal gluten particles determines the rheological properties of dough. By reference to the hyper-aggregation model it may be possible to provide an explanation for the results of our observations made with on different dough mixing regimes.

4.4.5.1 The optimally mixed regime

For optimally mixed doughs the Level II>III aggregation dominates the re-assembly of GMP during rest. Both the size and high K'of the glutenin particles at TTP enable re-assembly predominantly through physical interactions. Re-assembly starts immediately after the mixer is stopped (fig. 4.3a-

c). Table 4.4 shows that the HMW/LMW ratio of GMP after resting is not significantly different from that of GMP_{flour} (Skerrit et al. 1999).

Table 4.4   The HMW/LMW ratio of GMP from flour and mixed dough samples of varieties Estica, Soissons and Baldus.

Wheat variety	HMW/LMW ratio of GMP		
	Flour	TTP 60' rest	Overmixed 60' rest
Estica	0.62 ± 0.05	0.56 ± 0.04	0.34 ± 0.03
Soissons	0.78 ± 0.05	0.71 ± 0.05	0.57 ± 0.04
Baldus	0.83 ± 0.06	0.76 ± 0.05	0.61 ± 0.04

Between varieties differences exist in HMW/LMW ratio and leads to differences in the internal structure of the level II aggregates. We believe that these differences are responsible for the observation that with resting doughs, size differences in SDS insoluble glutenin particles re-appear. As suggested by Lefebvre and Van Vliet (2003), these differences may result in the observed differences in dough relaxation properties.

4.4.5.2 The under-mixed regime

In the under-mixed regime, glutenin particles are only partly dissociated and not evenly dispersed trough the dough. The size and interaction properties (K') of the particles (fig. 4.10-4.12, triangles) after mixing indicate a physical re-assembly mechanism, although less strong than with dough mixed to TTP. Re-assembly starts directly after mixing and results in aggregates after 90 min rest with similar K' and HMW/LMW composition (not shown) as in the optimally mixed regime. However, the final size of glutenin aggregates after resting is smaller. This is true for all three varieties tested, but cannot account for the systematic differences observed (fig. 4.2a-c), where dough relaxation times are consistently lower. Apparently in under-mixed doughs the aggregates are not homogeneously dispersed (Lee et al., 2001) and interactions between resulting aggregates are weaker (lower K') than with optimally dissociated particles. This may explain why subsequent partial re-assembly leads to larger aggregates, although they do not participate in a continuous particle network. The latter can also explain the lower $t_{1/2}$ observed.

4.4.5.3 The over-mixed regime

With over-mixing, glutenin particles are dissociated into even smaller fragments (fig. 4.1). The corresponding K'values (fig. 4.9) indicate that these fragments behave like free polymers in solution. At the chemical level the internal structure of the fragments is changed as is clear from the altered HMW/LMW ratio (table 4.4). As a consequence, re-assembly may now be governed by the transition between Levels I and II of the hyper-aggregation model and this can explain the 'lag time' observed in figure 4.3a-c, and why figure 4.5 shows how first larger oligomers are formed. The re-assembly of SDS insoluble glutenin particles (level II>III aggregation) occurs only when [η] > 0.10 l/g. After over-mixing and 60 min resting, the HMW/LMW ratio is changed indicating a different internal structure of the resulting glutenin particles. The extent of particle re-assembly is also less than with optimally mixed and under-mixed doughs. This may be explained by the end-blocker theory proposed by Kasarda (1999), which assumes that during mixing formation of covalent bonds responsible for the formation of large structures are blocked. The increased content of LMW glutenins in GMP is consistent with this explanation. The incomplete re-assembly and changed internal structure of the glutenin particles does not change the relationship between dough relaxation properties and glutenin aggregate size (fig. 4.7a-c). This relationship is the very much the same as in optimally mixed doughs. However, the relationship found with under-developed doughs is different and can be explained by differences in the ability to form a continuous particle network. Apparently, over-mixing does not prevent the formation of a continuous network. The effects of over-mixing on internal structure and final size of the particles are more important for dough rheology than the total amount of GMP obtained after mixing. Bloksma suggested (1972) that not every disulphide bond has a rheological effect, but this is still a matter of debate (Sutton et al., 2003). The over-mixing experiments show that K' largely determines the physical behaviour of the dissociated particles/oligomers during the re-assembly process. The changes in the internal structure of particles may determine the final properties of the network and hence dough rheological properties.

4.5   Conclusions

The systematic study of the effect of different mixing regimes on both glutenin particle breakdown and subsequent re-assembly has helped understanding of a number of important phenomena allowing us to propose a relationship between glutenin particle properties and resulting dough properties. This is based on two concepts: the hyper-aggregation model and the assumption that the glutenin particles are the structural elements that by forming an interacting network of deformable

particles determine dough properties. The time of optimal mixing is critical at this point particles are sufficiently dissociated and distributed to form a continuous network after resting, but still preserve the internal chemical network structure whose physical aggregation properties (K') allow re-assembly during resting. We suggest that in under-mixed doughs glutenin structures are still present in 'patches' and not able to form a continuous particle network. As a consequence, $t_{1/2}$ values of the resulting dough remain consistently lower. Finally, on over-mixing both the kinetics and extent of re-assembly and the internal structure of the particles are affected. The fact that, in contrast to glutenin particle size, the quantity of GMP in the rested dough does not correlate with $t_{1/2}$, indicates that the rheological behaviour of dough is perhaps more related to the size, structure and composition of level III aggregates rather than amount of glutenin. These aspects and the importance of the HMW/LMW ratio needs further study to strengthen the relationship between our observations and the postulates of the hyper-aggregation model.

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5

THE EFFECT OF MIXING ON GLUTENIN PARTICLES: HOW NEMI AFFECTS GLUTENIN INTERACTIONS AND GLUTENIN NETWORK FORMATION IN AN OPTIMALLY MIXED DOUGH

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Abstract

It is generally accepted that thiol-disulphide interchange reactions affect the mixing properties of dough. In most cases it is assumed that that breaking the disulphide bonds in the glutenin network will lead to extensive dough weakening. On the other hand, also strengthening effects have been reported, like the increase of the dough-mixing peak. In order to unravel the effect of –SH blocking we systematically investigated dough samples optimally mixed, with and without the –SH blocker NEMI. Apparently, the formation of an elastic network in dough is affected by NEMI, but rheologically the effects are insignificant. Under the special condition of optimally mixed dough, the elastic properties vs. rest of dough samples with NEMI are the same as the reference samples. It is clear that the mechanism of re-aggregation of glutenin has been affected. The covalent re-oxidation is not active in presence of NEMI, leading to smaller glutenin aggregates in dough. However, the physical aggregation properties are not significantly affected. Furthermore, a baking test indicated that NEMI insignificanly affected loaf volume. Hence, the quality of the glutenin aggregate interactions, are independent of glutenin particle size. Physical glutenin aggregation seems to have a relation with the internal composition HMW/LMW ratio of the glutenin aggregates.

Running Headline: How NEMI affects glutenin interactions

Key word index: GMP, GMP-particles, NEMI, dough rheology, hyperaggregation.

Abbreviations: SDS = Sodium Dodecyl Sulphate, GMP = Glutenin MacroPolymer, HMWGS = High Molecular Weight Glutenin Subunits, LMWGS = Low Molecular Weight Glutenin Subunits, CSLM = Confocal Scanning Laser Microscopy, FITC = Fluorescent Protein Label, PE = Petroleum-ether, SUP = supernatant fraction of GMP isolation, TTP = Time to Peak, ETP=Energy to Peak

5.1   Introduction

In chapter 4 we have demonstrated that mixing not only disrupts glutenin particles, but also the tendency of these particles to re-aggregate physically is positively affected by mixing to TTP. Mixing past TTP resulted in two effects: 1) it decreased the K'of glutenin particles 2) the glutenin particle size approached that of chemically reduced (DTT) GMP, hence indicating that over-mixing changes the physcio-chemical properties of the glutenin particle. The average size of the re-assembled GMP aggregates correlated positively with the elastic strength of the respective dough samples, revealing a link between meso-scopic glutenin aggregate size on one side and a dough rheological property like $t_{1/2}$ on the other side. The factors that affect glutenin particle interactions are its internal composition: reflected in the HMW-LMW ratio and mixing induced changes in glutenin particle shape (Don et al., 2005). This mixing-induced change in GMP-particles and the SDS soluble part of glutenins is thought to be accompanied an increase in their effective volume. Despite these interesting new clues on how deformation and compostion affect physical properties of glutenin particles, it is clear from many studies that –SH / S-S interchange reactions take place during mixing. For an example it has been demonstrated by Hoseney et al. (1984) how a -SH blocker like NEMI shortens the TTP, hence pointing to the importance of –SH chemistry in dough development and dough rheology. However, in rheological studies the blocking of –SH with NEMI did not always result in 'rheologically significant' differences (Bloksma, 1972). Recent observations on how mixing positively affects non-covalent particle interactions motivated us to investigate both GMP particle properties and dough rheology vs. rest of doughs optimally mixed in the presence of NEMI. In order to better understand the factors affecting the rheological properties of optimally mixed dough, both non-covalent factors and covalent interactions are discussed.

5.2   Experimental

5.2.1   Wheat flour samples

Flour was obtained from the following varieties: Amazon, Roblin and AC Phil, by using a Buhler mill, extraction rates were 76.3%, 76.1% and 78.1% respectively. Protein content (based on dry matter), HMW/LMW composition and TTP are presented in table 5.1. The respective TTP on a Z-arm mixer of the three flour varieties Amazon, Roblin and AC Phil was: 28, 15 and 3.5 minutes.

Table 5.1   Flour protein content and TTP for flour from wheat varieties
AC Phil, Roblin and Amazon.

Wheat variety	Flour protein content (%)	TTP (min)
AC Phil	12.7	3.5
Roblin	14.6	15
Amazon	15.8	28

5.2.2   Protein analysis

Flour protein content was measured by the Kjeldahl method. Protein content of GMP and GMP dispersions were measured using an UV absorption method (Hall, 1997). The UV method was successfully calibrated with a set of Kjeldahl protein values of GMP.

5.2.3   Isolation of GMP from flour

Flour defatted with petroleumether (percolation method) was suspended in 1.5% SDS (1.4 g flour in 28 mL, SDS of analytical grade from Merck™) and centrifuged at 80.000 g for 30 minutes at 20°C in a Kontron Ultracentrifuge (Graveland et al., 1979) The supernatant (SUP) was decanted and the gel-layer collected as GMP.

5.2.4   Isolation of GMP from mixed dough sample

The dough sample was freeze-dried after mixing. The freeze dried dough was powdered on a Retsch mill using a sieve of 0.25mm. Isolation of GMP was done as described for isolation from flour.

5.2.5   GMP dispersion

GMP dispersions were prepared by transferring 1g of GMP gel to a tube containing 10 mL 1.5% (w/v) SDS solution. The gel was mixed with the solvent by briefly stirring with a spatula. Then the tube was sealed and placed on a roller-bank for 3h at ambient temperature. This produced a visually homogenous, opalescent dispersion, indicating that part of the protein polymers are not dissolved, which was used for further characterisation.

5.2.6 Rheology of GMP gels

1 gram of material was carefully taken from the top of the gel and transferred into the measuring cell of a Bohlin VOR rheometer (Bohlin Instruments). The cell consisted of two parallel plates (d=30 mm) with a gap of 1 mm. Measurements were performed at 20°C in a strain sweep mode at amplitudes ranging from 1% to 100% at 0.15 Hz. Data were expressed as G' vs strain, G' plateau value or delta (δ) vs strain.

5.2.7 Viscometry of GMP particles

The viscosity of GMP dispersions was measured using an Ubbelohde capillary viscometer. The pass through time was in the order of 300-400 seconds to optimise accuracy. A so-called Huggins-plot was prepared using the reduced viscosity vs. GMP concentration data. From this Huggins-plot the intrinsic viscosity [η] and the Huggins constant K' were obtained. The [η], the suspended particle voluminosity, is estimated from the intersect point for GMP concentration → 0. K' the Huggins-constant reflecting particle interactions is calculated from the slope of the Huggins-plot.

5.2.8 Coulter Laser particle size analysis

Particle size analyses of diluted GMP dispersions from varieties Baldus, Soissons and Estica mixed to peak and rested for 90 minutes was performed with laser diffraction using a Coulter LS 130. In this instrument laser light is scattered by the suspended particles and the generated diffraction pattern, which is a composite of the diffraction patterns for all the particles, is measured. For GMP from flour a spherical shape for GMP particles was observed,.the instrument can convert the composite diffraction pattern into a particle size distribution with 72 classes ranging from 0.3 to 900μm using Fraunhofer theory.

5.2.9 Mixing experiments with a Z-blade mixer type

Mixing of defatted flour was performed using Brabender Plastograph equipped with a 10g Farinograph bowl. In the preparation of the dough 2% (w/w) NaCl and water were added and the mixing speed was 63 rpm. The required amount of water was determined according to ICC procedure 115/1[21]. Energy input (Nm) and TTP can be calculated from the recorded Torque vs. time plot with the Plastograph software. Energy input is an important parameter in practical dough development. In general the time to dough peak resistance (TTP) is a reference point. In the technical sense: 1) dough is under-developed or under-mixed before TTP, 2) dough is developed or

optimally mixed at TTP and 3) when mixing till after peak resistance the dough is broken down or over-mixed. The designation of under, optimal or overmixing is always somewhat arbitrary, since it varies with for example the variety or mixing conditions used.

5.2.10 Flow-relaxation measurements

Flow-relaxation measurements were carried out using a Bohlin VOR rheometer equipped with a serrated plate geometry with a cross section of 30mm. For flow relaxation measurement a piece of dough was placed in the rheometer geometry, after which the gap was set at 3 mm. Moisture loss from the dough piece was minimized by a plastic cover that was placed over the measuring geometry. Since the insertion of the dough piece ay cause some stress, an equilibrium time of 10 minutes was used before the actual measurement. The measuring temperature was 30°C. For the measurement the sample was deformed by a strain of 100% at a shear rate of 0.0208 1/s. The decrease of stress of the dough was recorded in time. The time necessary for the dough to relax to a stress of 50% of the initial stress was taken as the flow-relaxation halftime ($t_{1/2}$).

5.2.11 Kieffer extensograms

Dough was prepared as described in section 2.9. A teflon Kieffer block and teflon strips were used to prepare dough strips. A TA.XT2 texture analyzer was used to perform the measurements. Procedures were followed as described by Bekes et al. (2003)

5.2.12 RP HPLC

The HMW-LMW GS composition of GMP from flour and dough samples was assessed using a Waters HPLC with a C8 bonded phase column. 1g GMP was extracted with 1 mL 50 (v/v) % propanol, 0.02% (w/v)DTT followed by extraction with 1mL solvent A. These two extracts were combined as sample for HPLC analysis. Solvent A contains 6M Urea, 20 % (v/v), 0.1 %(v/v) TFA and 0.02% (w/v) DTT. Solvent B contains 5.5M Urea, 50% (v/v)ACN, 0.1 %(v/v) TFA and 0.02% (w/v)DTT. Loading was 10μL of GMP extract. Column temperature was maintained at 50°C. The column was eluted at a flow rate of 1mL/min, with a multistep gradient starting at 20% B to 80% B. Typical elution times for the HMW glutenins were 30-45 minutes and for LMW glutenins 70-85 minutes.

5.2.13 Simple baking test

Based on Roblin flour 2% salt and 1.67% dry yeast (Fermi Pan Red) was used. Water addition was according to ICC method. Reference without NEMI, test with 12 micromole/g flour NEMI. The dough was optimally mixed in a 300g Farinograph bowl at 20°C. Take out and shape dough, then proofing for 20 minutes at 30°C. Make up and shaping followed by 47 minutes proofing (30°). Baking for 15 minutes at 240°C.

5.3   Results

5.3.1   Glutenin particle disruption – physical and chemical mechanisms

Table 5.2 reflects various typical characteristics of GMP isolated from flour.

Table 5.2   Characteristics of GMP gels and GMP dispersions from flour samples

Wheat variety	GMP wet weight* (g)	GMP protein concentration* (mg/g)	HMW/LMW ratio*	G' plateau value* (Pa)	K'	[η] (L/g)
AC Phil	2.6 ± 0.2	10.2 ± 0.4	0.64 ± 0.04	22 ± 2	1.6	0.25
Roblin	2.9 ± 0.3	12.6 ± 0.7	0.87 ± 0.06	70 ± 3	0.5	0.33
Amazon	4.1 ± 0.7	12.1 ± 0.7	1.03 ± 0.09	165 ± 11	0.2	0.38

* n = 5, ± s.d., K' and [η] from linear regression of reduced viscosity vs. concentration plot with $R^2 > 0.95$

As in chapter 4 also for these three varieties clear differences in GMP quantity and protein concentration can be observed. Dispersion rheology shows that an increase in intrinsic viscosity values parallels an increase in the optimal mixing time (table 5.1). The [η] is a measure of the glutenin particle voluminosity and size (chapter 3, 4). Applying Kick's law to dough mixing, Don et al. (2003) have shown that required energy to optimally develop dough can be estimated from the glutenin particle size in flour. However, this 'mechanical-physical' approach does not include effects of thiol-disulphide interchange reactions in dough development (Goldstein, 1957; Hoseney, 1984). Nevertheless, such interactions are also important. Hoseney (1984) showed how addition of NEMI to the flour + water mixture decreases the TTP in dough mixing.
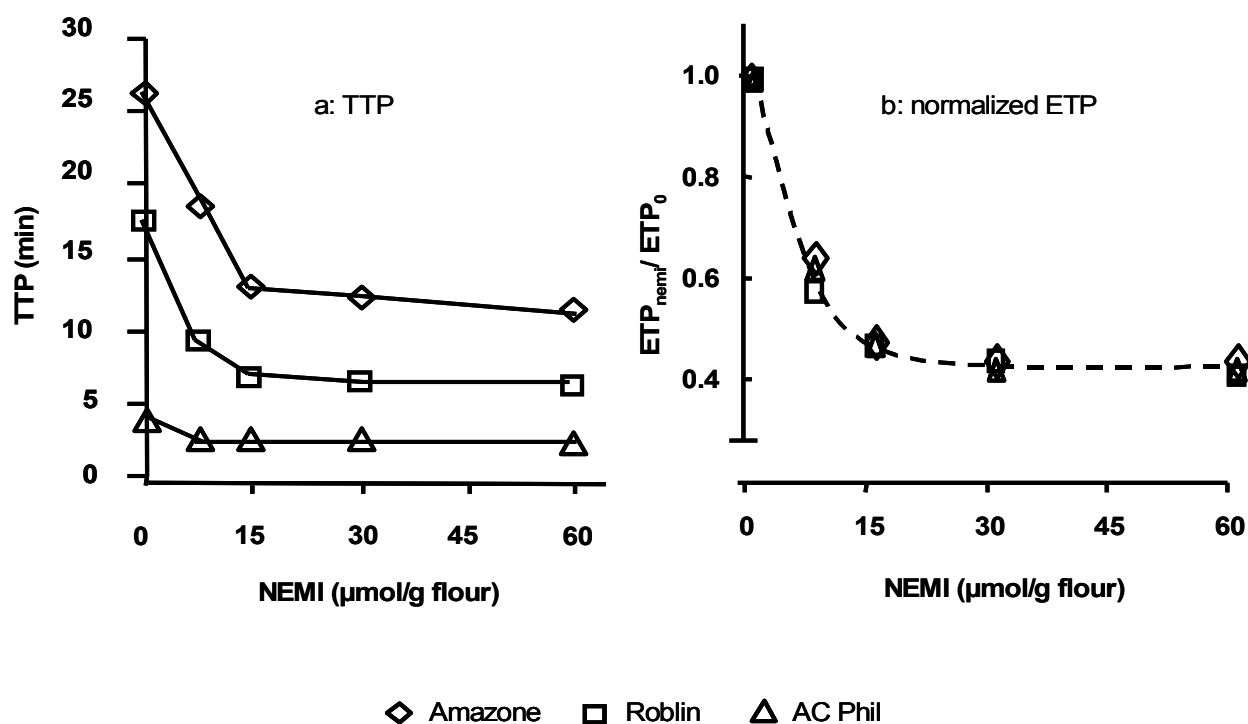
Figure 5.1a –b   *Effect of NEMI on wheat flour dough mixing time and energy. Figure 5a showing decrease of mixing time vs. NEMI. Figure 5b showing normalized energy to peak vs. NEMI (legend shows the wheat varieties used).*

Figure 5.1a shows how TTP decreases with increasing addition of NEMI. The TTP of Amazone, Roblin and AC Phil all decrease significantly. Addition of NEMI at concentration > 15µmol hardly affects TTP. Apparently, 15µmol of NEMI is sufficient to block both free thiols and thiols originating from disrupted disulphides. Interestingly, this applies to the three varieties tested. Figure 5.1b shows a normalized energy to peak (ETP) against NEMI. From this it can be observed that excess NEMI can reduce the ETP to ~45% of the control. Our results confirmed again that interfering with the thiol-disulphide interchange reactions affects mixing energy, it shortens TTP. Nevertheless, even at an excess of NEMI the respective ETP is: Amazon > Roblin > AC Phil, paralleling the initial particle voluminosities in table 5.2. It is apparent though; that by preventing the re-oxidation of disulphide bonds the energy required to disrupt the glutenin particles mechanically can be strongly reduced.

5.3.2 Effect of NEMI on flow relaxation of dough

Flow relaxation measurements require less time in dough preparation, allowing for getting an indication of the dough rheological state at 15' rest-time intervals starting at 15 minutes dough rest. We proceeded to compare the visco-elastic properties of dough mixed to TTP with NEMI (15μmol) and dough mixed to TTP without NEMI. For dough mixed with NEMI the TTP values were: 13.5, 8.0 and 1.9 minutes for Amazon, Roblin and AC Phil respectively. The TTP for dough mixed without NEMI are shown in table 5.1. Similar as in chapter 3, figure 2 shows how dough $t_{1/2}$ increases vs. rest. For all three varieties an increase of $t_{1/2}$ is noticed vs. rest, indicating that an elastic network is formed during dough rest.

Figure 5.2 a-c   *Increase of the flow relaxation half time ($t_{1/2}$) vs. rest time for optimally mixed resting dough prepared from Amazon (top left), Roblin (top right) and AC Phil (bottom right). At the bottom left the symbols and NEMI additions are explained*

Interestingly, the visco-elastic properties of dough are not significantly affected by mixing to TTP with NEMI. Extensogrammes are usually taken at 45' rest-time intervals, starting at 45 minutes

dough rest up to 135 minutes (ICC). Kieffer extensibility results may reveal how NEMI has affected dough rheological properties. Results are presented in table 5.3.

Table 5.3   The extensibility results for dough mixed to TTP, [η]$_{gmp}$ and t$_{1/2}$

Wheat variety	90'dough rest			
	R_{max} (N)	E_{max} (mm)	$[\eta]_{gmp}$ (L/g)	$t_{1/2}$
AC Phil	0.19 ± 0.02	101 ± 12	0.13	30 ± 2
Roblin	0.49 ± 0.05	54 ± 7	0.22	58 ± 3
Amazon	0.61 ± 0.07	77 ± 8	0.26	85 ± 5
AC Phil*	0.21 ± 0.02	109 ± 13	0.11	28 ± 2
Roblin*	0.51 ± 0.05	56 ± 8	0.14	57 ± 3
Amazon*	0.57 ± 0.06	81 ± 9	0.15	81 ± 4

* mixed to TTP with 15 μmol NEMI; $[\eta]_{gmp}$ from Huggins plot with $R^2 > 0.95$

The extensibility results are expressed as R_{max}, the maximal resistance of the dough and E_{max} the maximal extension of the dough. The results clearly indicate that the R_{max} follows Amazon > Roblin > AC Phil. When NEMI is used insignificant variation in average R_{max} is observable due to NEMI, but values are in the same order with NEMI, the R_{max} follows: Amazon* > Roblin* > AC Phil*. For E_{max} a slight but insignificant increase is observed with NEMI. Flow relaxation and extensibility are different rheological tests The results present a parallel between $t_{1/2}$ and R_{max}. This indicates that $t_{1/2}$ has a close parallel to dough strength parameter R_{max}. With the set of flour varieties used here variations in glutenin/gliadin, that are known to affect E_{max}, should be expected. We did not particularly study the gliadin part for this chapter, so we restrict this discussion to rheological parameters that are mainly affected by the glutenin fraction. The rheological results confirm earlier observations on gluten dough rheology of Lasztity et al. (1998). Nevertheless, the formation of a gluten-network in dough has often been hypothesized to depend on re-oxidation of disulphide bonds. The dough vs. rest data indicate that $[\eta]_{gmp}$ and $t_{1/2}$ respond during the first 60 minutes rest, after 90 minutes $[\eta]_{gmp}$ and $t_{1/2}$ seem to have reached a limit in increase.

When it is assumed that NEMI has blocked a large part of the free –SH groups, then these dough rheological results are perhaps not sensitive to a lack of some re-oxidised disulphide bonds. As Bloksma suggested (1964), not all disulphide bonds may be rheologically effective. Somehow, rheological results do not provide enough footing for understanding the effect of –SH blocking by NEMI. On one hand the effect on TTP in mixing is quite obvious, on the other hand the rheology of

the dough appears to 'restore' itself even in the presence NEMI. Lefebvre et al. (2000) showed that glutenin aggregate size parallels the elastic plateau (G_N^0) of gluten dough The size of re-assembled glutenin aggregates affect the trends observed in various rheological methods that aim to reveal the 'rheological strength' of a dough sample. It is also clear from these results that for mixing to TTP with NEMI, Kieffer extensibility parameters do not reveal a significant rheological effect of smaller GMP quantity and glutenin particle size.

In various studies it has been shown that parallels can be drawn between the presence and properties of GMP and dough properties (Weegels et al. 1996, 1997; Don et al., 2003; Kelfkens and Lichtendonk, 2000). Therefore, we studied the re-assembly of GMP vs. dough rest for dough mixed without and with NEMI.

5.3.3 Reassembly of GMP quantity

In figure 5.3 the re-assembly of GMP vs. rest is shown. For dough mixed without NEMI the typical re-assembly of GMP is observed (Don et al., 2004). Within 90 minutes $\geq$ 95% of the GMP originally present in flour is recovered. When the dough is mixed to TTP with NEMI the recovery of GMP is: 1) slower and 2) after 90 minutes GMP has not fully recovered. For dough mixed with NEMI: 75%, 68% and 59% of the original GMP quantity in flour is recovered for Amazon, Roblin and AC Phil respectively.
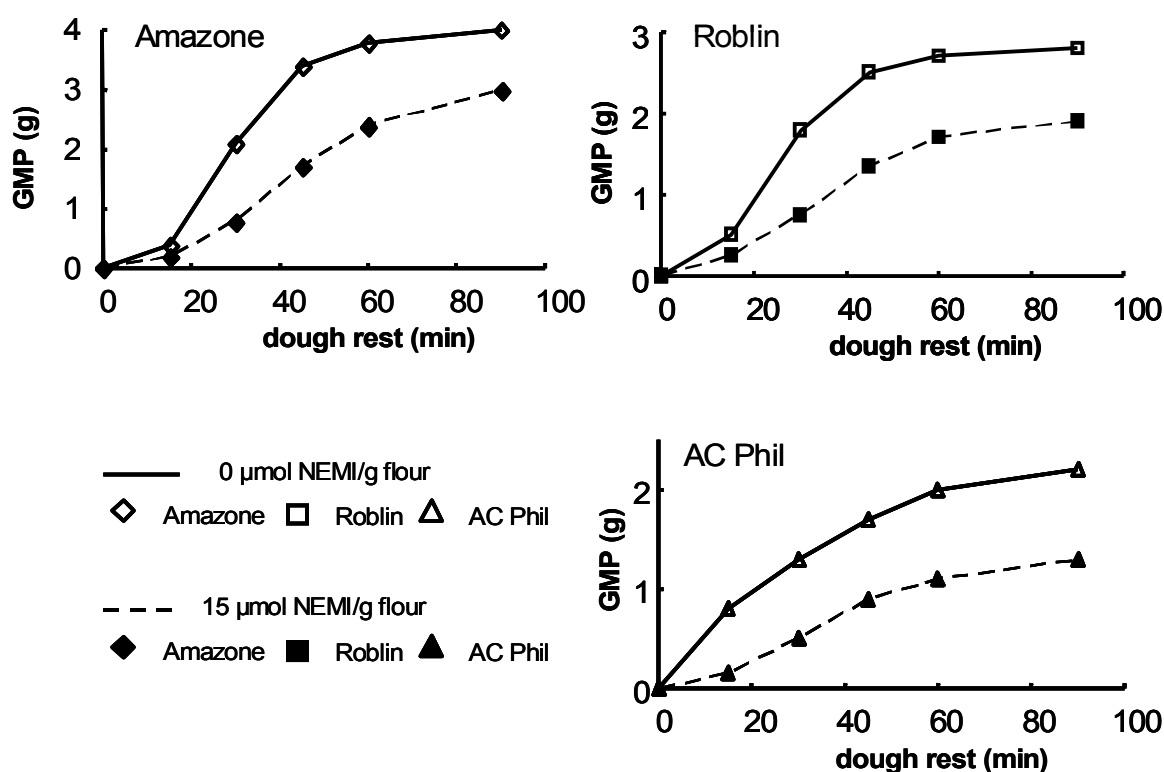
Figure 5.3 *The re-assembly of GMP against dough rest time for optimally mixed dough with NEMI (filled symbols) and optimally mixed dough without NEMI. Results for Amazone (top left), Roblin (top right) and AC Phil (bottom right).*

Comparing this with our results in chapter 4 (Don et al., 2005), mixing to TTP with NEMI appears to have a similar effect as overmixing. We observe that a simple parallel between GMP quantity and dough rheology can not be drawn. The decrease of GMP is not related to changes in rheology. The analyses on GMP confirm that –SH blocking with NEMI has affected the re-aggregation of GMP. Visually, GMP-gel that could be recovered from the dough mixed with NEMI was much weaker than gel recovered from dough mixed without NEMI. The SDS insoluble parts were dispersed; both the GMP dispersions and SUP from rested dough samples were further analysed viscometrically, analogous to our approach in chapter 4.

5.3.4 Glutenin particle size increase vs. rest

Figure 5.4 shows the result for re-aggregation of GMP isolated from dough mixed without NEMI. Figure 5.4 depicts similar features for Amazon, Roblin and AC Phil as observed for the re-aggregation of optimally mixed dough from Baldus, Soissons and Estica in (Don et al., 2005).

At TTP all varieties have roughly the same $[\eta]_{sup}$, this is at the starting point at 0' of dough rest. Again, differences in rate of change and final size of glutenin particles are observed. The weakest variety AC Phil shows a strong initial decrease of $[\eta]_{sup}$ vs. rest, but the $[\eta]_{gmp}$ does not increase as much as for Roblin and Amazon. The strongest variety Amazon appears to have slower initial decrease of $[\eta]_{sup}$, but $[\eta]_{gmp}$ increases more effectively. The glutenin particle voluminosity appears to parallel the dough $t_{1/2}$

Figure 5.4   *Re-aggregation process of SDS soluble and insoluble glutenin at various rest times for optimally mixed dough without NEMI. The x-axis shows the increase of insoluble glutenin aggregates. The y-axis shows the decrease of glutenin size in the supernatant phase.*

Glutenin aggregate size parameters and dough rheological properties have been shown to correlate (Lefebvre et al., 2000; Cornec et al., 1994; Don et al., 2005). As shown in figure 5.3, GMP quantity appears to be sensitive to the effect of NEMI. From figure 5.5 it can be seen whether re-aggregation for glutenin particles and in glutenin SUP is affected by NEMI.
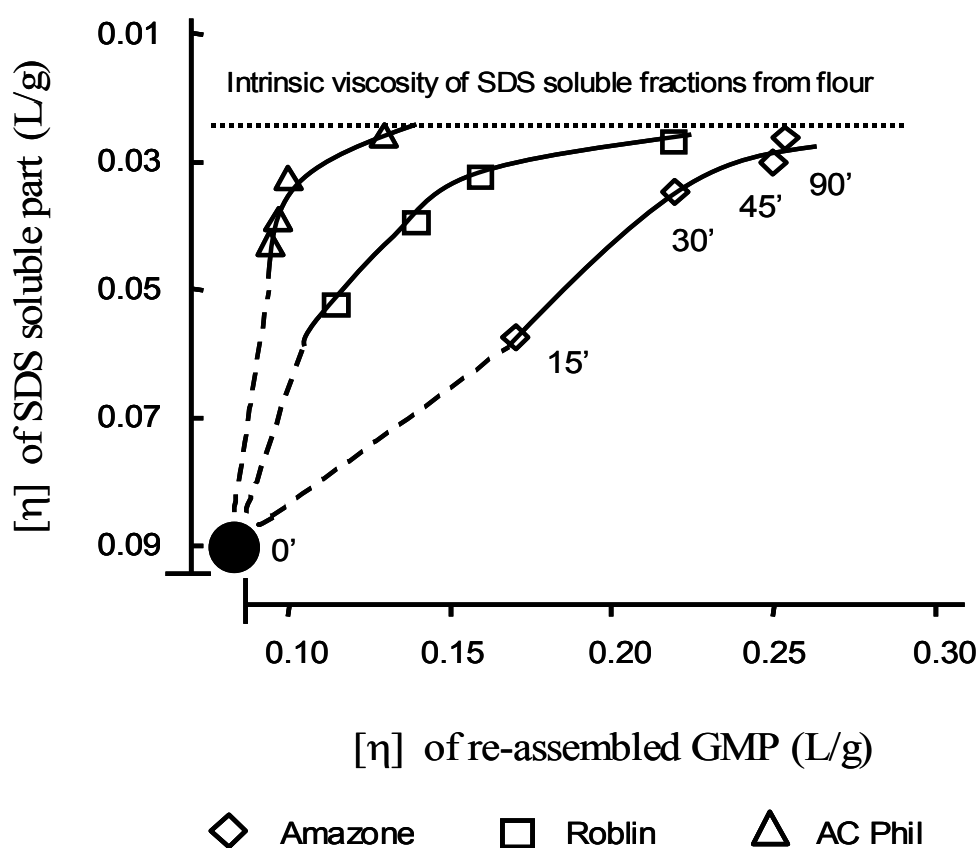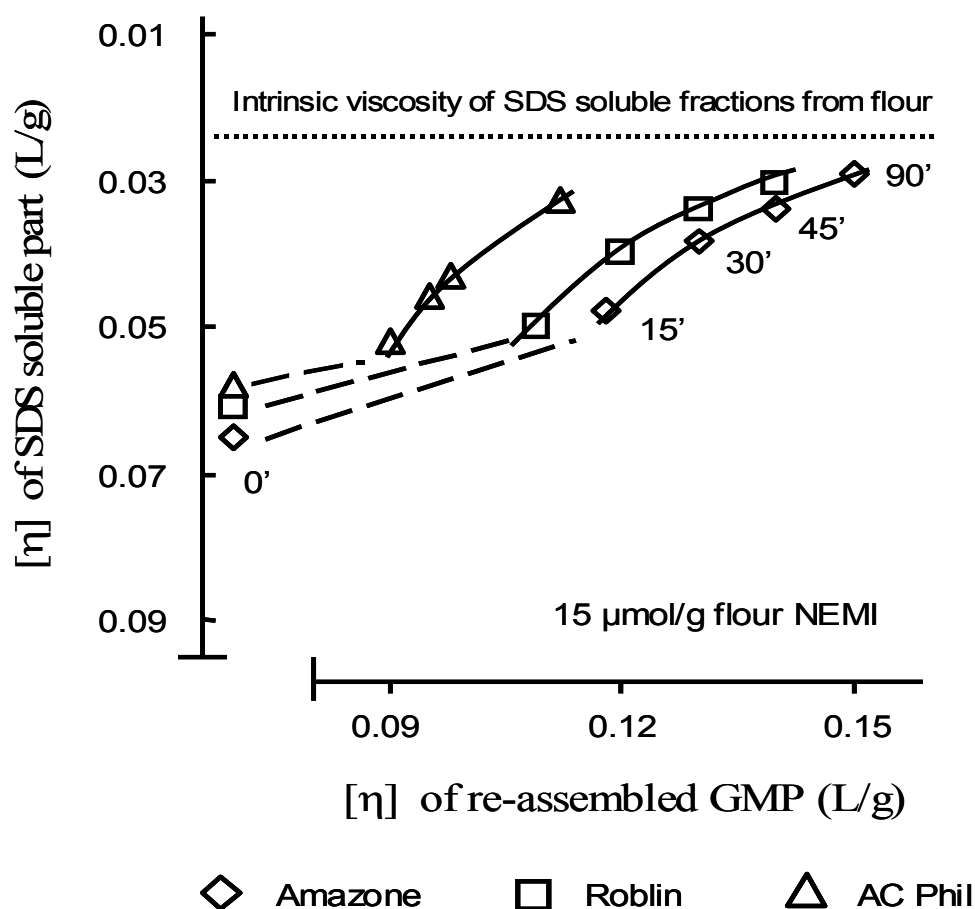
Figure 5.5   *Re-aggregation process of SDS soluble and insoluble glutenin at various rest times for optimally mixed dough with NEMI. The x-axis shows the increase of insoluble glutenin aggregates. The y-axis shows the decrease of glutenin size in the supernatant phase.*

With NEMI the average starting-point is not the same for each variety. A starting point < 0.09 L/g for the $[\eta]_{sup}$ has a resemblance to what was observed with over-mixed dough (Don et al., 2005). Over-mixing breaks down the glutenin particles further, but not to the extent of DTT then $[\eta]_{sup}$ decreases to ~0.02 L/g. Similar to over-mixing, use of NEMI lowers the starting-point of $[\eta]_{sup}$, values are ~0.06 L/g indicating that NEMI does not react te same on the GMP particles as DTT. However, contrary to over-mixed systems for the dough with NEMI the $[\eta]_{sup}$ does not increase in the first 15' of dough rest associated with disulphide bond formation. Clearly, NEMI prevents covalent repolymization. When NEMI is present the decrease of $[\eta]_{sup}$ starts immediately and increase of $[\eta]_{gmp}$ is observed. Also contrary to figure 5.4, in presence of NEMI the decrease of

$[\eta]_{sup}$ for AC Phil is initially less than for Roblin and Amazon. Finally, it can be seen that the $[\eta]_{gmp}$ values in figure 5.5 are smaller than in figure 5.4 and that $[\eta]_{sup}$ remains higher at 90' in figure 5.4 than in figure 5.5. The much lower $[\eta]_{gmp}$ in figure 5.5 corresponds to the observed weakness of GMP isolated from dough with NEMI as Don et al (2003) have shown that: $([\eta]_{gmp})^{3.3} \approx G'_{plateau}$. Together with GMP re-assembly shown in figure 3, the differences in re-aggregation behaviour (fig. 5.4 and 5.5) indicate that NEMI has blocked the disulphide reduction/re-oxidation mechanism for glutenin re-aggregation in dough, but not physical re-aggregation. Both SDS soluble glutenin and glutenin aggregate voluminosity are expected to have an effect on dough rheology (Weegels et al., 1996).

5.3.5 Particle voluminosity vs. dough $t_{1/2}$

From corresponding data-points in figures 5.2 and 5.4 it is possible to plot $t_{1/2}$ against aggregate size parameter $[\eta]_{gmp}$. The result is shown in figure 5.6. The open symbols and line in figure 5.6 show a clear parallel between $[\eta]_{gmp}$ and $t_{1/2}$ n=9 a R^2=0.89 is noticed, in agreement with previous results in chapter 4 where we found R^2=0.85, n=9 with three other varieties.
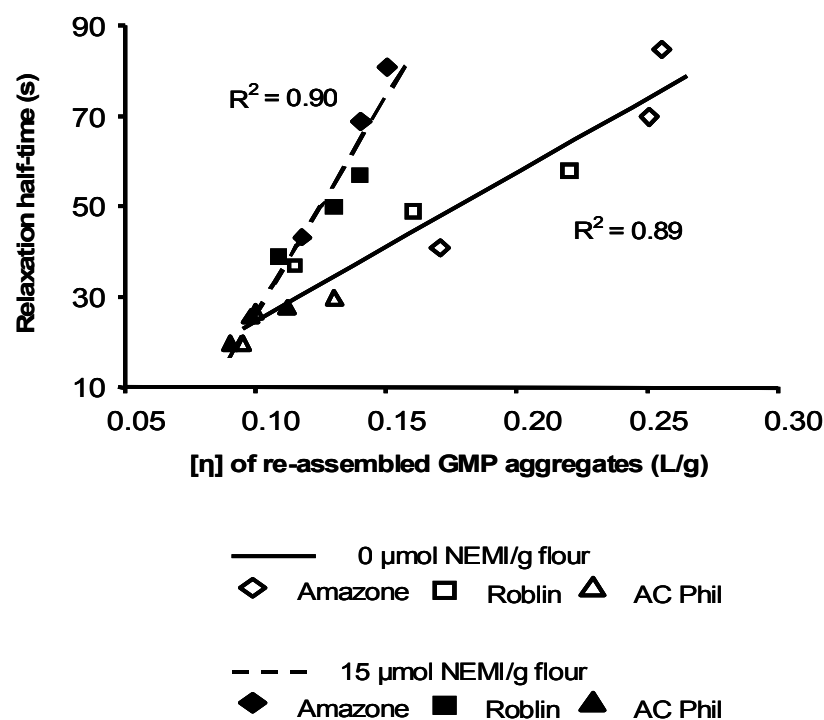

Figure 5.6   *The glutenin particle voluminosity plotted against dough $t_{1/2}$ for NEMI and non-NEMI mixed dough samples prepared with Amazone, Roblin and AC Phil flour.*

Figure 5.6 shows that also with NEMI a relation is found between $[\eta]_{gmp}$ and $t_{1/2}$. When dough is mixed with NEMI we find a completely different line for $[\eta]_{gmp}$ vs. $t_{1/2}$ as shown by the dotted line and filled symbols in figure 5.6. The $[\eta]_{gmp}$ is much lower for these dough samples, a correlation of $R^2=0.90$ appears between this $[\eta]_{gmp}$ in presence of NEMI and dough $t_{1/2}$. Clearly, other factors besides glutenin-particle size may have compensated for the loss of GMP quantity and glutenin particle size, enabling the dough to maintain similar $t_{1/2}$.

It is clear that by affecting covalent interactions with NEMI: TTP, GMP and $[\eta]_{gmp}$ are affected, however this can not explain the rheological similarities in dough $t_{1/2}$ with and without NEMI (figure 5.2). It has been shown in other papers that dough rheology could not present the quantitative footing required for sustaining the necessity of covalent interactions (Bloksma, 1972; Sutton, 2004). One of the factors not taken into account is K' an indicator of particle-particle interactions and glutenin composition, the HMW/LMW ratio.

5.3.6   The effect of NEMI on Huggins constant K'

In order to see whether other interactions are altered by NEMI we followed the K' of GMP for dough mixed to TTP. The results are shown in figure 5.7a and b.

Figure 5.7 a-b The glutenin particle K' against mixing energy for the non-NEMI (a) and NEMI (b) dough samples (see legend for flour samples and explanation of symbols).

In figure 5.7 it can be seen that the K' versus mixing follows a similar pattern as observed in the previous paper. Also, here there seems to be a variety dependence for the increase of K' versus mixing energy. Apart from the strong lowering of the mixing energy required to optimally mix the dough, the increase of K' vs. energy has an overall resemblance for what can be seen when dough is mixed without NEMI. Looking at figure 5.7 in more detail it appears that the initial rise in K' appears to go faster with NEMI and as mixing nears the optimal mixing energy the fast increase in K' appears to come to a stop. This is due to decrease in enery/time required to reach TTP. The

differences between figures 5.7a and 5.7b, suggest that NEMI affects the rate of breakdown of the glutenin particles, but not the effective aggregation properties of the particles. The increased rate of breakdown also explains why NEMI dough is more prone to over-mixing.

5.3.7 *The effect of NEMI on HMW-LMW glutenin composition*

Reflecting on the data so far, it is obvious that NEMI has a clear effect on the glutenins, it is most clear on GMP re-aggregation and $[\eta]_{gmp}$, Although average size is smaller, the level of interactions is similar with NEMI and rheologically the dough samples respond the same. In order to check differences in the chemical compostion of GMP we checked for changes in the HMW/LMW composition of GMP from dough samples rested for 60', the results are shown in table IV. As indicated earlier mixing to TTP minorly changes the HMW/LMW ratio. A lowering of HMW/LMW is noticed, but this remains < 10%. It may be argued whether this is really significant (Skerrit, 1999). For NEMI table 5.4 shows that mixing with NEMI lowers HMW/LMW about 20%. This is a more pronounced lowering of HMW/LMW with NEMI and is similar to overmixing (Skerrit, 1999, chapter 4). NEMI has a more significant lowering effect on the HMW-LMW composition of GMP.

Table 5.4   HMW/LMW ration of GMP for flour and dough

Wheat variety	HMW/LMW ration of GMP		
	Flour	TTP 60' rest	TTP + NEMI 60' rest
AC Phil	0.64 ± 0.04	0.58 ± 0.03	0.48 ± 0.03
Roblin	0.87 ± 0.06	0.82 ± 0.06	0.71 ± 0.05
Amazon	1.03 ± 0.07	0.97 ± 0.06	0.85 ± 0.06

For flour vs. NEMI the lowering of HMW/LMW bares significance

5.4   Discussion

This chapter is centered around two observations: First, that addition of NEMI leads to a strong decrease in energy required to reach TTP. This confirmed results on the effects of thiolchemistry in dough (Hoseney and Rogers, 1990; Kaufman et al., 1986) . Second, doughs mixed in the presence of NEMI but until TTP, show a similar reological behaviour as untreated dough. Interestingly Tkatchuk (1972) observed a shorter development time with iodoacetic acid, but increased loaf volumes. Kilborn and Tipples (1973) observed a decrease in optimal mixing time with cysteine, but as long dough was optimally mixed this did not show to be detrimental to "pup" loave volumes. We performed a bake test with optimally mixed NEMI dough using Roblin flour. The volumes were similar, NEMI (right picture). Also a finer structure was noticed.

Figure 5.8   "Pup" loaves baked without NEMI (left) and with NEMI (right). For both tests, dough was prepared by mixing to TTP. Gas production during proofing was the same for both tests (- / + NEMI).

We stress that it was not our goal to confirm with NEMI, that acting on -SH chemistry is not detrimental to baking performance, as this was already shown by Tkachuk (1972) and Kilborn and Tipples (1973). We used NEMI to help understand the physical chemical nature of mixing and resting, resulting in dough rheological properties. In previous chapters clear relations were observed between GMP, glutenin particle properties and mixing energy requirements and dough reology. To what extent are these relations still valid and can GMP and glutenin particle properties still help explain the phenomena observed in this chapter?

5.4.1 *Glutenin particle composition and dough $t_{1/2}$.*

In figure 5.2 we showed how dough $t_{1/2}$ seemed not affected by NEMI. The glutenin particle sizes are however clearly different as seen in figure 6. What is the factor that explains that the smaller glutenin particles can still aid in the formation of a network that has similar elasticity as dough containing larger particles? In figure 8 the dough elasticity is plotted against the HMW/LMW ratio.

Figure 5.9   The flow relaxation half-time of dough samples versus the respective HMW/LMW ratio of glutenin (see legend for explanation of symbols).

From figure 5.9 it becomes apparent that particle composition affects t1/2 and not particle size. In chapter 6 we have seen that HMW/LMW is related to glutenin particle size. NEMI changes this relation, but the particles still have network-forming properties. The K' of the particles increases to similar values for both dough systems (figure 5.7). In figure 5.10, K' is plotted against HMW/LMW.

Figure 5.10   The K' of GMP from dough versus HMW/LMW ratio (see legend for explanation of symbols).

From figure 5.10 it is clear that the increase of K'is related to HMW/LMW. NEMI has affected size, but HMW/LMW affects K' after mixing to TTP and $t_{1/2}$ at 60 minutes rest. We therefore consider that internal particle composition is more important than particle size for getting an elastic network. Probably K' reflects quality of particle interactions and this seems to be important for rheology and baking properties.

5.4.2   Hyperaggregation: both chemical and physical aspects of glutenin network formation

The results show the importance of SH-chemistry during dough mixing: TTP is lowered in a concentration dependent way and also the HMW/LMW ratio is significantly changed. During dough rest physical properties must be important because in the presence of NEMI reassembly is observed and dough rheological properties after resting are comparable to control dough. Our results show that the HMW/LMW ratio is the common factor that affects the aggregation properties of glutenin particles. Nevertheless, we observe a systematic effect of NEMI on K'. Reaction of free SH groups with NEMI will change the surface charge of glutenin particles. Although not studied in this paper, changes in surface charges will undoubtedly affect particle-particle interactions. This does not explain how the dough with NEMI has the same rheology with less GMP (figures 5.2 and 5.3). Variation in dough rheological properties is governed by (from general to specific) protein content, gluten content, ratio glutenin-gliadin, and quantity and properties of the highly aggregated glutenin fraction. This study specifically focuses on the role of the highly aggregated glutenins, since it is based on a pair-wise comparison of dough +/- NEMI. Clearly, in this case the variation in dough properties cannot be explained on the basis of only SDS insoluble glutenin protein (GMP). Weegels (1996) studied the relevance of total amounts of flour glutenin, flour GMP and dough GMP with respect dough extensibility and resistance against extension. Interestingly, variation in dough extensibility was better explained by flour GMP than by dough GMP. In contrast, resistance against extension was best explained by dough GMP. More recently, Wang et al. (2003) showed that for gluten, the glutenin particle size affected R_{max} and K' affected E_{max} of gluten. Since addition of NEMI leads to similar values of K' , this could explain why in this study similar E values are found for dough +/- NEMI. The size of particles after resting NEMI dough, however, is considerably smaller (η 0.09-0.15 versus 0.18-0.25 for non treated dough). Hence, with NEMI the relation between η and Rmax is changed.

Our results show that with NEMI, reassembly of glutenin structures and –likewise- dough rheological properties are largely governed by physical interactions. Their number and their quality determine these interactions. The number of interactions is dependent on the quantity of GMP and the average size of the glutenin particles. With NEMI both are reduced. Hence, this cannot explain the similar rheological properties found +/- NEMI. The quality of interactions is determined by the composition and structure of the particles. In this study, this is expressed as the ratio of HMW/LMW. Figure 5.9 shows that NEMI has a clear effect in changing the HMW/LMW ratio of glutenin particles after resting. Interestingly, HMW/LMW correlates well with t ½ independent of

the use of NEMI. A possible reason for this is provided by figure 5.10, where we demonstrate that the physical interaction parameter K' is also changed. Without NEMI we observe a lower K' at a lower HMW/LMW ratio. With NEMI, at a given HMW/LMW ratio a higher K' is observed. Although still based on statistical correlations, these results show that the quality of interactions in NEMI dough is significantly different from non-NEMI dough. We think this explains the rheological results found with NEMI dough.

5.5 Conclusions

Based on the results obtained we conclude that dough mixing time is determined by a balance between glutenin particle breakdown and S-S assisted reformation. Addition of NEMI leads to a shift in this equilibrium thus explaining shorter required dough mixing times. Provided that NEMI dough is mixed to TTP, similar dough rheological properties and product quality can be obtained as non-NEMI dough. The detailed analysis of this process at the level of GMP and glutenin particles has shown that while NEMI clearly affects the final properties of glutenin particles, still reassembly occurs, although less complete. We therefore conclude that reassembly is mainly driven by physical interactions. Physical interactions (K') are also important in explaining dough rheological properties. In the case of NEMI, especially the quality of interaction must be taken into account in explaining variations in dough properties. The correlation found between dough rheological properties and glutenin HMW/LMW ratio, irrespective of the use of NEMI, points at the underlying importance of glutenin network composition and structure. In the following chapters effects of composition on the glutenin particles are further studied.

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6

HMW-GS AFFECT THE PROPERTIES OF GLUTENIN PARTICLES IN GMP AND THUS FLOUR QUALITY

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Abstract

In previous studies we have shown that '5+10' varieties have larger glutenin particles than '2+12' varieties. Apparently, HMW-GS composition affects the size of these glutenin particles. This prompted us to study the possible link between HMW-GS, glutenin particle formation and glutenin particle properties. A unique set of deletion lines, (Olympic x Gabo) allowed us to demonstrate that the presence of glutenin particles in GMP is directly related to the presence of certain HMW-GS. In the absence of HMW-GS only a small amount of GMP could be recovered from flour that only existed of LMW-GS. No particles could be observed in this fraction. With the presence of one subunit (Glu-A1) some particles could be observed. In the presence of 2 or more HMW-GS particles could be clearly identified. The amount of GMP increased with increasing number of HMW-GS. In terms of composition, all particles had the same LMW-GS composition irrespective of HMW-GS-composition. Since the relative proportion of LMW-GS in GMP was dependent on the number of HMW-GS, we postulate that LMW-GS become part of GMP via disulfide cross-links with HMW-GS. GMP wet weight is correlated with HMW content over particle size (D3,2) ratio, the average HMW density of the glutenin particle.

We combined our data with published technological data obtained with the same set clear statistical relations were observed for 1) optimal mixing time vs. glutenin particle size 2) thimble -loaf height and GMP content. These studies demonstrate that glutenin HMW-GS composition affects flour technological properties via glutenin particles.

Key word index: SDS unextractable glutenins, GMP, particle network

Abbreviations: SDS = Sodium Dodecyl Sulphate, GMP = Glutenin Macro Polymer, HMW-GS = High Molecular Weight Glutenin Subunits, LMW-GS = Low Molecular Weight Glutenin Subunits, CSLM = Confocal Scanning Laser Microscopy, FITC = Fluorescent Protein Label, PE = Petroleum-ether

6.1   Introduction

The very high molecular weight fraction of gluten is considered important in determining wheat flour quality. Lefebvre et al. (2000) have shown that large gluten aggregates control the rheology of dough. A clear correlation was shown between aggregate size and dough strength. Both Shewry et al. (1989) and MacRitchie (1999) have pointed out that: 1) HMW-GS composition affect quantity of insoluble glutenin 2) HMW-GS composition affects flour mixing time and 3) The insoluble glutenin fractions are polymeric structures containing HMW-GS and LMW-GS. 4) That HMW-GS '5+10' and '17+18' play a key role in the 'polymeric' size and the quantity of insoluble glutenins, this also showed parallels with dough and bread-making properties.

Don et al. (2003a) have shown that the SDS insoluble glutenin fraction known as GMP (glutenin macro-polymer) consists of glutenin particles. It was observed that glutenin particles from 5+10 varieties are larger than from 2+12 varieties (Don et al., 2005). This is in agreement with Popineau who stated that both quantity and molecular size of the glutenin aggregates are largely determined by genetic factors (Popineau et al., 1994). Furthermore, it has been proposed that glutenin particles in GMP originate from protein particles in the wheat endosperm (Don et al., 2003).

These previous studies clearly point at a relation between   glutenin polymerisation and functionality is controlled by its HMW-GS composition. Controlled expression of HMW-GS in wheat (Lafiandra et al., 1993; Beasley et al., 2002) provides an opportunity to better understand how specific GS combine to form very high molecular weight glutenin fractions, like GMP.   We studied a set of eight well defined Olympic x Gabo lines with systematic deletions (Beasley et al., 2002) to reveal possible links between glutenin HMW-GS composition and properties of the GMP fraction. Further characterization of the glutenin particles that make up this fraction could also help unravel the relationships between glutenin HMW-GS composition, glutenin particle properties and wheat flour quality parameters. The latter have been previously reported for this set (Beasley et al., 2002; Uthayakumaran, 2002). This provided the opportunity to investigate and confirm possible links between glutenin particles and flour quality.

6.2   Materials and Methods

6.2.1 Materials

A set of eight near isogenic lines derived from the cross Olympic x Gabo was used. The set has been described previously (Uthayakumaran et al., 2002; Beasley et al., 2002) The set has been generated to have variations only in the Glu-A1, -B1 and -D1 loci. Table 6.1 shows the characteristics of the flour samples.

Table 6.1   Deletion lines: Olympic x Gabo

Loci Olympic x Gabo	A 1	B 17 + 18	D 5 + 10
Triple null	-	-	-
Double deletion	+	-	-
Double deletion	-	+	-
Double deletion	-	-	+
Single deletion	+	+	-
Single deletion	+	-	+
Single deletion	-	+	+
No deletion	+	+	+

6.2.2   Isolation of GMP

1.4g of petroleum ether defatted flour (by percolation) was suspended in 28 mL 1.5% SDS solution (SDS of analytical grade from Merck™) and centrifuged at 80.000 g for 30 minutes at 20°C in a Kontron Ultracentrifuge. The supernatant was decanted and the gel-layer collected as GMP. Typically, the GMP gel consists of HMW-GS and LMW-GS.

6.2.3   Rheology of GMP gels

Material (1g) was carefully taken from the top of the gel and transferred into the measuring cell of a Bohlin VOR rheometer (Bohlin, Sweden). The cell consisted of two parallel plates (d=30 mm) with a gap of 1 mm. Measurements were performed at 20°C in a strain sweep mode at amplitudes ranging from 1% to 100%. Data were expressed as G' vs strain or delta (δ) vs strain.

6.2.4   GMP dispersion

GMP dispersions were prepared by transferring ca 1g of GMP gel to a tube containing 10 mL 1.5% (w/v) SDS solution. The gel was mixed with the solvent by briefly stirring with a spatula (by hand). Then the tube was sealed and placed on a roller-bank at ambient temperature to homogenize the sample, but avoiding high energy input by mechanical stirring. This produced a visually homogenous, opalescent dispersion, indicating that at least part of the protein is not dissolved. The dispersion obtained was used for further characterisation.

6.2.5   Confocal Scanning Laser Microscopy

GMP was isolated from flour of the 8 wheat lines, followed by preparation of GMP dispersion. The dispersed GMP proteins were stained with FITC and observed using a Leica TCS NT Confocal Laser Scanning Microscope. In order to optimize contrast, GMP dispersions with protein concentrations varying from 0.1 mg/mL protein to 5.0 mg/mL protein were observed with CSLM.

6.2.6   Rheological characterisation of GMP dispersions

The viscosity of GMP dispersions could be measured using an Ubbelohde capillary viscometer. The pass through time was in the order of 300-400 seconds to optimise accuracy. The data were used to construct a reduced viscosity vs concentration plot. This plot was extrapolated to a protein concentration of zero to obtain the intrinsic viscosity. The intrinsic viscosity is a measure of the average voluminosity of the particles in the suspension. Data were expressed as reduced viscosity L/g.

6.2.7   Coulter Laser particle size analysis

Particle size distributions of diluted GMP dispersions were determined by laser diffraction using a Coulter LS 130. In this instrument laser light is scattered by the suspended particles and the generated diffraction pattern, which is a composite of the diffraction patterns for all the particles, is measured. For GMP from flour the composite diffraction pattern is calculated into a particle size distribution with 72 classes ranging from 0.3 to 900μm using Fraunhofer theory.

6.2.8   RP-HPLC

The HMW-LMW ratio of GMP from flour and dough samples was assessed with reversed phase HPLC (RP-HPLC) using a Waters HPLC with a C8 bonded phase column. 1g GMP was extracted twice with 1 mL 50 (v/v) % propanol, 0.02% (w/v)DTT (dithiothreitol) followed by extraction with

1mL solvent A. The two extracts were combined for HPLC analysis. Solvent A contains 6M Urea, 20 % (v/v), 0.1 %(v/v) TFA (trifluoroacetic acid) and 0.02% (w/v) DTT. Solvent B contains 5.5M Urea, 50% (v/v) ACN (acetonitrile), 0.1 %(v/v) TFA and 0.02% (w/v) DTT. Loading was 10μL of GMP extract. Column temperature was maintained at 50°C. The column was eluted at a flow rate of 1mL/min, with a multi step gradient starting at 20% B to 80% B. Typical elution times for the HMW glutenins were 30-45 minutes and for LMW glutenins 70-85 minutes. HMW/LMW ratios were taken as the ratio of the respective areas under the RP-HPLC chromatogram. The method used did not allow a distinction between LMW-GS and for example gamma-gliadin.

6.3   Results

6.3.1   GMP protein formed in deletion lines Olympic x Gabo

The samples from the set of Olympic x Gabo lines have systematic deletions at the Glu-A, Glu-B and Glu-D loci (Table 6.1). From each sample GMP was extracted. Table 6.2 contains the results in terms of GMP quantity and GMP-gel rheological parameters.

Table 6.2   GMP wet weight, protein and rheological properties

Sample	GMP (g)	Protein in GMP-gel (mg/g)	GMP protein (mg)	G' (Pa)	δ (°)
- - -	0.6 ± 0.1	11.9 ± 0.6	7.1 ± 1.5	n.d.	n.d.
+ - -	0.8 ± 0.1	11.4 ± 0.6	9.1 ± 1.6	2.1 ± 0.2	25.0 ± 1.2
- + -	2.6 ± 0.2	11.5 ± 0.5	29.9 ± 3.6	2.4 ± 0.2	23.4 ± 0.8
- - +	1.7 ± 0.2	13.6 ± 0.6	23.1 ± 2.9	9.4 ± 0.5	16.8 ± 1.2
+ - +	2.8 ± 0.2	12.5 ± 0.6	35.0 ± 4.1	21.0 ± 1.1	15.8 ± 0.6
+ + -	2.2 ± 0.2	13.6 ± 0.7	29.9 ± 3.9	7.1 ± 0.4	17.7 ± 0.5
- + +	3.0 ± 0.2	15.7 ± 0.8	47.1 ± 5.7	59.4 ± 2.2	12.1 ± 0.5
+ + +	3.5 ± 0.3	14.7 ± 0.8	50.4 ± 6.7	54.2 ± 2.1	12.8 ± 0.8

It is clear from table 6.2 that the specific deletions have an effect on the quantity of GMP recovered from flour. Figure 6.1 clearly shows how the quantity of GMP protein is related to the number and composition of HMW-GS present. This is in agreement with a recent paper by He et al. (2005) in which he found a clear relationship between the total amount of HMW subunits expressed and the % UPP. However, He et al were not able to draw conclusions on effects of specific HMW subunits.

With the materials we used in this study, differences become apparent with the double deletions. HMW-GS 17+18 have a more pronounced effect on GMP protein quantity than HMW-GS 5+10.
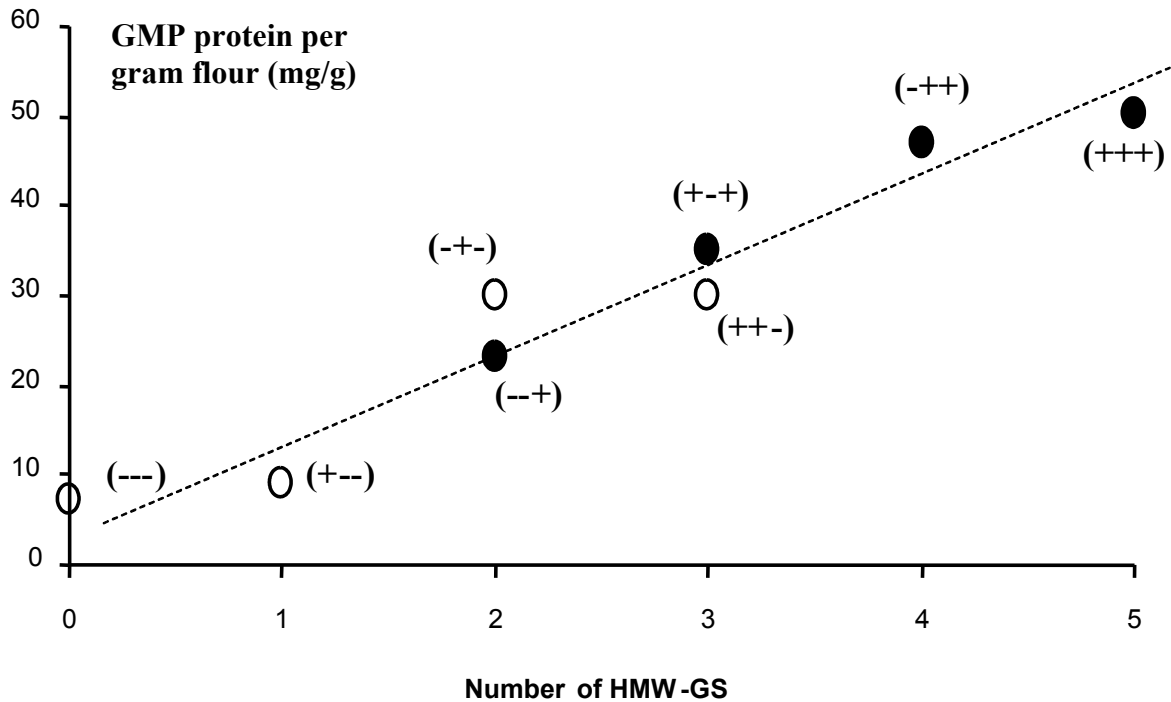

Figure 6.1   The quantity of GMP in wheat flour against the number of HMW-GS expressed in the respective wheat sample. The (+,-) refer to the deletions made in the respective wheat lines. See Table 6.1 for an explanation. Filled symbols contain HMW-GS '5+10'.

On the other hand, the HMW-GS 1 combined with HMW-GS 5+10 results in more GMP protein than HMW-GS 1 combined with HMW-GS 17+18. Lafiandra et al. (1993) have pointed at the importance of HMW-GS 5+10 for strengthening wheat flour dough. Since we expect that flour quality differences are both related to GMP quantity and GMP structure, we studied the rheological parameters of the GMP gels obtained.

6.3.2   GMP-gel rheology

There is a general consensus that HMW-GS play an important role in determining the glutenin protein network structure. We therefore expect that differences in HMW-GS composition are

reflected in differences in GMP rheological parameters. The GMP-gel stiffness (G') is an indicator of glutenin size (Don et al., 2003)). The triple null variant (---) could not be measured rheologically because of the very low quantity that could be recovered from flour. Visually, the gel that was recovered was viscous and slimy. For the other 7 Olympic x Gabo lines we have plotted the GMP-gel structural parameters G' and delta (fig. 6.2a and 6.2b) against the amount of HMW-GS present. Figure 6.2a shows that the value of G' increases with the he quantity of HMW-GS expressed.
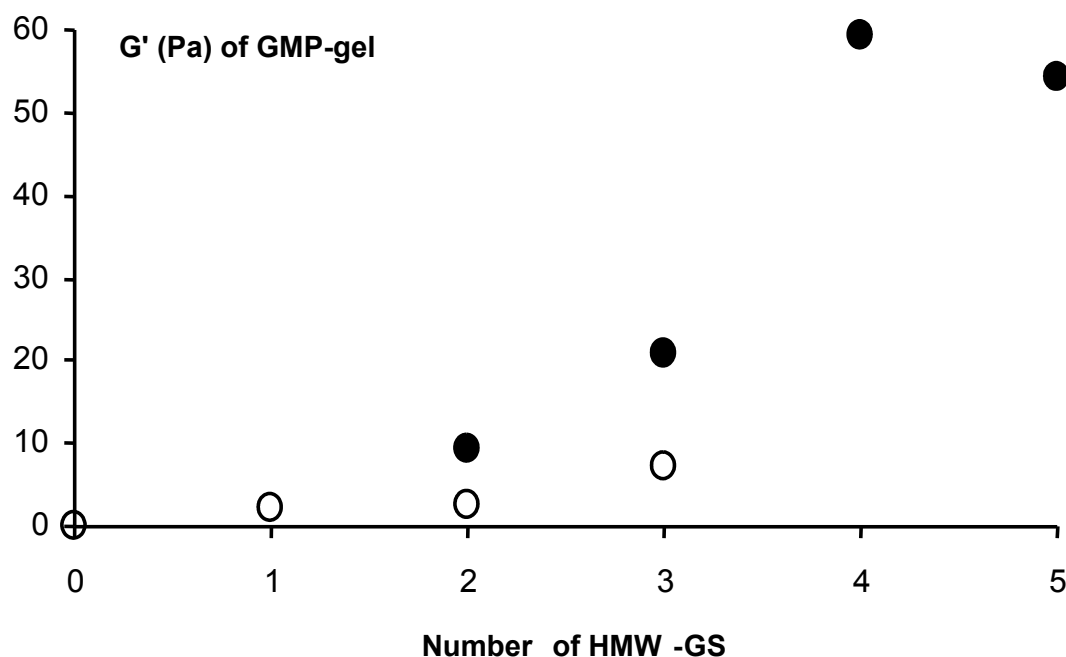

Figure 6.2a   The rheological gel stiffness (G') of GMP from flour vs. the number and type of HMW-GS expressed in the respective wheat sample. Filled symbols contain HMW-GS '5+10'.
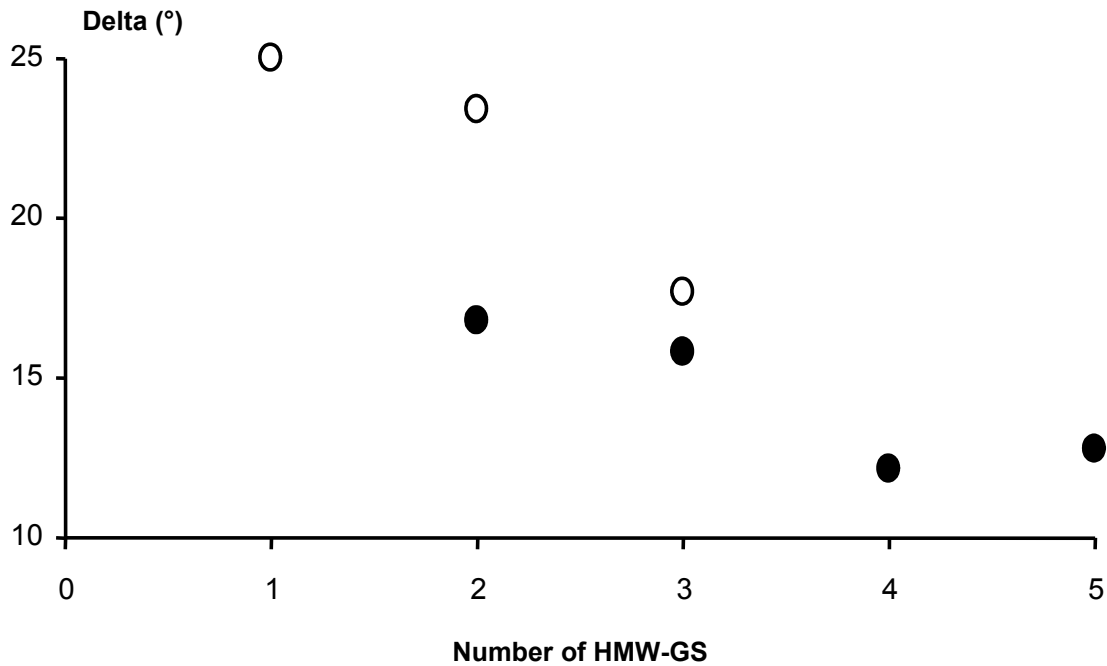
Figure 6.2b   *The small deformation elasticity (delta) of the GMP-gel from flour vs. number and*
                type of HMW-GS expressed in the respective wheat sample
                Filled symbols contain HMW-GS '5+10'.

If we consider the samples according to the number of deletions, some other observations can be made. For the double deletions, the (--+) line has the highest G'. For single deletions the order for G' is (-++) > (+-+) > (++-). The G' for the (-++) is the same as the (+++) line, considering the higher protein concentration of the (-++) (table 6.2). This agrees with findings of Popineau et al. (1994) that the presence of HMW-GS '5+10' strengthens gluten and dough elasticity. According to the authors this was not a result of protein content, but a result of HMW-GS (Popineau et al., 1994). We also note that with 2 and 3 HMW-GS, (4 lines), the GMP-gels containing HMW-GS '5+10' have higher G' values than those containing HMW-GS '17+18'. Also, with more HMW-GS, the phase angle delta is lower, indicating a higher gel elasticity. As a structural parameter, the phase angle does not depend on protein concentration, like the gel modulus G' does. Therefore, a lower delta can only indicate that the average cross-link density in the GMP-gel increases with a higher

number of HMW-GS. For this set of lines, the GMP rheology confirms that a relation between HMW-GS composition and glutenin structural properties.

6.3.3   RP-HPLC analyses on GMP from the Olympic x Gabo lines

We have reported previously that GMP consists of HMW and LMW glutenins (Don et al., 2003). In order to reveal how the GMP composition is affected by the systematic deletions of HMW-GS we analysed the GS composition of both flour and GMP using RP-HPLC. Figure 6.3 shows the traces for the respective wheat lines. The method used separates the HMW-GS peaks well from the LMW-GS that elute at later retention times.
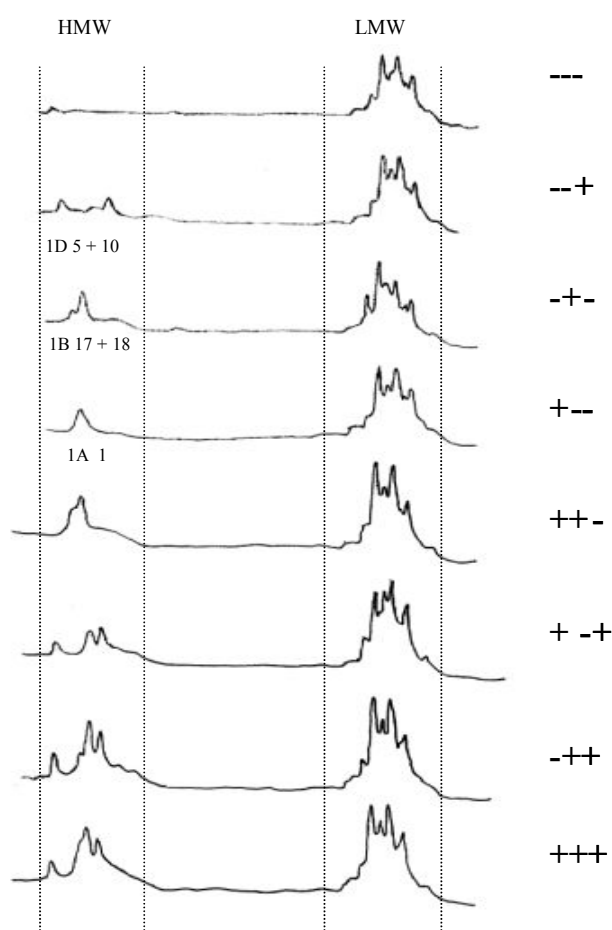

Figure 6.3   The RP-HPLC traces and HMW/LMW ratios of GMP extracted from flour of the set of wheat lines. The (+,-) refer to the deletions made in the respective wheat lines. See Table 6.1 for an explanation.

For the triple null sample, hardly any HMW-GS are found. In this case, GMP consists only of LMW-GS. Apparently, LMW-GS can also form aggregates insoluble in SDS. This finding is consistent with Courtin et al. (2004) who reported that LMW-GS can polymerise and form a larger structure. It is not clear from their paper of these LMW-GS polymers are also insoluble or partially soluble in SDS.

All the other lines show a GS composition that matches the GS composition of flour. For all the lines the pattern of the LMW glutenins is similar. Apparently, the composition of LMW-GS present in GMP is not affected by the differences in HMW-GS composition.

The RP-HPLC data allows us to calculate HMW/LMW ratios and study if the presence of HMW-GS affects the quantity of LMW-GS recovered in GMP. The expression of HMW-GS 5+10 is important for the HMW/LMW ratio, because the (+-+) and (-++) lines have a higher HMW/LMW than the (++-) line. As expected, the (+++) line has the most HMW glutenins in the GMP fraction. When we combine data from table 6.2 with the HMW/LMW ratios obtained from RP-HPLC, we can calculate the quantity of HMW and LMW (mg) in GMP. This comparison allows us to assess to what extent HMW-GS affects the presence of LMW-GS in GMP. Figure 6.4 shows the results of the amount of HMW-GS present in the sample and the HMW quantity compared to the LMW quantity in the GMP sample.
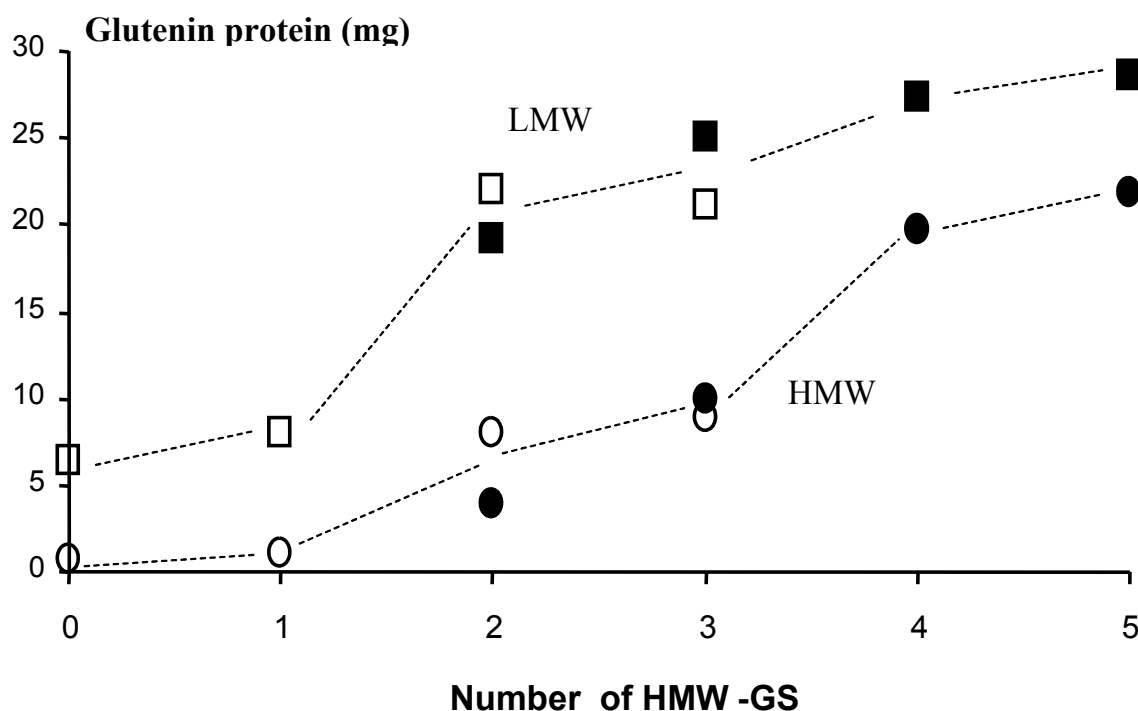
Figure 6.4   The quantity of HMW and LMW protein against the number and type of HMW-GS expressed in the wheat sample. Filled symbols contain HMW-GS '5+10'.

As more HMW-GS are expressed, also the absolute amount of LMW-GS in GMP increases. With the triple null line, RP-HPLCS only reveals the presence of LMW-GS in GMP. When HMW-glutenins are present, also the absolute quantity of LMW-glutenins in GMP increases further. We think this is due to the formation of cross-links between HMW-GS and LMW-GS as has been documented by Keck et al (1995).

If we assume the average molecular mass of HMW-GS to be 2.5 times the mass of LMW-GS (MS), we can calculate mol/mol ratios. From figure 6.4 it is clear that more LMW becomes part of GMP when only 2 HMW-GS are expressed than with 5 HMW-GS expressed. This can only be explained when HMW-GS can also form HMW-GS-HMW-GS cross-links, thereby limiting the possibilities for HMW-GS-LMW-GS cross-linking. The presence of HMW-GS-HMW-GS and HMW-GS-LMW-GS cross-links is well documented (Keck et al., 1995; Kasarda et al., 1999). Apparently, in the course of HMW polymerisation, the number of possibilities to link LMW-GS onto HMW-GS has become smaller. This could explain the higher HMW/LMW ratio found for GMP in comparison with the HMW/LMW ratio found for the respective flour (Beasley et al., 2002).

6.3.4   CSLM observation of glutenin particles

The specific HMW-GS in this unique set of deletion lines affect GMP quantity, rheology and composition. Don et al. (2003) have shown that GMP consists of glutenin particles. These particles are 1) heterogeneous in size, 2) the average size is different among varieties (Don et al, 2003a,b). To further unravel the effect of glutenin composition on the glutenin particle, it is necessary to analyse the GMP dispersion and study whether the particulate structures are present, or absent. In figure (6.5a-h) images obtained with CSLM are shown.
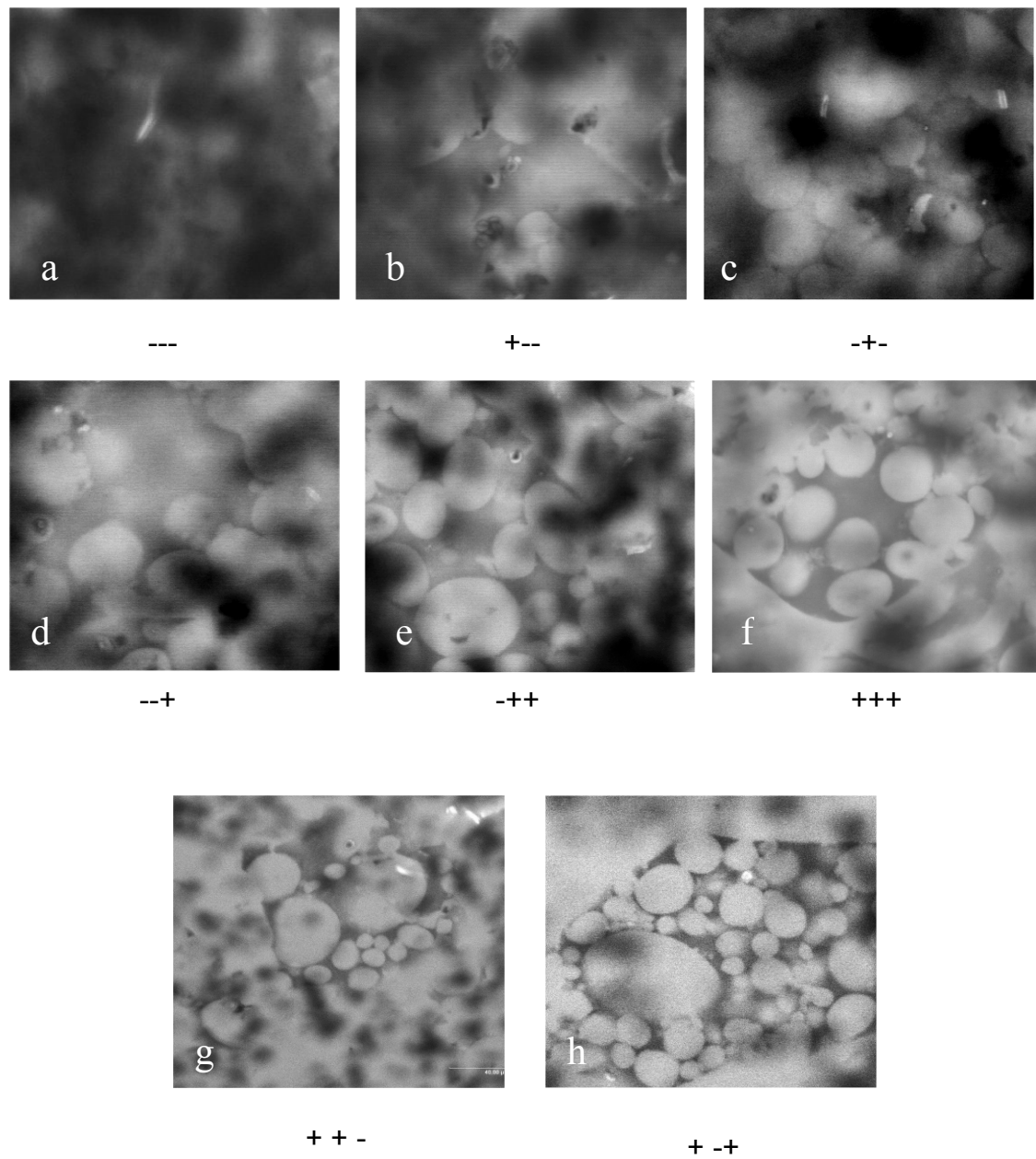
Figure 6.5a-h CSLM micrographs for the samples with systematic expressions / deletions of HMW-GS. The(+,-) refer to the deletions made in the respective wheat lines. See table 6.1 for an explanation.

Figure 6.5a does not show any glutenin particles. In spite of the fact that the process of GMP isolation would concentrate any particles present and the fact that CSLM is focused on the detection of particles, none were found. Some protein could be stained, possibly these are weak structures of LMW-GS, but not the glutenin particles. We therefore have no clear evidence of particles formed in the absence of HMW-GS. Dispersed GMP of the (+--) line displays some particulate structures shown in figure 6.5b. In figures 6.5c and 6.5d it can be seen that lines (-+-) and (--+) have more clear glutenin particle structures than (+--). With the lines (-++) and (+++) the glutenin particle structures are abundant. This demonstrates the importance of HMW-GS for the formation of glutenin particles.

6.3.5   The glutenin particle size

To provide further quantitative footing for our findings with CSLM we measured average glutenin particle size. In table 6.3 the particle size data have been summarized.

Table 6.3   *Voluminosity and average diameters for glutenin particles from Olympic x Gabo lines*

Type	$[\eta]$ (L/g)	D3,2	D4,3
- - -	0.10	4.3	8.3
+ - +	0.24	5.8	13.7
- + -	0.11	4.5	8.5
+ + -	0.15	5.0	10.9
- - +	0.16	5.1	10.2
+ - -	0.13	4.9	9.3
+ + +	0.28	7.2	25.6
- + +	0.27	6.6	21.2

We have taken $[\eta]$ as a measure of the average particle voluminosity. The samples containing HMW-GS 5+10 have $[\eta]$ values between 0.24 and 0.28 L/g, whereas samples lacking this pair of HMW-GS have smaller $[\eta]$ values of ca 0.14 L/g. The presence of HMW-GS 5+10 only does not lead to a large average particle size; this seems to require the presence of also HMW-GS 17+18. In figure 6.6 all the size distributions are shown, normalized for peak height.

Figure 6.6 The traces for size distribution patterns of glutenin particles extracted from flour. The (+,-) refer to the deletions made in the respective wheat lines. See table 6.1 for an explanation. The dotted line at 100 μm position is added to help compare the different volume distributions.

With the (---) sample little material was available leading to a larger contamination with small starch granules. These are clearly observed at ca 5.5 μm. In addition, no structures >50μm could be observed, indicating the absence of glutenin particles with the triple null line. In contrast, the (--+) contains particles in the region >100μ. The (-++) and (+++) have more large particles in the 10-100μ area than (--+). This is consistent with their higher [η]. These results are also in line with the higher G' values found with these gels.

We noticed a clear contrast between particles of the (--+) with >100µm sized particles and other double deletion lines in which particles are mainly < 100µm. This typical size distribution of (--+) provides supporting evidence for the important role of HMW-GS 5+10 in the size of glutenin, as mentioned by Popineau et al. (1994), Lafiandra et al. (1993). It stands to reason that the ability to form larger particles leads to more GMP. However, in this study GMP quantity and GMP particle size are only related in part (R^2=0.58). Apparently, GMP quantity is not only controlled by glutenin-particle size. With this set of samples a relation was found between HMW/LMW ratio in flour and the average size of the particles (R^2=0.75). This is in agreement with earlier results obtained, where a higher ratio between HMW/LMW subunits corresponded to a larger average size of the glutenin particles (Don et al., 2003a, b).

6.4   Discussion

Graveland et al. (1985) proposed that HMW glutenin polymers are key to GMP formation. To what extent are HMW-GS indeed required to form larger aggregates? Although not completely conclusive, the present study provides evidence from CSLM and laser light scattering that the triple null line does not appear to contain glutenin particles that are sufficiently stable in SDS. Don et al. (2003) have also shown with the double deletion line Galahad-7 that its GMP fraction contained instable structures. RP-HPLC analysis shows that this fraction of SDS insoluble protein consists of LMW-GS. It has been shown previously that LMW-GS can indeed form polymeric aggregates (Veraverbeke et al. 2000). We propose that in the absence of HMW-GS these weak aggregates can still become soluble in SDS. This could also explain why only a small amount of GMP is found.

Based on this set, it seems that HMW-GS are indeed prerequisite to form large SDS insoluble structures. Further note that GMP quantity or %UPP (Beasley et al., 2002; Uthayakumaran, 2002) is much more sensitive to HMW-GS variation than the flour protein content. This clearly indicates that the observed effects on glutenin cannot be explained on basis of flour protein.

Our second observation is that in the presence of HMW-GS, particles are formed that predominantly consist of HMW-GS and LMW-GS. We did not specifically analyse for the presence of gamma gliadins, that have a free SH-group allowing their cross-linking to glutenin structures, but RP-HPLC mainly shows HMW and LMW-GS. Fig. 6.3 provides a strong indication that LMW-GS and HMW-GS together form the internal structure of the glutenin aggregates, since the content of LMW-GS in GMP increases with increasing number of HMW-GS. This explains the observed correlation between HMW/LMW ratio and aggregate size (Table 6.3). This is also reported by

Southan and MacRitchie (1999) who point out that the HMW/LMW ratio affects the size of glutenins. In view of the large variation in HMW-GS, it is remarkable that the LMW-GS composition of GMP is constant. This can be explained either by a mechanism in which LMW-GS polymerise and become part of GMP independent of HMW-GS or a mechanism in which oligomers of LMW-GS cross-link to HMW-GS and thus become part of GMP. The presence of such oligomers has been demonstrated using partial reduction studies (Hamer and Weegels). Since we have already demonstrated that without HMW-GS no particles are formed, the latter explanation is more likely. Our results are in agreement with a model in which glutenin 'polymers' consist of a backbone of HMW-GS linked by disulphide bonds to which oligomers of LMW-GS are attached (Weegels et al., 1996; Graveland et al., 1985; Shewry et al., 1989; Keck et al., 1995). When interactions between HMW-GS and LMW-GS are involved, some HMW-GS can lead to larger structures than others. Interestingly, these observations on particles are supported by a number of studies on solubilised aggregates (e.g. via ultrasonication). For example, Ueno et al. (2002) have shown with FFF that acetic acid insoluble fractions contain glutenin aggregates with a larger Stokes diameter than the acetic acid soluble glutenins. The same authors also indicated that larger aggregates parallel an increase in dough development time and strength. Southan and MacRitchie (1999) report that the introduction of HMW-GS 5+10 and HMW-GS 17+18 are strongly associated with an increase of insoluble glutenin size. The HMW-GS 5+10 combination increases dough strength, and according to Lefebvre et al. (2000) this is related to an increase in the size of glutenin aggregates. More recently Don et al. (2005) confirmed that under non-stressed growing conditions Warigal and Lance with HMW-GS 5+10 had larger glutenin particles than Warigal and Lance 5+10. We conclude that HMW/LMW ratio and the presence of (specific) HMW-GS are the parameters that control the size of the glutenin particles formed in wheat. HMW-GS 5+10 and/or 17+18 have more pronounced effects on the glutenin particle structure and size. Veraverbeke et al. (2000) point at the role of certain x-type HMW-GS in the formation of larger glutenin aggregates. With the x-type HMW-GS 5 and 17 present we clearly observe a region of very large particles in figure 6. LMW-GS may affect glutenin aggregation (Veraverbeke et al., 2000; DuPont and Altenbach, 2003; D'Ovidio and Masci, 2004), but with this set the pattern of LMW-GS is always the same. The differences here are related to HMW-GS only. An interesting feature is that the phase angle delta of GMP fraction containing HMW-GS 5+10, have a lower phase angle (fig. 6.2b). The lower delta points at a higher cross-link density of the GMP-gel. In connection with the results of Veraverbeke et al. (2000) this confirms that certain HMW-GS affect the internal glutenin particle

structure. Our results combined with evidence from literature strengthen our view that HMW-GS control the physical properties of the glutenin particles. We therefore postulate that:

- HMW-GS are required for glutenin particle formation
- The size of the glutenin particles is affected by the HMW/LMW ratio.
- HMW-GS '5+10' shifts the size distribution pattern towards larger sizes, HMW-GS '17+18' combined with '5+10' also effectively shifts the size distribution to larger sizes.
- HMW-GS affect the internal structure of the glutenin particles (fig 6.2b).

As a consequence, we expect that these properties correlate with flour technological properties. Beasley et al. (2002) and Uthayakumaran et al. (2002) analysed the technological properties of these materials.

6.4.1 *Parallels between GMP, %UPP and dough*

In figure 6.7a it is shown how the GMP protein quantity parallels the %UPP determined by Uthayakumaran et al. (2002).
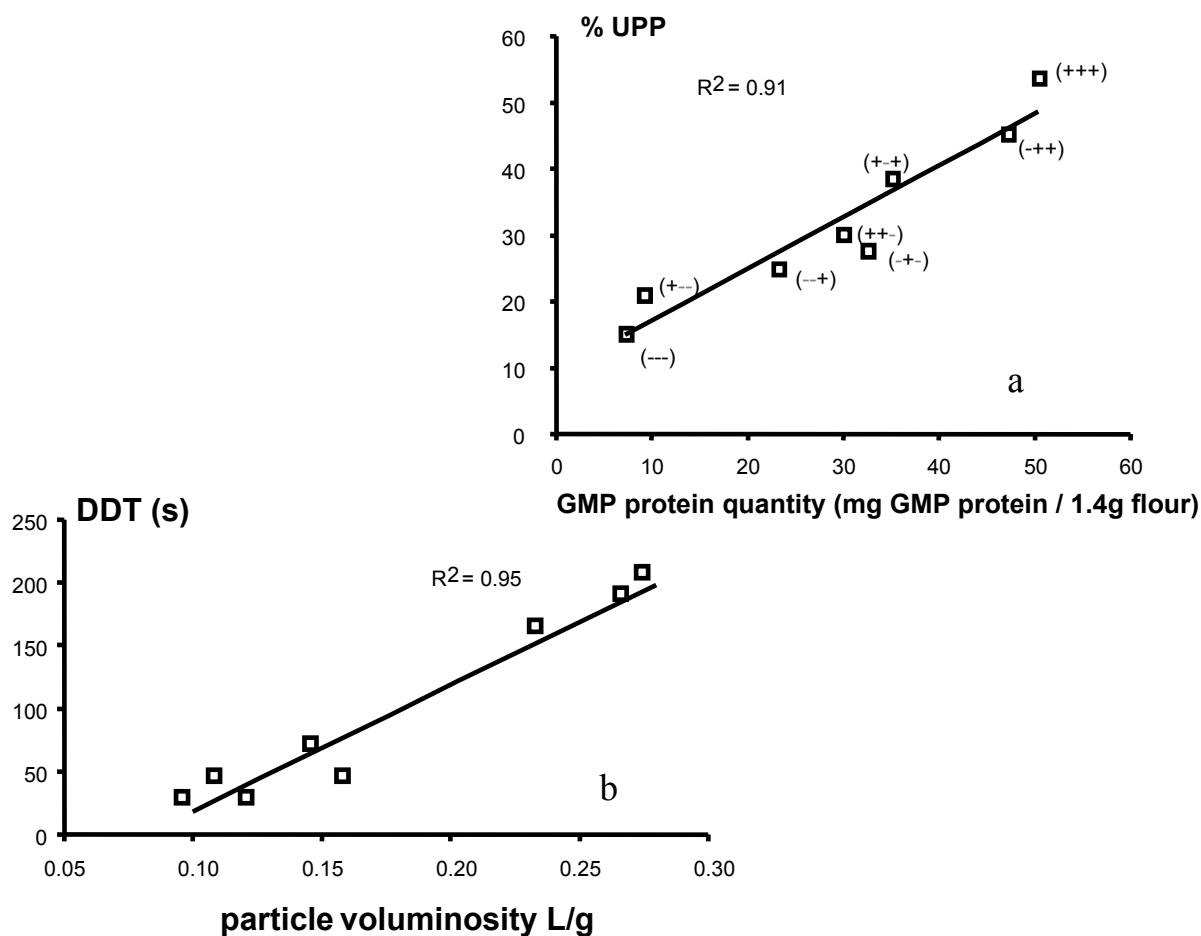
Figure 6.7a   The %UPP against the quantity of GMP isolated from the same flour (top right)

Figure 6.7b   The dough mixing time against the average glutenin particle size (lower left)

The (+,-) refer to the deletions made in the respective wheat lines. See Table 6.1 for

an explanation.

Dough rheological strength was shown to correlate with %UPP in the paper of Uthayakumaran et al. (2002). Don et al. (2003a,b) have demonstrated clear relations between GMP and dough rheological properties. The %UPP reflects the glutenins insoluble in propanol+SDS. In view of the clear correlation between %UPP and GMP (R^2=0.91) and the fact that they both relate to insoluble glutenin protein, we suggest that the UPP fraction will also contain glutenin particles. Following a different extraction method, Mecham et al. already suggested in 1965 that large aggregates insoluble in acetic acid strongly affect properties of gluten dough. Apparently, un-extractable glutenins is a recurring theme, independent of the solvent system used to separate extractable proteins from insoluble glutenins. The quantity of insoluble glutenin particles or aggregates has frequently been shown to correlate with flour end-use quality.

6.4.2   Dough development by particle disruption

For this set of lines the dough development time (DDT) on a Z-blade mixer was determined by Beasley et al. (2002). In figure 6.7b it is shown how the glutenin particle size that we have measured is related to this DDT. The relation between glutenin particle size and dough development has also been shown for other flour samples by Don et al. (2003). The quantity of GMP has an R^2 of 0.67 with DDT. Therefore, the disruption of large glutenin particles must be a controlling step for dough development. It is clear that here the HMW-GS determine the glutenin particle size of GMP from flour, whether LMW-GS affect the glutenin particle disruptions during mixing can not be seen from this set. This requires a GMP with variation in LMW-GS.

6.4.3   Loaf height of thimble loaves

Beasley et al. (2002) prepared thimble loaves with flour from the dedicated set of Olympic x Gabo lines. Although glutenin particle size clearly affect dough development time, the correlation R^2 between size and loaf height is just 0.60. GMP wet weight shows a correlation with loaf height of $R^2 = 0.92$. We realise the set is quite limited, but it spans a broad range of GMP wet weights (0.5 – 4 g). Figure 6.8a shows the lineair correlation plot. During dough rest, disrupted glutenin particles re-assemble (Weegels et al. 1996, Don et al. 2005). This plays a role in making the dough more elastic with increasing dough rest time (Don et al., 2005). The differences in formation of a glutenin network during resting may well be reflected in differences in thimble loaf height. Network formation and subsequent increase of dough elastic strength is not only related to glutenin particle size. The glutenin particle composition and internal structure (Don et al., 2005) also affect its effectiveness in forming a strong glutenin network.
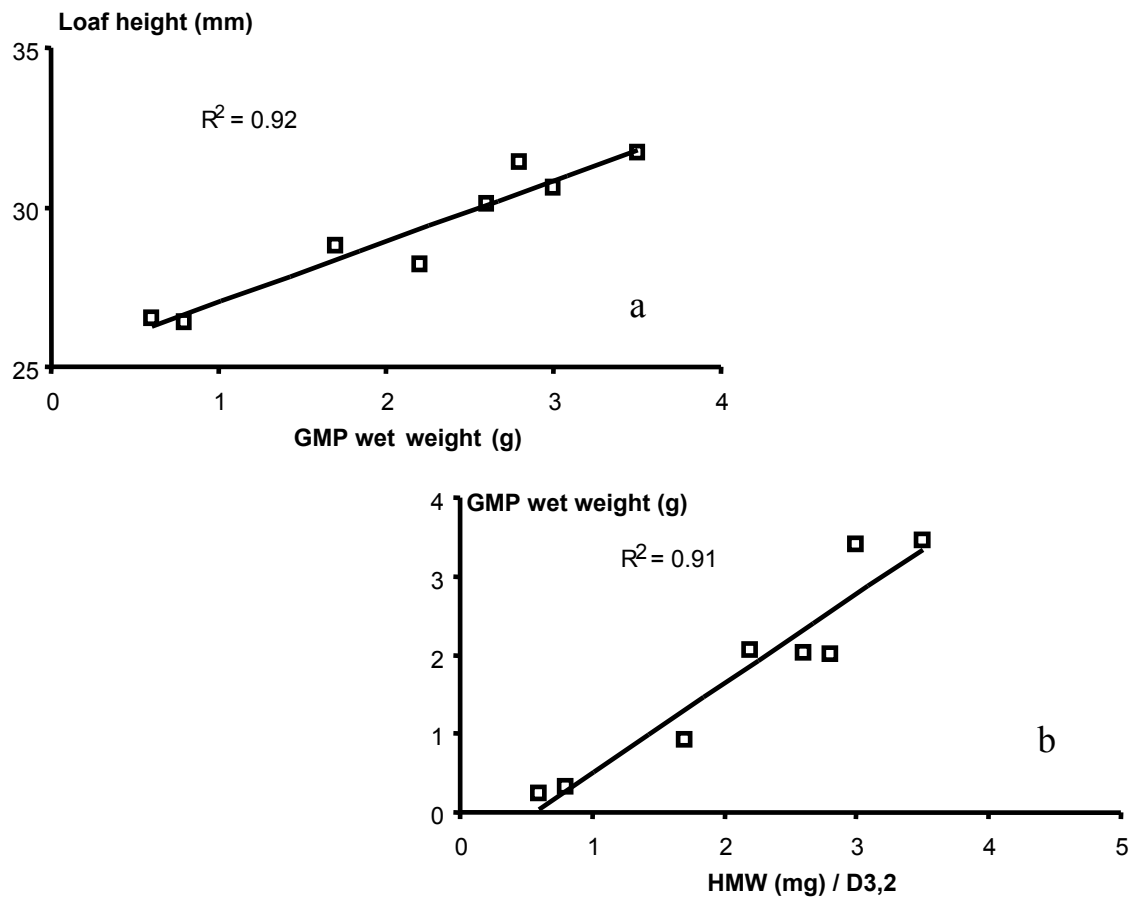
Figure 6.8a   The GMP wet weight plotted against loaf height (top left)

Figure 6.8b   The GMP wet weight plotted against the HMW quantity / size ratio (lower right)

The quantity of HMW-GS in GMP and the apparent glutenin particle volume over surface (D3,2) of the glutenin particles in GMP provide insight on how the number and type of HMW-GS affect the apparent average size of the glutenin particle. In figure 6.8b we plotted GMP wet weight against HMW/D3,2 ratio. The HMW/D3,2 ratio can be defined as an average HMW-GS density of the glutenin particles formed. This ratio correlates with the wet weight of GMP obtained (R^2=0.91) and also with %UPP (R^2=0.90). It has to be noted that we do not know how this relation is affected when also LMW-GS composition is varied. The significance of this relation is that the amount of GMP (or %UPP) is not only determined by the quantity of HMWGS in this fraction, but also by the relative size of the aggregates or particles. This will be determined by other factors like HMW-GS and perhaps also LMW-GS. Since the samples studied here have the same LMW-GS, no conclusions can be drawn on their role.

6.5   Conclusions

The formation of glutenin particles, stable in SDS-solution, requires the presence of HMW-GS. Within the set used, HMW-GS from either Glu-1B 17, 18 or Glu-1D 5, 10 proved essential. The size of the particles is related to the presence of Glu 1D 5+10. Our results are in agreement with a model in which HMW-GS form "backbone" structures to which polymers of LMW-GS become attached. The combination of our results on glutenin particle formation and the technological data of Beasley et al (2002) demonstrate that HMW-GS affect flour technological quality via glutenin particles. Further unravelling the internal glutenin particle structure will improve our understanding of glutenin quantity x glutenin particle structure relations in dough mixing and bread-making.

Finally, the combination of glutenin particle analysis and the use of near-isogenic wheat lines opens new possibilities to understand factors determining glutenin structure, aggregate size and their relation with flour end-use quality.

Acknowledgements

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7

HEAT STRESS AND GENOTYPE AFFECT THE GLUTENIN PARTICLES OF THE GMP-GEL FRACTION

Don C., Lookhart G., Naeem H., MacRitchie F., Hamer R.J, 2005. Journal of Cereal Science 42 69-80

Abstract

Both genetic and environmental factors affect wheat quality. It has been shown that the Glutenin Macro-Polymer consists of glutenin particles. Just recently, it was shown that these glutenin particles can only be formed when HMW-GS are present. To further unravel the factors that affect the formation of glutenin particles: Lance 5+10, Lance 2+12, Warigal 5+10 and Warigal 2+12 lines were grown under widely differing greenhouse conditions. We systematically studied the GMP characteristics and mixing properties of flour extracted from matured wheat kernels. The typical changes in processing requirements and glutenin HMW-LMW composition resulting from heat stress are not always understood. Also, some varieties seem to be more susceptible than others. In particular heat stress seems to strongly affect the hyper-aggregation of glutenins, resulting in the formation of larger glutenin particles. With very severe heat stress even fusion of glutenin particles is suspected. The new findings on how glutenin particle size is affected by heat stress could improve our understanding on how heat stress affects wheat quality.

Key word index: GMP, GMP-particles, heat stress and dough mixing

Abbreviations: SDS = Sodium Dodecyl Sulphate, GMP = Glutenin MacroPolymer, HMWGS = High Molecular Weight Glutenin Subunits, LMWGS = Low Molecular Weight Glutenin Subunits, CSLM = Confocal Scanning Laser Microscopy, FITC = Fluorescent Protein Label, PE = Petroleum-ether, SUP = supernatant fraction of GMP isolation, TTP = Time to Peak, ETP=Energy to Peak

7.1   Introduction

Differences in wheat quality are known to be affected by both genetic and environmental factors. Growing conditions can affect the quality of any variety. The Glutenin Macropolymer (GMP) quantity in flour parallels differences in wheat flour bread-making quality (Graveland et al., 1982, 1984; Weegels et al., 1996, 1997). GMP consists of HMW and LMW subunits. HMWGS and LMWGS present in glutenins are linked by disulphide bonds. Recently it was shown that GMP consists of spherical glutenin particles (Don et al., 2003a). Size measurements revealed that glutenin-particles can vary in average size. Flour from '2+12'variety Estica was shown to have on average less GMP and smaller glutenin particles than flour from the '5+10'variety Baldus. Clearly, genetic background affects GMP quantity, GMP particle size and, consequently, flour quality. Glutenins are formed during wheat kernel development (Carceller and Aussenac, 2001). Therefore, also growing conditions are expected to affect the formation of GMP. In order to study this in more detail in combination with effects of genetic background near isogenic lines: Lance C ('5+10'), Lance A ('2+12'), Warigal ('5+10') and Warigal ('2+12'), were grown under 6 different greenhouse conditions (Don et al. 2003c). These 6 growing conditions were used to mimic various heat stress conditions. The GMP characteristics of the Warigal and Lance lines were studied to better understand the effects of genetic background and heat stress on glutenin particle formation and mixing properties.

7.2   Experimental

7.2.1   Wheat materials

Four near-isogenic wheat lines were used, Lance C (HMWGS 2*, 17+18, 5+10) + Lance A (HMWGS 2*, 17+18, 2+12) and Warigal A (null, 7+8, 5+10) + Warigal B (null, 7+8, 2+12). Wheat was grown in pots under controlled conditions in greenhouse facilities at KSU. Each of the pots in the greenhouse was automatically watered three times a day using the local water supply. In the growth chambers, where the heat treatments were applied, the pots were kept under close surveillance to maintain the moisture supply and were watered at least once a day. The aim was to study the effects of heat stress free from conditions of water stress. The wheat lines were grown till maturity, using various temperature regimes – t °C day/ t °C night - to simulate six different stress levels (table 7.1). Protein contents of powdered kernel materials were determined by nitrogen combustion (LECO) on 200 mg of sample and averages of duplicates calculated.

*Table 7.1.   Greenhouse conditions #1 to #6, day / night temperature
              used with Lance and   Warigal lines*

#1. 20°C/16°C -- ------ 40 days → mature
#2. 20°C/16°C -----→16DAA --72h at 30°C/18°C -→ 20°C/16°C -----37 - 38 days → mature
#3. 20°C/16°C -----→16DAA --72h at 35°C/20°C-→20°C/16°C-----35 - 36 days → mature
#4. 20°C/16°C -----→16 DAA -- 35°C/20°C----------------- ---30 - 31 days → mature
#5. 20°C/16°C -----→16 DAA -- 40°C/25°C------------25 days → mature
#6. 20°C/16°C -----→25 DAA -- 40°C/25°C---------------28 days → mature

7.2.2   Wheat milling on Quadrumat JR

About 30g of wheat kernels were milled on a Quadrumat JR (Brabender). Kernel moisture was adjusted to 14-17% depending on the hardness of the grain; bran material was separated from endosperm flour by sieving over a 150μm sieve. Due to effects of growing conditions on kernel filling, extraction rates varied between 50-70%, noting that samples grown under condition #5 (table 7.1) had a typically low flour extraction of about 50%. These kernels had a shrivelled appearance. For samples from growing conditions #1, #2 and #3 extraction was ca 70%, for #4 and #6 it was 60%.

7.2.3   Dough Mixing

Mixing properties were determined with a National 2g Mixograph. Water addition was added according to AACC method 54-40 including addition of 2% salt (NaCl). The dough development time (DDT), Band Width at Peak Resistance (BWPR) and surface area (E) that parallels average energy-input for optimal development were recorded.

7.2.4 Characterization of GMP-gel and GMP dispersions

GMP was isolated by dispersing flour in 1.5% SDS followed by ultracentrifugation as described earlier by Graveland et al. (1982). The GMP gel-layer rigidity was measured with a Bohlin VOR (Bohlin Sweden) as described by Wang et al. (2003). Particle properties were determined viscometrically and with a Coulter Laser LS32 particle sizer as described by Don et al. (2003b). From The Coulter Laser pattern derived diameters can be calculated, such as D3,2 (weighted average surface area of the particles) and D4,3 (weighted average volume). Particles were also observed with confocal scanning laser microscopy (CSLM) using methods described by Don et al. (2003b)

7.2.5 Protein content of flour and GMP samples

Protein content of flour, GMP, GMP dispersions and SDS extractable proteins were measured using an UV absorption method. The UV method was successfully calibrated with a set of Kjeldahl protein values of GMP.

7.2.6 RP-HPLC analysis of flour glutenins and GMP

A Waters HPLC was used with C8 column. General procedure was according to Lookhart et al. (2003), with some adaptations as described earlier by Weegels (1996). The procedure was performed for 50% isopropanol + DTT extracted glutenins from flour as well as for glutenins present in the GMP-gel fraction.

7.3   Results

7.3.1 Effect of growing conditions on GMP protein in flour

The Lance and Warigal wheat samples were grown under the conditions shown in table 7.1. Flour was extracted from the grains as described in the experimental section. In this section we show that flour protein content and GMP content vary with growing conditions for the Lance and Warigal lines. Heat stress conditions are more severe going from treatment #1 to #5 (table 7.2). With treatment #6, the period of heat stress (40 °C day time) is started later than with treatment 5 (25 DAA instead of 16 DAA). In general, total flour protein content ranges between 12 – 17%. When comparing flour protein contents between the different samples under non-stressed and slightly stressed (#1 and #2) conditions, a systematic difference in protein content is observed, with the 5+10 lines having a higher flour protein content than the 2+12 lines. Since growing conditions are the same, this can only be due to genotypic differences.

Table 7.2   *The % of protein in flour and the % of GMP protein in flour protein for lines*

   Warigal and Lance grown under 6 different conditions

Growing condition	Lance 2+12		Lance 5+10		Warigal 2+12		Warigal 5+10	
	% protein in flour	% GMP in protein	% protein in flour	% GMP in protein	% protein in flour	% GMP in protein	% protein in flour	% GMP in protein
# 1	12.7 ± 0.4	20.2 ± 1.7	15.4 ± 0.7	34.6 ± 2.4	13.5 ± 0.6	21.1 ± 1.8	14.7 ± 0.6	27.2 ± 2.0
# 2	13.2 ± 0.5	18.6 ± 1.5	16.4 ± 0.8	34.7 ± 2.5	14.4 ± 0.7	22.0 ± 1.8	14.2 ± 0.7	28.6 ± 1.9
# 3	11.9 ± 0.6	19.1 ± 1.8	16.7 ± 0.8	29.3 ± 2.1	14.2 ± 0.7	23.3 ± 1.9	14.1 ± 0.6	30.0 ± 2.2
# 4	13.2 ± 0.5	15.1 ± 1.3	15.4 ± 0.6	23.3 ± 1.8	14.5 ± 0.6	16.0 ± 1.4	14.4 ± 0.6	22.9 ± 1.7
# 5	11.7 ± 0.5	9.0 ± 0.8	14.2 ± 0.7	19.5 ± 1.6	13.6 ± 0.6	9.7 ± 1.0	13.3 ± 0.5	13.3 ± 0.9
# 6	12.7 ± 0.4	16.4 ± 1.5	14.5 ± 0.7	30.2 ± 2.2	12.2 ± 0.6	15.6 ± 1.4	13.6 ± 0.5	20.2 ± 1.5

If we analyse table 7.2 for effects of stress, no systematic effect can be observed, perhaps with the exception of #5. It is known that heat stresses can affect starch biosynthesis more than protein biosynthesis (Dupont and Altenbach, 2003). This is also observed by Hengyong Xu et al. in our laboratory (2004, unpublished results) and Blumenthal et al. (1995). With the materials from this study, protein per kernel decreases with increasing heat stress and protein concentration increases (Naeem et al., 2002). Again, this demonstrates different effects of stress on protein and starch biosynthesis. In the flour extracted from these kernels, this is less clear, perhaps due to the different rates of extraction that were achieved. Now, only in condition #5 protein content is a little decreased. In the context of this paper we focused on protein parameters that can directly be related to quality. We therefore investigated effects of stress on GMP.

The effect of stress conditions on the content of GMP is already apparent at less severe stress conditions (from #3 and up, see table 7.2). Heat stress causes a reduction of GMP to a greater extent than with protein (up to 40-50 %) . Between the near isogenic lines also differences are noted. The 5+10 lines typically have a higher GMP content than the 2+12 lines. This is in accordance with other reports (Payne et al., 1987). Interestingly, Lance 5+10 seems to be more resistant to heat stress than the other three lines. These results confirm the positive effect of HMW-GS 5+10 on both protein and GMP formation. The variations observed underline that both
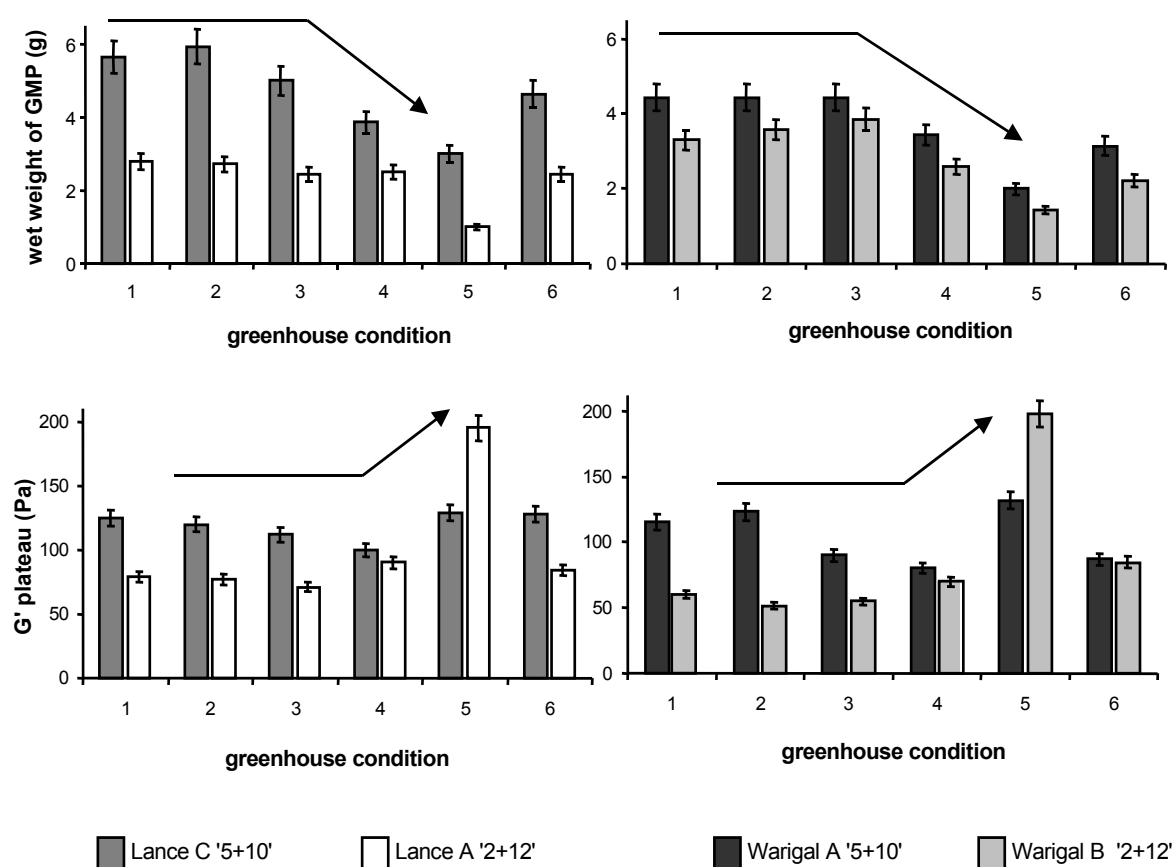
phenotypical effects, next to genotype effects are important. Where genotype affects the of protein and GMP levels under non-stressed conditions, stress conditions seem dominant with respect to GMP content.

Growing condition #5 clearly shows that application of heat stress from 16 DAA onwards negatively affects GMP formation. Again, this sharp decrease in GMP cannot be explained by a parallel decrease in flour protein or kernel protein content.

The effect of heat was not expected since Carceller and Aussenac (2001) indicated that heat is essential for the formation of GMP. They could simulate this by heating immature endosperm (without GMP) and thus induce GMP formation. Comparison of conditions #5 and #6, demonstrates that also the moment when heat stress is applied is essential. In condition #6 heat is increased at a later stage (from 25 DAA onwards) and significantly more GMP is formed (e.g. with Lance 5+10: 30.2 % vs 19.5 %). This points at a negative effect of heat in the early stages of glutenin synthesis.

7.3.2 *Effect of growing conditions on GMP-gel stiffness and GMP wet weight*

GMP and its rheological properties are reported to be good quality predictors (Weegels et al., 1996; 1997; Graveland et al., 1982, 1984; Pritchard, 1993; Kelfkens and Lichtendonk, 2000). To further unravel how growing conditions affected the internal structure of of the GMP, we investigated the rheological properties of GMP. Figures 7.1a-d show how GMP stiffness (G') and GMP wet weight vary with the different growing conditions (see #1 to #6 in table 7.1).

Figures 7.1 a-d   *The effect of growing conditions #1 - #6 on GMP wet weight (g) and GMP-gel stiffness (G') for wheat lines Lance 5+10, Lance 2+12, Warigal 5+10 and Warigal 2+12.*

As expected, GMP wet weight (7.1a-b) follows the trend for the %GMP proteins in table 7.2. GMP gel quantity (fig. 7.1a-b) and the %GMP protein (table 7.2) are highly correlated ($R^2 = 0.94$) for the whole set of lines and heat stress conditions (n=24). GMP gel stiffness is related to both the concentration of glutenin proteins and the average voluminosity of the glutenin particles in the GMP gel (Don et al 2003a). Gel stiffness initially (#1-3) parallels GMP quantity and is constant. Also, GMP values of 5+10 lines are considerably higher than for 2+12 lines. However, striking differences in GMP quantity and GMP stiffness are observed for growing regimes #4, #5 and #6. With treatments #4 and #6 a decrease in GMP quantity is observed, the GMP stiffness does not follow this trend. With treatment #5 a more dramatic decrease in GMP quantity is observed, but in contrast, the GMP stiffness even increases, especially for the 2+12 lines, where stiffness values are

higher than for the 5+10 lines (fig. 7.1c-d). Again, treatment #6, where the heat stress treatment is started later (from 25 DAA onwards) is more comparable to the less severe treatments. Heat stress between 16DAA and 25DAA appears to strongly affect the properties of the glutenin particles formed. As said, G' is governed both by protein concentration and glutenin particle size. With this treatment, and in contrast to the less severe treatments, we suspect that G' is more governed by size than concentration. This especially holds for the 2+12 lines, where G' values exceed values of the 5+10 lines. Parallel to the strong increase in G' of the 2+12 varieties, the phase angle δ decreases from 11° for #1 to 9° for condition #5. This decrease in δ confirms that the GMP-gel becomes more elastic with heat stress, indicative of a different internal structure of the glutenin particles.

In order to learn more about the specific nature of the glutenin particles present in GMP (Don et al. 2003a-b), we therefore studied how growing conditions affected the GMP-particle size in more detail.

7.3.3   *Glutenin particle size distributions.*

We have shown previously that GMP consists of glutenin particles that can be characterised with Coulter Laser analysis. Figures 7.2 and 7.3 give the different size distributions observed for the various wheat lines and treatments.
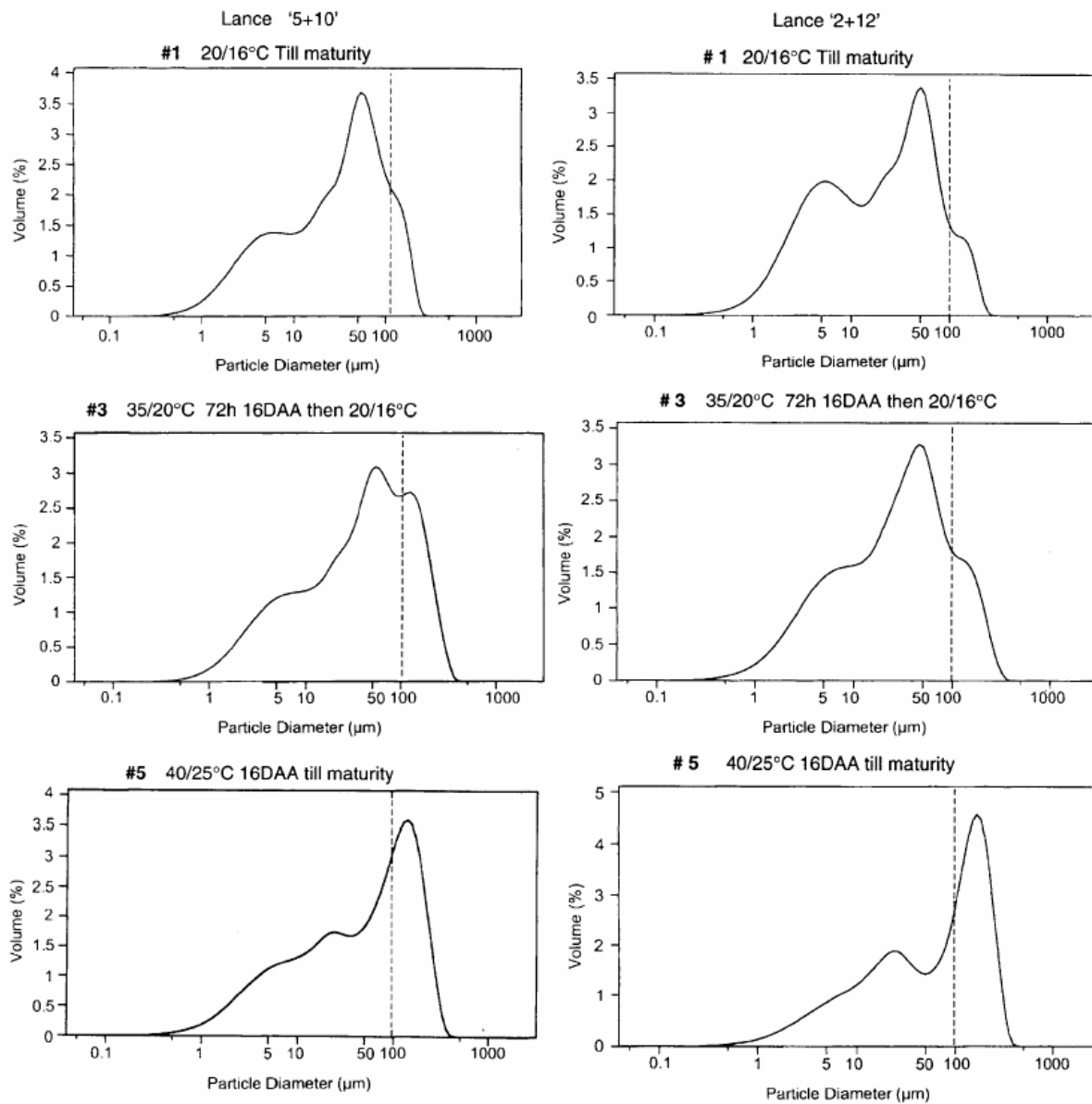
Figure 7.2   The effect of growing conditions #1, #3 and #5 on the glutenin particle size distribution of wheat lines Lance '5+10' and Lance '2+12'.

Figure 7.3   The effect of growing conditions #1, #3 and #5 on the glutenin particle size distribution of wheat line Warigal '5+10' and Warigal '2+12'.

Particles observed range between 1 to ca 300 μm. From the size distributions a characteristic parameter, the $D_{3,2}$ (the volume to surface ratio) can be calculated. In general, $D_{3,2}$ values increase with increasing heat stress. The distributions observed with treatment #5, however, stand out. Here, the distribution is characterised by a fraction of very large particles > 100 um. Although most

prominent with the 2+12 lines, this feature is observed with all wheat lines and seems characteristic for this treatment. This feature seems to be caused by the early application of heat and/or shorter exposure, since distributions obtained with treatment #6 do not have this feature.

7.3.3.1 Relation between G'and glutenin particle size

Figure 7.4a confirms the earlier results of Don et al. (2003b) that the GMP-particle voluminosity, determined from GMP dispersion viscosity, correlates with G' indicating that protein concentration is less important here. As expected, the $D_{3,2}$ data from the Coulter laser measurements and G' also show a positive correlation (fig. 7.4b).

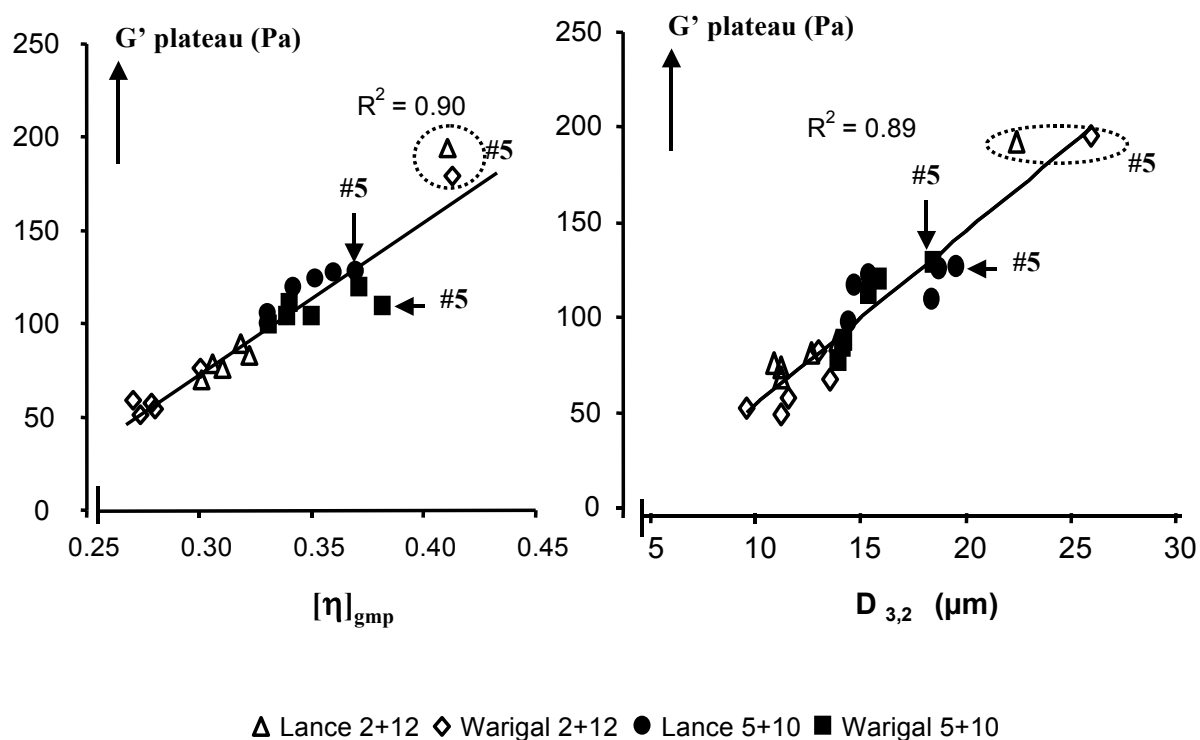△ Lance 2+12 ◇ Warigal 2+12 ● Lance 5+10 ■ Warigal 5+10

Figure 7.4a *Relation between G' plateau values of GMP-gel and the effective glutenin particle voluminosity [η] for wheat lines Warigal and Lance.*

Figure 7.4b *Relation between G' plateau values of GMP-gel and glutenin particle volume surface ratio $D_{3,2}$.*

Figure 7.4a reveals a number of interesting features regarding the effect of both genotype and heat stress. First, genotype seems to control the baseline level of size, with the 5+10 lines (filled symbols) giving larger particles than the 2+12 lines (open symbols). However, also between Warigal and Lance systematic differences exist. Lance 2+12 gives more voluminous particles than Warigal 2+12, Lance 5+10 gives larger particles than Warigal 5+10. This difference could be related to the differences in HMWGS composition.

The average glutenin particle sizes resulting from growing conditions were of the following order: #1 < #2 < #3 < #4 ≤ #6 << #5. In general, heat treatment leads to the formation of larger particles, with 5+10 lines giving larger particles than 2+12 lines. The most stressful #5 treatments are indicated in figures 7.4a and b . The combination of treatment #5 and 2+12 resulted in extremely large particles and is an exception to this rule (encircled data points). Here, the 2+12 lines form larger particles than the 5+10 lines. For clarity we have summarized the particle size data of all treatments (table 7.3). The table shows that less, smaller particles ($D<10\mu$) are formed with more heat stress and that area% of $D>100\mu$ increases with stress. $D3,2$ (weighted average surface area of the particles) is useful for predicting particle gel structural properties (fig. 7.4). The parameter $D4,3$ (weighted average volume) takes the larger particles better into account. The table shows that $D4,3$ responds sensitively to stress effects. $D3,2$ and $D4,3$ correlate ($R^2=0.77$). Also the viscometrically determined voluminosity parameter η also correlates with $D3,2$ and $D4,3$ (R^2 0.78 and 0.62 respectively).

Table 7.3   *Summary of the Coulter Laser glutenin particle size data for all 24 GMP samples analysed (Lance and Warigal wheat lines).*

	D<10 (area%)	10<D<100 (area%)	D>100 (area%)	$D_{3,2}$ (μm)	$D_{4,3}$ (μm)
Lance 2+12					
1	36.4	55.1	8.6	10.8	42.8
2	29.1	59.7	11.2	11.0	44.5
3	26.7	58.3	15.0	11.1	46.8
4	29.0	50.2	20.9	12.0	55.1
5	16.2	41.3	42.5	22.3	94.7
6	29.7	52.3	18.0	12.2	60.5
Lance 5+10					
1	26.4	59.4	14.2	14.4	47.1
2	26.8	61.6	11.7	14.7	46.2
3	22.8	54.3	22.9	15.1	63.9
4	20.9	51.0	28.1	18.0	67.9
5	22.1	45.4	32.5	19.8	77.0
6	20.3	55.0	24.8	18.1	65.5
Warigal 5+10					
1	27.1	63.9	9.0	13.8	43.8
2	28.2	64.6	7.2	13.9	42.3
3	27.1	66.7	6.2	14.1	39.6
4	26.5	57.6	15.9	15.0	50.2
5	14.2	46.8	39.0	18.1	86.1
6	27.7	56.7	15.6	15.6	50.3
Warigal 2+12					
1	32.8	58.2	9.0	9.0	41.1
2	28.1	63.8	8.1	11.2	41.3
3	27.9	62.8	9.4	11.8	42.0
4	27.5	54.5	18.0	13.1	52.1
5	15.2	40.4	44.4	25.1	96.4
6	29.7	46.8	23.5	14.0	59.3

7.3.3.2 CSLM observations of glutenin particles.

In order to confirm the trends indicated with Coulter and viscosity measurements, we also observed the glutenin particles using CSLM. Figure 7.5a-d shows the most contrasting samples (treatment #1 vs #5 for Lance). Especially with the 2+12 variety some very large and more irregularly shaped particles can be observed (arrows in figure 7.5d), that seem to consist of fused smaller particles. Again, this is only observed with treatment #5 and 2+12 lines. This confirms the very large diameters observed with Coulter laser.
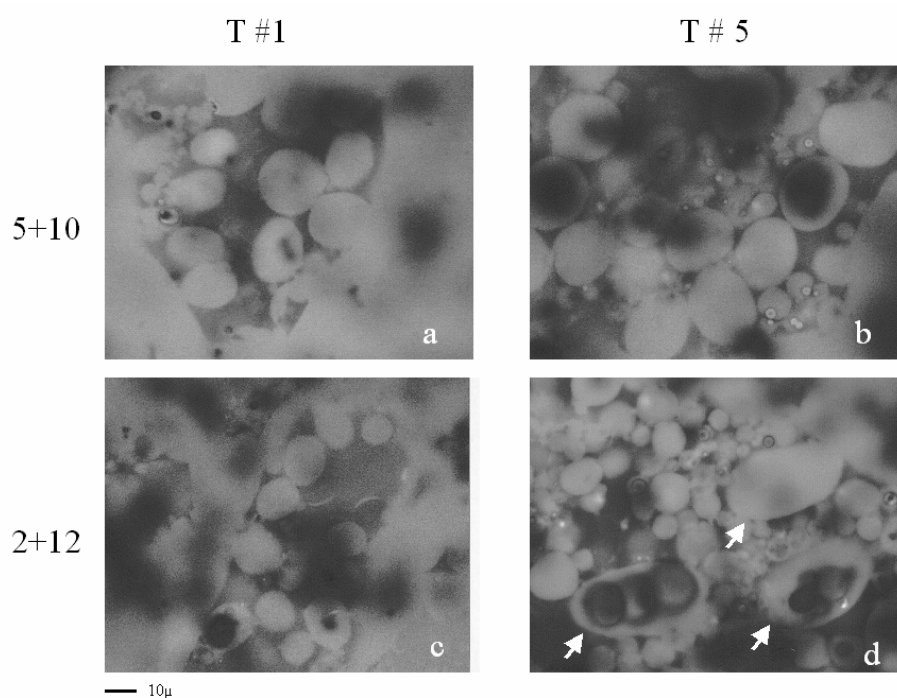

Figure 7.5a-d   *Examples of CSLM pictures that show how glutenin particle sizes are strongly affected by both HMWGS composition (2+12 vs. 5+10) and growing conditions (#1 vs. #5)*

7.3.4   HMWGS-LMWGS ratio and effect on glutenin-particle size

Heat stress could have an effect not only on size, but also on the composition of the glutenin particles could be affected. We therefore analysed the HMWGS and LMWGS composition of both flour and GMP for all samples. Figure 7.6 shows some relevant examples of RP-HPLC traces, comparing treatment #1 and #5.
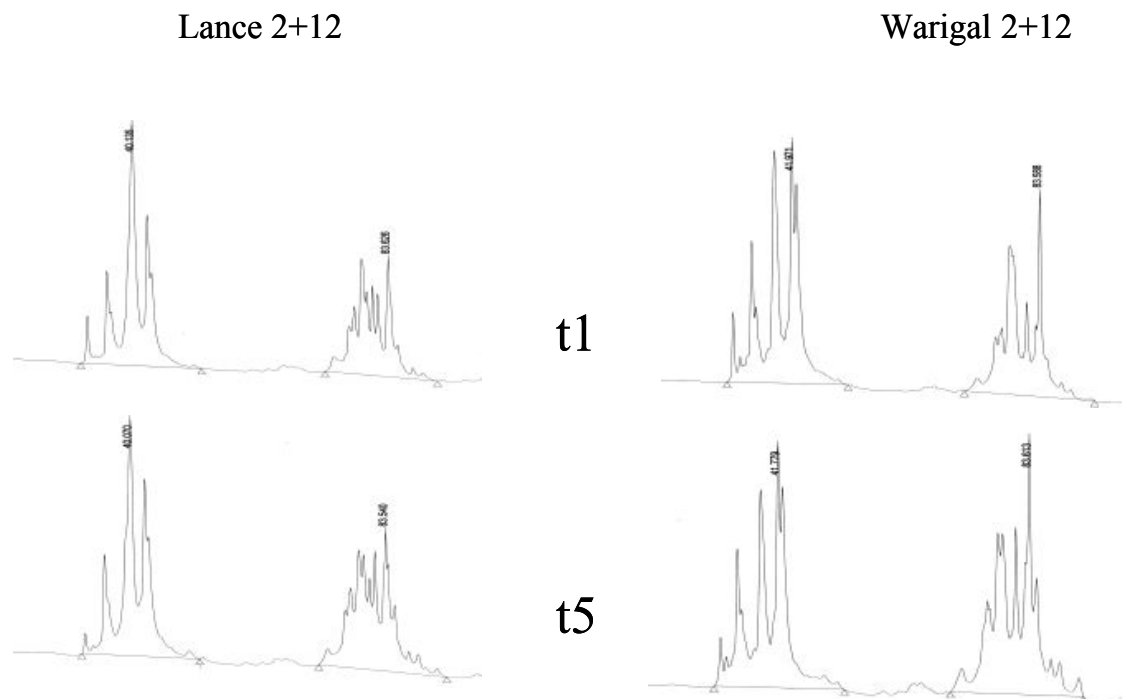
Figure 7.6   Examples of RP-HPLC traces that show how the two most differing growing conditions (#1 vs. #5) affect the HMW/LMW of lines Lance 2+12 and Warigal 2+12.

Qualitatively, the patterns are the same. Consequently, heat treatment did not result in different levels of expression of specific HMW or LMW glutenin subunits. This is confirmed by DuPont and Altenbach (2003) who did not find any evidence that expression of *specific* glutenin subunits should change with heat stress. However, they did find that heat stress could lead to a decrease in the glutenin / gliadin ratio.

Based on our HPLC data we did observe a change in the *relative amount* of HMWGS over LMWGS (HMW/LMW ratio). We have calculated HMW/LMW ratio's for both HMWGS/LMWGS in flour and GMP. In general, HMW/LMW ratio's for GMP and flour are the same. This is contrary to earlier findings (Don et al., 2003) where an average ratio of 1.6 was reported for GMP HMW/Flour HMW. We cannot provide a conclusive explanation for this difference. However, we note that the ratio of 1.6 was found with a group of European wheat varieties. The US lines used in this study, and also other US lines we have characterized in our laboratory, have a significantly higher content of HMW-GS in flour in comparison to LMW-GS (US vs EU: HMW/LMW 0,9-1,4 (n=7) vs 0,2-0,7 (n=10)). This may explain why in this case the GMP HMW/LMW ratio for these US samples is similar to the HMW/LMW in respective flour. For

EU samples HMW/LMW is lower, perhaps also larger differences in GMP HMW/LMW and flour HMW/LMW (ca 1 vs 1,6). With increasing heat treatment, the relative amount of LMW increases, leading to a lower HMW/LMW ratio (#1 vs # 5 = 1.3 vs 1.0). We could not detect differences between Warigal and Lance or between 5+10 and 2+12 lines. We therefore consider this decrease in ratio to be an effect of the heat stress. Interestingly, with treatment #6 where heat stress is applied at a later stage and consequently for a shorter duration, HMW/LMW ratio is ca 1.2. This points at a mechanism where heat stress, applied as early as 16 DAA, effects HMW or LMW glutenin protein synthesis differently, lowering HMW/LMW ratio. If we consider all treatments, a relation is observed between $D_{3,2}$ and the HMW/LMW ratio (fig. 7.7a-b). With decreasing HMW/LMW, the $D_{3,2}$ increases.
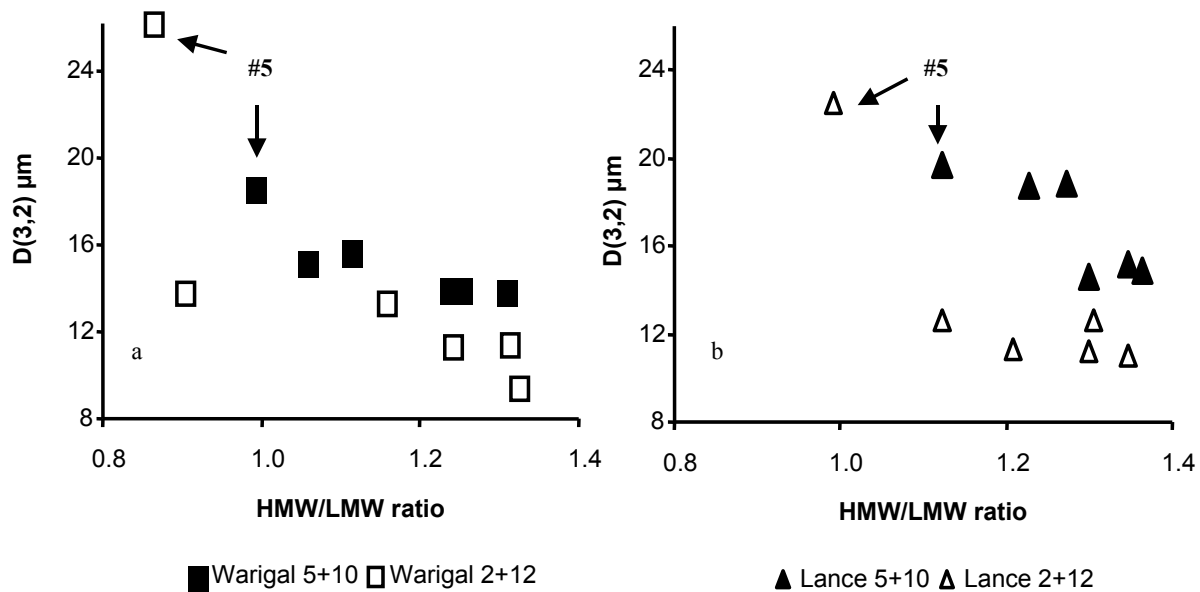

Figure 7.7a-b The effect of growing conditions #1 - #6 on GMP HMW/LMW ratio and how this affects the glutenin particle $D_{3,2}$ of wheat lines Lance 5+10, Lance 2+12, Warigal 5+10 and Warigal 2+12.

We do not exclude however that both an increased size and a decreased ratio are the result of heat stress. The combination of treatment #5 and 2+12 again, stands very much in contrast. Here, HMW/LMW ratio is significantly changed (< 1) and the $D_{3,2}$ is sharply increased ($D_{3,2} > 20$). Glutenin particles consist of both HMWGS and LMWGS. We have demonstrated earlier that HMWGS are key to the formation of glutenin particles (Don et al., 2003d), LMWGS cannot form stable particles by themselves and only end up in glutenin particles as a part of a structure with HMWGS. It is not unlikely that variation in the ratio between HMWGS and LMWGS will affect particle size. Recent results show that under unstressed growing conditions, increasing HMW/LMW ratios result in increasing glutenin particle sizes (Don et al. 2003d). Nevertheless, we believe that the enormous increase in size observed with treatment #5 is the result of a different process and must be considered the abnormal effect of very high temperature stress.

7.3.5   *Micro-scale mixing experiments*

Mixogrammes are an important tool in assessing technological quality differences on a small scale. Dough development time (DDT) is related to glutenins and recognized as an indicator of dough strength. The bandwidth at peak resistance (BWPR) has been indicated to parallel extensogram Rmax (Uthayakumaran et al., 1999). The heat stress conditions induced a unique variation in GMP quantity and glutenin particle size. On basis of increasing glutenin-particle size, DDT is expected to increase with heat stress according to Don et al. (2003b). On the other hand, DDT is also reported to increase with GMP quantity (Graveland et al., 1984; Weegels et al., 1996, 1997). Consequently, DDT would decrease with heat stress. As expected, the results with the 2g mixograph revealed a large variation in DDT values.
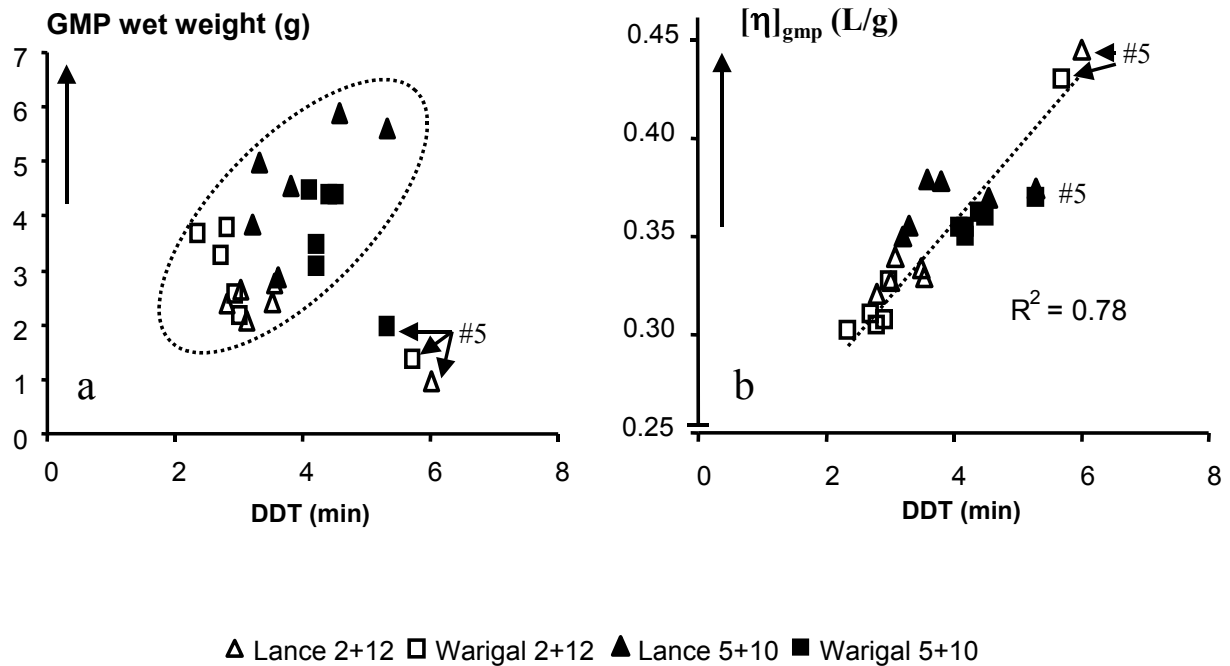
Figure 7.8a   Relationship between DDT and GMP wet weight for Lance 5+10, Lance 2+12, Warigal 5+10 and Warigal 2+12 grown under conditions #1 - #6

Figure 7.8b   The relationship between DDT and glutenin particle voluminosity [η] for Lance 5+10, Lance 2+12, Warigal 5+10 and Warigal 2+12 grown under conditions #1 - #6.

In figure 7.8a we have plotted DDT against GMP wet weight. If we take all samples together, no correlation is found. However, if we exclude the treatment #5 samples, we can observe a weak relation ($R^2 \sim 0.5$). With decreasing GMP, DDT also decreases. However, if we plot DDT against glutenin particle size as shown in figure 7.8b, a better correlation is observed, even when all samples are included ($R^2 = 0.78$). Apparently, glutenin particle size is more important than GMP wet weight for DDT. This is in agreement with Don et al. (2003) who proposed a mechanism in which optimal dough mixing is governed by glutenin particle disruption. Nevertheless, GMP wet weight is still relevant for dough properties.
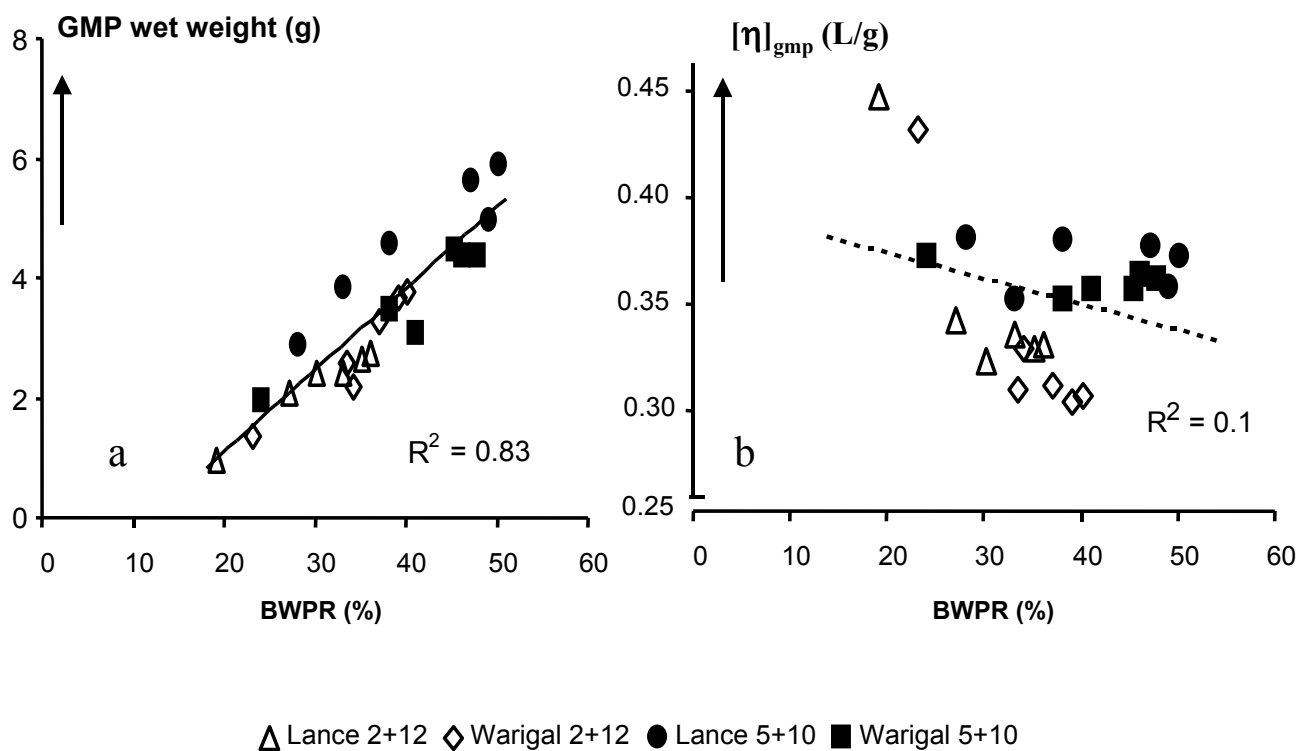
Figure 7.9a   *The relationship between BWPR and GMP wet weight for Lance 5+10, Lance 2+12, Warigal 5+10 and Warigal 2+12 grown under conditions #1 - #6*

Figure 7.9b   *The relationship between BWPR and glutenin particle voluminosity [η] for Lance 5+10, Lance 2+12, Warigal 5+10 and Warigal 2+12 grown under conditions #1 - #6.*

Figure 7.9a shows a correlation of $R^2 = 0.83$ between BWPR and GMP wet weight. Glutenin particle size does not correlate with BWPR (see figure 7.9b).

In conclusion: heat stress can lead to a decrease in GMP at an increase of glutenin particles. The increase in glutenin particle size will lead to an increase in DDT, whereas the decrease in GMP will lead to a lower dough stability (BWPR). Whether these typical effects on glutenin quantity, glutenin particles and dough mixing are also reflected in bread-making quality could not be part of this study due to the small quantities of wheat grain material available.

7.4   Discussion

7.4.   Heat stress effects on dough mixing properties

It is generally accepted that wheat quality is the result of genotype x growing conditions. This causes a large variation in wheat quality, not only between varieties, but also between different batches/growing locations of the same variety. Although flour millers aim to produce a constant flour product, the baker is still faced with variations, especially with respect to dough mixing requirements. For this reason there is a strong interest in understanding the nature of these variations. In general, with non-stressed wheat samples, a relation is found between gluten, glutenin or GMP- content and dough mixing requirements (Gupta et al., 1992; Lafiandra and MacRitchie 1997; Weegels et al., 1996, 1997; Pritchard, 1993; Bekkers et al., 2000). Using the unique set of heat stressed samples in this study we could add important details to this relationship. It is not only the quantity of GMP that is important, but also the size of the glutenin particles contained in this fraction. This information could help manage heat stress effects, on the condition that we understand how heat stress effects glutenin particle formation. Heat stress influences both glutenin biosynthesis and glutenin particle formation. In general, we observe that with increasing heat stress, (treatments #4-6), a smaller amount of GMP is formed, but that this fraction contains larger glutenin particles. The decrease in polymeric glutenins like GMP is confirmed by other research labs, who measured %UPP (Unextractable Polymeric Protein), Naeem et al. (2002) or %IPP (Insoluble Polymeric Protein) Ciaffi et al. (1996). We share the explanation of Ciaffi et al, who stated that this was presumably due to a decrease in HMW-GS synthesis. The observation that larger particles are formed is new, and in our view helps explain how stress affects dough-mixing time. Carceller and Aussenac (2001) suggested that dehydration and heat induce a faster and more effective disulphide bond formation and glutenin aggregate formation. However, aggregation phenomena are also

related to quantity, and we expect that changes in the HMW/LMW ratio will also play a role. So, how can we understand the effects of heat stress on glutenin particle formation?

7.4.2   *Hypothetical formation of glutenin particles*

If we apply the hyper-aggregation model (Hamer and Van Vliet, 2000; Carceller and Aussenac, 2001) for the formation of a glutenin particle, we can distinguish three steps:

1) Biosynthesis of Glutenin subunits (HMW plus LMW)
2) Polymerisation and formation of larger oligomer clusters (Carceller and Aussenac, 2001), observable protein storage vacuoles ~14-17 DAA (Bechtel and Wilson, 1997)
3) Further assembly into larger insoluble glutenin particles (Carceller and Aussenac, 2001), and also fusion of protein particles sometimes observable during maturation (Bechtel and Wilson 1997)

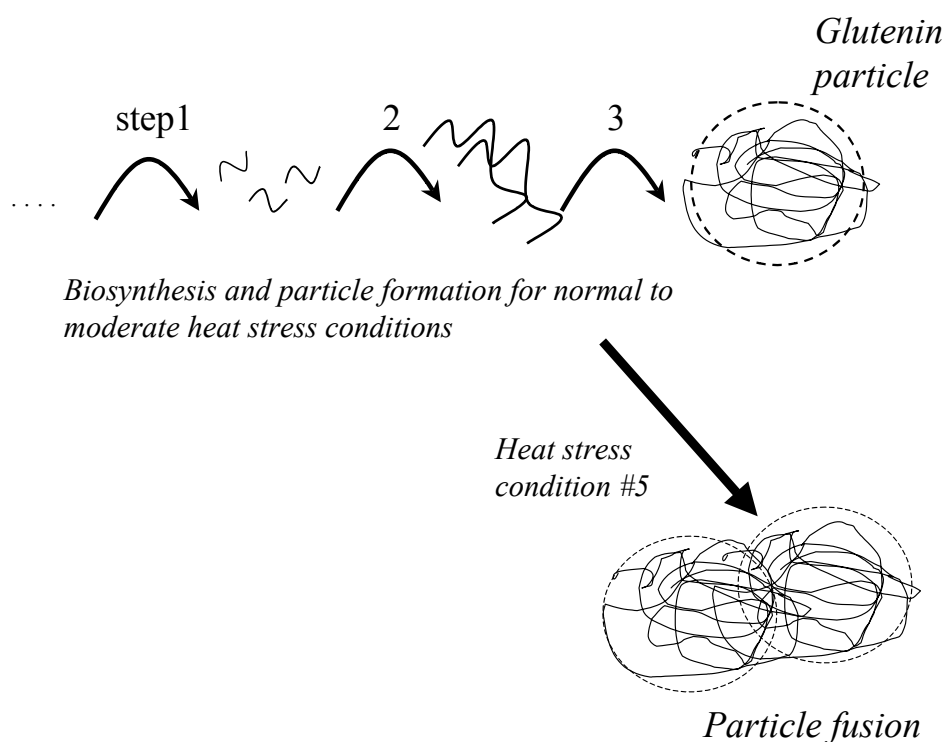We have depicted a hypothetical assembly process in figure 7.10.

Figure 7.10   Hypothetical formation of glutenin particles by hyper-aggregation and suspected particle fusion induced by heat stress condition #5.

We suggest that assembly of the glutenins during steps 2 and 3 is important in controlling the final size of the glutenin-particles. When heat stress starts at 16 DAA as in #5, the hotter regime slows down the biosynthesis of glutenin (step 1). This agrees with Dupont and Altenbach (2003) who have reported this effect of heat on decreasing of glutenin biosynthesis. This explains the overall decrease in GMP observed. On the other hand, a higher temperature can cause the hyper-aggregation in steps 2 and 3 to progress faster (Carceller and Aussenac, 2001). This could explain why from a smaller quantity of glutenin, still larger particles are formed.

In general, the 5+10 varieties have more GMP than 2+12 varieties, irrespective of growing regime used. This could result from a higher content of glutenins as reported by DuPont and Altenbach (2003). This would affect all steps of the hyperaggregation model. We also note that with the exception of treatment #5, 5+10 varieties produce larger particles than 2+12 irrespective of GMP content. This could be due to the specific polymerisation properties of 5+10 lines (Lafiandra et al., 1993; Popineau et al., 1994). Also, 5+10 lines typically have a higher HMW/LMW ratio than 2+12 lines. This feature has been observed earlier by (Don et al., 2003b). Apparently, these features

enable 5+10 lines to form larger network structures in steps 2 and 3. The same holds for the differences between Lance and Warigal observed. Lance-lines are able to form larger particles than Warigal-lines. We believe this is due to differences in HMWGS composition.

Our observations indicate that the results with treatment #5 and 2+ 12 lines stand out and do not fit this model. Here, the 2+12 lines show a different behaviour from the 5+ 10 lines. With the 2+12 lines in treatment #5 more LMW subunits combined with heat may have had an unexpected affect on glutenin structure. For an example D'Ovidio and Masci (2004) found that heat stress can effect the aggregation behaviour of LMW-GS. That the glutenin particle structure is different is clear from our results with glutenin particle size, GMP rheology and CSLM. We propose that the large particles observed with this treatment are the result of fusion between individual particles. It is important to note that this only occurs when heat stress is applied from 16 DAA. In condition #6, where stress is applied 25 DAA, this phenomenon is not observed. In the greenhouse experiment with the Lance and Warigal lines, the first small quantities of 'polymeric' glutenin were observed about 16 DAA. Perhaps, already at this time, first particles are formed that could fuse under high temperature conditions.

7.5   Conclusions

GMP quantity is more sensitive to growing conditions than protein quantity. With the exception of severe heat stress conditions, 5+10 varieties produce larger glutenin particles than 2+12 varieties. The HMW/LMW ratio of GMP is lowered by heat stress, but glutenin particles become larger. We have demonstrated that this effects dough mixing properties, where glutenin particle size is more relevant than GMP for DDT and GMP is more relevant for BWPR. Using the hyperaggregation model, we have combined our data with those of others studying glutenin formation and presented a hypothetical model to explain how heat stress controls glutenin particle formation. In general, heat stress leads to a lower amount of GMP, but favours hyperaggregation, (larger particles). In addition, we propose that severe heat stress conditions could lead to fusion of glutenin particles leading to long mixing times at a low dough strength. This will help better understand effects of growing conditions on glutenin particle size distributions and variations in flour quality.

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8

GENERAL DISCUSSION

In the previous chapters the new insights gained on glutenin particle properties and aggregation factors affecting glutenin functionality have been described. In this chapter we will discuss to what extent it has been possible to:

1) Close in on the objectives set out in chapter 1
2) Reveal experimental evidence that supports the hypothesis of hyper-aggregation and combine these experimental results into a single model relating: composition to structure, to function.

8.1 Methodology for studying relations between wheat flour proteins, dough and flour quality

Even today, a better understanding of the role of glutenins in dough strength and bread making impels substantial research efforts. From literature, four main types of research approaches in cereal science become apparent. These can be categorized into:

A. <u>Statistical approaches</u>. Studies that assess correlations between protein characteristics, dough and bread-making quality;
B. <u>Model studies</u> that study the physico-chemical properties of isolated glutenins or reconstituted systems to assess their role in for example dough strength and flour quality;
C. <u>Breeding and genetic modification studies</u> leading to specific materials that can be used for composition-functionality studies;
D. <u>Processing studies</u>, where changes in gluten are induced in a controlled way for example through mixing and resting.
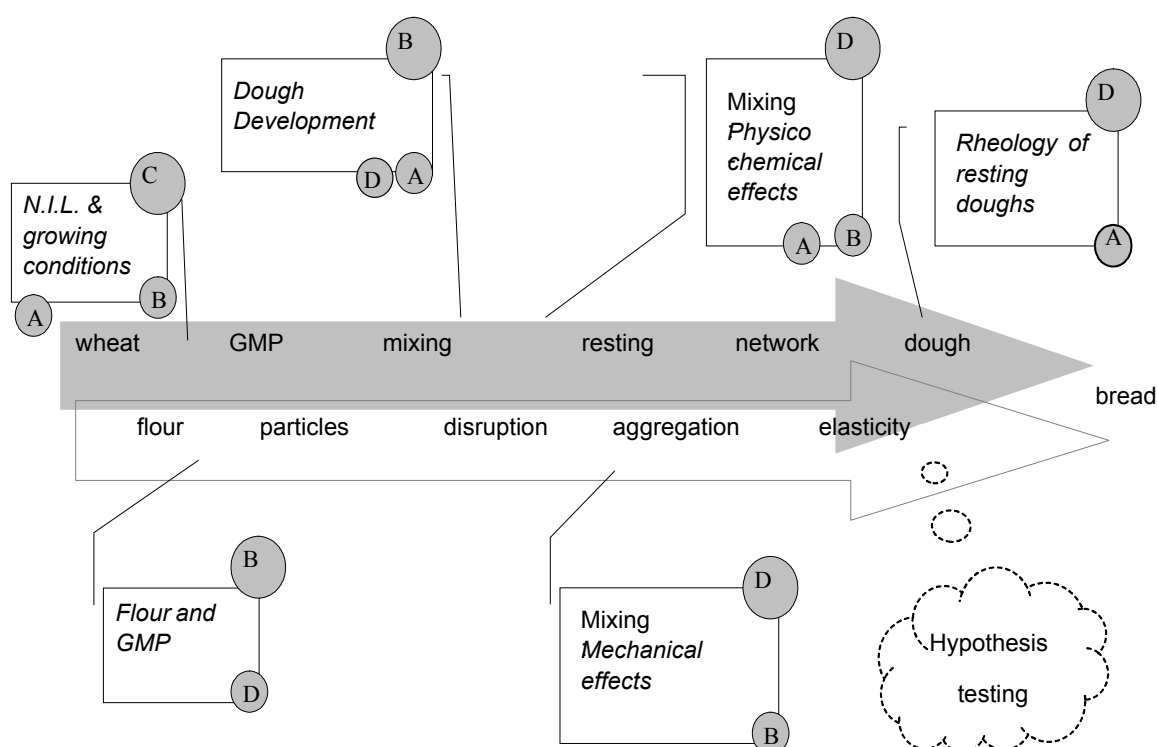
Figure 8.1   Research approaches followed: The figure shows in schematic manner the topics addressed and their connections from wheat and glutenin particles to models to processing and dough. The double arrows indicate the glutenin factors at different levels. Letters refer to the research approaches listed earlier. The methods of research applied are indicated by letters, the size of the circle indicates the importance for that specific topic of the study. N.I.L: near-isogenic lines

Figure 8.1 demonstrates that this study combined these approaches in various ways, with a main emphasis on model and processing studies (B and D). Good use was made of near- isogenic lines (category C methodology). This finds itself in high interest in the recent literature (He et al. 2005). Statistical correlation studies between quantity of protein fractions and loaf volume are valuable, but do not help develop our mechanistic insight.

Correlation studies (category A) were used to provide additional support together with the main methods. Long-standing methodologies in cereal science were used to provide experimental grounds for studying GMP and challenging the hyper-aggregation model. On the other hand it is not overlooked that newer or alternative methods are also advocated (Belton 2005). It is not intended to

extensively review these here, but the advantages and disadvantages of three important ones will be discussed shortly:

- Non-invasive, spectroscopic methods (Belton, 2005)
- Sonication in combination with SE-HPLC (Gupta, 1993)
- HMW-GS composition (Payne, 1987)

Spectroscopic methods hold promise of studying the native situation of proteins, because dough handling before analysis and extraction of GMP are not required. However, proteins in flour are not functional and dough is a very complex mixture containing components of highly varying solubility and even degree of hydration. This makes a correct interpretation of spectral data from dough very difficult.

Sonication in combination with SE-HPLC is frequently used as a technique to study the size distribution and quantity of glutenin fractions (Singh et al., 1990; Gupta et al., 1993). However, we believe that sonication disrupts glutenin aggregates too rigourously, leading to a loss of important information on glutenin network structure. Hamer et al. (1995) for example have shown that mixing and sonication lead to different disruption patterns of glutenins. Furthermore, it is unclear whether sonication only disentangles glutenin polymers or also breaks covalent bonds. It is sometimes noticed that sonication also enhances the foaming properties of proteins. This points at a structural change. Therefore, we believe that sonicated glutenin samples cannot be considered to represent the key aggregated fraction that is functional in dough. HMW-GS are important glutenin constituents, but these can only be studied after reduction of an aggregated glutenin fraction. The present study focuses on the role of very highly aggregated glutenin proteins as an intermediary level between composition and function. Non-sonicated and non-reduced fractions are therefore clearly preferred. GMP is such a fraction.

GMP isolation has been collaboratively tested (Law et al., 2005). Quantity and GMP-gel stiffness are industrially agreed as predictors of wheat flour end-use quality (Law et al., 2005). This is of practical importance. There is however recent debate on the fundamental relevance of the stiffness of the GMP fraction (Belton, 2000). This impels us to further improve our understanding of the key elements governing GMP-gel stiffness and dough properties. According to Lefebvre et al. (2003) dough elastic properties are conveyed by very large glutenin aggregates. Although the glutenins are just a small fraction of flour (~3%) and dough (~2%), their aggregates are viewed as main elements

of the glutenin aggregate network. Every protein has its own unique functional properties, and it is not uncommon that ~2% of a highly functional protein can tremendously affect macro-scopic properties. Our own results provide experimental evidence to support this:

- Chapter 3:effect on mixing requirements,
- Chapter 4: effect of for example over-mixing on dough rheology,
- Chapter 5: effect of NEMI on mixing requirements,
- Chapter 6/7: effect of composition on glutenin particles and mixing requirements

In view of the various methodologies, advantages and disadvantages discussed, in-depth analysis of the composition, structure and physical properties of GMP appears the appropriate course for this study. It allows a direct observation and measurement of the functional glutenin aggregates / particles.

8.2 Main findings

8.2.1 Glutenin particles

Until ca 2000, most groups considered the gluten network as a complex chemical structure, consisting of disulfide linked glutenin subunits. Ewart was perhaps one of the exceptions in this respect. He already in 1968 pioneered the notion that entanglements are also of importance. The entanglement model of MacRitchie (1999) and the hyper-aggregation model of Hamer and Van Vliet (2000) are more recent models. The hyper-aggregation model represents the first attempt towards a full integration of approaches, including physical and chemical interactions. At this time, it is generally accepted that such an approach is required to understand the structure-function relationships of the glutenin network. However, the debate continues if the glutenin network can be best described as a network of entangled flexible polymers (Belton 2005), or a colloidal particle network (Lefebvre and van Vliet 2003, Hamer, Van Vliet and Lefebvre 2005). Therefore, our first objective was to find out what structural elements underlie GMP and it's rheological properties. In chapter 2 it is demonstrated with CSLM that GMP is a gel that consists of glutenin particles. The four varieties tested all exhibited such particles, but differences in stability were clear, presumably related to differences in HMW-GS composition.

It was also established that both glutenin quantity and glutenin particle size determine the stiffness of the gel: Large differences in glutenin particle voluminosities [η] affect the G' plateau values of the respective GMP-gels. Coulter Laser experiments on water-dispersed GMP-gel show aggregate

sizes in a 0.1 – 300 µm range. The Coulter laser size results depend on the assumption that it is correct to use the Fraunhofer model. The particle sizes detected with Coulter Laser parallel voluminosity values that were measured by viscometry of particle dispersions in SDS. More importantly, size estimates from CSLM photographs are consistent with the glutenin particle size measurements done on GMP from flour. Also, CSLM demonstrates that particles isolated from flour are spherical. In this respect, the condition to use the Fraunhofer model is met.

The glutenin particles observed in the typical 1.5 % (w/v) SDS extract used for GMP isolation are swollen. Exchanging SDS for methanol for example, leads to shrinkage. On average, the protein concentration in a GMP-gel is about 10-15 mg/mL (~1-1.5wt%). This concentration is not uncommon for gels consisting of hydrated particles. Dough contains roughly 2 wt% glutenin protein. Our observation of particles may stand in contrast with earlier findings. In the developing wheat kernel, gluten proteins are initially stored in protein bodies (Evers & Bechtel 1988). Since protein bodies are no longer visible in mature grains, it was concluded that they have become part of a homogenous protein matrix.

The particle concept also met with opposition from another perspective. Belton (2005) stated that the particles observed in GMP were clearly an artifact induced by using a high concentration of SDS. According to Belton, this 'artifact' of SDS resembles techniques used in the manufacture of detergent particles. This extrapolation to glutenin particle formation is not supported with evidence. On the contrary, Hamer and Van Herpen (2005, personal communication) have recently demonstrated that protein bodies, isolated from immature endosperm are dissolved in 1.5 % SDS, leading to a loss of particles. In flour of mature grains the protein bodies are expected to be denser and more internally cross-linked (disulphide bonds). Such particles would no longer be soluble in SDS and can be observed after isolation.

Thus far, the experimental evidence here is based on one type of extraction, the GMP-method. This extraction allows us to separate SDS-soluble matrix proteins (e.g. gliadins, but also SDS soluble glutenin polymers) from insoluble glutenin structures, leaving them as 'skeleton particles'. In our opinion this also explains why these particles are not visible by microscopic methods. Glutenin particles could be present in a continuous matrix, but are not revealed because they embedded in similar types of wheat protein. This tentative explanation given in chapter 2 has not been refuted. Moreover, two earlier studies (Mecham et al. (1965), and Graveland & Henderson (1987), also provided experimental data that suggest the existence of particulate structures originating from protein bodies in wheat endosperm. Mecham et al. (1965) used acetic acid extractions, Graveland & Henderson (1987) used water extractions.

The proposed relation between protein bodies and glutenin particles leads to a possible conflict with respect to size. EM images of immature reveal dense protein bodies, of roughly 5μm (Evers & Bechtel 1988) in diameter. The particles isolated in water are appr. 10-25 μm (Graveland & Henderson 1987). Mecham et al. (1965) reported that they could visually observe the large glutenin aggregates in acetic acid solution. In 1.5% SDS, the particles are estimated to range from 20 – 80μm based on CSLM observation. With Coulter Laser also some particles appear to be > 100μm, but this is not the majority of the protein. Furthermore, the particles must have a very heterogeneous distribution, as shown in chapters 6 and 7. Varieties without deletions and grown under normal conditions have particles with a weighed average volume (D4,3) between 25 – 40 μm. The D4,3 is the average volume estimate taking the largest particles into account. Swelling is a typical property of protein gels, the extent being dependent on the properties of the protein gel and the type of solvent used. Addition of SDS will lead to swelling of the glutenin network. If we assume that a glutenin particle would be the result of swelling of a protein body, the average diameter increases with a factor of ca. 5 - 8. Consequently, the volume would increase by a factor of 125 – 512. Is this amount of swelling possible? In a GMP-gel the deformable glutenin particles are compressed: the gel contains appr. 10-15mg/ml protein. It requires dilution to observe the non-compressed particles. The swollen particles are observed in a GMP-gel dispersion that has 1-2mg/mL protein. We estimate the average protein concentration in a swollen particle to be 3 – 6 mg/mL protein. The density of non-swollen protein bodies has been reported to be 1.2 – 1.5 g/mL (Rosenberg et al. 1993). Based on this calculation a swelling factor of 200 – 500 is possible.

8.2.2   Dough development

The dough mixing step is one of the key steps in process and product quality control. It is at this step that variations in raw material quality have to be taken into account. Insight in dough development is therefore very important. Bloksma & Bushuk (1988) thoroughly described the phenomenological factors involved in developing dough. The observation of peak consistency after a certain mixing period is still regarded as the moment the gluten structure has 'developed' (ICC method). Between different batches of wheat flour considerable differences in dough mixing requirements exist. Explanations for varying dough development times have been based on correlations between gluten or glutenin content and mixing time (Gupta et al. 1993, MacRitchie 1999). However, when we studied the fate of glutenin particles during mixing, a new view on development was developed. In chapter 3 it is explained that it is not the quantity of glutenin, but primarily the size of the glutenin particles that determine dough mixing requirements. Dough

development coincides with the *disruption* of glutenin particles. This process could be modeled using Kick's law demonstrating the relation between initial (flour) glutenin particle size and energy input required to reach time to peak. This relation holds for heat stressed samples, N.I.L. lines and additional set of 9 industrial flour samples (Chpt. 6, Chpt. 3). Especially the heat stressed samples provided an opportunity to distinguish effects of protein content and gluten content from effects of glutenin particle size. Only the latter correlated with dough mixing requirements (Chpt. 7). For both breeders and industry this relation is very useful, as effects on quality and dough processing can be interpreted based on glutenin particle size. The results presented in chapter 3, although in complete agreement with earlier literature (Graveland et al. 1987, Weegels et al. 1994, Mecham et al. 1965), seem in conflict with a common belief that protein structures are formed during dough mixing (Lee et al 2004, Belton 2005). When studying GMP we can only conclude that mixing disrupts this highly aggregated fraction. It is important to note that our findings reflect the situation in dough directly after stopping the mixer. Our dough samples are frozen in liquid Nitrogen, whereas other authors report on results from dough rheology. In rheology, the dough is typically rested for at least 30-45 min after mixing. Based on our biochemical data this does not reflect the situation in dough directly after stopping the mixer.

8.2.3 Glutenin network formation after mixing

Chapter 4 studies the fate of glutenin particles when resting a dough after mixing. Three mixing regimes were used: under-, optimal, and over-mixing. The phenomena of under, optimal and over-mixing on dough rheological properties and bread-volumes have been extensively described by Kilborn & Tipples (1975) and Frazier (1975). The results in this thesis confirm their observation of a relation between mixing regime and subsequent dough properties. Kilborn & Tipples (1975) also stated effects of un-mixing, hinting that the gluten network must be regarded as a dispersed phase. Later Kieffer et al (2000) confirmed this view. But, how and to what extent do glutenin particles play a role here? Decrease in size is apparent, but in chapter 4 the concept of K' is added to the picture. The K' or Huggins constant, reflects the overall aggregative properties of the (disrupted) glutenin particles. An increase of K' indicates that physical particle interactions increase. Chapter 4 extensively discusses the effects of under-mixing, optimal mixing and over-mixing. Mixing changes glutenin particle structures, this is reflected in size, K' and with over-mixing also in the ratio HMW/LMW. CSLM observation of glutenin particles under shear shows that particles can be deformed. During dough rest new aggregated structures are formed. Their ability to confer elastic properties to dough is linked with K', size and HMW/LMW. With under-mixing disruption is

incomplete, glutenin structures are not yet homogenously distributed, the size of the glutenin aggregates is high (10 – 100 μm), and K' is low. These aggregate structures are not able to form an elastic network throughout the dough. With optimal mixing, gluten structures are homogenously distributed, the size of glutenin aggregates is lowered (0.1 – 20 μm) and K' is increased. These glutenin structures present the optimal starting point to achieve a visco-elastic dough after resting. With over-mixing the particles are too small in size, and HMW/LMW is affected. Only after re-polymerisation glutenin particle structures are formed that can give dough elasticity.

SH <-> S-S reactions also affect dough mixing. Effects of reducing agents and oxidizing agents have been observed very clearly by several authors (Hoseney et al. 1990, Goldstein et al. 1957). However, most studies focus on mixing, and not on resting. The results in chapter 5 agree with previously reported effects of NEMI: addition of this SH-blocker speeds-up dough development (Hoseney et al. 1990). This clearly points at the importance of chemical reactions involving SH groups. Nevertheless, initial glutenin particle size remains important demonstrating that the principle of KicK's law still holds. Therefore, during mixing both chemical and physical processes are important. Chapter 5 reports new information on the subsequent effect of NEMI on the properties of dough after resting. Our findings of Chapter 4 showing a relation between particle size after resting and dough relaxation properties was clearly challenged by these results. In the presence of NEMI still a dough could be produced with comparable rheological properties as a non -NEMI dough. First of all, the re-assembly of glutenin particles is also driven by physical interactions. Second, glutenin particle properties play a role. A relation was shown between HMW/LMW and dough $t_{1/2}$. However the size and quantity of the glutenin particles is decreased by NEMI. If the glutenin network controls rheology, how can the smaller particles form a network that gives the same rheological properties. Several options are possible:

- NEMI alters the physical interactions of the particles (available surface, positive charge)
- Not only SDS insoluble glutenin particles should be taken into account, also the SDS soluble glutenin help in the formation of a glutenin network in dough

The study in chapter 5 does not allow firm conclusions on the first option. The second option is supported by Weegels et al. (1996): all GMP present in flour participates in the glutenin network that controls dough rheological properties. However, this reasoning should then also apply to over-mixed dough, where also a significant amount of flour GMP is not recovered after resting. This is

not the case. The option of NEMI altering the physical interactions in our opinion holds more promise. It is clear that NEMI affects the internal composition (HMW/LMW ratio) of the particles and their ability to reassemble into larger structures stabilized by SS bonds. A chemical reaction with NEMI will also change the surface charge of the particle and thus the quality of its interaction. The one parameter, that could be used as an indicator for this is the Huggins constant K'. Typically K' values are the same or increased when using NEMI. Although much remains to be proven, the first option seems to provide a more likely explanation for the results observed.

The glutenin particle HMW/LMW ratio and K' are clearly important factors in glutenin network formation. These factors govern the quality of the particle-particle interactions, resulting in a network structure of soluble and insoluble glutenin. In this respect it will be important to further unravel the biochemical basis of physical parameters like K'.

In conclusion: Both physical and chemical reactions are important during mixing. TTP is determined by the balance between physical disruption and physical-chemical processes leading to aggregate formation. The size of the glutenin particles is determined by the HMW/LMW ratio. The K' of the particles during all steps of processing are determined by internal particle composition.

8.2.4   *How are glutenin particles formed? Genetic and environmental effects*

We have proposed that glutenin particles revealed in GMP, have their origination in the endosperm of the wheat kernel. Already during the early stages of a developing kernel glutenins are formed (Dupont & Altenbach 2003) that are deposited as protein bodies (Evers & Bechtel 1988). These proteins typically have a poor water-solubility. The GMP extraction method separates glutenin from other proteins, enabling observation of glutenin particles with CSLM. The HMW-GS are important in glutenin formation (Kasarda 1999, Graveland 1987). For glutenin particles this is further shown in this thesis by using a set of deletion lines from Olympic x Gabo (chapter 6). The HMW-GS affect the presence of glutenin particles (CSLM) and size (CSLM, viscometry and Coulter Laser). Results for 5+10 and 2+12 lines Warigal and Lance (chapter 7) confirm that the presence of HMW-GS 5+10 results in an increased glutenin particle size.

In a previous section we discussed the swelling capacity of glutenin particles in SDS dispersion. Can the HMW-GS affect the particles in such a manner that swelling properties / solubility properties are different? Based on current data this is difficult to prove, but it is known that composition and internal cross-links (disulphide bridges in case of glutenin) affect swelling capacity

(Flory, 1953). Since the presence of HMWGS 2+12 or 5+10 leads to a different network in terms of number of cross-links it is not unlikely that this will also lead to a difference in the extent of swelling. In this respect it is important to note the observations in Chapter 2 where the behaviour of different glutenin particles was studied as a function of dilution. If particles would only be stabilized by physical interactions, a higher dilution would lead to more solubilization. This is clearly observed with '2+12'-type particles, but not by '5+10' type particles. The latter particles are presumably more internally cross-linked allowing them to swell to a larger extent without dissolving. An interesting effect of heat stress is shown in chapter 7. Heat stress lowers glutenin quantity but increases glutenin particle size. This may be an effect of disulphide bonds, because according Rhazi et al. 2003 the formation of disulphide bonds is related to heat. The evidence presented here strengthens the idea that glutenin particles are formed in the immature wheat endosperm. As more deletion lines become available the role of HMW-GS, LMW-GS and growing conditions in glutenin particle formation and properties will be pinpointed more precisely. Also, as larger quantities of flour from deletion lines can be obtained, to back up these findings with more dough rheological and bread-making tests. Recent publications by other authors on this subject (He et al. 2005; Bedo et al. 2005), agree with the importance of glutenin particles in understanding factors affecting flour, dough and bread-making quality.

8.2.5   *Input parameter vs. output parameter*

Clearly the findings discussed support the concept of a particle network as proposed by Lefebvre and Van Vliet (2003). If we make an effort to explain dough rheological properties on the basis of particle properties, what parameters are important and by what factors are they in turn determined? In this respect it is important to distinguish input and output parameters. HMWGS, LMWGS composition, their content, the relative amount of gliadin vs glutenin etc. are clearly input parameters. The amount of GMP and particle properties are output parameters, the same holds for the final dough rheological parameters. In this thesis a number of relations between input and output parameters were found. These are listed below:

- HMW-GS ~ HMW/LMW -> chapters 6 and 7
- HMW/LMW ~ glutenin particle size
- HMW/LMW ~ glutenin particle K'
- HMW/LMW ~ SDS insoluble glutenin in flour

- HMW/LMW ~ dough $t_{1/2}$

Using HMW/LMW as input parameter, we can observe that this controls the glutenin network properties on: the glutenin particle level, the aggregation level, the GMP level and the dough level (figure 1.1 in Chapter 1).

The glutenin particle clearly is an important intermediate that helps understand the relations between molecular and macroscopic properties. Shape, K' and glutenin quantity / average glutenin particle size ratio, play a role in the physical interactions. A challenge for the future is to get a better grip on glutenin particle shape. Also the specific type of interactions (H-bond, hydrophobic, ionic) needs to be understood in relation to particle composition and structure. This may be a difficult task as glutenin particles are typically heterogeneous both in size and composition.

8.3   Relations between observations and HA-model

8.3.1 Glutenin particle origin and the HA-model

Various findings have demonstrated that glutenin particles exist. How do these results fit in the concept of hyperaggregation? Rhazi et al. (2003) explain the formation of GMP in wheat as a process of hyper-aggregation. Figure 8.2 depicts the different steps of hyper-aggregation for the formation of glutenin.
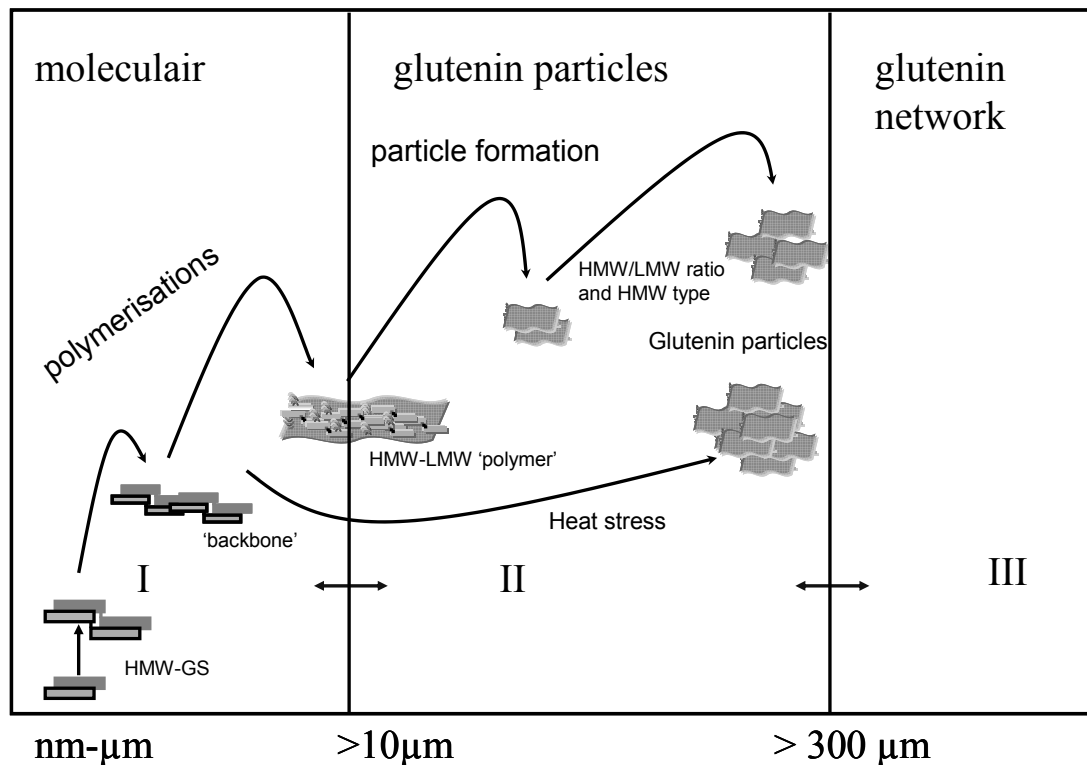
Figure 8.2   Schematic of the hyper-aggregation model for glutenin particle formation in developing wheat kernel

At level I subunits are synthesized in the ER that will almost directly form oligomers. Our results fit best with a model in which HMW-GS form a backbone to which LMW-GS clusters are attached. These oligomers are stored in protein storage vacuoles that 'bud off' from the ER (Evers and Bechtel 1988). Due to the higher concentration, a level II type aggregation will take place, leading to the formation of larger structures stabilized by additional SS bonds. Only at kernel maturation, presumably due to a further increase in concentration (dehydration) and oxidation further covalent and non-covalent aggregation occurs. The sizes of these structures are on the border of level I and level II: polymer and particle behavior coincide. However, as the grain matures, aggregation proceeds from level II>III. The glutenin particles increase in size. The glutenin particles formed swell in SDS but retain the spherical appearance of the original protein body. HMW-GS are required to form these particles and the particle size increases with increasing HMW/LMW ratio. This is clearly found in chapter 6. In chapter 7 the 2+12 have a lower HMW/LMW than the 5+10

lines; the latter has larger final glutenin particle size. Therefore we assume that the glutenin particle HMW/LMW ratio is mediated by specific HMW-GS.

With severe heat stress this process can be changed. Growing at high temperatures (30-40 ´C) results in less glutenin, but level I and level II aggregation go very fast, taking an abnormal course, leading to fusion of particles. Dupont and Altenbach (2003) and Ovidio and Masci (2004) reviewed that LMW-GS also affect glutenin properties. Due to the materials used in this thesis, apart from the effect of HMW/LMW ratio on glutenin particle size, this aspect was not investigated.

8.3.2 From particles in flour to function

In figure 8.3 the framework of the HA-model is shown for mixing, resting and glutenin network formation.

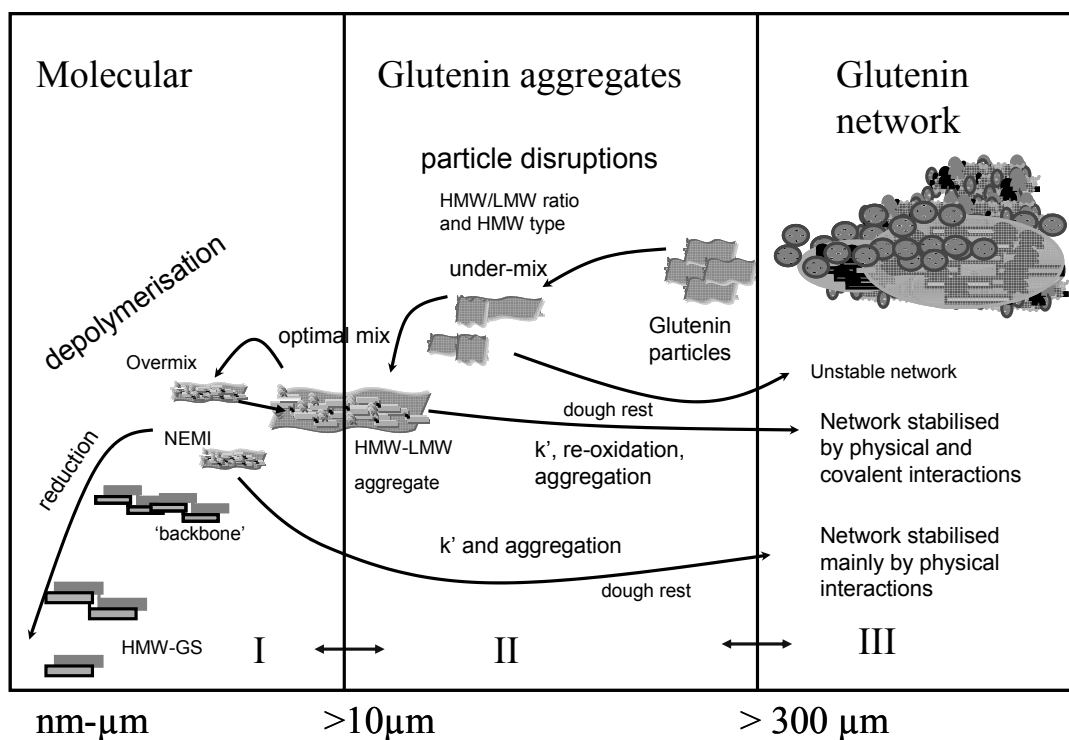*Figure 8.3   Schematic of the hyper-aggregation model for relating dough processing with glutenin particles*

When dough is mixed, the glutenin particles are disrupted. In a under-mixed stage the fragments are still large and have low K'. Furthermore, the proteins are not well distributed at this stage. The network formed during dough rest is inhomogeneous and unstable resulting in a low elasticity. At

the optimal development stage the glutenin particle has reached a voluminosity of 0.09 L/g, K' is optimal as well at this stage. This appears the most appropriate initial size and K' for re-aggregation into an elastic network. Physical aggregation and re-oxidation control network formation. The effect of re-oxidation became apparent from the over-mixing part of the systematic mixing study in chapter 4. Over-mixing changes particle composition because HMW/LMW is lower. This results in a different type of aggregate at the start of re-aggregation during dough rest. The re-aggregation clearly involves oxidation and physical aggregation steps as outlined in the hyper aggregation model. The importance of physical aggregation is also reflected in the systematic mixing study with NEMI. This resembles the over-mixing situation, but size and K' are different. Therefore another starting particle is sketched in figure 8.3 The re-oxidation is blocked in the presence of NEMI but as K' seems optimal these NEMI treated particles can still form a network, mainly through interaction at level II>III of the hyper- aggregation model. It is clear that the HA-model helps with the understanding of the phenomena observed. It is however realized that oxidation and K' are just part of the whole range of interactions. The K' is governed by internal particle composition and particle shape (effective volume). More factors that affect K' should be unraveled to understand the scope of the physical interactions reflected in the K' values.

8.4 Final conclusions and impact

The scope of this study was to determine the properties of isolated glutenins and assess its structure - functionality relationships during processing. The results discussed support the H.A. model and the findings on glutenin particle properties already have a broad impact in wheat production and applications. The glutenin particle size distribution requires endosperm material of just ~5 mature or nearly mature grains. Breeders can now effectively use the knowledge about the most important factors that control glutenin particle formation, size and network forming potential. Thereby more stable lines can be developed and environmental effects on end-use quality can be predicted.

Based on the particle size concept, early selection strategies can be set-up, since the particle size predicts mixing properties and baking potential. Furthermore, glutenin particle size information can be used by millers in new approaches for blending.

Processing properties and glutenin particles are clearly related. The particle disruption concept together with optimal K' provided:

- A set-up to improve gluten-starch separation processing.
- Application of process tolerance of particles in simple shear (Peighambardoust (2005).

- The concept of K' and glutenin particle size proved very useful in explaining the effect of pentosans on gluten agglomeration (Wang et al., 2004).

- Barley processing can benefit by using the mechanical disruption concept on particulate barley proteins (Patent Application, Malt Modification for Brewing, PCT/NL08/00675, 2003).

- The action of enzymes on dough properties could be better explained ((Primo et al., 2005))

These applications confirm that particulate protein characteristics are key to better understand various processes in which cereals are used.

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SUMMARY

Summary

Wheat is an important agricultural commodity. The products made from wheat and wheat flour are part of our daily menu. From wheat flour a leavened product like bread can be prepared. Mixing of flour, water and salt results in a visco-elastic mass called: dough. Only with flour from wheat the unique gasholding consistency is achieved. Upon further mixing the dough feels more homogeneous and elastic. It is commonly known that the proteins of wheat are responsible for the properties of wheat flour dough. The famous chemist stated the following on this:

"*Of the protein substances used as food, none is of more importance than those contained in the seeds of wheat*"

The protein content of wheat and wheat flour is still an important factor in selection. However, next to protein content, also protein type and protein quality play a role. The dough consistency is followed on a so-called Farinograph, the result is a dough-mixing curve. The peak, also referred to as time-to-peak (TTP), is generally agreed as the moment of optimal gluten development. Processing requirements of flour, like: TTP, dough consistency and baking properties are related to gluten content and gluten quality. A lot of effort has been devoted to understand the unique functionality of wheat gluten and the gluten network. This brings us to the constituents of gluten: the network forming glutenins and the monomeric gliadins. Osborne defined the gliadins as the protein group soluble in 70% ethanol. Glutenins are poorly soluble, only in strong base these proteins (partially) dissolve. Various researchers have found, independently from each other, that the quantity of insoluble glutenin is a measure of dough and baking properties. The type of extraction solutions used to discern soluble from insoluble glutenin does not change the final conclusion: insoluble glutenin holds the key to explain wheat flour quality differences.

The Glutenin Macro Polymer is a glutenin fraction that is insoluble in 1.5% SDS. The quantity of this GMP-gel and its rheological stiffness are related to flour quality. The stiffnes of the GMP-gel is related to biological factors: variety, climate and soil. Glutenins consist of High Molecular Weight Glutenin Subunits (100 – 70kD) and Low Molecular Weight Glutenin Subunits (30-60kD). There are ca. 20 HMWGS known and classified. In short, the varieties with HMWGS 5+10 are breadmaking varieties , the feed of biscuit varieties contain HMWGS 2+12. Unraveling the glutenin structure is however difficult due to its highly insoluble nature. HMWGS and LMWGS can only be analysed after chemical reduction. It is agreed that glutenin consist of disulphide linked HMWGS and LMWGS. Various scientists have proposed a moleculair structure for glutenin, but even tody

there is no concensus about the structure of glutenin. This makes it difficult to find structure-function relationships.

Recently, it has been put forward that from physical and rheological points of view that the gluten network is a particle network. The functional properties of this particle network essentially rests upon glutenins. In this thesis it has been revealed that the Glutenin Macro Polymer consists of glutenin particles. The initial size of the glutenin particles determine the mixing energy required for optimal dough development. This provided a new insight: glutenin has particulate properties. Further following the paradigm of glutenin particles, it was discovered that mixing disrupts, downsizes and deforms the glutenin particles. This in turn affects the physical properties of the glutenin particles. This has been measured with particle size analyses and the Huggins constant. With optimal mixing the glutenin particles reach the same size, irrespective of variety. The Huggins constant however does differ among the varieties. This Huggins constant is a particle property that also affects the re-aggregation properties of glutenin particles during dough rest. The Huggins constant (K') is a new parameter for this area of cereal science, it typifies the particle-particle interactions. Glutenin particles with a higher K' after dough mixing re-aggregate into larger particles than particles with a lower K'. This difference in re-aggregation, affects the rheologcial properties of dough. As particles with higher K' associate to large glutenin structures, the dough elasticity increases. Of course, also the covalent interactions play a role (SH <-> SS), this is revealed by mixing with SH-blocker (NEMI). However, for the rheology of rested dough samples the K' appears to be important as well. The use of SH-blockers results in smaller re-aggregated glutenin structures in dough, but the rheology of the respective dough is the same as dough optimally mixed without SH-blocker. It appears that the K' could be a factor that helps interactions of smaller particles to form a network that gives the dough the same elasticity as a reference dough with larger glutenin particles. The glutenin particle composition is the intrinsic factor that governs the glutenin particle properties. For example the increase of K' with mixing has a relation with the HMW/LMW ratio.

The HMW/LMW ratio also affects the glutenin particles in flour. Especially with deletions lines (Olympic x Gabo) it can be observed how specific HMWGS affect the glutenin particle size. The particle properties revealed for the deletion lines show relationships with end-use quality. Climate affects the glutenin particles as well: heat stress increases the glutenin particle size, but glutenin quantity is decreased by heat stress. From the experiment with extreme heat stress it was learned

that HMW/LMW ratio was lowered by heat, but glutenin particles sizes were considerably larger than for normal growing conditions. Hence heat induces also an oxidative mechanism that affects aggregation of glutenin into large glutenin particles. New insights are hereby obtained for the formation of glutenin particles in wheat kernel endosperm. The experiments with the special deletion lines (Olympic x Gabo; Lance and Warigal) strengthen the idea that glutenin particles originate from protein bodies is wheat endosperm.

Finally, the results obtained fit with the framework of the hyper-aggregation model. The model has to be used separatedly on particles and dough. Mixing brings the particles from level III>II, to partly level I. Without SH-blocker there is re-oxidation, and via level II the particles aggregate towards macroscopic proportions (level III). With SH-blocker NEMI the K' seems to affect re-aggregation and the quality of the interactions in level II aggregation results in a dough that has comparable elasticity as a reference dough.

Formation of glutenin particles is mainly in levels I>II, resulting in large particles in II>III type aggregation. The size of the glutenin particle is determined by genetic factors, as well as aggregation factors. Especially the oxidative disulphide bond formation in level one appears to have a large effect on final glutenin particle size.

Summary

SAMENVATTING

De diverse producten uit tarwe vormen een belangrijk deel van ons dagelijks voedsel. Sinds mensenheugenis is het bekend dat tarwebloem eigenschappen heeft die uniek zijn. Precies daardoor is tarwebloem zo geschikt voor het maken (gerezen) brood. Wanneer je tarwebloem met water en zout kneedt, ontstaat er een plastische massa: deeg. Alleen met tarwebloem ontstaat deeg met een unieke consistentie. Bij doorkneden zul je merken dat het deeg homogener en elastischer aanvoelt. Het is bekend dat de eigenschappen van een tarwebloemdeeg m.n. bepaald worden door de eiwitten in de bloem. De beroemde chemicus Osborne verwoorde dit in 1902 –vrij vertaald - als volgt:

"De tarwe-eiwitten zijn de meest belangrijke ewitten voor de bereiding van ons dagelijks voedsel"

Het eiwitgehalte van tarwe en tarwebloem is nog altijd een belangrijk gegeven voor selectie. Naast eiwitgehalte, spelen eiwittype en eiwitkwaliteit een belangrijke rol. Tijdens een kneedproef, op een Farinograaf, wordt de deegconsistentie gevolgd in de tijd, met als resultaat een kneedcurve. De 'piek' in de kneedcurve is het moment van optimale gluten-ontwikkeling. Verwerkingseigenschappen van bloem zoals: optimale kneedtijd, deegconsistentie en bakeigenschappen, worden toegeschreven aan het gluten-gehalte en de glutenkwaliteit.

Veel werk is verricht aan het begrijpen van de unieke functionaliteit van gluten en het gluten-netwerk. Dit brengt ons bij: de netwerkvormende gluteninen en de monomere gliadinen, de complexe eiwittengroep waaruit gluten bestaat. Osborne definieerde gliadinen als de 70% ethanol-oplosbare fractie. Gluteninen zijn slecht oplosbaar, alleen in sterke loog lossen deze enigszins op. Diverse onderzoekers concludeerden onafhankelijk van elkaar, dat de hoeveelheid onoplosbaar glutenine, voorspellend is voor deeg - en bakeigenschappen. Het type extractiemiddel, om onoplosbaar glutenine te meten doet niets af aan hun eindconclusie: onoplosbaar glutenine bevat de sleutel tot het verklaren van kwaliteitsverschillen. Het Glutenine Macro Polymeer (GMP), is een glutenine-fractie onoplosbaar in 1.5% SDS. Zowel de hoeveelheid GMP is belangrijk als de stevigheid van het GMP-gel, een steviger gel duidt op een betere glutenine-kwaliteit. De gluteninekwaliteit is weer gerelateerd aan biologische factoren: zoals tarweras, klimaat en bodem. Glutenine bestaat uit Hoog Molgewicht Glutenine Subunits (100-70 kD) en Laag Molgewicht Glutenine Subunits (30-60kD). Er zijn ca. 20 HMWGS bekend, en geclassificeerd. Kortweg: tarwerassen met de zog. HMWGS 5+10 zijn 'bakrassen', de 'voer -'of 'biscuit -' rassen bevatten HMWGS 2+12.

Het ontrafelen van de gluteninestructuur is echter lastig door de slechte oplosbaarheid. HMWGS en LMWGS kunnen alleen geanalyseerd worden na reductie (verbreken) van disulfidebruggen. De glutenine is opgebouwd uit door disulfidebruggen verbonden HMWGS en LMWGS. Diverse onderzoekers hebben een voorstel voor de structuur van het eiwit glutenine gepubliceerd. Er is echter geen consensus over de precieze moleculaire structuur van glutenine. Dit belemmert het vinden van structuur-functie relaties in ernstige mate. Recentelijk is vanuit fysisch en rheologisch oogpunt aangedragen dat het glutennetwerk feitelijk een deeltjesnetwerk is. De gluteninen hebben hier de belangrijkste dragende functie. Uit dit onderzoek blijkt nu dat GMP bestaat uit gluteninedeeltjes. De initiële grootte van de sferische deeltjes bepalen de kneedenergie benodigd voor optimale ontwikkeling. Dit levert al een belangrijk nieuw inzicht, glutenine heeft een deeltjeskarakter. Doorgaand op dit thema dat GMP-gel is opgebouwd uit deeltjes, geeft aan dat kneden de gluteninedeeltjes verkleint en vervormt. Dit heeft zijn effect op de fysische eigenschappen van de deeltjes. Dit is zichtbaar aan de deeltjesgrootteverdelingen en een verhoogde Huggins-constante van de gluteninedeeltjes. Door optimaal kneden krijgen deeltjes dezelfde gemiddelde grootte, onafhankelijk van ras/soort tarwebloem. De Hugginsconstante verschilt echter wel per bloemtype. Deze Hugginsconstante blijkt effect te hebben op de re-aggregatie van gluteninedeeltjes tijdens de deegrust. De Huggins constante is een, voor dit type onderzoek, nieuwe meetbare parameter die mogelijk licht werpt op deeltjesinteracties. De deeltjes met een hoge K' direct na kneden, aggregeren tot grotere deeltjes tijdens rust, dan deeltjes met een lagere K'. Dit heeft weer effect op de elasticiteit van het deeg. Deeltjes met hogere K' aggregeren tot grotere gluteninestructuren die op hun beurt weer zorgen voor een hogere deegelasticiteit. Uiteraard spelen nog altijd covalente interacties (SH <-> SS) een rol, dit blijkt uit kneden met SH-blocker (NEMI). Voor de deegrheologie tijdens rust blijkt K' echter ook belangrijk, met kleinere deeltjes wordt een netwerk gevormd dat nog altijd even elastisch is als bij deeg zonder NEMI. De kwaliteit van de deeltjesinteracties lijkt dus van belang, maar ook de grootte van de gluteninedeeltjes.

De intrinsieke factor is de HMW/LMW ratio van de deeltjes, deze bepaalt in sterke mate de hoogte van K' van de deeltjes na kneden. De HMW/LMW ratio heeft ook effect op de gluteninedeeltjes in bloem. Vooral bij gebruikmaking van lijnen met deleties op HMWGS (Olympic x Gabo) zien we het effect van HMW-GS en HMW/LMW op grootte van de gluteninedeeltjes in bloem. Vooral HMW-GS 5+10 blijken effect te hebben op de grootte van de gluteninedeeltjes in het GMP. De deletie van HMW-GS 5+10 leidt tot kleinere glutenine-deeltjes in het GMP. Verder kunnen de

deeltjeseigenschappen in verband gebracht worden met verwerkingseigenschappen van deze deletielijnen. Zoals kneedtijd en rheologie van het deeg.

Het effect van klimaat: hittestress, heeft ook een effect op de gluteninedeeltjes. Belangrijke vondst is dat hittestress de gluteninedeeltjes vergroot, terwijl de hoeveelheid glutenine aanzienlijk lager wordt. Een nieuw inzicht op de vorming van deeltjes is hiermee verkregen. Op het niveau van de gluteninesamenstelling (HMW/LMW ratio) blijkt dat bij extreme hittestress de gluteninedeeltjes een lagere HMW/LMW ratio hebben, maar aanzienlijk groter zijn. Dit terwijl onder normale groeicondities grotere gluteninedeeltjes samengaat met hogere HMW/LMW ratio. Het verbeteren van het inzicht in de condities die de vorming gluteninedeeltjes beinvloed, geeft op diverse manieren een doorkijk naar tarwekwaliteit. De uitgevoerde experimenten met speciale lijnen (Olympic x Gabo; Lance & Warigal) versterken het beeld dat gluteninedeeltjes oorspronkelijk als zog. 'eiwitlichamen' gevormd worden in het endosperm van de tarwekorrel.

Tenslotte blijken de gevonden resultaten vrij goed te passen in het hyper-aggregatiemodel. Het model moet dan wel apart op: deeg en gluteninedeeltje behandeld worden. Het kneden brengt de glutenine-particle van level II, deels naar level I. Zonder NEMI, is er enige re-oxidatie van SH-> S-S en via level II aggregeert het deeltjesnetwerk door naar macroscopische level III. Met NEMI lijkt de K' de kwaliteit van de interacties te beïnvloeden in level II aggregatie, het netwerk is in level III nog altijd dusdanig dat de deegelasticiteit nagenoeg gelijk is aan die van degen zonder NEMI.

De vorming van het glutenineparticle past met name in level I en II van het hyperaggregatiemodel. HMWGS 5+10 hebben hier duidelijk invloed op de grootte van het glutenineparticle. Maar ook de oxidatieprocessen die leiden tot meer disulfidebruggen lijken een effect te hebben op de grootte van de gluteninedeeltjes die uit bloem te isoleren zijn.

DANKWOORD

Goed, het is gedaan. Maar 'het' is natuurlijk al lang geleden begonnen. Nee, niet het werk voor dit proefschrift! Met 'het' bedoel ik, het onderzoek aan het mooiste eiwit ter wereld. Gluten, en dan weer in het bijzonder de glutenine. Laten we dus in 2005 niet vergeten, dat ook dit werk voortbouwt op een lange traditie bij TNO. Mede door deze traditie in de granen was het niet vreemd dat er een hoogleraar in de technologie van graaneiwitten uit de gelederen van TNO opstond. Ik was al een paar jaar werkzaam bij TNO in de zogenaamde Eiwitafdeling van Martin Hessing, en ik zag het voormalig divisiehoofd Rob Hamer zijn inaugurele rede geven in de Aula van Wageningen. Ik herinnerde mij het korte gesprek met Rob Hamer tijdens de sollicitatie nog wel, en zelfs toen in 1997 kwam het onderwerp 'promoveren' ter sprake. Ik was echter alweer enige jaren aan het werk en met mijn voorliefde voor technologische applicaties hield ik mij verre van 'academisch geneuzel'. Ware het niet dat in mijn werkomgeving twee heren: August Bekkers en Wim Lichtendonk mij halstarrig meenamen in de wereld van gluten en GMP. En als je een keer aan gluten zit, dan blijf je eraan plakken. Die plakkerigheid van gluten is mij nog wel een paar keer getoond door Han Marseille. Dan was er in het prille begin bij TNO nog de vriendelijke Pieter Bosveld met wie ik meereed. Meer en meer hoorde ik de verhalen over gluten en tarwe, en als je even de literatuur in dook vond je deze namen terug samen met andere oud-TNO-ers. De tijd was nog niet rijp voor gluten, want eerst begon ik bij nestor Gerrit Wijngaards. Ik vestigde mijn aandacht op eiwitten en technologische eiwit-functionaliteit. Een zeer leerzame periode, die ik maar kort 'de surimitijd' zal noemen. De aanpak via modellen en rheologische metingen leverde veel inzicht op in de eigenschappen van diverse dierlijke en plantaardige eiwitten, toch door nabije collega's bleef gluten naar mij lonken. Met een hoogleraar aan de WUR en een TNO afdeling met eiwitkennis, moest het toch mogelijk zijn een promotie te doen? En ja in 2001 startte 'de promotieconstructie' zoals die kort werd genoemd. Als niemand begrijpt wat ik bedoel met de 'promotieconstructie' dan is dat prima. Ik denk dat het het doel belangrijker was, dan het hoe.

Mogelijk is het bovenstaande een vreemd begin van het dankwoord, maar ergens is dit toch een weergave van hoe het ooit begon. Het mag niet onvermeld blijven dat inspiratie, vasthoudendheid en traditie de elementen zijn geweest naar het startpunt toe. Na dit startpunt ging de tocht verder, maar misschien wel wat eenzamer. Een paar jaar flink ploeteren om te meten, want normaal gesproken: 'meten = weten'. En om Wilbert Oostrom te citeren, niet zomaar meten: goed meten. Een waar woord en zonder de gevraagde en ongevraagde hulp van o.a.: Toos Gröneveld, Anne v/d Pijpekamp, Wim Lichtendonk, Jerry van Maanen, Albert Jurgens, Martin Bos, Cees Heddes, Han Marseille, Piet Sluimer, Bertus Dunnewind, Therese Maarschalkerweerd, Franklin Zoet en Jan Klok

was dat niet gelukt. Na het meten, interpreteren, discussies en manuscripten maken voor publicatie. Natuurlijk met promotoren, maar ook met Ton van Vliet die een paar moeilijke manuscipten voor publicatie heeft helpen stroomlijnen. Verder natuurlijk de vrienden van de glutenclub: Hadi, Cristina, Eva, Neeleke en Teun. Helaas kon ik steeds minder erbij zijn, het samen bespreken van resultaten is leuk, en daarbij was te zien dat het glutenin particle doorgaat, ook in andere gebieden. Ondanks de vele veranderingen in mijn directe werkomgeving hebben alle afdelingshoofden tot aan de huidige: Carlien Krist en Edwin van Unen de 'promotieconstructie' gesteund. Daarvoor ben ik zeer dankbaar, de voorbije jaren waren we als IIP niet altijd in rustig vaarwater. Heel veel geluk heb ik gehad dat ik tussen zulke goede collega's bij IIP het werk heb kunnen volbrengen, want nooit is er negatief gedaan over mijn promotie. Iedereen heeft het altijd in een positief licht gezien en gunde mij de kans om mijzelf de verbeteren, dank daarvoor!. Het zal moeilijk zijn, want met iedereen binnen IIP heb ik wel interactie gehad, maar goed, ik noem toch nog een paar namen van de 'Noorderlingen', want ook uit Groningen kwamen goede tips van bijvoorbeeld: Peter Steeneken, Harold Helmens en Albert Woortman. Wie weet, ligt na eiwitten de toekomst in de zetmeel?
Na nog een dank aan alle collega's, oud-collega's van TNO, van ATO-DLO en WCFS, maak ik toch even ruimte hieronder om dank te betuigen aan andere mensen uit mijn privéleven.

Mijn vrienden van de HEMP (Heteromannenpraatgroep): Ruud, Willem, Niels, Rob, Arthur, Erik en Sander, de wekelijkse kijk op het leven en bespiegelingen waren waardevol. Ik hoop dat we nog veel meer HEMP-avonden zullen hebben. Het is uitgegroeit tot iets unieks.

Mijn vrienden van QUAST of eigenlijk oud-QUAST(?), tja de tijd in Delft is alweer jaren geleden, toch ons college is nu een doctor rijker. Al zijn de ontmoetingen schaarser, jullie tomeloze vertrouwen in mijn kunnen heb ik als verfrissend ervaren. Het gaf mij dikwijls weer 'de spirit' om weer die stap verder te gaan. Is de promotie in december van lustrumjaar 2005 toeval? Ik weet het niet, maar het maakt alles weer net een stukje feestelijker!

Mijn moeder en mijn lieve familie, bedankt voor de interesse en onvoorwaardelijke steun door alle jaren heen.

Lieve Lotje, mijn levenspartner, jij nog het meest bedankt voor alle hulp.

Dankwoord

Dankwoord

CURRICULUM VITAE

Clyde Don werd op 16 juni 1968 geboren te Welland (Canada). In 1987 behaalde hij het VWO diploma aan de Dalton SG te Den Haag. In 1992 haalde hij zijn HBO diploma tot Chemisch Technoloog (Ing.) aan de Haagse Hogeschool. In 1994 werd het doctoraal diploma in de Scheikunde (Drs.), met specialisatie Chemische Technologie, behaald aan de Universiteit van Amsterdam. Het universitaire afstudeerwerk in de bio-proceskunde groep van Prof. Ir. Simon Ottengraf wekte bij hem interesse voor zowel de applicatie als de onderzoekskant van de chemie en chemische technologie. De geliefde combinatie van onderzoek en applicaties trof hij aan bij zijn eerste baan, bij het ATO-DLO in Wageningen bij de afdeling Industriële Eiwitten. In de periode 1995 – 1997 heeft hij daar als contractonderzoeker gewerkt aan diverse onderwerpen te weten: eiwitfilms en coatings, glutenlijm en extrusie. In 1997 startte hij als junior projectleider bij TNO te Zeist, in de Afdeling Eiwittechnologie. Technische functionaliteit van eiwitingrediënten en eiwitcomponenten: 'van dierlijk tot plantaardig' in applicaties, is in de jaren bij TNO uitgegroeid tot zijn specialiteit. Vanaf 2001 werd ten dele tijd vrijgemaakt om bij de leerstoel van Prof. Dr. Rob Hamer, een promotieonderzoek te doen aan het tarwe-eiwit genaamd GMP (Glutenine Macro Polymeer). Het resultaat is dit proefschrift. Anno 2005 werkt Clyde nog altijd met plezier bij TNO in de afdeling Innovatieve Ingrediënten en Producten.

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