Protein Quality in Wheat:

Changes in Protein Polymer Composition during Grain Development and Dough Processing

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This Thesis is dedicated to the memory of my father, Stasiui Kuktai.

Everything should be as simple as possible, but not simpler.

Einstein

Abstract

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The overall objective of the present studies was to increase the understanding about protein quality in wheat, *i.e.* to make it possible to improve the production of wheat with desired quality for different end-uses. Studies of changes in protein polymer composition during grain development and dough processing were used as one track to investigate the protein quality in the wheat material.

Accumulation and build-up of the proteins in developing wheat grain was found to be a predetermined process and independent of variation in temperature and nitrogen regimes. For determination of amount and size distribution of polymeric proteins, the length of maturation time together with availability of nutrients during the latter half of the grain development are of highest importance.

By the ultracentrifugation of dough specific low molecular weight (LMW) glutenins were found only in the gluten phase, indicating the importance of these proteins for bread-making. Also, specific water soluble proteins were found in the gluten phase.

The genetically and environmentally determined amount and size distribution of polymeric proteins form the basis for the build-up (cross-linking) of protein polymers during dough formation. The omega-gliadins interact with the glutenins during dough processing through non-covalent interactions.

Prolonged mixing time caused an increase in the water content of gluten, changed the rheological properties of dough and made the gluten surface smoother. The storage modulus (G', representing the gluten network density) was uninfluenced by prolonged mixing. Overmixing (from optimum to overmixing) had a smaller effect on strong flour compared to weaker flours.

Differences in extractability behaviour between the SDS-extractable and –unextractable proteins indicated a more branched and complex structure in the SDS-unextractable protein. Omega gliadins showed a tight link with the glutenins in the gluten macropolymer.

The use of proteomics, for investigating the whole protein composition and structure of the gluten polymer, are still rather limiting. One possible use of this technique is to investigate presence of disulphide bonds through the comparison of digested and digested+reduced samples.

Taken together, the results presented in this thesis provide new insights into polymeric gluten protein structure specificity and changes from grain development to dough processing in general.

Keywords: wheat, proteins, composition, gluten polymer structure, bread-making quality.

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Appendix

Papers I-V

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Johansson, E., Kuktaite, R., Andersson, A., Prieto-Linde, M., 2004. Protein polymer built-up during wheat grain development: influences of temperature and nitrogen timing. Journal of the Science of Food and Agriculture (in press).

II. Kuktaite, R., Larsson, H., Johansson, E., 2003. Protein composition in different phases obtained by the centrifugation of dough. Acta Agronomica Hungarica, 51(2), 163-172.

III. Kuktaite, R., Larsson, H., Johansson, E., 2004. Variation in protein composition and its relationship to dough mixing behaviour in wheat flour. Journal of Cereal Science, 40(1), 31-39.

IV. Kuktaite, R., Larsson, H., Marttila, S., Johansson, E. Effect of mixing time on gluten recovered by ultracentrifugation studied by microscopy and rheological measurements. (submitted)

V. Kuktaite, R., Di Giorgi, S., Garozzo, D., Johansson, E., 2004. Differences in composition of SDS-extractable and SDS-unextractable gluten protein: possibilities using SDS-PAGE, RP-HPLC and Proteome. (manuscript)

Papers were reprinted with kind permission from Journal of the Science of Food and Agriculture (Paper I), Acta Agronomica Hungarica (Paper II) and Journal of Cereal Science (Paper III).

Introduction

Similarly as in many other parts of the world, wheat is one of the major cultivated crops in Southern Sweden (Jordbruksverket, 2004). Wheat, barley and oats have the largest production volume and a wider food application compared to many of the other cereals (Jordbruksverket, JO 10 SM 0401, 2004). During the last 15 years the Swedish wheat production has been between 1.5 and 2.5 million tons/year. During the same period the yearly export has varied between 504 000 and 750 000 tones and the import has changed between 50 000 to 80 000 tones/year. Human consumption of wheat in Sweden reaches around 1/3 of the Swedish produced wheat (FAO data base, 2004).

Wheat is used for many different purposes and in Sweden the largest part is used for feed (Jordbruksverket, 2004). According to wheat food applications, bread-manufacturing is still the most essential use of wheat in Southern Sweden (Belderok *et al.*, 2000). For bread production, the right quality is important and thus, bread-making quality surveys are of fundamental nature.

Both the type of wheat cultivar and the climatic and other conditions differing between cultivations, influences the quality characteristics of wheat, in Sweden and also in other countries such as U.K. (Kettlewell *et al.*, 2003), Germany (http://www.bsb.org.uk/members/library/conferences/2000autumn/paper_442.htm) and Australia (http://www.seedquest.com/News/releases/2004/october/10168.htm). The large proportion of Swedish wheat, produced as feed, do not fulfil the quality requirements outlined by the millers and bakers for the manufacturing of bread. However, sometimes also the bread wheat has problems to fulfil the bread quality criteria. The characteristics that determine the suitability of wheat for the bread production are: the milling properties, the dough properties and the bread properties. The proportion of wheat imported to Sweden, is often used as high quality wheat with the intention to strengthen the dough properties within the Swedish bread production. The desire to better understand the reasons and background of variations in bread-making quality is beyond the scope of this thesis.

Wheat fields in Alnarp, original picture by *R. Kuktaite.*

1. Wheat

The earliest known grains of domesticated wheat dates from approximately 7500 – 6500 B.C. Between 6000 and 5000 B.C. hexaploid wheat (*T. aestivum)* and cultivated emmer wheat (*T. timopheevi var. araraticum* and *T. turgidum var. dicoccoides*) penetrated into the irrigated agriculture of Mesopotamia and western Iran (Feldman, 1976). In Sweden, wheat has been grown since 4000 B.C., *i.e.* since the Neolithic period (Jensen, 1991).

As well as other cereals, such as rye and barley, wheat belong to the tribe *Triticeae*. This is one of the largest and most important tribes in the grass *Poaceae* family (Dewey, 1984). The number of genera in the tribe *Triticeae* varies considerably between different classifications (Löve, 1984; Stebbins, 1956) although, the five and same genera, *Aegilops*, *Elymus*, *Hordeum*, *Secale* and *Triticum* are included in the *Triticeae,* independent of classification system.

The basic chromosome number is x = 7 for *Triticum* and all the species in the tribe. The wild species are diploids (2n = 2x = 14), *e.g.* with genome designation AA (*Triticum monococcum*), DD (*T. tauschii*) and SS (*T. speltoides*), or tetraploids (2n = 4x = 28), *e.g.* with the genomes AABB (*e.g. T. turgidum*) or AAGG (*T. timopheevii*) (Feldman, 1976). The most common cultivated hexaploid wheat nowadays is *T. aestivum*, AABBDD, (2n = 6x = 42) (Zohary and Hopf, 1993).

2. Bread-making quality

The application of wheat flour is broad and it is widely used for bread, pasta and noodles, breakfast cereals and fermented drink. Other applications are the starch and gluten industry. Bread-making quality is both an important and complex character of bread wheat (*T. aestivum*) (Pomeranz, 1988). First of all, the consumers' demands, the bread-making technology (MacRitchie, 1984; Mesdag, 1985) and the cultural traditions differ largely between the countries. Also the bread-making quality tests of flour are not easy to repeat, because baking tests depend on the baker and technological differences of the process (MacRitchie, 1984). Therefore, it is complicated to formulate universally applicable criteria for bread-making quality. Traditionally, the volume of the bread loaf has been considered as the most important criterion for produced bread (MacRitchie, 1984). The desirable rheological characteristics of dough (stickiness, mixing requirement, water absorbance) together with the colour and appearance of loaf are of importance as well (Finney *et al.*, 1987).

The balance and interaction between different flour components as proteins, starch, lipids, water, pentosans etc., are of high importance for the bread-making quality.

Starch

Starch is the main component in the carbohydrate fraction accounting for 65-75% of the grain dry weight. A series of enzymes synthesize the amylase and amylopectin chains that comprise starch. Differences in wheat starch particle size

distribution and amount of damaged starch granules has been shown to be important for the baking performance (Hoseney *et al.*, 1971). The shape and size of granules vary with species and cultivar origin (Soulaka and Morrison, 1985). The starch damage depends mainly on the milling conditions and the grain hardness (Bass, 1988). However, the differences in protein concentration have stronger influence on bread-making quality than the starch granule size (Lelievre *et al.*, 1987). In baking technology starch is mostly associated with staling, the processes that are responsible for the aging of bread.

Lipids

The wheat lipids constitute about 1-2% of the total flour weight. Despite the small amounts, lipids affect bread-making quality (Morrison, 1988). One part of the lipids is bound (not covalently) to starch, *i.e.* the starch lipids, in the amylose-lipid complex (Morrison, 1988). Another part has interactions with the flour proteins (MacRitchie *et al.*, 1990). Both mentioned connections are important for bread-making quality (Frazier *et al.*, 1981). Based on their ability to interact with water, wheat flour lipids can be classified into a polar (glycolipids and phospholipids) and non-polar lipid fraction (mainly triglycerides) (Eliasson and Larsson, 1993). The polar lipids have positive effects in baking whilst non-polar lipids distinctly deleterious effects (MacRitchie, 1984).

Proteins

The protein fraction is known to play the most essential role for bread-making (Morrison, 1988; Wall, 1979). Bread-making quality correlates with the presence or absence of specific proteins and protein subunits (Gupta *et al.*, 1989; Johansson, 1996; Johansson *et al.*, 1993; Payne *et al.*, 1987a). In addition, the quality depends on the ratio of monomeric to polymeric proteins (Sapirstein and Fu, 1998) and amount and size distribution of polymeric proteins (Gupta *et al.*, 1993; Johansson *et al.*, 2001).

3. Wheat proteins

The ability of wheat flour to be processed into different foods is largely determined by the gluten proteins (Weegels *et al.*, 1996a). Therefore, the gluten proteins have been the subject of intensive studies for a period exceeding 250 years. This has revealed gluten proteins having unusual structures and properties, making them of special interest for studies as well as applied work about their functional properties (Shewry and Tatham, 2000).

All gluten proteins are synthesised on the endosplasmic reticulum (ER). They all contain a signal peptide, which through analogy with animal systems (Kreil, 1981), were found to direct the nascent chain into the lumen of ER (Grimwade *et al.*, 1996). Some wheat storage proteins appear to follow the secretory pathway from ER over Golgi to protein bodies and lose their integrity as the grain matures forming a protein matrix in the mature dry tissue (Parker, 1980). However, other proteins accumulate in the ER and are incorporated in vacuole-like compartments

that surround the protein bodies (Levanony *et al.*, 1992). Beside that, specifically for wheat, protein in the vacuole-like compartments is compressed between the starch granules (Levanony *et al.*, 1992; Shy *et al.*, 2001). The precise mechanism of intracellular transport of storage proteins from their site of synthesis to their site of deposition is still uncertain (Grimwade *et al.*, 1996). Protein folding together with both inter and intra-chain disulfide bond formation are considered to occur within the lumen of the ER, and may be assisted by molecular chaperones and by the enzyme protein disulfide isomerase (PDI) respectively (Roden *et al.*, 1982; Shimoni *et al.*, 1995).

Many attempts to reveal the structure of the gluten proteins have been carried out, although they have been troubled by the low solubility and lack of crystallinity of the proteins. The solubility properties of gluten proteins are determined by the primary structures of the individual proteins and their interactions by non-covalent forces (notably hydrogen bonds and hydrophobic interactions) (Belton *et al.*, 1998) and by covalent disulphide bonds (Shewry *et al.*, 2002). The whole protein structure is still far from being clear (Veraverbeke and Delcour, 2002).

Mature wheat grains contain 8-20% proteins. The gluten proteins, the gliadins and glutenins, constitute up to 80-85% of total flour protein, and confer properties of elasticity and extensibility that are essential for functionality of wheat flours (Shewry *et al.*, 1995). The gliadins and glutenins constitute each around 50% of the gluten proteins.

Osborne was the first to classify wheat grain proteins on the basis of their solubility: albumins (soluble in water), globulins (salt), gliadins (aqueous water) and glutenins (dilute acid or alkali) (Osborne, 1907). Due to findings of the Osborne fractions being heterogeneous and containing protein types overlapping each other, the methods of protein fractionation have been improved nowadays (MacRitchie *et al.*, 1990). The today used protein classifying system is based on biological characteristics of the proteins together with their chemical and genetic relationship, leading to different states of aggregation in dissociating solutions (Shewry and Tatham, 1990; Shewry *et al.*, 1986). Thus, gliadins are a mixture of monomeric polypeptides and glutenins consist of polypeptides aggregated by disulphide bonds (Sapirstein and Fu, 1998; Shewry and Tatham, 1990; Singh and MacRitchie, 2001a) (Fig. 1).

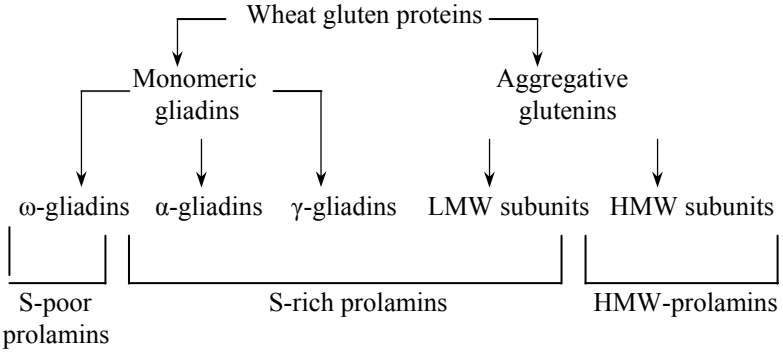

Figure 1. Traditional classification of gluten proteins (Shewry *et al.*, 1986; Shewry and Tatham, 1990).

Both types of wheat storage proteins, the gliadins and glutenins, are the main components, building the gluten polymer and determine bread-making properties (Branlard and Dardevet, 1985a, b).

Albumins and globulins

Apart from the gluten proteins, water-soluble albumins and salt-soluble globulins constitute from 10 to 22% of total flour protein (Singh and MacRitchie, 2001a). Albumins such as α-amylase/trypsin inhibitors (Buonocore *et al.*, 1985; Shewry *et al.*, 1984), serpins (Østergaard *et al.*, 2000) and purotionins (Garcia-Olmedo *et al.*, 2002) may have dual roles as nutrient reserves for the germinating embryo and as inhibitors of insects and fungal pathogens prior to germination. Puroindolines influence grain hardness (Morris, 2002). Generally, albumins and globulins are not thought to play a critical role in flour quality, although, minor importance on bread-making quality has been reported (Schofield and Booth, 1983). Both protein fractions are important from nutritional point, because of rather high amounts of essential amino acids.

Gliadins

The gliadins are divided into four groups, alpha- (α-), beta- (β-), gamma- (γ-), and omega- (ω-) gliadins, based on their electrophoretic mobility at low pH (Woychik *et al.*, 1961). More than 30 components are separated by two-dimensional (2D) electrophoresis (Friedli, 1996). The amino acid compositions of the α- , β- , γ- and ω- gliadins are similar to each other (Tatham *et al.*, 1990), although, the ω-gliadins contain little or no cysteine or methionine and only small amounts of basic amino acids. All gliadins are monomers with either no disulphide bonds (ω-gliadins) or intra- chain disulphide bonds (α-, β-, and γ- gliadins) (Müller and Wieser, 1995, 1997). The molecular weights of ω-gliadins are between 46,000 and 74,000, and the α-, β- and γ-gliadins have lower Mrs, ranging from 30,000 to 45,000 by SDS-PAGE and amino acid sequencing (Kasarda *et al.*, 1983). The latter approach has shown that the α- and β-gliadins are closely related and thereby they are often referred to as α-type gliadins. Most α- type gliadins contain six cysteine residues. Because of the monomeric character of α-type gliadins, and the absence of free sulphydryl groups, it has been assumed that the cysteine residues are linked by three intra-molecular disulphide bonds (Kasarda *et al.*, 1987).

The γ-type gliadins are single monomeric proteins with intra-chain disulphide bonds and are considered to be the ancestral type of the S-rich prolamins (Shewry *et al*., 1986). Complete amino acid sequences of several γ-gliadins have been deduced from genomic and cDNA sequences (Okita *et al.*, 1985; Scheets and Hedgcoth, 1988). These sequences showed a clear domain structure, with a non-repetitive sequence of 14 residues at the N-terminus, an N-terminal repetitive domain based on a heptapeptide repeat motif (consensus Pro Gln Gln Pro Phe Pro Gln) and a non-repetitive C-terminal domain which contained all the cysteine residues. Structural studies, using circular dichroism and structure prediction, indicated that the two domains adopt different conformations. While the repetitive

domain adopts a β-reverse turn rich conformation, the non-repetitive domain is rich in α-helix (Tatham *et al*., 1990).

In dough formation, the gliadins are thought not to become covalently-linked into large elastic networks as the glutenins but act as a 'plasticiser', promoting viscous flow and extensibility which are important rheological characteristics of dough. They may interact through hydrophobic interactions and hydrogen bonds (Belton, 1999).

Glutenins

The glutenin fraction is formed of a mixture of polymers, high-molecular-weight glutenin subunits (HMW-gs) and low-molecular-weight glutenin subunits (LMW-gs). The large glutenin polymers are stabilised by inter- chain disulphide bonds (Field *et al.*, 1983). The HMW-gs have molecular weight ranging from 80-160,000 Da and the LMW-gs weights are 30-51,000 using SDS-PAGE (Payne *et al.*, 1980). The HMW-gs account for about 5-10% of the total protein (Payne, 1986). The LMW-gs most closely resemble γ-gliadins in sequence (Müller *et al.*, 1998) and comprise about 20-30% of the total protein (Gupta *et al.*, 1992). Three to six HMW-gs (Margiotta *et al.*, 1996; Payne and Corfield, 1979) and 15-20 different LMW-gs proteins are recognised in 1 and 2D gels of hexaploid wheat (Lew *et al.*, 1992).

The HMW-gs consist of nonrepetitive domains of 88-104 and 42 residues at the N- and C-termini, respectively, separated by a longer repetitive domain (481-690 residues). Variation in the repetitive domain is responsible for most of the variation in the size of the whole protein, and it is based on random and interspersed repeats of hexapeptide and nonapeptide motifs, with tripeptides also present in x-type subunits only. Structure prediction indicated that the N- and C-terminal domains are predominantly α-helical, while the repetitive domains are rich in β-turns (Shewry *et al.*, 1989) (Fig. 2). Many partial and full-length sequences of HMW-gs and LMW-gs have been determined (Shewry and Tatham, 1997). Despite the high degree of similarity in general structures and amino acid sequences of x- and y- type HMW-gs, some important differences are potentially critical for the structure and functionality of glutenin polymers (Shewry *et al.*, 1992). There are three differences in: 1) molecular weight (x-type are bigger then y-type) due to a difference in length of the central repetitive domain; 2) the repeat structures in central domain; 3) the number and distribution of cysteine residues (Shewry *et al.*, 1992).

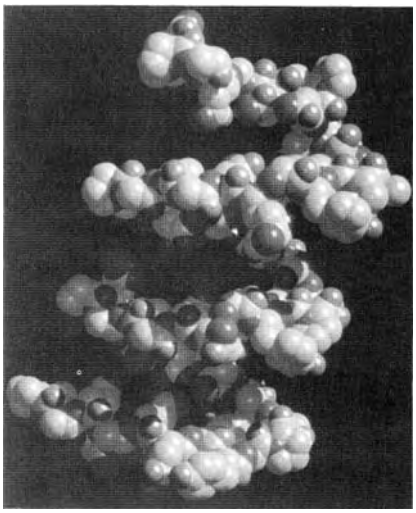

Figure 2. Molecular model developed for a β-spiral structure based on the amino-acid sequence of repetitive domain of a HMW subunit (Shewry *et al.*, 2002).

4. Gluten Polymer Structure

Several models for the structure of wheat gluten polymer have been proposed. According to earlier models, glutenin has only intra- chain disulphide bonds. The intra- chain disulphide bonds were thought to force glutenin molecules into specific conformations that facilitated interaction of adjacent glutenin molecules through non-covalent bonds, thereby causing aggregation (Kasarda *et al*., 1976).

Ewart (1979) proposed an alternative model in which the adjacent polypeptide chains of glutenins were thought to consist of linear polymers and two adjacent chains were connected to each other with one disulphide bond (Ewart, 1979). In this model, the rheological properties of dough are dependent on the presence of rheologically active disulphide bonds and thiol groups as well as on secondary forces in the concatenations (Ewart, 1979).

Another model was proposed by Khan and Bushuk (1979) in which the functional glutenin complexes contained both inter- and intra- chain disulphide bonds. On the basis of results from SDS-PAGE, they proposed an aggregate of two types of glutenin complexes, I and II. In their model, glutenin I comprised subunits of molecular weight 6.8×10^4 and lower, held together through hydrogen bonds and hydrophobic interactions: glutenin II comprised crosslinked subunits of molecular weights above 6.8×10^4, linked by inter- chain disulphide bonds.

More recent studies have shown that gluten protein polymers have a wide range of size distribution, ranging from dimers to polymers with molecular weights up to millions (M_rs possibly exceeding 1×10^7) (Larroque *et al.*, 1996; Wrigley, 1996) (Fig. 3). The proteins with the highest molecular weight are reported to have the strongest correlation with strong dough properties (MacRitchie, 1984). A certain amount of these polymers remains unextractable in various extracting systems (acetic acid solution or SDS phosphate buffer). The

%UPP (percentage unextractable polymeric protein in total polymeric protein) is often used as a measurement of the amount and size distribution of the polymeric protein (Gupta *et al*., 1992; Field *et al*., 1983). High %UPP values are related to a greater proportion of glutenin that is insoluble in SDS and for that reason are thought to be of the highest molecular weight (MacRitchie and Singh, 2004). Thus, wheat with a greater percentage of UPP are expected to have a greater dough resistance (elasticity) and a longer mixing requirement than those with a greater proportion of extractable polymeric protein (Gupta *et al.*, 1993).

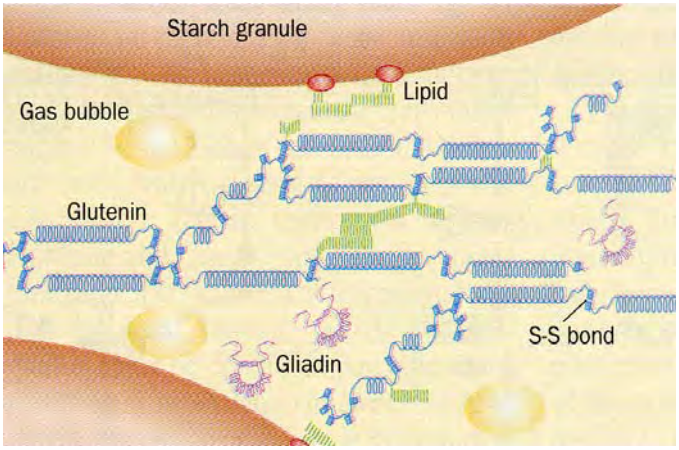

Figure 3. The disulphide-bonded polymeric structure of wheat glutenin: the protein matrix between the starch granules and gas bubbles in dough (Wrigley, 1996).

Today, both intra- (formed by gliadins and glutenins) and inter- (formed by glutenins) chain disulfide bonds, as well as non-covalent bonds are thought to be important in the formation of the gluten polymer complex (Fig. 3). Additionally, the polymeric proteins are stabilised by inter-chain hydrogen bonding (Belton, 1999).
Both x-type and y-type of HMW-gs have a typical three-domain structure consisting of relatively small N- and C- terminal domains flanking a major central domain (Fig. 4)

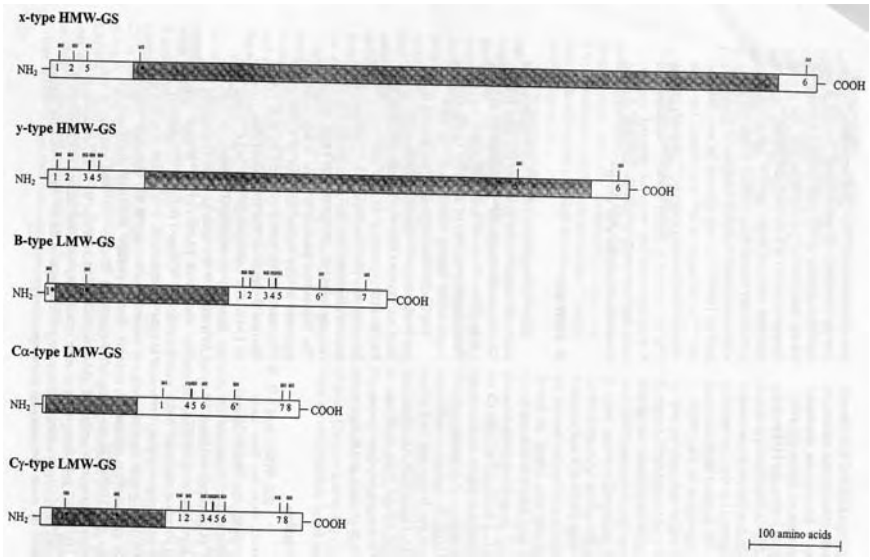

Figure 4. The structure of the main classes of wheat glutenin subunits (D-type and positions of the conserved (numbers) and unconserved (numbers with the prime) cysteine residues. *- unconserved cysteine in glutenin subunits (Veraverbeke and Delcour, 2002).

Differences in the disulfide bonding properties of gluten subunits impact their role in establishing gluten structure and function (Lindsay and Skerritt, 1999) (Fig. 5). The knowledge gained from polymer studies can be applied to understand dough behaviour (MacRitchie and Singh, 2004).

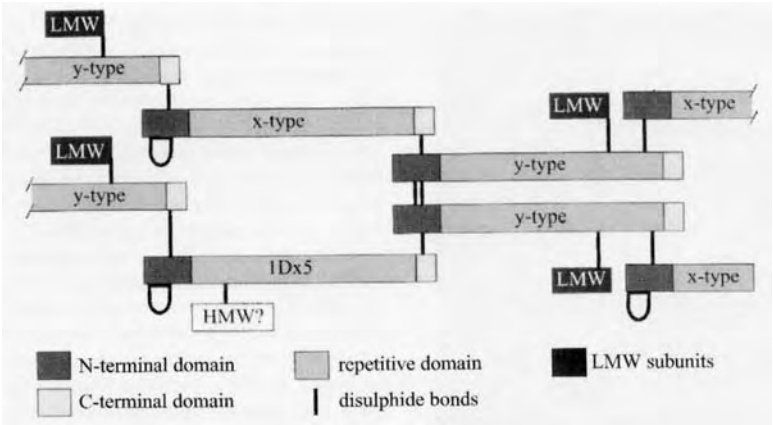

Figure 5. Schematic model of the structure of HMW subunit polymers, based on mapped disulphide bonds (Shewry *et al.*, 2002).

Unique properties of the gluten polymer

Segmental motion. Specific portions or segments of large molecules have been found to be the main reason for the activation energy of that molecule (Kauzmann and Eyring, 1940). To some extent, these segments of polymer molecules can move independently as kinetic units. The size of such protein segments are around 6-10 amino acid residues (MacRitchie, 1998).

Molecular weight distribution. Gliadins are single chain polypeptides with similar molecular weight, relatively to the glutenins forming polymers. The polymers are formed from glutenin subunits by a post-translational polymerisation. The polymers are present in wheat grains, flours and dough with a wide molecular weight distribution.

Entanglements. Rheological properties (viscosity) usually increase with increasing molecular weight. However, the viscosity increases more sharply, at a critical molecular weight (Bueche, 1962). The reasons for this are entanglements, widely spaced points along the chain with additional resistance to flow. The entaglements act as cross links and contribute to the strength of the gluten polymer (Singh and MacRitchie, 2001a).

Factors influencing solubility of gluten proteins

The difficulty in solubilising gluten proteins arises mainly from a lack of ionisable groups and the very high molecular weight of the glutenins (Singh and MacRitchie, 2001a). The proteins contain both polar and non-polar amino acid chains. The non-polar side chains contribute to the lack of solubility in aqueous solutions and this is referred to as the hydrophobic effect (Singh and MacRitchie, 2001a). Non-polar side chain frequency is a parameter that provides a measure of the hydrophobic effect. The non-polar side chain frequency is defined as the number of tryptophan, isoleucine, tyrosine, phenylalanine, proline, leucine and valine residues divided by the total number of residues. Another parameter influencing solubility is charged group frequency defined as the number of aspartic and glutamic acids, histidine, lysine and arginine expressed as a fraction of the total number of residues (Nelson and Cox, 2003).

The repetitive domains of the wheat gluten polymer molecules contain many hydrophilic glutamine residues that can interact with the solvent (water) or form intermolecular hydrogen bonds (β sheets), leading to nonentropic interactions. When the number of arrangements with solvent molecules decreases it leads to an increased insolubility of the gluten proteins due to a negative entropy change (Belton, 1999).

5. Factors Influencing Gluten Polymeric Protein Size Distribution

Four main protein compositional factors are governing the gluten polymeric protein size distribution:

1) the HMW/LMW-gs ratio (also the ratio of B-range to C-range LMW-gs, but to a lesser extent), (MacRitchie, 1999)

2) allelic variation at *Glu*-1 loci (*e.g.* the presence of HMW-gs 5+10 vs. 2+12), (MacRitchie, 1999)

3) the presence of chain terminators (ω-gliadins, which contain a single cysteine residue (MacRitchie, 1999), and α- and γ-LMW-gs (Masci *et al.*, 1995)).

4) the amount and allelic forms of LMW-gs (cysteine residues) (Consalvi *et al.*, 2004; Dachkevitch and Autran, 1989).

These factors influence the amount and size distribution of polymeric proteins *e.g.* the %UPP. In addition to the mentioned genotypic factors, also environment shows strong influence of the amount and size distribution of polymeric proteins (MacRitchie, 1999; Altenbach *et al.*, 2003; Johansson *et al.*, 2001, 2002).
The most important environmental conditions influencing the amount, composition and/or polymerization of the gluten proteins, are temperature and fertilizer (Johansson *et al.*, 2001, 2002, 2003, 2004; Müller *et al.*, 1998). Prior anthesis, environment affects mainly yield, through wheat grain germination, photosynthesis, tiller formation, and inflorescence development, thereby impacting grain number (Herzog, 1986). After anthesis, environmental conditions primarily affect kernel size, protein concentration and composition. Increases in grain protein content and gliadin to HMW-gs and LMW-gs ratios were observed with increased nitrogen fertilizer (Johansson *et al.*, 2001, 2003; Gupta *et al.*, 1992; Müller *et al.*, 1998). Timing of N application influences the %UPP (Johansson *et al.*, 2004). Temperature during grain filling under Swedish conditions correlates significantly with %UPP (Johansson *et al.*, 2003). Higher temperatures, above 30 ºC showed instead a negative correlation with %UPP (Randall and Moss, 1990).

6. Protein Behaviour during Dough Processing

Dough development and breakdown

Three factors are required to form dough - flour, water and energy. Doughs are concentrated systems where shear and tensile forces imparted by mixing or sheeting cause gluten proteins join together and form a network in the dough (Singh and MacRitchie, 2001a). During development, the dough acquires viscoelastic properties which become optimum at peak consistency. At a molecular level, the dough development involves the use of shear and tensile forces to extend the large glutenin molecules from their equilibrium conformations. Extended molecules give rise to elastic restoring forces similar to what occurs in rubber elasticity. The high elasticity arising mainly from the entanglement coupling of glutenin molecules retards the molecular retraction and maintains the elasticity during resting. At a molecular level, during mixing glutenins retract from extended conformations. Mixing stresses produce orientation of molecules. Polymer molecules respond to the application of stress by three main processes, disentanglement, chain orientation and bond rupture. All three can occur during dough mixing. Scission of the largest glutenin molecules results from chains not being able to slip free at entanglements points quickly enough in response to the stress (Singh and MacRitchie, 2001a).

The covalent bonds that are broken are the disulphide bonds between glutenin subunits. Important is that the highest stresses occur at the centres of molecules where the probability of chain scission is greatest (Singh and MacRitchie, 2001a).

Proteins in dough

The roles of the individual gluten components in dough functionality are complex (Gupta *et al.*, 1991, 1992; Khatkar *et al.*, 2002). When isolated gliadins are mixed with starch and water, a purely viscous material is formed and there is no development stage as in common dough. In contrast, pure glutenin forms a rubbery material with low extensibility. The elastic properties that appear in dough during mixing are due to glutenin (MacRitchie and Singh, 2004).

Many approaches have attempted to explain the molecular basis of interactions of dough proteins (Létang *et al.*, 1999). Undeveloped dough is defined as wheat flour that has become fully hydrated without being deformed (*i.e.* subjected to no mechanical action). Developed dough is described as a transformation of undeveloped dough through some appropriate deformational energy input, to form the developed protein matrix (Campos *et al.*, 1997).

Dough mixing

Most of the studies regarding polymeric proteins are related to the impact of individual protein classes, HMW-gs and LMW-gs, or the genes encoding them (Bekes *et al.*, 2001). The molecular models used to describe the gluten development involve glutenin proteins and crosslinks between them. Schematic mechanisms involved in dough formation are shown in Figure 6.

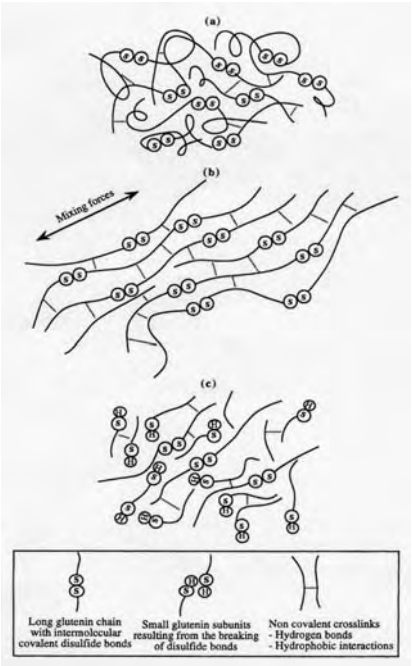

Figure 6. Molecular interpretation of the gluten development: (a) beginning of the mixing, (b) optimum development, (c) overmixing (Létang *et al.*, 1999).

Variation in the composition of polymeric proteins between wheat cultivars during dough mixing has been demonstrated (Gupta *et al.*, 1996; Johansson *et al.*, 2001; Kuktaite *et al.*, 2000; Lindsay and Skerritt, 2000a). The relative amount of polymeric protein increases with increasing gluten strength (Johansson *et al.*, 2001; Kuktaite *et al.*, 2000). However, protein composition explains only a small proportion of the variation in quality. The effects of the glutenin polymer function in dough and for flour end-use quality have also been investigated (Bekes *et al.*, 1994; Uthayakumaran *et al.*, 1999). Specific changes in molecular weight and composition of polymeric glutenin occur during dough development and break-down (Lindsay and Skerritt, 1999; Weegels *et al.*, 1996, 1997).

Many protein structural studies, as well as mixing and baking studies, have postulated that disulphide bonds contribute to the process of dough formation through the disulphide-sulphydryl exchange (Lindsay *et al.*, 2000a; Tilley *et al.*, 2001) (Fig. 6). However, a full understanding of the structure of the gluten polymer during dough processing, as well as of the changes in molecular associations, is still far from being reached.

7. Rheological behaviour

General concepts

Rheology is the study of the flow and deformation of materials (Barnes, 2000). In “A handbook of elementary rheology” Barnes described, what is flow using an example of water, that is carried carefully in a bucket, and it is moving but not flowing. Let’s look at the dough: when the piece of dough lying on the table, carrying its own weight instantly, it starts to flow in a while and ends up like a “floated mass” after long time.

There are two kinds of flows: shear (liquid elements flow over or past each other) and extensional (adjacent elements flow towards or away from each other) (Fig. 7ab). A shear flow can be visualised as the movement of hypothetical layers sliding over each other (Fig. 7c).

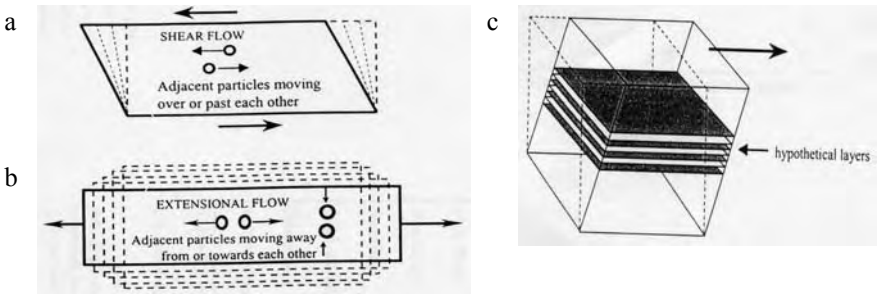

Figure 7. Particle motion in shear (a,c) and extentional (b) flows (Barnes 2000).

All flows are resisted by viscosity, *e.g.* the flow of water poured out from a bucket is quicker than the flow of motor oil poured out from the same bucket.

The gradient of the velocity (force making the liquid to flow) in the direction at right angles to the flow is called the shear rate (γ) and the force produced by the flow per unit area is called the shear stress (σ). The shear viscosity (η) is given as the proportion between the shear stress and the shear rate:

$$\sigma = \eta \gamma \qquad \text{(Newton's postulate)}$$

When η is constant, the material shows Newtonian behaviour.

The potential energy and the energy which is dissipated as heat, can be separated into the storage modulus (G') and the loss modulus (G"), respectively. Dynamic oscillation measurements can be performed either at constant strain or stress. This means that when a sample is subjected to a sinusoidal strain ($\gamma = \gamma_0 \sin \omega t$), the material responds with a sinusoidal stress ($\sigma = \sigma_0 \sin \omega t$) which depends on the properties of the material. The strain (γ) is applied with a given angular frequency (ω), and σ_0 with γ_0, are the shear stress and strain amplitudes, respectively. The phase angle (δ) gives information on the phase shift, the ratio of the viscous to the elastic properties in the sinusoidal deformation:

$$\tan \delta = G'' / G'$$

For an elastic solid the resulting stress is in phase with the strain ($\delta = 0$), and for a viscous liquid the stress is 90 º ahead of the strain. The two moduli are by the relationships:

$$G' = (\sigma_0 / \gamma_0) \cos \delta$$

and

$$G'' = (\sigma_0 / \gamma_0) \sin \delta$$

Mechanical spectra

A mechanical spectrum, *i.e.* the frequency sweep of both moduli (G' and G") in dynamic oscillation can be used to distinguish between the elastic and viscous properties of material within the time. When the viscous properties dominate, G" > G', and G' > G"when the elastic properties govern. From long to short times (low to high frequencies) for a non-cross-linked or with covalent links (transient network) polymer the terminal zone when G' < G" represents liquid properties, the plateau zone with G' > G", rubbery properties, and the transition zone, where the rheological behaviour changes from rubbery to glasslike. The viscoelastic behaviour in the transition zone is relatively similar for all polymers, and is independent of polymer molecular weight and weight distribution (Ferry, 1970). Rheological properties of polymeric glutenins in wheat, with the multiple chain polymers in which the individual peptides or subunits are linked by disulphide bonds, have been shown to give more information about the relative size of the polymeric aggregates and their interactions (Tronsmo *et al.*, 2002).

Viscoelasticity of gluten and factors influencing rheological properties of dough

By measuring the rheological properties of dough not much information about the structure of gluten proteins can be obtained, since the rheological properties of gluten are masked by the large amount of starch and water content of dough. Gliadins are responsible for the viscous and extensible properties, while glutenins confer elastic properties and resistance to expansion to the system. A balance between the two protein fractions is important for the rheological behaviour of gluten (Janssen *et al.*, 1996a, b).

The viscoelastic properties of the glutenins have been postulated to govern the good mixing properties of dough and the quality of the final bread (MacRitchie, 1992). However, an appropriate relationship between rheological properties and baking performance is difficult to find. A great number of studies showed, that a lot of factors influencing rheological properties of dough and baking quality. Water content and flour type have a significant effect on storage modulus (G') and phase angle measured by an oscillatory test both in linear viscoelastic region and as a function of stress (Autio *et al.*, 2001). Oscillatory measurements are known to be very sensitive to water content (Dreese *et al.*, 1988; Hibberd, 1970; Navickis *et al.*, 1982).

Also changes in rheological properties of dough based on granule size distribution might be expected because an increase in the proportion of the small B granules provides a much higher surface area for the binding of proteins (including amylases), lipids and water (Rahman *et al.*, 2000). In a study of the effect of granule size on dough extension it was found that small starch granules increase extensibility of the dough, whereas large granules increase resistance to extension (Larsson and Eliasson, 1997). Also starch containing only purified B granules show markedly longer mixing times and higher water absorption compared to reconstituted flour containing only A granules (Rahman *et al.*, 2000).

8. Wheat Proteomics

In mass spectrometry, the sample (*e.g.* a tryptic digest of a protein) is ionised and mass per charge is analysed. The most commonly used ionisation techniques for polypeptides are electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI). In ESI, polypeptides are ionised amino acid by amino acid, and thereby sequenced according to mass per charge (*m/z*). In MALDI, whole peptides are charged and their *m/z* is measured. More elaborate techniques include ion trap mass spectrometry (ionising a complex mixture of proteins, trapping them according to *m/z* and sending them one by one for further analysis in a tandem coupled mass spectrometer). To decrease the level of complexity, a preseparatory technique such as HPLC is often used when peptide mixtures are studied. Presence of peptide fragments matching known sequences from data bases will identify the protein. Two problems in database matching are that some different amino acids have the same mass per charge ratio, what makes them indistinguishable from one another, and reported sequences in the data bases may contain a substantial amount of erroneously annotated entries, thus making protein with incorrect reference data. Sequencing errors may also make the

databases incorrect. Mass spectrometry is gaining further in popularity due to lowered costs of analysis. It is not limited to protein identification, but in proteomics it has had one of the greatest impacts though it is new accurate possibilities of identification (Kjell, 2004).

New developments in proteomics make possible to identify hundreds of wheat endosperm proteins using mass spectrometry (Van Wijk, 2001). Identification of proteins is based on: 1) separating proteins by 2D-PAGE, HPLC, or other methods; 2) subjecting proteins to protease digestion or fragmentation; 3) measuring the masses of the resulting peptides; 4) matching masses of peptides to masses predicted by known gene and protein sequences.

A complementary approach involves micro sequence analysis of proteins and peptides. Both methods rely on availability of extensive databases of gene and protein sequences from wheat, rice, maize, *Arabidobsis* etc. Approximately 1300 proteins extracted from wheat endosperm at mid-development were resolved by 2D-PAGE (Clarke *et al.*, 2000). Extensive structure/function relationship studies on amino-acid sequences of HMW glutenin subunits provided some information on the primary structures of glutenins using matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOFMS) (Cozzolino *et al.*, 2001; Foti *et al.*, 2000; Cunsolo *et al.*, 2001, 2003). The reports demonstrated differences in glycosylation and other post-translational modifications of HMW glutenin subunits. Furthermore, the combine use of RP- or SE- HPLC and MALDI-MS techniques can be successfully applied in obtaining rapid mapping information of HMW-gs peptides.

Proteomics techniques provide an opportunity to add greatly to information about patterns of protein accumulation and can provide information about pathways of signal transduction by identifying regulatory modification of proteins, such as phosphorylation (Vener *et al.*, 2001). However, the link of all this information to the exact biochemical mechanisms of the entire (unreduced) gluten polymer and variation during wheat processing is still not clear. Thus, proteomic analysis will be the most useful tool when combined with other functional genomics approaches such as microarray analysis and opens new possibilities for the investigations of gluten protein polymer structures.

Objectives

The overall objective of the present studies was to increase the understanding about the protein quality in wheat, *i.e.* to make it possible to improve the production of wheat with desired quality for different end-uses. Studies of changes in protein polymer composition during grain development and dough processing were used as one track to investigate the protein quality in the wheat material.

More specifically, the objectives were to investigate:

- formation of gluten protein polymers during grain filling, and influences of environmental factors such as temperature and nitrogen.
- formation and break-down of gluten protein polymers in dough system (phases) during dough mixing.
- structure-function and extractability changes in protein polymers.
- structural differences in the gluten network of SDS-extractable and –unextractable (extractable only with sonication) polymeric proteins.
- gluten network of wheat flours of different quality during dough mixing using small deformation oscillatory measurements, light and scanning electron microscopy.
- genetical and environmental influences on protein structure and composition.
- possibilities to use proteomics for examination of the complex wheat polymeric protein.

Results and General Discussion (Survey of papers I to V)

Formation of the gluten polymers: from plant to product (flour) (papers I and III)

The gluten polymer formation in wheat differs not only between the cultivars but also due to climatic and environmental alterations (Johansson *et al.*, 1998). In field studies, mean temperatures below 30 ºC, have been correlated with %UPP (Johansson *et al.*, 2002; 2003).

High temperature, above 30 ºC, was reported to increase the proportion of gliadins to glutenins and decrease the proportion of large polymer in flour from several wheat cultivars grown in controlled environment experiments (Blumenthal *et al.*, 1995; Panozzo and Eagles, 2000). At low temperatures, cultivars containing HMW-gs 2+12 were found to be more stable in quality (Johansson *et al.*, 2000) and polymeric protein composition (Johansson *et al.*, 2003) compared to those containing 5+10. Less variability in storage protein composition in response to high temperatures were found for 5+10 cultivars compared to the cultivars with the HMW-gs 2+12 (Blumenthal *et al.*, 1995).

Concerning N application, an increased N have been found to enhance the total amount of all protein components containing gliadins and glutenins, while timing of N was found influencing the %UPP (Johansson *et al.*, 2004).

Reasons for the variation in amount and size distribution of polymeric protein in mature wheat are not fully understood and can be searched for during or within the protein polymer formation period, *i.e.* during grain maturation.

The general pattern of protein accumulation and built-up was found to be similar in the investigated cultivars and did not depend on temperature and applied N (paper I). This pattern (paper I) was similar to the patterns that have been reported also by other authors (Altenbach *et al.*, 2003; Daniel and Triboi, 2002), with increasing built-up of polymeric protein in the middle of grain-filling period.

Temperature was not found to be the main reason for variation in amount and size distribution of polymeric protein in the mature wheat (paper I). Instead N availability seemed to play a greater role (paper I) for the protein accumulation and build-up during grain filling, resulting in differences in amount and size distribution of polymeric protein in mature wheat (paper I).

A certain combination of temperature and cultivation was found to cause changes in the amount and size distribution of polymeric protein, due to variation in N availability in mature grain (paper I). The reason for the differences in amount and size distribution of polymeric protein at maturity seems to be related to amount of up-taken N (available in the plant for redistribution) before flowering, length of grain filling period, and availability of N during the later half part of grain development (paper I).

The formation of %UPP fraction is closely related with the water loss from the grain (Aussenac and Carceller, 2000). However, the water loss is a continuous process, taking place in a steady rate during the whole grain development time (Salgo and Gergely, 2001), while the formation of the polymeric proteins mainly

happens during the latter half of the grain filling period (paper I) (Panozzo *et al.*, 2001; Daniel and Triboi, 2002).

For plant showing differences in amount and size distribution of polymeric proteins due to availability of N, there was a positive relationship between the N availability and the amount of lately accumulated polymeric (LPP, SPP) and monomeric (LMP) gluten proteins. However, these lately accumulated gluten proteins appeared mainly as an increase in SDS-extractable proteins, *i.e.* not too strongly bound proteins, indicating differences in the disulphide bond formation between these, and earlier formed proteins (paper I). Since, intermolecular disulphide formation in SDS-unextractable glutenins seems to occur when gluten protein synthesis ends and dehydration of kernel starts, differences in disulphide bond formation and extractability of polymeric proteins might be due to either alterations in synthesised proteins or in amounts of protein disulphide isomerase (PDI) (paper I).

Protein disulphide isomerase is known as a catalysator during the formation and reorganization of disulphide bonds in developing wheat grains (Every *et al.*, 2003; Kasarda, 1999; Shewry, 1999).

Differences in number of cystein residues between HMW glutenin subunits, and thereby differences in possible formations of disulphide bonds, is thought to be one explanation for the variation in amount and size distribution of polymeric proteins between wheat cultivars containing specific HMW glutenin subunit compositions (Shewry and Tatham, 1997). As described above, the main environmental effect on the amount and size distribution of polymeric protein is thought to be N availability, leading to alterations in protein synthesis or amounts of protein disulphide isomerase (paper I). Whatever reasons (cultivar or environment) alterations in amount and size distribution of polymeric protein (%UPP) in mature grains leads to differences in bread-making quality (Gupta *et al.*, 1993; Johansson *et al*., 2003), although the reasons for alterations might lead to different changes in the bread-making quality. The amount and size distribution of polymers in the mature grains, forming the flour, is the basis for the build-up and cross-linking of the protein polymers in the dough during bread baking (paper III).

The %UPP in a flour is positively correlated to gluten strength (Kuktaite *et al.*, 2000), and the size and amount of HMW-gs is related to the protein matrix presented in the dough (Lee *et al.*, 2002). During bread-making the gluten polymer structure and function changes (Bekes *et al.*, 1994). Amount and size distribution of polymeric proteins changes and disulphide bond formation is rearranged (Lindsay *et al.*, 2000b). For three investigated flours of various quality (paper 3), %UPP increased at mixing, compared to in flours. Also %large UMP (large unextractable monomeric protein in total large monomeric proteins) increased at mixing compared to in the flours (paper III). The pattern of the cross-linking was similar independent of flour, although the original differences in %UPP and %large UMP between the flours persisted throughout the mixing treatments (paper III). Also, extractability of the different protein types did not vary between the different flours.

Comparing the gluten polymers within a grain/flour and dough showed strikingly differences (papers I and III). In a grain/flour, %UPP and extractability differ according to maturity, cultivar, and environment and the polymer seems mainly to consist of gluten subunits. In the dough, also gliadins seems to be bound/catched more tightly in the polymer, ionic forces influences the polymer to be more equal between different flours, but differences in amount and size distribution of polymeric proteins available already in flours seems to persist (papers I and III). Thus, although the similarities in the polymers among doughs from flours of different origin and with different baking properties, differences in disulphide bonds and maybe also other bonds present already in the wheat grain was found to be the main reason for differences in bread-making quality (paper III).

Breakdown of gluten polymers (paper III)

Breakdown of gluten polymers happen in nature when the grain is germinating or sprouting. When the grain is germinating, proteases are attacking the protein polymers, breaking them down into small peptides and/or amino acids. The amino acids are then used as building material in the new, germinating plant (Shewry *et al.*, 1995). If sprouting has started before harvest, the break-down of the proteins have already started, leading to bad bread-making quality (Johansson *et al*., 2002).

Overmixing of dough leads to a breakdown of the gluten polymers (Skerritt *et al.*, 1999a, b). After overmixing of dough the percentages of large UPP, total UPP and large UMP in gluten decreased to lower levels than in the flour (paper III). The reason for the breakdown of polymers in the dough is most likely not related to an attack of proteases, but to a breakdown of disulphide bands and disruption of protein matrix through mechanical forces. Mixing stresses produce reorientation within the gluten polymer molecules (Skerritt *et al.*, 1999a). Polymer molecules respond to the application of stress and cause chain orientation and disulphide bonds breakdown during overmixing (Singh and MacRitchie, 2001a). Upon prolonged mixing the viscosity of the SDS-extractable protein decreases (Danno and Hoseney, 1982), indicating that not only the SDS-unextractable proteins becoming more easily extractable (paper III), but also the SDS-extractable proteins becoming smaller (Danno and Hoseney, 1982). Moreover, a significant decrease in large UMP at overmixing (paper III) seems to be related to that the branching gluten polymer structure that "traps" the monomeric proteins, *i.e.* omega-gliadins, through hydrogen bonds (Belton, 1999) and ionic-electrostatic interactions (Hamer and van Vliet, 2000), are "releasing" those proteins when the breakdown of the polymer begins.

Extractability of proteins and impact on quality (papers I, III, V)

The relations of SDS-extractable versus SDS-unextractable proteins influence baking quality (Gupta *et al*., 1993; Johansson *et al*., 2003). The SDS-extractability of the polymeric proteins differ depending on cultivar (Johansson *et al*., 2001) and environment (Johansson *et al.*, 2002, 2003, 2004), where timing of N and N availability are important parameters (paper I, Johansson *et al*., 2001). The relationship between SDS-extractable and –unextractable proteins changes during

mixing (paper III). Also, changes in glutenin extractability have been observed during dough resting, assuming that repolymerization of glutenin polymer takes place (Hamer and Lichtendonk, 1987). The rate of the increase in protein unextractability was greater for weak flours than for strong ones (Tanaka and Bushuk, 1973; Tsen, 1967).

What is the difference between SDS-extractable and –unextractable polymeric protein? What does actually happen when the proteins are shifting between SDS-extractable and –unextractable polymeric protein?
One can imagine changes in protein conformation, amount of disulphide bonds, types of proteins involved in the polymer, and polymer size. According to Eckert *et al.* (1993), an increase in extractability of the polymer is an effect of conformational rearrangements and not of a reduction in the size of protein polymer. The more extended the structure is (*e.g.* after mixing), the greater will the extractability be and a lower amount of unextractable glutenin polymer will be found (Eckert *et al.*, 1993). In the present work, an attempt to understand the polymeric conformation was carried out (paper V).

In the SDS-extractactable protein, the main part of the albumins and globulins were found, while gliadins and glutenins were found in both the SDS-extractractable and -unextractable (sonicated) samples (paper V). The proteins extractable with propanol + DTT found in the SDS-extractable protein seemed to be more "gliadin-like" compared to those found in the SDS-unextractable protein (paper V) and thereby these proteins might be cysteine residue connected to the glutenin polymer (Masci *et al.*, 1993, 1999). The largest protein polymers of both the SDS-extractable and –unextractable protein contained, however, similar types of proteins (paper V). Thus, changes in types of proteins involved in the large polymeric proteins are not the main reasons for differences in extractability of polymeric proteins (paper V). Instead, the reason for variation in extractability might be changes in number of disulphide bonds or conformations - causing disulphide bonds being more difficult to disrupt both eventually leading to larger polymers. However, only larger polymers do not seem to be the main reason for differences in extractability of SDS-extractable and –unextractable polymeric protein (paper V).

A higher complexity of the polymer with increased strength, was as well indicated when various solvents were used to extract different protein types in three flours of different quality (paper III). In the weakest flour (biscuit flour), the highest amount of the glutenins was extracted with propanol + DTT, while acetic acid increased the amount of extracted glutenin in the stronger flour (standard flour) (paper III). Furthermore, significantly higher amounts of extracted glutenins were found for strong flour compared to the other flours when SDS + DTT were used for the extraction (paper III). DTT in general breaks the disulfide bonds, while in the strong flour, the structure of the protein polymers needed to be rearranged, using SDS, before DTT was able to reach the disulfide bonds. Thereby, a more complicated conformation of the polymer structure, alternatively a larger protein polymer (as stated by some authors; *e.g.* (Singh and MacRitchie, 2001a)), with probably a higher amount of disulfide bonds is indicated. The results from the

studies in this thesis could not prove whether structure, size or amount of disulfide bonds were the main reasons.

A high extractability of smaller molecules (called SMP) early during grain development was seen in paper I. These smaller molecules were partly consisting of small proteins, likely albumins and other enzymatic proteins important at grain development. Part of the molecules in the SMP peak were, however, very small and could possibly be peptides or even amino acids, transported to the grain for accumulation of proteins during maturity (paper I).

Rheological behaviour and polymer structure (paper IV)

A number of studies have been performed on fundamental gluten rheological behaviour (Khatkar *et al.*, 1995), indicating the importance of intermolecular disulphide bonds in dough/gluten rheological behaviour (Shewry and Tatham, 1997). There is strong evidence that the specific subunit composition plays an important role in determining the rheological properties of dough (Payne, 1987b). In addition, the molecular size and structure of the gluten polymer are related to the polymer rheological properties. Interactions between polymer chain entanglements and branching are seen to be key mechanisms determining the rheological behaviour of gluten polymers (Dobraszczyk and Morgenstern, 2003). Also, the phenomenon of repolymerization of gluten polymer during dough resting confirm the existence of a close link between glutenin molecular distribution and the rheological properties of dough (Bangur *et al.*, 1997). According these remarks, seems that only polymeric proteins above a certain molecular size contribute to dough properties such as dough strength (Aussenac *et al.*, 2001).

Differences of mixing time on rheological properties of dough, *i.e.* the storage modulus G', depends on the type of the flour (paper IV). Since, the G' value represents the solid properties of the sample at small deformations, the increase in storage modulus indicates an increase in the network density of the gluten. The storage modulus G' is higher for the higher in gluten strength (strong) flour compared to the weak flour (biscuit) (paper IV). Thus, the higher G' value signifies the higher gluten network density for the strong flour compare to the biscuit flour at Optimum mixing time (paper IV) and specific interactions in the gluten polymer. However, rheological properties of dough during mixing appear influenced differently from the gluten. The strongest effect of mixing time was found for the durum flour dough, where G' increased linearly from min to overmixing and the frequency dependence of G' (n') of dough decreased (paper IV).

Concerning water content, both storage modulus (G') and loss modulus (G'') are known to decrease as the water content of doughs increase (Autio *et al.*, 2001). However, contrasting observations of the increase in water content of gluten with increased mixing time did not show an evident relation and effect on the storage modulus of gluten (paper IV). The explanation seems to be related to the properties and microstructure of gluten. The fact that rheological properties of dough were uninfluenced and the large starch granules appeared in the gluten after overmixing, seem to indicate more complex gluten network in the strong flour

compare to the weaker flours and probable an incomplete overmixing effect for this flour (paper IV).

Also, the increase in the gluten water content for the standard flour did not influence the G' modulus of the gluten (paper IV). The explanation seems to be related to a relatively high amount of cultivar carrying 1BL/1RS translocation in the standard flour (paper IV), thus the water uptake of the dough is increasing and that causes the dough stickiness. Furthermore, the negative pentosans effect in such flour is also possible. Hence, the flour type affects the rheological properties of dough (Autio *et al.*, 2001). This is related with the genetical cultivar background, for instance, the G' values of both dough and gluten were highest for the durum flour compared to bread wheat at increasing mixing (paper IV).

Protein composition in gluten – influences of genetics and environment (paper II, III and IV, unpublished results and from posters)

Specific variations in gluten protein composition are related to differences in gluten strength between cultivars (Johansson *et al.*, 1995, 1996). Correlation have been established between particular proteins and protein subunits and different bread-making quality parameters (Johansson *et al.*, 1996; Payne *et al.*, 1987a). Also, dough treatment influences the amount and size distribution of polymeric proteins (paper III). Most of the HMW-gs and LWM-gs found in the gluten phase were present in the bottom phase as well (paper II), likely indicating that those proteins appeared in the bottom phase due to the large size during centrifugation. The specific LMW-gs detected only in the gluten phase (Fig. 1b) under all mixing conditions may indicate specificity of these proteins within the polymeric network formation in gluten phase (paper II).

Presence of water-extractable proteins was found both in the liquid and gluten phase after ultracentrifugation of dough (paper II). This seems to indicate, that albumins are trapped, or somewhat included in the gluten network during mixing. However, the type of albumins found in the gluten phase was different from those found in the liquid phase (paper II). Thus, specific types of water-soluble proteins somehow were a part of the network. Water extractable proteins within the liquid phase were found using Western blotting analysis. The used antibodies (derived from J. Hejgaard, Biocentrum DTU, Lyngby, Denmark) were typical albumin type. Water extractable proteins in the gluten phase were instead found to be of gliadin type (antibodies from J. Hejgaard) (Fig. 8).

Figure 8. Water-soluble gliadins found in gluten phase after ultracentrifugation of dough.

The eventual role of water and salt soluble proteins in gluten network formation is not properly known yet.

The amount of polymeric protein in the gluten phase was mostly related to the amount of N applied to the crop, but was also influenced by the cultivar and the cultivation year (Table 1a, b). This indicates a cultivar and environment influence on amount of polymeric protein in the gluten. Cultivar and environment influence amount of polymeric protein in the cultivar (Johansson *et al*., 2001, 2002). A higher amount of polymeric protein in the cultivar does not necessarily mean a higher amount in the gluten, but the present investigations showed that this was in fact the case (Table 1b).

Table 1. *Mean squares from the combined analysis of variance across cultivars, nitrogen application and dough mixing time (treatment). The measured parameters are: (a) the sizes of different phases after ultracentrifugation of dough, (b) different protein parameters from SE-HPLC analyses*

a)

Source	**Liquid**	**Gel**	**Gluten**	**Starch**	**Bottom**
Cultivar	20.4	2.0	12.2*	31.1	33.9
Nitrogen	1.9	1.1	21.0*	44.1	19.9
Treatment	4.3	14.3***	11.8*	72.1*	315.4***
Error	6.5	1.1	2.6	11.5	21.9

b)

Cultivar	**SDS-extractable**			
	LPP	**SPP**	**LMP**	**SMP**
Gel x10^{10}	38.0*	52.6	13590.0**	2825.0**
Gluten x10^{11}	18.0	185.5***	1762.0***	2.4
Bottom x10^{10}	12.7*	0.9	117.3	3.5
Nitrogen				
Gel x10^{10}	4.5	0.2	140.0	4361.0**
Gluten x10^{11}	37.7	211.6***	1476.0***	0.0
Bottom x10^{10}	3.2	30.0**	272.5	1.4

Cultivar	**SDS-unextractable**					
	LPP	**SPP**	**LMP**	**SMP**	**LUPP**	**TUPP**
Gel x10^{10}	21.6***	83.2**	223.6**	4.0	0.02**	0.03*
Gluten x10^{11}	1974.8	186.6*	441.8	1.1	0.0	0.0
Bottom x10^{10}	120.5***	1660.0***	4.4	20.1	0.1***	0.05***
Nitrogen						
Gel x10^{10}	0.5	17.0	413.6**	2.8	0.0	0.0
Gluten x10^{11}	286.9	82.0	218.1	0.1	0.0	0.0
Bottom x10^{10}	17.8**	130.0	5.4	46.7	0.0	0.0

Gliadins and glutenin subunits combine to form gluten polymers, which give wheat dough its unique viscoelastic properties. Protein film in undermixed dough generally had a rougher structure than in overmixed dough (paper IV). This is related to increasing dough elasticity with increasing mixing time, resulting a smoother gluten network. According to earlier results, prolonged mixing untangles inter-chain disulphide linkages, modifies chain orientation, ruptures bonds between LMW-gs and HMW-gs and promotes new polymer-polymer interactions. The explanation to the changes in protein composition during mixing seems to be dependent on the genetic composition of the flours (paper IV).

Proteomics and polymer structure (paper V)

The proteome is composed of the whole set of proteins present in a given tissue, cell, or in sub-cellular components of a living organism at a given time. The identification of proteins expressed (gene location and sequence), post-translational modifications, function in physiological metabolism are the major tasks of the proteomic approach. Gluten proteomic approach could be used for: 1) characterisation of the gene products, 2) study the expression of wheat storage protein genes, 3) study interactions between chromosomes, 4) accumulation of storage proteins in protein bodies, 5) post-translational modifications (a result of genetic and environmental influence), 6) environmental influences on storage proteins (Branlard *et al.*, 2004). Proteomic analysis is an extremely useful analytical tool for the separation, the quantification and identification of proteins from complex protein mixtures. However, difficulties in studying of the entire gluten polymer structure appear (paper V). Proteomics results were generally interesting and comparisons of digested versus digested+reduced samples could contribute to expanding understanding of disulphide bond formation and break-down pattern within the gluten macropolymer. However, SDS-PAGE and RP-HPLC techniques were simpler to use and more informative on differences in SDS-extractable and –unextractable protein compositions compared to the proteomics (paper V).

Concluding remarks

Accumulation and built-up of the proteins in the developing wheat grain is independent of variation in temperature and nitrogen regimes.

The grain protein concentration is influenced by the temperature through a higher starch accumulation rate at temperatures around 15-20 ºC.

Nitrogen and sulphur availability are important factors in determining the amount and size distribution of polymeric proteins, especially during the latter half of grain development.

Variation in temperature did not influence the %large UPP, total UPP and large UMP.

The specific LMW-gs found in gluten phase during dough mixing are involved in gluten macropolymer formation and bread-making quality.

The amount and size distribution of polymeric proteins are genetically and environmentally determined, and form the basis for the build-up (cross-linking) of protein polymers during dough formation.

The omega-gliadins interact with the glutenins during dough processing through non-covalent interactions.

During dough formation the protein polymer have hydrophilic residues orientated on the outside, and therefore, only small differences in protein extractability appear.

The general effects of prolonged mixing of dough (from opt to overmixing) were an increase in water content of gluten, changes in the rheological properties of dough, smoothening of the gluten surface and distribution of starch granules in gluten.

The storage modulus (G', representing the network density) of gluten was uninfluenced by prolonged mixing.

Overmixing resulted in a smaller effect on the strong flour compared to the weaker (biscuit and standard) flours.

The variation in gluten protein network formation during dough mixing is caused by the genetic source of flour, as well as by the protein composition of the polymeric proteins.

Differences in the extractability behaviour of SDS-extractable and –unextractable proteins in various solvents indicated the different roles of those proteins in the gluten macropolymer structure.

The specific ω-gliadins seem to have a tight link with the HMW- and LMW gs and are a part of the gluten macropolymer.

The large polymeric protein (LPP) fraction of the gluten macropolymer has a branched and complex structure with possibly conformation differences compared to the rest of the macropolymer and an increased number of disulphide bonds.

Differences in size of the LPP fraction seems not to be the main reason of variation in extractability between the SDS-extractable and –unextractable protein.

The specific albumins have a certain function by being involved in polymer structure.

The attempts of using proteome technique for investigating the entire gluten macropolymer composition and structure are limiting.

Protein Future Application

Molecular mechanisms involved in wheat grain development are yet poorly understood despite the importance of cereals as a major source of nutrition for human kind (Altenbach *et al.*, 2003). Until now it has been impossible to answer questions such as: what are the differences in gluten network formation between cultivars and the influence of environmental variations? How do the protein polymers change during processing for different cultivars and during different grain treatments? Also, there is still a lack of understanding about post-translational protein modifications, such as glycosylation, that occur and can have major effects on properties of the gluten proteins.

Genomics and proteomics techniques provide considerable opportunities for understanding these processes. By comparing profiles of gene expression and protein accumulation pattern under different environmental conditions, it should be possible to reveal basic molecular mechanisms that are influenced by environment and affect productivity and quality.

Understanding the molecular basis and behaviour of gluten proteins is an important pre-requisite for breeding in order to improve the quality for traditional uses and to develop new properties for novel uses in both the food and non-food industries, *e.g.* gluten biodegradable plastics. For future gluten protein work, the synthesis of already known information about the gluten proteins and new techniques, *i.e.* proteomics, create new possibilities for the investigations of gluten protein complexity in order to understand the biochemical basis and reactions behind the structure of the gluten polymer. This work in a broader context may have suitable information outside direct study field, *e.g.* not only improving wheat cultivar breeding, bread-making, pasta and baking goods, but also solving the gluten allergy and intolerance problems. Also, knowledge for the expanding field of non-food applications in the development of coatings, adhesives and disposables may be anticipated.

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