The effects of salts on the formation of gluten structure during hydration

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis/project is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Helen Cynthia Dewi Tuhumury

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“The fear of the LORD is the beginning of knowledge, but fools despise wisdom and discipline” (Proverbs 1:7, NIV).
Publications and presentations

Journal publications


Fully refereed conference proceedings papers


Other conference presentations


Abstract

Health-related issues regarding high salt intake including high blood pressure and associated cardiovascular diseases have been the reason for the growing research on salt reduction in different food products. However, in dough-based products, reducing or eliminating salt from the formulation is highly challenging. This is because salt (in the form of NaCl) is used in the processing of wheat-based foods, not only for enhancing sensory taste, but also for its technological functions. Previous studies have been conducted to examine the effect of NaCl on wheat flour dough properties and the properties of gluten protein fractions and the amount of solubilised or aggregated gluten proteins as affected by salts. However, it is not clear whether the rheological properties and structural changes of the gluten in the presence of NaCl are due to the contribution of either sodium or chloride ions. Therefore, a basic understanding of how salt influences the formation of gluten network and the rheological behaviour of the dough is needed. This can lead to developing ways to maximise the technological functionality of these salts in replacing NaCl.

Accordingly, this thesis is based on the need to establish a knowledge and understanding of the function of salts on gluten structural network during hydration at the molecular level. Rheological, microstructural, and chemical properties of the gluten as a function of salt have been investigated. In addition, the effects of salts belonging to the Hofmeister series on gluten and wheat flour dough have been evaluated to enhance the understanding the functionality of gluten as it is controlled by salts.

The effect of salt particularly NaCl on the structure and rheological properties of gluten were investigated by obtaining gluten samples from two wheat flours, with different levels of total protein, in the presence or absence of sodium chloride (2% flour base). The dynamic oscillation rheology, large extensional deformation, confocal laser scanning microscopy, transmission electron microscopy and chemical analysis of disulfide bond linkages and the ratio of polymeric glutenins and monomeric gliadins were used to investigate the effect of salt on the structure and rheological properties of
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gluten. The effect of NaCl on the gluten structure and rheology has shown that NaCl increased the non-covalent interactions of the gluten and the β-sheet structure which results in different molecular conformation, fibrous network structure, hence differences in rheological properties. On the basis of these results, it is proposed that NaCl causes conformational changes as water molecules are drawn away from the gluten to interact with sodium and chloride ions. The hydrogen bonding and hydrophobic interactions could be the reason for the increase in the β-sheet structure within gluten. This proposed mechanism results in the formation of the gluten with a typically more closely aligned structure.

The rheological properties of the gluten with and without NaCl during heating have also been investigated. Both the rehydrated and fresh gluten samples were prepared in the presence and absence of NaCl during mixing and washing. In addition, the starch was added back to the gluten samples with and without NaCl to determine the effect of residual starch in the gluten network as influenced by NaCl. Gluten network formed in the presence of NaCl determine its rheological properties during heating. Changes in the gradual decrease in G’ and G’’ values up to certain temperature and the onset of the sharp increase in those values during heating are the results of the extent of hydrogen bond formation as a function of NaCl. The delay of the sharp increase in the G’ to higher temperature during heating is the result of the formation of the gluten network in the presence of NaCl, rather than vii of the presence of the residual starch in the gluten network.

Different cation salts were used to investigate the effects of the Hofmeister salt series on gluten network formation. The work was carried out by comparing the effects of cation salts on both wheat flour dough mixing properties, as well as the rheological, and the chemical properties of the gluten extracted from the dough with the respective salts. The effects of different cations on dough and gluten different flours generally followed the Hofmeister series. Despite the differences observed in mixing properties and microstructure of dough with different cation salts, the impacts of cations on gluten structure and dough rheology at large deformation at the levels tested were relatively
small. Among the different cations, the $K^{+}$ gave a similar microstructural resemblance and effect on large deformation rheological properties of both dough and gluten samples including the extensibility, resistance to extension, and the strain hardening behaviour to that of NaCl.

Different anion salts were used to investigate the effects of Hofmeister salt series on gluten network formation. The work was carried out by comparing the effects of anion salts on both wheat flour dough (mixing properties and microstructure), and the properties of the gluten extracted from the dough with the respective salts (chemical and rheological properties). Hofmeister anion salts influence the gluten network formation in a way that they cause changes in gluten protein composition, as well as the percentage of the unextractable polymeric protein fraction by interacting directly with specific amino acid residues. These changes consequently result in the remarkable differences in the dough mixing profile, microstructural features of the dough, the small deformation rheological properties of the gluten, as well as the strain hardening behaviour of dough and gluten samples.

The effect of Hofmeister anion salts on gluten network formation are more pronounced than the Hofmeister cation salts. These effects of the anions compared to the cations on gluten structure may be due to the ability of the anions to bind with the more positively charged of the amino acid residues which are present in the gluten protein composition and hence the aggregation of gliadins to glutenins, while cations are weakly bound or do not bind at all and contribute to the changes in structure by interfering with water structure.

Based on the microstructural, dough mixing profile, rheological properties at small deformation and large deformation as well as the gluten size distribution studies with two different series of Hofmeister salts, this study for the first time provides a strong basic understanding on how NaCl influences gluten network formation. Overall, the implications from this present study indicate that anions are important in determining the structure and functionality of the gluten as well as wheat flour dough. Therefore, the
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chloride ions of NaCl cannot be readily replaced by other anions of sodium salts. On the other hand, the NaCl could be replaced by other cations of chloride salts, particularly KCl. This is because the cations of chloride have relatively similar effects on dough rheology and functionality. These findings now contribute significantly to the formulation of strategies to reduce the sodium intake in the production of wheat-based foods without causing deleterious effects on the functionality of the dough.
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Abbreviations

A Absorbance
A Area
AACC American Association of Cereal Chemists
ANOVA Analysis of variance
ATR Attenuated total reflectance
b Cell path length
C Concentration
CaCl₂ Calcium chloride
CCD Charge-coupled device
CLSM Confocal laser scanning microscope
Da Dalton
DMAE Dimethylethanolamine
Dₘₐₓ Maximum distance
DTNB 5,5’-Dithiobis-(2-Nitrobenzoic Acid)
EDTA Ethylenediaminetetraacetic acid
ERL Cycloaliphatic Epoxide Resin
ESEM Environmental scanning electron microscope
F Force
F Phenylalanine
FITC Fluorescein isothiocyanate
Fₘₐₓ Maximum force
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSANZ</td>
<td>Food Standards Australia New Zealand</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infra-red</td>
</tr>
<tr>
<td>G</td>
<td>Glycine</td>
</tr>
<tr>
<td>G’</td>
<td>Storage modulus</td>
</tr>
<tr>
<td>G’’</td>
<td>Loss modulus</td>
</tr>
<tr>
<td>Gli</td>
<td>Gliadins</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutenins</td>
</tr>
<tr>
<td>GU</td>
<td>Gasograph units</td>
</tr>
<tr>
<td>HMW-GS</td>
<td>High molecular weight-glutenin subunits</td>
</tr>
<tr>
<td>k</td>
<td>Strain hardening coefficient</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>L</td>
<td>Length</td>
</tr>
<tr>
<td>L</td>
<td>Leucine</td>
</tr>
<tr>
<td>LMW-GS</td>
<td>Low molecular weight-glutenin subunits</td>
</tr>
<tr>
<td>LVR</td>
<td>Linear viscoelastic region</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MCR</td>
<td>Modular compact rheometer</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>N</td>
<td>Newton</td>
</tr>
<tr>
<td>n</td>
<td>Strain hardening index</td>
</tr>
<tr>
<td>NaBr</td>
<td>Sodium bromide</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>Amonium chloride</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>Sodium dihydrogen phosphate</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Disodium hydrogen phosphate</td>
</tr>
<tr>
<td>NaI</td>
<td>Sodium iodide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>Na₂SO₃</td>
<td>Sodium sulphite</td>
</tr>
<tr>
<td>NSA</td>
<td>Nonenyl succinic anhydride</td>
</tr>
<tr>
<td>NSW</td>
<td>New South Wales</td>
</tr>
<tr>
<td>NTSB²⁻</td>
<td>2-nitro-5-thiosulfobenzoate</td>
</tr>
<tr>
<td>P</td>
<td>Proline</td>
</tr>
<tr>
<td>PES</td>
<td>Polyethersulfone</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>S-rich</td>
<td>Sulfur rich</td>
</tr>
<tr>
<td>S-poor</td>
<td>Sulfur poor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SE-HPLC</td>
<td>Size exclusion-high performance liquid chromatography</td>
</tr>
<tr>
<td>SH</td>
<td>Sulfhydryl</td>
</tr>
</tbody>
</table>
Abbreviations

SS  Disulfide
SW  Salt-washed
T   Threonine
Tan δ  Phase angle
TEM  Transmission electron microscope
Tris-HCl  Tris hydrochloric acid
UK  United Kingdom
UPP  Unextractable polymeric protein
USA  United States of America
UV  Ultra violet
VIC  Victoria
VIS  Visible
WW  Water-washed
Y   Tyrosine
Δl  Change in length
v/v  Volume by volume
w/w  Weight by weight
α   Alpha
β   Beta
γ   Gamma
ω   Omega
ε   Strain
ε   Molar extinction coefficient
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sigma$</td>
<td>Stress</td>
</tr>
<tr>
<td>$\sigma_0$</td>
<td>Stress amplitude</td>
</tr>
<tr>
<td>$\gamma_0$</td>
<td>Strain amplitude</td>
</tr>
<tr>
<td>$\delta$</td>
<td>Loss angle</td>
</tr>
<tr>
<td>$\omega$</td>
<td>Oscillation frequency</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Dynamic viscosity</td>
</tr>
</tbody>
</table>
Explanatory notes

The purpose of these notes is to briefly describe the approaches adopted during the preparation of this thesis. These include issues of spelling and expression as well as formatting styles including those used in the scientific literature:

1. Where alternative spellings are in common use then the British rather than the American approach has been adopted in the text. Examples include the term colour (rather than color), words ending with –ise (rather than –ize) and some technical terms.

2. For presentation of experimental results, SI units have been used throughout this thesis.

3. In the citation and listing of references and information sources, the current recommendations to authors for Food Chemistry (published by Elsevier) have been applied throughout except for the chapter 6 which has been published in the Journal of Cereal Science.

4. In relation to a number of other specific formatting issues, it was also decided that, for this thesis, the style would be adopted from the instructions to authors currently recommended for manuscripts submitted the Journal of Food Science. This approach was used on the basis that most of other well recognised journals do not clearly define requirements to cover these issues. The specific formatting adopted from this source include:

   - Space between number and degree sign (for example: 120 °C);
   - Space between ± and number (for example: 29 ± 1 °C);
   - Space between measurement and number (for example: 13 mm); and
   - No space between % and number (for example: 94%).
Chapter 1

Introduction

The purpose of this chapter is to provide a brief overview of the research program described in this thesis regarding the effects of salts on the formation of the gluten network. The work has involved investigating the development of gluten structure during hydration, network formation at a molecular level and the resultant functional properties as it is affected by salts. This project has been developed on the basis of the following issues:

- High dietary salt intake has been identified as a significant issue for human health globally. The amounts required by adults for good health are 6 g/day and, despite the reported risks, salt consumption often exceeds these levels in Australia and other developed nations;

- It has been estimated that around 75% of the salt we eat is already in the foods we buy. The salt is primarily contained in processed food and as much as 25% of the salt intake in western diets is from cereal products;

- It is increasingly important for food manufacturers to develop products with reduced salt contents. However, sodium reduction is difficult to achieve since salt generally has a significant technological role in the processing and stability of these products as well as contributing to the appealing taste;

- In dough-based products, reducing or eliminating salt from the formulation has adverse effects on network formation and rheological properties and subsequently the product quality including loaf volume, crumb structure and texture.

- Salt plays an important role in the processing of cereal based foods. Salt strengthens the mechanical properties of hydrated gluten protein network, decreases yeast
activity in dough, thus retarding gas production, while influencing final product quality and enhancing flavour;

- There has been a variety of studies carried out regarding the effect of salts on rheological properties of gluten or dough. It has been shown that salt concentrations and particular salts belonging to the Hofmeister series affect dough/gluten rheology and gluten properties. Salts increase dough strength and stability as determined by mixing (Mixograph and Farinograph) and extension parameters of the dough and are cultivar specific. These reports are described further in subsequent chapters;

- Models describing gluten molecular structure have been developed, along with others outlining changes in the molecular structure of gluten proteins during hydration and mixing;

- One of the challenges of studying the roles of the molecules in a dough matrix during processing/mixing has been the availability of methods to characterise protein structure forming in a continuously changing environment. Various studies have focussed on microstructural changes of dough/gluten proteins using microscopic methods;

- In spite of the large number of excellent studies dedicated to characterising structure and functionality of gluten proteins, most, if not all, have investigated the situation at the completion of mixing, after the gluten structure and network has been formed. The important processes of gluten protein network formation during the first initial hydration have not been elucidated;

- There are still limited findings regarding the effects of salts on gluten at a molecular level, and the protein network and hierarchical structure formation during the very first key step of processing which is dough hydration. Gaps remain in our knowledge and understanding of the relationship between the structure (observed at various scales of magnification) and functionality of gluten proteins, particularly how it is controlled by salt;
• NaCl is the combination of the sodium and chloride ions. Since the relatively important health implication of salt is directly associated with the sodium ion, it is therefore necessary to understand the function of both of the two different ions on the formation of gluten structure and its functionality; and

• The common strategy to reduce the sodium content in wheat based foods is to replace a proportion of NaCl with other chloride salts of different cations including potassium chloride in the formulation. Although the strategy has provided some success, a basic understanding of how different salts influence the formation of the gluten network and the rheological behaviour of the dough currently lacking. Such an understanding is needed in order to maximise the technological functionality of these salts in replacing NaCl. It is not clear whether the rheological properties and structural changes of the gluten in the presence of NaCl are due to the contribution of the sodium or the chloride ions.

Accordingly, this thesis is based on the need to establish a knowledge and understanding of the function of salts on the gluten structural network during hydration at the molecular level. Rheological, microstructural and chemical properties of the gluten as a function of salt are investigated. In addition, the effects of salts belonging to the Hofmeister series on gluten and wheat flour dough have been evaluated to enhance our understanding of the functionality of gluten as it is controlled by salts.
Chapter 2

Background and literature review: An overview of wheat gluten properties, rheology and microstructure

This chapter provides background and review of previous scientific research relevant to the studies reported in this thesis. The areas covered consist of properties of wheat gluten, particularly focusing on rheology and microstructure.

2.1. Gluten properties

Wheat flour is almost unique amongst cereal flours in possessing characteristics that enable it to be used for preparing an extensive range of baked products. The principal characteristic that governs its wide usage is its viscoelasticity when hydrated. The component in the flour that possess the property of viscoelasticity is the gluten (Attenburrow, Barnes, Davies, & Ingman, 1990; Day, Augustin, Batey, & Wrigley, 2006). Knowledge of the gluten structure is essential for understanding the way gluten proteins interact with each other and with other flour constituents.

The term gluten maybe defined as the cohesive, viscoelastic proteinaceous material remaining when dough is washed to remove starch and water soluble constituents (Day, Augustin, Batey, & Wrigley, 2006; Weiser, 2007). In practice, the term gluten refers to the hydrated state of the storage proteins present in wheat which then determine the viscoelastic properties of the dough (Letang, Piau, & Verdier, 1999; Shewry, Halford, Belton, & Tatham, 2003; Weiser, 2007). The dry solid material of prepared gluten contains 75-85% protein and 5-10% lipids, while most of the reminder is starch and non-starch carbohydrate (Weiser, 2007). The storage proteins in wheat flour which become a part of the gluten matrix when hydration occurs are the gliadins and glutenins. However, it is noted that in common usage, the storage proteins of the wheat endosperm are sometimes referred to as gluten. Reflecting their significance during breadmaking, the gluten proteins have been widely studied over an extended period of time in order to
determine their structures and properties as well as providing a basis for manipulating and enhancing end use product quality (Shewry, Halford, Belton, & Tatham, 2002).

### 2.1.1. The classification of gluten proteins

Wheat protein was first analytically separated into four fractions by Osborne (1907), and the resultant materials were then termed as the Osborne fractions. In this approach, wheat flour proteins were characterised according to differences in their solubility: albumins which are soluble in water; the globulins being soluble in water and NaCl solutions; gliadins soluble in 70% ethanol and glutenins, partly soluble in dilute acid or alkali.

As knowledge of the gluten proteins expanded, it was ultimately found that these are responsible for the unique ability of wheat to be made into bread (Baier-Schenk, Handschin, von Schönau, Bittermann, Bächli, & Conde-Petit, 2005). As a result, gluten proteins have been widely studied. The gluten proteins are the major storage proteins of wheat and two functionally important and distinct groups of gluten proteins can be differentiated: monomeric gliadins and polymeric glutenin (Lindsay & Skerritt, 1999; Shewry & Halford, 2002).

Both fractions are important contributors to the rheological properties of dough and are largely responsible for the ability to process wheat into a range of food products including bread, pasta, and noodles. However, their functions are different. Gliadins when hydrated give the viscosity and extensibility properties to the dough system, while glutenins are elastic and cohesive and determine dough strength and elasticity. A suitable mixture or balance of both fractions is necessary to provide the viscoelastic properties of dough and the quality of the final product (Weiser, 2007).

Based on their mobilities on sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE), the gliadins have been classified into three classes, the α, γ, and ω-gliadins, while the glutenins are categorised as either high molecular weight glutenin subunits (HMW-GS) or low molecular weight glutenin subunit (LMW-GS). In
addition, both groups have also been classified according to three broad groups called sulphur rich (S-rich), sulphur poor (s-poor), and high molecular weight prolamin (Figure 2.1).

Figure 2.1  Classification of gliadin and glutenin subunits in wheat flour
Sourced from Lindsay and Skerritt (1999)

2.1.1.1. Gliadins

Gliadins are essentially monomeric proteins since they are present as monomers. They can be classified as ω5-, ω1,2-, α/β-, and γ-gliadins based on their mobility during gel electrophoresis as well as on evaluation of complete or partial amino acid sequences (Cornell, 2003; Shewry, Halford, Belton, & Tatham, 2002; Weiser, 2007). Gliadins have extremely low solubility in water and neutral buffers but are largely soluble in 70% v/v ethanol. The extracted products are of sticky structure. They consist of single polypeptide chain capable of some intra-molecular disulfide bonding (Cornell, 2003). The characteristics of different gliadins are presented in Table 2.1.

Within each type of gliadin, there are small structural variations as a result of substitution, deletion and insertion of single amino acid residues. Studies on the
secondary structure of gliadins have pointed out that the N-terminal domains α/β-gliadins, γ-gliadins and ω-gliadins are characterised by β-turn conformation (Tatham, Miflin, & Shewry, 1985).

### Table 2.1 Characterisation of gliadins

| Type         | Molecular Weight $\times 10^3$ | Proportion *(|%|) | Partial amino acid composition (%) |
|--------------|--------------------------------|----------------|-----------------------------------|
| ω5-gliadins  | 49-55                          | 3-6             | Q: 56, P: 20, F: 9, Y: 1, G: 1   |
| ω1,2-gliadins| 39-44                          | 4-7             | Q: 44, P: 26, F: 8, Y: 1, G: 1   |
| α/β-gliadins | 28-35                          | 28-33           | Q: 37, P: 16, F: 4, Y: 3, G: 2   |
| γ-gliadins   | 31-35                          | 23-31           | Q: 35, P: 17, F: 5, Y: 1, G: 3   |

Notes 1 * with respect to total gluten proteins  
2 Sourced from Weiser (2007)

#### 2.1.1.2. Glutenins

Extensive research on gluten proteins has clearly revealed the molecular characteristics of glutenin fraction and has shown that this fraction is an important factor in understanding and controlling those characteristics which determine end-use quality. It has been found that the subunits, HMW-GS and LMW-GS can be obtained after treatment of glutenin with a disulfide reducing agent such as mercaptoethanol. HMW-GS have apparent molecular weights within the range of 80,000-120,000 Da, while the molecular weights of LMW-GS are approximately 40,000-55,000 Da (Shewry, Halford, Belton, & Tatham, 2002; Veraverbeke & Delcour, 2002; Weiser, 2007). However, any biochemical approaches which may be used to elucidate glutenin structure are obstructed due to the extremely large size and insolubility of the component proteins. The best explanation obtained so far is that glutenins are a heterogenous mixture of HMW-GS and LMW-GS that form disulfide-linked polymers. The polymeric features
are determined by the both intra- and inter-molecular disulfide bonds, a feature which clearly differentiates them from the gliadin proteins (Shewry, Halford, & Tatham, 1992).

In relation to HMW-GS, currently the locations of the genetic materials have been reported and these are encoded in single loci within three parts of the wheat genome (A, B, and D), with each locus comprising two genes encoding subunits which differ in their properties and are called x-type and y-type subunits. These two types can be differentiated by both their N-terminal and C-terminal residues and the central repetitive domains which confer elasticity to the structures formed by the protein molecules. The N- and C-terminal are richer in charged residues and contain most of the cysteine residues which are responsible for the disulfide bond in the subunits. The N-terminal sequence of the X-type subunits contains 4 cysteine units and that of the N-terminal part of Y-type subunits contains 5 cysteine residues. Repeating sequences of hexa- and nona-peptides in the central domain also characterise y-type subunits (PGQGQQ and GYYPTSLQQ), while the x-type subunits are characterised by hexa-, nona-, and tripeptides (PGQGQQ and GYYPTSLQQ, and GQQ) (Anjum, Khan, Din, Saeed, Pasha, & Arshad, 2007; Shewry, Halford, Belton, & Tatham, 2003). These repeating sequences are responsible for the hydrogen bonding interactions with other gluten proteins (Kasarda, 1999). The HMW-GS have been reported to account for 12% of the total grain protein, corresponding to 1-7% of the flour dry weight. These are minor components in term of quantity, but they are key factors in the process of bread making because they are major determinants of gluten elasticity (Shewry, Halford, Belton, & Tatham, 2003; Tatham, Miflin, & Shewry, 1985).

The studies of the HMW-GS have received considerable attention, but glutenins also contains LMW-GS and their significance appears to have been underestimated. Despite less attention on its characterisation, there are four sub-fractions of LMW-GS and these are referred to as A, B, and C, D. The important features in LMW-GS are that they can
act as chain extender and chain terminator of the glutenin polymerisation processes. The D-LMW-GS, with 1 free cysteine functions as chain terminator, hence some of the LMW-GS may determine the size of the glutenin (D'Ovidio & Masci, 2004; Kasarda, 1999). The characteristics of glutenin proteins are shown in Table 2.2.

### Table 2.2 Characteristics of glutenins

<table>
<thead>
<tr>
<th>Type</th>
<th>Molecular Weight $\times 10^3$</th>
<th>Proportions * (%)</th>
<th>Partial amino acid composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>x-HMW-GS</td>
<td>83-88</td>
<td>4-9</td>
<td>Q: 37, P: 13, F: 0, Y: 6, G: 19</td>
</tr>
<tr>
<td>y-HMW-GS</td>
<td>67-74</td>
<td>3-4</td>
<td>Q: 36, P: 11, F: 0, Y: 5, G: 18</td>
</tr>
</tbody>
</table>

Notes:  
1. *According to total gluten proteins  
2. Sourced from Weiser (2007)

### 2.1.2. Gluten network development

A great deal of work has been conducted on how to explain the structural network of the gluten. So far, it has been generally agreed that the structure of this network basically relies upon glutenins which provide the structural backbone. Glutenin subunits are aggregated in some way to form large polymeric features and polymerisation of the glutenin chains occurs via inter-molecular disulfide bonds (Lindsay & Skerritt, 1999). It was first hypothesised by Ewart (1968) that the gluten structure was due to the interactions of the long linear glutenin polymers that contribute elasticity. The HMW-GS and LMW-GS formed covalently linked polymers. The first aspect of HMW-GS structure that it is relevant to elasticity is the number and distribution of cysteine residues available to form intermolecular cross-links. This is because the degree of the cross-linking will determine the bulk elastic properties. With a low degree of cross-linking the extensibility will be high, but with increased cross-linking the material would become more rubber-like. Changes in the number of cross-links would be
expected to have major effects on the physical properties of the HMW-GS polymers (Shewry, Halford, & Tatham, 1992).

However, this structure with the disulfide cross-links cannot fully explain the elasticity of the gluten. The existence of some cross-linking may explain the resistance to extension but does not explain the elasticity. Beside the disulfide bonds as the backbone of the structure, the high level of glutamine residues has a very high capacity to form both intra- and inter-molecular hydrogen bonds. This feature then may be involved in elasticity through the formation of inter-molecular hydrogen bonds (Belton, 1999).

Taking into account these proposed mechanisms of gluten network development, a model of the glutenin aggregation has been developed by Hamer and van Vliet (2000). The model proposed the hyperaggregation of the glutenin and consisted primarily of three levels of aggregation (Figure 2.2). At level one, the HMW-GS and LMW-GS form a covalent polymer through disulfide links. Therefore, only covalent bonds are encountered in this level of aggregation. The position of the bonds is determined by protein conformation, position of the sulfhydryl groups and other stabilizing bonds including hydrogen bonds. The presence of individual subunits and their ability to proliferate or terminate the network will determine the extent of aggregation.

At the next level, larger aggregates will be formed by previously formed covalent aggregates through physical interactions and also stabilised by hydrogen bonds as well as other bonds including those involving electrostatic and hydrophobic interactions. The size of the aggregates at level one will still determine the size and incidence of the aggregates in this level. At the third level, further aggregation occurs only by physical interactions which mean that covalent bonds do not play a role. It is thought that processing conditions such as shear, stress and the role of other polymers impeding physical interactions are the predominant influences affecting the formation of aggregates at this level.
Overall, it can be summarised that differences in glutenin functionality result from differences in composition, structure or distribution of glutenin polymers (Goesaert, Brijs, Veraverbeke, Courtin, Gebruers, & Delcour, 2005). Firstly, differences in glutenin composition may result in the non-covalent interactions that determine the elasticity of glutenin. Secondly, differences in the structure of glutenin largely affect glutenin functionality during breadmaking. To a certain extent, differences in structure of glutenin may also result from differences in glutenin composition. Thirdly, based on polymer theories, only those polymers above the certain size would contribute to the elasticity of the glutenin network. Differences in glutenin size distribution may also be attributed to differences in GS composition (Goesaert, Brijs, Veraverbeke, Courtin, Gebruers, & Delcour, 2005; Veraverbeke & Delcour, 2002).

![Figure 2.2 A model for glutenin hyperaggregation](image)

Figure 2.2 A model for glutenin hyperaggregation: LMW-GS, HMW-GS, gliadin, starch, pentosans
2.1.3. Gluten network formation during dough mixing

The interaction or the aggregation model can be extended to explain events that occur during dough mixing. Dough is a complex mixture of flour constituents, water along with other ingredients. It is a primary intermediate stage in the transformation of wheat, through flour, into bread (Bloksma & Bushuk, 1988). It also can be regarded as a composite material: a continuous protein phase (gluten matrix) in which starch granules and other insoluble flour constituents are dispersed (Chiotelli, Rolé, & Le Meste, 2004; Petrofsky & Hoseney, 1995). A simplified model of the dough structure is shown in Figure 2.3.

![Figure 2.3](image_url)  
**Figure 2.3** A simplified method of the dough structure  
Sourced from Eliasson (1993)
In flour, gluten proteins exist in structures known as protein bodies (Sapirstein & Fu, 2000). Conformational arrangements of gluten proteins are evident when mechanical energy is applied to the dough during mixing (MacRitchie, 1986). There are several stages in the formation of the dough: hydration, blending, dough development, and breaking down or over-mixing (MacRitchie, 1986; Stauffer, 2007).

The suggested importance of the hydrogen bonds as mentioned by Belton (1999) has led to the development of the “loops and trains” model to describe the gluten structure that forms upon hydration (Figure 2.4). The gluten molecular model as explained by Belton (1999), firstly sees that in the dry state glutenin chains will tend to bond to each other via hydrogen bonds to form a dense mass in their native folded conformation. The assembly of hydrogen bonded chains are referred to as the train region of the structure. On addition of water, the originally closely packed protein bodies hydrate and there is an increase in the number of water-protein hydrogen bonds formed. However, the large number of remaining inter-chain hydrogen bonds will ensure that not all of these bonds are likely to break at the same time. The water-protein hydrogen bonds result in the formation of the loop structure of the model. The train regions which are regions where there are surface interactions of a group of polymer units, while the loops are the regions where there are a significant number of polymer-solvent interactions. These correspond to the $\beta$-sheet structure and to the extended $\beta$-turn structure in the NMR patterns, respectively. The proportion of the train regions decreases and the hydrated loop region increases as the hydration process continues (Belton, 1999).

Hydration of the proteins determines the development of the technologically important structure of the gluten. Hence, not only the interactions within and between the polymers but also between the polymers and water determine the structural stability of gluten. Covalent bonds, ionic forces, hydrophobic interactions and hydrogen bond are types of interactions that stabilise gluten polymers directly (Belitz, Kieffer, Seilmeier, & Wieser, 1986). On the other hand, gluten proteins can be indirectly stabilised by the structure of the water present within the gluten network (Balla, Razafindralambo, Blecker, & Paquot, 1998).
As the dough is further mixed, the network formed during hydration undergoes several changes. The properties of glutenin proteins have also been used to describe the gluten development during dough formation. A sketch of the mechanisms involved in dough formation is provided in Figure 2.5. Glutenins are more or less folded at the beginning of the mixing process (a). As mixing time increases (b), the polymers tend to align because of the shear and stretching forces applied. Cross-links between proteins are formed and enhanced which result in increased dough strength. Longer mixing times (c) can lead to the breaking of disulfide bonds which are holding the polypeptide subunits together and this results in the partial depolymerisation of the glutenin proteins.
Biochemical studies have clearly demonstrated that the glutenin polymer is composed of aggregates of the glutenin subunits that are susceptible to cleavage during mixing especially at the disulfide bonds between HWW-GS and LMW-GS rather than those bonds within an aggregate of a single class of glutenin subunits. It has also been suggested that the B-LMW-GS are more susceptible to cleavage than C-LMW-GS because the latter re-associate with the glutenin polymer after being released (Lindsay & Skerritt, 1999).

Overall, gluten network development will result from disaggregation or depolymerisation of the glutenin component and its further interaction with gliadin. In the mixing process, the gliadin which interacts with glutenins acts as a plasticiser by weakening the interaction occurring within glutenin aggregates (Sapirstein & Fu, 2000; Stauffer, 2007). The involvement of the gliadin interacting with the glutenin aggregates can be described as shown in Figure 2.6.

Figure 2.5  Molecular interpretation of gluten development during mixing
Sourced from Letang et al. (1999)
2.2. Rheology of dough and gluten

2.2.1. The importance and the principles of dough rheology

Rheology is the study of the behaviour of a material or deformation of matter under a force which is governed by stress, strain and time (Dobraszczyk & Morgenstern, 2003; Sahin & Sumnu, 2006; Steffe, 1996). In order to measure the rheological behaviour, a controlled, well-defined deformation or strain is applied to a material over a given time and the resulting force is measured. Alternatively, it can be the other way around to give an indication of material parameters including stiffness, modulus, viscosity, hardness, strength or toughness of the material (Dobraszczyk & Morgenstern, 2003).

The terms stress and strain have different meaning. On one hand, stress (\(\sigma\)) is the intensity of force components acting on a body. This parameter is expressed in units of force per unit area and it is independent of the size and shape of the specimen.

\[
\sigma = \frac{F}{A} \quad \text{Equation 2.1}
\]
Where $\sigma$ is stress (N/m\(^2\)), $F$ is the force applied (N), and $A$ is the area of the face of the specimen where the force is applied (m\(^2\)). There are three common types of stresses: compressive (directed toward material), tensile (directed away from the material), and shearing (directed tangentially to the material). On the other hand, strain is the change in size and shape of a body in response to the applied force.

$$\varepsilon = \frac{\Delta l}{L} \quad \text{Equation 2.2}$$

Where $\varepsilon$ is strain, $\Delta l$ is the change in length of the specimen (m), and $L$ is the initial length of the specimen. There are also three types of strains: compressive, tensile, and shear (Bushuk, 1985).

An elastic material will deform if a stress is applied to it, and if the stress is removed the material will recover to its original dimensions (Menjivar, 1990; Mohsenin, 1986). On the contrary, a viscous material will also deform when the stress is applied but it never regains its original dimensions on removal of stress (Mohsenin, 1986). This is caused by the irreversible deformation of the material, which for a viscous material is time dependent.

Viscosity of a material refers to its resistance of flow as indicated by the ratio of shear stress to shear rate in the fluid. The relevance of rheology lies in its application for both industry and cereal science. Rheological measurements have traditionally been used to give some indication of the probable baking quality of dough (Khatkar, Bell, & Schofield, 1995; MacRitchie, 1992). Rheological properties of materials depend on the structural arrangement of constituents and forces between them.

The fundamental rheological properties of wheat flour doughs are important in determining both the handling properties of the dough during processing and the quality of the finished product. This is useful because rheology can be related to product functionality. Many rheological tests have been used to attempt to predict final product
quality and these include mixing behaviour, sheeting and baking performance (Dobraszczyk & Morgenstern, 2003).

An important point is that two types stress are involved in dough rheology: shear and extensional stresses (Figure 2.7). In shear stress, opposing forces are applied parallel to each other, in opposite directions to the matrix element. In extensional stress, the opposing forces are applied in the opposite directions, but at the opposite faces of the matrix element. The rheological instruments which use shear stress as the dominant mode are the Mixograph and Farinograph, whereas the Extensograph and Alveograph use extensional stress to the dough.

**Figure 2.7**  **Diagram of shear and extensional deformation**  
Sourced from Staufer (2007)

On the basis of studies of the rheological response of wheat and gluten doughs, they are considered as non-linear viscoelastic material (Faubion & Hoseney, 1990). The viscoelasticity of gluten arises from interactions between the protein molecules via hydrogen bonds and disulfide cross-links (Bloksma, 1975).
2.2.2. Rheological measurement of dough and gluten

2.2.2.1. Empirical rheology

There has been a long history of using descriptive empirical measurements of rheological properties with a remarkable range of creative devices which include the Penetrometer, Texturometer, Consitometer, Amylograph, Farinograph, Mixograph, Extensigraph, Alveograph, and many others. The empirical instruments most widely used to study dough rheology include two mixers (which are the Farinograph and Mixograph) and two load-extensional instruments (Extensigraph and Alveograph).

These empirical methods have several advantages in that they are easy to perform and often used in practical factory situations because they usually provide data which is useful in evaluating performance during processing. In addition, the applications of those instruments do not require highly skilled or technically trained personnel. However, there are limitations for these instruments in that they do not provide rheological properties of dough in fundamental units. The samples used in empirical methods usually have varied geometries and these are not well defined. Moreover, the stress and strain states are uncontrolled, complex, and non-uniform (Dobraszczyk & Morgenstern, 2003).

The Mixograph and Farinograph determine resistance to mixing, with the former instrument assessing optimal mixing time, and the latter assessing optimal water absorption. Mixograph curves represent the torque (%) exerted by the dough on the blades or pins of the laboratory mixers as a function of time (min) of the test. Mixograms show a maximum torque after a specific period of mixing (peak time), which indicates the time when the dough has the strongest properties and this can be used as an indicator of flour quality (i.e. its optimal development has been reached) (Hoseney, 1992; MacRitchie, 1987; Weipert, 1992). Several Mixograph studies have been conducted over the years with the objective to study gluten quality as affected by different factors (including type of cultivars, mixing time, water absorption, absence of oxygen, addition of chemicals, gluten properties, mixing rate) (Bloksma, 1990; Holmes
& Hoseney, 1987; Janssen, van Vliet, & Vereijken, 1996; Kokelaar, van Vliet, & Prins, 1996; Uthayakumaran, Newberry, Phan-Thien, & Tanner, 2002; van Vliet, Janssen, Bloksma, & Walstra, 1992). The Extensigraph and Alveograph determine resistance to extension of a dough and flour strength. The former instrument subjects the dough to the uni-axial extension, while the latter applies a biaxial extension (Hoseney, 1992; Weipert, 1992).

2.2.2.2. Fundamental rheology

Just as empirical rheology has some advantages and disadvantages, the same applies to fundamental rheology. Among the advantages of fundamental rheology assessments are: results are independent of size and shape of the material being tested, small samples can be used, the applied stress (or strain) is controlled, and the results are obtained in absolute units, as well as being described by defined rheological parameters (stress, strain, strain rate, modulus or viscosity). The disadvantages of fundamental techniques include: complex instrumentation which is expensive, the tests are time consuming and of limited use in industrial environments, the instruments require a high level of technical skills to operate and the results are difficult to interpret (Dobraszczyk & Morgenstern, 2003; Weipert, 1992).

There are different types of tests within fundamental rheology which can be mentioned as follows (Bloksma & Bushuk, 1988; Eliasson & Larsson, 1993; Gras, Anderssen, Keentok, Bekes, & Appels, 2001):

- Viscosity measurements
- Stress relaxation measurements
- Creep measurements
- Dynamic measurements
- Surface rheological behaviour that characterises the bulk behaviour of a material

The objective of viscometry is to determine the relationship between the rate of shear and shear stress in a material in a steady state. The material is called a Newtonian fluid
if the stress is proportional to the rate of shear. In dough, the ratio between stress and the rate of shear is called the apparent viscosity since dough is considered as non-Newtonian fluid (Bloksma & Bushuk, 1988).

Stress relaxation measurements are considered as large strain rheological methods. In these measurements, deformation is held constant and the force response is measured. Bloksma and Bushuk (1988) reviewed the experimental results from the literature for stress relaxation measurements for a number of doughs. Those results indicated that the relaxation process within the dough is not determined by a single type of molecular interaction but by a broad distribution of molecular mechanisms. This may be related to the wide molecular distribution of gluten. When Bohlin and Carlson (1980) measured the stress relaxation on dough and gluten in shear, there were two relaxation processes that could describe the relaxation behaviour of the dough, i.e. a rapid relaxation and a slower one which occurred over 0.1-10 s and 10-10,000 s, respectively. The former has been associated with small polymer molecules which relax rapidly while the latter has been linked to the HMW polymers. In addition, relaxation properties of dough relate well to molecular weight distribution and especially to entanglements of HMW glutenins. The evaluation of these can be used as a rapid method of differentiating variations in molecular weight distribution between varieties which vary in baking quality (Li, Dobraszczyk, & Schofield, 2003).

The other large-strain rheological methods are the creep and recovery measurements. However, they differ from the stress relaxation measurements in that they consist of the sudden application of a stress which is held constant while the deformation is measured as a function of time (Amemiya & Menjivar, 1992; Dobraszczyk & Morgenstern, 2003). Doughs with different baking quality showed no significant differences in relaxation behaviour when small strain amplitudes (0.1%) were applied. However, when a range of large strains (up to 29%) were applied, the creep and relaxation behaviour had a close correlation with the baking behaviour of the dough (Safari-Ardi & Phan-Thien, 1998). Moreover, not only do these tests require small amounts of material but also provide information about fundamental structural behaviour of the material. Day et al. (2005)
developed a small-scale deformation test (creep and recovery) to assess the rheological properties of isolated gluten using a set of commercial dry-gluten samples. The results showed that, with less than one gram of dry gluten, the methods can provide information correlating closely to dough extensibility data obtained by the traditional empirical methods.

The most popular and widely used fundamental rheological techniques for measuring dough properties involve dynamic measurements which apply a small amplitude stress or strain in an oscillatory manner to measure elastic and viscous moduli with time (Bloksma & Bushuk, 1988; Dobraszczyk & Morgenstern, 2003). There are two parameters which can be obtained from fundamental small strain rheological tests and can be used to characterise the properties of flour doughs. They are the shear modulus, $G'$ sometimes called elastic or storage modulus and $G''$ (viscous or loss modulus) (Mirsaeedghazi, Emam-Djomeh, & Mousavi, 2008; Steffe, 1996; Weipert, 1992). $G'$ is related to the elasticity of the material which gives an indication of the solid-like characteristics of the material, whereas $G''$ is related to viscosity which gives an indication of liquid like characteristics of the material (Bohlin & Carlson, 1980; Mirsaeedghazi, Emam-Djomeh, & Mousavi, 2008).

$$G' = \left( \frac{\sigma_0}{\gamma_0} \right) \cos (\delta) \quad \text{Equation 2.3}$$

$$G'' = \left( \frac{\sigma_0}{\gamma_0} \right) \sin (\delta) = \omega \eta \quad \text{Equation 2.4}$$

Where $\sigma_0$ is the stress amplitude (Pa), $\gamma_0$ is the strain amplitude, and $\delta$ is the loss angle, $\omega$ is the oscillatory frequency (rad/s), and $\eta$ is the dynamic viscosity. A function that involves both moduli is called the loss tangent and is defined as the ratio between the two shear moduli:

$$\tan \delta = \frac{G''}{G'} \quad \text{Equation 2.5}$$
The loss tangent, defines the relative contributions of the viscous (\( G'' \)) and elastic (\( G' \)) characteristic of the material.

If the elastic character (\( G' \)) goes beyond the viscous character (\( G'' \)), hence \( \tan \delta < 1 \), then the material behaves more like a solid which is when the deformation within the linear range is essentially elastic and recoverable. On the contrary, if \( \tan \delta > 1 \), the material behaves more like a liquid (Khatkar, 2004). In addition, when strain applied is below 0.1\%, \( G' \) is greater than \( G'' \), but when greater strain is used, this ratio becomes reversed due to viscoelastic solid conversion to elasto-viscous liquid (Mirsaeedghazi, Emam-Djomeh, & Mousavi, 2008).

The oscillatory test with low strain offers rheological information which can be related to the native structure of the material. The small deformation allows stretching but does not disrupt bonds and entanglements within the sample, thus providing the information on the native undisturbed structure of the material (Gras, Anderssen, Keentok, Bekes, & Appels, 2001). The tests can also be performed with stress or strain controlled using rheometers within the range of low frequency \((0.01<f<100 \ Hz)\). However, for measurements in the high frequency range, wave propagation methods such as ultrasound can be used (Letang, Piau, & Verdier, 1999).

2.3. Microscopy and its application to evaluation of gluten properties

2.3.1. Importance of the microscopic techniques in studying food microstructure

The microstructure of food products determines to a large extent physical, textural, and sensory properties of these products. Therefore, developing a thorough understanding of the microstructure, particularly the spatial distribution and interaction of food components is a key tool in developing products with desired mechanical and organoleptic properties (Frisullo, Laverse, Marino, & Nobile, 2009; Kalab, Allan-Wojtas, & Miller, 1995). An increased interest in food microstructure has been observed
due to the fact that consumers have changed their preferences to foods with more aesthetic appeal, superior taste, and convenient products and that most elements determining these food qualities usually exists at a micro-level (Aguilera, 2005; Lim & Barigou, 2004). Moreover, in foods, measured rheological responses are directly affected by the changes and properties at the microscopic level although those responses are seen at the macroscopic level (Rao, 2007).

Microscopy and other imaging techniques are the techniques which can be used to study food microstructure. They are analytical methods that produce results in the form of images rather than numbers, hence they have become the most appropriate techniques for evaluating food structure (Kalab, Allan-Wojtas, & Miller, 1995). Microscopy techniques vary in terms of the method of image production, resolution, and type of signal detected and different combinations of these give a particular type of structural information. There are two broad areas regarding microscopy, i.e: optical or light, and electron microscopy (Kalab, Allan-Wojtas, & Miller, 1995). Other imaging techniques are also available and these facilitate non-invasive imaging of the microstructure. Computerised tomography and magnetic resonance imaging are examples and these techniques are based on the use of X-ray and magnetic pulses, respectively (Ferrando & Spiess, 2000).

Light and electron microscopy have been widely applied to study the microstructure in a wide range of raw materials as well as processed products. Light microscopy uses a light or photon beam which is focused by glass lenses to form the magnified images. On the other hand, in electron microscopy, an electron beam is used as the illumination source and this is focused by magnetic lenses in order to form the image (Ferrando & Spiess, 2000). The basic techniques of light microscopy which are most frequently applied in food science include bright-field, polarising, and fluorescence microscopy (Ferrando & Spiess, 2000; Kalab, Allan-Wojtas, & Miller, 1995).

The difference between those three techniques relies on the tools which are used to transmit the light. In bright-field microscopy, a real image is produced that is upside
down and then reversed through the illumination which is transmitted sequentially through a condenser, the specimen and the objective. Two polarisers are inserted in the light path of the polarising microscopy. One between the light source and the specimen, while the other one between the viewer and the objective. Fluorescence microscopy is used for specific molecules that are present in the specimen to absorb light of a specific wavelength and the energy is re-emitted as light of a longer wavelength and lower intensity (fluorescence) (Kalab, Allan-Wojtas, & Miller, 1995).

Further progress which has been made in light microscopy is the development of confocal laser scanning microscopy (CLSM). Originally, the confocal microscope was invented in 1957, but the commercial production of the instruments and widespread use of the technology only became possible during the decade of the 1980s, after undergoing many innovations and combinations in technology. CSLM has a great ability to provide sharp, well resolved images from particular levels within three dimensional objects which have relatively thick features. The results of sharp and enhanced images are due to the placement of the pinhole at the focal plane of the image which has the effect of removing out-of-focus light. This pinhole placement marks the difference between CLSM and other conventional light microscopes (Blonk & van Aalst, 1993; Dürrenberger, Handschin, Conde-Petit, & Escher, 2001; Ferrando & Spiess, 2000; Kalab, Allan-Wojtas, & Miller, 1995; Paddock, 2000).

The other microscopy technique used to determine the microstructure of food is electron microscopy. The resolution of images is significantly improved with electron compared to light microscopy. The image formation in both is similar, however, they differ in relation to illumination sources and lenses. The source in electron microscopy is electrons focused with magnetic lenses rather than photons are focused with glass lenses (Kalab, Allan-Wojtas, & Miller, 1995). The scanning electron microscopy (SEM) and the transmission electron microscopy (TEM) are the two main techniques applied to study food microstructure.
SEM and TEM differ basically in the method of image formation. SEM is usually applied to study surfaces of the specimen while TEM typically visualises internal structures. However, the main limitation of these techniques is that both may require extensive sample preparation, particularly for products with high moisture content. This can be achieved by drying and freezing the sample before examination. In addition, the presence of artefacts presents another limitation to these techniques, despite the advances achieved in recent times in the preparation of samples (Ferrando & Spiess, 2000; Kalab, Allan-Wojtas, & Miller, 1995).

2.3.2. Microstructure of dough and gluten

A range of microscopic and imaging techniques have been applied to study the microstructure of wheat products and their changes during processing (Aguilera, Stanley, & Baker, 2000; Kalab, Allan-Wojtas, & Miller, 1995; McDonough & Rooney, 1999; Peighambardoust, Dadpour, & Dokouhaki, 2010; Roman-Gutierrez, Guilbert, & Cuq, 2002). Direct light microscopy, whether it involves bright field, polarising or fluorescence, allows resolution close to 0.2 µm and selective staining of flour components is possible. However, these approaches cannot be readily applied to thick specimens, especially at high magnifications, and they provide limited resolution.

Very high resolution and high magnification images of wheat structures including starch granules, proteins and protein aggregates have been obtained by TEM (Autio & Laurikainen, 1997; Roman-Gutierrez, Guilbert, & Cuq, 2002). Furthermore, SEM has been a useful tool for investigating the microstructures of cereal grains, wheat flours and derived products (Roman-Gutierrez, Guilbert, & Cuq, 2002). SEM photos of optimally mixed dough have been used to describe the microstructure of gluten. These indicate that most of the starch is readily removed, but a small number of granules appear to have protein fibrils strongly attached to them and an example of such an image can be seen in Figure 2.8 (Amend & Belitz, 1990).
Figure 2.8  Protein network after starch removal from hydrated flour, observed with SEM
Sourced from Amend and Belitz (1990)

The structure of gluten has also been investigated by Amend and Belitz (1990). They revealed that the formation of the gluten is basically the process of aggregation and mechanical transformation of protein films and results in a layer-like arrangement in dough or gluten. Jiang et al (2008) has also tried to relate the structure of the glutenin and gliadin to its rheological properties using TEM. The gluten structure observed with TEM can be seen in Figure 2.9.

Limitations in the use of older SEM systems have been overcome by Environmental Scanning Electron Microscopy (ESEM) and CLSM. The ESEM has recently been used in cereal applications (McDonough & Rooney, 1999). For instance, the examination of the microstructure of the gluten network in wheat flour dough and the extent of starch gelatinisation in moist and partially cooked products were possible using the ESEM (McDonough & Rooney, 1999).
Recently, many researchers have preferred to utilise the CLSM as an alternative to SEM for visualising the changes of starch-gluten network architecture in dough as influenced by different treatments due to the key feature of CLSM in depth imaging in non-deformed thick samples (Dürrenberger, Handschin, Conde-Petit, & Escher, 2001; Kalab, Allan-Wojtas, & Miller, 1995; Peighambardoust, Dadpour, & Dokouhaki, 2010; Peighambardoust, van der Goot, van Vliet, Hamer, & Boom, 2006). For example, in Figure 2.10, the CLSM results revealed that increasing mixing time led to development of interconnected gluten network covering starch granules throughout the dough and that over-mixing led to formation of a homogenous dough microstructure in which gluten phase showed a fine distribution throughout the dough (Peighambardoust, Dadpour, & Dokouhaki, 2010).
Figure 2.10 CLSM results of dough network with different mixing times, undermixed dough (A); optimal mixed dough (B) and over mixed (C)
Sourced from Peighambardoust et al.(2010)

Advances in microscopy and imaging techniques are therefore important to study the microstructure of dough and gluten network since changes in dough microstructure and its viscoelastic properties will affect the rheological behaviour of the dough (Peighambardoust, van der Goot, van Vliet, Hamer, & Boom, 2006). It has been shown that the composition and microstructure such as spatial arrangement of its constituents determine the macroscopic behaviour of certain doughs (Bloksma, 1990).

In addition, Dobraszczyk and Morgenstern (2003) showed a correlation between the molecular structure of dough, i.e. the presence or absence of long chain branching in glutenin subunits and large deformation rheology. Some studies have confirmed that the rheological behaviour of wheat flour dough at large deformations is dominated by the gluten fraction (Dobraszczyk & Morgenstern, 2003; Peighambardoust, van der Goot, van Vliet, Hamer, & Boom, 2006). Therefore, large deformation rheology has the
potential to provide a basis to study structural changes in the protein phase of the dough microstructure, which has been shown to account for its viscoelastic behaviour as well as final product quality (Peighambardoust, van der Goot, van Vliet, Hamer, & Boom, 2006).

The ESEM has recently been used in cereal applications (McDonough & Rooney, 1999). For example, ESEM imaging permitted the study of the microstructure of the gluten network in wheat flour dough (Bache & Donald, 1998) and the extent of starch gelatinisation in moist and partially cooked products (McDonough & Rooney, 1999). In addition, enzymatic degradation of starch granules has been observed using these instruments. ESEM was also used to describe the in situ microstructure of hydrated cereal products, such as tortilla dough and chips (Roman-Gutierrez, Guilbert, & Cuq, 2002). In addition, ESEM observations of wheat flour and flour components during hydration showed that there were slight changes in surface particles that are associated with apparent swelling effects. The formation of a continuous aqueous phase between particles was observed for long hydration times (Roman-Gutierrez, Guilbert, & Cuq, 2002).
References


Chapter 3

Background and literature review: salts and their effects on dough and gluten properties

The purpose of this chapter is to provide background and review of previous scientific research relevant to the studies reported in this thesis. The areas covered in this chapter consist of the principle of different salts belonging to the Hofmeister series, and their significance, as well as the effects of salt on the processing of wheat-based foods.

3.1. Effects of salt on processing of wheat based food

3.1.1. Effects of salt on dough rheology and dough handling properties

As it has been mentioned in the previous chapter, dough is a complex mixture of flour constituents, water, yeast, salt, and other ingredients. It is a primary intermediate stage in the transformation of wheat, through flour, into bread (Bloksma & Bushuk, 1988). It also can be regarded as a composite material: a continuous protein phase (gluten matrix) in which starch granules and other insoluble flour constituents are dispersed (Chiotelli, Roleile, & Le Meste, 2004; Petrofsky & Hoseney, 1995).

One of the key steps during the production of dough-based products is mixing. Flour, water, and other ingredients are allowed to be assimilated during the mixing step which in turn forms a coherent mass. Changes in conformational arrangements of gluten proteins are evident when mechanical energy is applied to the dough during mixing (MacRitchie, 1986; Stauffer, 2007). Therefore, during this procedure, the addition of salt may also influence the conformation of the gluten proteins. Basically, salt in form of sodium chloride is one of the four essential ingredients (flour, salt, yeast and water) in the bread-making industry since it improves the flavour and loaf volume of the final product (Eliasson & Larsson, 1993; Miller & Hoseney, 2008). There are several stages
of dough processing during bread making, i.e: mixing, dividing, rounding, moulding, proofing and baking (Belton, 2003). Salt is usually added to form dough with other ingredients before it is exposed to the step of baking in an oven. Dough processing is influenced by salt in that it affects mixing and proofing. In the mixing process, salt usually strengthens the dough and increases dough mixing time (Miller & Hoseney, 2008). Those effects on dough are commonly studied by looking at dough rheology when salt is added into the formulation for bread-making.

On the basis of studies of the rheological response of wheat and gluten doughs, they are considered as non-linear viscoelastic material (Faubion & Hoseney, 1990). The rheological properties of wheat flour doughs are important in determining both the handling properties of dough during processing and the quality of the finished product. This is because rheological measurements can be readily related to product functionality. Many rheological tests have been used to attempt to predict final product quality such as mixing behaviour, sheeting and baking performance (Dobraszczyk & Morgenstern, 2003). Rheological techniques are commonly categorised according to both the type of strain imposed (e.g. compression, extension, shear, torsion) and also the relative magnitude of the imposed deformation (e.g. small or large deformation) (Dobraszczyk & Morgenstern, 2003). The main techniques to asses dough rheology are either empirical or fundamental rheological instruments (Dobraszczyk & Morgenstern, 2003; Eliasson & Larsson, 1993; Menjivar, 1990). Fundamental instruments differ from empirical ones in that the strain and stress are known and are well characterised in the whole of the test piece (Eliasson & Larsson, 1993). Although there has been extensive work using both empirical and fundamental rheological studies of dough, the definition of a constitutive model for dough still represents a major challenge (Bagley, Dintzis, & Chakrabarti, 1998).

As the rheological properties of the dough have been considered important, a number of studies regarding the effect of salts on the rheological properties of wheat flour dough have also been investigated. Most of the studies have focused on the effect of NaCl on wheat flour dough, both empirically and fundamentally. Empirical rheology of dough
systems has shown that NaCl generally increases dough development time, resistance to extension and dough extensibility (Butow, Gras, Haraszi, & Bekes, 2002; He, Roach, & Hoseney, 1992; Kinsella & Hale, 1984; Preston, 1989; Salovaara, 1982). The earlier explanation of the effects of NaCl addition on dough empirical rheology are primarily attributed to the interaction of NaCl with gluten protein (Fu, Sapirstein, & Bushuk, 1996; He, Roach, & Hoseney, 1992; Larsson, 2002; Salovaara, 1982). When dough is prepared without NaCl, the gluten protein has positive net charges in the flour-water system. These positive charges repel each other and limit the ability of the protein molecules to interact thereby resulting in a weaker dough network. When salt is present, it shields the charges on the gluten protein, reducing electrostatic repulsion between proteins and allowing them to associate, thus producing a stronger dough.

Salt affects dough properties in terms of mixing requirements. Various studies showed that salt increases the mixing time of the dough as shown by Farinograph results (Preston, 1989; Salovaara, 1982). Dough prepared without NaCl results in gluten proteins hydrate more rapidly since they tend to repel each other, so that the dough is more readily mixed in a relatively short time. But, when salt is added to the formulation, the gluten proteins associate each other, so that they hydrate more slowly, resulting in longer mixing times (Miller & Hoseney, 2008).

A study conducted by McCann and Day (2013) has shown that there are significant differences in terms of rheological properties of a dough mixed with salt (2%) and without salt. The development of the gluten network has been also observed. At a particular time of mixing when observed with confocal microscope, gluten in the dough mixed with salt has developed a fine and continuous network compared to the dough without salt. The latter treatment resulted in a discontinuous network with much of the starch incorporated in the form of starch granules (Figure 3.1).

On the contrary, the results on the fundamental rheology of doughs have shown apparently contradictory effects of NaCl at different concentrations upon G’ values (Larsson, 2002; Lynch, Dal Bello, Sheehan, Cashman, & Arendt, 2009; Salvador, Sanz,
& Fiszman, 2006). Larsson (2002) found that the G’ of dough increased as salt was added in increasing concentrations. Similar results were also reported recently by Beck et al. (2012). On the other hand, Lynch et al. (2009) found decreasing G’ of the dough with increasing NaCl addition. These results indicate that the effect of salt on dough rheology is relatively complex and measurement of small deformation rheology may not provide adequate explanations. This might be due to the differences in protein concentration between flours and the possible contribution of starch components which are a larger fraction of the dough than the protein (McCann & Day, 2013).

Figure 3.1 The development of the gluten network in the dough as function of salt: a. FSB flour and b. Yellowbase flour. i. 0% NaCl, ii. 1% NaCl, iii. 2% NaCl. Sourced from (McCann & Day, 2013)
3.1.2. Effect of salt on gluten properties

The effect of salt may appear to influence the interactions occurring in the gluten molecular structures. The major covalent bonds of the wheat proteins which determine the rheological properties of flour-water systems are the peptide bond and the disulfide bond. The disulfide bonds are the naturally occurring covalent side chain cross-links found in proteins. They usually stabilise the folded structure of the protein, once they are formed and can form intramolecular as well as intermolecular cross-links (Bloksma & Bushuk, 1988; Cornell & Hoveling, 1998). The other interactions that may influence the interaction among dough constituents are electrostatic interactions although they may not be the primary force in protein folding, hydrogen bonds, hydrophobic interactions, and van der Wahls interactions (Damodaran, 1996). The typical interactions in flour-water dough systems can be seen in Figure 3.2.

Figure 3.2  Typical interactions in the flour-water dough systems
Sourced from Cornell and Hoveling (1998)
Explanations of the effect of salt on the dough rheological properties have generally been proposed on the basis of effects on the gluten proteins. These explanations derive from studies of the effect of different salts on gluten protein properties. When salt is present in low concentrations, it shields the charges of the gluten molecules, thereby reducing electrostatic repulsion between proteins, allowing them to associate and produce a stronger dough (Kinsella & Hale, 1984; Miller & Hoseney, 2008). At higher concentrations, salt interacts more with solvent molecules and its effect on gluten proteins is primarily determined by the type of ions present. Electrostatic effects such as non-specific charge shielding of the polar groups in the protein happen at relatively low salt concentrations of 0.05-0.15 M. These effects are called direct interactions, because the ions of the salts interact directly with the chemical groups of the protein such as amino acid residues, peptide bond, amino and carboxyl group in order to neutralise their charge. These interactions depend primarily on the type of anion or cation of the salt, as well as its valence (Balla, Razafindralambo, Blecker, & Paquot, 1998). At higher salt concentrations (> 0.5 M), specific-ion interactions predominate and the extractability of gluten protein is highly dependent upon anion type. These were observed to follow the order described by Hofmeister which gave rise to the sequence known as the Hofmeister series: this, in increasing order, consists of F<sup>−</sup>, Cl<sup>−</sup>, Br<sup>−</sup>, ClO<sub>4</sub><sup>−</sup> and SCN<sup>−</sup>.

Other interactions in the gluten structure due to the effects of salt are indirect interactions which are called lyotropic interactions. They are indirect because the ions primarily affect bonding within the protein by interacting with solvent molecules (Butow, Gras, Haraszi, & Bekes, 2002; Kinsella & Hale, 1984). Non-chaotropic ions have the effect of making the structure of water more ordered thereby promoting the development of hydrophobic interactions between gluten polymers (Kinsella & Hale, 1984). For metal chloride salts, gluten strength is observed to increase as a function of the charge density of the metal ion. This is due to higher charge densities which result in an increase in the hydrogen bonding associated with the water. Therefore, it can be implied that increasing the hydrogen bonding capacity of the solvent will increase the gluten strength (Belton, 2003). Moreover, the presence of divalent cations also
strengthens the gluten proteins. However, strengthening effects have been related to the formation of additional cross-links between the gluten polymers by the cations (Balla, Razafindralambo, Blecker, & Paquot, 1998).

Salt also affects the extractability of the gluten proteins as well as their susceptibility to external factors. For example, it was observed that the proteins extractable in acetic acid solutions were precipitated by addition of salt at various concentrations (Kim & Bushuk, 1995). The results from electrophoretic studies showed that the higher the NaCl concentration, the less extractable were the high molecular weight glutenins and gliadins, and they were also more readily precipitated. The sensitivity of the gluten proteins to NaCl also depends on the type of flour. The stronger flour was more responsive to NaCl concentration than the weaker flour. Moreover, differences in the molecular size and subunit composition of glutenin polymer contributed to differences in NaCl susceptibility (Kim & Bushuk, 1995). For example, the relative proportions of high/low molecular weight glutenin subunits and the relative proportion of X/Y type molecular weight glutenin subunits was found to be important. Preston (1984) found that there were large differences in both the quantitative and qualitative properties of the gluten extracts due to anion type and that their extractabilities increased dramatically across the lyotropic series (F < Cl < Br < ClO₄ < I < SCN).

The solubility of the gluten proteins is also affected by treatment with salts. Fu et al. (1996) reported that treatment of gluten with NaCl solutions changed protein solubility during successive treatments. It was first considered that salt induced aggregation between gliadins and glutenins and also induced conformational changes in the proteins. The interactions were then decreased as the salt was removed and the proteins became more accessible to water and caused them to be more soluble. Therefore, certain ionic solvents might reduce gluten solubility by aggregating gliadins with glutenins.

Apart from the obvious effects of salt mentioned, the role of salts is difficult to understand and there has been much discussion and conjecture in the scientific literature. However, spectroscopic results on gluten at constant water content indicate
that the series of sodium salts increase counter-ion size and hence the capacity of water to change the structure of the gluten matrix. There is also an increase in the amount of beta-turn present and the amount of mobile protein present (Belton, 2003). Wellner et al. (2003) investigated the effect of selected salts (NaCl, NaBr, NaI) with increasingly chaotropic anions on gluten prolamin secondary structure and dynamics using Fourier Transform Infra-Red (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy. FTIR spectra indicated a small increase in intermolecular $\beta$-sheet structures when gluten samples were incubated with low levels of NaCl and NaBr. The $\beta$-sheet content remained constant with further NaCl addition, but was reduced again with higher NaBr concentrations. Overall, it can be said that in the presence of chaotropic anions, the protein tended to prefer the $\beta$-turn helix structure characteristics of glutenin subunits in solution. These results are consistent with those previously reported by Preston (1989).

Recent studies on the effect of NaCl on the structure of gluten proteins during the formation of gluten were interpreted as showing firstly that monomeric $\alpha/\beta$-gliadins and $\gamma$-gliadins became more soluble in distilled water. Secondly, salt appeared to induce the aggregation of gliadins (Ukai, Matsumura, & Urade, 2008). The gliadin aggregation may result from the interaction of a certain ion with the specific amino acid residues of a given protein, rather than from an increase in ionic strength. Because there were limited differences in aggregation among chloride salts and large differences among sodium salts, it was presumed that the interactions of anions with specific amino acid residues may be important for aggregation (Ukai, Matsumura, & Urade, 2008).

### 3.1.3. Effect of salt on yeast functions

Salt inhibits, or at least controls, fermentation rate by decreasing the rate of gas production which results in requirement for longer proof times (He, Roach, & Hoseney, 1992). This appears to be the result of increased osmotic pressure and the effects of sodium and chloride ions on the membranes of yeast cells (Miller & Hoseney, 2008). The growth of the cells is then retarded so that fermentation and dough development is controlled, whereas if dough is made without salt, the yeast ferments excessively, resulting in gassy and sour dough. The dough with these properties when baked may
result in products with open grain and poor texture (Miller & Hoseney, 2008). He et al., (1992) highlight the effects due to production of doughs with and without salt along with the influence of different types of salts on the gas production. Indeed, dough without salt has higher gas production (67.8 GU) and was significantly different compared to dough treated with sodium chloride which had 61.8 GU. In addition, salt with different cations has lower gas production than sodium chloride, with the exception of potassium chloride.

Another study using a Chopin Rheofermentometer was applied to obtain information on gas expansion and retention capabilities of dough during fermentation. The effect of different salt concentrations in reducing series on dough properties and yeast growth were measured simultaneously (Lynch, Dal Bello, Sheehan, Cashman, & Arendt, 2009). The results showed that the maximum height of dough, that can be considered as a marker for baking quality, increased as salt level decreased. As mentioned earlier salt inhibits yeast growth, so a reduction in salt level increases yeast activity and hence leads to higher CO₂ production. In order to achieve good quality bread, it is necessary to control gas production to a minimum level and, therefore, reducing salt levels may result in poorer quality products. However, even though reduced salt level result in higher gas production, the capability of a dough to retain gas was lower as shown by lower retention coefficient when salt content of the dough was reduced (Lynch, Dal Bello, Sheehan, Cashman, & Arendt, 2009).

3.2. The Hofmeister series

3.2.1. The principle of the Hofmeister series

The term Hofmeister series originates from the ranking of various ions toward their ability to participate a mixture of egg white proteins. During the 1880s and 1890s Franz Hofmeister and some of his co-workers demonstrated that salts having a common cation but differing in anion have different effectiveness in stabilising protein suspensions.
Therefore this series of salts are still widely referred to in the current scientific literature as the Hofmeister after their first investigator, and reflecting the pioneering work done at that time (Boström, Tavares, Finet, Skouri-Panet, Tardieu, & Ninham, 2005; Der, 2008; Kunz, Lo Nostro, & Ninham, 2004; Lyklema, 2009; Zhang & Cremer, 2006). The salts can be arranged in a sequence and subsequent studies have confirmed the universality of the effects of the series. The Hofmeister series, are sometimes called lyotropic series, refers to the relative effectiveness of anions or cations to influence or determine a wide range of phenomena. The typical ordering of the cation and anion series and some of its related properties are presented in Figure 3.3.

Regarding the hydration properties of the ions, the cations series extends from the weakly hydrated ions on the left to those which are strongly hydrated on the right. On the contrary, the anions series goes from the strongly hydrated ions to weakly hydrated ones. The difference in the effects of the series can be partly explained by the interactions involving the various charged groups present on the surface of biological molecules particularly proteins (Kunz, 2010).
Generally, the effects of the various are more pronounced for anions than for cations, because anions have stronger interactions with water than cations of the same size and absolute charge density. The effects of the anions can be seen in Figure 3.4. The species to the left of $\text{Cl}^-$ are referred to kosmotropes while those to the right are called chaotropes. These terms originally referred to the ability of an ion to alter the hydrogen bonding network formed between water molecules. The kosmotropes, which were believed to contribute to developing the structure of water, are strongly hydrated and have stabilising and salting-out effects on proteins and other macromolecules. On the
other hand, chaotropes, water structure breakers, are known to destabilise folded proteins and give rise to salting-in behaviour (Der, 2008; Zhang & Cremer, 2006). These effects are only relevant in instances where ion-water interactions are dominant for the specific ion effects. However, specific cation effects can be of the same order magnitude as specific anion effects when direct ion-ion or ion-charged headgroup interactions are dominant (Kunz, 2010).

Figure 3.4 The effects of specific anions belonging to the Hofmeister series
Sourced from Zhang and Cremer (2006)

3.2.2. Understanding the specific effects of the Hofmeister series on macromolecules

Over the years, the effects of the Hofmeister series have been observed in a very large number of systems: solubility of salts; solubility of oxygen; surface tension of salt solutions; pH measurements, ion binding to micelles, protein and membranes; colloid stability and many more (Kunz, Lo Nostro, & Ninham, 2004). Despite the fact that the Hofmeister series plays a significant role in a wide range of phenomena, the exact origin of the action of the ions in the series has not yet been clarified and no general
explanations exist at the molecular level. Up until now, explaining or predict of these
effects remains contentious and there is debate regarding whether the effects are caused
by direct ion-ion interactions or ion-water interactions.

Several different ideas about the nature of specific ion effects on macromolecules have
been proposed recently. Originally, explanations of the influence of particular ions on
macromolecular behaviour were that ions modify the structure and properties of water.
The character of the water as the solvent for macromolecules would then change in
specific ways in presence of electrolytes. In this context it has been a standard practice
to regard ions as chaotropes and kosmotropes (Aroti, Leontidis, Dubois, & Zemb, 2007;

However, it is now thought that these explanations may be somewhat misleading.
According to recent experimental findings, it seems that the structure of the water is not
heavily perturbed by monovalent ions beyond the first hydration shell, and that there is
no correlation between the impact of a solute on water structure and its effect on the
stability of bio-molecules (Batchelor, Olteanu, Tripathy, & Pielak, 2004; Otma,
Kropman, Woutersen, & Bakker, 2003). The study by Otma et al. (2003) revealed that
ions do not affect the bulk water properties. The anions of Cl\(^-\), Br\(^-\), I\(^-\), ClO\(_4\)^- , or SO\(_4\)^2- had no influence on the dynamics of bulk water, even at very high concentrations of
both kosmotropic ions and chaotropic ions. It showed that correlation times for first
hydration shell water molecules of these anions where much slower than for bulk water.
Therefore, it can be said that the anions did not change the hydrogen bonding network
outside the direct surrounding area of the anion. As a result, it was concluded that there
was no long-range structure-making or structure-breaking for either chaotropes or
kosmotropes take place (Zhang & Cremer, 2006).

Another different approach assumes that ion interactions with specific groups on
surfaces can explain the Hofmeister series. Some years ago Baldwin (1996) argued that
specific ion effects on protein stability could be described in terms of the ability of ions
to salt-in the polar peptide group and salt-out the non polar side chains. This approach
might be relevant for a variety of phenomena to a certain extent, but cannot be the sole explanation of specific ion effects. This is because such effects are also observed in the absence of specific surface groups. In addition, Sedlak, et al. (2008) have found a correlation between the effects of a range of cations and anions on thermal stability of proteins and on the ion surface/bulk partition coefficient. These results indicated that direct protein-ion interactions were responsible for the Hofmeister effects of the ions on protein stability. However, the effect of cations and anions did not depend on the net charge of the proteins which may imply that the peptide bond is responsible for the interactions with ions.

A theory regarding the origin of ionic specificity was also previously proposed by Ninham and Yaminsky (1997). The specificity could be due to the usually neglected dispersion interactions between ions and surfaces. It was suggested that an ionic dispersion potential acting between ions or ions and water or ions and interfaces must be included in the usual electrostatic theory to explain specific ion effects. Therefore, dispersion forces need to be taken into account. However, it turned out that it is not possible to describe adequately the experimentally determined surface tension in relation to ion polarisabilities.

Clues to more accurate explanations for specific ion effects are emerging from studies of the behaviour of ions near interfaces. In addition, Jungwirth and Tobias (2006) have performed simulations of air/electrolyte interfaces in the presence of salt in which they included the polarisabilities of ions. For example, molecular dynamics simulations have predicted that the tendency for anions to adsorb to the air-water interface follows an inverse Hofmeister series (SO_4^{2-}, NO_3^-, I^-) and is correlated with specific ion effects on surface tensions and surface potential (Jungwirth & Tobias, 2006). The results indicate that specific ion effects could reflect differences in the hydration of ions near surfaces whether they are air-water interfaces or biomolecular surfaces compared to bulk solution. Figure 3.5 shows sample ion distributions from aqueous/air interface for sodium halide solutions (at 1.2 M), obtained by molecular dynamic simulations and these can also be used to explain this feature. The results show that including
polarisability is critical to obtain the correct ion distribution as a function of distance from the interface.

![Aqueous/air interfaces for 1.2 M sodium halide solutions obtained by molecular dynamic simulations](current-opinion-in-chemical-biology)

**Figure 3.5** Aqueous/air interfaces for 1.2 M sodium halide solutions obtained by molecular dynamic simulations
Sourced from Zang and Cremer (2006)

Although the dispersion forces and polarisability theory have been taken into account, still there are doubts about how the specific ions can affect macromolecules concerning interfacial properties. Kuntz et al. (2004) have stated that including polarisability to describe ion effects at the protein/water interfaces is less important than that at the air/water interface.

According to Kunz (2010), there are some important factors that should be considered concerning the specific ion effects on macromolecules. Firstly, the charge density which is the ratio between charge and ionic radius play an important role as the modeling of ion specificities significantly depends on these values. For instance, Sedlak, et al. (2008) concluded that Hofmeister effects on protein stability are due to anion charge density. Since ion charge density is directly related to hydration, it seems that ion hydration modulates ion binding to the peptide group which then result in those effects. Secondly, the environment of the ions may be involved, in particular the counterions or headgroups in their vicinity, since ion properties depend strongly on these parameters.
Thirdly, the structure and chemical composition of macromolecules should be known in order if we are to have a chance to predict any specific ion effects. For example, ion-protein interaction cannot be described adequately by simplified models that consider the protein as a sphere with uniform charge distribution. Finally, the concentration and pH of the solutions are likely to be important since specific ion effects are dependent upon salt concentration and, especially in the case of proteins, they are strongly pH dependent.

Taking together all these explanations regarding the origin of the specific ion effects on macromolecules and all possible factors which are important about these effects, it seems that such complex phenomena including specific ion effects cannot be fully described within a simple model. However, there is an alternative model proposed by Collins (2004) which is simple, convincing, and understandable for many experimental results in biology and colloidal science.

The latter model is known as the concept of matching water affinities. Initially, ions are considered as a sphere with a point charge in the centre, the surrounding water molecules will be tightly bound when the ions are small which in turn will make the ions kosmotropic. On the other hand, when the ions are relatively large, the hydration shell will be loosely bound and hence the ions can be regarded as chaotropic. The difference between these types results from the relative strength of the ion-water interactions compared to the water-water interactions. It has been suggested that this concept can serve as a starting point to understand the interactions of ions with polar, but uncharged polymers which is important in food chemistry (Kunz, 2010).

There have been various studies conducted to explain the molecular mechanisms of the specific ion effects on biomolecules. The effects of the ions have been found to be depended on the molecular characteristics of the biomolecules. For example, the effect of the cations and anions are different on the amino acids in the aqueous solution (Tomé, Pinho, Jorge, Gomes, & Coutinho, 2013), and the specific binding site for cations and anions on the particular protein molecules are dependent on the charge of
the protein and the peptide bonds in the protein (Rembert, Paterová, Heyda, Hilty, Jungwirth, & Cremer, 2012). Previous studies on the Hofmeister ions have shown that anions have more pronounced effects on proteins than do the cations. The binding site for anions with polypeptide backbones has been shown to involve the combination of the polar nitrogen atom and the adjacent hydrocarbon group, whereas the binding site for cations usually involve the carbonyl oxygen of the amide. It has also been found that the cations binds weakly to the amides in aqueous solutions (Okur, Kherb, & Cremer, 2013).

### 3.2.3. Specific ion effects in food systems

The effects of the Hofmeister series in foods have been investigated over a number of years and most studies have been concerned with sugars or starch and proteins. Salts have a significant influence on the gelatinisation and rheological properties of starch. The interactions are more subtle than interactions of ions with charged headgroups since starch is not charged. A study by Ahmad & Williams (1999) showed that effects of salts are due to both anion mediated modified water-polymer interactions, and disruption of polymer chain aggregation by the interaction of cations with the hydroxyl group of the starch molecules.

A considerable number of studies on the effect of salts on the structural properties of proteins have been carried out in the past. The effects of the Hofmeister series of several protein isolates have been found to be in agreement with their old classification, which suggest that at least two effects of salt make major contributions to the stabilizing properties of proteins i.e: their effects on water structure and electrostatic interaction with the charged groups of the protein. In particular, their rheological behaviour, water absorption capacity, gelation concentration, and foaming behaviour were investigated (Lawal, Afolabi, Adebowale, Ogunsanwo, & Bankole, 2005).
References


Chapter 4

Summary of background, significance of the project and description of the project aims

The purpose of this chapter is to summarise the context in which this study has been developed and to describe the aims of the project.

4.1. Summary of current situation and significance of the project

High dietary salt intake has been identified as a significant issue for human health globally including blood pressure and cardiovascular diseases. The amounts required by adults for good health are 6 g/day and, despite the reported risks, salt consumption often exceeds these levels in Australia and other developed nations. In fact, 25% of the salt intake in the typical western diet is from cereal products (Miller & Hoseney, 2008). The foods that contribute the most to salt consumption of Australians are bread and bread rolls (25%), meat, poultry & game products and dishes, including processed meat (21%), other cereal products and cereal based dishes (e.g. biscuits and pizza) (17%), savoury sauces and condiments (8%) and cheese (5%). Breakfast cereals contribute approximately 4% of total salt consumption from processed foods and dried soup mixes less than 3% (FSANZ, 2012).

Against this background, it is increasingly important for food manufacturers to develop products with reduced salt content. However, sodium reduction is difficult to achieve since salt not only has an important role in taste of the food but also for preservation, and in some products, structuring or other purposes. Reduction in the sodium content of processed foods presents challenges because food manufacturers usually perceive the sodium content to be vital to the flavour and overall consumer acceptability of the product. Salt in semi-solid foods and dry powder formulations generally has a significant technological role in the processing and stability of these products as well as contributing to the appealing taste. In dough based products, reducing or eliminating
salt from the formulation has adverse effects on network formation and rheological properties and subsequently the product quality including loaf volume, crumb structure and texture. This is because salt acts as a co-solute and influences the hydration of wheat proteins and starch under defined moisture conditions during the manufacture of bread, cereal, pasta, noodles and other cereal-based foods. The hydration of these components allows the development of appropriate dough rheology and mechanical behaviour of biopolymer networks to ensure processability as bakery goods as well as sheeted and extruded products.

The common strategy to reduce the sodium content in wheat based foods is to replace a proportion of the NaCl with the chloride salts of different cations particularly potassium chloride in the formulation and to modify the processing conditions as required. Although this approach has provided some success, a basic understanding of how different salts impact the formation of gluten network and the rheological behaviour of the dough is needed. This is needed in order to maximise the technological functionality of these salts in replacing NaCl. In addition, it is not clear whether the rheological properties and structural changes of the gluten in the presence of NaCl are due to the contribution of each sodium or chloride ions.

By studying the effect of salts on the formation of gluten structure during hydration at the molecular level and determining the relationship between microstructure, chemical properties and its rheological properties, an understanding of the function of salts can be established. This knowledge can then be applied to the design and control of the protein/starch matrix by replacing the salt with other healthier ingredient(s). Alternatively, it may be possible to manipulate the functionality of the raw material, wheat flour, through controlled milling in order to support innovation and the development of novel reduced-salt cereal based products.
4.2. Hypothesis

The research reported in this thesis has been based upon the hypothesis that salts affect structural differences of the gluten proteins during initial hydration which influences the specific rheological properties and dough handling properties during processing.

4.3. Project aims

The broad aim of this project has been to investigate the structure of gluten proteins and their network formation during hydration at different microscopic levels and to evaluate the effects of salts on the functional properties of the gluten/dough. Specific objectives have been to:

1. To investigate the effects of sodium chloride (NaCl) on gluten network formation and rheological/mechanical properties of gluten during hydration;

2. To investigate the effect of NaCl on gluten structural changes during hydration and network formation as a function of heating;

3. To investigate and clarify the effect of each sodium and chloride ions on gluten network formation (selected from the Hofmeister series). First series was the chloride salts with different cations and second was the sodium salts with different anions.

References


Chapter 5

Materials and methods

The objective of this chapter is to describe all the materials, including chemical reagents, equipment, instruments along with the methods used in this study. The procedures applied for gluten sample preparation are outlined. Moreover, the methods of analysis chemical properties of gluten proteins including SE-HPLC, FTIR, as well as their rheological properties and microstructure are also described.

5.1. Chemical reagents

All of the chemicals used in this study were of analytical reagent grade unless otherwise specified. The details of the chemicals used, including a brief description and their suppliers are listed in Table 5.1. It is specifically noted that Milli-Q water was used for the preparation of solutions as well as for all the other procedures applied in this study.

5.2. Equipment

Information regarding the equipment used in this study is presented in Table 5.2.

5.3. Consumable and ancillary items

The sources and details of consumables and ancillary items are provided in Table 5.3.
Table 5.1 List of chemicals

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<td>Urea</td>
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<td>Sigma, Australia</td>
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<td>Lot no. 109H02001</td>
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<td>Ethylene diamine tetraacetic acid (EDTA)</td>
<td>Product code: E-5134</td>
<td>Sigma, Australia</td>
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<td>Lot no. 116H0184</td>
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### Glycine
- Product code: G7126
- Lot no. 070M0183V
- Sigma Aldrich, Australia

### Uranyl acetate
- Product code: 569
- Ajax chemicals, Australia

### Lead nitrate
- Product code: 29038AR
- BDH, Australia

### Sodium citrate
- Product code: 467
- Ajax chemicals. Australia

#### Table 5.2  List of equipment and instrument

<table>
<thead>
<tr>
<th>Equipment/instrument</th>
<th>Description</th>
<th>Manufacturer</th>
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<tr>
<td>Hobart mixer</td>
<td>Model N-500</td>
<td>Hobart Corporation, USA</td>
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<tr>
<td></td>
<td>Serial No. 14037601</td>
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</tr>
<tr>
<td>Micro-doughLAB</td>
<td>Serial No. 2092311-udM</td>
<td>Newport Scientific, Australia</td>
</tr>
<tr>
<td>Analytical balance</td>
<td>Sartorius Model BP 2105</td>
<td>AG, Gottingen, Germany</td>
</tr>
<tr>
<td></td>
<td>Serial No. 50907315</td>
<td></td>
</tr>
<tr>
<td>Freeze dryer</td>
<td>Model FD-5</td>
<td>Dynavac, Melbourne, Australia</td>
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<tr>
<td>Coffee grinder</td>
<td>Model HMC GK</td>
<td>Homemaker. Australia</td>
</tr>
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<td></td>
<td>Cat. No. C6.2006</td>
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<tr>
<td>Rheometer</td>
<td>Paar Physica MCR 300</td>
<td>Messtechnik GmbH, Stuttgart, Germany</td>
</tr>
<tr>
<td></td>
<td>Model No. 538824</td>
<td></td>
</tr>
<tr>
<td>Instron</td>
<td>Model 5564</td>
<td>Instron, UK</td>
</tr>
<tr>
<td></td>
<td>Serial No. H1576</td>
<td></td>
</tr>
<tr>
<td>Colorview III high resolution CCD camera</td>
<td>Serial No. 26610216</td>
<td>Olympus Life Science, Germany</td>
</tr>
</tbody>
</table>
| **Confocal laser scanning microscopy** | Leica TCS SP-5  
Serial No. 510000500 | Leica Microsystem, Germany |
<table>
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<th></th>
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<th></th>
</tr>
</thead>
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<tr>
<td><strong>Ultramicrotome</strong></td>
<td>Leica Ultra Cut</td>
<td>Leica Microsystem, Austria</td>
</tr>
<tr>
<td><strong>Diamond knife</strong></td>
<td>Ultra 45° DiATOME</td>
<td>DiATOME, USA</td>
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<tr>
<td><strong>Transmission electron microscope</strong></td>
<td>Model JEOL 1010</td>
<td>JEOL Australasia, Australia</td>
</tr>
<tr>
<td><strong>LEICA embedding machine</strong></td>
<td>Leica EM TP 709202</td>
<td>Leica Microsystem, Germany</td>
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<tr>
<td></td>
<td>Serial No. 530182</td>
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</table>
| **HPLC shimadzu**                    | Prominance HPLC system,  
Equipped with LC-10A  
model pump, SIL-20A  
automatic sampler and a  
SPD-20A UV-Visible detector | Shimadzu, Japan            |
| **Platform mixer**                   | Ratek Model OM6         | Ratek Instruments, Pty.  
Ltd., VIC, Australia       |
|                                     | Serial No. 605018133    |                           |
| **Vortex mixer**                     | MS1 Minishaker          | IKA Works Inc., USA       |
|                                     | Serial No.*008538*      |                           |
| **Microcentrifuge**                  | Model 1-14              | Sigma, Germany            |
|                                     | Serial No. 111477       |                           |
| **Sonicator**                        | Model Soniprep 150      | MSE Scientific Instruments, Fisons Ltd, Sussex, England |
|                                     | Serial No. 693          |                           |
| **Hot plate with magnetic stirrer**  | Cat No. 2091-001        | Industrial Equipments &  
Controls Pty. Ltd.,  
Australia                 |
| **FTIR**                             | Perkin Elmer Spotlight 400  
ATR/FTIR  
Product no: L1860116 | Perkin Elemer, USA         |
<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oven</strong></td>
<td>Contherm oven</td>
<td>Contherm Scientific Ltd., NZ</td>
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<tr>
<td><strong>LECO protein content</strong></td>
<td>Trumac N</td>
<td>LECO Corporation, USA</td>
</tr>
<tr>
<td></td>
<td>Model 630-300-300</td>
<td></td>
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<td></td>
<td>Serial No. 4183</td>
<td></td>
</tr>
<tr>
<td><strong>Agitation shaker</strong></td>
<td>Multireax</td>
<td>Heidolph, Germany</td>
</tr>
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<td></td>
<td>Model 545-10000-00-0</td>
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<tr>
<td></td>
<td>Serial No. 07040020</td>
<td></td>
</tr>
<tr>
<td><strong>UV VIS spectrophotometer</strong></td>
<td>Shimadzu Model UV-1700</td>
<td>Shimadzu, Japan</td>
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<td></td>
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**Table 5.3 Description of consumable and ancillary items used in this study**

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
<th>Manufacturer</th>
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<tr>
<td><strong>BIOSEP-SEC S4000 column</strong></td>
<td>Part No. 00H-2147-KO</td>
<td>Phenomenex, NSW, Australia</td>
</tr>
<tr>
<td></td>
<td>Serial No. 370057-1</td>
<td></td>
</tr>
<tr>
<td><strong>Micropipette 1-5 mL</strong></td>
<td>BioPette™ P3960-5000A</td>
<td>Labnet International Inc., USA</td>
</tr>
<tr>
<td><strong>Micropipette 100-1000 μL</strong></td>
<td>BioPette™ P3960-1000A</td>
<td>Labnet International Inc., USA</td>
</tr>
<tr>
<td><strong>Syringe 3 cc/mL</strong></td>
<td>Terumo Syringe</td>
<td>Grace Davison Discovery Sciences, VIC, Australia</td>
</tr>
<tr>
<td></td>
<td>SO. 488978 000050</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cat No. AU-OTH-1-100</td>
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<tr>
<td><strong>Microcentrifuge tubes 1.5 mL</strong></td>
<td>1.5 mL MaxyClear Snaplock</td>
<td>Axygen, Australia</td>
</tr>
<tr>
<td></td>
<td>Product No. #MCT-150-A</td>
<td></td>
</tr>
<tr>
<td><strong>Nylon HPLC syringe filter 0.45 μm</strong></td>
<td>Cat No. 2167</td>
<td>Grace Davison Discovery Sciences, VIC, Australia</td>
</tr>
<tr>
<td></td>
<td>Lot No. 86045756D2</td>
<td></td>
</tr>
</tbody>
</table>
### Materials used in this study

Two commercial flours were used in this study: FSB flour and Redbase flour. The flours were kindly provided by Allied Mills (Kensington, Victoria, Australia). The gluten samples were prepared from these two flours according to the specified procedures mentioned in the next section. The properties of the flours used in this study are summarised in Table 5.4. The protein and moisture contents were determined by the AACC method 46-30 and the AACC method 44-15a, respectively (AACC International, 2000).
Table 5.4 Properties of the wheat flour materials

<table>
<thead>
<tr>
<th>Wheat flour</th>
<th>Protein content (%)&lt;sup&gt;ab&lt;/sup&gt;</th>
<th>Moisture content (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Glu:Gli</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSB</td>
<td>13.2</td>
<td>12.8</td>
<td>0.71</td>
</tr>
<tr>
<td>Redbase</td>
<td>10.4</td>
<td>12.9</td>
<td>0.71</td>
</tr>
</tbody>
</table>

<sup>a</sup>: All values presented as % values on a w/w basis.
<sup>b</sup>: N × 5.7

5.5. Preparation of the gluten samples

Gluten samples were prepared by hand-washing method. For determination of the effect of sodium chloride on gluten during hydration, two different samples with different mixing procedure were prepared. Preparation of water-washed gluten (WW) and salt-washed gluten (SW) was carried out according to the method previously described by Day et al (2009). Briefly, flour (300 g) was mixed with water (180 mL) with or without 2% NaCl (flour base) in a Hobart mixer at setting 1 (63 rpm) for 2 min followed by setting 2 (111 rpm) for a further 2.5 min to form a dough. The dough was then rested for 30 min in either water or 2% NaCl solution, then washed 3 times by hand in 5 L water or salt solution (150 g/5 L). The wet gluten was then freeze-dried for 72-96 h. The freeze-dried gluten was ground to a powder using a coffee grinder and sieved through a 250 µm sieve. The gluten samples were prepared in two batches for each flour. The moisture and protein content of dried gluten powders were determined according to the AACC methods (AACC International, 2000). Freeze dried gluten (0.5 g) was rehydrated with 0.75 mL water or NaCl solution using a mortar and spatula to obtain a rehydrated gluten dough containing approximately 63% w/w water content for rheological measurement. The schematic diagram of the preparation of gluten samples can be seen in Figure 5.1.
Fresh wet gluten samples were also obtained by mixing flour (4 g) with 2.4 mL water or 2% NaCl solution for 4.5 min, using a Micro-doughLAB (Perten Instruments, NSW, Australia). The gluten sample was then washed in either water for WW gluten or salt solution for SW gluten. Schematic procedures can be seen in Figure 5.2. The similar
method in the above paragraph was also applied to prepare the fresh wet gluten samples with different salts belong to the Hofmeister cation and anion series. Doughs from both flours were mixed and also hand-washed with each salt solutions (KCl, NH₄Cl, NaCl, MgCl₂, CaCl₂, NaF, NaH₂PO₄, NaBr, and NaI). Solutions of various chemical salts were prepared so that the number of moles of each corresponded with that of a 2% (flour base) sodium chloride solution. A proportion of the fresh wet gluten (approx. 1.5 g) was used for the rheological measurement. The remainder of the wet gluten was used for the determination of protein and moisture contents. The schematic procedures to prepare gluten with different salts and the analysis approaches can be seen in Figure 5.3.

![Schematic diagram of the preparation of the gluten samples with Micro-doughLAB to study the effect of NaCl during hydration](image-url)

**Figure 5.2** Schematic diagram of the preparation of the gluten samples with Micro-doughLAB to study the effect of NaCl during hydration
5.6. Microstructural investigation

Microstructures of the gluten and dough samples in this study were investigated using two microscopic techniques, the confocal laser scanning microscopy (CLSM) and the transmission electron microscopy (TEM). CLSM was utilised for both gluten and dough samples, while the TEM was used only to investigate the gluten samples. Firstly, CLSM was used to investigate the structure of the gluten as function of NaCl. A small piece of
each rehydrated or fresh gluten sample with and without NaCl was placed on a glass slide and stained with a drop of rhodamine B solution (0.1% w/w in MilliQ water). Rhodamine B was excited by the 543 nm laser and the emission fluorescence was detected at 500–535 nm. The CLSM was utilised to investigate the microstructure of dough with different cation salts. Secondly, CLSM was used to examine the microstructure of dough with different Hofmeiter salts. Dough samples as prepared using a Micro-dough LAB from flours with different salt solutions and 0.2 mL of mixed fluorescein isothiocyanate (FITC, 0.025% in dimethyl sulfoxide) and rhodamine B (0.01% in MilliQ water) were added into the mixing solutions. The microstructure of the sample was examined after mixing for 8 min. FITC was excited by a 488 nm laser and rhodamine B by a 543 nm laser. The emission fluorescence was detected at 505-550 nm and 565-620 nm for FITC and rhodamine B, respectively. The samples were then covered by a cover glass and viewed using a 20× objective on a Leica SP5 Confocal Laser Scanning Microscope (CLSM) (Leica Microsystems, Germany) (Figure 5.4).

Figure 5.4  CLSM instrument (Leica SP5) used for microstructural characterisation
For TEM investigation, the rehydrated or fresh wet gluten samples (63% w/w water content) were fixed overnight at room temperature by immersion in 2% glutaraldehyde 0.07 M phosphate buffer (pH 6.8) followed by rinsing three times with the buffer over a period of 20 min for each change. Dehydration and embedding of the sample were performed in a LEICA EM TP instrument (Leica Microsystems, Germany). Samples were sequentially dehydrated in 50, 70, 90 and 100% ethanol for 10 min each. For each solvent the process was repeated 3 times and the final step (100% ethanol) was repeated four times. Embedding was conducted using a Low Viscosity SPURR’S kit (Electron Microscopy Sciences, Hatfield, USA). This involved immersion of dehydrated gluten samples sequentially in mixtures of ethanol and SPURR resin. Then samples were placed in the moulds, covered with the resin and polymerised at 60 °C overnight. Sample blocks were sectioned using an ultramicrotome (Leica Microsystem, Vienna, Austria) and collected on the Copper finder grids 200 mesh. Sections were stained with uranyl acetate in 50% ethanol for 10 min followed by Reynold’s citrate for another 10 min and examined using a TEM Jeol 1010 microscope (Jeol Australasia, NSW, Australia) with accelerating voltage set at 80 kV and 4.85 amps objective current lens. Images were acquired using a Gatan CCD Digital Camera at 4000× magnification.

5.7. Protein extraction by SE-HPLC and determination of the Glu:Gli

Protein extraction was performed on flour and gluten samples in the study of the effect of NaCl as well as the effect of Hofmeister ion series on gluten and analysed using SE-HPLC according to the modified method of McCann et al. (2009). Briefly, the gluten sample (10 mg) was dispersed in 1 mL of 0.5% SDS 0.05 M phosphate buffer pH 6.9, and vortexed for 1 min followed by 10 min of mixing on a shaker at room temperature. The sample was then sonicated for 30 s using a sonicator (Soniprep 150 MSE Scientific Innstruments, Fisons Ltd, Sussex, England) equipped with a 3 mm probe tip at amplitude level 6, followed by centrifugation for 15 min at 16,000 ×g. The supernatant was filtered through a 0.45 µm filter. The extracts (20 µL) were then injected into a Biosep-SEC S4000 column (Phenomenex, NSW, Australia) connected to a Shimadzu
HPLC system (Shimadzu, Japan) equipped with LC-10A model pump, SIL-20A automatic sampler and a SPD-20A UV-Visible detector. Samples were eluted under isocratic conditions using a solvent containing 0.1% trifluoroacetic acid in 50% acetonitrile at a flow rate of 0.5 mL/min, monitored by UV detection at 214 nm. Glutenin was associated with peak area of the polymeric fraction while gliadin was associated with the monomeric protein fraction. The preparation of the solutions for protein extraction and SE-HPLC can be seen in Appendix 1.

Figure 5.5 TEM instrument (JEOL 1010) used for microstructural characterisation
5.8. Determination of the % unextractable polymeric protein (UPP)

The % UPP was determined by the method of Gupta et al. (1993) with modification. The SDS extractable protein was obtained using the same dispersion method described above without sonication. After centrifugation, the supernatant was designated as SDS-extractable protein. The residue was then resuspended in 0.9 mL 0.5% SDS 0.05 M phosphate buffer pH 6.9 and sonicated for 30 s, to ensure that the sample was completely dispersed within the first 5 s. The mixture was then centrifuged and filtered through a 0.45 μm filter. This fraction was designated SDS-unextractable protein. Both SDS-extractable and SDS-unextractable protein fractions were analysed using SE-HPLC outlined above. The percentage of unextractable polymeric protein (%UPP) in the sample was determined as the ratio of polymeric peak area (unextractable) to the sum of polymeric area of both extractable and unextractable × 100.

Figure 5.6  HPLC Shimadzu system
5.9. Disulfide analysis

Disulfide (SS) and sulphydryl (SH) contents of the gluten samples were determined according to the modified direct colorimetric assay method of Chan and Wasserman (1993). For free SH content, gluten samples (10 mg) were suspended in 1.0 mL of reaction buffer containing 8 M urea, 3 mM EDTA, 1% SDS, and 0.2 M Tris-HCl, pH 8.0 (Buffer A). Samples were vortexed for 30 second and mixed on a constant platform shaker at room temperature for 1 h. Buffer B which contains 10 mM 5,5’Dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 M Tris-HCl, pH 8.0 (0.1 mL) was then added to each sample and mixed for a further period of 1 h. Samples were then centrifuged at 13,600 × g for 15 min at room temperature. The absorbance of the supernatant was determined at 412 nm using a UV-VIS spectrophotometer (Shimadzu UV-1700, Kyoto, Japan) against a blank containing 1.0 mL of buffer A and 0.1 mL of buffer B.

Total sulphydryl content was determined by the method of Thannhauser et al. (1987) as modified by Chan and Wasserman (1993). Reaction buffer (1.0 mL, 8 M urea, 3mM EDTA, 1% SDS and 0.2 M Tris-HCl pH 9.5 with 0.1 Na₂SO₃ and 0.5 mM 2-nitro-5-thiosulfobenzoate (NTSB²⁻) synthesised from DTNB according to the method of Thannhauser et al. (1987)) was added to 10 mg of each sample. The samples were then vortexed for 30 s and constantly mixed in a dark room for 1 h followed by centrifugation at 13,600 × g for 15 min at room temperature. An aliquot of the supernatant (0.1 mL) was diluted with 0.9 mL buffer without NTSB²⁻ (8 M urea, 3mM EDTA, 1% SDS and 0.2M Tris-HCl with 0.1 Na₂SO₃ pH 9.5). Both free SH and total SH content were calculated using a molar extinction coefficient (ε) of 13,600 M⁻¹ cm⁻¹ as \( A = εbc \), where \( A \) is the absorbance, \( ε \) is the extinction coefficient, \( b \) is the cell path length, and \( c \) is the concentration. The disulfide content was calculated as: \( SS = (TS-SH)/2 \), where SS is disulfide content, TS is total sulphydryl content (free SH+reduced SS), and SH is free sulphydryl content. The preparation of all solutions used in the disulfide content analysis was presented in Appendix 2.
5.10. FTIR measurements

FTIR measurements were conducted to determine the effect of NaCl on gluten samples. FTIR spectra were recorded using a Perkin-Elmer Spotlight 400 ATR/FT-IR spectrometer equipped with an ATR accessory. For this, the freeze-dried gluten samples were used. A total of 128 scans were taken at an interval of 4 cm$^{-1}$ to give an optimal signal-to-noise ratio, in the range of 650-4000 cm$^{-1}$ against the background of an empty ATR crystal. Two replicate spectra were obtained for each sample. Each spectrum was automatically corrected by linear baseline correction determined by the Spectrum™ software integrated with the FTIR spectrometer. The secondary structure of the gluten samples based on the FTIR spectra were determined in the amide I region of the spectra (1600-1700 cm$^{-1}$). The assignment of the particular secondary structure of the protein was conducted based on the method previously described by Byler and Susi (1986). To measure the relative areas of the resolved amide I region, the deconvoluted spectra were curve fit using Peak Analyser Software Origin Pro 8.0 (Hearne Scientific Software). The secondary derivatives, the deconvoluted and relative peak areas were presented in Appendix 3.

Figure 5.7 Perkin-Elmer Spotlight 400 ATR/FT-IR spectrometer equipped with an ATR accessory
5.11. **Dynamic rheological measurements**

Small amplitude oscillatory sweeps were conducted both on freeze-dried gluten samples and also on freshly prepared wet gluten in order to determine the effects of NaCl on structure. In addition, tests were conducted on fresh gluten samples prepared with the incorporation of different Hofmeister salts. The measurements were conducted using a Paar Physica rheometer MCR 300 (Figure 5.8) and dynamic oscillation measurements were performed using parallel-plate geometry. The top (25 mm) and bottom plates were both serrated to prevent the sample from slipping during measurement. The rehydrated samples were prepared as follows: freeze dried gluten (0.5 g) was mixed with 0.75 mL of either water or salt solution using a mortar and spatula to obtain a dough piece containing approximately 63% w/w water.

![Figure 5.8 Rheometer Paar Physica MCR 300 for dynamic rheological measurements](image)
In addition, a proportion of the fresh wet gluten (approx. 1.5 g) was used for rheological measurements both in the study of the effect of NaCl and also the effect different Hofmeister salt series. The gluten dough was then wrapped in plastic film and allowed to rest for 1 hr. Following this, the gluten dough was placed between the plates and the upper plate was lowered to a fixed gap of 2 mm and allowed to rest for 10 min after loading. A purpose-built compartment with a water-saturated filter paper was used to minimise dehydration of the gluten sample during measurement (Day, et al 2009). Oscillation measurements were performed at strain values within the range of 0.01-1000% at a constant frequency of 1 Hz at 25 °C. Once the strain sweep curves were obtained, the linear viscoelastic region was determined. Further, the $G'$, $G''$, and tan delta values were measured within the strain of 0.05-10%. Measurements were carried out in duplicate for each gluten preparation.

Temperature sweep was conducted on gluten samples, rehydrated and fresh wet gluten sample as well as the gluten starch blend. The gluten dough and gluten starch dough was then wrapped in plastic film and allowed to rest for 1 hr. Dynamic oscillation measurements were performed on a controlled stress-strain rheometer (Paar Physica MCR 300, Messtechnik GmbH, Stuttgart, Germany), using parallel-plate geometry. Top (25 mm) and bottom plates were both serrated to prevent the sample from slipping during measurement. The gluten dough was placed between the plates and the upper plate was lowered to a fixed gap of 2 mm and allowed to rest for 10 min following loading. A purpose-built compartment with a water-saturated filter paper was used to minimise dehydration of the gluten sample during measurement (Day, et al 2009). Oscillation measurements were performed at 1% strain values, and constant frequency of 1 Hz (in Linear Viscoelastic Region). For the temperature sweep, samples were heated in the rheometer cell from 25 to 95°C at heating rate 1°C per minute. Measurements were carried out in duplicate for each gluten preparation.
5.12. Uniaxial extensional rheology

Uniaxial extensional rheology was carried out on both wheat flour dough samples and rehydrated gluten samples as well as freshly prepared gluten samples using a Universal Testing Machine (Instron 5546, Instron, UK) with a 100 N load cell using the modified methods described by McCann and Day (2013). Dough samples from FSB and Redbase flours prepared with different salts after mixing for 8 min and hand-washed gluten samples from the respective doughs, were rested for 30 min in plastic wrap before measurement. Samples were shaped using a purpose-made cylindrical PTFE mould with an inner diameter of 6.8 mm and height of 26 mm. After resting in the mould for a further 30 min, the sample was then glued onto the base and the 25 mm probe using instant adhesive. The mould was then carefully removed. Uniaxial extension was carried out by raising the probe at a rate 60 mm/min and 300 mm/min for dough and gluten samples, respectively. The extension was stopped when the samples fractured. Images of the sample were taken during extension using a Colorview III high resolution CCD camera (Olympus Life Science, Germany) connected with Cell D software. Each image was analysed using the AnalySIS® software (Soft Imaging System, Germany) to calculate the true stress ($\sigma$) and true strain ($\varepsilon$) based on the formula described by McCann and Day (2013). The stress-strain curve was then fitted with the exponential equation $\sigma = k e^n$, (Dobraszczyk & Salmanowicz, 2008; T. H. McCann & Day, 2013) where $k$ is the strain hardening coefficient and $n$ the strain hardening index. Four replicate analyses were performed. The exponential curve fitting were conducted using Origin Pro 9.0 software (Hearne Scientific Software). The fitting curves were presented in Appendix 4.
Figure 5.9  Instron Universal Testing Machine
References:


Chapter 6

Results and discussion

The effect of sodium chloride on gluten network formation and rheology

Journal article published in *Journal of Cereal Science* as Chapter 6
Chapter 7

Results and discussion

Effects of Hofmeister series on gluten network formation:
Part I. Cation series

Manuscript prepared for publication in *Food Chemistry*, presented as Chapter 7
Chapter 7

Effects of Hofmeister salt series on gluten network formation: Part I. Cation series

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Abstract

Different cation salts were used to investigate the effects of Hofmesiter salt series on gluten network formation. The work was carried out by comparing the effects of cation salts on both wheat flour dough mixing properties, the rheological, and the chemical properties of the gluten extracted from the dough with the respective salts. The aim of this work was to understand how different cation salts influence the formation of the gluten structure during dough mixing and whether the different cations have similar effects on gluten formation compared with the sodium ion. The effects of different cations on dough and gluten of different flours mostly follow the Hofmeister series (NH$_4^+$, K$^+$, Na$^+$, Mg$^{2+}$ and Ca$^{2+}$). The impacts of cations on gluten structure and dough rheology at levels tested were relatively small. Therefore, the replacement of sodium is possible from technological function, however the sensory attributes of the cation salts need to be taken into consideration.

Keywords: Hofmeister cation series, mixing properties, microstructure, uniaxial tension rheology, wheat flour dough, gluten.
1. Introduction

Consumer’ concerns over health-related issues regarding high salt intake have been the reason for the growing research on salt reduction in different food products. However, in dough based products, reducing or eliminating salt from the formulation is highly challenging. This is because salt (in the form of NaCl) is used in the processing of wheat-based foods, not only for enhancing sensory taste, but also for its technological functions (Belz, Ryan, & Arendt, 2012; Lynch, Dal Bello, Sheehan, Cashman, & Arendt, 2009; Uthayakumaran, Batey, Day, & Wrigley, 2011). This includes providing required dough strength and stability for processing which can have a profound impact on the textural characteristics of final products (Miller & Hoseney, 2008). Reducing or eliminating NaCl from the formulation may cause adverse effects on the gluten network formation and its rheological property which subsequently influences the product quality and characteristics such as the loaf volume, crumb structure and texture.

Studies have been conducted to examine the effect of NaCl on wheat flour dough properties (Butow, Gras, Haraszi, & Bekes, 2002; He, Roach, & Hoseney, 1992; Kinsella & Hale, 1984; Preston, 1989; Salovaara, 1982). The more recent studies have shown that the changes in the rheological properties in dough as influenced by NaCl primarily determined by its influence on the structure and formation of gluten matrix (Beck, Jekle, & Becker, 2012; T. H. McCann & Day, 2013). By examining the dough structure, McCann and Day (2013) found that the gluten network formation was delayed by the addition of NaCl during mixing and that the formation of the elongated fibril protein structure enhanced the dough strength during large deformation rheology. Our previous study on the effect of NaCl on the gluten structure and rheology has shown that NaCl increased the non-covalent interactions of the gluten and the β-sheet structure which results in different molecular conformation, fibrous network structure, hence differences in rheological properties (Tuhumury, Day, & Small, 2014). NaCl affect the gluten structure during the initial hydration when wheat flour is mixed with water. NaCl causes conformational changes in gluten proteins as water molecules are drawn away from the gluten to interact with sodium and chloride ions. The hydrogen bondings and hydrophobic interactions could be the reason for the increase in the β-sheet structure
within gluten. This proposed mechanism result in the formation of the gluten with typical more closely aligned structure (Tuhumury, Day, & Small, 2014).

Several explanations have been proposed regarding the effect of salts on gluten. These explanations have been based mostly on the effect of different salts belonging to the Hofmeister series (Balla, Razafindralambo, Blecker, & Paquot, 1998; Butow, Gras, Haraszi, & Bekes, 2002; Kinsella & Hale, 1984; Preston, 1989). The term of Hofmeister series originates from the ranking of various ions toward their ability to precipitate a mixture of egg white proteins (Kunz, 2010; Zhang & Cremer, 2006). During the 1880s and 1890s Franz Hofmeister and few of his co-workers demonstrated that salts with common cation but differing in anion have different effectiveness in stabilising protein suspensions (Hofmeister, 1888). Therefore they are called Hofmeister after their first investigator (Boström, Tavares, Finet, Skouri-Panet, Tardieu, & Ninham, 2005; Der, 2008; Kunz, Lo Nostro, & Ninham, 2004; Lyklema, 2009; Zhang & Cremer, 2006). From these series, two terms have been developed for the ions. These terms applied to both cation and anion series and originally referred to an ion’s ability to alter the hydrogen bonding network of water. The kosmotropes, which were believed to be water structure makers are strongly hydrated and have stabilising and salting-out effects on proteins and macromolecules. On the other hand, chaotropes, water structure breakers, are known to destabilise folded proteins and give rise to salting-in behaviour (Der, 2008; Zhang & Cremer, 2006). Recent studies on the effect of Hofmeister ions on different proteins have shown that there are direct and indirect effects of the ions on protein structural properties hence altering its functional properties. The effects are primarily depended both on the ion properties as well as the structure and chemical properties of the protein (Kunz, 2010).

The explanations on the effects of different salts on gluten have been based on the studies utilising the salts of Hofmeister series with different anion and cation series as well as different concentrations (Butow, Gras, Haraszi, & Bekes, 2002; Charlton, MacGregor, Vorster, Levitt, & Steyn, 2007; He, Roach, & Hoseney, 1992; Kinsella & Hale, 1984). Most of the studies have focused on gluten aggregation behaviour in flour.
particularly based on mixograph, farinograph and baking properties. The results from these studies have suggested that each salt has an important but different role in determining the mixing and extensional properties of dough. Salt addition, regardless of the cationic type, caused an increase in the mixing time and the effect of different cation salts on the mixing properties were determined by inter-cultivar variation of the wheat flour (Butow, Gras, Haraszi, & Bekes, 2002).

Others have also studied the properties of gluten protein fractions and the amount of solubilised or aggregated gluten proteins as affected by salts (Preston, 1981, 1989; Ukai, Matsumura, & Urade, 2008; Wellner, Mills, Brownsey, Wilson, Brown, Freeman, et al., 2004). The most recent study by Melynk et al (2011) applied both Hofmeister cation and anion series to study development time and the strength of the gluten network between flours with different protein contents using a gluten peak tester. They found that ions of the Hofmeister series, either cations or anions at high concentrations, clearly influence gluten aggregation in dough particularly peak maximum time. The effects were in accordance to their position in the series. Moreover, they suggested that flour specific differences such as protein content, protein composition, and hydrophobic composition may determine the effect of these salts on the gluten aggregation in dough. However, the effects of different salts on gluten protein network at the molecular level and resulting rheological properties still remain unclear.

The common strategy to reduce the sodium content in wheat based foods is to replace a proportion of NaCl with other chloride salts of different cations such as potassium chloride in the formulation and changes in production parameters (Farahnaky & Hill, 2007). Although the strategy has provided some success, a basic understanding of how different salts impact the formation of the gluten network and the rheological behaviour of the dough is necessary. This understanding is needed in order to maximise the technological functionality of these salts in replacing NaCl. It is not clear whether the rheological properties and structural changes of the gluten in the presence of NaCl are due to the contribution of either sodium or chloride ions. In order to understand and clarify the effect of sodium and chloride ions on gluten network formation, two series of
studies were carried out. First series was the chloride salts with different cations and second was the sodium salts with different anions.

In this study, several different cation salts were selected to investigate their effects upon gluten network formation. The work was carried out by comparing the effects of cation salts on both wheat flour dough mixing properties and the rheological properties of the gluten extracted from the dough with the respective salts. The aims of this work was to understand how different cation salts influence the formation of the gluten structure during dough mixing follow the Hofmeister series and to determine whether the different cations could have similar effects compared with the sodium on gluten formation. This in turn could provide an insight to the potential for sodium replacement and the knowledge can then be applied to the design and control of the protein/starch matrix by replacing the NaCl with other ingredient(s).
2. Materials and methods

2.1. Wheat flour samples

A high protein commercial wheat flour (FSB) and a low protein flour (Redbase) were kindly provided by Allied Mills (Kensington, Victoria, Australia). The protein contents of the flours were 13.2 and 10.4% for FSB and Redbase, respectively, determined by the AACC method 46-30 (AACC International, 2000). The moisture contents were 12.8 and 12.9% for FSB and Redbase, respectively, measured by AACC method 44-15a (AACC International, 2000). The Glu:Gli ratio of both flour were similar, i.e. 0.7 (Tuhumury, Day, & Small, 2014). Chemicals used were of analytical grade and the salts (NH₄Cl, KCl, NaCl, MgCl₂, and CaCl₂ were purchased from Chem-Supply (Sydney, Australia). Solutions of various chemical salts were prepared so that the number of moles of each cation corresponded with that of a 2% (flour base) sodium chloride solution. For monovalent cations, the same number of moles was used (0.057 moles), whereas for divalent species, half of the number of moles (0.029 moles) was used so that the same number of positive charges was utilised.

2.2. Dough mixing properties

Dough mixing properties was evaluated using a Newport Micro-doughLAB mixer (Perten Instrument, Australia). Flour sample FSB and Redbase were mixed with 2.4 mL of each salt solution at a constant speed of 63 rpm and 30 °C. The consistency was recorded using DLW version 1.0.0.56 software.

2.3. Confocal laser scanning microscopy (CLSM)

The CLSM was utilised to investigate the microstructure of dough with different cation salts. Dough samples were prepared using a Micro-doughLAB from flours with each salt solutions (NH₄Cl, KCl, NaCl, MgCl₂, and CaCl₂) and 0.2 mL of mixed fluorescein isothiocyanate (FITC, 0.025% in dimethyl sulfoxide) and rhodamine B (0.01% in MilliQ water) were added into the mixing solutions. The microstructure of the sample was examined after mixing for 8 min using a Leica SP 5 confocal microscope (Leica, ...
Microsystem, Germany) equipped with 20× oil immersion objective. FITC was excited
by a 488 nm laser and rhodamine B by a 543 nm laser. The emission fluorescence was
detected at 505-550 nm and 565-620 nm for FITC and rhodamine B, respectively. The
sample was then covered by a cover glass and viewed using a 20× objective on a Leica
SP5 Confocal Laser Scanning Microscope (CLSM) (Leica Microsystems, Germany).

2.4. Gluten washing

Fresh wet gluten samples were obtained by mixing flour (4 g) with 2.4 mL each salt
solution for 8 min, using a Micro-doughLAB mixer (Perten Instruments, NSW,
Australia). The gluten samples were then washed in salt solutions three times with the
same concentrations as for in the mixing procedure. A proportion of the fresh wet gluten
(approx. 1.5 g) was freeze-dried and used for the rheological measurements. The
remainder of the wet gluten was used for the determination of protein content and water
content of the wet gluten.

2.5. Determination of the Glu:Gli ratio and percentage of unextractable polymeric
protein (% UPP)

Protein extraction was performed on gluten samples and analysed using SE-HPLC
according to the method by McCann et al. (2009). The Glu:Gli ratio and % UPP were
determined using the methods described in McCann et al. (2009) and Tuhumury et al.
(2014), respectively.

2.6. Dynamic rheological measurement

Dynamic rheological measurement was conducted on gluten samples as described in
Tuhumury et al. (2014).

2.7. Uniaxial extensional rheology

Uniaxial extensional rheology was carried out on both wheat flour dough samples and
gluten samples using a Universal Testing Machine (Instron 5546, Instron, UK) with a
100 N load cell using the modified methods described by McCann and Day (2013).
Dough samples from FSB and Redbase flours prepared with different cation salts after mixing for 8 min and hand-washed gluten samples from the respective doughs, were rested for 30 min in plastic wrap before measurement. Samples were shaped using a purpose-made cylindrical PTFE mould with an inner diameter of 6.8 mm and height of 26 mm. After resting in the mould for a further 30 min, the sample was then glued onto the base and the 25 mm probe using instant adhesive. The mould was then carefully removed. Uniaxial extension was carried out by raising the probe at a rate 60 mm/min and 300 mm/min for dough and gluten samples, respectively. The extension was stopped when the samples fractured. Images of the sample were taken during extension using a Colorview III high resolution CCD camera (Olympus Life Science, Germany) connected with Cell D software. Each image was analysed using the AnalySIS® software (Soft Imaging System, Germany) to calculate the true stress ($\sigma$) and true strain ($\varepsilon$) based on the formula described by McCann and Day (2013). The stress-strain curve was then fitted with the exponential equation $\sigma = k e^n$, (Dobraszczyk & Salmanowicz, 2008; T. H. McCann & Day, 2013) where $k$ is the strain hardening coefficient and $n$ the strain hardening index. Four replicate analyses were performed.

### 2.8. Statistical analysis

Results of replicate analyses are presented as mean value ± standard error. Data were analysed by one way ANOVA for each set of flour and Tukeys test was used to determine significance of differences among the samples. MINITAB® 16 (Minitab Inc., USA) statistical program was used for this purpose.
3. Results and discussion

3.1. Effects of Hofmeister cation salts on dough mixing properties and microstructure

The mixing profiles of the FSB and Redbase flour dough with different Hofmeister cation salts were determined using MicrodoughLAB (Fig 1). Doughs which were prepared with monovalent cations, i.e. NH₄Cl, KCl, and NaCl exhibited a flat and more stable torque profile following peak maximum, compared with the mixing profiles of those with divalent cations (MgCl₂ and CaCl₂). The dough prepared with divalent cations displayed curves that reached the maximum peak at an earlier time (Table 1) than those of monovalent cations and followed by a steady decline in torque profile. The wheat dough without salt from the two flours have higher torque values and were significantly different to dough cation salts. The distinct differences in the mixing profiles between the monovalent and divalent cations were observed for both FSB and Redbase flour (Fig. 1). The more chaotropic is the cation salt in the Hofmeister series (e.g. NH₄⁺, K⁺, Na⁺, Mg²⁺, and Ca²⁺, respectively) used in the mixing of FSB flour, the higher is the maximum peak value and the shorter are the development time and stability (Table 1). Among all cation salts used, the mixing parameters of both flours obtained using KCl were the closest to those obtained with NaCl. This was not surprising as K⁺ is the closest to Na⁺ in the series.

These results imply that, firstly the divalent cations were less effective in maintaining the stability of the dough. Secondly, the differences in the dough mixing profiles influenced by different cations were relatively small, less than the differences between the flours, or in other words the protein content of the flour (13.2 and 10.4% for FSB and Redbase flour, respectively). Although the difference in dough mixing profile with different cations per each four were relatively small, the Redbase flour with lower protein content showed lower response than FSB flour. The work by Butow et al. (2002) also showed that response pattern of mixing time with mixograph to the addition of different cations was similar, in the sense that the mixing time increase with the order of the cations in series, and this was the case for both a low and high protein content of
wheat flour. Lower torque value hence lower dough strength was associated with the Redbase flour which had a lower protein content than the FSB flour. The importance of the protein content in determining the mixing profile of wheat flour dough with NaCl has also been suggested by McCann and Day (2013).

The microstructure of the fully developed FSB flour dough mixed for 8 min with different cation salts were examined by CLSM and presented in Fig 2. In the course of 8 min mixing time, the gluten structure with monovalent cation salts (NH$_4^+$, K$^+$, and Na$^+$) showed a typical protein network structure with interconnected gluten strands. However, there are obvious differences between the structures of the gluten within the monovalent cations. The gluten network in the dough prepared with NaCl has more fibril and extended structure (Fig. 2C). Some fibrous structures were also visible in the gluten network of the dough prepared with KCl. There were less fibrous structures observed in gluten network of the dough prepared with NH$_4$Cl. On the contrary, the gluten appeared as homogenous, continuous large aggregates gluten phase in the dough prepared with the divalent chaotropic cations (Mg$^{2+}$ and Ca$^{2+}$), which relatively had quite similar structure to gluten in the dough without salt, except for the distribution of the starch within the network. The fibrous stranded gluten network in the dough prepared with the monovalent cations is thought to result from the enhanced hydrogen bonding between glutenin molecules because of the competition of the ion with water. The degree of the effect followed the order of the cation in Hofmeister series. Monovalent kosmotropic ions tend to make water structure more ordered hence less available for flour hydration and thus delayed the unfolding of the protein (Melnyk, Dreisoerner, Bonomi, Marcone, & Seetharaman, 2011). This in turn resulted in decreased water absorption of the flour and lower torque values, respectively as shown in the dough mixing properties (Table 1).
Figure 1  Micro-dough LAB mixing curves for dough from two different flours with different Hofmeister cation salts.
### Table 1. Dough mixing properties with different cation salts from two flours.

<table>
<thead>
<tr>
<th>Salts</th>
<th>Peak maximum (mNm)</th>
<th>Water absorption (%)</th>
<th>Arrival time (min)</th>
<th>Development time (min)</th>
<th>Stability (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 1 FSB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>113.5 ± 0.9 c</td>
<td>61.8 ± 0.3 c</td>
<td>4.6 ± 0.2 a</td>
<td>9.7 ± 0.2 a</td>
<td>13.4 ± 0.7 b</td>
</tr>
<tr>
<td>KCl</td>
<td>112.0 ± 0.7 c</td>
<td>61.5 ± 0.1 cd</td>
<td>3.6 ± 0.1 b</td>
<td>9.5 ± 0.2 a</td>
<td>16.4 ± 0.1 a</td>
</tr>
<tr>
<td>NaCl</td>
<td>108.5 ± 0.9 d</td>
<td>61.1 ± 0.1 d</td>
<td>3.5 ± 0.1 b</td>
<td>8.9 ± 0.4 a</td>
<td>16.5 ± 0.1 a</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>122.3 ± 0.4 b</td>
<td>62.9 ± 0.0 b</td>
<td>3.5 ± 0.1 b</td>
<td>6.6 ± 0.1 b</td>
<td>8.2 ± 0.1 c</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>123.0 ± 0.7 b</td>
<td>62.8 ± 0.2 b</td>
<td>3.7 ± 0.1 b</td>
<td>6.7 ± 0.2 b</td>
<td>6.4 ± 0.2 d</td>
</tr>
<tr>
<td>No salt</td>
<td>137.5 ± 0.9 a</td>
<td>64.7 ± 0.1 a</td>
<td>4.5 ± 0.1 a</td>
<td>7.3 ± 0.3 b</td>
<td>6.4 ± 0.5 d</td>
</tr>
<tr>
<td>Set 2 Redbase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>106.8 ± 0.3 cd</td>
<td>60.9 ± 0.1 cd</td>
<td>1.6 ± 0.2 c</td>
<td>7.1 ± 0.3 bc</td>
<td>17.2 ± 0.1 c</td>
</tr>
<tr>
<td>KCl</td>
<td>105.0 ± 0.9 d</td>
<td>60.6 ± 0.1 de</td>
<td>1.9 ± 0.1 bc</td>
<td>9.6 ± 0.1 a</td>
<td>18.1 ± 0.1 b</td>
</tr>
<tr>
<td>NaCl</td>
<td>103.8 ± 0.9 d</td>
<td>60.4 ± 0.2 e</td>
<td>1.4 ± 0.2 c</td>
<td>7.4 ± 0.2 b</td>
<td>18.6 ± 0.2 a</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>109.5 ± 0.9 c</td>
<td>61.2 ± 0.1 c</td>
<td>2.4 ± 0.1 b</td>
<td>6.1 ± 0.3 d</td>
<td>7.3 ± 0.1 d</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>116.0 ± 0.6 b</td>
<td>62.0 ± 0.1 b</td>
<td>1.5 ± 0.1 c</td>
<td>5.9 ± 0.1 d</td>
<td>6.6 ± 0.1 e</td>
</tr>
<tr>
<td>No salt</td>
<td>122.0 ± 0.4 a</td>
<td>62.8 ± 0.1 a</td>
<td>3.3 ± 0.2 a</td>
<td>6.3 ± 0.1 cd</td>
<td>5.1 ± 0.1 f</td>
</tr>
</tbody>
</table>

Means within a column per each set of flour with the same letter are not significantly different (p < 0.05).

The divalent cations cause the breakage of water molecules (Melnyk, Dreisoerner, Bonomi, Marcone, & Seetharaman, 2011), thus allowing better penetration of water into the gluten structure and enhance the hydrogen bondings between glutenin and water molecules. This may result in the appearance of the continuous gluten phase. Higher water absorption and torque values observed in dough mixing profile for these ions could be due to the extent of protein unfolding at the early stage of dough mixing.
Figure 2  CLSM micrograph of FSB flour dough at 8 min mixing time with different cation salts: A. NH₄Cl; B. KCl; C. NaCl; D. MgCl₂; E. CaCl₂; and F. No salt.
3.2. Effects of Hofmeister cation salts on gluten proteins

The size distribution of the gluten as affected by the presence of Hofmeister cation salts upon mixing and washing of the flour dough was studied by extraction in SDS-phosphate buffer and SE-HPLC. Different cation salts did not show any significant influence on the Glu:Gli of SDS-extractable proteins from both flours. However, the Glu:Gli of the total protein in Redbase flour gluten were significantly affected by the cation salts, but not in the case of the gluten from FSB flour (Table 2). The Glu:Gli ratio of the total protein of gluten from Redbase flour decreased in the following order NH4\(^+\)\(\text{=}\)Mg\(^{2+}\)\(\text{=}\)Ca\(^{2+}\)\(\text{=}\)Na\(^+\)\(\text{=}\)K\(^+\). To characterise the changes in polymeric structure of gluten proteins in the presence of different cation salts, the results from the SE-HPLC were applied to determine % UPP.

The different cation salts did not cause any significant differences in the % UPP in the gluten samples from both flours, indicating that cation salts cause the gluten to have relatively similar proportion of the glutenin that is insoluble in SDS. In other words the cation salts mainly cause the glutenin to have similar molecular weight distribution. The recent studies regarding the protein composition of the wheat gluten (Vensel, Tanaka, & Altenbach, 2014) using a size exclusion chromatography to separate the monomeric and polymeric fractions and further analysis by quantitative two dimensional gel electrophoresis, have shown that most HMW and LMW glutenin partitioned into the polymer fractions, while most gliadins were found in the monomer fractions. In addition, there are the α-, γ-, and ω-gliadins containing the odd numbers of cysteine residues which were detected in all fractions, but present the largest proportion in polymeric fraction of SDS-extractable. These gliadins and ω-gliadins are incorporated into polymer and influence the overall amount of polymeric protein. Because of their role as chain terminating proteins, they may determine the size of the polymer, thus the % UPP. The study by Ukai, Matsumura and Urade (2008) has indicated that salt (NaCl) influences the interaction of gliadins to glutenins, as well as the aggregation of the gliadins. They have also shown that there were limited differences in aggregation of gliadins among chloride salts with different cations. In this study, no significant effect on the % UPP may be attributed to the influence of cation salts on the aggregation of the
gliadins and therefore the incorporation of the odd number cysteine gliadins and ω-
gliadins into the polymeric fractions. Thus, salts with different cations did not show
any significant role in determining the aggregation behaviour of the polymeric proteins
which made them unextractable.

Table 2. Gluten properties as function of different Hofmeister cation salts.

<table>
<thead>
<tr>
<th>Salts</th>
<th>Water content of wet gluten (%)</th>
<th>Protein content of freeze-dried gluten (%)</th>
<th>Glu:Gli SDS extractable</th>
<th>Glu:Gli total protein %</th>
<th>%UPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 1 FSB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>72.5 ± 0.2 b</td>
<td>81.6 ± 0.0 a</td>
<td>0.43 ± 0.00 bc</td>
<td>0.68 ± 0.00 a</td>
<td>49.6 ± 2.6 a</td>
</tr>
<tr>
<td>KCl</td>
<td>70.4 ± 0.1 d</td>
<td>70.8 ± 0.0 c</td>
<td>0.39 ± 0.00 c</td>
<td>0.66 ± 0.04 a</td>
<td>54.1 ± 1.0 a</td>
</tr>
<tr>
<td>NaCl</td>
<td>74.0 ± 0.2 a</td>
<td>69.9 ± 0.2 d</td>
<td>0.44 ± 0.00 ab</td>
<td>0.68 ± 0.00 a</td>
<td>45.0 ± 0.1 a</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>73.9 ± 0.4 a</td>
<td>68.9 ± 0.1 e</td>
<td>0.46 ± 0.01 ab</td>
<td>0.69 ± 0.00 a</td>
<td>47.4 ± 0.3 a</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>71.5 ± 0.2 bc</td>
<td>70.6 ± 0.0 c</td>
<td>0.48 ± 0.01 a</td>
<td>0.67 ± 0.04 a</td>
<td>44.0 ± 3.2 a</td>
</tr>
<tr>
<td>No salt</td>
<td>70.6 ± 0.2 cd</td>
<td>77.1 ± 0.1 b</td>
<td>0.46 ± 0.02 ab</td>
<td>0.71 ± 0.00 a</td>
<td>47.8 ± 4.0 a</td>
</tr>
</tbody>
</table>

Set 2 Redbase

| NH₄Cl     | 73.9 ± 0.4 a                    | 80.7 ± 0.3 a                            | 0.47 ± 0.02 a          | 0.53 ± 0.01 a           | 40.6 ± 3.6 a |
| KCl       | 72.2 ± 0.2 bc                   | 70.7 ± 0.2 b                            | 0.44 ± 0.03 a          | 0.42 ± 0.00 a           | 45.9 ± 3.9 a |
| NaCl      | 75.0 ± 0.3 a                    | 70.6 ± 0.1 b                            | 0.44 ± 0.02 a          | 0.50 ± 0.00 a           | 46.6 ± 5.2 a |
| MgCl₂     | 75.1 ± 0.1 a                    | 66.8 ± 0.4 c                            | 0.52 ± 0.02 a          | 0.61 ± 0.06 a           | 38.6 ± 1.0 a |
| CaCl₂     | 72.3 ± 0.4 b                    | 68.2 ± 0.2 c                            | 0.55 ± 0.05 a          | 0.60 ± 0.07 a           | 37.4 ± 1.2 a |
| No salt   | 70.7 ± 0.3 c                    | 79.8 ± 0.1 a                            | 0.48 ± 0.03 a          | 0.54 ± 0.01 a           | 42.5 ± 3.8 a |

a: All values presented as % values on a w/w basis.
b: N × 5.7.
Means within a column per each set of flour with the same letter are not significantly different (p < 0.05).
3.3. Effects of Hofmeister cation salts on dynamic rheological properties of gluten

In order to understand the effect of different cation salts on gluten structure, the gluten samples were prepared by mixing for 8 min with the series of cation salts and were hand-washed in the salt solutions of the same concentrations used for mixing the dough. The small deformation rheological properties of washed gluten samples were measured. The storage modulus $G'$, loss modulus $G''$, and tan $\delta$ values at 1% strain (in the LVR region) of the gluten with different cation salts are shown in Fig. 3. In all cases, the $G'$ values of the gluten samples were higher than the $G''$ values indicating that gluten samples prepared with these cations persisted a solid or elastic like behaviour.

However, different cation salts appear to have significant effects on the absorption $G'$, $G''$ values, but not on their ratios, i.e. the tan $\delta$ values (Fig. 3). These results indicated that different cation salts contribute significantly on both the elasticity and viscosity of the gluten network. Differences in $G'$ and $G''$ values of the gluten with different cations may arise from differences in water content of the gluten dough. Studies have showed that rheological properties of wheat gluten are influenced by a number of factors. Water content and flour type have been shown to have significant effect on $G'$ measured by dynamic oscillatory test in linear viscoelastic region (Autio, Flander, Kinnunen, & Heinonen, 2001; Dreese, Faubion, & Hoseney, 1988). The gluten samples prepared without salt had highest $G'$ and $G''$ values for both flours. Kosmotropic cations ($\text{NH}_4^+$, $\text{K}^+$, and $\text{Na}^+$) have similar $G'$ and $G''$ values. They were higher compared with the $G'$ and $G''$ values of the gluten samples prepared with chaotropic divalent cations ($\text{Mg}^{2+}$ and $\text{Ca}^{2+}$). The $G'$ and $G''$ values of the gluten without salt were not different to the gluten with kosmotropic salts but were different to those with chaotropic salts. Our previous results have also showed that gluten without salt had similar $G'$ and $G''$ to gluten with NaCl. Most of the studies regarding the effect of salts on the dynamic rheological properties have been focused on the effect of NaCl with varying concentrations on the wheat flour dough. There seemed to be contradictory results on the $G'$ and $G''$ values of the dough with increasing NaCl concentrations (Beck, Jekle, & Becker, 2012; Larsson, 2002; Lynch, Dal Bello, Sheehan, Cashman, & Arendt, 2009). Such measurements have not been applied to study the effects of different salts on...
Therefore, this study emphasise that gluten structural network were influenced by different cation salts. These results may indicate that the cations do influence the gluten network structures indirectly by altering the water structure hence the gluten structure. It is obvious also from the microstructure of the dough with different cations, that there were structural differences of gluten structure in dough matrix with different cations. This may consequently influence the $G'$ and $G''$ when gluten samples were measured using small deformation oscillation sweep.

Moreover, the $G'$ and $G''$ values of gluten from both flours with NH$_4$Cl did not follow the correct series of the earlier series proposed based on its effect to solvent structure. However, these values were not different statistically to both K$^+$ and Na$^+$. Kunz (2010) has mentioned that the nature of the geometry of NH$_4^+$ (organic ion) compared to others which are inorganic ions and the characteristic of charged headgroups which acts as counterions can significantly alter the position of this ion in the series. Therefore, the nature of the NH$_4^+$ may be the reason for the altered position in series on their effects on gluten molecules, but still remain similar with other kosmotropic cations used in this study. The different series can also be possibly encountered as it has been mentioned by Kunz (2010) that based on the published data there seems to be no single and unique series. The position of the ions can be altered in series and the series can be all reversed depending on the properties of the sample being examined.
Figure 3  G’, G’’ and tan δ values at LVR of gluten from two flours with different Hofmeister cation salts generated from small deformation oscillatory shear. Columns with same letter column per each set of flour with the same letter are not significantly different (p < 0.05).
The decrease in $G'$ values of the gluten with different cation salts were accompanied by
the decrease in $G''$ values proportionally, hence resulting in the tan $\delta$ values that did not
differ significantly. This means that there were no differences in the relative
contributions of the viscous and elastic components to the rheological properties of
samples as affected by cation salts. Overall, the viscoelastic properties of the gluten
with different cation salts were similar. This indicates that the tan $\delta$ values of gluten
with cation salts depend on the aggregation behaviour of the gluten and the molecular
weight distribution of gluten which remain similar in the presence of different cations.

3.4. Effects of Hofmeister cation salts on uniaxial rheology

3.4.1. Wheat flour dough

The large deformation extension was conducted to determine the strength of the dough
samples with different cations of the chloride salts. The maximum distance ($D_{\text{max}}$) and
maximum force ($F_{\text{max}}$) values at fracture of dough samples of the two flours were
determined (Table 3). The dough samples prepared in the presence of NH$_4$Cl and CaCl$_2$
exhibited the lowest $D_{\text{max}}$ although they were not statistically different to the dough
prepared with other cations salts in the case of Redbase flour. This may be due to large
variation in the measurements (Table 3). The $F_{\text{max}}$ values of the dough samples were
greatly affected by the addition of chloride salts with different cations. The cations were
ordered in series based on their effect on the $F_{\text{max}}$ values. $F_{\text{max}}$ values decreased in this
order $K^+=Na^+>NH_4^+>Mg^{2+}=Ca^{2+}$ and occurred in both dough samples of the two flours.
The monovalent cations appeared to have a relatively similar effect on the $F_{\text{max}}$ values
and they showed different effects on the values compared to the divalent cations.

By taking into account the changes in the dough sample geometry during the tests, the
stresses and strains were determined from the force-displacement measurement (Fig. 4). The strain hardening coefficients ($k$) and hardening indices ($n$) obtained from the
exponential fitting of the stress-strain curve were also summarised in Table 3. Both
dough samples with different cation salts showed a strain hardening behaviour, i.e. an
increase in stress values as the strain is increased, however all the dough samples
showed similar stress-to-strain profiles for both flours (Fig. 4). There was little
difference statistically in either the n values or the k values of the flour dough from both
flours. In comparison with the dough without salt, the n and k values were not different
statistically, however it tend to have lower values. The results from the study by
McCann & Day (2013) also showed that FSB dough without NaCl has lower n and k
values than the dough with NaCl. The n and k values in their study were higher than
what was found in our study. This is because the dough in the previous method applied
total displacement of 52 mm, while the displacement in this study was set at maximum
displacement at fracture. However, the trend with lower n and k values with dough
without NaCl still applied. When comparison was made between the dough with
different cation salts, the results indicated that different cations of chloride salts did not
appeared to have insignificant influence on the strain hardening behaviour during
extension of the flour dough.

The results from the uniaxial extensional rheology imply that cation salts largely affect
the resistance of the dough to extension and the extensibility but not the strain
hardening behaviour. It has been widely known that the rheological behaviour of flour
dough at large deformations is mainly determined by the gluten fraction (Kokelaar, van
Vliet, & Prins, 1996; Sliwinski, van der Hoef, Kolster, & van Vliet, 2004;
Uthayakumaran, Newberry, Phan-Thien, & Tanner, 2002). The subtle differences in the
microstructure of the gluten network in dough with different cation salts with typical
fibrous and extended structure observed for NaCl and becoming less apparent in KCl
and NH₄Cl, subsequently may contribute to the dough having less resistance to
extension during large deformation extension. Therefore, the resistance of the dough to
extension as function of different cation salts may be more influenced by the
microstructure of the gluten in the dough for example the number of gluten strands or
bundles formed during mixing. On the other hand, the strain hardening behaviour may
be largely influenced by the size of the glutenin aggregates. The theory of glutenin
predicts that there is a critical molecular weight of glutenin above which it begins to
impart contribution to dough strength (MacRitchie, 2014). From these results, cation
salts cause changes in the gluten structure which results in the gluten that have similar
fraction of higher molecular weight glutenin. This in turn will result also in the dough having similar strain hardening behaviour. In order to further elucidate this hypothesis, the uniaxial rheological properties of the glutens that were isolated by washing the dough with the respective salt solutions as used in the mixing process, were also examined.

3.4.2. Isolated gluten

Table 3 also shows the maximum distance ($D_{\text{max}}$) and maximum force ($F_{\text{max}}$) values at fracture of gluten samples of the two flours, and their stress-to-strain profiles are shown in Fig. 4. $D_{\text{max}}$ and $F_{\text{max}}$ values of gluten with NaCl and without NaCl were not different significantly and are in agreement with our previous study (Tuhumury, Day, & Small, 2014), although the values are lower for this present study. The nature of the samples, the rehydrated gluten samples in the previous study and the freshly prepared wet gluten samples in this present study were the reason of difference in the values.

Unlike the uniaxial results for the dough samples, the $D_{\text{max}}$ and $F_{\text{max}}$ values of gluten from both flours were found to be significantly influenced by the type of chloride salts (Table 3), although there was no clear relationship between the order in the position of the cation in the series for the $D_{\text{max}}$ values. However, the cations were ordered in series based on their effect on the $F_{\text{max}}$ values, which for this particular study decreased the order of $K^+ = Na^+ > NH_4^+ > Mg^{2+} = Ca^{2+}$. This trend also occurred in dough samples of the two flours, which indicates that the effect of cation salts on the resistance to extension in uniaxial rheology of the dough was largely related to their effects on gluten proteins.

Gluten samples with different cation salts also showed a strain hardening behaviour at higher stress levels at the same strain compared with the dough samples. However, similar to that found for the dough samples, there were no differences statistically in the strain hardening coefficients ($k$) and hardening indices ($n$) of the gluten samples (Table
These results indicated that different cation salts in the Hofmeister cation series did not influence the strain hardening behaviour of the gluten samples.

The results from the uniaxial extensional rheology of both the dough and gluten samples indicated that the effects of cation salts on the dough were determined by their effects on gluten network formation. In addition, the cation salts affect the resistance of the gluten to extension and the extensibility but not the strain hardening behaviour. The effects on the resistance to extension and extensibility may be likely caused by subtle microstructural differences in the gluten network. The different amount of apparent fibrous structure as an indicative of the hydrogen bonding between glutenin molecules formed during hydration and mixing may regulate the degree of the resistance to extension during large deformation extension. These has been in the agreement with the model proposed by Belton (1999) that the hydrogen bondings or the loops and trains ratio in the gluten network are responsible for the resistance to extension and that the NaCl cause the enhanced hydrogen bondings between the glutenin molecules thus its resistance to extension (Tuhumury, Day, & Small, 2014).

However, from these results it also appeared that the strain hardening behaviour as determined by the n and k values of the dough as well as gluten samples remain unaffected by salts with different cations. It has been mentioned previously that the strain hardening behaviour in this study may be determined by the unextractable part of the HMW glutenin, because the % UPP of the gluten with different cation salts also did not show any significant differences. Cation salts cause changes in the gluten structure with similar aggregation of gliadins which results in the gluten that have similar fraction of highest HMW glutenin. Therefore, the results from this study suggest both the non-covalent interactions (Belton, 1999) and the entangled polymer network with critical molecular weight of glutenin above which it begins to impart contribution to dough strength (MacRitchie, 1992, 2014) contributes to the formation of the gluten network and its respective rheological properties.
### Table 3  Effects of different cation salts on uniaxial tension parameters of the wheat flour dough and gluten from two flours

<table>
<thead>
<tr>
<th>Salts</th>
<th>Wheat flour dough</th>
<th>Wheat gluten</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D_{\text{max}}$ (mm)</td>
<td>$F_{\text{max}}$ (mN)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 1 FSB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>101 ± 8 b</td>
<td>185 ± 12 ab</td>
</tr>
<tr>
<td>KCl</td>
<td>153 ± 10 a</td>
<td>213 ± 8 a</td>
</tr>
<tr>
<td>NaCl</td>
<td>136 ± 5 a</td>
<td>204 ± 9 a</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>146 ± 3 a</td>
<td>154 ± 2 bc</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>122 ± 4 ab</td>
<td>125 ± 3 c</td>
</tr>
<tr>
<td>No salt</td>
<td>150 ± 11 a</td>
<td>133 ± 8 c</td>
</tr>
<tr>
<td>Set 2 Redbase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>135 ± 17 a</td>
<td>130 ± 3 a</td>
</tr>
<tr>
<td>KCl</td>
<td>153 ± 6 a</td>
<td>149 ± 9 a</td>
</tr>
<tr>
<td>NaCl</td>
<td>148.5 ± 23 a</td>
<td>135 ± 8 a</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>145.5 ± 14 a</td>
<td>100 ± 6 b</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>129 ± 6 a</td>
<td>86 ± 1 b</td>
</tr>
<tr>
<td>No salt</td>
<td>145 ± 3 a</td>
<td>92 ± 3 b</td>
</tr>
</tbody>
</table>

$a$: Strain hardening index.

$b$: Strain hardening coefficient.

Means within a column per each set of flour with the same letter are not significantly different ($p < 0.05$).
Figure 4  Stress-strain curves of the dough and gluten from two flours with different cation salts generated from uniaxial extensional rheology measurement.  
Note: Different scales are used for the dough and gluten samples.
Moreover, in presence of cation salts, firstly, the most apparent feature in the gluten network were non-covalent interactions that determine the differences in the microstructures, the dough mixing profile, small deformation rheology, and the resistance to extension during large deformation. Secondly, the aggregation of gliadins which determine the aggregate size of the glutenins was not influenced by cation salts. This in turn caused similar strain hardening behaviour. Good strain hardening characteristics should results in finer crumb structure and larger bake volume (Dobraszczyk & Morgenstern, 2003). The strain hardening behaviour and thus the crumb and volume of the baked of the product from gluten with NaCl is similar to other cation salts.

4. Conclusion

The effects of different cations on dough and gluten of different flours mostly follow the Hofmeister series. Monovalent kosmotroph cations (NH$_4^+$, K$^+$, and Na$^+$) tend to have similar rheological properties both the dough mixing profile as well as the gluten dynamic rheological properties. However, the chaotroph cations (Mg$^{2+}$ and Ca$^{2+}$) showed different rheological properties measured by both compared to the kosmotroph one. These resulting rheological properties may arise from the effect of these salts upon the water structure. The kosmotroph cause the water structure more ordered which made water less available for gluten upon hydration resulting in more time to reach peak maximum (fully developed dough) during mixing and lower torque values in the mixing profile. Whereas, the divalent cations (Mg$^{2+}$ and Ca$^{2+}$) break the structure of the water which made it available and aid faster hydration and protein unfolding which has higher water absorption and faster time to reach maximum peak, and resulting the gluten to form honeycomb like network around starch granules. Though the kosmotroph showed primarily similar rheological properties, the microstructure observed among them were different. However, the microstructure of the dough with KCl showed a closer structural resemblance to that of the NaCl.

The effect of Hofmeister cation salts on the rheological behaviour of wheat flour dough at large deformations were primarily determined by their effects on gluten structure.
The presence of cation salts in the formation of gluten network during hydration influence the resistance to extension and extensibility of the structure but not strain hardening behaviour, when applied under large deformation. Among the different cations, the $K^+$ was having the similar effect on large deformation rheological properties of both dough and gluten samples including the extensibility, resistance to extension, and the strain hardening behaviour to that of NaCl.

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References:


Chapter 7


Chapter 8

Results and discussion

Effects of Hofmeister series on gluten network formation: Part II. Anion series

Manuscript prepared for review and publication in *Food Chemistry*, presented as Chapter 8
Effects of Hofmesiter series on the formation of gluten network: Part II. Anion series

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Abstract

Different anion salts were used to investigate the effects of Hofmeisiter salt series on gluten network formation. The work was carried out by comparing the effects of anion salts on both wheat flour dough (mixing properties and microstructure), and the properties of the gluten extracted from the dough with the respective salts (chemical and rheological properties). The aim of this work was to understand how different anion salts influence the formation of the gluten structure during dough mixing. Hofmeister anion salts influence the gluten network formation in a way that they cause changes in gluten protein composition, as well as the percentage of the unextractable polymeric protein fraction (% UPP) by interacting directly with specific amino acid residues. These changes consequently result in the remarkable differences in the dough mixing profile, microstructural features of the dough, the small deformation rheological properties of the gluten, as well as the strain hardening behaviour of dough and gluten samples.

Keywords: Hofmeister anion series, gluten, dough, microstructure, dynamic, uniaxial tension rheology.
1. Introduction

Wheat-based foods have formed a basic staple diet for many people around the world and have contributed to mankind’s nutritional needs. However, over the recent years, the wheat-based foods have been criticised for having high salt content, particularly in bread. In a typical western diet, bread products contribute to 30% salt intake and are the major food source of dietary sodium (Belz, Ryan, & Arendt, 2012). High sodium intake has been linked to serious health problems including elevated blood pressure and associated cardiovascular diseases (Tuomilehto, Jousilahti, Rastenyte, Moltchanov, Tanskanen, Pietinen, et al., 2001). There is an increasing need for food manufacturers to produce food products with reduced salt levels. Nevertheless, salt, specifically sodium chloride (NaCl), has important technological functions in the processing and quality characteristics of wheat-based foods, not only that it enhances the flavour of the baked product but also the dough strength and its handling during production (Miller & Hoseney, 2008).

Studies have been conducted to determine the importance of NaCl on the functional and baking properties of wheat flour dough (Butow, Gras, Haraszi, & Bekes, 2002; He, Roach, & Hoseney, 1992; Kinsella & Hale, 1984; Preston, 1989; Salovaara, 1982). Many of them were carried out by measuring dough strength with Mixograph and Farinograph. Several hypotheses have been proposed regarding the effect of salts on gluten. They are typically based on the effect of different salts belonging to the Hofmeister series. Recent studies on the effect of Hofmeister ions on different proteins including lysozyme and bovine serum albumin have also shown that there are direct effects of the ions on protein structural properties e.g.: the ions of the salts interact directly with the chemical groups of the protein including amino acid residues, the peptide bonds, as well as amino and carboxyl groups in order to neutralise their electrical charges (Kunz, 2010; Lo Nostro & Ninham, 2012; Zhang & Cremer, 2006). The effects are primarily dependent on the ion properties as well as the structure and chemical properties of the protein (Kunz, 2010). When dough is prepared without salt, the gluten protein has positive net charges in the flour-water system because the distribution of ionic amino acid residues is higher for the basic amino acid residues.
including arginine, histidine, and lysine (Galal, Varriano-Marston, & Johnson, 1978).

Under this condition, lysine and arginine would exist entirely in the positively charged form. The positive charge at the protein surface makes the protein molecules repel each other and this restricts the extent to which protein molecules interact, thereby resulting in a weaker dough network. When salt is present, it shields the charges on the gluten protein, reducing electrostatic repulsion between proteins and allowing them to associate thus producing a stronger dough (Kinsella & Hale, 1984; Miller & Hoseney, 2008). These interactions depend mainly on the type of anion or cation of the salt and are more pronounced at low salt concentrations (Balla, Razafindralambo, Blecker, & Paquot, 1998).

Our previous study on the effect of sodium chloride on the gluten structure and rheology has shown that the presence of NaCl in comparison with the absence of NaCl during dough mixing has increased the non-covalent interaction which resulted in different molecular conformation and network structure of gluten proteins and contributed to the differences in the rheological properties (Tuhumury, Day, & Small, 2014a). In addition, the effect of NaCl on the gluten structure is controlled by the extent of protein hydration during the initial stage of mixing of flour with water. By examining the dough structure, McCann and Day (2013) found that the gluten network formation was delayed by the addition of NaCl during mixing and that the formation of the elongated fibril protein structure enhanced the dough strength during large deformation rheology.

NaCl is the ionic combination of the sodium and chloride. Studies with different sets of cation as well as the anion salt series are necessary to investigate the effects of salts on gluten structure. With regard to the effects of salt with different cations, although the earlier study by Butow et al. (2002) showed that salts with different cations affect the dough mixing profile measured by the Mixograph, the more recent study by Ukai et al. (2008) suggested that there were limited difference in aggregation among chloride salts with different cations. The similar extractability of polymeric protein from gluten
isolated from dough with different cation salts indicated that gluten macromonomer structure in these dough systems was largely unchanged.

In Part I of this study, we also investigated the effect of different cation salts on gluten network formation (Tuhumury, Day, & Small, 2014b). The results show that Hofmeister cations have little influence on the % UPP (aggregation of the polymeric protein fraction) and the strain hardening behaviour of both wheat flour dough and gluten samples. However, there were subtle differences in the gluten network microstructures as observed by CLSM influenced by different cation salts. The differences in the gluten network microstructures explained the differences in the dough mixing profile, small deformation rheological properties, as well as the D_max and F_max value during extension. Hofmeister cation salts appear to influence gluten network formation indirectly by altering the water structure and thus hydrogen bondings and hydrophobic interactions. Hofmeister kosmotrophic cation K⁺ has similar effect on the functional properties of the gluten to that of NaCl (Tuhumury, Day, & Small, 2014b).

The effects of different anion salts have also been investigated. Preston (1989) showed that the presence of chaotropic anions reduced dough strength and increased Farinograph water absorption. The author went on to suggest that the much larger effect of chaotropic anions may be related to their ability to induce conformational changes in gluten protein that could further enhance inter-protein interactions (Preston, 1989). The most recent study by Melynk et al (2011) applied both Hofmeister cation and anion series to study development time and strength of the gluten network between flours with different protein contents using a gluten peak tester. They found that ions of the Hofmeister series either cations or anions at mostly high concentrations (> 0.3 M) clearly influence gluten aggregation in dough, particularly the peak maximum time. The effects were in accordance to their position in the series. Moreover, they suggest that flour specific differences such as protein content, protein composition, and hydrophobic composition may determine the effect of these salts on the gluten aggregation in dough. However, they did not provide the effect of salts on the composition of protein fractions which can be a determinant factor for gluten aggregation and dough properties. Other
studies have also suggested that when it comes to specific ions effects (the effect on the protein molecule directly), anions are more pronounced than cations in their specific ion effects (Kunz, 2010; Lo Nostro & Ninham, 2012; Rembert, Paterová, Heyda, Hilty, Jungwirth, & Cremer, 2012). This is because anions have little influence on the dynamic of bulk water as solvent, but are more effective at associating with protein and screening of the electrostatic repulsion.

In this part II of the study, several different anion salts were selected to investigate their effects upon gluten network formation. This present study was aimed to study the effects of different anion salts on gluten formation by comparing their effects on wheat flour dough mixing properties, gluten and dough rheological properties, as well as gluten composition and aggregation prepared from the dough with the respective salts. The hypotheses were that the different anion salts influence formation of the gluten structure during dough mixing follow the Hofmeister anion series and different anions may have more pronounced effect on gluten structure than the cations.
2. Materials and methods

2.1. Wheat flour samples

A high protein commercial wheat flour (FSB) and a low protein flour (Redbase) were kindly provided by Allied Mills (Kensington, Victoria, Australia). The protein contents of the flours were 13.2 and 10.4% for FSB and Redbase, respectively, determined by the AACC method 46-30 (AACC International, 2000). The moisture contents were 12.8 and 12.9% for FSB and Redbase, respectively, measured by AACC method 44-15a (AACC International, 2000). The Glu:Gli ratio of both flour were similar, i.e. 0.7. Chemicals used were of analytical grade and the salts (NaH$_2$PO$_4$, NaF, NaCl, NaBr, and NaI were purchased from Chem-Supply (Sydney, Australia). Solutions of various chemical salts were prepared so that the number of moles of each (0.057 moles) corresponded with that of a 2% (flour base) sodium chloride solution.

2.2. Dough mixing properties

Dough mixing properties were evaluated using a Newport Micro-dough LAB mixer (Perten Instruments, Australia). Flour samples FSB and Redbase were mixed with 2.4 mL of each salt solution at a constant speed of 63 rpm and 30 °C. The consistency was recorded using DLW version 1.0.0.56 software.

2.3. Confocal laser scanning microscopy (CLSM)

The CLSM was utilised to investigate the microstructure of dough with different anion salts. Dough samples were prepared using a Micro-dough LAB from flours with each salt solutions (NaH$_2$PO$_4$, NaF, NaCl, NaBr, and NaI) and 0.2 mL of mixed fluorescein isothiocyanate (FITC, 0.025% in dimethyl sulfoxide) and rhodamine B (0.01% in MilliQ water) were added into the mixing solutions. The microstructure of the sample was examined after mixing for 8 min using a Leica SP 5 confocal microscope (Leica Microsystem, Germany) equipped with 20× oil immersion objective. FITC was excited by a 488 nm laser and rhodamine B by a 543 nm laser. The emission fluorescence was detected at 505-550 nm and 565-620 nm for FITC and rhodamine B, respectively. The
sample was then covered by a cover glass and viewed using a 20× objective on a Leica SP5 Confocal Laser Scanning Microscope (CLSM) (Leica Microsystems, Germany).

### 2.4. Gluten washing

Fresh wet gluten samples were obtained by mixing flour (4 g) with 2.4 mL each salt solution for 8 min, using a Micro-dough LAB mixer (Perten Instruments, NSW, Australia). The gluten samples were then washed in salt solutions three times with the same concentrations as for in the mixing procedure. A proportion of the fresh wet gluten (approx. 1.5 g) was used for the rheological measurements. The remainder of the wet gluten was used for the determination of protein and moisture contents.

### 2.5. Determination of the Glu:Gli ratio and percentage of unextractable polymeric protein (% UPP)

Protein extraction was performed on gluten samples and analysed using SE-HPLC according to of McCann et al. (2009). The Glu:Gli ratio and % UPP were determined using the methods described in McCann et al. (2009) and Tuhumury et al. (2014a), respectively.

### 2.6. Dynamic rheological measurement

Dynamic rheological measurement was conducted on gluten samples as described in Tuhumury et al. (2014a)

### 2.7. Uniaxial extensional rheology

Uniaxial extensional rheology was carried out on both wheat flour dough samples and gluten samples using a Universal Testing Machine (Intron 5546, Intron, UK) with a 100 N load cell using the modified methods described by McCann and Day (2013). Dough samples from FSB and Redbase flours prepared with different anion salts after mixing for 8 min and hand-washed gluten samples from the respective doughs, were rested for 30 min in plastic wrap before measurement. Samples were shaped using a purpose-made cylindrical PTFE mould with an inner diameter of 6.8 mm and height of
26 mm. After resting in the mould for a further 30 min, the sample was then glued onto the base and the 25 mm probe using instant adhesive. The mould was then carefully removed. Uniaxial extension was carried out by raising the probe at a rate 60 mm/min and 300 mm/min for dough and gluten samples, respectively. The extension was stopped when the samples fractured. Images of the sample were taken during extension using a Colorview III high resolution CCD camera (Olympus Life Science, Germany) connected with Cell D software. Each image was analysed using the AnalySIS® software (Soft Imaging System, Germany) to calculate the true stress ($\sigma$) and true strain ($\varepsilon$) based on the formula described by McCann and Day (2013). The stress-strain curve was then fitted with the exponential equation $\sigma = k\varepsilon^n$, (Dobraszczyk & Salmanowicz, 2008; T. H. McCann & Day, 2013) where $k$ is the strain hardening coefficient and $n$ the strain hardening index. Four replicate analyses were performed.

### 2.8. Statistical analysis

Results of replicate analyses are presented as mean value ± standard error. Data were analysed by one way ANOVA for each set of flour and Tukeys test was used to determine significance of differences among the samples. MINITAB® 16 (Minitab Inc., USA) statistical program was used for this purpose.
3. Results and discussion

3.1. Effects of Hofmeister anion salts on dough mixing properties and microstructure

The mixing profiles of the wheat flour dough with different anion salts were determined by Micro-doughLAB and shown in Fig 1. For each flour, the sodium salt with different anions showed remarkable differences in the mixing profiles. The more chaotropic is the anion in the Hofmeister series ($\text{H}_2\text{PO}_4^-$, $\text{F}^-$, $\text{Cl}^-$, $\text{Br}^-$ and $\text{I}^-$, respectively) used in the mixing of flour, the higher is the maximum peak value and water absorption but the lower is the development time and stability (Table 1). Both wheat flours showed similar mixing profile in response to the presence of different anion salts. Dough mixing profile (peak maximum torque, water absorption, and stability) of wheat flour without salt were in between the values obtained for flour with NaBr and NaI. When the cation salts were used in our previous study, the flour without salts had the higher torque values and were significantly different to dough with cation salts. In addition, there were large differences between mixing profile of dough with different anion salts compared to the dough with different cations in our previous study (Tuhumury, Day, & Small, 2014b). This indicates that different anion salts have more pronounced impact on the structure of gluten proteins during mixing in comparison to doughs with different cations of chloride salts.

The effect of the anion on the protein molecules have been reported to have more pronounced effect than the cations (Kunz, 2010; Lo Nostro & Ninham, 2012; Rembert, Paterová, Heyda, Hilty, Jungwirth, & Cremer, 2012). This is because anions have little influence on the dynamic of bulk water as solvent, but are more effective at associating with protein and screening of the electrostatic repulsion on specific binding sites. However, most cations usually excluded from the polypeptide, and this exclusion is the reason why direct cation effects are less pronounced than direct anion effects.
Figure 1  Micro-dough LAB mixing curves for dough from two different flours with different Hofmeister anion salts.
Table 1. Dough mixing properties with different anion salts from two flours.

<table>
<thead>
<tr>
<th>Salts</th>
<th>Peak maximum (mNm)</th>
<th>Water absorption (%)</th>
<th>Arrival time (min)</th>
<th>Development time (min)</th>
<th>Stability (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Set 1 FSB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>104.0 ± 1.0 d</td>
<td>60.4 ± 0.2 d</td>
<td>6.2 ± 0.2 a</td>
<td>10.0 ± 0.0 a</td>
<td>13.8 ± 0.1 b</td>
</tr>
<tr>
<td>NaF</td>
<td>106.0 ± 1.0 cd</td>
<td>60.8 ± 0.2 cd</td>
<td>5.5 ± 0.1 b</td>
<td>9.9 ± 0.1 ab</td>
<td>14.5 ± 0.1 b</td>
</tr>
<tr>
<td>NaCl</td>
<td>108.5 ± 0.9 c</td>
<td>61.1 ± 0.1 c</td>
<td>3.5 ± 0.1 d</td>
<td>8.9 ± 0.4 b</td>
<td>16.5 ± 0.1 a</td>
</tr>
<tr>
<td>NaBr</td>
<td>119.5 ± 0.7 b</td>
<td>62.4 ± 0.1 b</td>
<td>4.5 ± 0.2 c</td>
<td>9.5 ± 0.2 ab</td>
<td>11.7 ± 0.3 c</td>
</tr>
<tr>
<td>NaI</td>
<td>142.0 ± 1.0 a</td>
<td>65.3 ± 0.2 a</td>
<td>4.7 ± 0.1 c</td>
<td>6.8 ± 0.1 c</td>
<td>5.0 ± 0.3 e</td>
</tr>
<tr>
<td>No salt</td>
<td>137.5 ± 0.9 a</td>
<td>64.7 ± 0.1 a</td>
<td>4.5 ± 0.1 c</td>
<td>7.3 ± 0.3 c</td>
<td>6.4 ± 0.5 d</td>
</tr>
<tr>
<td><strong>Set 2 Redbase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>100.3 ± 0.5 de</td>
<td>60.0 ± 0.1 de</td>
<td>5.7 ± 0.2 a</td>
<td>9.5 ± 0.4 a</td>
<td>14.3 ± 0.2 b</td>
</tr>
<tr>
<td>NaF</td>
<td>96.0 ± 1.0 e</td>
<td>59.6 ± 0.2 e</td>
<td>5.1 ± 0.1 a</td>
<td>9.9 ± 0.1 a</td>
<td>15.1 ± 0.2 b</td>
</tr>
<tr>
<td>NaCl</td>
<td>103.8 ± 0.9 d</td>
<td>60.4 ± 0.2 d</td>
<td>1.4 ± 0.2 d</td>
<td>7.4 ± 0.2 bc</td>
<td>18.6 ± 0.2 a</td>
</tr>
<tr>
<td>NaBr</td>
<td>115.8 ± 0.8 c</td>
<td>62.0 ± 0.1 c</td>
<td>2.9 ± 0.3 c</td>
<td>8.0 ± 0.6 b</td>
<td>11.6 ± 0.7 c</td>
</tr>
<tr>
<td>NaI</td>
<td>135.5 ± 0.9 a</td>
<td>64.5 ± 0.1 a</td>
<td>4.2 ± 0.1 b</td>
<td>6.0 ± 0.2 c</td>
<td>3.8 ± 0.3 d</td>
</tr>
<tr>
<td>No salt</td>
<td>122.0 ± 0.4 b</td>
<td>62.8 ± 0.1 b</td>
<td>3.3 ± 0.2 c</td>
<td>6.3 ± 0.1 c</td>
<td>5.1 ± 0.1 d</td>
</tr>
</tbody>
</table>

Means within a column for each set of flour with the same letter are not significantly different (p < 0.05).

Previous studies on the effect of the anion series on the dough mixing profile have shown that more chaotropic anions reduced the dough strength and Farinograph water absorption (Preston, 1989). The results from this study are in agreement with the present study mentioned previously that the more chaotropic is the anion in the series, the higher is the peak torque value (reduced strength) and the water absorption (Table 1).

CLSM images of the fully developed dough (8 min mixing time) were obtained to investigate the dough structural differences that resulted in the specific mixing profile mentioned before (Fig. 2). There were clear structural differences in the gluten network prepared with kosmotropic anion salts (H₂PO₄⁻, F⁻, and Cl⁻). The kosmotropic anions resulted in the formation of gluten network with fibril structure, with H₂PO₄⁻ having larger strand surface. The more aligned fibril structure gave the dough more stable and flat torque values during mixing. On the contrary, the chaotropic anions (Br⁻ and I⁻)
caused the gluten to form a homogenous, continuous honey-comb like network, similar to that gluten structure formed without salt. This suggests that chaotropic anions have little influence on the hydration properties of gluten and its network formation. This also explains the mixing properties/curves of chaotropic anions were similar to no salt.

Kosmotropic anions binds to the gluten molecules and were more effective in shielding the electrostatic repulse during flour hydration and experienced less unfolding of the protein with enhanced hydrogen bonding within glutenin molecules. While the chaotropic anion may be less effective in shielding the repulsive forces which favour more protein unfolding. The unfolding of the protein and disruption of the native polypeptide interactions within the gluten proteins would lead to honey-comb like structure of gluten with chaotropic anions.

The remarkable structural differences in gluten network and mixing profile of the flours observed between the kosmotropic anions were contrast to the much less differences within the kosmotropic cations (Tuhumury, Day, & Small, 2014b). This suggested that the dough mixing properties with different anions are the results of structural differences of gluten with different anions and that the effects were more pronounced than different cation salts. The effect of the anions on gluten structure may be associated to specific ion effect on gluten molecule. It has been shown that most of the biomolecules including proteins also experienced the specific ion effect, which is the direct effect of ion on the molecules. The ions of the salts interact directly with the chemical groups of the protein including amino acid residues, the peptide bonds, as well as amino and carboxyl groups in order to neutralise their electrical charges (Kunz, 2010; Lo Nostro & Ninham, 2012; Rembert, Paterová, Heyda, Hilty, Jungwirth, & Cremer, 2012).
Figure 2  CLSM micrograph of FSB flour dough at 8 min mixing time with different anion salts: A. NaH$_2$PO$_4$; B. NaF; C. NaCl; D. NaBr; E. NaI; and F. No salt.
Previous study by Okur et al. (2013) have suggested that the cations usually bind weakly to the carbonyl oxygen of the amide of the protein, but the anion affinity for amide binding sites is very strong nearly 2 orders of magnitude higher compared to cations. In the case of the gluten proteins, it has been shown the gluten proteins have more positive charges which are contributed by positively charged amino acid residues particularly lysine and arginine in the C-terminal area of the gliadins (Delcour, Joye, Pareyt, Wilderjans, Brijs, & Lagrain, 2012; Weiser, 2007; Weiser & Keiffer, 2001). In addition, the gluten proteins are unique as both gliadins and glutenins contain approximately 35% amide from the amide side chain of the amino acid glutamine. Therefore, anions could bind directly with both the polar nitrogen atoms of the positively charged amino acid residues and the anion-amide interactions of the gluten proteins. While the effect of cations on the gluten structure formation may occur indirectly through their effect on water structure, because there are less numbers of negatively charged amino acid residues, thus the effectiveness of cations to shield the protein surface charge is negligible. All these may contribute to the particular formation of the gluten structure and resulting rheological properties.

3.2. Effects of Hofmeister anion salts on gluten proteins

The water content of the wet gluten and the protein content of the freeze-dried gluten samples from dough mixed and washed with different anion salts are shown in Table 2. The water content of the gluten samples varied significantly. The more kosmotropic is the anion in Hofmeister series used in the mixing and washing of the dough, the lower is the water content of the gluten dough. This result emphasises the important role of the ions in interfering with the solvent molecules (water). The kosmotropic anions are more effective in shielding the electrostatic repulses of the gluten proteins and the loss of these repulsive forces allow glutenin to form intermolecular hydrogen bondings between themselves rather than hydrogen bonding with water. This makes water structures more ordered and less available for protein hydration resulting in lower water content of the wet gluten dough compared to the chaotropic anions which are less
effective in shielding the electrostatic repulses, and thus favour the gluten to hydrogen bonding with water molecules and make them more readily available for protein to be hydrated.

The protein content of the dried gluten with kosmotropic anions were lower compared to the chaotropic anions. In addition, the protein contents of the gluten without salt were significantly higher than gluten with anion salts. Solubility of the gluten protein without salt was found to be lower than the gluten with salt (Mejri, Rogé, BenSouissi, Michels, & Mathlouthi, 2005). Therefore, it would be possible that gluten prepared with anion salts have lower protein content than gluten without salts, due to the solubilisation.

The freeze-dried gluten protein samples prepared from the dough mixed with different anion salts by the hand-washing procedure with the respective salt solutions were subjected to extraction procedure and size distribution measurement using the SE-HPLC method. Similar Glu:Gli ratio of the total protein was observed for the gluten samples with different anion salts except for the \( \Gamma \). Moreover, the Glu:Gli of the SDS-extractable protein extract were significantly lower for the chaotropic than the kosmotropic anions.

The % UPP was also varied significantly in the gluten samples prepared by the addition of the different anion salts (Table 2). The more kosmotropic is the anion in the series, the higher is the % UPP. The work by Fu et al. (1996) has shown the importance of salts on the aggregation of the gliadins to the glutenin molecules. This reflects their findings that indicated the glutenin fraction was substantially contaminated with gliadins and other monomeric proteins when salt is used in flour protein fractionation. It is likely that the loss of the repulsive forces allow the protein to form intermolecular hydrogen bonds that result in their aggregation (Fu, Sapirstein, & Bushuk, 1996). In addition, Ukai et al. (2008) has also shown that gluten prepared with NaCl allowed for gliadin solubilisation in water after NaCl removal. This suggests a change in gliadin structure since native gliadin is not soluble in water.
Table 2. Gluten properties as function of different Hofmeister anion salts.

<table>
<thead>
<tr>
<th>Salts</th>
<th>Water content of wet gluten (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Protein content of dry gluten (%)&lt;sup&gt;ab&lt;/sup&gt;</th>
<th>Glu:Gli SDS extractable</th>
<th>Glu:Gli total protein</th>
<th>% UPP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Set 1 FSB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaH&lt;sub&gt;2&lt;/sub&gt;PO₄</td>
<td>63.6 ± 0.5 d</td>
<td>58.9 ± 0.1 f</td>
<td>0.35 ± 0.00 b</td>
<td>0.66 ± 0.00 a</td>
<td>51.9 ± 0.2 ab</td>
</tr>
<tr>
<td>NaF</td>
<td>64.2 ± 0.3 d</td>
<td>64.7 ± 0.0 d</td>
<td>0.29 ± 0.00 c</td>
<td>0.64 ± 0.00 a</td>
<td>59.8 ± 0.4 a</td>
</tr>
<tr>
<td>NaCl</td>
<td>71.5 ± 0.2 c</td>
<td>69.9 ± 0.2 b</td>
<td>0.44 ± 0.00 a</td>
<td>0.68 ± 0.01 a</td>
<td>45.0 ± 0.1 b</td>
</tr>
<tr>
<td>NaBr</td>
<td>74.5 ± 0.3 b</td>
<td>67.4 ± 0.1 c</td>
<td>0.23 ± 0.01 d</td>
<td>0.41 ± 0.05 b</td>
<td>48.5 ± 1.8 b</td>
</tr>
<tr>
<td>NaI</td>
<td>77.1 ± 0.6 a</td>
<td>60.6 ± 0.2 e</td>
<td>0.20 ± 0.01 d</td>
<td>0.38 ± 0.01 b</td>
<td>29.2 ± 0.6 c</td>
</tr>
<tr>
<td>No salt</td>
<td>70.6 ± 0.2 c</td>
<td>77.1 ± 0.1a</td>
<td>0.46 ± 0.02 a</td>
<td>0.71 ± 0.00 a</td>
<td>47.8 ± 4.0 b</td>
</tr>
<tr>
<td><strong>Set 2 Redbase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaH&lt;sub&gt;2&lt;/sub&gt;PO₄</td>
<td>63.5 ± 0.5 d</td>
<td>60.4 ± 0.0 e</td>
<td>0.41 ± 0.00 abc</td>
<td>0.60 ± 0.00 a</td>
<td>35.6 ± 0.7 ab</td>
</tr>
<tr>
<td>NaF</td>
<td>65.1 ± 0.4 d</td>
<td>67.4 ± 0.1 c</td>
<td>0.39 ± 0.01 bc</td>
<td>0.56 ± 0.06 a</td>
<td>43.7 ± 0.7 a</td>
</tr>
<tr>
<td>NaCl</td>
<td>72.3 ± 0.4 c</td>
<td>70.6 ± 0.1 b</td>
<td>0.44 ± 0.03 ab</td>
<td>0.50 ± 0.00 ab</td>
<td>46.6 ± 5.2 a</td>
</tr>
<tr>
<td>NaBr</td>
<td>76.1 ± 0.6 b</td>
<td>66.3 ± 0.3 d</td>
<td>0.34 ± 0.01 cd</td>
<td>0.40 ± 0.02 bc</td>
<td>47.4 ± 1.5 a</td>
</tr>
<tr>
<td>NaI</td>
<td>76.1 ± 0.4 a</td>
<td>60.1 ± 0.1 e</td>
<td>0.29 ± 0.03 d</td>
<td>0.31 ± 0.02 c</td>
<td>22.3 ± 3.7 b</td>
</tr>
<tr>
<td>No salt</td>
<td>70.7 ± 0.3 c</td>
<td>79.8 ± 0.3 a</td>
<td>0.48 ± 0.03 a</td>
<td>0.54 ± 0.02 a</td>
<td>42.5 ± 3.8 a</td>
</tr>
</tbody>
</table>

a: All values presented as % values on a w/w basis.

b: N × 5.7.

Means within a column per each set of flour with the same letter are not significantly different (p < 0.05).

The result from our previous study also suggested the conformational changes of the gluten protein with increasing intermolecular β-sheet structure compared to gluten without salt (Tuhumury, Day, & Small, 2014a). Ions affect gliadins in a way that allows gluten network formation either through solubilisation or a conformational changes (Ukai, Matsumura, & Urade, 2008). Moreover, they have shown that the aggregation of the gliadins was induced by various salts. Therefore, the differences in % UPP of the gluten with anion salts may be attributed to the aggregation of the gliadins and interaction of the gliadins to glutenins.

The anions of the salts could interact with specific amino acid residues of the gluten proteins, especially the gliadins, because gliadins contain some positively charged...
amino acids such as lysine and arginine in the C-terminal region. This may be necessary for conformational changes and their aggregation. Therefore, the more kosmotropic is the anion in the series, the more is the aggregation of the gliadins. This in turn will decrease the availability of the odd number cysteine gliadins and ω-gliadins to interact with the glutenins and act as the chain terminating agent in the polymerisation of the glutenins. As a result, polymerisation of the glutenin were enhanced and thus the % UPP of the gluten with kosmotropic anions. On the other hand, the chaotropic anions may lower the aggregation of the gliadins, and the chain terminating gliadins and LMW glutenins may become more available to interact with glutenins thus reducing the polymerisation of the glutenin and lower the % UPP.

The increasing Glu:Gli ratio of total protein of the gluten with chaotropic anions and the decreasing % UPP in this study suggest that anions induced changes in the aggregation, and interaction of the gliadins to glutenins during mixing and washing of the flour. The more chaotropic is the anion in series used in the mixing, the less aggregation in gliadins and thus the more available are the chain terminating gliadins to interact with glutenins as indicated by less gliadins eluted in the monomeric fraction of the total protein during SE-HPLC determination. This could be the reason for the increasing Glu:Gli ratio with the more chaotropic anions. But because the gliadins that are incorporated into the glutenins as function of the chaotropic anions are the chain terminating gliadins, the % UPP appear to decrease.

In comparison with our previous results, in which there was little difference in aggregation among chloride salts with different cations (Tuhumury, Day, & Small, 2014b), large differences among sodium salts with different anions were found in this study. This suggests that the interactions of anions with specific amino acid residues (specific ion effects) are important for the aggregation behaviour of gluten proteins. These results were in agreement with those of Ukai et al. (2008) who suggested that the gliadin aggregation may be induced by the interaction of certain ion with the specific amino acid residues of a given protein. Specific ion effect of the Hofmeister ion series have also been widely observed across a number of protein and macromolecules (Kunz,
and are depending on several factors including the properties of the ions and the nature and structure of the macromolecules.

3.3. Effects of Hofmeister anion salts on dynamic rheological properties of gluten

The rheological properties of wet gluten samples prepared using different anions were measured by dynamic oscillational strain sweep. The storage modulus \(G'\), loss modulus \(G''\), and \(\tan \delta\) values at 1% strain (in the LVR region) of the gluten with different anion salts are shown in Fig. 3. Overall, the \(G'\) values of the gluten samples were higher than the \(G''\) values indicating all gluten samples showing a solid or elastic like behaviour.

Different anion salts had significant effects on \(G'\) and \(G''\) values of the wet gluten samples. The more kosmotropic are the anions, the higher are the \(G'\) and \(G''\) values (Fig. 3). One of the factors that might influence the \(G'\) and \(G''\) values of the gluten could be the water content of the gluten (Autio & Laurikainen, 1997; Berland & Launay, 1995; Dreese, Faubion, & Hoseney, 1988). The more chaotropic are the anions, the higher are the water contents of the wet gluten (Table 2), which were correspondent to the lower \(G'\) and \(G''\) values. Although there were subtle structural differences of the gluten network in dough matrix with different anions (Fig 2), the effects of the anion salts on \(G'\) and \(G''\) values depend on the water content of the gluten.

The \(\tan \delta\) values were similar across different anion salts except for the \(\Gamma^-\). This means that the relative contributions of the viscous and elastic components to the rheological properties of samples as affected by anion salts were similar. The relatively similar \(\tan \delta\) values may result from equal contribution of the hydrogen bonding between glutenin molecules as the results of the shielding of the electrostatic repulsion and the aggregated gliadins.
Figure 3  
$G'$, $G''$ and tan $\delta$ values at LVR of gluten from two flours with different Hofmeister anion salts generated from small deformation oscillatory shear. Columns with same letter column for each set of flour with the same letter are not significantly different ($p < 0.05$).
Although the anions salts may cause the aggregation of the gliadins, hence the interaction of the gliadins to glutenins and % UPP, the aggregated gliadins still act as the molecular bearing. The movement of the glutenin molecules with the aggregated gliadins as molecular bearing during small strain measurement confers the similar viscous flow to the network. The different tan δ value of the I− can be seen in the consistency of the gluten samples with NaI which appeared more runny and viscous, which then result in higher tan δ value. These results specify that different anion salts contributes significantly on both the elasticity and viscosity of the gluten network.

The part I of this study using salts with different cations on wet gluten showed that the G’ and G’’ were also influenced by different cations (Tuhumury, Day, & Small, 2014b). However, the extent of the difference in the G’ and G’’ values of gluten samples prepared using different anion salts found in this part II study was greater than with the cation salts. When chloride salts with different cations were present in the mixing, the gluten structure was formed predominantly by the non-covalent interactions hence the water content of the gluten. However, by the aggregation of gliadins and aggregation of the polymeric fractions was not affected, therefore G’ and G’’ values were affected to lesser extent. On the other hand, when the sodium salts with different anions were present, gluten network was formed not only by changes in non-covalent interactions and the water content but also the changes in aggregation behaviours (% UPP), which signify the effect on G’ and G’’ values of the gluten.

### 3.4. Effects of Hofmeister anion salts on uniaxial rheology

Wheat flour doughs mixed with different anion salts as well as the isolated gluten samples with respective salt solutions were subjected to large deformation rheology. The large deformation properties including the maximum distance obtained at fracture during uniaxial extension (D_max); maximum force at fracture (F_max); strain hardening index (n); and strain hardening coefficient (k) were determined (Table 3). The measurements on the dough and gluten samples with NaI were not determined, because
the dough and gluten samples were excessively runny and viscous. The samples could
not form correct and similar cylindrical dimension when shaped in the PTFE mould.

D\text{max} values indicating the extensibility of both dough samples with different anion salts
were relatively similar, except for H\text{2}PO\text{4}^- which have the lowest value (Table 3). This
trend can also be seen in Fig.4 that the dough with H\text{2}PO\text{4}^- had the lowest fracture
strain, while the others have similar fracture strain. D\text{max} values of the gluten samples
with NaCl were significantly different to gluten with either kosmotropic or chaotropic
anion salts, with again the H\text{2}PO\text{4}^- having the lowest value, and they are different to the
gluten without salt (Table 3). In our previous study, D\text{max} value of the gluten with NaCl
was not significantly different to gluten without NaCl (Tuhumury, Day, & Small,
2014a). This may have been due to the different sample preparation. In that study, the
rehydrated gluten samples were utilised with similar water content and protein content.
In this present study, the freshly prepared gluten resulting in higher water content
contributed to lower D\text{max} values compared to rehydrated gluten samples in the previous
study. However, there was also a tendency that the D\text{max} values of the wet gluten
samples related to the protein content (Table 2).

In the case the F\text{max} values which indicate the resistance of the samples to extension, the
dough samples were not significantly affected by the addition of salts with different
anions, this time with the exception for H\text{2}PO\text{4}^- on the dough from Redbase flour.
Whereas, the F\text{max} values of the gluten with NaCl were significantly different to other
anion salts, but similar to gluten without salt. This was found to be similar to the
observation for rehydrated gluten samples as the F\text{max} values of gluten with and without
NaCl also showed no significant difference (Tuhumury, Day, & Small, 2014a).
Table 3. Effects of different anion salts on uniaxial tension parameters of the wheat flour dough and gluten from two flours.

| Salts       | Wheat flour dough | | | | | | Wheat gluten | | | |
|-------------|-------------------|---|---|---|---|---|---|---|---|---|---|---|
|             | $D_{\text{max}}$ (mm) | $F_{\text{max}}$ (mN) | $n^a$ | $k^b$ | $D_{\text{max}}$ (mm) | $F_{\text{max}}$ (mN) | $n^a$ | $k^b$ |
| Set 1 FSB   |                   |               |     |     |               |               |     |     |
| NaH$_2$PO$_4$ | 51 ± 5 b         | 198 ± 13 a    | 0.8 ± 0.1 b | 11.0 ± 2.0 a | 98 ± 6 c        | 873 ± 48 b    | 2.1 ± 0.1 b | 5 ± 2 a |
| NaF         | 123 ± 8 a        | 208 ± 14 a    | 1.5 ± 0.1 a | 2.9 ± 0.4 b | 145 ± 3 ab      | 1023 ± 34 a   | 2.1 ± 0.0 b | 4.2 ± 0.2 a |
| NaCl        | 136 ± 5 a        | 204 ± 9a      | 1.7 ± 0.1 a | 1.8 ± 0.3 b | 167 ± 17 ab     | 345 ± 20 c    | 2.3 ± 0.1 ab | 0.8 ± 0.3 b |
| NaBr        | 128 ± 14 a       | 220 ± 12 a    | 1.7 ± 0.1 a | 2.0 ± 0.3 b | 134 ± 6 bc      | 203 ± 13 d    | 2.3 ± 0.1 ab | 0.5 ± 0.1 b |
| NaI         | nd               | nd            | nd         | nd         | nd              | nd            | nd         | nd |
| No salt     | 150 ± 11 a       | 133 ± 8 b     | 1.5 ± 0.1 a | 1.5 ± 0.4 b | 175 ± 6 a       | 345 ± 19 c    | 2.5 ± 0.2 a | 0.5 ± 0.1 b |
| Set 2 Redbase |               |               |     |     |               |               |     |     |
| NaH$_2$PO$_4$ | 45 ± 2 b         | 230 ± 18 a    | 0.6 ± 0.1 b | 25.0 ± 5.0 a | 93 ± 3 d        | 655 ± 20 b    | 1.7 ± 0.0 c | 6.8 ± 0.5 a |
| NaF         | 123 ± 12 a       | 173 ± 9 b     | 1.4 ± 0.1 a | 2.6 ± 0.5 b | 116 ± 2 cd      | 775 ± 48 a    | 2.2 ± 0.0 b | 3.0 ± 0.2 b |
| NaCl        | 149 ± 23 a       | 135 ± 8 b     | 1.7 ± 0.1 a | 1.0 ± 0.2 b | 153 ± 9 ab      | 313 ± 21 c    | 2.4 ± 0.1 ab | 0.7 ± 0.1 c |
| NaBr        | 118 ± 2 a        | 150 ± 4 b     | 1.5 ± 0.1 a | 2.0 ± 0.3 b | 128 ± 3 bc      | 150 ± 4 d     | 2.5 ± 0.1 a | 0.33 ± 0.1 c |
| NaI         | nd               | nd            | nd         | nd         | nd              | nd            | nd         | nd |
| No salt     | 146 ± 3 a        | 92 ± 3c       | 1.7 ± 0.1 a | 0.7 ± 0.1 b | 170 ± 11 a      | 310 ± 16 c    | 2.6 ± 0.1 a | 0.4 ± 0.1 c |

$a$: Strain hardening index.
$b$: Strain hardening coefficient.
nd: not determined.

Means within a column per each set of flour with the same letter are not significantly different ($\rho < 0.05$).
Figure 4  Stress-strain curve of the dough and gluten from two flours with different anion salts generated from uniaxial extensional rheology measurement. The scales for the dough samples are different to the gluten samples.
The more kosmotropic are the anions in the series, the higher the $F_{\text{max}}$ values ($\text{Br}^->\text{Cl}^->$ $\text{F}^->\text{H}_2\text{PO}_4^-$). These results indicated that the gluten structure formed during the mixing with various anion salts has caused the isolated gluten samples to have increasing resistance to extension following the Hofmeister series from chaotropic to kosmotropic. However, the significant differences in gluten samples were diminished when wheat flour dough was measured due to large variation in extensibility and resistance to extension during uniaxial extension.

Stress and strain curves of the samples with different anion salts were constructed by taking into account the changes in the sample geometry during the test. Dough samples with different anion salts showed a strain hardening behaviour, i.e. an increase in stress values as the strain is increased (Fig 4). Stretching of the dough or gluten samples will induce stretching and orientation of protein chains and therefore a stress increase. The stress values of the dough samples from both flours seem to increase according to order of the anion in the series with the increasing strain in FSB flour but altered in the Redbase flour. There are large differences in the stress levels between each anion salts. In addition, the fracture strain were also different among anion salts with $\text{H}_2\text{PO}_4^-$ being the extreme lowest. The lowest fracture strain of the dough may be the result of the hydration behaviour of the gluten proteins with salts. Isolated gluten samples with anion $\text{H}_2\text{PO}_4^-$ showed the lowest water content and protein content which may explain lowest fracture strain.

The strain hardening coefficients ($k$) and hardening indices ($n$) obtained from the exponential fitting of the stress-strain curve were also summarised in Table 3. There was little difference statistically in either the $n$ values or the $k$ values of the flour dough with different anions from both flours. In comparison with the dough without salt, the $n$ and $k$ values were not different statistically, apart from dough with $\text{H}_2\text{PO}_4^-$ . The results from the study by McCann & Day (2013) showed that FSB dough without NaCl has lower $n$ and $k$ values than the dough with NaCl. The results were also in line with the results from this study, although the values are lower compared to their study. This is because the dough in their method applied total displacement of 52 mm, while the
displacement in this study was set at maximum displacement at fracture. However, the
trend with lower n and k values with dough without NaCl still applied.

The n and k values of gluten samples were influenced by salts with different anions.
The chaotropic anions cause gluten samples to have relatively little difference in n
values, but significant lower k values. This indicates that anion salts largely determine
the strain hardening coefficients (k values). The n and k values of gluten with and
without NaCl were similar, and this was also agree with rehydrated gluten samples in
our previous study (Tuhumury, Day, & Small, 2014a). The relatively similar n values of
the rehydrated gluten samples with and without NaCl, the wet gluten with different
cations, as well as little difference in wet gluten with different anions suggested that
strain hardening index were not determined by water content and that salts generally did
not cause any difference in strain hardening index.

On the contrary, k values of NaCl were similar to gluten without NaCl in either
rehydrated gluten samples (Tuhumury, Day, & Small, 2014a) or wet gluten samples
with different cation salts (Tuhumury, Day, & Small, 2014b). Although the k values of
gluten with NaCl were similar to those without NaCl, these values were significantly
different to gluten with other anion salts. There were more significant differences in k
values within a set of various anion salts compared to insignificant differences in the set
of cation salts (Tuhumury, Day, & Small, 2014b). These results specified that the effect
of anions on gluten structure formation resulting in the significant effect on strain
hardening coefficients of gluten as well as the dough samples. Therefore, the cations did
not cause any difference in the % UPP indicating that aggregation of the polymeric
protein fraction were not affected by cations and as the result the strain hardening
coefficient of the gluten during extension were not influenced as well. Anions on the
other side, cause substantial differences in % UPP indicating the aggregation of the
polymeric protein fraction were greatly impacted which in turn result in also remarkable
differences in strain hardening coefficients.
Sliwinski et al. (2004) have indicated that the large deformation behaviour of gluten may be determined by the amount of both the domain within the gluten structure with protein-protein interactions and also region where protein-solvent interaction dominate. The higher values in stress levels, $F_{\text{max}}$, and strain hardening coefficients, all as affected by kosmotropic anion salts may be attributable to the protein-protein interactions domains which dominate the structure than the protein-solvent region. This may lead to gluten having less water content and typical fibrous structure as observed in the microstructural investigation. Higher force was therefore necessary for the extension the gluten. During extension the loop regions (protein-solvent interactions) has to be deformed first then followed by the breaking of the interchain hydrogen bondings in the train regions (protein-protein interactions) so that the chain slip over each other and the fracture occurs (Belton, 1999). Since the amount of the protein-solvent interactions via hydrogen bondings, and thus the water content was higher for the chaotropic salts, the honey-comb structure was encountered and the extensibility and the fracture strain of the isolated gluten were also higher. It is because these glutens firstly have to experience the deformation of the more extensive loop regions than the gluten with kosmotropic salts, followed by the breaking of the interchain hydrogen bonds which lead to fracture. From these results, it can be suggested that strain hardening coefficients of the gluten during uniaxial extension were determined by the effect of the ions on gluten molecules which cause changes in $\%$ UPP during hydration of the proteins.

4. Conclusion

The effect of Hofmeister anion salts on gluten network formation are more pronounced than the Hofmeister cation salts. The functionality of the gluten and hence the wheat flour dough were greatly determined by the anions. Different anions showed different effect on the structure and functionality of gluten as well as the dough compared to what is achieved by NaCl, and the effect follows the Hofmeister anion series. These anion salts influence the gluten network formation in a way that they cause changes in the percentage unextractable polymeric protein fraction ($\%$ UPP). This may be the results of
interaction of anions directly with specific amino acid residues which cause changes in gluten protein composition, aggregation of the gliadins, and interaction of gliadins to glutenins. These changes consequently result in the remarkable differences in the dough mixing profile, microstructural features of the dough, the small deformation rheological properties of the gluten, as well as the strain hardening behaviour of the dough and gluten samples.

The more pronounced effect of anions compared to the cations on gluten structure may be due to the ability of the anions to bind with the more positively charged of the amino acid residues which present in the gluten protein composition by shielding the electrostatic repulses and consequently hydrogen bondings formation as well as the aggregation of gliadins to glutenins. Whereas the effect of cations on the gluten structure formation may occur indirectly through their effect on water structure, because there are less numbers of negatively charged amino acid residues, thus the effectiveness of cations to shield the protein surface charge is negligible.

Overall, the implications from our previous report (Tuhumury, Day, & Small, 2014b) and this present study suggest that anions are important in determining the structure and functionality of the gluten as well as wheat flour dough. Therefore, the chloride ions of NaCl cannot be replaced by other anions of sodium salts. On the other hand, the NaCl could be replaced by other cations of chloride salts, particularly KCl. This is because the cations of chloride have relatively similar impact on dough rheology and functionality. These findings contribute significantly to the strategies in reducing the sodium intake in the production of wheat-based foods without causing deleterious effect on the functionality of the dough. However, the issue with the changes of the taste with the replacement should also be taken into account in the further studies.
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References:


Chapter 8


Rheological properties of gluten as function of sodium chloride during heating

Abstract

Both rehydrated and fresh gluten samples were prepared in the presence and absence of NaCl during mixing and washing. The samples were subjected to the temperature sweep to determine the rheological properties of gluten during heating as a function of NaCl. In addition, the starch was added back to the gluten samples with and without NaCl to determine the effect of residual starch in the gluten network as influenced by NaCl. Gluten network formed in the presence of NaCl determine its rheological properties during heating. NaCl caused the enhanced hydrogen bonding in the formation of the gluten network during hydration, which may cause the onset in the sharp increase in $G'$ at higher temperature (above 60 °C) during heating compared to without NaCl at 55 °C. The delay of the sharp increase in the $G'$ value to higher temperature during heating is the result of the formation of the gluten network in the presence of NaCl not of the presence of the residual starch in the gluten network.

Keywords: Salts, gluten, small deformation rheology, starch, temperature sweep.
9.1. Introduction

Wheat flour contains two forms of proteins, gliadins and glutenins, that contribute and strongly influence the end-product functionality of wheat-based foods. When wheat flour is mixed with water, the gliadin and glutenin proteins are hydrated leading to the formation of a continuous network known as gluten. This network forms the structure of the dough and makes it elastic and extensible. The rheological properties of the gluten protein network, their impact on handling properties of the dough during processing and the resultant end-product quality, depend on a number of factors. These include the quantity and quality of the gluten proteins, their structure at the molecular level, conformational re-arrangements effected by the solvent environment upon hydration as well as physical strain induced by mechanical shear as mixing proceeds (Delcour, Joye, Pareyt, Wilderjans, Brijs, & Lagrain, 2012).

Salt determines the functional rheological properties of the gluten network formed when flour is mixed with water, which in turn affects the dough strength and dough-handling properties (Butow, Gras, Haraszi, & Bekes, 2002). Several studies have been conducted to study the effect of salt on the rheological properties of wheat flour dough and breadmaking quality. Most of the results have proposed that the resulting rheological properties of the dough as influenced by salts are due to the effect of salt on the gluten proteins both indirectly through their effect on water structure and directly upon gluten protein molecules. These effects are primarily dependent on the type of the salts belonging to the Hofmeister series as well as the concentration of the salts (Balla, Razafindralambo, Blecker, & Paquot, 1998; Butow, Gras, Haraszi, & Bekes, 2002; Charlton, MacGregor, Vorster, Levitt, & Steyn, 2007; He, Roach, & Hoseney, 1992; Kinsella & Hale, 1984; Miller & Hoseney, 2008; Preston, 1989).

Our previous study on the effect of sodium chloride on the gluten structure and rheology has shown that the presence of NaCl in comparison with the absence of NaCl during dough mixing has increased the non-covalent interaction which resulted in different molecular conformation and network structure of gluten proteins and contributed to the differences in the rheological properties. In addition, the formation of
the gluten structure as a function of NaCl is governed during initial hydration of the flour (Tuhumury, Day, & Small, 2014).

Therefore, the formation of the gluten network during hydration and mixing as function of salt will also determine the behaviour during processing including the baking stage. One particularly important process in breadmaking is the heat denaturation of wheat gluten proteins and the accompanying rheological and functional changes (Schofield, Bottomley, Timms, & Booth, 1983). Heating the wet gluten usually increases the molecular size of the glutenin aggregates and decreases protein extractability indicating increased crosslinking and polymerisation of the gluten polymer, which was attributed to increased sulphhydryl-disulphide interchange reactions (Kieffer, Schurer, Köhler, & Wieser, 2007; Schofield, Bottomley, Timms, & Booth, 1983; Stathopoulos, Tsiami, David Schofield, & Dobraszczyk, 2008; Stathopoulos, Tsiami, Dobraszczyk, & Schofield, 2006). Changes occurring during heating have been found to be similar based on the results of most of the rheological studies. Some studies have suggested that rheological properties of gluten during heating with steady decrease of G’ and G” up to 55 °C, prior to the a sharp increase beyond 55 °C, resembling the changes that occur in the parent dough (Lefebvre, Popineau, & Cornec, 1994).

This present study has been conducted to investigate the effect of NaCl on the rheological properties of the gluten during heating. The hypothesis has been that the gluten network formed with NaCl during hydration determines the rheological properties during heating.

9.2. Materials and methods

9.2.1. Wheat flour samples

Two commercial wheat flours (FSB and Redbase) were kindly provided by Allied Mills (Kensington, Victoria, Australia). The protein contents of the flours were 13.2 and 10.4% for FSB and Redbase, respectively, determined by the AACC method 46-30 (AACC International, 2000). The moisture contents were 12.8 and 12.9% for FSB and
Redbase, respectively, measured by AACC method 44-15a (AACC International, 2000). A commercial wheat starch was used in trying to confirm the effect of the residual starch. Chemicals used were of analytical grade and NaCl was purchased from Chem-Supply (Sydney, Australia).

9.2.2. Gluten washing

Preparation of water-washed gluten (WW) and salt-washed gluten (SW) was carried out according to the method previously described by Day et al. (2009). Briefly, flour (300 g) was mixed with water (180 mL) with or without 2% NaCl (flour base) in a Hobart mixer at setting 1 (63 rpm) for 2 min followed by setting 2 (111 rpm) for a further 2.5 min to form a dough. The dough was then rested for 30 min in either water or 2% NaCl solution, then washed 3 times by hand in 5 L water or salt solution (150 g/5 L). The wet gluten was then freeze-dried for 72-96 h. The freeze-dried gluten was ground to a powder using a coffee grinder and sieved through a 250 µm sieve. The gluten samples were prepared in two batches for each flour. Freeze dried gluten (0.5 g) was rehydrated with 0.75 mL water or salt solution using a mortar and spatula to obtain a rehydrated gluten dough containing approximately 63% w/w water content for the rheological temperature sweep.

Fresh wet gluten samples were also obtained by mixing flour (4 g) with 2.4 mL water or 2% salt solution for 4.5 min, using a Micro-doughLAB (Perten Instruments, NSW, Australia). The gluten sample was then washed in either water for WW gluten or salt solution for SW gluten. A proportion of the fresh wet gluten (approx. 1.5 g) was used for the rheological measurement.

9.2.3. Reconstitution studies to confirm the effect of residual starch

In order to confirm the effect of salt during heating on gluten itself, the starch was added back to the gluten with increasing concentration to decrease the amount of gluten. The amount of water used is based on the amount of gluten. 10% decrease in gluten weight equals to 15% decrease in the amount of water. The gluten starch blend was
prepared so the samples contain both WW gluten and SW gluten in 100% gluten + 0% starch, 20% gluten + 80% starch, and 100% starch of the total solid. The preparation of the samples was summarised in Table 9.1.

Table 9.1. Preparation of the gluten starch blend.

<table>
<thead>
<tr>
<th>Gluten (g)</th>
<th>Starch (g)</th>
<th>Water (mL)</th>
<th>Water : total solid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0</td>
<td>0.75</td>
<td>1.5</td>
</tr>
<tr>
<td>0.1 (20%)</td>
<td>0.4 (80%)</td>
<td>0.4125</td>
<td>0.6</td>
</tr>
<tr>
<td>0</td>
<td>0.5</td>
<td>0.8</td>
<td>0.4</td>
</tr>
</tbody>
</table>

a: The amounts of the gluten and starch are the percentage of total solids.

9.2.4. Dynamic rheological measurement (temperature sweep)

Temperature sweep was conducted on gluten samples, rehydrated and fresh wet gluten sample as well as the gluten starch blend. The gluten dough and gluten starch dough was then wrapped in plastic film and allowed to rest for 1 hr. Dynamic oscillation measurements were performed on a controlled stress-strain rheometer (Paar Physica MCR 300, Messtechnik GmbH, Stuttgart, Germany), using parallel-plate geometry. Top (25 mm) and bottom plates were both serrated to prevent the sample from slipping during measurement. The gluten dough was placed between the plates and the upper plate was lowered to a fixed gap of 2 mm and allowed to rest for 10 min following loading. A purpose-built compartment with a water-saturated filter paper was used to minimise dehydration of the gluten sample during measurement (Day, Augustin, Pearce, Batey, & Wrigley, 2009). Oscillation measurements were performed at 1% strain values, and constant frequency of 1 Hz (in Linear Viscoelastic Region). For the temperature sweep, samples were heated in the rheometer cell from 25 to 95 °C at heating rate 1 °C per minute. Measurements were carried out in duplicate for each gluten preparation.
9.3. Results and discussion

9.3.1. Effects of NaCl on the rheological properties of gluten during heating

Gluten samples prepared with and without NaCl during mixing and washing of the dough were subjected to temperature sweep. The rheological profile of the rehydrated gluten samples of the two flours during heating can be seen in Figure 9.1. Gluten samples from both flours either the rehydrated or the freshly prepared showed a small decrease in both $G'$ and $G''$ up to a certain temperature at which there appeared to be a sharp increase in the $G'$ and $G''$ values. The temperature where the sharp increase in values occurred seemed to be determined by gluten network formed during hydration as a function of NaCl. The gluten network formed with NaCl have caused the $G'$ and $G''$ values to be sharply increased at higher temperature (above 60 °C) compared to the gluten network formed without NaCl (around 55 °C). After the slump increase, the $G'$ and $G''$ values seemed to be levelled off above the temperature of 85 °C with the gluten prepared with NaCl having lower values compared to gluten without NaCl. These results indicated the function of NaCl in the formation of the gluten network during hydration determine further changes in the structure of the gluten during heating.

Most rheological studies on dough and gluten without NaCl have found similar changes upon heating and have also shown that any affects of heating on dough were the result of what happened to gluten during heating (Lefebvre, Popineau, & Cornec, 1994). It is the steady decrease in $G'$ and $G''$ up to 55 °C, followed by a steep increase following this particular temperature, which also in agreement with this current results. The steep increase shifted to higher temperature when the gluten was prepared in the presence of the NaCl during hydration. Our previous study on the effect of NaCl on the gluten structure and rheology has shown that NaCl increased the non-covalent interactions of the gluten and the $\beta$-sheet structure which results in different molecular conformation, fibrous network structure, hence differences in rheological properties. NaCl caused the enhanced hydrogen bondings between the glutenins and aggregation of the gliadins to glutenins (Tuhumury, Day, & Small, 2014). The increased hydrogen bonding and aggregation of gliadins to glutenins may be the reason for the shifting to the steep
increase of the $G'$ and $G''$ values to higher temperature. Some studies have also suggested that changes in the gluten structure in the heating process were due to the broken hydrogen bondings (Wang, Belton, Bridon, Garanger, Wellner, Parker, et al., 2001), as a result the structure of the protein opens up causing a gradual decrease in $G'$ and $G''$ values. Because NaCl caused the enhanced hydrogen bondings in the formation of the gluten network during hydration, the higher temperature during heating was therefore encountered to break the enhanced bonding compared to gluten without NaCl.

![Figure 9.1](image_url)

**Figure 9.1**  $G'$ and $G''$ values of the rehydrated and freshly prepared water-washed (WW) and salt-washed (SW) gluten from two flours
Other studies have shown that with increasing temperature, gluten protein extractability decreased which indicated the heat-induced aggregation of gluten proteins (Stathopoulos, Tsiami, David Schofield, & Dobraszczyk, 2008). Singh and MacRitchie (2004) also showed that upon heating at higher temperatures, gliadins become more polymerised into glutenins through sulfhydryl interchanges as it resulted in a decrease in the gliadin peak with a corresponding increase in the glutenin peak. Therefore the increase of the G’ and G” values at higher temperature may also due the enhanced aggregation of gliadins to glutenins in the presence of NaCl during gluten network formation.

However, some studies have also found out that there are two processes which are responsible for the thermal behaviour of the dough: starch gelatinisation and protein denaturation. The starch granules which are initially entrapped as filler in the gluten network, may escape the gluten network as it is heated and cause the decrease in the moduli (Salvador, Sanz, & Fiszman, 2006). Since the gluten network may contain residual starch, the effect of the residual starch has to be taken into account. The next section reports on the effect of adding starch into the gluten samples prepared with and without NaCl.

9.3.2. Effects of added starch on gluten prepared with and without NaCl during heating

In order to confirm the effect of salt during heating on gluten itself, the starch was added back to the gluten with increasing concentration to decrease the amount of gluten. Figure 9.2 shows the G’ values of the WW and SW gluten with 0% starch, 20% starch, and the G’ values of the 100% wheat starch. Upon the addition of starch to the WW and SW gluten samples in the amount of 80%, the starting G’ values during heating were higher than 100% WW and SW gluten. The gluten samples from different flour with and without NaCl showed different response of G’ when starch was added. However, similar rheological profiles were encountered, i.e. a small decrease in both G’ and G” up to a certain temperature where there appeared to be a sharp increase in those particular values.
Figure 9.2  

G’ values of the rehydrated water-washed (WW) and salt-washed (SW) gluten from two flours with added starch during heating

The gradual decrease of G’ during initial heating has been thought to be the result of the escape of the starch granules which were initially entrapped as filler in the gluten network (Salvador, Sanz, & Fiszman, 2006). If the gradual decrease in G’ value is the result of the starch escaping the gluten network, then the increasing amount of starch in the samples should have shown as a sharp decrease in the G’ values to certain temperatures. It can be seen from these results that the similarity in gradual decrease of G’ values is still observable during initial heating. This result confirmed that the change in G’ during initial heating is determined by the gluten structure. The presence of NaCl during mixing has also determined how the gluten structure was formed and thus resulted in these specific rheological properties during heating. The results are in agreement with the suggestion that changes in the gluten structure in the heating process were due to the broken hydrogen bondings (Wang, et al., 2001). Therefore, the opening
of the structure of the protein may cause a gradual decrease in $G'$ and $G''$ values. On the contrary, the amount of starch in the blend proportionally influences the starting point of $G'$ values of the samples during heating. The temperature characteristic showed that with less gluten, the temperature for the onset of the sharp increase in $G'$ of the gluten with and without NaCl became more similar with decreasing amount of gluten. On the contrary, the 100% gluten with and without NaCl showed that the sharp increase in $G'$ values occurred at higher temperature when gluten was prepared with NaCl. If the delay in sharp increase of the $G'$ values occurred at higher temperature as the results of the presence of residual starch in the gluten network, the samples with higher starch content should also delay this increase to be occurred at higher temperature, however this is not what it looked like in the rheological properties above. These results suggested that the delay of the sharp increase in the $G'$ to higher temperature during heating is the results of the formation of the gluten network in the presence of NaCl not of the presence of the residual starch in the gluten network. Therefore, NaCl which caused the enhanced hydrogen bondings in the formation of the gluten network during hydration, caused the onset in the sharp increase in $G'$ at higher temperature during heating. It is because higher temperature was necessary to break the enhanced bonding compared to gluten without NaCl.

### 9.4 Conclusion

The gluten network formed in the presence of NaCl determines its rheological properties during heating. Changes in the gradual decrease in $G'$ and $G''$ values up to a certain temperature and the onset of the sharp increase in those values during heating are the results of the amount of the hydrogen bond formation as a function of NaCl. This salt caused enhanced hydrogen bonding in the formation of the gluten network during hydration, which subsequently caused the onset in the sharp increase in $G'$ at higher temperature during heating. The delay of the sharp increase in the $G'$ to higher temperature during heating is the result of the formation of the gluten network in the presence of NaCl not of the presence of the residual starch in the gluten network.
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References


Chapter 10

General discussion and conclusions

This chapter briefly summarises the results obtained during the current study, draws final conclusions and make recommendations for further research that might extend and develop the results presented in this thesis.

10.1. Introduction

Consumer concerns over health-related issues regarding high salt intake have been the reason for the growing research on salt reduction in various food products. However, in dough based products, reducing or eliminating salt from the formulation is particularly challenging. This is because salt (in the form of NaCl) is used in the processing of wheat-based foods, not only for enhancing sensory taste, but also for its technological functions. These include providing required dough strength and stability for processing which can have a profound impact on the textural characteristics of the final food products. Reducing or eliminating NaCl from the formulation may cause adverse effects on the gluten network formation and its rheological properties which subsequently influences the product quality and characteristics including the loaf volume, crumb structure and texture.

Studies have been conducted to examine the effect of NaCl on wheat flour dough properties. Most of these investigations have been focused on the rheological properties. The more recent studies have shown that the changes in the rheological properties as influenced by NaCl are primarily determined by its influence on the structure and formation of the gluten matrix. The explanations on the effects of different salts on gluten have been based on the studies utilising the salts of Hofmeister series with different anion and cation series as well as different concentrations. Most of the studies have focused on gluten aggregation behaviour in flour using the Mixograph, Farinograph as well as baking properties. The results from these studies have indicated
that salts of different types have important roles in determining the mixing and extensional properties of dough.

Others have also studied the properties of gluten protein fractions and the amount of solubilised or aggregated gluten proteins as affected by salts. Ions of the Hofmeister series, either cations or anions, and particularly at high concentrations, clearly influence gluten aggregation in dough particularly peak mixing time and the effects were in accordance to the position of the ions in the series. However, the effects of different salts on gluten protein network at the molecular level and resulting rheological properties still remain unclear.

The common strategy to reduce the sodium content in wheat based foods is to replace a proportion of NaCl with other chloride salts of different cations such as potassium chloride in the formulation and changes in production. Although the strategy has provided some success, a basic understanding of how different salts impact the formation of gluten network and the rheological behaviour of the dough is needed. This understanding is needed in order to maximise the technological functionality of these salts in replacing NaCl. It is not clear whether the rheological properties and structural changes of the gluten in the presence of NaCl are due to the contribution of either sodium or chloride ions.

Accordingly, in the current project we have sought to establish a knowledge and understanding of the function of salts on gluten structural network during hydration at the molecular level. Therefore the broad aim of the research reported in this thesis has been to investigate the structure of gluten proteins and their network formation during hydration at different microscopic levels and to evaluate the effects of salts on the functional properties of the gluten/dough. The specific objectives were: to investigate the effects of sodium chloride (NaCl) on gluten network formation and rheological/mechanical properties of gluten during hydration; to investigate the effect of NaCl on gluten structural changes during hydration and network formation as a function of heating; and to investigate and clarify the effect of each sodium and chloride ions on
gluten network formation (selected from the Hofmeister series). The first series studied was the chloride salts with different cations and second was the sodium salts with different anions.

10.2. Major conclusions

The final conclusions of this study are summarised as follow:

1. The effect of NaCl on the gluten structure and rheology has shown that NaCl increased the non-covalent interactions of the gluten and the β-sheet structure which results in different molecular conformation, fibrous network structure, hence differences in rheological properties. The gluten matrix formed with salt resulted in higher tan δ values corresponding with a less elastic network when measured using oscillatory rheometry. Large deformation extensional measurements showed that the maximum force to fracture were lower for the gluten samples prepared in the presence of NaCl. This affects the gluten structure during the initial hydration when wheat flour is mixed with water. We have therefore proposed that NaCl causes conformational changes as water molecules are drawn away from the gluten to interact with sodium and chloride ions. The hydrogen bonding and hydrophobic interactions could be the reason for the increase in the β-sheet structure within gluten. These proposed mechanisms result in the formation of the gluten with typically more closely aligned structure.

2. The gluten network formed in the presence of NaCl determines its rheological properties during heating. Changes in the gradual decrease in G’ and G” values up to certain temperature and the onset of the sharp increase in those values during heating are the results of the amount of the hydrogen bond formation as function of NaCl. NaCl caused the enhanced hydrogen bonding in the formation of the gluten network during hydration, which caused the onset in the sharp increase in G’ at higher temperature during heating. The delay of the sharp increase in the G’ to higher temperature during heating is the result of the formation of the gluten.
network in the presence of NaCl not of the presence of the residual starch in the gluten network.

3. The effects of different cations on dough and gluten in different flours generally followed the Hofmeister series. Monovalent kosmotroph cations (NH$_4^+$, K$^+$, and Na$^+$) tend to have similar rheological properties for both the dough mixing profile as well as the gluten dynamic rheological properties. However, the chaotrophic cations (Mg$^{2+}$ and Ca$^{2+}$) showed different rheological properties measured by both compared to the kosmotrophs. Though the kosmotrophs showed primarily similar rheological properties, the microstructure observed among them were different. However, the microstructure of the dough with KCl showed a closer structural resemblance to that of the dough in which NaCl was incorporated.

4. The effect of Hofmeister cation salts on the rheological behaviour of wheat flour dough at large deformations were primarily determined by their effects on gluten structure. The presence of cation salts in the formation of gluten network during hydration influenced the resistance to extension and extensibility of the structure but not strain hardening behaviour, when applied under large deformation. Among the different cations, K$^+$ had similar effects on large deformation rheological properties of both dough and gluten samples including the extensibility, resistance to extension, and the strain hardening behaviour to that of NaCl.

5. The effect of Hofmeister anion salts on gluten network formation are more pronounced than the Hofmeister cation salts. The functionality of the gluten and hence the wheat flour dough were greatly determined by the anions. Different anions showed significant effect on the structure and functionality of gluten as well as the dough compared to what was achieved by NaCl. Hofmeister anion salts influenced the gluten network formation in a way that caused changes in gluten protein composition, aggregation of the gliadins, and interaction of gliadins to glutenins, as well as the percentage of the unextractable polymeric protein fraction (% UPP) by their interaction directly with specific amino acid residues. These
changes consequently resulted in the remarkable differences in the dough mixing profile, microstructural features of the dough, as well as the small deformation rheological properties of the gluten.

6. The more pronounced effect of anions compared to the cations on gluten structure may be due to the ability of the anions to bind with the more positively charged of the amino acid residues which are present in the gluten protein composition hence the aggregation of gliadins to glutenins, while cations are weakly bound or do not bind at all and contribute to the changes in structure by interfering with water structure.

7. Based on the microstructural, dough mixing profile, rheological properties at small deformation and large deformation as well as the gluten size distribution studies with two different series of Hofmeister salts, the basic understanding on how NaCl influences gluten network formation can be proposed (Figure 10.1). NaCl primarily influences the non-covalent interactions of the gluten proteins. When the NaCl is present during hydration of the wheat flour, on one hand, the cation (Na⁺) primarily interfere with the structure of the water, which make the water become unavailable for the hydration of the gluten, hence the glutenin molecules tend to hydrogen bonding with each other due to the high amount of the glutamine residues. The cations have been found to have little influence on the aggregation of gluten proteins. On the other hand, the anions (Cl⁻), bind directly the gluten molecules to the positively charged amino acids of the gluten, since the gluten proteins tend to have positive net charge. The binding of the Cl⁻ to gluten proteins have caused conformational changes and also the shielding of the repulsive forces of gluten proteins, thus enhancing the aggregation of the proteins, especially the gliadins. The aggregation of gliadins will therefore determine the polymerisation of the glutenins during hydration and mixing and consequently its % UPP. The simultaneous effect of both Na⁺ and Cl⁻ on gluten proteins have resulted in the formation of the gluten network with the typical fibrous extended structure and the resulting rheological and functional properties.
Overall, the implications from this present study suggest that anions are important in determining the structure and functionality of the gluten as well as wheat flour dough. Therefore, the chloride ions of NaCl cannot be replaced by other anions of sodium salts. On the other hand, the NaCl could be replaced by the chloride salts of other cations, particularly KCl. This is because the cations of chloride have relatively similar impact on dough rheology and functionality. These findings contribute significantly to the selection of strategies in reducing the sodium intake in the production of wheat-based foods without causing deleterious effect on the functionality of the dough.

10.3. Possible areas for future research

This study has concentrated on the effects of different salts type on gluten protein and the network formation during by looking on the microstructural, rheological and functional properties, as well as the gluten aggregation and size distribution. It would be of value to extend this work to the different salts with different concentration range. Subsequently, confirmation of these results for the end-product including bread and breadmaking properties should be undertaken to evaluate how end-product will behave due to the incorporation of the different salts. Such work may also lead to further insights on the effect of salts on wheat-based foods.

The other significant outcome of this study has been that anions are important in determining the structure and functionality of the gluten as well as wheat flour dough. NaCl could be replaced by other cations of chloride salts, particularly KCl, but cannot be replaced by other anions of sodium salts. However, a replacement of sodium with larger amount of KCl has caused the product to remain bitter and with a metallic taste, although both the structural and functionality of KCl were very similar to NaCl.
Therefore, future work should systematically investigate the use of sodium replacers in combination with bitter taste blockers such as sugars.

As an overall conclusion, this study has sought to present the first fundamental conceptual structure function model of the effect of salts particularly NaCl on gluten network formation. This knowledge can then be applied to the design and control of the protein/starch matrix by replacing the salt with other healthier ingredient(s). Alternatively, it may be possible to manipulate the functionality of the raw material, wheat flour, through controlled milling in order to support innovation and the development of novel reduced-salt cereal based products.

Finally, it is my hope that the research reported in this thesis can form a strong foundation for future studies of this important but challenging area of study. May the current work contribute to the endeavours of the many in industry and in our global community who are seeking to enhance the health and wellbeing of our ever-expanding world population.
Appendix 1

Preparation of solutions for SE-HPLC

Na$_2$HPO$_4$ 0.05 M solution: 1.78 g Na$_2$HPO$_4$.2H$_2$O was dissolved in 200 mL Milli Q water.

NaH$_2$PO$_4$ 0.05 M solution: 1.56 g NaH$_2$PO$_4$.2H$_2$O was dissolved in 200 mL Milli Q water.

Phosphate buffer 0.05 M solution: The NaH$_2$PO$_4$ 0.05 M solution (55 mL) was mixed with the Na$_2$HPO$_4$ 0.05 M solution (100 mL). The pH was adjusted to 6.9 using either NaH$_2$PO$_4$ 0.05 M for decreasing pH or Na$_2$HPO$_4$ 0.05 M for increasing pH.

Sample buffer 0.05 M phosphate buffer, pH 6.9 containing 0.5% SDS: prepared by dissolving 0.5 g SDS in 100 mL 0.05 M phosphate buffer.

Eluent solution of acetonitrile-water, 1:1 containing 0.1% trifluoroacetic acid: 500 mL acetonitrile and 1 mL trifluoroacetic acid were mixed and diluted to 1000 mL using Milli Q water.
Appendix 2

Preparation of the solutions for disulfide analysis

0.2 M Tris-HCl pH 8.0 solution: 17.76 g TRISMA HCl and 10.60 g TRISMA base were weighed and dissolved in 1 L Milli Q water. pH was adjusted to 8.0 with either 0.1 M HCl or 0.1 M NaOH

Stock solution (8 M urea with 0.2 M tris-HCl pH 8 and 1% SDS): 480.48 g urea and 10.00 g SDS were weighed and dissolved with 700 mL 0.2 M Tris-HCl pH 8.0 until completely dissolved. The volume was adjusted to 1 L with the same solution.

Working buffer A (50 mL 8 M urea, 0.2 M Tris-HCl, pH 8.0, 1% SDS and 3 mM EDTA): 0.0438 g EDTA was weighed and dissolved and dissolved in 50 mL stock solution.

Working buffer B (10 mM DTNB in 0.2 M Tris-HCl pH 8.0): 0.198 g DTNB was weighed and dissolved in 50 mL 0.2 M Tris-HCl pH 8.0.

Synthesis of NTSB from DTNB: 29.8 mg of DTNB was weighed and dissolved in 1 M Na₂SO₃. pH was adjusted to 9.0-9.5 using 1 M Na₂SO₃. O₂ was bubbled into the solution until appear a decrease of colour to a yellow colour. The final solution is 50 mM NTSB and stable for 6 month at -20°C
Appendix 3

The second derivatives, deconvoluted spectra and relative peak areas of the FTIR

The secondary structure of the gluten samples based on the FTIR spectra were determined in the amide I region of the spectra (1600-1700 cm\(^{-1}\)).

Secondary derivatives of the gluten powder from FSB flour of water-washed (WW) and salt-washed (SW) gluten

Only component detected by second derivatives spectra were considered.
To measure the relative areas of the resolved amide I region, the deconvoluted spectra were curve fit.

The curve fitting of the deconvoluted spectra of the gluten samples with the assigned peaks to determine the percentage of relative peaks of certain secondary structure.
Band assignment for the secondary structure of the gluten

<table>
<thead>
<tr>
<th>Peak centre</th>
<th>Band assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1612</td>
<td>Intermolecular β-sheet</td>
</tr>
<tr>
<td>1620</td>
<td>Intermolecular β-sheet</td>
</tr>
<tr>
<td>1631</td>
<td>Intramolecular β-sheet</td>
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<td>β-turn</td>
</tr>
<tr>
<td>1680</td>
<td>Intermolecular β-sheet</td>
</tr>
</tbody>
</table>

The amount of the β-sheet structure was determined by the sum of all the β-sheet peaks.
Appendix 4

Curve fitting of the stress-strain curves to get the strain hardening index (n) and strain hardening coefficient (k) values in the uniaxial extensional rheology measurements

The determination of the n and k values was applied to each replicate of gluten as well as the wheat flour dough samples. For example, the curve fitting of one replicate of gluten samples from FSB flour with NaCl are presented below. The similar methods were also applied for the WW and SW gluten samples as well as the gluten and dough samples with different Hofmeister salts.

The stress-strain curve fitted with the exponential equation $\sigma = k\varepsilon^n$. Therefore, the n values equal to $1/t_1$ in the exponential Gro 1 fitting curve above, whereas the k values equal to A1 values.