

Tailoring the Structure-Function Relationship in Wheat Gluten

Processing, Genotype and Environment Effects in Bio-
Based Materials

Faiza Rasheed

*Faculty of Landscape Architecture, Horticulture and Crop Production Science
Department of Plant Breeding
Alnarp*

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Cover: Protein secondary structures at ~4-10 nm and protein morphologies at 40-80 nm in gluten-based materials

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Abstract

Gluten proteins ranging in size from 30 000 to several million daltons form one of the largest and most complex polymers in nature. The giant molecular nature and intricate network of the over 100 types of proteins in gluten make structural studies rather challenging. This thesis examines the molecular crosslinking and structural properties of variously sourced gluten and its gliadin and glutenin protein fractions, both as unprocessed proteins and in films and foams.

Protein modification through chemical additives, separation procedure and genotype (G) and environmental (E) interactions had an impact on protein polymerization, nano-structure morphology, secondary structures and the mechanical properties of films. The extent of denaturation in the starting material for film formation was of relevance for the development of specific nano-scale morphology and improved mechanical properties of films. When molded into films, non-aggregated starting material such as gliadin with additives and mildly separated gluten indicated both hydrogen- and disulfide-bonded protein network, with some non-reducible covalent crosslinks. These films also showed bi-structural morphology at nano scale. The gliadin films revealed hexagonal structures and additional not previously observed structural units. The films from mildly separated gluten also showed hexagonal and lamellar structural morphology. The films from glutenin and industrially sourced gluten proteins showed a high content of non-reducible covalent crosslinks and unorganized morphology at nano scale. The G and E interactions were associated with strong and weak gluten, resulting in films with various structural and mechanical properties. The mechanical properties of films were found to be influenced by protein structure development. Structural attributes such as relatively high number of disulfide crosslinks compared with non-reducible crosslinks, high β -sheet content and specific nano-scale morphology also led to high mechanical performance of films.

Keywords: wheat gluten, gliadin, glutenin, nano-scale morphology, mechanical properties, protein polymerization, β -sheets

Author's address: Faiza Rasheed, SLU, Department of Plant Breeding,

P.O. Box 101, 230 53 Alnarp, Sweden

E-mail: Faiza.Rasheed@slu.se

Dedication

To Prophet Muhammad Peace be Upon Him,
The mentor of all times,

*Seeking knowledge is obligatory for every man and woman (Prophet
Mohammad Peace be Upon Him)*

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Johansson, E., Malik, A.H., Hussain, A., **Rasheed, F.**, Newson, W.R., Plivelic, T.S., Hedenqvist, M.S., Gällstedt, M. and Kuktaite, R. 2013. Wheat gluten polymer structures: The impact of genotype, environment, and processing on their functionality in various applications. *Cereal Chemistry* 90: 367-376.
- II **Rasheed, F.**, Newson, W.R., Plivelic, T.S., Kuktaite, R., Hedenqvist, M.S., Gällstedt, M. and Johansson, E. 2014. Structural architecture and solubility of native and modified gliadin and glutenin proteins: non-crystalline molecular and atomic organizations. *RSC Advances* 4: 2051-2060.
- III Blomfeldt, T.O.J., Kuktaite, R., Plivelic, T.S., **Rasheed, F.**, Johansson, E. and Hedenqvist, M.S. 2012. Novel freeze-dried foams from glutenin- and gliadin-rich fractions. *RSC Advances* 2: 6617-6627.
- IV **Rasheed, F.**, Newson, W.R., Plivelic, T.S., Kuktaite, R., Hedenqvist, M.S., Gällstedt, M. and Johansson, E. 2014. Wheat protein structure: Nano-scale refinements to modulate the functionality upon chemical modification. Submitted.
- V **Rasheed, F.**, Hedenqvist, M.S., Kuktaite, R., Plivelic, T.S., Gällstedt, M. and Johansson, E. Mild gluten separation – a non-destructive approach to fine tune the structure and mechanical behavior of wheat gluten films. Submitted.

VI **Rasheed, F.**, Kuktaite, R., Hedenqvist, M.S., Plivelic, T.S., Gällstedt, M. and Johansson, E. Can we tune wheat gluten structure and functionality in bio-based films via genetic and environmental interactions? Manuscript.

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Papers II and III are reproduced with the permission of *Royal Society of Chemistry*.

The contribution of Faiza Rasheed to the papers included in this thesis was as follows:

- I Participated in some of the experimental work and provided input to writing the manuscript.
- II Performed the main experimental work, evaluated and analyzed the data and wrote the manuscript with the input of co-authors.
- III Participated in some experimental work and contributed to writing the manuscript along with co-authors.
- IV Performed the main experimental work, evaluated and analyzed the data and wrote the manuscript with the input of co-authors.
- V Planned the experiment together with the supervisor, carried out most of the experimental work, evaluated and analyzed the data and wrote the manuscript with the input of co-authors.
- VI Planned the experiment together with the supervisor, carried out most of the experimental work, evaluated and analyzed the data and wrote the manuscript with the input of co-authors.

Abbreviations

CD	Circular dichroism
CLSM	Confocal laser scanning microscopy
DHA	Dehydroalanine
DTT	Dithiothreitol
E	Environment
E-Modulus	Young's modulus
FT-IR	Fourier transform Infra-red
G	Genetics
Glia	Gliadin
Glut	Glutenin
Gly	Glycerol
GMP	Grain maturation period
HMW	High molecular weight
IDPs	Intrinsically disordered proteins
I-Gluten	Industrial gluten
I-Gluten-Gly	Industrial gluten film
LAL	Lysinoalanine
LAN	Lanthionine
LMW	Low molecular weight
Max. Stress	Maximum stress
M-Gluten	Mildly separated gluten
M-Gluten-Gly	Film from mildly separated gluten
MP	Monomeric proteins
N	Nitrogen
N1, N2	No and low nitrogen (spike formation) respectively
N3	High nitrogen (spike formation+ anthesis)
N4	High nitrogen (anthesis)
NaOH	Sodium hydroxide

NH ₄ OH	Ammonium hydroxide
PP	Polymeric proteins
RP-HPLC	Reverse phase high performance liquid chromatography
s	Second
SA	Salicylic acid
SAXS	Small angle X-ray scattering
SDS	Sodium dodecyl sulfate
SE-HPLC	Size exclusion high performance liquid chromatography
SH	Thiol group
SH/SS	Thiol-sulfur interchange
T	Temperature
T1	High temperature 25/19 °C
T2	Low temperature 18/14 °C
WAXS	Wide angle X-ray scattering

1 Introduction

1.1 Protein structure

Proteins are biological macromolecules made up of organic subunits called amino acids. There are 20 ‘standard’ amino acids used as main building blocks in the synthesis of proteins (Whitford, 2005; Voet & Voet, 2001). Each amino acid is unique from others due to the presence of different side chains or functional groups (R) attached to its central carbon atom (Figure 1a). The functional group can be *e.g.* a single hydrogen atom, carboxylic acid, imine, a variety of basic groups, alkyl group, thiol and aromatic groups *etc.* (Kuchel *et al.*, 1998). Owing to the various functional groups, the amino acid side chains provide opportunities for protein modification, both *in vivo* and *in vitro*, at certain points. Amino acids are combined in varying numbers and orders through peptide bonds (Figure 1b) to form protein chains. Therefore proteins are also called polypeptides (Kessel & Ben-Tal, 2012; Berg *et al.*, 2002).

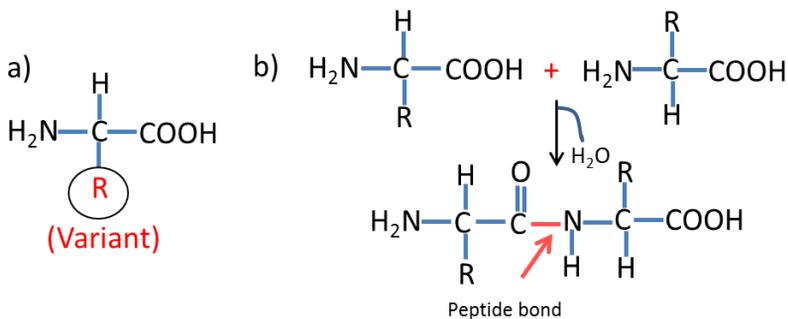


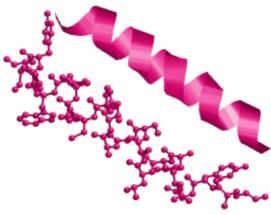
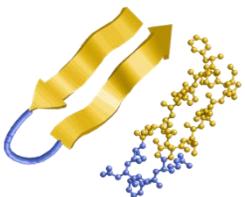
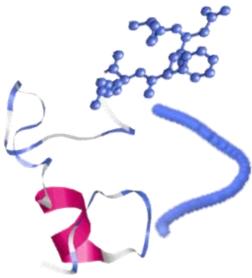
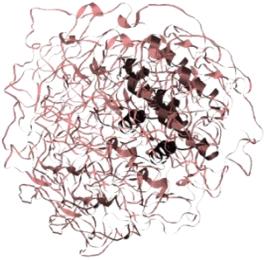
Figure 1. a) Molecular formula of an amino acid (b) peptide bond formation. Redrawn from Voet & Voet (2001).

Protein structure is generally determined at four levels: primary, secondary, tertiary and quaternary. The quaternary level is only achieved if the protein possesses multiple subunits (dimers, trimers, oligomers, polymers *etc.*). The linear sequence of amino acids in a polypeptide chain is classically referred to as the primary structure of proteins. When portions of a protein or proteins come into close proximity to each other, protein-protein interactions occur. These interactions can be non-covalent type (hydrogen bonding, electrostatic and *van der Waals* interactions) or covalent type (generally disulfide, dityrosine and, in specific conditions, non-reducible isopeptide, lanthionine, lysinoalanine interactions *etc.*) (Rombouts *et al.*, 2010; Whitford, 2005; Gerrard, 2002). These types of interactions also result in the secondary, tertiary and quaternary structures of proteins.

Specific regions within polypeptide chains folded into different structural elements, usually α -helices, β -sheets and turns held together by hydrogen bonds, are known as the secondary structures of a protein. Table 1 describes the different types of protein secondary structures and their corresponding conformation.

The secondary structure of proteins is determined to some extent by the specific order and types of amino acids in a polypeptide chain. For instance, frequent occurrence of proline residue impedes the formation of α -helix or β -sheet structures due to its cyclic ring (Whitford, 2005; Voet & Voet, 2001). Some amino acids such as methionine, leucine, alanine and glutamic acid preferably adopt a helical conformation (Kuchel *et al.*, 1998). Others such as tryptophan, tyrosine, phenylalanine, valine and threonine possess a high propensity for β -sheet conformation (Voet & Voet, 2001; Chou & Fasman, 1978). The amino acids glycine, aspartate and proline have a high predictive probability of adopting β -turn conformation (Whitford, 2005).

Table 1. *Secondary structures of proteins (structure concepts adopted from Whitford, 2005).*

Type	Description	Conformation
α -helices	<p>In an α-helix, the polypeptide backbone follows a helical path like a spring coil, with 3.6 residues in each turn of the helix. Within the helix the backbone carbonyl oxygen of one residue (i) is hydrogen-bonded with the amine hydrogen of the fifth residue ($i+4$) along the polypeptide chain (Kuchel <i>et al.</i>, 1998).</p> <p>Hydrogen bond$_{\alpha\text{-helix}} = i*(i+4)$.</p>	
β -sheets	<p>In a β-sheet, strands of polypeptide chains (β-strands) lie adjacent to one another, interacting laterally through hydrogen bonds (Kuchel <i>et al.</i>, 1998). In β-sheets, there is hydrogen bonding between different segments of the polypeptide chain rather than between adjacent residues as in α-helices. The arrows show the direction of the β-sheets, which can be parallel or antiparallel.</p>	
Turns and loops	<p>A turn is a form of secondary structure where the polypeptide chain folds back on itself. Loops and turns also occur in polypeptide chains to connect α-helices and/or β-sheets (Rose <i>et al.</i>, 1985). β-turns are usually four residues long and are commonly found in proteins. There are hydrogen bonds between the first and fourth ($i+3$) residues if a favorable arrangement occurs between donor and acceptor, but the middle two residues ($i+1$, $i+2$) never form hydrogen bonds.</p>	
Random coils	<p>The unordered regions of polypeptide chains without regular structural units are called random coils. Random coils are not true secondary structures, but are folded conformations of unstructured regions of a polypeptide chain (Kuchel <i>et al.</i>, 1998).</p>	

The tertiary structure of proteins is a unique three-dimensional (3D) overall folding of one polypeptide chain stabilized by intra-chain molecular interactions of either a non-covalent or covalent nature (Figure 2) (Whitford, 2005; Voet & Voet, 2001). The quaternary structure exists for proteins with more than one polypeptide chain, and is stabilized by intermolecular interactions (Whitford, 2005).

When two cysteine molecules in one polypeptide chain, or in two different polypeptide chains, react with each other, an intra-chain, or inter-chain, disulfide crosslink occurs respectively (Shewry & Tatham, 1997). A disulfide bridge is formed due to oxidation of the thiol functional group (SH) in cysteine residues. The two cysteine molecules crosslinked via a disulfide formation are conventionally named cystine. The disulfide formation imparts structural stability and functional characteristics (for instance, strength) to proteins. Another type of covalent crosslink among proteins is dityrosine. Dityrosine crosslinks connect a polypeptide chain or chains *via* tyrosine residues (Tilley *et al.*, 2001). Severe heat treatment of proteins can also lead to isopeptide crosslinking between the amino group of lysine residues and the carboxyl group of some other amino acid (*e.g.* aspartate, glutamate) (Gerrard, 2002). Alkali-induced crosslinking of amino acids also exists, through the formation of modified amino acids when proteins are treated at high temperature with alkaline additives. For example, an intermediate, dehydroalanine (DHA), is formed by β -elimination of cystine (Rombouts *et al.*, 2013). DHA further reacts with a cysteine molecule, resulting in the formation of a non-reducible lanthionine (LAN) crosslink.

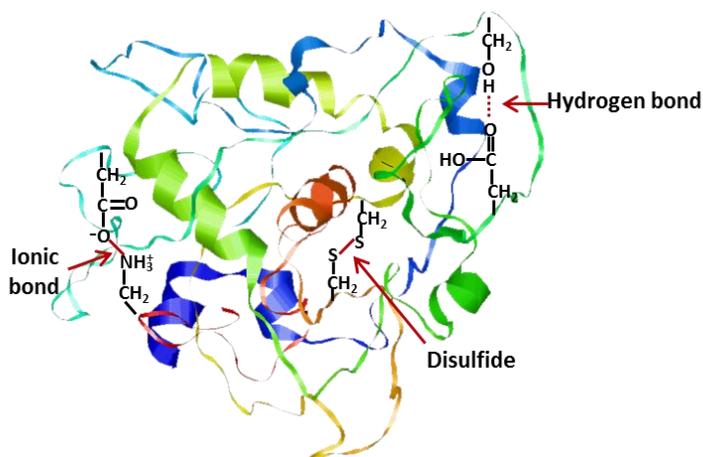


Figure 2. Schematic representation of interactions in a polypeptide chain. Protein structural model generated with Phyre2 (Kelley and Sternberg, 2009).

1.2 Intrinsically disordered proteins

For a long time, protein denaturation was considered to be a reversible ‘all-or-none’ transition between a folded native state and an unfolded denatured state. The native state was regarded as the 3D compact globular conformation, specifically folded into a rigid and well-defined structure (Anson, 1945; Mirsky & Pauling, 1936). This rigidity gives proteins the ability to form crystals and opportunities for structural determination of the proteins down to the atomic level based on X-ray crystallographic information. The distinct structure-function paradigm developed in the 20th Century can be described as follows:

Amino acid sequence → *3D structure* → *Function*

The 3D folded conformation was regarded as obligatory for protein function and equated with the native structure of proteins (Wright & Dyson, 1999). However, the scenario changed about two decades ago with the discovery of intrinsically disordered proteins (IDPs), which are partially or fully disordered but still have the ability to perform specific functions (Wright & Dyson, 1999). Since the main criterion for a protein to be regarded as ‘native’ is its ability to perform its biological role, IDPs are essentially regarded as native (Turoverov *et al.*, 2010).

Examples of IDPs from plants are the cereal proteins, *i.e.* gluten proteins, in which some protein regions possess structural domains (Tatham & Shewry, 1985) and some are disordered (Blanch *et al.*, 2003). Due to the presence of repeat sequences consisting mainly of non-polar amino acids, the gluten proteins possess large domains which lack specific structural conformation. This partly ‘unstructured’ conformation of gluten proteins is energetically favorable for their biological role as storage proteins, meaning that their function is primarily to provide nutrients for the developing wheat seed embryo.

Among disordered proteins, some gain specific structural features *in vivo* when they react with other molecules and can thereafter perform a specific biological function (Dedmon *et al.*, 2002; Demchenko, 2001). Structural organization can also be fully or partly obtained *in vitro* for IDPs, by modifying the proteins through physical or chemical means.

1.3 Supramolecular structure of proteins

Proteins possess specific supramolecular morphologies (~1-10 nm) of different shapes and sizes under the effects of various physiological and chemical environments. Small angle X-ray scattering (SAXS) is a powerful technique to

study the morphology (shapes) of macromolecules. SAXS probes the structure of macromolecules in the nanometer range ($\sim 1-10$ nm) by measuring scattering intensity at an angle 2θ close to 0° (Schmidt, 1995). A specific tetragonal and hexagonal morphology at supramolecular (6-8 nm) level has been reported for modified wheat gluten proteins (Kuktaite *et al.*, 2012; Kuktaite *et al.*, 2011). The essential components in scattering experiments are an X-ray source, a sample and an analyzing detector, as shown schematically in Figure 3. The X-rays from the source are confined into a fine beam, often by slits, and strike the sample. A small fraction of the incident beam is thus scattered and bounces in other directions, making an angle 2θ to the direction of the incoming beam (Figure 3). Scattering beams are recorded by a 2D detector (Schmidt, 1995; Brumberger, 1994). The scattering curves obtained through SAXS data are used to obtain a low resolution model of protein morphology (shape), as shown by the exemplifying diffractogram in Figure 3. The SAXS pattern apparent in the resulting diffractogram (relationship between intensity I and distance q) arises from the shape and interaction of scattering objects in the sample (Berk, 1991).

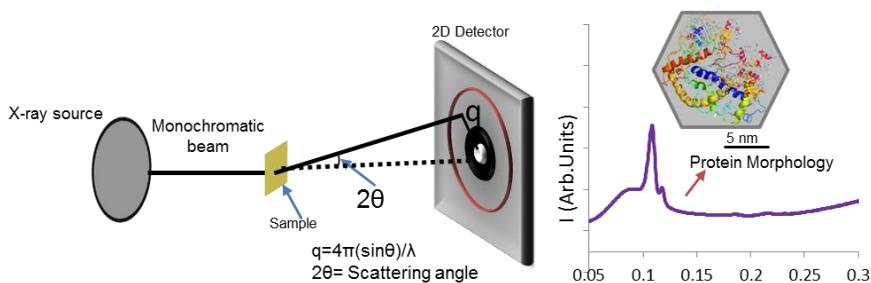


Figure 3. Schematic representation of SAXS set-up and morphology detection for proteins. Based on information from Schmidt (1995). The diffractogram is from Paper V.

2 Background

2.1 Wheat proteins

The protein content in mature wheat grains varies from 8 to 20 %. Among the protein types in grain, albumins and globulins constitute about 15-20 % and the gluten proteins constitute up to 80-85 % of total proteins.

2.1.1 Wheat gluten

Gluten is a rubbery mass of proteins separated from flour by simple dough washing under a stream of water. Gluten confers the elasticity and extensibility to wheat dough (Shewry *et al.*, 1995), and these properties make the gluten proteins one of the key determinants of bread-making quality. Gluten can be separated into over 100 different proteins (either monomeric or polymeric) by isoelectric focusing or gel chromatography (Wieser, 2007). Gluten proteins have been studied over the past three centuries in order to determine their structures and the relevance of these structures for functional properties in various end-use products. The gluten proteins are unique, with their amino acid composition being rich in glutamine and proline and low in charged amino acids (Gianibelli *et al.*, 2001).

Another important aspect of gluten is the presence of a relatively high amount of cysteine residues, which imparts important structural and functional properties via the formation of intra- and inter-molecular disulfide crosslinks (Wieser, 2007; Shewry & Tatham, 1997; Nielsen *et al.*, 1962).

2.1.2 Major protein groups in gluten

The two major subgroups of gluten proteins are gliadin and glutenin, which are further sub-classified into three and two protein types, respectively (Figure 4).

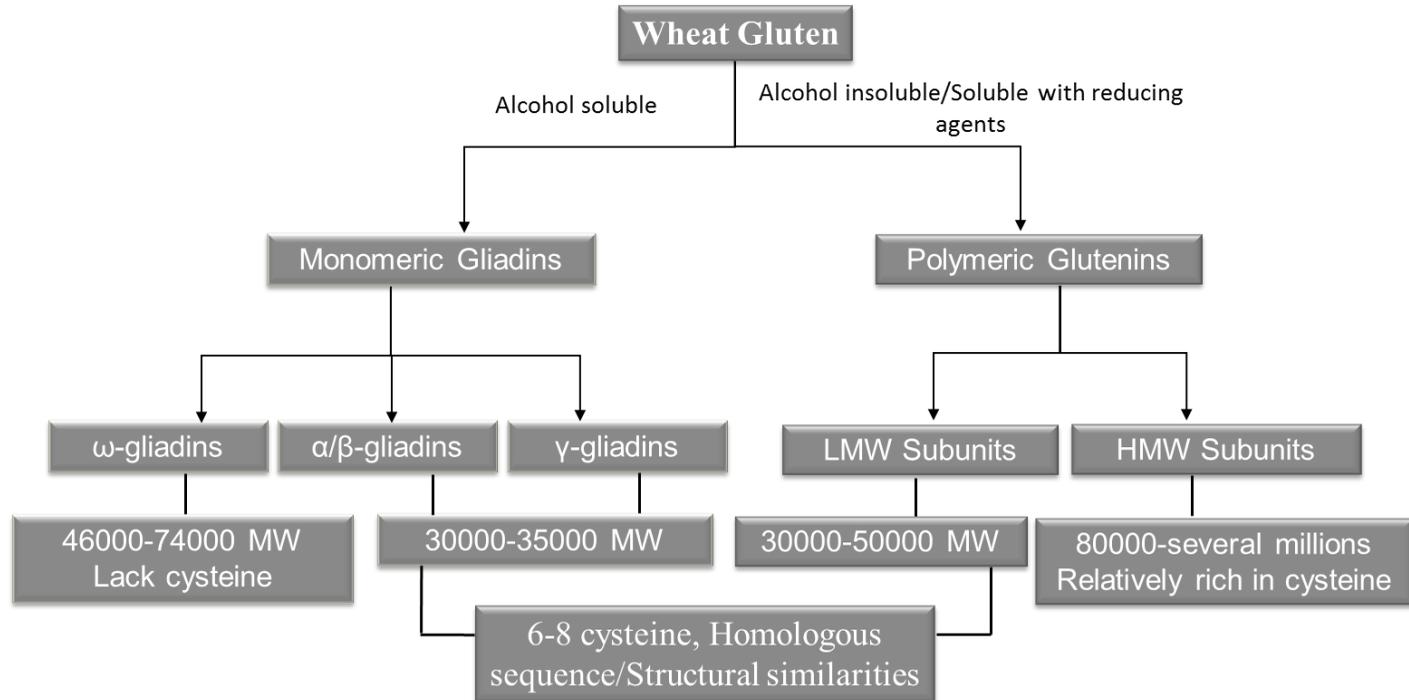


Figure 4. Classification of wheat gluten proteins (based on findings by Wieser, 2007; Shewry *et al.*, 1984; Kasarda *et al.*, 1976; Aykroyd & Doughty, 1970) LMW: low molecular weight, HMW: high molecular weight.

2.1.3 Structure characterization of gluten

Structural studies of gluten proteins have been of focus over the past four decades, but a satisfactorily defined structural model has not yet been established. A recent study on gluten protein structure in ethanol/water mixture suggests a loose structure in the dilute phase, similar to branched polymers, and structural similarities with polymeric gels in the dense phase (Dahesh *et al.*, 2014). Obstacles to defining a peculiar structural model for gluten proteins are the high molecular weight of the proteins, sequence redundancy and complexity due to several polypeptide chains. Owing to the complex nature of gluten, X-ray crystallographic studies have been challenging, although useful structural information has been obtained through infra-red (IR), circular dichroism (CD), microscopic and X-ray scattering techniques (Kuktaite *et al.*, 2011; Thomson *et al.*, 1999; Miles *et al.*, 1991; Tatham *et al.*, 1985).

2.1.4 Structure of gliadin proteins

Clearly distinguishable N- and C-terminal domains are present in α/β - and γ -gliadin (Wieser, 2007). The N-terminal domain for α/β -gliadins is rich in glutamine, proline, phenylalanine and tyrosine amino acids. The C-terminal domains of α/β - and γ -gliadin are homologous and contain fewer proline and glutamine amino acids than the N-terminal domains. Moreover, the C-terminal domain of α/β -gliadins contains 6 cysteines and that of γ -gliadin contains 8 (Wieser, 2007). These cysteine amino acids form three intra-chain disulfide crosslinks in α/β -gliadins and four intra-chain disulfide crosslinks in γ -gliadin in flour and dough (Wieser, 2007; Beckwith *et al.*, 1965). Circular dichroism studies of α/β - and γ -gliadins in aqueous ethanolic solution have indicated the presence of some α -helices and β -sheets, and also aperiodic structures such as β -turns and random coils (Tatham & Shewry, 1985). Considerable amounts of random coil conformation have also been reported for aggregated gliadins (Tatham *et al.*, 1987). The ω -gliadins are unique among the other gluten proteins in that they lack the cysteine amino acids (Tatham & Shewry, 1985) and are deprived of disulfide linkages. The ω -gliadins have been found to be rich in β -turns and random coils (Tatham & Shewry, 1985).

2.1.5 Structure of glutenin proteins

The glutenin proteins are subdivided into low molecular weight (LMW) glutenin subunits and high molecular weight (HMW) glutenin subunits. The LMW glutenin subunits are similar to α/β -gliadins in terms of amino acid sequence and structure of the N- and C-terminal domains. The HMW glutenin subunits are unique among all glutenin proteins in terms of their structure, amino acid composition and functional properties. In the literature, the HMW

glutenin subunits are reported to be the main determinant of gluten strength and elasticity and several models explaining the elastic properties of HMW-GS have been proposed (Shewry *et al.*, 1992; Tatham *et al.*, 1985). Structural studies based on CD measurements (HMW glutenin subunits dissolved in ethanol-trifluoro-ethanol) and secondary structure predictions have shown that the central domain of the HMW glutenin subunits is rich in β -turns and that the N- and C-terminal domains are most likely globular, with few α -helices (Tatham *et al.*, 1984). A structural model has been proposed based on the elastin model, since there are structural similarities between the hexapeptide repeat motif of elastin and the repetitive domain of HMW glutenin subunits (Tatham *et al.*, 1985). Both are predicted to be rich in β -turns (Tatham *et al.*, 1984; Venkatachalam & Urry, 1981). The β -spiral conformation; a linear helical structure consisting of repeating β -turns, is suggested for elastin (Venkatachalam & Urry, 1981). The central repetitive domain of HMW glutenin subunits, which is rich in β -turns, is also suggested to adopt the β -spiral conformation, similar to elastin (Shewry *et al.*, 1992; Tatham *et al.*, 1985; Tatham *et al.*, 1984). Being rich in cysteine residues, glutenin subunits in flour and dough occur as polymers and are mainly crosslinked through inter-chain disulfide bonds (Wieser, 2007; Shewry *et al.*, 1992).

2.2 Industrial production of wheat gluten and food uses

The use of gluten for animal feed has been practiced from 1840, where the starch isolation from flour was the primary objective (Anon, 1912). The industrial potential of gluten was not realized until 1930, when Harry Maltwood (a pastry maker) from New Zealand and Nigel Love from Australia independently isolated gluten from flour and used it to enrich their bread, resulting in increased bread volume and improved texture (Wrigley, 2000). These developments in bread quality led to gluten production on a substantial scale in Australia with the help of separation methods producing 'vital' gluten, rendering its unique viscoelastic properties viable (Day *et al.*, 2006). Since then, 'vital gluten' has been a significant trade commodity world-wide for the bread industry.

With the recent increased use of wheat for production of starch and bioethanol, gluten has become industrially available as a co-product on relatively abundant scale (~50 000 tons/year). A fraction of this industrially produced gluten is utilized in bakery products to improve texture and quality, as a protein source for humans and also in animal feed. However, the use of gluten as a protein source for humans is not strongly advocated, owing to its low index of essential amino acids and the risk of coeliac toxicity.

The distinguishable characteristics of gluten compared with other cereal proteins are its viscosity, strength and elasticity. The viscoelastic characteristics of gluten have therefore been exploited in edible packaging film, due to its film formation and biodegradable abilities (Aydut *et al.*, 1991; Anker *et al.*, 1972).

2.2.1 Non-food uses

The idea of edible films leads further to the exploitation of gluten for several non-food applications in the packaging industry. There is a need to replace some of today's petroleum-derived plastic packaging due to increasing environmental concern and potential limitation of petroleum resources (Heralp *et al.*, 1995). Owing to the wide-scale production of gluten as a relatively inexpensive industrial co-product, its utilization for production of some value-added products would be important. Such use would also help a future bio-economy to be viable.

The gluten proteins have shown their potential for use in several non-food applications, such as insulating foams, adhesives, coatings and packaging films (Blomfeldt *et al.*, 2012; Lei *et al.*, 2010; Ullsten *et al.*, 2009; Anker *et al.*, 1972).

2.3 Structure-function relationship of gluten proteins

The unique functional properties of gluten, both for food and non-food applications, are largely dependent on the structure and polymerization of the proteins. During mixing, dough processing and bread making, gliadin and glutenin proteins interact in various ways through hydrogen-bonded networks stabilized by covalent interactions (disulfide and isopeptide) and form polymers of various sizes and shapes (Belton, 1999; Larroque *et al.*, 1996). Several structural models based on molecular interactions have been proposed in relation to gluten viscoelastic properties in dough and baked products such as loops and trains model by Belton (1999) and β -spiral model by Shewry *et al.* (2009).

The structure of the gluten proteins has been found to be of relevance in relation to their mechanical and barrier properties when processed into bio-based films (Kuktaite *et al.*, 2011; Sun *et al.*, 2007). Hierarchical structural arrangements of gluten into tetragonal and hexagonal assemblies have been reported for bio-based films with improved performance in terms of mechanical and oxygen barrier properties (Kuktaite *et al.*, 2012; Kuktaite *et al.*, 2011-; Ullsten *et al.*, 2009).

2.4 Impact of genotype and environmental interactions on gluten structure

Genotype (G) and environmental (E) interactions have been shown to have a significant impact on gluten protein polymerization, structure and quality in flour, dough mixing and bread-making (Johansson *et al.*, 2001; Ciaffi *et al.*, 1996; Gupta *et al.*, 1993; Lawrence *et al.*, 1987). The genotypes with HMW glutenin subunits 5+10 are reported to possess relatively strong gluten in dough and bread-making and the genotypes with HMW glutenin subunits 2+12 are reported to have relatively weaker gluten (Johansson *et al.*, 1999; Payne *et al.*, 1987). The differences in gluten quality due to the presence of different subunits are related to the number of cysteine residues present in the HMW glutenin proteins. The 5+10 subunits have 12 cysteine residues, which result in a greater amount of disulfide crosslinks compared with the 2+12 subunits, which have 11 cysteine residues (Shewry *et al.*, 1992).

Furthermore, plant development time or grain maturation period (GMP) can be manipulated by altering environmental parameters such as temperature (T) and nitrogen (N) to influence the grain protein concentration and composition (Johansson *et al.*, 2004; Dupont & Altenbach, 2003). Low T is reported to increase the length of GMP (Dupont & Altenbach, 2003). The increased GMP results in low protein concentration, but high gluten strength due to increased protein polymerization. High temperature results in shorter GMP and increased protein concentration, and also increases the gliadin to glutenin ratio (Sofield *et al.*, 1977). The increased amount of gliadin usually leads to low protein polymerization and a decrease in the complexity of the gluten polymer, resulting in relatively weak gluten (Gibson *et al.*, 1998).

3 Objectives

The overall aim of this doctoral thesis was to determine how the structure of gliadin, glutenin and gluten proteins could be fine-tuned through various modifications and thereby modulate the protein functionality in bio-based materials (films and foams). The structure and polymerization of gliadin, glutenin and gluten in proteins were elucidated on a length scale ranging from micro (20 μm) to nano (0.45-8 nm), using a range of techniques including confocal laser scanning microscopy (CLSM), X-ray scattering, infrared spectroscopy and high performance liquid chromatography (HPLC).

Specific objectives of this thesis were to:

- Study the structural conformation of industrially sourced gluten and subsequently extracted gliadin and glutenin proteins and develop a structural model for the gliadin and glutenin proteins.
- Modify the structure of gliadin, glutenin and gluten proteins with glycerol and chemical additives (NH_4OH and salicylic acid), and evaluate the effect of structural changes on mechanical properties of the corresponding films.
- Evaluate polymerization and structural differences in bio-based films and foams produced from gliadin and glutenin proteins.
- Evaluate the impact of gluten separation procedure on the structure and functional properties of gluten in bio-based films.
- Determine the interactive effect of G and E on gluten protein polymerization and structure in bio-based films, and evaluate how the structural changes influence the functionality of bio-based films.

4 Methodology

4.1 Protein sources

Industrially sourced gluten (I-Gluten) was kindly supplied by Lantmännen Reppe, Sweden. This I-Gluten was further fractionated into gliadin and glutenin proteins, based on the gliadin fraction being soluble in 70 % ethanol and the glutenins being left as a 'residual fraction' after gliadin extraction (Papers II-IV). During industrial separation of gluten from flour, shear force and high temperature are employed. In Paper V, a mild separation treatment without shearing and drying at high temperature was used to separate gluten from flour. Furthermore, two genotypes (Diskette and Puntari) differing in HMW glutenin subunit composition were cultivated in the greenhouse and biotron under different N and T regimes to produce gluten of various strengths (Paper VI).

4.2 Bio-based material production

Gliadin and glutenin proteins were dispersed in distilled water, the pH was adjusted to 11 and the mixture was heated to 75 °C. Parallelepiped-shape foams were produced by placing the dispersed proteins in silicon molds and freeze-drying them for at least 24 h, as described by Blomfeldt *et al.* (2011). Various sourced gliadin, glutenin and gluten films were produced by mixing the proteins with 30 % glycerol and compression molding at 130 °C for 10 minutes in a hydraulic press. In the samples where NH₄OH and salicylic acid (SA) were used as additives, the NH₄OH (5 %) was mixed with glycerol and SA (1 %) was mixed with protein powder. The protein-Gly-NH₄OH-SA blend was then molded into films (Paper IV).

4.3 Analytical tools

Size exclusion high performance liquid chromatography (SE-HPLC)

Previous studies have developed a three-step extraction procedure by using sodium dodecyl sulphate (SDS)-phosphate buffer in the first extraction and repeated sonication in the second (30 s) and third (30+60+60 s) extractions to investigate the polymerization and size distribution of gluten proteins (Blomfeldt *et al.*, 2011). The extractions were analyzed by SE-HPLC (Papers II-VI), which provided information on both the protein size distribution and solubility in the three steps.

Reverse phase high performance liquid chromatography (RP-HPLC)

Six serial extraction steps were used to determine the protein solubility in various solvents (Papers II-VI). Data on the solubility of proteins in various solvents gave insights into the type of crosslinking and protein polymerization pattern. A relationship between protein solubility in different solvents and type of crosslinking present in proteins was also established (Figure 5).

Confocal laser scanning microscopy (CLSM)

The films and foams were analyzed by CLSM, gliadin proteins and the HMW-glutenin subunits immunolabelled with polyclonal and monoclonal antibodies, respectively, were used to study the microstructural pattern (polymerization) of proteins (Papers III and VI).

Fourier transform infra-red (FT-IR) spectroscopy

Structural changes in the amide I spectra ($1600\text{-}1700\text{ cm}^{-1}$) of proteins were determined by FT-IR (Papers II-VI). Based on these changes, the secondary structure of proteins was investigated.

Small-angle X-ray scattering (SAXS)

The MAX IV Laboratory synchrotron, Lund, Sweden, was used to study the morphological changes in proteins at nano-scale (Papers II-VI).

Tensile testing

The tensile properties of films were tested by conditioning the samples at 50 % relative humidity for at least 48 h and then cutting the films into a minimum of eight dumbbell-shaped tensile specimens. The specimens were tested on an Instron machine using either the 50 or 100 Newton load cell (Papers IV-VI). The stress strain curves were recorded and the data obtained were processed to calculate Young's modulus (E-modulus), maximum stress and strain at break.

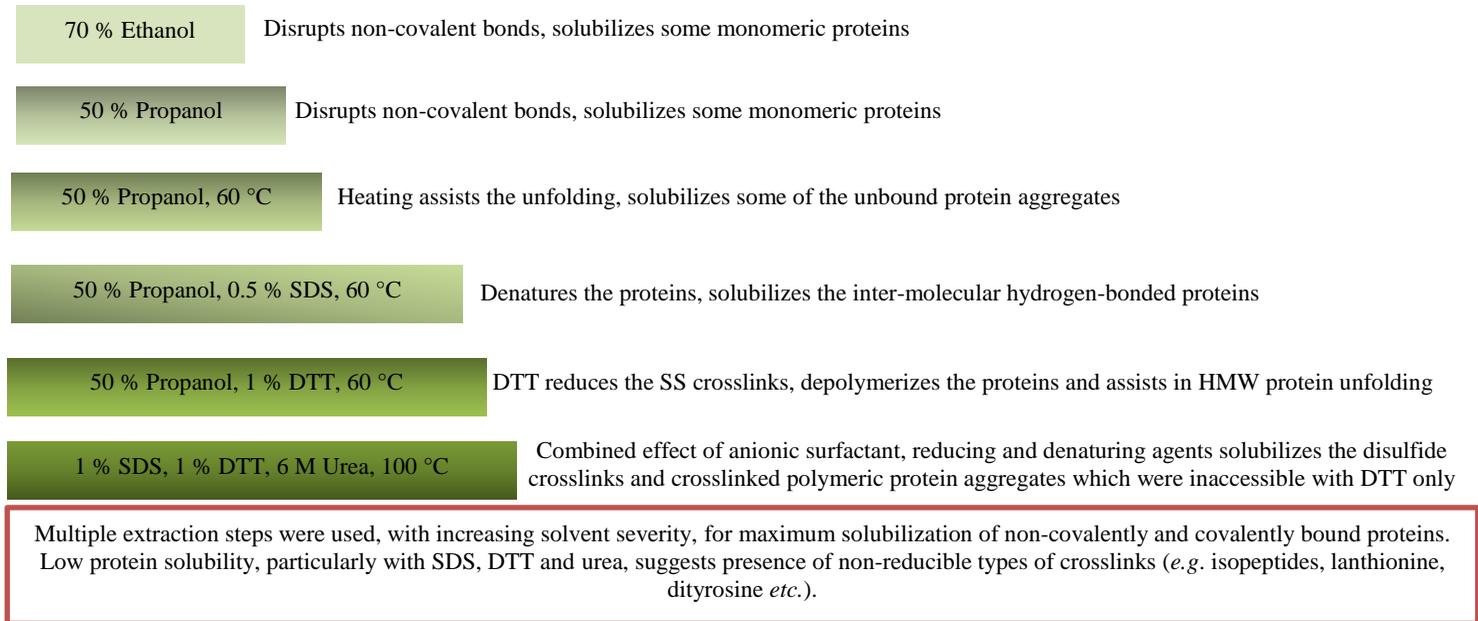


Figure 5. Protein solubilization in various solvents and relationship to protein crosslinking behavior.

5 Results and Discussion

5.1 Structural architecture of unprocessed gluten proteins

A number of factors such as processing, G and E factors during wheat cultivation influence the gluten polymer structure in various applications, which is related to end properties (Paper I). During the formation of the gluten polymer in dough mixing and bread making, both the gliadin and glutenin proteins take part in polymer formation through thiol-sulphur interchange (SH/SS) reactions. When the gluten is used for the production of bio-based materials, additional structural changes due to processing and chemical modifications led to formation of polymers of various sizes and shapes, such as tetragonal and hexagonal assemblies (Paper I).

To understand the mechanism which triggers the various structural changes in gluten polymer, detailed understanding and structural modelling of its components gliadin and glutenin proteins are important (Paper II). Proteins of both monomeric and polymeric sizes were found to be present in gliadin, glutenin and I-Gluten in unprocessed form (dry powder) (Figure 6). However, total amount of extractable proteins was lower in glutenin and gluten than in gliadin. The gliadin proteins in unprocessed form were found to be non-aggregated, and thereby readily extractable in SDS-phosphate buffer with and without sonication (Figure 6). The glutenin proteins were mainly of a polymeric nature, although some non-covalently bound monomers were also extracted in SDS-phosphate buffer without sonication. Gluten is a mixture of gliadin and glutenin showing intermediate extractability, although lower than for gliadin proteins (Figure 6). Owing to low solubility of glutenin and gluten proteins even with sonication, the SE-HPLC profile indicated aggregation of the proteins in unprocessed glutenin and gluten. However, solvents with reducing and denaturing agents solubilized these proteins by accessing and dissolving the inter-molecular SS crosslinks (Paper II). The IR spectra showed α -helical or random coil conformation of unprocessed gliadin, glutenin and I-

Gluten (Paper II). The literature also suggests mainly α -helical or random coil conformation of gluten proteins while in dry/unhydrated form (Zhu *et al.*, 2006; Shewry *et al.*, 2002). The SAXS and wide angle X-ray scattering (WAXS) data (down to 0.45 nm) also revealed the amorphous nature of unprocessed gliadin, glutenin and gluten proteins and the unorganized nano-structural pattern (Paper II). Based on the above findings and structure predictions through the bioinformatics algorithms I-TASSER (Roy, 2010) and Phyre2 (Kelley and Sternberg, 2009), in Paper II a structural model was proposed for unprocessed gliadin and glutenin proteins extracted from I-Gluten powder (Figure 7).

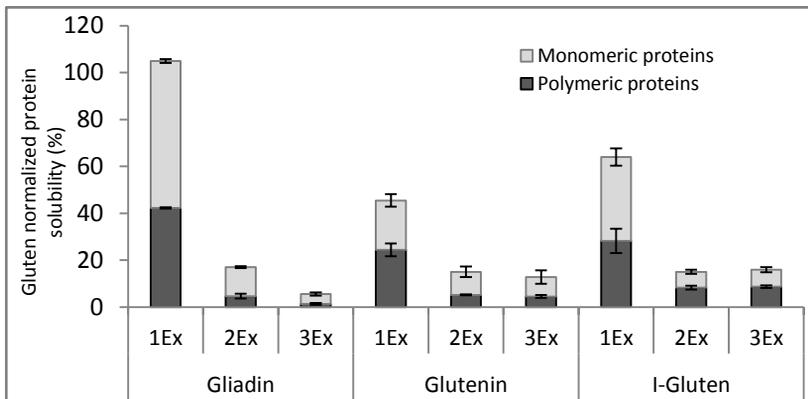


Figure 6. Protein solubility and size distribution of gliadin, glutenin and industrial gluten (I-Gluten) proteins. 1Ex-3Ex = extractions 1-3.

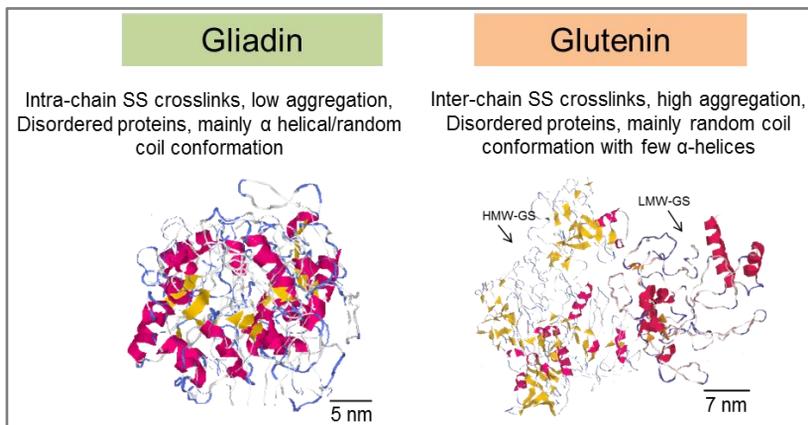


Figure 7. Structural properties of gliadin and glutenin proteins. Structural models reproduced from Paper II with the permission of the publisher.

5.2 Structural rearrangements in gliadin- and glutenin-based films and foams

The structure of gliadin and glutenin proteins showed molecular crosslinking rearrangements and various degrees of aggregation when molded into films with 30 % glycerol and processed into foams (Papers II and III).

Compared with unprocessed gliadin and glutenin proteins (Figure 6), the films from gliadin and glutenin revealed substantially higher polymerization of both the monomeric and polymeric protein content, as indicated by low solubility in all three extraction steps (Figure 8). The low solubility of proteins indicates an aggregated protein matrix where both the monomeric and polymeric proteins polymerize through inter-molecular SS crosslinks in gliadin films and additional irreversible crosslinks for glutenin films (Papers II and IV).

Compared with films, gliadin and glutenin foams indicated a lower degree of aggregation for both the monomeric and polymeric protein content (Figure 8), although the aggregation was higher than for unprocessed gliadin and glutenin proteins. The high degree of aggregation and crosslinking in films compared with foams can be attributed to the high molding temperature (130 °C; Papers II and IV) applied during the film formation process, compared with the low temperature (75 °C) applied during the foaming process (Paper III). Compared to unprocessed material, transition from random coil/ α -helical conformation to formation of β -sheet structures in films and foams was also observed in this thesis (Papers II and III). A transition from random coil to formation of some secondary structures in gluten proteins while in solubilized form has been reported previously (Blanch *et al.*, 2003). The secondary structure of films and foams was also related to the degree of aggregation, particularly in terms of β -sheet formation (Papers II and III). A positive correlation between protein aggregation and formation of secondary structure, particularly β -sheet formation, was observed, showing consistency with previous studies (Blomfeldt *et al.*, 2011; Kuktaite *et al.*, 2011). The gliadin films revealed periodic nano-structural organization in the form of hexagonal assemblies (Figure 9). The presence of hexagonal structures in protein-based films not treated with chemical additives was reported for the first time in Paper II. Although, extruded gluten films treated with chemical additives such as urea, NH_4OH and SA displayed hexagonal morphology in previous studies (Kuktaite *et al.*, 2012; Kuktaite *et al.*, 2011). Films from glutenin proteins and foams from both gliadin and glutenin proteins did not show organized nano-scale morphology (Figure 10). The molecular crosslinking and structural

changes in gliadin and glutenin proteins processed into films and foams are summarized in Figure 10.

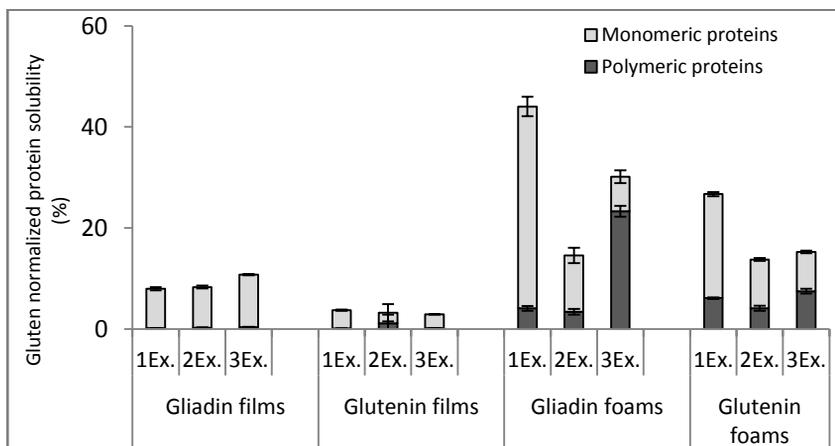


Figure 8. Protein solubility and size distribution measured by SE-HPLC in glycerol-plasticized films and freeze-dried foams 1Ex-3Ex= extractions 1-3.

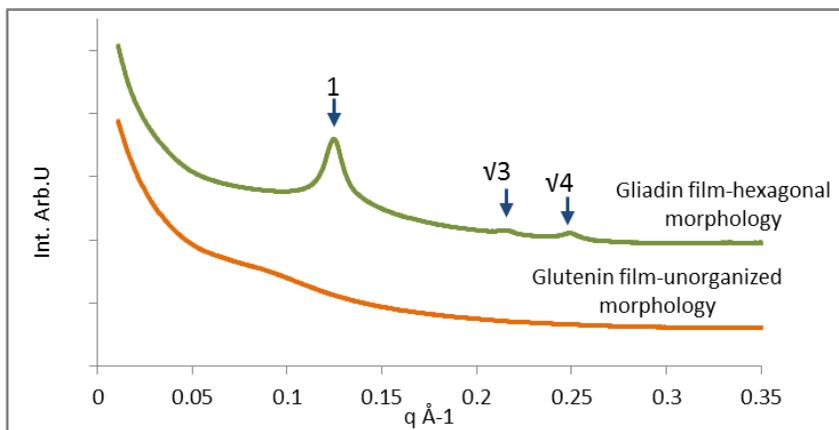


Figure 9. Results of SAXS analysis of gliadin and glutenin films.

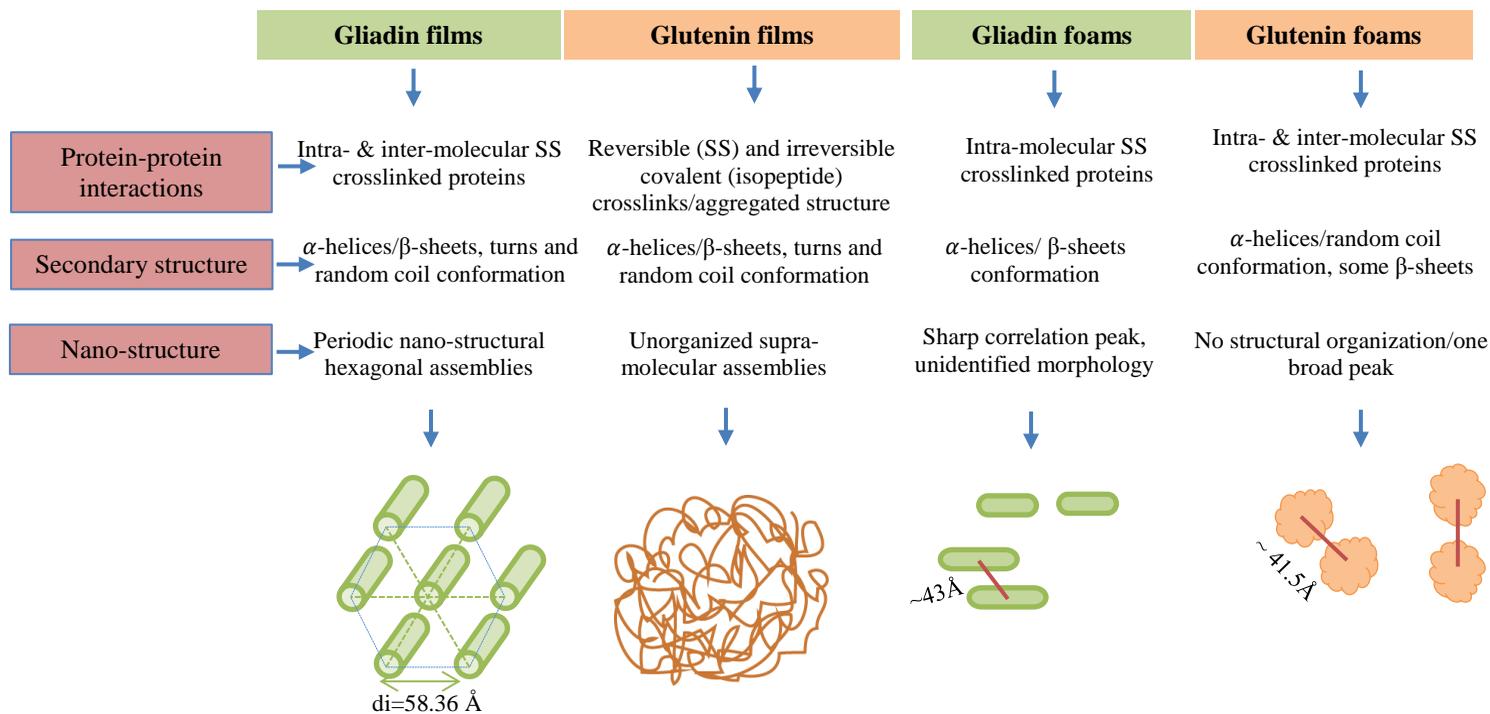


Figure 10. Molecular crosslinking and structural properties of gliadin-based and glutenin-based films and foams.

5.3 Impact of alkaline additives on protein structure

A glycerol-NH₄OH-SA blend was used as an additive to modify the structure of gliadin, glutenin and I-Gluten while molded into films (Paper IV). Higher protein solubility was recorded for gliadin, glutenin and I-Gluten films with the glycerol-NH₄OH-SA additive compared with films with glycerol only (Figure 11). However, the solubility was drastically lowered compared with the unprocessed proteins (Figure 11 compared with Figure 6). The changes in protein network formation due to addition of chemicals suggest low solubility of gluten proteins (Micard *et al.*, 2001). When the solubility of films was studied by RP-HPLC in solvents with reducing and denaturing agents (Paper IV), the results revealed that the additives NH₄OH-SA unfolded the polypeptide chains and provided the opportunities for rearrangements of molecular crosslinks. Films showed presence of both reducible covalent crosslinks through thiol-sulphur interchange (SH/SS) reactions and also non-reducible LAN or lysinoalanine (LAL) crosslinks. Gliadin films with a glycerol-NH₄OH-SA additive showed a higher number of SS crosslinks than glutenin and gluten films with the same additive. Glutenin and I-Gluten films with the additives glycerol-NH₄OH-SA were rich in non-reducible crosslinks. For I-Gluten films, the results found were consistent with previous reports of formation of LAN and LAL crosslinks at alkaline pH or with alkaline additives (Rombouts *et al.*, 2013; Ullsten *et al.*, 2009).

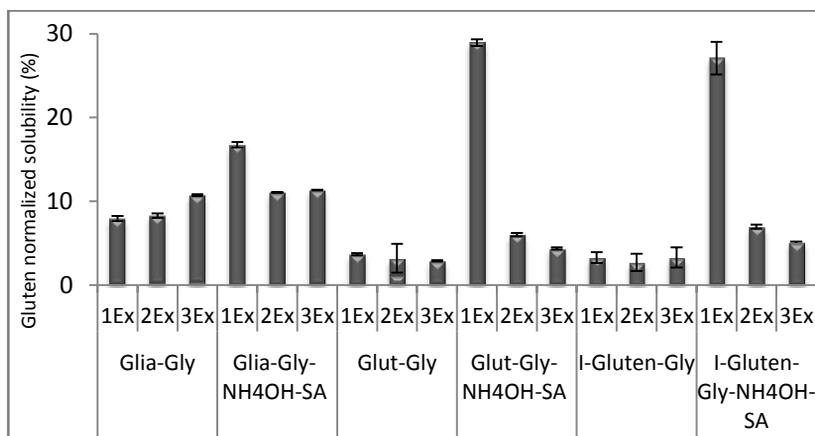


Figure 11. Protein solubility in gliadin (glia), glutenin (glut) and I-Gluten films with glycerol (Gly)-ammonium hydroxide (NH₄OH)-salicylic acid (SA) additives. Protein solubility normalized to unprocessed I-Gluten. 1Ex-3Ex = extractions 1-3.

Gliadin films containing glycerol-NH₄OH-SA showed both the hexagonal structural features ($d_1=65 \text{ \AA}$), and also additional not previously observed structural features with an interphase distance of 50 \AA at nano-structural level (Figure 12). The glutenin and I-Gluten films with the glycerol-NH₄OH-SA showed a tendency toward organized structures (Figure 12), but the scattering objects were not co-related to defined morphologies (Paper IV).

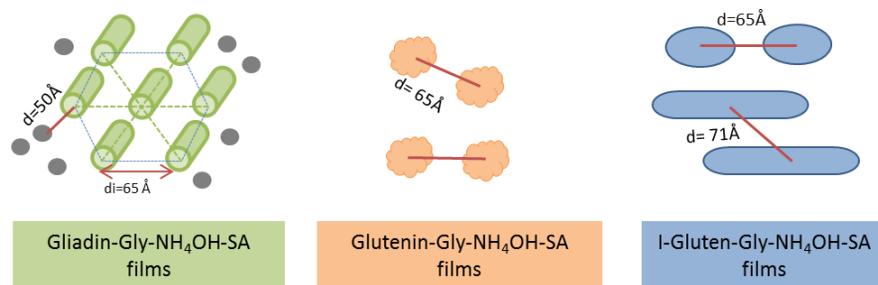


Figure 12. Schematic representation of gliadin, glutenin and gluten proteins morphology in films with glycerol (Gly)-ammonium hydroxide (NH₄OH)-salicylic acid (SA) additives.

The morphology of glutenin and gluten films (Paper IV) was concluded to be a polymerized and crosslinked protein structure where the crosslinks consisted of both disulfide and irreversible bonds. For both glutenin and gluten, the use of glycerol-NH₄OH-SA as an additive together with compression molding did not seem to be sufficient to break and rearrange the original structure of the polymerized proteins (Paper IV). However, when the same additive blend was used with gluten and the films were extruded instead of compression molded, this resulted in hexagonal structures as reported by Kuktaite et al. (2011), indicating that the original crosslinking of the proteins was broken and rearranged. The gliadin protein, which is non-aggregated (unfolded) in unprocessed form (see section 5.1), underwent molecular rearrangement when molded with glycerol-NH₄OH-SA additive, resulting in defined nano-structures such as hexagonal assemblies and additional structural units (Paper IV). Thus, it is of relevance to evaluate whether glutenin and I-Gluten proteins can be depolymerized to a certain extent before film processing or during film formation in order to obtain specific supra-molecular arrangements at nano-scale.

The use of glycerol-NH₄OH-SA additive with gliadin and glutenin proteins during film formation resulted in considerably higher β -sheet content compared with films without additives, particularly for gliadin films (Figure 13). The gliadin-glycerol-NH₄OH-SA films displayed a broad shoulder in the 1624-

1632 cm^{-1} region that corresponds to amide bonds in extended β -sheet conformation (Surewicz & Mantsch, 1988).

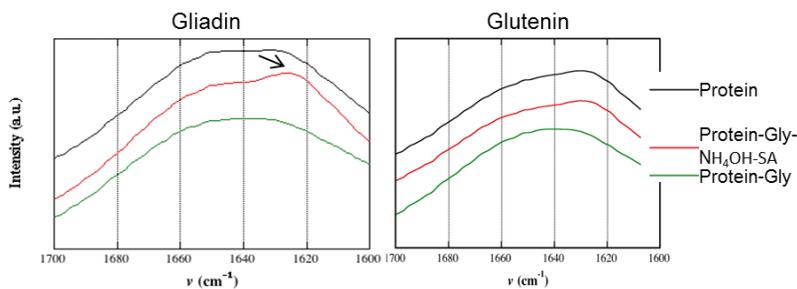


Figure 13. Amide I spectra of gliadin and glutenin proteins with and without glycerol (Gly)-ammonium hydroxide (NH_4OH)-salicylic acid (SA). Arrow indicates the β -sheet signal.

5.4 Effect of separation procedure on gluten polymerization and structural morphology

High solubility was recorded for unprocessed mildly separated gluten (M-Gluten), compared with the relatively lower solubility of I-Gluten (Figure 14). This solubility difference indicates that the separation treatments affected the gluten structure, with the M-Gluten being non-aggregated and the harshly treated (at T: $\sim 70^\circ\text{C}$) I-Gluten being of pre-crosslinked/aggregated structure (Paper V). Previous studies suggest aggregation and molecular crosslinking rearrangements of gluten proteins treated at high temperature (Sofield *et al.*, 1977).



Figure 14. Protein solubility and size distribution in mildly separated (M-Gluten) and industrially sourced (I-Gluten) gluten proteins. 1Ex-3Ex = extractions 1-3.

Proteins of monomeric and polymeric size were both extractable in films from mildly separated gluten (M-Gluten-Gly), while only proteins of monomeric size were extractable from I-Gluten film (I-Gluten-Gly) (Figure 15). This indicates that the pre-crosslinked protein network in I-Gluten influenced protein polymerization during film formation, limiting the opportunities for molecular crosslinking among monomeric and polymeric proteins (Paper V).

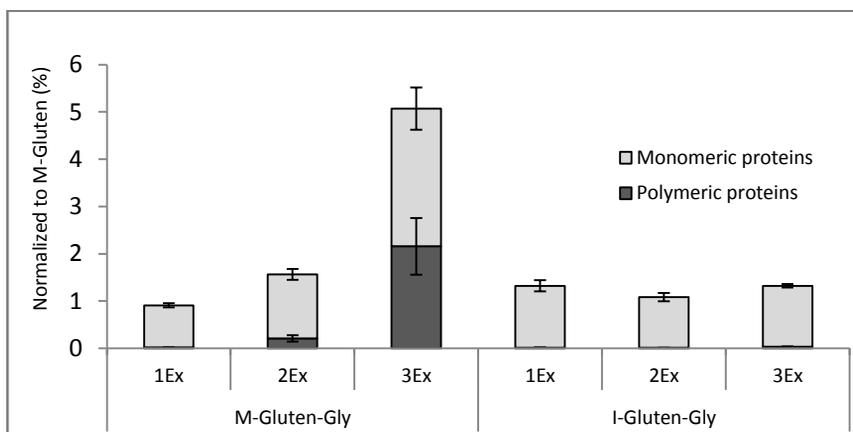


Figure 15. Protein solubility and size distribution in the films produced from mildly separated gluten (M-Gluten-Gly) and industrially sourced gluten (I-Gluten-Gly) with added glycerol (Gly). 1Ex-3Ex = extractions 1-3.

Furthermore, SAXS analysis revealed a series of well-defined peaks for films from mildly separated gluten (Figure 16). A bi-structural morphology with both hexagonal and lamellar structures (indicated by green and orange arrows, respectively, in Figure 16) was revealed for films from mildly separated gluten (Paper V). Lamellar morphologies have been observed previously in starch-based systems (Oostergetel & van Bruggen, 1989), while Paper V is the first study to report them for proteins. Furthermore, the two-phase system for gluten-based films has not been reported previously. No defined nano-scale morphology was revealed for films from I-Gluten. It can be speculated that the pre-crosslinked polypeptide chains in the unprocessed I-Gluten restricted the molecular rearrangements and defined nano-structural morphology when molded into films (Paper V).

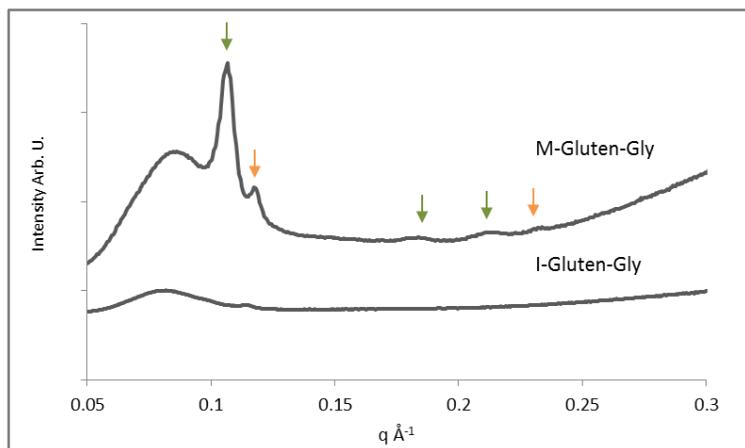


Figure 16. Results of SAXS analysis for films produced from mildly separated gluten (M-Gluten-Gly) and industrially sourced gluten (I-Gluten-Gly).

5.5 Protein nano-structural morphology and its relationship to protein unfolding (denaturation)

As stated in section 5.3, using a non-aggregated starting material for gliadin proteins assisted the formation of bi-structural nano-scale morphology, both as hexagonal and additional decoupled structural units. The same phenomenon was seen for M-Gluten, where the non-aggregated starting material when molded into films resulted in the formation of defined nano-structural morphology, both as hexagonal and lamellar structures. It can be expected that use of non-aggregated or denatured starting material of mildly separated gluten provides opportunities for molecular and structural rearrangements while being processed into films, resulting in the two-phase system also seen in the case of the Gliadin-Gly-NH₄OH-SA film (Figure 17). In general, non-aggregated starting material can be considered important to obtain materials with organized structural assemblies. It is reported in the literature that a certain degree of unfolding or denaturation in proteins is important to rearrange the structure and obtain required functionality, such as strength and water barrier properties (De Graaf, 2000).

Furthermore, high total solubility of the proteins from the films was also seen in both cases (Figure 18a). The protein solubility in various solvents measured by RP-HPLC was also similar in both film types (Figure 18b). A relationship between protein solubility in various solvents and crosslinking pattern in proteins was established in the present thesis (Figure 5; Papers II, IV-VI). The solubility and crosslinking pattern of both types of films indicated a

non-covalently (hydrogen bonding, *van der Waals* interactions) and covalently bound protein matrix (mainly SS crosslinks), as demonstrated by SE-HPLC and RP-HPLC (Figure 18). The films with defined nano-structures, such as M-Gluten films and Gliadin-Gly-NH₄OH-SA films, also exhibited higher mechanical properties than films without defined nano-scale morphologies.

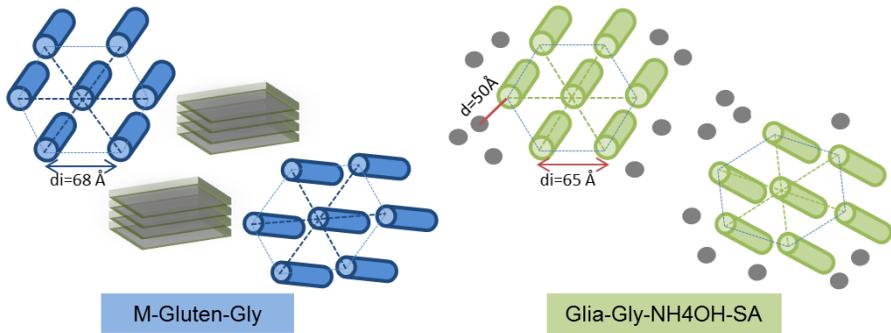


Figure 17. Morphology of gluten films prepared from mildly separated gluten (M-Gluten) and gliadin with glycerol (Gly)-ammonium hydroxide (NH₄OH)-salicylic acid (SA) additives.

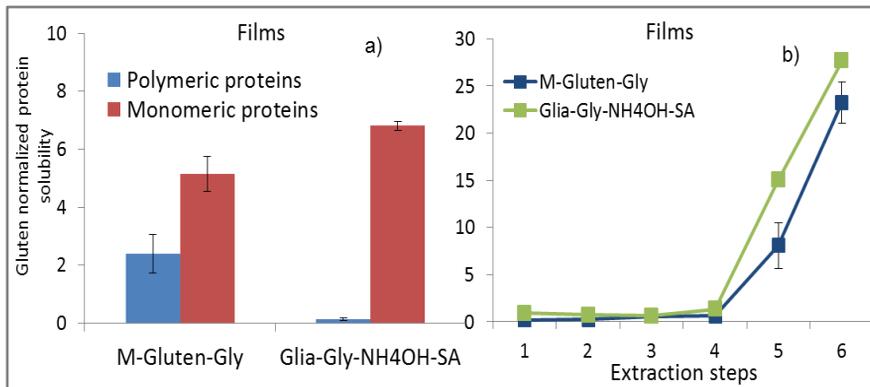


Figure 18. Solubility of protein films measured by SE-HPLC and RP-HPLC.

5.6 Genetic and environmental interactions governing gluten structure in films

The impact of various G and E interactions was found to influence percentage of unextractable polymeric proteins (%UPP) and structure of gluten polymer in flour, dough and bread making (Paper I). This inspired us to examine how G and E interactions affect gluten structure and quality in films (Paper VI). The %UPP value is also an indicator of gluten strength in flour, with higher %UPP values indicating relatively greater gluten strength for dough and bread making (Malik *et al.*, 2013; Johansson *et al.*, 2005).

The highest variability in %UPP for gluten films from various treatments and genotypes was observed for G x E interactions rather than the independent impacts of each factor (Figure 19). The impact of E on variation in %UPP was higher in films from the Diskette (5+10) gluten than in films produced from the Puntari (2+12) gluten (Figure 20).

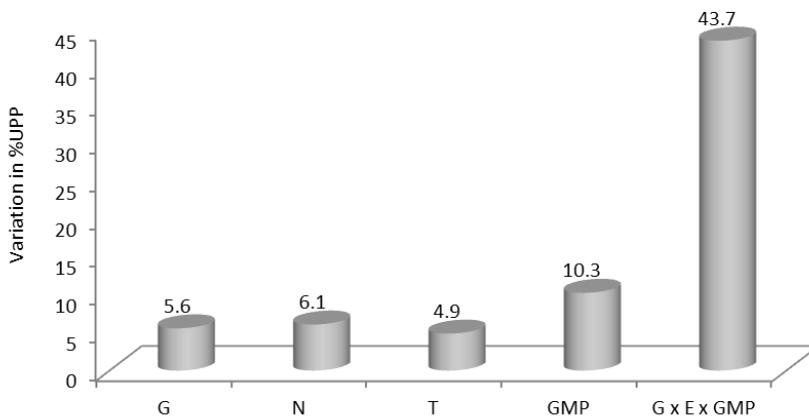


Figure 19. Variation in percentage of polymeric proteins extractable with sonication (%UPP) in total polymeric proteins in films. G: genetics, N: nitrogen, T: temperature, GMP: grain maturation period, E: environment (T and N).

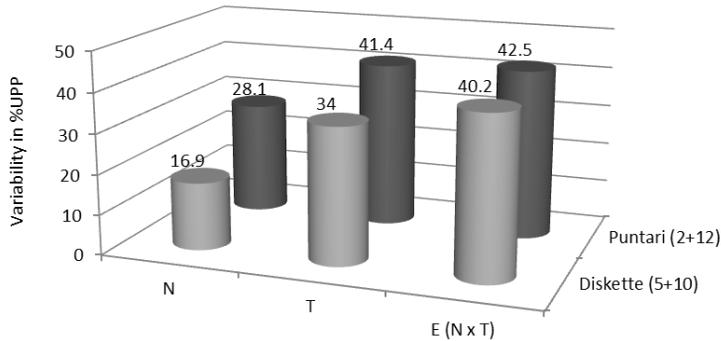


Figure 20. Variation in percentage of polymeric protein extractable with sonication (%UPP) in total polymeric proteins in films. N: nitrogen, T: temperature, E: environment.

Interactions between G and E also caused differences between films in formation of protein nano-structures and secondary structures, particularly β -sheets. Films produced from Diskette (5+10) gluten under low N and high T showed relatively high β -sheet content (Figure 21), while the E-modulus and tensile strength were higher than for all other films (Paper VI). Films from Puntari (2+12) gluten under high N and low T treatment (Figure 21) showed relatively high β -sheet content and higher E-modulus and tensile strength compared to other Puntari gluten films (Paper VI). The differences observed in structural and functional properties of films under the impact of various G and E interactions are summarized in Figure 21.

5.7 Functionality of proteins in bio-based films

The structural variations in gliadin, glutenin and gluten proteins were also correlated to the mechanical properties of films. Changes induced in protein aggregation and molecular crosslinking rearrangements have been reported in previous studies to influence the mechanical properties of films (Sun *et al.*, 2007; Pommet *et al.*, 2005). Our results showed that proteins rich in disulfide crosslinks and with specific structural assemblies at nano-scale also have high mechanical performance.

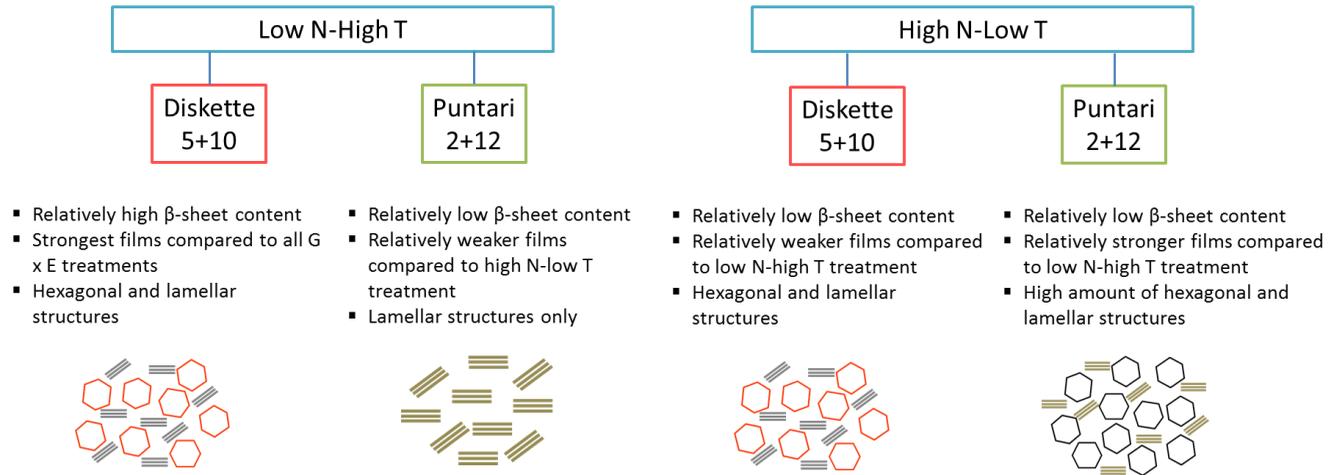


Figure 21. Influence of interactions between genetic (G) factors (Diskette and Puntari wheat genotypes) and environmental (E) factors [nitrogen (N), temperature (T)] on the structural and functional properties of gluten films.

5.7.1 Strength and stiffness

Among variously produced gluten films, the highest E-modulus was observed for films produced from mildly separated gluten (Figure 22, Table 2). Elastic modulus is related to stiffness of a material. An increase in stiffness or E-modulus can be expected due to increased protein crosslinking density, mainly through non-covalent (hydrogen bonds) and covalent crosslinks (mainly intermolecular SS bonds). The strength (max. stress) of gluten was in general high for the films produced from mildly separated gluten or from gluten produced from genotypes grown under various environments and also extracted by the mild separation treatment (Table 2). The strength of films is co-related to local deformation stability. The cohesive protein network through intermolecular crosslinking reactions decreases the likelihood of local failure such as crack formation, resulting in increased tensile strength (Woerdeman *et al.*, 2004).

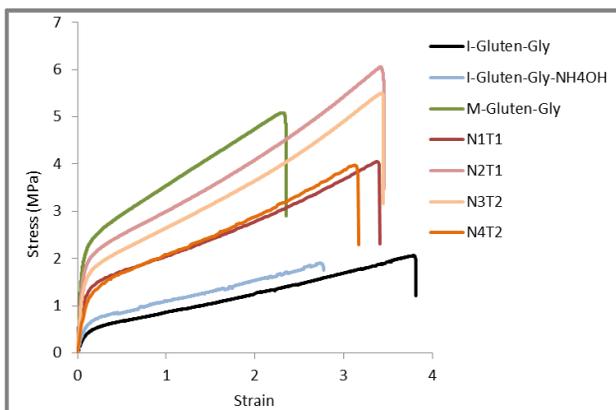


Figure 22. Representative tensile curves for various gluten films. N1, N2: No and low nitrogen, N3, N4: high nitrogen, T1: high temperature (25/19 °C), T2: low temperature (18/14 °C).

The films from the same genotype grown under different nitrogen and temperature regimes showed significant variations in mechanical properties, particularly for strength and stiffness (Paper VI) (Table 2). This indicates that G and E interactions, resulting in differences in gluten quality, also governed differences in the mechanical performance of films.

The films with high strength and stiffness showed bi-structural morphology at nano-scale. For example, hexagonal and additional structural units in the case of the Gliadin-Gly-NH₄OH-SA film and the hexagonal and lamellar structures in the case of the M-Gluten-Gly film in combination with different G and E interactions were correlated to high strength and stiffness. This structural

morphology is unique in protein-based systems and to our knowledge has not been reported previously, particularly not in relation to functional properties. The amount of β -sheets was also substantially higher in these films. A structure-function relationship can be suggested here, whereby films with organized morphology at nano-scale, such as hexagonal and lamellar structures and high β -sheet content, also possess high mechanical properties.

Table 2. *Mechanical properties of gliadin (Glia), glutenin (Glut) and gluten films with glycerol (Gly)-ammonium hydroxide (NH₄OH)-salicylic acid (SA) additives. N1,N2: No and low nitrogen, N3,N4: high nitrogen, T1: high temperature (25/19 °C), T2: low temperature (18/14 °C). All films were produced with same glycerol level (30 %). Standard deviation shown in brackets.*

Film type	E-modulus (MPa)	Max. stress (MPa)	Elongation (%)
Glia-Gly	4.1 (1.1)	2.1 (0.2)	489 (44)
Glia-Gly-NH ₄ OH-SA	14.2 (2.2)	4.3 (0.3)	398 (37)
Glut-Gly	12.1 (1.7)	3.0 (0.2)	215 (31)
Glut-Gly-NH ₄ OH-SA	15.2 (0.9)	3.1 (0.2)	203 (10)
I-Gluten-Gly	5.6 (0.4)	2.0 (0.1)	389 (32)
I-Gluten-Gly-NH ₄ OH-SA	6.9 (0.4)	1.8 (0.1)	293 (18)
M-Gluten-Gly	43.4 (4.8)	5.2 (0.4)	242 (13)
Gluten-N1T1	19.0 (1.3)	4.0 (0.3)	321 (33)
Gluten-N2T1	34.3 (3.8)	6.1 (0.3)	343 (19)
Gluten-N3T2	24.0 (1.5)	5.1(0.5)	315 (30)
Gluten-N4T2	12.7 (1.4)	3.5 (0.2)	299 (17)

5.7.2 Extensibility

The gliadin films in general showed higher elongation to break compared with the gluten or glutenin films (Table 2). The glutenin films showed the lowest elongation to break values of all films tested. This may be co-related to degree of aggregation, as the highest aggregation was observed for glutenin films and gliadin films showed relatively lower aggregation. Thus, presence of a highly aggregated protein network may influence the extensibility of protein chains, resulting in relatively lower elongation to break. The films with high elastic modulus in general exhibited lower elongation to break (Figure 22, Table 2), which suggests a typical elastomer relationship with the exception of films from gluten at low nitrogen and high temperature and Gliadin-Gly-NH₄OH (Table 2). In general, the behavior of films studied in this thesis was similar to rubbery materials which usually possess Max. stress ~5 MPa with high elongation to break (Pommet *et al.*, 2005).

5.7.3 Color

Other qualitative characteristics, beside mechanical properties, which are often considered important, particularly for packaging purposes, are film transparency and color. The films produced from various sources in the present thesis exhibited a range of colors from transparent to dark-colored, without the use of coloring agents (Figure 23). The gliadin films were fairly transparent when molded with glycerol. The glutenin and gluten films molded with glycerol only were light brown. The gliadin, glutenin and gluten films showed a dark brown color when molded with the Gly-NH₄OH-SA. Maillard reactions occurring among amino acids and reducing sugars are responsible for browning of products (Fogliano *et al.*, 1999). Maillard reactions are a complex cascade of several chemical reactions and many reactive intermediate compounds are formed, leading to generation of polymerized brown pigments mainly known as “melanoidins” (Fayle & Gerrard, 2002). The dark color of films with additives observed in this thesis (Figure 23) was most likely due to Maillard reactions owing to exposure of amino acid functional groups to saccharides (present in minor amount in gluten), as previously reported for whey protein coatings (Trezza & Krochta, 2000).

The range of functional properties such as strength, stiffness, extensibility and color described in this thesis make the gluten proteins a potential candidate for use in state-of-the-art applications in the materials industry, either as protein coatings, packaging films or insulating foams.



Figure 23. Films with various colors produced in this thesis work. Photo: Faiza Rasheed.

6 Concluding remarks

Natural polymers such as gluten proteins are remarkably diverse in their structural, chemical and mechanical properties. Specifically, their potential to undergo a wide range of structural and chemical modifications makes them outstanding for uses in a range of products such as films, foams, coatings and adhesives. The structural architecture of gliadin, glutenin and gluten proteins was determined here at various scales from polymeric aggregates up to molecular crosslinking level (Figure 24). The results clearly show that the structure of polymers such as proteins is an indicating force, modulating the functionality of end-products. The main conclusions and key findings in this thesis were as follows:

- The protein solubility and size distribution of gliadin, glutenin and gluten proteins were an indication of protein aggregation in both unprocessed proteins and processed materials; the lower the solubility, the higher the protein aggregation.
- The gliadin films showed unique nano-structure morphologies such as hexagonal structures when no chemical additives were added, and showed additional structural units with addition of $\text{NH}_4\text{OH-SA}$. The structural morphology observed for gliadin-based films has not been reported previously.
- Structural models for unprocessed and modified gliadin and glutenin proteins were proposed for the first time.
- The gliadin films produced with additives $\text{NH}_4\text{OH-SA}$ exhibited high SS crosslinks, but the glutenin and gluten films were rich in non-reducible crosslinks.
- Addition of $\text{NH}_4\text{OH-SA}$ resulted in high tensile performance of films, particularly gliadin films.

- A positive correlation between protein crosslinking density (polymerization) and amount of β -sheets was observed for most of the films.
- Gluten separated through a mild separation procedure while in unprocessed form exhibited non-aggregated structure, in contrast to the aggregated structure of unprocessed industrial gluten. The impact of separation treatment on gluten structure and functionality is reported for the first time in this thesis and was found to be of high relevance for gluten performance in materials.
- The mildly separated gluten in unprocessed form exhibited nano-structural morphology as lamellar structures not reported previously for proteins.
- The mildly separated gluten-based films showed up to 600 % higher elastic modulus values and up to 160 % higher tensile strength compared with industrial gluten-based films.
- The proteins with the lowest degree of aggregation in starting material (such as gliadin or mildly separated gluten) exhibited organized nano-scale morphology when processed into films.
- Factors such as plant development time, nitrogen amount and timing, and temperature were found to influence grain protein polymerization and concentration, resulting in variations in gluten quality.
- The varying quality glutes obtained through different G x E interactions showed polymerization and structural differences when processed into bio-based films. The gluten separated from cultivars grown at low temperature and with high nitrogen showed high β -sheet content and nano-scale morphology in the form of hexagonal and lamellar structures when processed into films.
- Environmental factors had a more pronounced influence on protein polymerization and structural properties of gluten in films than genetic factors.
- The films with bi-structural nano-scale morphology showed high mechanical properties compared with the films with unorganized morphology.

The qualitatively different proteins, protein blends, protein-additive blends, separation techniques, and G and E interactions tuned the gluten proteins structure into various morphologies, resulting in a range of mechanical properties.

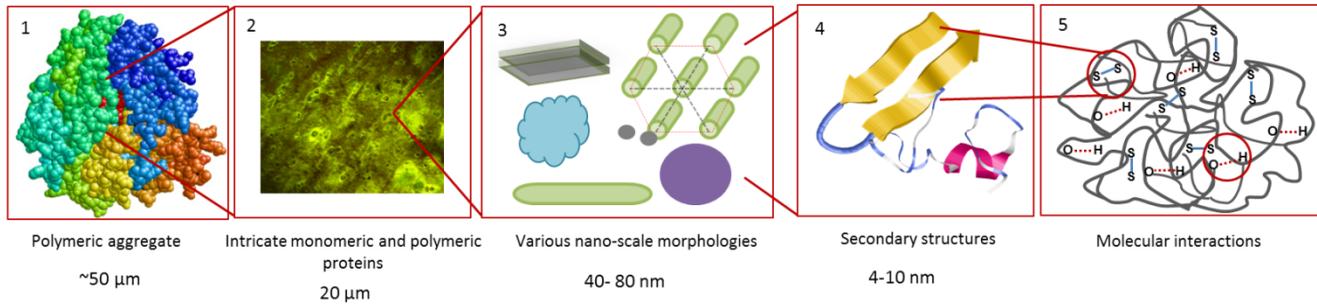


Figure 24. Schematic representation of protein structure hierarchy at length scale range from 50 μm to 1 nm. 1) Aggregated protein model generated with Rasmol, 2) protein microstructure by confocal laser scanning microscopy (CLSM), 3) various nano-scale morphologies of gluten proteins, 4) protein secondary structures generated with Rasmol and 5) protein-protein interactions at atomic scale.

7 Future prospects

- It would be interesting to expand the structure-function relationship of gluten proteins for applied products in the bio-materials and materials industry, particularly for packaging films and medical healthcare devices.
- Studies are needed on how biological organisms such as bacterial systems, viruses and cells can be used to synthesize gluten proteins, to potentially eliminate the food versus materials conflict.
- New ways to fabricate high performance materials and devices from gluten proteins through biotechnological processes by specifically increasing the strength of gluten also need to be developed.
- With the formation of specific nano-structural features, gluten proteins offer great potential to be utilized in bio-inspired nanomaterials and nanotechnology. However, a detailed understanding of the origin of nano-structural features in gluten proteins through high throughput microscopic techniques is needed.
- Keeping in mind the coeliac toxicity effect of gluten proteins, a careful evaluation of the impact of using gluten proteins for food packaging purposes should be made (due to possible traces in food).
- It would be interesting to evaluate whether ribonucleic acid interference technology such as RNAi can be used to silent the genes responsible for the synthesis of the potentially allergen part of gluten proteins, in order to safely implement the proteins for packaging purposes.

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ڈاکٹر کا جو ٹائٹل مجھے ملے گا وہ حقیقتاً آپ کے لئے ہے۔