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Immunochemical analysis of prolamins in gluten-free foods

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ACADEMIC DISSERTATION

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ABSTRACT

People with coeliac disease have to maintain a gluten-free diet, which means excluding wheat, barley and rye prolamin proteins from their diet. Immunochemical methods are used to analyse the harmful proteins and to control the purity of gluten-free foods. In this thesis, the behaviour of prolamins in immunological gluten assays and with different prolamin-specific antibodies was examined. The immunoassays were also used to detect residual rye prolamins in sourdough systems after enzymatic hydrolysis and wheat prolamins after deamidation. The aim was to characterize the ability of the gluten analysis assays to quantify different prolamins in varying matrices in order to improve the accuracy of the assays.

Prolamin groups of cereals consist of a complex mixture of proteins that vary in their size and amino acid sequences. Two common characteristics distinguish prolamins from other cereal proteins. Firstly, they are soluble in aqueous alcohols, and secondly, most of the prolamins are mainly formed from repetitive amino acid sequences containing high amounts of proline and glutamine. The diversity among prolamin proteins sets high requirements for their quantification. In the present study, prolamin contents were evaluated using enzyme-linked immunosorbent assays based on ω- and R5 antibodies. In addition, assays based on A1 and G12 antibodies were used to examine the effect of deamidation on prolamin proteins. The prolamin compositions and the cross-reactivity of antibodies with prolamin groups were evaluated with electrophoretic separation and Western blotting.

The results of this thesis research demonstrate that the currently used gluten analysis methods are not able to accurately quantify barley prolamins, especially when hydrolysed or mixed in oats. However, more precise results can be obtained when the standard more closely matches the sample proteins, as demonstrated with barley prolamin standards. The study also revealed that all of the harmful prolamins, i.e. wheat, barley and rye prolamins, are most efficiently extracted with 40% 1-propanol containing 1% dithiothreitol at 50 °C. The extractability of barley and rye prolamins was considerably higher with 40% 1-propanol than with 60% ethanol, which is typically used for prolamin extraction.

The prolamin levels of rye were lowered by 99.5% from the original levels when an enzyme-active rye-malt sourdough system was used for prolamin degradation. Such extensive degradation of rye prolamins suggest the use of sourdough as a part of gluten-free baking. Deamidation increases the diversity of prolamins and improves their solubility and ability to form structures such as emulsions and foams. Deamidation changes the protein structure, which has consequences for antibody recognition in gluten analysis. According to the resuts of the present work, the analysis methods were not able to quantify wheat gluten after deamidation except at very high concentrations. Consequently, deamidated gluten peptides can exist in food products and remain undetected, and thus cause a risk for people with gluten intolerance.

The results of this thesis demonstrate that current gluten analysis methods cannot accurately quantify prolamins in all food matrices. New information on the prolamins of rye and barley in addition to wheat prolamins is also provided in this thesis, which is essential for improving gluten analysis methods so that they can more accurately quantify prolamins from harmful cereals.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals. In addition, previously unpublished data are presented.

I Kanerva, P., Sontag-Strohm, T. and Lehtonen, P. 2005. Determination of prolamins in beers by ELISA and SDS-PAGE. Journal of the Institute of Brewing 111:61-64.

II Kanerva, P., Sontag-Strohm, T., Ryöppy, P., Alho-Lehto, P. and Salovaara, H. 2006. Analysis of barley contamination in oats using R5 and ω-gliadin antibodies. Journal of Cereal Science 44:347-352.

III Loponen, J., Kanerva, P., Zhang, C., Sontag-Strohm, T., Salovaara, H. and Gänzle, M. 2009. Prolamin hydrolysis and pentosan solubilization in germinated-rye sourdoughs determined by chromatographic and immunological methods. Journal of Agricultural and Food Chemistry 57:746-753.

IV Kanerva, P., Sontag-Strohm, T., Brinck, O. and Salovaara, H. 2011. Improved extraction of the prolamins for gluten detection from processed foods. Agricultural and Food Science 20:206-216.

V Kanerva, P., Brinck, O., Sontag-Strohm, T., Salovaara, H. and Loponen, J. 2011. Deamidation of gluten proteins and peptides decreases the antibody affinity in gluten analysis assays. Journal of Cereal Science 53:335-339.

Contribution of the author to publications I to V:

I, II, IV, V Päivi Kanerva planned the study together with the other authors. She had the main responsibility for interpreting the results, and she acted as the corresponding author of the paper.

III Päivi Kanerva planned the immunological part of the study together with the other authors. She performed the experiments for this part and had the main responsibility for interpreting the experimental results.

ABBREVIATIONS

AACC	American Association of Cereal Chemists
AP	alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
CCNFSDU	Codex Committee on Nutrition and Foods for Special Dietary Uses
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
EP-B2	cysteine endoprotease from germinating barley
FDA	The U.S. Food and Drug Administration
HLA	human leukocyte antigen
HMW	high molecular weight
HMW-GS	high molecular weight glutenin subunit
HPLC	high-performance liquid chromatography
HRP	horse radish peroxidase
IEL	intraepithelial leukocyte
LMW	low molecular weight
LMW-GS	low molecular weight glutenin subunit
MMW	medium molecular weight
MS	mass spectrometry
mTG	microbial transglutaminase
NBT	nitroblue tetrazolium
PCR	polymerase chain reaction
PEP	prolyl endoprotease
POP	prolyl oligopeptidase
PWG	Prolamin Working Group
RT	room temperature
SDS-PAGE	sodium dodecyl sulphate-polyacryl amide gel electrophoresis
TCEP	*tris*(2-carboxyethyl)phosphine
tTG	tissue transglutaminase

1 INTRODUCTION

The main storage proteins of wheat, barley and rye are called prolamins. The function of storage proteins is to store nitrogen, carbon and sulphur in the grain endosperm. Prolamins of wheat have the special characteristic of forming viscoelastic dough, which is important in wheat baking, whereas prolamins of other cereal species lack this property. Unfortunately, these same proteins are also harmful for gluten-sensitive people, e.g. for people with coeliac disease. In the context of coeliac disease, prolamins of wheat, barley and rye are often called gluten, and hence the term gluten-free is generally used. This thesis research focused on the immunological analysis of these proteins.

Many studies have been published on the relationship between prolamins and coeliac disease, and extensive knowledge of the pathogenesis of coeliac disease has been gained in recent years (reviewed in, e.g., Koning et al. 2005, Briani et al. 2008). Coeliac disease is initiated by the ingestion of prolamin-containing food. During digestion, gastric and pancreatic enzymes break proteins down into small peptides. In people with coeliac disease, these peptides initiate a reaction chain that leads to mucosal villous atrophy and crypt hyperplasia. In order to avoid this, coeliac patients have to maintain a diet free of prolamins, i.e. a gluten-free diet. Such a diet is currently the only treatment for coeliac disease. Gluten-free products are usually made from rice, maize or buckwheat. Industrially purified wheat starch is also commonly used in gluten-free baking. This special starch is manufactured gluten-free and is therefore suitable for most coeliac patients. Nevertheless, traces of prolamins remain in the starch. In addition, some contamination from the harmful cereals may occur in various gluten-free food products (Thompson et al. 2010). The studies of present thesis focused on the special circumstances that occur when prolamins are analysed from different food matrices and from processed foods.

The quantitative analysis of prolamins is mainly based on immunological methods, but mass spectrometric and chromatographic techniques have also been introduced (e.g. Wieser et al. 1998, Sealey-Voyksner et al. 2010). Most of the immunological methods that are used today are based on the antibody recognition of wheat gliadin or peptides derived from wheat. Wheat is the most common cereal throughout the world, and wheat proteins have received a considerable amount of research attention. Although prolamins of barley and rye are also considered harmful for people with coeliac disease, they have not been studied as much in this context. Since prolamins of all of the harmful cereals resemble each other, it is incorrectly assumed that they can be analyzed in the same way.

Prolamins can be degraded to reduce their harmfulness to gluten-intolerant people. This has been carried out by degrading them with specific enzymes into small peptides that no longer have immunological activity (Mitea et al. 2008a). Because of the tight structure of prolamins caused by their high proline content, the enzymes of gastrointestinal system are incapable of efficiently hydrolysing prolamins (Shan et al. 2005). However, cereal grains themselves contain enzymes that are able to degrade prolamins under optimal conditions (Loponen et al. 2004). These enzymes have been suggested to be used as an oral therapy, or they could offer a tool when developing new gluten-free cereal-based products, an approach that was examined in this thesis research.

Wheat storage proteins, which are also known as gluten, have many special characteristics that favour their use in various food products. Because a large amount of gluten is generated during the manufacture of starch, it has a relatively low price. The low solubility of gluten, however, limits its use. Numerous techniques have been developed to modify proteins to have more desired characteristics, such as increased solubility. Deamidation increases the potential use of proteins in various food products, e.g. to improve the structure or increase the protein content. This may turn out to be problematic for people on a gluten-free diet, since gluten proteins may be found in unexpected sources such as meat, fish or milk products (Day et al. 2006). Deamidation also changes the immunological behaviour of proteins, and the deamidation of gluten peptides by a tissue transglutaminase (tTG) is described to be an important part of the pathogenesis of coeliac disease (Molberg et al. 1998). It is not precisely known whether industrial deamidation increases or decreases their harmfulness to people with gluten intolerance, but either way, deamidation influences the detection of proteins by antibody-based immunological assays. A study included in this thesis focused on this phenomenon.

This thesis reviews the literature on the characteristics of prolamin proteins of wheat, barley, rye and oats and the literature relating to prolamins and coeliac disease. In addition, the different gluten-detecting antibodies and immunological gluten analysis methods are reviewed. Finally, the literature on gluten-free legislation and recommendations and their influence on the gluten-free diet and the variety of gluten-free products is reviewed. The experimental part focuses on qualitative and quantitative studies on prolamins using immunological and electrophoretic analysis methods. The aim of this study was to improve gluten detection in the analysis of different gluten-free foods.

2 REVIEW OF THE LITERATURE

2.1 Prolamins in cereals

Prolamins of wheat, barley and rye are storage proteins located in the grain endosperm. They belong to the prolamin superfamily together with several plant food allergens such as 2S albumins, nonspecific lipid transfer proteins and cereal alpha-amylase/trypsin inhibitors (Breiteneder and Radauer 2004). Osborne was first to suggest the name prolamins for this group of cereal proteins because of their high content of proline and amide nitrogen (Shewry and Tatham 1999). The prolamin superfamily was named after the cereal prolamins. Osborne characterized cereal prolamins as freely soluble in relatively strong ethyl alcohol, but insoluble in absolute alcohol, slightly soluble in water, and easily soluble in very dilute acids and bases (Osborne 1907).

Wheat storage proteins are also known as gluten. The term gluten is sometimes used with similar proteins from other cereals, such as barley and maize, even though they lack the rheological characteristics of wheat gluten. In the context of coeliac disease, however, gluten refers to the harmful proteins of wheat, barley and rye. In legislation, the term gluten is defined in different ways. The Codex definition of gluten is a "protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5M NaCl" (Codex Stan 118-1979). The U.S. Food and Drug Administration (FDA) defines gluten more from the clinical perspective as "the proteins that naturally occur in a prohibited grain and that may cause adverse health effects in persons with coeliac disease (e.g., prolamins and glutelins)" (FDA 2007). The term 'prohibited grain' refers to wheat, rye and barley, including any species belonging to their genera, or their crossbred hybrids. In this thesis, the term prolamin is preferred over gluten for the cereal proteins that are harmful to coeliac patients. The prolamin fraction of each cereal has a specific name: wheat prolamins are termed gliadins and glutenins, barley prolamins are hordeins and those from rye are secalins. The prolamin fractions of cereals that are not harmful to people with coeliac disease also have their own names: oat prolamins are called avenins, maize prolamins are zeins and rice prolamins oryzeins.

The function of storage proteins is to store carbon and nitrogen for germination. They contain high amounts of the amino acids glutamine and glutamic acid (about 40 mol %), and proline (about 20 mol %), but are low in lysine (less than 1 mol %) (Shewry et al. 1992). A special characteristic of proline is its ability to make β-turns. These turns form a tighter helix than an α-helix and thus enable proteins to be packed more efficiently into a small space. This is convenient for a plant to store vital amino acids, but makes it difficult for enzymes to hydrolyse the tight structures of prolamins. As a consequence, these proteins are poorly degraded by the gastrointestinal enzymes and remain relatively large peptides when entering the small intestine, where coeliac disease is manifested. The ability of prolamins to resist degradation was suggested to be one reason for their harmful effect on susceptible people (Shan et al. 2002).

All cereals contain prolamins. However, only the prolamins of wheat, barley and rye are harmful to people with gluten intolerance. One explanation could be phylogenetic. Wheat, barley and rye are

closely related to each other and they belong to the tribe of *Triticeae* (Figure 1). The prolamins of wheat have been extensively studied and their association with coeliac disease has been confirmed. The other harmful cereal species, barley and rye, have not gained such attention. However, their prolamins resemble those of wheat so closely that their harmfulness has not been seriously questioned.

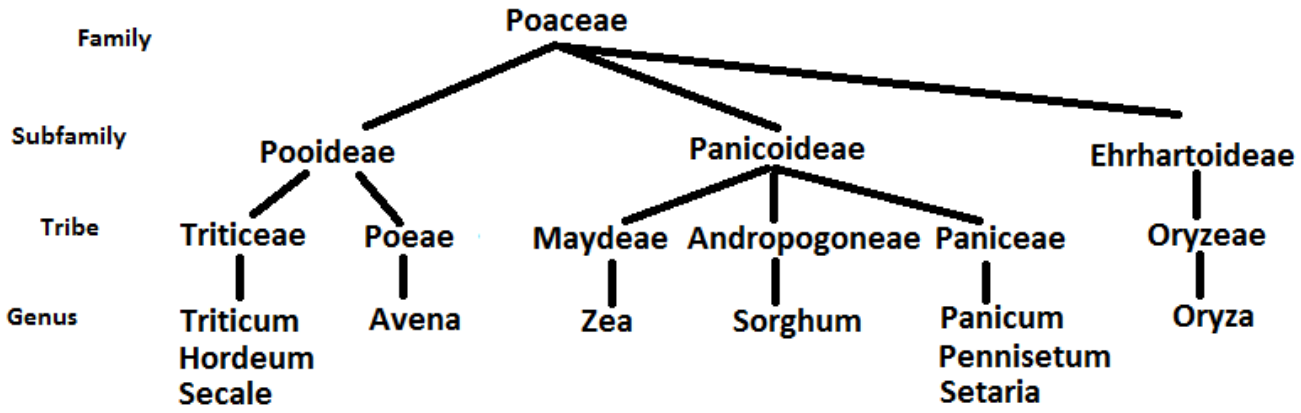

Figure 1. The common cereals in the grass family. Wheat (Triticum), barley (Hordeum) and rye (Secale) belong to the same tribe of *Triticeae*, whereas oats belong to *Poeae*. Maize (Zea), sorghum (Sorghum) and millet (Panicum, Pennisetum, Setaria, etc.) belong to the separate subfamily of *Panicoideae*, and rice (Oryza) to the *Ehrhartoideae* (data from www.uniprot.org/taxonomy).

Wheat is one of the main components of Western diets. Wheat gluten has special characteristics that no other cereal protein group have. It is able to form a viscoelastic dough, which is the basis for wheat baking. Because of the unique characteristics of wheat gluten, in addition to the increased demand for vegetable protein, it is used in many food products, including as a thickener in sauces and soups and an extender or filler in meat and fish products (Day et al. 2006). The gluten protein fraction can also be modified to increase its food-use potential. In addition to the wide use of wheat-based products, barley has recently also attracted new interest as a food ingredient because of its nutritional value as a wholegrain material (Baik and Ullrich 2008). The wholegrain foods overall have become more popular, since they are associated with increased satiety and weight loss. Barley contains β-glucan, which has found to have a lowering effect on blood cholesterol levels and the glycemic index. Rye-based products are also widely used, especially in Eastern Europe. Consequently, our diet is very much based on the harmful cereals, and maintaining a gluten-free diet can be quite cumbersome.

Prolamins consist of multiple proteins that can be divided to monomeric and polymeric groups. Monomeric prolamins are soluble in aqueous alcohols, whereas polymeric prolamins need the reduction of interchain disulphide bonds before they can be solubilized in aqueous alcohol. The disulphide bonds can be broken by reductive agents or by acid or enzyme treatments. Disulphide bonds are typical in the structure of storage proteins. Monomeric prolamins contain intramolecular disulphide bonds, whereas polymeric prolamins contain intermolecular disulphide bonds in addition to intramolecular bonds (Figure 2). The disulphide bonds hold the proteins closer together and improve the packing of proteins in a smaller space. The occurrence and formation of disulphide bonds in wheat storage proteins were reviewed by Shewry and Tatham (1997).

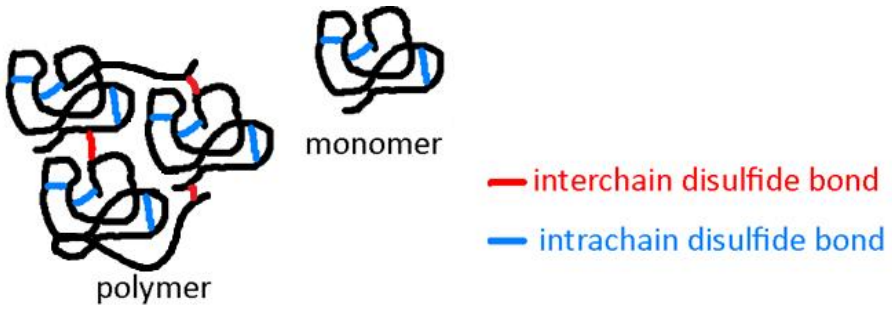

Figure 2. Positions of disulphide bonds in monomeric and polymeric prolamins.

Prolamins can be divided into groups based on their sulphur content, size or sequence homologies. Shewry and Tatham (1990) divided prolamins based on their sulphur content into S-poor, S-rich and high molecular weight (HMW) prolamins, whereas Wieser (2000) divided prolamins into three groups based on their size: HMW (80 000–120 000 g/mol), medium molecular weight (MMW) (52 000–80 000 g/mol) and low molecular weight (LMW) (30 000–52 000 g/mol) groups. The HMW group consists of HMW glutenin subunits of wheat, HMW secalins and D-hordeins. The MMW group consist of ω-type gliadins and secalins and C-hordeins of barley. The LMW group consists of α/β- and γ-gliadins, γ-secalins (monomeric γ-40 and polymeric γ-75) and γ-hordeins. The prolamin subgroups are described in more detail below.

2.1.1 Wheat

Wheat grain usually contains about 12–14% of protein. Approximately 80% of the total protein content of wheat grain is prolamins. About half of these are gliadins, the other half being glutenins (Huebner 1970). There is, however, considerable variation in these relative proportions (Wieser and Koehler 2009). Gliadins consist of monomeric proteins whereas glutenins have a polymeric nature.

Wheat prolamins are divided into α-, β-, ω- and γ-gliadins and HMW and LMW glutenin subunits according to the electrophoretic mobility at acid pH. The α- and β-gliadins are often combined together and simply referred to as α-gliadins because of the high similarity of their N-terminal amino acid sequences.

Gliadins

Monomeric wheat prolamins, gliadins, are divided into subgroups of α-, γ- and ω-gliadins.

The group of α-gliadins is generally the major group, comprising between 44 and 60% of the total gliadin content. The second largest group is γ-gliadins (31–46%), and together these groups account for about 80% of wheat gliadins (Shewry and Tatham 1990). The sizes of α- and γ-gliadins are approximately 36 000–44 000 g/mol, they contain about 250–300 amino acid residues and are rich in sulphur. The α-gliadins are typical for wheat and they are thought to be the most harmful fraction for people with coeliac disease. Although rye and barley are considered as harmful for coeliacs as wheat, they do not contain similar proteins (Shewry and Tatham 1990). The α-gliadins contain long repeats of

glutamine residues along with typical repetitive domains (Table 1). The γ-gliadins are also repetitive and mostly monomers; however, polymeric forms also exist (Shewry and Tatham 1990).

The rest of the gliadins, about 10–20%, are sulphur-poor ω-gliadins (Shewry and Halford 2002). Their sizes are between 44 000–78 000 g/mol. Due to the highly repetitive nature of ω-gliadins, about 80% of their amino acid content is glutamine, glutamic acid, proline and phenylalanine residues. Since ω-gliadins are poor in sulphur and do not therefore contain disulphide bonds in their structure, they retain their solubility after heat treatment. Their solubility was reported to remain the same in processed samples as the solubility of sulphur-containing α- and γ-gliadin decreased considerably (Skerritt and Smith 1985). Immediate allergic reactions have been associated with ω-gliadins (Palosuo et al. 2001).

Altogether, about 30 individual proteins can be distinguished by two-dimensional electrophoresis of the gliadin fraction of wheat (Madgwick et al. 1992).

Glutenins

Glutenins are polymeric, consisting of low molecular weight glutenin subunits (LMW-GS) and high molecular weight glutenin subunits (HMW-GS).

The LMW-GS can be divided into B-, C- and D-types based on their electrophoretic mobility (Jackson et al. 1983). B-type subunits are the major group of LMW glutenins. C-type LMW-GS are similar to α- and γ-gliadins, whereas D-type LMW-GS resemble ω-gliadins. The molecular weights of LMW-GS are similar to α- and γ-gliadins: the molecular weights of B-type subunits are 40 000–50 000 g/mol, those of C-type subunits are 30 000–40 000 g/mol, and the weights of D-type subunits are slightly higher than the weights of B-type subunits (Lew et al. 1992). The LMW glutenins are closely related to gliadins, the main difference being their higher tendency to aggregate. Many antibodies raised against gliadins also recognize LMW glutenins (Skerritt and Robson 1990).

About 10% of wheat storage proteins are HMW glutenins (Shewry and Tatham 1990). The subunits of glutenins are located in seeds in large polymers stabilized with disulphide bonds. These polymers are about 10^6–10^7 g/mol in size, and belong to the largest polymers in nature (Wahlund et al. 1996). The HMW-GS consist of approximately 600–800 amino acids, with high amounts of glycine, glutamine and proline. The HMW-GS can be divided into x-types and y-types. The size of the x-types is typically 83 000–89 000 g/mol and the y-types 68 000–73 000 g/mol, as determined by mass spectrometry (MS) (Hickman et al. 1995). The variability in HMW-GS between wheat cultivars is high. About 20 different subunits were distinguished by sodium dodecyl sulphate-polyacryl amide gel electrophoresis (SDS-PAGE) (Payne et al. 1981). However, most cultivars contain from 3 to 5 subunits with molecular weights between 82 000 and 125 000 g/mol.

Polymeric prolamins have an important role in the formation of the gluten network. The relative amount of polymeric proteins in gluten correlates with dough strength, and the composition of subunits of HMW glutenins has been used to predict the baking qualities of wheat cultivars (Payne et al. 1979).

2.1.2 Barley

Estimates of the amount of prolamin in barley grain vary considerably. The reported figures are between 30 to 50% of the total protein content. However, the prolamin content is most likely higher, since only about 10–20% of the total proteins belong to the water- and salt soluble albumins (9–13%) and globulins (2–6%) (Rhodes and Gill 1980). Barley storage proteins are termed hordeins and they can be divided into four groups: B-, C-, D-and γ-hordeins. Most of the barley storage proteins (80%) belong to the sulphur-rich proteins, mainly to the B-hordeins.

Monomeric hordeins

The C- and γ-hordeins are monomeric prolamins of barley.

C-hordeins are approximately 55 000–70 000 g/mol in size (Shewry and Tatham 1990). They are the second largest group of hordeins in barley, comprising about 10–20% of the hordeins (Tatham and Shewry 1995). Their sequence consists of about 440 amino acid residues and is almost entirely composed of repeats of the octapeptide PQQPFPQQ (Shewry and Tatham 1990). The amino acid content of C-hordein is comparable to this sequence: 40 mol-% glutamine, 30 mol-% proline and 8–9 mol-% phenylalanine. C-hordeins are poor in sulphur and do not contain cysteine (Shewry and Tatham 1990). The isoelectric points of the C-hordeins are in the pH range 5.0–6.0 (Shewry et al. 1978). Studies using circular dichroism spectroscopy have suggested C-hordeins to have an unusual secondary structure with regularly repeated β-turns in the absence of an α-helix and β-sheet (Tatham et al. 1985, Field et al. 1986). The structure seems to be stabilized by strong hydrophobic interactions and hydrogen bonding.

The γ-hordeins are approximately 36 000–44 000 g/mol in size, consisting 250–300 amino acid residues (Shewry and Tatham 1990). Similar to the γ-gliadins of wheat, some of the γ-hordeins are found in polymers, most of them being monomeric (Shewry et al. 1985). Similar repetitive units are found in γ-hordeins and C-hordeins (Shewry and Tatham 1990). Only minor amounts of γ-hordeins are present in barley.

Polymeric hordeins

Polymeric prolamins of barley are termed B- and D-hordeins.

Sulphur-rich B-hordeins are the main group of hordeins, making up about 70–80% of the prolamin content of barley (Shewry et al. 1985). They are found in polymers, and as subunits they are about the same size as γ-hordeins (36 000–44 000 g/mol) (Shewry and Tatham 1990). B-hordeins are composed of a highly polymorphic group of proteins and they are not as repetitive as other hordeins. Less than 30% of their amino acid sequence contains repeats of PQQP (Shewry and Tatham 1990). The isoelectric points of the B-hordeins are in the pH range 6.0–8.0 (Shewry et al. 1978). The B-hordeins are most closely related to the low molecular mass subunits of wheat glutenin.

D-hordeins are similar to the HMW subunits of wheat. The molar masses of D-hordein subunits are about 90 000–110 000 g/mol. D-hordeins are high in glycine, glutamine and proline (Shewry and Tatham 1990), and because of the unique repetitive units D-hordeins contain considerable amounts of

threonine (Shewry and Tatham 1999). D-hordeins make up about 7–8% of the hordeins (Marchylo et al. 1986).

2.1.3 Rye

The protein content of rye grain is somewhat lower than those in wheat and barley, being about 10% of the grain. Rye has a unique composition of proteins, and contains significantly higher proportions of soluble proteins, i.e. albumins and globulins, compared to wheat and barley. In studies on cereal protein compositions, it has been observed that nitrogen soluble in NaCl and water made up 40% of the total nitrogen of rye flour, whereas only 20% of wheat nitrogen was soluble under the same conditions (Charbonnier et al. 1981). Based on high-performance liquid chromatography (HPLC) studies on the protein composition of rye, about 26% of total proteins were found to fall into the group of salt-soluble proteins (Gellrich et al. 2003), which is about twice as much as found in wheat flour.

Rye prolamins are called secalins. They are divided into four types: HMW, γ-75, ω and γ-40 secalins. All of these groups have been thoroughly studied by Gellrich et al. (2003, 2004a, 2004b, 2005). In earlier studies, rye prolamins were divided similarly to barley prolamins into three groups based on their size: A-secalins (about 16 000 g/mol), B-secalins (about 29 000 g/mol, similar to α- and γ-gliadins) and C- secalins (about 38 000 g/mol, similar to ω-gliadins) (Charbonnier et al. 1981).

Monomeric secalins

The monomeric secalins are made up of γ-40 and ω-secalins.

The monomeric γ-40 secalins account for about 24% of the total secalin fraction, and their molecular weights are about 36 000–44 000 g/mol (Shewry and Tatham 1999, Gellrich et al. 2003). The γ-40 secalins appeared homologous to γ-gliadin of wheat (Shewry et al. 1982), which was later confirmed by a study on N-terminal amino acid sequences (Gellrich et al. 2005).

Monomeric ω-secalins account for about 17% of the total secalin fraction of rye (Gellrich et al. 2003). Based on the N-terminal amino acid sequences, ω-secalins are homologous to corresponding ω-gliadins of wheat. The molecular weight of ω-secalins is about 48 000–53 000 g/mol (Shewry and Tatham 1990, Gellrich et al. 2003). The ω-secalins are poor in sulphur. They are almost entirely composed of the repetitive sequence PQQPFPQQ, similar to C-hordein in barley (Shewry and Tatham 1999). Approximately 80 mol-% of their amino acid composition is made up of glutamine, glutamic acid, proline and phenylalanine (Shewry and Tatham 1990).

Polymeric secalins

Polymeric secalins are made up of γ-75 and HMW secalins.

The γ-75 secalins made up about 46% of the total secalins and their molecular weights are about 70 000 g/mol (Gellrich et al. 2003). The γ-75 secalins are similar to γ-gliadin, but their glutamine and proline contents are higher. Therefore, they form a unique group of prolamins. The higher glutamine and

proline contents are thought to be due to the higher contents of repetitive sequences in γ-75 secalins than in γ-40 secalins. The γ-75 secalins contain one cysteine residue in the N-terminal, which makes them able to form similar bonds and structures to LMW glutenins (Gellrich et al. 2004b). However, γ-75 secalins do not contain cysteine in the C-terminal, which prevents the formation of larger protein aggregates similar to the gluten network.

HMW secalins account for about 7% of the total secalins. The molecular weights of HMW secalin subunits are about 100 000 g/mol (Shewry et al. 1983). HMW secalins appeared similar to HMW gliadins when comparing their N-terminal amino acid sequences (Gellrich et al. 2003). Rye, however, does not contain aggregated sulphur-rich prolamins, as do wheat LMW glutenins and barley B-hordeins (Shewry and Tatham 1990). HMW secalins consist of high amounts of glycine, glutamine and proline (Shewry and Tatham 1990).

2.1.4 Oats

The storage proteins of oats are different from those of wheat, barley and rye. Oat storage proteins are divided into salt-soluble globulins and alcohol-soluble prolamins, avenins. About twice as many genes encode globulins compared to avenins (Chesnut et al. 1989), which is also seen in the amount of proteins found in the grain. Avenins comprise about 10% and globulins about 70–80% of the total protein of oats. However, the amount of avenins in oats varies considerably, between 4 and 15% of the total seed nitrogen. Oat avenins are not considered harmful for people with coeliac disease (e.g. Janatuinen et al. 1995).

Avenins

Avenins are monomeric and can be divided into two groups based their molecular weights. The molecular weights of α-avenins are about 12 000–18 000 g/mol and those of β-avenins about 22 000–35 000 g/mol (Jussila et al. 1992). The isoelectric points are between pH 4.5 and 8.0 (Robert et al. 1983).

Oat avenins are similar to α- and γ-gliadins of wheat. Barley B-hordeins and rye γ-secalins are also similar prolamin subgroups (Chesnut et al. 1989). Avenins are high in glutamine and glutamic acid (up to 30 mol%), and fairly high in proline (about 10 mol%) and leucine (10-15 mol%) (Chesnut et al. 1989).

Table 1. Prolamins of wheat, barley, rye and oats. The one-letter code for amino acids is used in the repetitive domains: F = phenylalanine, G = glycine, H = histidine, I = isoleucine, L = leucine, P = proline, Q = glutamine, S = serine, T = threonine, V = valine, Y= tyrosine.

Cereal	Prolamin subgroup	MW x10 g/mol	%	Repetitive domain
Wheat	α/β-Gliadins	28–35	28–33	PQPQPFP and PQQPY (35%)
	γ-Gliadins	31–35	23–31	PQQPFPQ (40%)
	$\omega_{1,2}$-Gliadins	39–44	4–7	PQQPFPQQ (90%)
	ω_5-Gliadins	49–55	3–6	QQQ-F/I/L-P
	LMW	32–39	19–25	PQQPPPFS and QQQQPVL (26%)
	HMW subunit x	83–88	4–9	PGQGQQ, GYYPTS-P/L-QQ and GQQ
	HMW subunit y	67–74	3–4	PGQGQQ and GYYPTS-P/L-QQ
Barley	B-Hordeins	36–44	70–80	PQQP (<30%)
	C-Hordeins	55–70	10–20	PQQPFPQQ
	D-Hordeins	90–110	<10	P-G/H-QGQQ, GYYPSXTSPQQ and TTVS
	γ-Hordeins	36–44	<10	PQQPFPQ
Rye	ω-Secalins	48–53	17	PQQPFPQQ
	γ-40 Secalins	36–44	24	QPQQPFP
	γ-75 Secalins	70	46	QQPPQQPFP
	HMW secalins	~100	7	QQPGQG
Oats	α-Avenins	12–18	Minor	PFVQQQ
	β-Avenins	22–35	Major	

Data collected from Tatham et al. 1985, Shewry and Tatham 1990, Jussila et al. 1992, Shewry et al. 1995, Shewry and Tatham 1999, DuPont et al. 2000, Shewry and Halford 2002, Wieser 2005b, and Wieser and Koehler 2008. MW denotes molecular weight.

2.2 Prolamin proteins and coeliac disease

Prolamin proteins of wheat, barley and rye contain multiple proteins, and among them specific sequences exist that are harmful for coeliac patients. A common feature for the harmful sequences is that they contain high amounts of proline and glutamine. Most of the harmful sequences are found from monomeric prolamins, such as α-gliadin, but the harmfulness of HMW glutenin subunits has also been observed. Harmful sequences have additionally been demonstrated from barley and rye by *in vitro* and *in silico* studies.

2.2.1 Coeliac disease in brief

Even though the first observations of coeliac disease date back hundreds of years, it was only about 60 years ago when W.K. Dicke wrote in his thesis that wheat gliadin was a harmful substance for coeliac patients (Wieser and Koehler 2008). Soon after, barley, rye and oats were also proposed to be harmful due to their close relation to wheat. Today, oats are no longer considered harmful for coeliacs. In addition, major progress has been made in explaining the pathogenesis of coeliac disease.

Coeliac disease (gluten intolerance) is a small intestinal enteropathy caused by an inflammatory response to prolamins of wheat, barley and rye. The immune reaction induced by ingested prolamins causes flattening of the villi in the small intestine and thus leads to inflammation and malabsorption of nutrients. Typical symptoms in coeliac disease include chronic diarrhoea, abdominal pain, bloating, flatulence, indigestion, weight loss and mouth ulcers; however, long-term malnutrition may lead to osteoporosis, developmental delay, educational underperformance, infertility, headaches, predisposition to other autoimmune diseases, neurological diseases, fatigue, hair loss, nutrient deficiencies and malignancies (Green et al. 2003, Brousse and Meijer 2005, Corazza et al. 2005, Lohi et al. 2009).

The recommended diagnosis of coeliac disease in the Current Care Guidelines in Finland includes gastroenteroscopy to affirm damage in small intestine mucosa (www.kaypahoito.fi). However, the presence of certain antibodies is screened when testing for possible coeliac disease. For example, serum immunoglobulin A antibodies against tTG or endomysium are good indicators of the disease (Sulkanen et al. 1998, Dieterich et al. 1998). There is also a rapid test that detects antibodies from a blood sample and can be performed at home if coeliac disease is suspected (Raivio et al. 2007).

Pathogenesis of coeliac disease

Coeliac disease has an environmental and a genetic factor. Both factors are needed for the disease to occur. The environmental factor is dietary gluten, whereas the most important genetic factor is the expression of HLA-DQ2 or HLA-DQ8 molecules. These molecules are found from over 95% of all coeliac patients (Sollid et al. 1989). However, about 25% of population carries these genes without developing the disease (Sollid et al. 1989) and they cannot therefore be the only genes contributing to the development of the disease. The function of HLA molecules is to present gluten antigens to T-cells. If the presented gluten antigens are recognized by the gluten-specific T-cells, an immune reaction starts, leading to the production of antibodies and cytokines. The HLA-DQ2 molecules are more

common in coeliac patients than HLA-DQ8 molecules and the role of HLA-DQ2 molecules in presenting gluten peptides to T-cells has been examined in detail in several studies (Johansen et al. 1996, Costantini et al. 2005, Bergseng et al. 2008). The HLA-DQ2 and HLA-DQ8 molecules present different gluten-derived peptides to the T-cells (Mazzarella et al. 2003, Henderson et al. 2007). Pathogenesis was described in detail by Koning et al. (2005), and recent advances were reviewed by van Heel and West (2006).

An alternative pathway of coeliac disease to the above-described adaptive immunoreaction is an innate reaction to gluten proteins. Innate immunereaction is a rapid reaction that leads to the secretion of interleukin 15 and intraepithelial lymphocytes (IELs). This pathway also results in the destruction of the villous structure in the small intestine of people with coeliac disease (reviewed by Jabri et al. 2005). Furthermore, gluten-induced coeliac-type autoantibodies have been implicated in the pathogenesis (Caja et al. 2011).

Prevalence

Coeliac disease is most prevalent in Western countries. People with coeliac disease are estimated to comprise about 1% of the European population as well as that in North America (Kagnoff 2007, Mustalahti et al. 2010). Coeliac disease is also found in populations in the Middle East, India and North Africa (Malekzadeh et al. 2005). However, it appears be a rare disease in Russia and North Asia (Kondrashova et al. 2008). The prevalence of the disease appears to be increasing. In the latest studies, the estimated prevalence in Finland has been doubled to 2% (Lohi et al. 2007). The increase is suspected to be due to environmental factors and not just to a better detection rate, which has also increased. This has raised discussion on whether our modern, highly hygienic lifestyle induces autoimmune diseases to occur.

2.2.2 Immunogenic proteins and peptides

Proteins involved in coeliac disease

After the immunogenicity of wheat was established, barley, rye and oats were also included among the cereals prohibited for coeliac patients. However, the harmfulness of barley and rye prolamins to coeliac patients has scarcely been tested, although, since they are so closely related to wheat prolamins, their harmfulness has not seriously been questioned. Recently, the possibility of T-cell testing has offered an easier way to study specific peptides, and peptides derived from barley and rye prolamins have consequently also been tested. Indeed, peptides homologous to the T-cell stimulatory peptides have been found in barley, rye and oats (Vader et al. 2003). In addition, barley and rye prolamins have been shown in *in vitro* studies to produce similar levels of interferon-γ mRNA in coeliac duodenal tissue to wheat prolamins (Bracken et al. 2006).

Because of the differences between wheat gliadins and glutenins, the immunoactive role of HMW and LMW glutenins in coeliac disease has been questioned. Ciclitira et al. (2005a) separated and mixed four HMW glutenin subunits and tested their reactivity with T-cells. Altogether, 11 of the 17 tested T-

cell lines reacted to the mixture of glutenins. Experiments were also carried out *in vivo* on three coeliac patients. They all developed increased levels of IELs and their villous hight/crypt depth ratio decreased (Dewar et al. 2006). The harmfulness of the HMW subunits was also demonstrated by T-cell stimulation by van de Wal et al. (1999) and Molberg et al. (2003). Epitopes triggering coeliac disease were found in the LMW glutenins of wheat by database searching, and some of these peptides were tested to have T-cell stimulatory activity (Koning and Spaenij-Dekking 2005). It is therefore likely that all types of wheat prolamins have T-cell stimulatory properties. A closer study of rye prolamins revealed that the ω-type secalin and γ-type secalins in addition to two low molecular mass glycoproteins of 15 000 and 18 000 g/mol from rye exhibited coeliac serum antigenicity (Rocher et al. 1996). These small glycoproteins are most likely not prolamins, although able to trigger similar symptoms.

The harmfulness of oats has been debated for years. Oats were initially considered harmful, but many clinical studies have subsequently shown the suitability of oats for coeliac patients (reviewed in Salovaara et al. 2009). However, oats have caused symptoms in some studies, indicating a harmful character (Lundin et al. 2003, Arentz-Hansen et al. 2004). It is possible that a subgroup of coeliac sufferers exists who cannot tolerate oats.

Peptides involved in coeliac disease

Typical immunogenic prolamin peptides contain large amounts of proline and glutamine, and it seems that most of them are located in the repetitive domains. Their structure appears to be dominated by disordered random-coil formations and β-turns (Darewicz et al. 2007), which are suggested to be relevant to coeliac disease (Tatham et al. 1990). However, β-turns are common in storage proteins because of their high proline content, and are also found in parts of the sequence that are not active in coeliac disease. Therefore, β-turns cannot be the only cause of the harmfulness, although they are a seemingly important characteristic of immunogenic peptides.

Most of the harmful sequences are found in α-gliadin, but the harmfulness of other gliadin groups has also been confirmed *in vivo* (Ciclitira et al. 1984b). Difficulties in the identification of harmful peptides have resulted in findings that people with coeliac disease react to varying peptides (Vader et al. 2002b). Enzymatic digestion and heating appear to increase the T-cell stimulation capacity of the peptides (Lundin et al. 1997). Some of the peptides that are found to be active in coeliac disease are presented in Table 2.

It has turned out to be an immense task to identify the dominant peptides causing coeliac disease to manifest itself. About ten years ago, a peptide of 33 amino acids was found to be very resistant to degradation and was therefore suggested to be the most important peptide involved in coeliac disease (Shan et al. 2002). However, this finding did not explain the overall pathogenesis of coeliac disease, and many more peptides triggering T-cell activation have since been found. Despite this, the peptide of 33 amino acids has often been used as a model immunogenic peptide. The peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) is commonly shortened to 33mer. It consists of repeats with a large amount of proline and glutamine and sequences that have been tested harmful for coeliac patients. Although the pathogenesis of coeliac disease is not entirely explained by the presence

of 33mer, the inflammatory character of gluten is suggested to be due to the proteolytic resistance of gluten peptides (Shan et al. 2005). At least 60 peptides that are similar to 33mer and which may all be active in coeliac disease have been identified by *in silico* protein analysis (Shan et al. 2005).

Table 2. Some immunogenic peptides found in wheat prolamins.

Method	Peptide	Amino acid sequence*	Reference
In vivo	α-Gliadin 31–43	LGQQQPFPPQQPY	Marsh et al. 1995
	α-Gliadin 31–49	LGQQQPFPPQQPYPQPQPF	Sturgess et al. 1994
	α-Gliadin 44–55	PQPQPFPSQQPY	Marsh et al. 1995
	α-Gliadin 56–75	LQLQPFPQPQLPYPQPQLPY	Fraser et al. 2003
	α-Gliadin 206–217	LGQGSFRPSQQN	Mantzaris and Jewell 1991
In vitro	α-Gliadin 1–30	VRVPVPQLQPQNPSQQQPQEQVP LVQQQQF	De Ritis et al. 1988
	α-Gliadin 3–24	VPVPQLQPQNPSQQQPQEQVPL	Wieser et al. 1986
	α-Gliadin 57–68	QLQPFPQPELPY	Arentz-Hansen et al. 2000
	α-Gliadin 62–75	PQPELPYPQPQLPY	Arentz-Hansen et al. 2000
	α-Gliadin 198–222	QYPSGQGSFQPSQQNPQA	Van de Wal et al. 1998a
	α-Gliadin 203–220	QYPSGQGSFQPSQQNPQA	Mazzarella et al. 2003
	γ-Gliadin	QPFPQPQLPY, QPFPQPQQTF	Arentz-Hansen et al. 2002
	γ-Gliadin 60–79	LQPQQPFPQQPQQPYPQQPQ	Arentz-Hansen et al. 2002
	LMW glutenin	QQQQPPFSQQQQSPFSQQQQ	Vader et al. 2002b
	LMW glutenin	QQPPFSQQQQQPLPQ	Vader et al. 2002b
	HMW glutenin	GQQGYYPTSPQQS	Van de Wal et al. 1999

*A= alanine, E = glutamic acid, F = phenylalanine, G = glycine, L = leucine, P = proline, Q = glutamine, S = serine, T = threonine, Y= tyrosine

Since the amino acid content, arrangement of proline residues and specific deamidation by tTG are important for T-cell recognition, computational methods can be used to detect harmful sequences with reasonable accuracy. A database of known T-cell stimulatory sequences and prolamin epitopes was created by O'Brien and Feighery (2008). Information on a total of 101 epitopes has been collected from published results.

2.2.3 Modified prolamins

Proteins are modified to improve their functionality and increase their usage in different applications to form and stabilize structures. In addition to traditional baking improvers, gluten proteins are used to make biofilms (Olabarrieta et al. 2006, Guillaume et al. 2010), and are reported to be good candidates as a clarifying agent of red wine (Marchal et al. 2002). Proteins can be modified by various techniques, such as deamidation, transamidation and degradation. All of these modifications also happen naturally due to enzymes in cereal seeds. Deamidation of prolamin peptides has been found to be a significant part of the pathogenesis of coeliac disease, in which the deamidation is caused by a tTG. This enzyme is able to deamidate specific glutamines to glutamic acid residues and, as a consequence, enhances the binding of the peptides to HLA-DQ2 and -DQ8 molecules. In certain conditions, the transglutaminase enzyme may also crosslink. Crosslinking by microbial enzymes is used to improve protein structures in food applications. Crosslinking has also been suggested as a tool to reduce the immunoactivity of prolamin peptides.

2.2.3.1 Heating

Since the formation of a gluten network has a major role in baking, the changes occurring in gluten proteins during heating have been extensively studied. During heating, new disulphide bonds are formed and they are reorganized from intramolecular to intermolecular bonds, leading to large aggregates with lowered solubility (Schofield et al. 1983). The prolamin subgroups undergo changes during the baking process that reduce their extractability. The subgroups that contain high amounts of sulphur, such as α- and γ-gliadins, are more susceptible to heat-induced changes compared to those with a low sulphur content. γ-Gliadins have been shown to be more sensitive to heating than α-gliadins (Lagrain et al. 2005). The extractability of α- and γ-gliadins was observed to be lost after heating wheat samples for 1 h at 45–110 °C (Lavelli et al. 1996). The extractability was, however, returned by reduction. The solubility of ω-gliadins, which are low in sulphur, is not affected by heating (Schofield et al. 1983, Skerritt and Smith 1985). In addition to an increase in the number of intermolecular disulphide bonds during heating, other bonds such as isopeptide bonds are formed between the proteins (Rombouts et al. 2011). These bonds are not broken by reduction and consequently further decrease the solubility of the proteins.

2.2.3.2 Enzymatic degradation

Prolamins are not easily degraded by gastrointestinal enzymes. One reason for this is that they contain large amounts of the amino acid proline. Proline is a structurally unique amino acid and causes such bends in the peptide chain that only specific enzymes are able to cleave the chain (Simpson 2001). Proline has a specific character, as it is able to make β-turns in the peptide chain. These turns form a tighter helix than in a normal α-helix, and enable tighter packing of proteins in the endosperm of seeds. However, β-turns also hinder the action of hydrolysing enzymes. Consequently, relatively large peptides are absorbed from the lumen to the gut mucosa.

Hydrolysis of the proteins during the processing of food could be one way to reduce or abolish the harmfulness of prolamin proteins and thus increase the variety of gluten-free cereal foods. The role of

sourdough lactic acid bacteria in reducing the harmfulness of fermented foods to coeliac sufferers has been examined. It has been claimed that the majority of ethanol-soluble rye polypeptides are almost totally hydrolysed by lactic acid bacteria, and no prolamins were recognized after 48 h fermentation of rye flour when measured by R5-Western blot analysis (De Angelis et al. 2006). The role of endogenous enzymes was not, however, determined in that study. These enzymes are most likely to account for the majority of prolamin degradation during fermentation. Similar results were obtained with fermented durum wheat with lactic acid bacteria in which the gluten content was reduced by 83% with the help of fungal enzymes (Di Cagno 2004). However, after successful hydrolysis of the majority of prolamins, considerable levels of gluten (1045 ppm) were still detected. By mixing fermented wheat flour with other ingredients, however, it was possible to make sweet breads with 8 ppm of gluten. These breads did not cause any serological changes or deterioration in the small bowel of people with coeliac disease (Greco et al. 2011).

Endogenous enzymes have a more important role in prolamin degradation than lactic acid bacteria. Lactic acid bacteria are thought to adjust the pH to low levels, which are optimal for the endogenous enzymes of cereals to function. Therefore, their role is more in pH adjustment rather than protein hydrolysis. The extent of prolamin degradation during sourdough fermentation has been studied by Loponen et al. (2004) and Tuukkanen et al. (2005). Prolamins were as efficiently degraded during fermentation without starter microorganisms (lactic acid bacteria). The most rapid degradation of prolamins occurs in rye (Hartmann et al. 2006, Loponen et al 2011). Glutelins were degraded faster at 30 °C, and prolamins at 15 °C (Hartmann et al. 2006). Under optimum conditions, at pH 4.5 at 50 °C or at pH 6.5 between 50 °C and 60 °C, the synthetic gliadin peptides were degraded into peptides of less than 9 amino acids within two hours. Such small peptides are not considered to have T-cell stimulation activity (Sollid 2002). Proteases preferred cleavage sites between P-Q, Q-P, P-F, L-P and P-Y. Loponen et al. (2007) and Stenman et al. (2009) also demonstrated the efficient degradation of wheat prolamins by proteases from germinating cereals.

In addition to endogenous cereal enzymes, various microbial enzymes have been reported to degrade prolamins. Although endogenous enzymes are very efficient in degrading prolamin proteins, levels still harmful for coeliacs remain. These remaining prolamins have successfully been degraded by prolyl endoprotease of *Aspergillus niger* (AN-PEP) (Spaenij-Dekking et al. 2006), *Sphingomonas capsulata* (SC-PEP) (Tye-Din et al. 2010) and prolyl oligopeptidase of *Flavobacterium meningosepticum* (POP) (Shan et al. 2004, Marti et al. 2005). AN-PEP was shown to degrade prolamins efficiently in stomach conditions, where POP lost its functionality (Mitea et al. 2008a). However, good results have been obtained when combining a recombinant cysteine endoprotease from germinating barley (EP-B2) with POP (Siegel et al. 2006). AN-PEP is a serine protease that cleaves the peptide chain after proline (Edens et al. 2005). It has a pH optimum between 4 and 5 and remains stable at pH 2. It was found to be completely resistant to pepsin, which enables its use in the stomach environment, whereas the POP enzyme was found to be degraded by pepsin and only be active at a neutral pH. AN-PEP was found to efficiently degrade T-cell stimulatory epitopes from intact gluten as well as from digested peptides (Stepniak et al. 2006). AN-PEP has also been shown to be very efficient in degrading gluten from white bread and in fast food meal to such an extent that no immunoactivity remained. The degradation was modelled using a dynamic gastrointestinal model that mimics the human stomach and small intestine (Mitea et al. 2008a). Another advantage of AN-PEP (commercially available as Brewers

Clarex, DSM, the Netherlands) is that it is food grade and can therefore potentially be used orally as an enzyme therapy after ingestion of gluten-containing food as well as endogenous cereal enzymes.

2.2.3.3 Deamidation

Deamidation of proteins can be carried out using enzymatic or chemical methods. Enzymatic reactions are preferred in food processes, since they are considered safer (Shih 1996). Deamidation means that the amide group of glutamine or asparagine is removed and replaced by a hydroxyl group. As a consequence, they are changed to glutamic acid and aspartic acid, respectively, and ammonium is released (Figure 3).

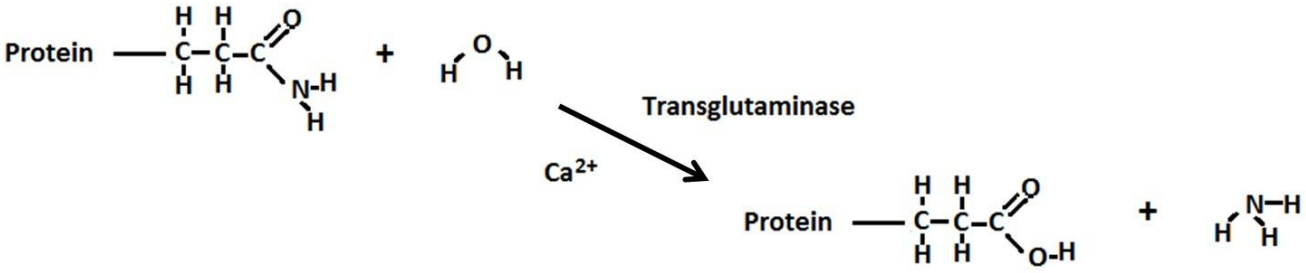

Figure 3. Deamidation of glutamine to glutamic acid. Deamidation is preferred at low pH values and with high relative amounts of glutamine.

Even limited (2–5%) deamidation may improve the functional properties of the proteins (Shih 1996). As the level of deamidation increases, the surface hydrophobicity of the protein increases (Matsudomi et al. 1982), consequently increasing the emulsifying potential and emulsion stability (Wu et al. 1976). The foam-forming capacity of deamidated gliadin is slightly better than the capacity of egg white (MacDonald and Pence 1961).

The tTG-induced deamidation of prolamin proteins has been shown to considerably increase their binding to HLA-DQ2 or -DQ8 molecules (Molberg et al. 1998, Sjöström et al. 1998). As a consequence, deamidation increases the T-cell stimulating activity of prolamin peptides (Arentz-Hansen et al. 2000, Ellis et al. 2003). tTG is a calcium-dependent enzyme that catalyses the posttranslational modification of proteins by transamidation and deamidation. The enzyme belongs to the family of transglutaminase enzymes and is also known as transglutaminase 2. It is a cytoplasmic enzyme and is found in many cells. The function of the enzyme is not known, but it is involved in stabilizing molecules in physiological and pathological processes (Greenberg et al. 1991). It has a role in the regulation of the cytoskeleton, cell adhesion and cell death, while a mouse model has shown that the enzyme is involved in impaired wound healing, autoimmunity and diabetes (Fesus and Piacentini 2002). In addition to its important role in the pathogenesis of coeliac disease, tTG is also involved in fibrosis, Alzheimer's disease and Huntington's disease (Fesus and Piacentini 2002, Jabri et al. 2005). People with coeliac disease develop increased levels of autoantibodies against tTG. These antibodies are important in diagnosis (Sulkanen et al. 1998, Sblattero et al. 2000, Reif and Lerner 2004), since they are found from almost everyone with untreated coeliac disease (Dieterich et al. 1997, 1998).

Binding to the human gliadin antibodies found from the sera of patients with coeliac disease is enhanced after the deamidation of gliadin peptides, in which tripeptide PEQ has been found to be important for antibody binding (Osman et al. 2000). Since similar effects on T-cell stimulation can be obtained by the acid deamidation of prolamins and by enzymatic deamidation with transglutaminase, it has been speculated that harmful deamidation of gluten peptides could occur in the acid environment in the stomach (Sjöström et al. 1998). However, in the studies of Wieser (2005a) and Kahlenberg et al. (2005), deamidation was not observed to occur in the acidic conditions of the stomach or in the conditions for peptic digestion. However, the results of Kahlenberg et al. (2005) indicated that food processing under acidic conditions may reduce the detectability of gluten proteins with antibody assays used for detecting the gluten content.

2.2.3.4 Transamidation

Transamidation results in crosslinks between lysine (or another amine donor) and the glutamine residues of proteins by covalent bonding (Figure 4). Crosslinking is catalyzed by tTG at higher pH values and with high lysine concentrations. The covalent isopeptide bond between the protein and lysine is stable and resistant to proteolysis, and therefore improves the stability of proteins.

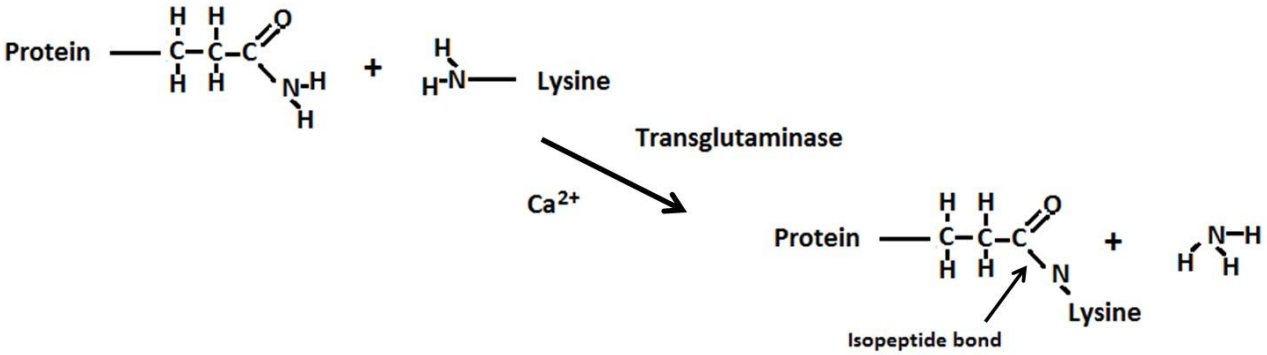

Figure 4. Transamidation between glutamine and primary amine donors.

Microbial transglutaminase (mTG) does not have such a catalysing effect on deamidation as tTG (Gianfrani et al. 2007). In cereal food applications, mTG has been used for crosslinking to improve the baking qualities of weak wheat flours. The effects of mTG on the structure, cross-linking and rheological properties of gluten proteins have been investigated by Bauer et al. (2003a and 2003b). The treatment of wheat flour with mTG and lysine methyl ester was an effective inhibitor of intestinal gliadin-induced IFN-γ production, which suggests the use of amine donors in blocking T-cell acitivity in the treatment of coeliac disease (Gianfrani et al. 2007). The addition of mTG to wheat flours was not observed to affect quantification using the sandwich R5 antibody, and only a minimal decrease in results was observed with high amounts of the enzyme (Wieser et al. 2006).

2.3 Analysis of prolamin proteins

Based on the standard for gluten-free foods, the method to determine gluten should be based on an immunological method or other method providing at least equal sensitivity and specificity (Codex Standard 118-1979). The antibody should recognize the harmful peptides and should not react with other food proteins. The detection limit of the method should be at least 10 mg of gluten / kg of food. Although an immunological method is recommended for quantitative gluten analysis, the use of other methods is not excluded. Many analytical methods have been used to either qualify or quantify prolamins. Prolamins have multiple roles in food manufacturing and are therefore often analysed from cereal material for other purposes than simply for their presence in gluten-free products. For example, wheat prolamins play an important role in dough quality, and barley prolamins are involved in beer haze formation. Prolamins have also been demonstrated to be good for cultivar identification, since each cultivar has a unique prolamin composition (Shewry et al. 1978, Jussila et al. 1992, Kubiczek et al. 1993, Dvořáček et al. 2003).

2.3.1 Chemical analysis

Many methods for detecting prolamin proteins have been developed over the years. The complexity of prolamin proteins, however, creates an enormous challenge in accurately quantifying the total prolamin content. Some of the methods, including the polymerase chain reaction (PCR), HPLC and MS, are used simultaneously with immunological methods. One of the most recent methods in gluten analysis is a potentiometric electronic tongue, which was able to detect 1–2 mg/kg of gliadin from different food matrices (Peres et al. 2011).

Gel electrophoresis has a long history in protein analysis. Prolamin subgroups were originally identified based on their mobility in acid electrophoresis, and SDS-PAGE is used widely to qualify prolamin proteins. Currently, the subgroups are also separated by HPLC, which additionally enables their quantification (Wieser et al. 1994). The qualification of proteins by SDS-PAGE is further improved by adding another dimension that separates the proteins based on their isoelectric points, creating one form of a 2D-PAGE system. This is very useful, since most of the protein bands of prolamins separated by SDS-PAGE contain multiple proteins, and an additional dimension enables their separation and identification. A relatively new method based on the same separation technique is known as lab-on-chip electrophoresis. In this method, the separation gel is in a small capillary in a chip and the proteins are detected with fluorescence detection as they pass the detector. The data are simultaneously collected with a computer program. This method is much faster to perform than traditional gel electrophoresis, since it takes only 30 min for 10 samples to be analysed. However, the separation of proteins is not as good, and not as many choices of gel material are available as with PAGE. Lab-on-chip electrophoresis has been used to identify wheat varieties based on the differences in their HMW and LMW glutenin subunits (Uthayakumaran et al. 2005, Bradová and Matejova 2008), and it has proven to be useful in screening breeding lines for dough quality (Uthayakumaran et al. 2006).

Another technique used in prolamin analyses is PCR, which is based on the detection of deoxyribonucleic acid (DNA) from the studied species. However, the method does not recognize proteins, as the other detection methods do, which causes a major problem. DNA can exist in a sample without any proteins being present. For example, the addition of wheat starch can add wheat DNA to the product without a significant increase in protein. This situation can be resolved by combining the PCR method with immunological analysis (Allmann et al. 1993, Dahinden et al. 2001). DNA is also easily destroyed during food processing and might therefore give a negative result, despite harmful proteins being present. Mujico and Méndez (2006) observed that results from PCR and prolamin contents obtained with the R5 antibody did not correlate in heat-processed samples. However, the PCR method appeared more sensitive than immunological ELISA methods when wheat contamination in oats was determined (Köppel et al. 1998). Despite its weaknesses in gluten analysis, PCR has been extensively used in the analysis of contamination of gluten-free products (Sandberg et al. 2003, Olexová et al. 2006) or to identify species and varieties (Terzi et al. 2005).

2.3.2 Immunological analysis

Enzyme-linked immunosorbent assays (ELISAs) have become the recommended methods in gluten analysis. Immunological methods are based on the antibodies raised against the different prolamin fractions or specific sequences found in prolamins. The requirement for the assays is that they should measure the harmful proteins and peptides, regardless of the type of food or manufacturing process (Denery-Papini et al. 1999). The methods for gluten analysis are summarized in Table 3.

2.3.2.1 Prolamin-specific antibodies

Various mono- and polyclonal antibodies have been developed against prolamin proteins. Almost without exception, they have been raised against wheat gliadin or its subfractions. In most cases, the antibodies raised against one prolamin fraction recognise all the other prolamin fractions (Brett et al. 1999, Rumbo et al. 2000). Because of the structural homology of prolamins, the antibodies find multiple reaction sites from different prolamins. However, reaction intensities differ between the subgroups. Despite the development of multiple potential prolamin-specific antibodies, only two of them, the ω-gliadin antibody and the R5 antibody, have gained widespread approval and have been used in recommended gluten analysis assays.

Antibody against ω-gliadin

The ω-gliadin antibody was produced by Skerritt in the late 1980s. It is a monoclonal antibody that was raised against wheat gliadin and observed to recognize the heat-stable ω-fraction of gliadin. The antibody mainly recognizes ω-type prolamins and HMW subunits (Skerritt and Hill 1990, van Eckert et al. 2008). The antibody has also been referred to as 401.21.

Before development of the assay, Skerritt developed multiple antibodies and tested their reactivity against prolamins from different cereals. Among other tests, Skerritt and Smith (1985) examined how the antibody reacted with prolamins in wheat flour samples mixed with water and heated for different

periods of time. They noticed the disappearance of other gliadins apart from ω-gliadins as the heating time increased. The recognition of ω-gliadins by the antibody was not altered by heating, which made it highly suitable for gluten detection in processed foods. The ω-type prolamins lack cysteine and are very low in lysine, which makes them highly resistant to heat. This antibody was a very important development at the time, since for the first time the prolamin proteins could also be quantified from processed foods.

However, the antibody has weaknesses. For example, the quantitative analysis is based on the relative amount of ω-gliadins, which varies between cereal species and varieties. As a consequence, the results can vary depending on the relative ω-gliadin content. In addition, the reaction of the antibody to barley prolamins is very weak, and only less than 10% of hordeins can be quantified.

Antibody against pentapeptide QQPFP (R5)

R5 antibody was raised against an ethanol extract of rye (Sorell et al. 1998). The R5 antibody mainly recognizes α- and γ-gliadins, whereas a somewhat weaker response was obtained for ω-gliadins and proteins of over 75 kD (van Eckert et al. 2010). More specific analysis has revealed that the antibody mainly recognizes the epitope QQPFP. It is also able to recognize homologous epitopes such as LQPFP, QLPYP, QLPTF, QQSFP, QQTFP, PQPFP, QQPYP and PQPFP, although with weaker reactivity (Osman et al. 2001). Scanning of the complete sequence of ω-secalin demonstrated the reactivity of R5 against the epitopes QQPFPQ, QQPFPL, PQQPFP, SQQPFP, QLPFPQ, QRPFAQ and QQSFPQ (Kahlenberg et al. 2004). QQPFP occurs in the repetitive domains of prolamins and has been found to occur multiple times in ω-type prolamins (Kahlenberg et al. 2004). Therefore, the antibody is a very good candidate for the detection of prolamins. ELISA methods based on the R5 antibody are currently recommended for gluten analysis in the Codex Alimentarius (Codex Stan 118-1979).

The R5 antibody has weaknesses. It also recognizes other food proteins, and not only harmful prolamins, such as soya and lupin proteins (Immer and Haas-Lauterbach 2005a). However, using a cocktail solution (60% ethanol with 2-mercaptoethanol and guanidine hydrochloride) for the soya samples instead of ethanol, false positive reactions with antibodies were no longer observable with the soya samples (Immer and Haas-Lauterbach 2005a). Mena et al. (2007) found in their study that soya proteins were able to create an emulsion in 60% ethanol, and could not be separated from the supernatant during centrifugation. When reduction is performed prior to extraction, which is the case when using the cocktail solution, soya proteins were separated into pellets and the emulsion disappeared. The antibody also reacts unexpectedly strongly with barley prolamins (Malmheden-Yman 2006). This characteristic especially jeopardizes the addition of oat products to the selection of gluten-free products.

Antibody against 19-mer of A-gliadin (PN3)

New approach to gluten analysis is to develop antibodies against harmful fragments of prolamins. PN3 is a monoclonal antibody raised against an epitope of 19 amino acids of A-gliadin (19mer). This epitope has been shown to be harmful *in vivo* (Sturgess et al. 1994). PN3 antibody reacts strongly with α- and γ-gliadins, but only weakly with ω-gliadins (van Eckert et al. 2010). The antibody also reacts

with LMW glutenins, rye secalins and barley hordeins, but not with HMW glutenins, oat avenins or maize zeins. The main recognition epitope is QQQPFP (Ciclitira and Ellis 1999).

Antibodies against 33-mer (G12 and A1)

Two antibodies, G12 and A1, have been raised against 33mer. The G12 antibody was developed by Morón et al. (2008a) and it especially recognizes the hexapeptide QPQLPY. The antibody is highly selective for 33mer and similar peptides found in barley and rye. It also detects similar sequences from oats. Another antibody against 33mer, A1, was developed by the same research group. The A1 antibody recognizes the heptameric sequence QLPYPQP, which is also a part of 33mer. A1 has a higher sensitivity for gluten detection that the G12 antibody; however, G12 has a better affinity for 33mer (Morón et al. 2008a).

2.3.2.2 Enzyme-linked immunosorbent assays

Since the mid-1980s, multiple immunochemical gluten analysis methods have been developed. The earlier methods were reviewed by Howdle and Losowsky (1990). Two ELISA formats, sandwich and competitive, are widely used in gluten analysis (Figure 5). The sandwich method is based on two antibodies. The first is called a coating antibody and the second a detecting antibody. The coating antibody is bound to the bottoms of the microplate wells and the detecting antibody is used to detect the antigens (proteins of interest) attached to the coating antibody. An enzyme is linked to the detecting antibody. Commonly used enzymes include horse radish peroxidase (HRP) and alkaline phosphatase (AP). The purpose of the enzyme is to induce a colour reaction involving chromogen, which can be measured by spectrophotometric methods. The coating and detecting antibody can be the same antibody or they can be different. For the method to work, the sample protein must be large enough for two antibodies to attach to it at the same time. Therefore, the sandwich technique is not suitable for hydrolysed proteins.

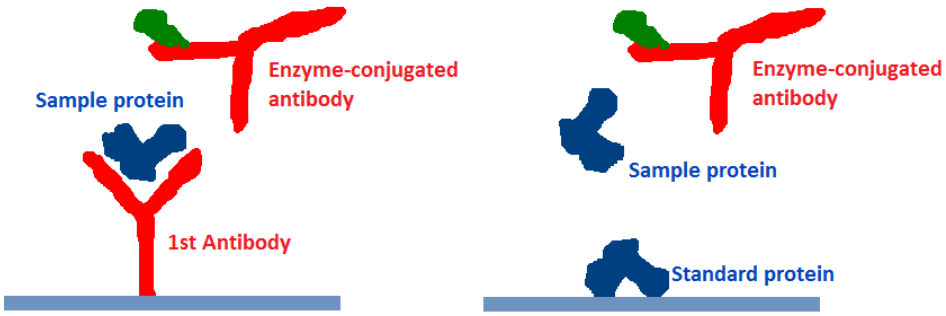

Figure 5. The sandwich (left) and competitive ELISA (right) systems. Antibodies are in red, antigens in blue and enzymes in green.

The competitive method, on the other hand, is based on the competition between sample proteins and standard proteins. Only one antibody is used in this assay, which makes it suitable for also detecting small, hydrolysed proteins and peptides. However, the specificity of the method may not be as good as that obtained with the sandwich format, since nonspecific binding is more likely when only one binding

site is needed for detection. The enzyme in competitive systems can be conjugated with the antibody or with the standard peptide/protein. Either way, in the competitive assay format, the intensity of the colour reaction is inversely proportional to the amount of antigen in the sample.

Many ELISA assays, both sandwich and competitive, are commercially available (Table 3). The ELISA based on the R5 antibody is currently recommended by the Codex standard, and is therefore practically the only method in use. The R5 ELISA method replaced ELISA based on the ω-gliadin antibody, which was recommended earlier. These two methods and also some emerging methods in gluten analysis are described here in more detail.

ω-Gliadin ELISA– the Skerritt method

This method was developed in the 1990s by Skerritt and co-workers. After development and successful collaborative testing, the method was approved by the AOAC (Association for Official Analytical Chemists), and it was used for many years in gluten analysis. The method is based on an antibody that recognizes the heat-stable ω-gliadin fraction. This is an advantage, since that fraction remains unchanged during the processing of food. However, the disadvantage of the method is that the different relative contents of the ω-fraction among cereal species causes considerable variation in the quantitative results. Not only different varieties, but also the availability of nitrogen during grain development alters the content of the ω-fraction (Shewry and Tatham 1999). Therefore, fertilization may have strong effect on the protein composition of the grain. These changes in protein composition affect the immunological analysis results, especially with ω-specific antibody. The other disadvantage is that method has only a weak response to barley hordeins.

Before developing an ELISA assay, Skerritt developed a 'disc-in-tube' method, since the binding of gliadin to plastic plates was noted to be low and strongly affected by solvents (Skerritt 1985). Nitrocellulose was better in binding gliadin proteins. The discs of nitrocellulose were soaked with the gliadin-containing sample and blocked with bovine serum albumine. The gliadins attached to the discs were detected by monoclonal anti-gliadin antibody conjugated to horseradish peroxidase. This method successfully quantified prolamins from different foods including starch, soya, milk and egg powder, raw meats, different soups and baked goods.

In 1990, Skerritt and Hill published a monoclonal antibody sandwich enzyme immunoassay. The method was based on the monoclonal antibody immunoglobulin G_1, which recognizes the heat-stable ω-gliadins and related prolamins of rye and barley. The results of a collaborative study with ELISA were published in 1991 (Skerritt and Hill 1991). The repeatability of the method was 16–22% and the reproducibility 24–33%. The assay was semiquantitative for processed meat products, but quantitative for cereal and soup products. No false positive results were obtained. Samples included meat-gluten blends, wheat flours, wheat flour cookies, gluten-free baking mixes, maize starch and soup thickened with wheat flour (Skerritt and Hill 1991). Prolamins from various types of foods were extracted with 40% ethanol.

This method is no longer in general use, since it was replaced by R5 ELISA. However, it can still be obtained from Biocontrol Systems, USA, under the name Transia Plate Gluten, from ELISA Systems,

Australia, under the name Gliadin assay, from ELISA Technologies, USA, under the name GlutenELISA, from Neogen, USA, under the name Tepnel Biokits Gluten Assay Kit and from Diagnostics Innovations Ltd., UK, under the name HAVen Gluten Kit.

R5 ELISA – the Méndez method

The R5 ELISA method was developed by Méndez and co-workers (Valdés et al. 2003). It quickly replaced ω-gliadin ELISA, since it was able to detect barley hordeins and was not as affected by the cultivars as the previous method. The international ring trial of the method was organized by the Prolamin Working Group (PWG) during 2002 (Méndez et al. 2005) and involved 20 laboratories and two test systems (Ingenasa and R-Biopharm). The samples studied in the trial contained heated and spiked maize bread samples, unheated spiked rice bread samples and contaminated gluten-free samples. The repeatability and reproducibility were found to be 20% and 32% for the Ingenasa kit and 18% and 30% for the R-Biopharm kit. These values were considered acceptable for immunological analysis.

The first step in developing the R5 ELISA was an ELISA method that was based on an antibody cocktail of three antibodies: Rye3, Rye5 and 13B4 (Sorell et al. 1998). Later on, the group developed the method further and limited it to only one single antibody, R5 (Valdés et al. 2003). The cocktail extraction procedure is recommended to be used with sandwich R5 ELISA. This means that prolamins are reduced before extraction by 2-mercaptoethanol. The extraction is further enhanced by guanidine hydrochloride (a disaggregating agent). The cocktail solution was introduced in 2000 at the 15th Meeting of the Prolamin Working Group and published in 2005 (García et al. 2005). Later, the group of Méndez compared extraction with the cocktail solution to that with aqueous ethanol (García et al. 2003). The recoveries obtained with the cocktail solution were from 70 to 98%, while the recoveries of gluten from the same samples with 60% ethanol were about 30 to 50%.

Competitive R5 ELISA was introduced at the 18th PWG meeting in 2003 (Ferre et al. 2004). It detected prolamins from beer and syrup samples more efficiently than the sandwich method. The standards used in the first competitive assay were PWG gliadin digested with pepsin, trypsin, pepsin+trypsin, chymotrypsin or with a pool of these enzymes. The results for the beer samples were from 1.9 to 17 times higher with competitive R5 than with sandwich R5. However, higher results were obtained for breakfast cereals with sandwich method than the competitive method (Hernando et al. 2005). This was suggested to occur because of heat treatment. Extraction with the cocktail solution is not compatible with competitive ELISA (Immer and Haas-Lauterbach 2005b), and heat-treated proteins may therefore remain unextracted when ethanol extraction without reduction is used.

The method is available from BioControl Systems, USA (Transia Plate Prolamins), Ingenasa, Madrid, Spain (Ingezim Gluten), Neogen, USA (Veratox for Gliadin R5) and R-Biopharm, Darmstadt, Germany (Ridascreen Gliadin and Ridascreen Gliadin Competitive). It should be noted, however, that the gliadin standard in the ELISA test of BioControl is from the wheat variety Timgalen, the intensity of which is about a half of that of PWG gliadin (Hasselberg et al. 2004). PWG gliadin is used in the ELISA by Ingenasa, and the standard of R-Biopharm is calibrated to PWG gliadin. The standards are described in more detail below.

Others

Other immunological ELISA systems based on different antibodies have also been developed. McKillop et al. (1985) and Troncone et al. (1986) developed ELISAs based on polyclonal rabbit antisera against gliadin with very low detection limits. The assay of McKillop was not tested with heated samples and that of Troncone reacted with proteins from rice and maize that are not harmful for coeliac patients. Friis (1988) also developed an ELISA using a polyclonal rabbit antibody; however, this antibody additionally recognized proteins from buckwheat. Furthermore, the author was concerned about the accuracy when detecting prolamins from heated samples. Freedman et al. (1987) developed an ELISA based on a monoclonal mouse IgM antibody. This method was promising, since it detected all prolamin fractions of wheat and also barley and rye. Unfortunately, the antibody also recognized oat proteins. The same problem occurred with the ELISAs developed by Chirdo et al. (1995, 1998). Ellis et al. (1998) developed an ELISA based on the PN3 antibody, in which a polyclonal gliadin antibody was used as the capture antibody. The sensitivity of the assay was 4 ng/ml for gliadins, 500 ng/ml for secalins and 1000 ng/ml for avenins and hordeins. Subsequently, a competitive ELISA was developed with the same antibody (Bermudo Redondo et al. 2005). The competitive assay based on the PN3 detected equally harmful peptides from wheat, barley, rye and oats, and it was claimed to be specific for the cereal proteins that cause coeliac disease, and to be capable of detecting hydrolysates. The detection limit of the assay was reported to be 0.128 ppm with good reproducibility. Neither of these above-mentioned methods are commercially available. The ones that are available are described in more detail below.

ImmunoTech a Beckman Coulter Company (Pardubice, Czech Republic) developed a gliadin ELISA kit based on two monoclonal antibodies against two different epitopes of gliadin and one polyclonal antibody (Gabrovská et al. 2004, 2006, Sánchez et al. 2007). The detection limit of the assay is 3 ng/ml, and range of the calibration scale is to 320 ng/ml. It recognizes wheat, rye and spelt with the same efficiency, but barley with an efficiency of only about 20–30%. It does not cross-react with oat, maize, rice or buckwheat proteins. Extraction is carried out with 40% ethanol. The method is based on the PWG gliadin standard. However, the method gives considerably higher results than obtained with the Ridascreen assay of r-Biopharm (Gabrovská et al. 2003).

An ELISA method for detecting α-gliadins was developed by Koning and co-workers. Initially, the research group developed several antibodies against T-cell stimulatory epitopes. The antibodies were raised against synthetic peptides that represented T-cell stimulatory epitopes in α-gliadin, γ-gliadin, LMW glutenin and HMW glutenin (Spaenij-Dekking et al. 2006, Mitea et al. 2008b). The antibodies were very specific to the epitopes they were raised against and were able to detect homologous epitopes in other cereals (barley, oats, wheat, rye and triticale). However, as the method was further developed, only the α-gliadin antibody was selected for inclusion in the final ELISA. Because of this, the results of the method are expressed as α-gliadin contents, which are difficult to relate to total gluten levels. The method is sold by Europroxima (the Netherlands) under the name GlutenTek ELISA.

Morón et al. (2008b) developed ELISA methods based on antibodies against 33mer. They developed two monoclonal antibodies, G12 and A1, which both are highly selective for 33mer. A competitive method and two different sandwich methods based on these antibodies are now available. The

competitive ELISA is based on the G12 and sandwich methods either on G12-G12 or G12-A1. The detection limit for wheat, barley and rye prolamins was under 1 μg/ml with the sandwich method and 0.5 μg/ml with the competitive method (Morón et al. 2008b). The methods are sold by Biomedal Diagnostics of Romer Labs (UK) under the name GlutenTox ELISA.

Table 3. Enzyme-linked immunosorbent assays for gluten detection.

Antibody raised against	Specific name of antibody	Main recognition epitope	Type of antibody[1]	Type of test	Sensitivity[2] (LOD)	Kit manufacturers	Notes	Reference
α-Gliadin Gliadin	-	-	pAb	Sandwich Competitive	1-20 ng/ml[3]	-	Not tested with other cereals	Windemann et al. 1982
Gliadin	-	-	pAb	Sandwich	22 ng/ml	-	Low reactivity with barley and oats	McKillop et al. 1985
Gliadin	-	-	pAb	Sandwich	5 ng/ml	-	Also detects oats, rice and maize	Troncone et al. 1986
Gliadin	-	-	mAb	Sandwich	15 ng/ml	-	Low reactivity with barley, rye and oats	Freedman et al. 1987
Gliadin	-	-	pAb	Competitive	13 ng/ml	-	Also detects buckwheat	Friis et al. 1988
ω-Gliadin	401/21	-	mAb	Sandwich	100-150 ng/ml	BioControl Systems Inc., ELISA Technologies, ELISA Systems, Diagnostics Innovations Inc., GenProbe, Neogen	Specific to ω-gliadins and HMW-glutenins	Skerritt and Hill 1990
Gliadin	-	-	pAb	Competitive	1 ng/ml	-	Also suitable for processed foods	Chirdo et al. 1995
Gliadin	13B4 12A1	-	mAb	Sandwich (12A1) Competitive (13B4) Competitive (12A1)	1 ng/ml 20 ng/ml 5 ng/ml	-	Specific to wheat, barley and rye; no reactivity with oats, rice, soy or maize	Chirdo et al. 1998
Secalin	R5	QQPFP	mAb	Sandwich Competitive	1.5 ng/ml 1.2 ng/ml	BioControl Systems inc., Ingenasa, Neogen, R-Biopharm	Claimed to detect wheat, barley and rye equally	Valdés et al. 2003, Ferre et al. 2004
19mer	PN3	QQQPFP	mAb	Sandwich Competitive	4 ng/ml 128 ng/ml	-	Also detects oats; lower sensitivity to heated foods	Ellis et al. 1998, Bermudo Redondo et al. 2005

α-Gliadin γ-Gliadin	Glia-α2/9 Glia-γ1	LQPFPQPQ QQRPFI	mAb mAb	Competitive	12 ng/ml	EuroProxima	Detects T-cell stimulatory peptides of wheat, barley and rye	Spaenij-Dekking et al. 2004
Gliadin	8D4 7C6	QQSFPQQ QQTFPQP QPFRPQ	2 x mAb 1 x pAb	Sandwich	5 ng/ml	Immunotech A Beckman Coulter Company	Antibodies recognize the same sequences as anti-gliadin antibodies of coeliac sera	Gabrovská et al. 2006, Sánchez et al. 2007
33mer	G12 A1	QPQLPY QLPYPQP	mAb	Sandwich Competitive	0.6 ng/ml 0.4 ng/ml	Biomedal Diagnostics	Highly specific for 33mer and similar peptides in barley and rye	Morón et al. 2008b
Gliadin	-	-	pAb	Sandwich	0.3 μg/ml	Morinaga Institute of Biological Science, Inc., Crystal Chem Inc.	Wheat Protein ELISA Kit (Gliadin), gives results as the quantity of wheat protein	-
Not indicated	-	-	-	Sandwich	5 μg/ml	Neogen	Veratox for gliadin for the quantification of prolamins	-
Not indicated	-	-	-	Sandwich	0.6 μg/ml	Romer Labs	AgraQuant Gluten for the quantification of gliadin in food	-

[1]pAb denotes polyclonal antibody, mAb monoclonal antibody

[2]Sensitivity for gliadin

[3]Limit of quantification

Standards

The standard is a very important part of quantitative analysis and it should represent the sample as closely as possible. Although (or because) this is very well known, many different standards are used in gluten analysis. Among the many different gliadin preparations, the most common standards are gliadin separated from the Australian wheat variety Timgalen, RM8418, Sigma gliadin and PWG gliadin.

The gliadin fraction separated from the Australian wheat variety Timgalen has been used in ω-gliadin ELISAs produced by R-Biopharm, Neogen and BioControl Systems. It contains wheat prolamins from only one wheat variety, as well as reference material 8418, which is separated from Canadian wheat. When only one variety is represented in the standard, it does not take into account the differences between the varieties, which may cause inaccuracies in the results. Sigma gliadin and PWG gliadin, on the other hand, are prepared from several wheat varieties. Sigma gliadin is isolated from 12 different German wheat varieties and is therefore more suitable for broader use. It has been used in the ELISA of Riedel-de-Häen (van Eckert and Jordan 2003). PWG gliadin, Sigma gliadin, RM8418 and Timgalen gliadin have been reported to express very similar patterns in 2D electrophoresis (van Eckert and Jordan 2003). PWG gliadin had the highest gliadin content and RM8418 contained more glutenins, albumins and globulins, but the differences were not so clear that the behaviour of the standards in different analysis assays could be explained.

PWG gliadin is currently the recommended reference material, and is therefore described here in detail. Other names used in the literature for this material are European wheat gliadin and IRMM-480. In this thesis, the name PWG gliadin is preferred. In addition to the widely used PWG gliadin, some emerging peptide standards are described.

PWG gliadin

PWG gliadin was produced in international collaboration by the PWG in 2000. The standard is a representative of European wheat varieties, since it was isolated from the 28 most frequently grown wheat varieties from three main wheat-producing European countries: France, Germany and Britain. The gliadin fraction was isolated from the mixture of the cultivars according to Wieser et al. (1994). A flour mixture was first defatted with n-hexane and vacuum-dried. The albumins and globulins were removed by 0.4M NaCl and the gliadins were extracted with 60% ethanol. The gliadin extracts were concentrated and dialysed by ultrafiltration against 0.01M acetic acid and freeze-dried. The chemical characteristics are summarized in Table 4. The final protein content of the standard was 86.4% after albumins and globulins were subtracted. The immunochemical reactions of the standard were tested with various monoclonal and polyclonal prolamin-specific antibodies.

Table 4. Characteristics of PWG gliadin (van Eckert 2002, van Eckert et al. 2006)

Character	Value
Solubility	0.5mg gliadin/200µl 60% ethanol
Protein content	87.7% (Kjeldahl), 89.4% (Dumas)
Gliadin content	67.7%
Gliadin composition	40.4% α, 48.7% γ, 6.2% ω_{12} and 4.7% ω_5
Albumin/globulin	3.4%
Glutenin contents	28.9%

The certification process by the EC Institute for Reference Materials and Measurements (The Methodology Institute of the European Commission, IRMM) was started soon after the material was separated from wheat varieties. Considerable effort was put into characterizing the standard in detail to obtain the certified reference material status for it. However, the IRMM did not accept this standard as a certified reference material due to its high glutenin content, which prevents it from being a pure gliadin standard.

Peptide standards

Since a pure gliadin standard turned out to be impossible to separate from cereal material, synthetic peptide standards are of interest. The composition of synthetic material is always the same and can be known in detail, which is not the case with natural prolamin fractions. Chambers et al. (2001) successfully used a synthetic multiantigenic peptide mixture as the standard in gluten analysis. The competitive R5 ELISA by R-Biopharm is also calibrated with the synthetic peptide QQPFP. However, the results obtained using peptide standards are peptide concentrations instead of the desired protein concentrations. Since the limits in gluten-free products are based on the total gluten content and not on the peptide content, it is very difficult to compare the peptide concentrations with the total gluten content of the sample.

Another type of peptide standard was developed by Gessendorfer et al. (2009), as they created a reference material by hydrolysing a mixture of wheat, barley and rye prolamins. The results obtained with this type of standard can more easily be related to the total gluten contents. However, the hydrolysis of proteins is difficult to optimize so that no differences between batches occur.

2.4 Gluten-free diet and products

2.4.1 Legislation

The gluten standard and other regulations concerning gluten-free products can be found in the Codex Alimentarius (www.codexalimentarius.net). The Codex Alimentarius is a collection of international food standards, advisory codes of practice, guidelines and other recommended measures. The Food and Agriculture Organization and the World Health Organization established the Codex Alimentarius Commission in 1963. The development of the standards is performed in various committees. Three of the committees working under the Commission have specific importance with regard to gluten-free foods. These are: 1) the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU), 2) the Codex Committee on Food Labelling and 3) the Codex Committee on Methods of Analysis and Sampling. The official reports of the committee meetings are called alinorms.

The standard for gluten-free products dates from 1979, when the Codex Alimentarius Commission adopted the draft standard as a formal Codex text (Codex Stan 118-1979). At that time, no specific methods were available for gluten detection, and the maximum gluten level allowed in gluten-free products was set according to the nitrogen content of the sample. The total nitrogen content of a gluten-containing product that has been rendered gluten-free was not allowed to exceed 0.05g nitrogen per 100 g of food on a dry matter basis. This was, however, misleading, since many food products contain high amounts of endogenous proteins, which increase the total nitrogen level. Most of the nitrogen is present in proteins, but gluten proteins make up only a proportion of the total protein content of the sample. With the methods based on the nitrogen content, gluten proteins cannot be separated from other proteins. Food products that were made of non-gluten-containing cereals were then advised to be labelled naturally free from gluten and therefore suitable for a gluten-free diet (Codex Alimentarius 1979).

During the last 30 years, knowledge and analysis methods have considerably developed. In the 1990s, new methods based on the immunological detection of gluten proteins were developed, including methods based on antibodies against ω-gliadin and R5. Because the new methods were able to separate gluten proteins from other proteins, revision of the standard became important. New levels for the gluten content and new regulations concerning the addition of gluten were subsequently suggested.

The first draft of the revised standard is from 1996 (Codex Alimentarius 1997). At that time, the maximum gluten levels were discussed. Three alternative approaches were considered: zero-tolerance, with the lowest possible level in gluten-free products being the same as the lowest possible level that can be detected, and one or two different levels for the gluten content. In the two-level model, one level was suggested for products that are naturally gluten-free (20 ppm) and the other for products that have been rendered gluten-free (200 ppm). In the one-level model, the maximum gluten amount of 200 ppm was suggested to cover both food groups. However, the one-level model was not accepted by the Committee and development of the standard proceeded with the two levels for gluten-free products. In the meeting of CCNFSDU in 2006, the gluten threshold for products rendered gluten-free was lowered from 200 ppm to 100 ppm. Oats were also moved from the list of prohibited grains to the list of cereals for which use may be determined at the national level.

In 2007, the standard was given a new name, the *Codex Standard for foods for special dietary use for persons intolerant to gluten*. At that time, CCNFSDU continued the discussion concerning the thresholds for gluten, but also about the labels and the role of oats (Codex Alimentarius 2008). In 2008, the new standard was finally released. The key issues described in the standard were that oats were considered gluten-free; however, the gluten content of oats should not exceed 20 ppm. There are two categories for gluten-free products: gluten-free and products with a low gluten content. Products that belong to the gluten-free category are the ones previously labelled as naturally gluten-free, and products with a low gluten content are those rendered gluten-free. The gluten content of gluten-free products should not exceed 20 ppm, whereas products with a very low gluten content should not exceed 100 ppm. The method for the determination of gluten should be "an enzyme-linked immunoassay (ELISA) R5 Mendez Method." The Codex Standard was used as a basis for EU regulation (Commission Regulation (EC) No 41/2009) concerning gluten-free foods, which was adopted at the beginning of 2009 and has to be fully endorsed by 2012. All members of the European Union are therefore obliged to follow this new revised standard concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten.

Other issues relating to the addition of gluten to otherwise gluten-free products have also gained some attention in the Codex Commission. The Codex Alimentarius Commission adopted the amendment to the General Standard for the labelling of prepackaged foods that foods and ingredients known to cause hypersensitivity shall always be declared, including cereals containing gluten; i.e., wheat, rye, barley, oats, spelt or their hybridized strains and products of these (Codex Stan 1-1985). In 2001, it was stated in the draft standard for wheat protein products including wheat gluten that "wheat gluten of wheat protein products should not be used for technical reasons e.g. coating or processing aids for foods which are gluten-free by nature" (Codex Alimentarius 2001). It was declared in the Codex Stan 1-1985 (Section 4.2.2), that if it is not possible to provide information on allergens transferred to the product, the food should not enter the market. Gluten is on the list of allergens. A permanent exception was, however, granted for wheat-based glucose syrups including dextrose, wheat-based maltodextrins and products thereof (Commission Directive 2007/68/EC). The granting of this exception was based on a study by Kaukinen et al. (2008), which demonstrated that the consumption of these products did not cause any harmful effects in people with coeliac disease.

The genetic modification of foods has also raised concerns. There have also been studies aiming to transfer wheat genes to naturally gluten-free cereals to improve their baking quality. However, it is recommended by Codex that if genes of allergenic sources are transferred, their role in the elicitation of coeliac disease should be evaluated. Transfer from commonly allergenic foods should be avoided, unless it has been documented that the gene does not code for an allergen.

2.4.2 Daily amount

The tolerable daily amount of gluten has raised discussion. A few clinical studies have been carried out on different gliadin levels. Catassi et al. (1993) examined the effect of 100–500 mg of gliadin in the daily diet and observed some changes in the small intestinal mucosa of coeliac patients, but without serological or clinical symptoms. With a 50 mg daily dose a minimal decreasing trend in the villous

height / crypt depth ratio was observed, but no serological changes (Catassi et al. 2007). Due to these results, they recommended that the gluten intake be kept under 50 mg/day.

Collin et al. (2004) took a different approach to estimate the safe amount of gluten for coeliacs. They measured the gluten content of naturally gluten-free and wheat starch-based gluten-free products and calculated the daily intake from the results. About one quarter of the products contained more than 20 ppm of gluten, but less than 200 ppm. The flour intake was about 80 g per day. Since the patients who were on this diet had a good mucosal recovery, the safe threshold was suggested to be set at 100 ppm. The data from a study by Espadaler (2006) showed that people with coeliac disease eat between 200 and 300 g of gluten-free foods each day in Spain. With a gluten content of 200 ppm, this would mean about 40 to 60 mg of gluten is ingested each day. However, Gabrovská et al. (2007) reported in their study that the average daily intake among Czech coeliac patients was between 1.5 and 5.5 mg of gliadin, which is significantly less than if it was assumed that all the gluten-free products would contain 200 ppm of gluten. Most of the higher values for gluten intake are caused by random dietary mistakes, such as unexpectedly high contamination in gluten-free flour mixes. Gibert et al. (2006) calculated that a rather large amount of gluten may be consumed daily if the limit is 200 ppm, and they therefore recommended that the limit should be lowered to 20 ppm for products naturally gluten-free and 200 ppm for products rendered gluten-free to ensure recovery. Biagi et al. (2004) observed that even the consumption of a very small amount of gluten, such as in a communion wafer, may prevent the mucosa from recovering. However, their study included no control to confirm the result.

Gluten thresholds have been reviewed by Hischenburger et al. (2006) and Akobeng and Thomas (2008), and based on studies conducted in the field, they concluded that amounts lower than 10 mg of gluten are unlikely to cause significant symptoms in people with coeliac disease. As a comparison, the average amount of gluten ingested daily in a gluten-containing diet is about 13 g (Ciclitira et al. 2005b).

2.4.3 Compliance

Compliance with a gluten-free diet is the main problem in the treatment of coeliac disease, and regular follow-up is often needed. Poor compliance may be due to ignorance or a lack of knowledge, but the varying labelling of gluten-free products also causes misunderstandings. Efforts to overcome the problem with labelling have been made by developing gluten-free labelling systems, legislations and recommendations obligating most countries to follow the same guidelines (e.g. EU legislation, Codex, FDA). Adherence to the diet may also be jeopardized because gluten may be found in unexpected sources such as pharmaceuticals, desserts, flavourings, sauces and soups. Gluten is also used in meat and fish products as an extender or in protein enrichment.

It is probably impossible to offer coeliac patients a wide variety of foods totally free from gluten. Trace amounts of gluten are found in wheat starches and in naturally gluten-free cereals. Trace amounts of gluten may also be found in products that are manufactured in the same facilities as gluten-containing products. Therefore, the label ‘may contain wheat’ can be found on many food products. It is, of course, important to ensure that coeliac patients remain healthy; however, this has a downside. The

variety of foods in a gluten-free diet is dependent on the accepted gluten levels. If zero tolerance is preferred, many food products must be excluded from the diet, including wheat starch-containing products. The experience gained so far indicates that the industry producing gluten-free products is reluctant to use the label 'very low gluten content' and, therefore aims to develop gluten-free products of under 20 mg of gluten/kg. If this is not reached, it remains to be seen whether the manufacturers will choose not to label their products as gluten-free at all. Compliance with the diet is improved when the variety of suitable foods is as wide as possible. Most of the coeliac patients in Finland use wheat starch-based gluten-free foods, and compliance with the diet has been good, ranging between 85–90% (Collin and Kaukinen 2006). This is high compared to the more typical levels of 50–80% (Ciclitira et al. 2005b). One factor increasing compliance might be the free use of oat products in a gluten-free diet. These increase the variety of products substantially. However, some patients are very sensitive to residual gluten or oats. These patients should be treated accordingly, but their sensitivity should not restrict the diet of other people with coeliac disease.

2.4.4 Gluten-free products

Wheat starch

Wheat starch has a controversial role in the gluten-free diet. It has been the basis of the gluten-free diet in Europe for about 30 years because of its good technological qualities, but is still not recommended in the USA. When wheat starch was included in the gluten-free diet, the amount of residual gluten was not known. However, it was found that people with coeliac disease recovered on a gluten-free diet despite the use of wheat starch.

In the 1980s, no test was available to determine the gluten content of the samples, so it was decided that if the nitrogen content of a starch-containing product remained under 50 mg / 100 grams of food (dry weight), the product would be suitable for a gluten-free diet (Codex standard 118-1979 before revision). Since 1992, wheat starch has been considered gluten-free if it contains less than 100 ppm of gliadin (dry weight). When the wheat starch Sanostar (Kröner-Stärke) was studied in the production year 2000 (Kröner 2003), only 50% of the starches contained less than 100 ppm of gliadin. The next year the company was able to optimize production so that all of its starches were below 100 ppm. However, year-to-year variation in gliadin contents was found to be inevitable, since the gliadin content in wheat starches depends on the production process and the wheat quality. The content is higher in a difficult harvest year and with unfavourable wheat quality, and levels above the current limit of 20 ppm may occasionally be found (Kasarda et al. 2008). Nevertheless, a standardized production process is in place for gluten-free wheat starch that ensures its safety for people with coeliac disease.

Wheat starch-based gluten-free products in the treatment of coeliac disease have been reported not to cause symptoms relating to coeliac disease, and wheat starch-based gluten-free flour products can therefore be safely consumed by coeliac patients (Kaukinen et al. 1999, Peräaho et al. 2003). No harm was observed when eating wheat-starch containing gluten-free bread for a one-week follow-up period (Ciclitira et al. 1984a).

Beer

Whether beer made from barley can be included in a gluten-free diet is still unclear. With most gluten-detecting methods, very low levels of coeliac-harmful peptides have been measured in most beers. The competitive method based on the R5 antibody detects the prolamin levels in beers. The current recommendations for beers in a gluten-free diet are based on this method, even though it is based on the peptide standard and no factor to relate the results to the total gluten level has officially been provided. Since barley has been used as a raw material, it is evident that some hordeins will end up in the final beer. However, most of the hordeins are removed by filtration and only a very small proportion of them are left in the beer. The majority of beer peptides are derived from albumins, which are water-soluble proteins and not harmful for coeliac patients (Picariello et al. 2007).

Oat products

The allowance of oats in a gluten-free diet differs between countries. The Codex standard for food for special dietary use for persons intolerant to gluten states that oats may be included in a gluten-free diet if their purity from wheat, rye and barley has been checked. The EU Commission has adopted this, and oats that are free from contamination are consequently allowed for people with coeliac disease in the countries of the EU. Most studies carried out on oats have proven their suitability for coeliac patients (Janatuinen et al. 1995, Picarelli et al. 2001, Kilmartin et al. 2003). However, in some published studies, some of the patients have developed gastrointestinal symptoms or serological changes typical of coeliac disease or even subtotal villous atrophy while on an oat-containing diet (Lundin et al. 2003, Arentz-Hansen et al. 2004, Silano et al. 2007a,b). These cases seem to be exceptions, since no harmful effect caused by oats have been found in several long-term trials (Janatuinen et al. 2000, Holm et al. 2006, Kemppainen et al. 2007). It is possible that there is a subgroup of coeliac patients who are intolerant of oats, since similarities between some avenin sequences and harmful gliadin sequences have been found (Vader et al. 2003). The possibility of harmful and unharmful oat varieties has also been suggested (Mujico et al. 2011, Comino et al. 2011). It is not, however, wise to prohibit oats from the diet of all coeliac patients simply because some coeliacs cannot tolerate them. The suitability of oats and their use in a gluten-free diet has been reviewed in several publications (Garsed and Scott 2007, Dickey 2008, Salovaara et al. 2009).

The main problem with oats is their contamination with wheat and barley. Very high contamination levels have been measured from gluten-free foods including oat products (Storsrud et al. 2003, Hernando et al. 2006 and 2008, Gélinas et al. 2008). However, some of the contamination levels reported in these studies indicate such high levels of barley that the samples must have been flour mixtures.

Oats have been a part of the gluten-free diet in Finland for several years without any cases of clear symptoms of coeliac disease, i.e. flattening of the small intestinal mucosa. Oats offer a good addition to the gluten-free diet. About 86% of coeliac patients in Finland use oats in their diet (Alden 2010). Oats have a mild flavour in contrast to many other gluten-free cereals, which makes them more palatable. The use of oats, including kilned oats, increases the dietary intake of fibre, vitamin B, iron and magnesium in coeliac patients (Kemppainen et al. 2010). In addition, by having oats in a gluten-free diet, the overall gluten intake is reduced (Løvik et al. 2009).

2.4.5 New developments

The only treatment currently recommended for coeliac disease is the exclusion of products containing wheat, barley and rye from the diet. However, new approaches are being tested to develop novel gluten-free products, including genetic removal of the harmful sequences, enzymatic degradation of gluten proteins by oral supplements and blocking of the epitopes or parts of the immune system.

Plant breeding

Some researchers are aiming to develop new variants of cereals that are no longer harmful for people with coeliac disease. If the immunodominant peptide sequences are known, the immune response may be modulated and new plants without these sequences may be developed. The aim is to develop new varieties from the old ones which lack genes encoding the gluten subgroups that are harmful for coeliac patients. Spaenij-Dekking et al. (2005) found that large variation exists in the amount of T-cell-stimulatory peptides present in wheat. Based on their results from database searches, it would be possible to select wheat varieties with low amounts of T-cell-stimulatory sequences. The focus is on developing varieties that do not contain any of the harmful sequences without losing the baking qualities. Before this is possible, the harmful sequences in gliadins and glutenins as well as corresponding sequences in rye and barley have to be exactly known.

If there was not so much resistance against genetic modification, it could be a potential way to reduce the harmfulness of wheat varieties for coeliac patients. To date, genetic modification of wheat has focused on improving herbicide or fungal resistance, but also on improving the dough properties and baking qualities of wheat.

Oral supplements and modifiers

An alternative therapeutic strategy for coeliac disease is inhibition of the abnormal immune response (Lundin and Sollid 2003). The immune response can be inhibited with the use of agents that reduce intestinal permeability (Watts et al. 2005), that block HLA-DQ2 or -DQ8 (Bergseng et al. 2005, Jüse et al. 2010) or that inhibit tTG (Hausch et al. 2003). Another approach is to break down the harmful sequences using enzymes such as POP combined with EP-B2 as an oral supplement or as a pre-treatment for food (Siegel et al. 2006).

Polymeric binders are able to reduce the immunoactivity of gliadins. Complexing gliadins with binders such as poly(hydroxyethylmethacrylate-*co*-styrene sulphonate) was shown to hinder the digestion of gliadin proteins and consequently decrease the amount of immunogenic peptides produced by gastroinstestinal enzymes (Pinier et al. 2009). In addition, a decapeptide (QQPQDAVQPF) from durum wheat prevented the activation of coeliac peripheral blood lymphocytes by gliadin peptides (Silano et al. 2007c). Przemioslo et al. (1995) prevented the harmful effects of gliadin-sensitive T-cell clones by pre-incubating them with anti-interferon γ antibody.

3 AIMS OF THE STUDY

The overall aim was to study the prolamin compositions of wheat, barley and rye and the suitability of immunological analysis methods to determine the prolamin content accurately from various food materials. The specific aims were to:

- improve the quantification of hydrolysed barley hordeins from beer using a barley malt standard (I);
- improve the accuracy in the quantification of barley contamination in oats using a barley standard (II);
- follow degradation of rye prolamins in sourdough systems with prolamin-specific R5 antibody (III);
- optimize the extraction of prolamins from wheat, barley and rye for immunological analysis (IV);
- study the effect of the deamidation of gluten proteins and peptides on immunological quantification (V).

4 MATERIALS AND METHODS

This section summarizes the materials and methods, which are presented in more detail in the original publications (I–V).

4.1 Materials

4.1.1 Cereal materials and products

Raw material

Barley malt used for the extraction of the malt protein standard (**I**) was obtained as a ground meal from Sinebrychoff (Kerava, Finland). Oat, wheat, barley and rye grains (**II, IV**) were obtained from Raisio plc (Raisio, Finland) and MTT Agrifood Research Finland (Jokioinen, Finland). The germinated and native rye grains for sourdoughs (**III**) were obtained from Laihian Mallas (Laihia, Finland). The vital gluten material was provided by Raisio plc (Raisio, Finland) and synthetic peptides were purchased from GenScript Corporation (Piscataway, NJ, USA) (**V**).

Products

The selection of beers was provided by Alko Inc. (Finland). Details of the beer samples are summarized in Table 5. Three bread samples that were used for the optimization of prolamin extraction (**IV**) included a wheat bread that was a yeast-leavened toast bread (Vaasan arki vehnäpaahto, Vaasan Oy, Estonia), a self-made flat barley bread, which was made using barley meal (Sunnuntai, Raisio, Finland), water and salt (no yeast added) and a rye loaf bread made using a sourdough process (Uotilan Aito Pälkäneen Maalaislimppu, Uotilan leipomo Oy, Finland). The details of meal and bread samples are summarized in Table 6.

4.1.2 Preparation of meal samples

Dehulling of oat grains

The oat grains were dehulled using the Nipere impact dehuller (Teuva, Finland). This separates the hulls and grains by impacting the grains against the outer walls by centrifugal force. Lighter hulls are separated by air from the heavier grains. The dehuller was carefully cleaned after each use to avoid any contamination from other cereal grains. On certain occasions, the oat grains were dehulled by hand to ensure the absence of contamination (**II**).

Milling

The grains were milled with a sample mill (Koneteollisuus Oy, KT-30, Finland) (**IV**), a coffee mill (Krups KM75, Celaya, Mexico) (**II**) or with a Retsch ZM-200 ultracentrifugal mill (Retsch, Haan, Germany) (**III**). Different sieves were used after or during the milling to either separate seed coat parts from the ground barley meal (**IV**) or to obtain meal with a certain particle size (**III**).

Table 5. Details of the beer samples.

Beer	Type	Malt type	Dry extract (mass-%)
Koff Velvet III	ale	barley	4.90
Guinness Special Export IVB	stout	barley	5.92
Old Engine Oil IVB	ale	barley and oat	6.54
Leffe Radieuse IVB	ale	barley	6.54
Urho IVA	lager	barley	4.13
Legenda IVA	lager	barley	4.69
Nokian Vaalea IVA	lager	barley	-
Singha IVB	lager	barley	3.62
Corona Extra III	lager	barley, rice and maize	4.29
Sol III	lager	barley	3.26
Zhujiang Beer IVA	lager	barley and rice	3.56
Bi-Aglut IVA	lager	buckwheat and maize	4.19
Weihenstephaner Hefeweissbier IVA	wheat beer	barley and wheat	-
Franziskaner Hefe-Weissbier IVA	wheat beer	barley and wheat	4.01
Franziskaner Weissbier Kristallklar IVA	wheat beer	barley and wheat	3.81
Münchner Kindl Weisse IVA	wheat beer	barley and wheat	3.70

Table 6. The moisture, ash and protein contents of wheat, barley and rye meal and bread samples (**IV**). Meal samples were made of a mixture of ten varieties.

Sample	Moisture %[1]	Ash %[2]	Protein %[3]
Wheat meal	11.6	1.55	14.5
Barley meal	11.4	2.24	13.8
Rye meal	11.3	1.57	11.9
Wheat toast-bread	29.2	n.d.	12.8
Barley flat bread	41.2	n.d.	9.7
Rye sourdough bread	42.9	n.d.	11.2

[1]AACC 44-15A, [2]AACC 08-02, [3]Dumas method (N x 5.7 for wheat and N x 6.25 for barley and rye), n.d. = not determined

Sourdough fermentation (III)

The rye sourdoughs were prepared by mixing 5.0 g of flour with 2.5 ml of tap water and 5 ml of starter culture. The chemical controls were prepared by mixing 5.0 g of flour with 7.5 ml of water containing 0.5% acetic acid and 1.8% lactic acid. The sourdoughs were fermented for 24 h at a temperature suitable for each starter organism. Starter organisms included four lactobacilli strains (*Lactobacillus sakei* LS8, *Lactobacillus sanfranciscensis* DSM20451^T, *Lactobacillus sanfraciscensis* DSM20451, and *Lactobacillus reuteri* TMW1.103). The chemical sourdough controls were incubated for 24 h at 30 °C and 37 °C. Sourdoughs were lyophilized for immunological analysis.

Deamidation of vital gluten (V)

Vital wheat gluten was deamidated using a modified procedure of Ma et al. (1986). Deamidation was started by adding 200 ml of 0.1M HCl to 5 g of the vital gluten. The suspension was heated at 100 °C for 2 h. After the treatment, the suspension was neutralised with sodium hydroxide, dialysed against distilled water and the contents were lyophilized.

4.2 Protein analyses

4.2.1 Extraction

A modified Osborne fractionation was used as a basis of the extractions. However, after noting that some of the prolamins may be extracted with water and salt-soluble albumins and globulins, extraction with aqueous alcohols only was preferred for the separation of prolamins. In the preparation of the hordein standard from barley malt, pre-extraction with water and salt solutions was performed (**I**), but this was omitted in the preparation of the hordein standard from barley meal (**II**). Modified Osborne fractionation was used in study **IV** when prolamin-detecting antibodies were compared.

Before the extraction conditions for wheat, barley and rye prolamins were optimized in study **IV**, varying alcoholic solutions with elevated extraction temperatures were used. Hordeins were separated from barley malt with 55% 2-propanol at 60 °C (**I**), but from barley meal with 60% ethanol at 40 °C (**II**). Rye prolamins for a secalin standard were extracted with 50% 1-propanol containing 1% dithiothreitol (DTT) at 50 ºC. The cocktail solution and 60% aqueous ethanol were used according to the recommendations of the manufacturers for the extraction when the samples were intended for ELISA analysis (**II**, **III**, **V**). Extraction with a SDS buffer (2% (w/v) SDS, 10% (v/v) glycerol, 62.5 mM tris(hydroxymethyl)aminomethane, 1% (w/v) DTT) was performed for the lyophilized samples before electrophoretic analysis (**III**, **IV**). Reducing agents such as 2-mercaptoethanol and DTT were added to alcoholic solutions to improve the extraction, except when the samples were extracted for competitive ELISA analysis (**III**, **V**). Concentrations of 2–5% 2-mercaptoethanol or 1% DTT were considered sufficient.

Because prolamins are already solubilized in beer samples, no further extraction was needed. Instead, the protein fraction was purified using desalting gel columns (Sephadex G-25, Amersham Biosciences) and dried prior to SDS-PAGE analysis or evaporated down to 8% of the original volume prior to ELISA analysis (**I**).

4.2.2 Total protein content

Three methods were used for the quantification of total proteins. Two of them, the Kjeldahl and Dumas methods, were based on the detection of total nitrogen in the sample. The protein content of pearled barley was quantified by the Kjeldahl method, whereas the protein contents of the extracts were quantified using the method of Lowry (Lowry et al. 1951) (**II**). The Kjeldahl method includes three steps: degradation, distillation and titration. During the degradation the nitrogen of the proteins is converted to ammonia by sulphuric acid. The released ammonia is separated by distillation and quantified by titration with hydrochloric acid. The Lowry method, on the other hand, is based on the oxidation of aromatic amino acid residues such as tyrosine and tryptophan by the acids of the assay. A bovine γ-globulin was used as a standard (**II**, **IV**, **V**). For the optimization of the most efficient extraction solution (**IV**), the Dumas method (Vario MAX CN, Germany) was used to determine the protein contents of different extracts using N x 5.7 for wheat and N x 6.25 for barley and rye (AACC Method 46-30). Since the cocktail solution contains guanidine hydrochloride, which makes the measurement of total nitrogen irrelevant, the Lowry method was used when quantifying the protein contents of the samples that were extracted with the cocktail solution.

4.2.3 Prolamin content by electrophoresis and Western blotting

SDS-PAGE and Western blotting with prolamin detecting antibodies were used to study beer prolamins (**I**), degraded rye prolamins (**III**) and different prolamin subgroups (**IV**). The separation with SDS-PAGE was based on a modified Laemmli procedure using 10–20% Tris-HCl gels (BioRad Laboratories, USA) (**I**), isocratic (12%) or gradient (4–12%) Bis-Tris gels (NuPage, Invitrogen, USA) (**III**, **IV**). Prolamin extracts or wheat, barley and rye meals were also analysed by an automated gel electrophoresis system, a lab-on-chip technique (Experion, BioRad Laboratories, USA). Prolamins were extracted with 40% 1-propanol at 50 ºC without reducing and separated using Pro260 chips by following the instructions of the manufacturer.

Dried samples were dissolved in an SDS sample buffer to a concentration of 60 mg/ml (**I**) or 100 mg/ml (**III**). The sample buffer contained 2% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) glycerol, 62.5 mM tris(hydroxymethyl)aminomethane, 1% (w/v) DTT, and bromophenol blue, pH 6.8 (**I**) or pH 8.5 (**III**, **IV**). The electrophoretic separation was carried out using the Bio Rad MiniProtean II electrophoresis system (**I**) or the electrophoresis system of Invitrogen (**III**, **IV**). The running conditions were 20 mA/gel for 90 min in the BioRad system and 150 V for 70 min in the Invitrogen system.

After electrophoretic separation, the proteins were transferred to polyvinylidene fluoride membranes using a current of 30 V for 60 min (**III, IV**) (58 V in Study **I**). The transfer buffer contained 25 mM

tris(hydroxymethyl)aminomethane, 192 mM glycine, and 20% (v/v) methanol. After transfer, the membrane was blocked with bovine serum albumin. It was then incubated with either a polyclonal anti-gliadin antibody (**I**, **IV**), a monoclonal R5 antibody (**III**, **IV**) or a monoclonal ω-gliadin antibody (**IV**). The polyclonal rabbit anti-gliadin antibody was detected using a goat anti-rabbit antibody conjugated with AP. The R5 and ω-gliadin antibodies were already conjugated with HRP enzyme, which enabled a direct colour reaction after antibody incubation. The colour reaction with HRP was obtained using tetramethylbenzidine (Promega, USA), whereas the colour reaction with an alkaline phosphatase conjugate of anti-rabbit antibody was induced by 5-bromo-4-chloro-3-indolyl phosphate (BCIP) / nitroblue tetrazolim (NBT). The colour reaction with AP is based on the dephosphorylation of BCIP, which consequently dimerizes. The dimer reduces NBT to form an insoluble dark blue diformazan precipitate, which becomes visible on the membrane. Tetramethylbenzidine, on the other hand, forms a blue colour when oxidized by HRP in the presence of an oxidizing agent of hydrogen (or urea) peroxide.

4.2.4 Prolamin content measured by ELISA

Five different ELISA assays were used in the studies included in this thesis: 1) a sandwich assay based on the ω-gliadin antibody (**I**, **II**), 2) a sandwich assay based on the R5 antibody (**II, III, V**), 3) a sandwich assay based on a combination of A1-G12 antibodies (**V**), 4) a competitive assay based on the R5 antibody (**III**, **V**), and 5) a competitive method based on the G12 antibody (**V**).

The extraction of the samples is described in section 4.2.1.

Unless otherwise mentioned, two different dilutions were prepared in duplicate for each sample. The results with sandwich ELISA were multiplied by two to obtain the total gluten content, except with the rye samples (**III**), since the majority of rye sourdough proteins are considered to be soluble and, therefore, present in the sample solution.

4.2.5 Immunoprecipitation of prolamins

Immunoprecipitation was used to study more closely the peptides degraded in sourdough systems, since ELISA methods cannot distinguish prolamins from each other or indicate the size of the detected prolamins. The qualitative detection of prolamins by immunoprecipitation is based on their specific separation from the extraction solution by complexing them with an antibody. The antigen-antibody complexes are incubated with silicon particles coated with protein G, which reacts with the complexes by binding them to the surface of particles. Bound antigen-antibody complexes can be removed from the particles and analysed further.

The residual proteins were extracted from wheat, barley and rye samples after acid-incubations with 60% ethanol, for 30 min at room temperature (RT), following the recommended extraction of the competitive R5 ELISA (R-Biopharm, Germany). Immunoprecipitation was carried out by incubating the sample extract with HRP-conjugated R5 antibody for 60 min at RT. After incubation with the antibody, Sepharose material (6511-5, Biovision Research, USA) was added to the solution and

incubated with antigen-antibody complexes for 30 min at RT. Sample proteins were separated from Sepharose by concentrated Laemmli buffer (0.0625M Tris-HCl, pH 8.5, 10% glycerol, 2% SDS, bromophenol blue and 2% 2-mercaptoethanol) and boiling for 2–4 min. The separated proteins were analysed by SDS-PAGE using 4–12% Bis-Tris gel (NP0323, Invitrogen, USA). The gel was stained with Coomassie Brilliant Blue.

5 RESULTS

5.1 Determination of barley prolamins in beers (I)

We developed a new standard by extracting hordeins from barley malt to quantify barley prolamins from lager beers. At the time of present study, only an ELISA method based on the ω-gliadin antibody was available. The antibody had a very low reactivity with barley prolamins, and only less than 10% of hordeins could be quantified with the commercially available methods. Unfortunately, obtaining stable standard curves was relatively difficult, leading to inconsistent results. The ω-gliadin antibody was not able to recognise barley hordeins at the same concentrations as wheat gliadins (Figure 6). However, when approximately 12 times higher concentrations of hordeins were used, a similar standard curve to that with gliadin was obtained. This demonstrates that the method can be used in quantifying hordeins, although with much higher prolamin concentrations.

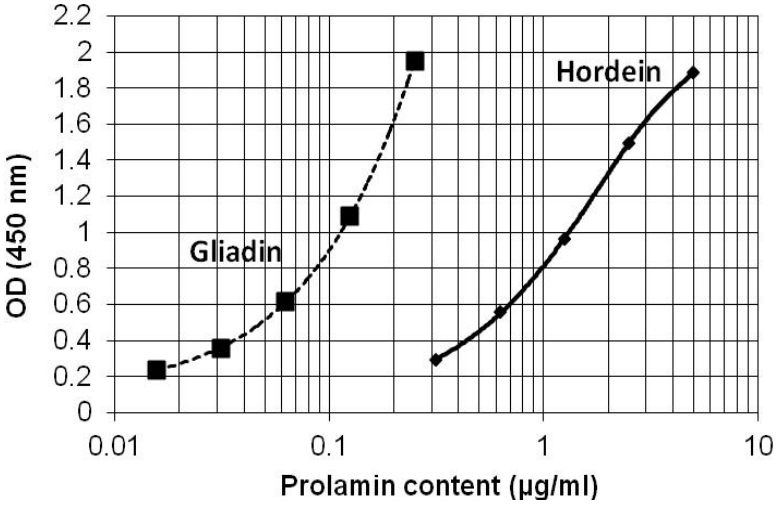

Figure 6. Comparison of hordein and gliadin standard curves in a sandwich ω-gliadin ELISA.

The standard made of barley malt was used in the ω-gliadin ELISA for the quantitative analysis of hordein contents in various lager beers (Figure 7). Dark ale-type beers contained significantly more hordeins than clear lager beers. All of the beers that were studied contained less than 20 ppm prolamins and could therefore be categorised as gluten-free. The prolamin contents of selected wheat beers were also analysed using a gliadin standard in the assay. They contained about 1000 times more prolamins compared to the barley-based lager beers (Figure 8).

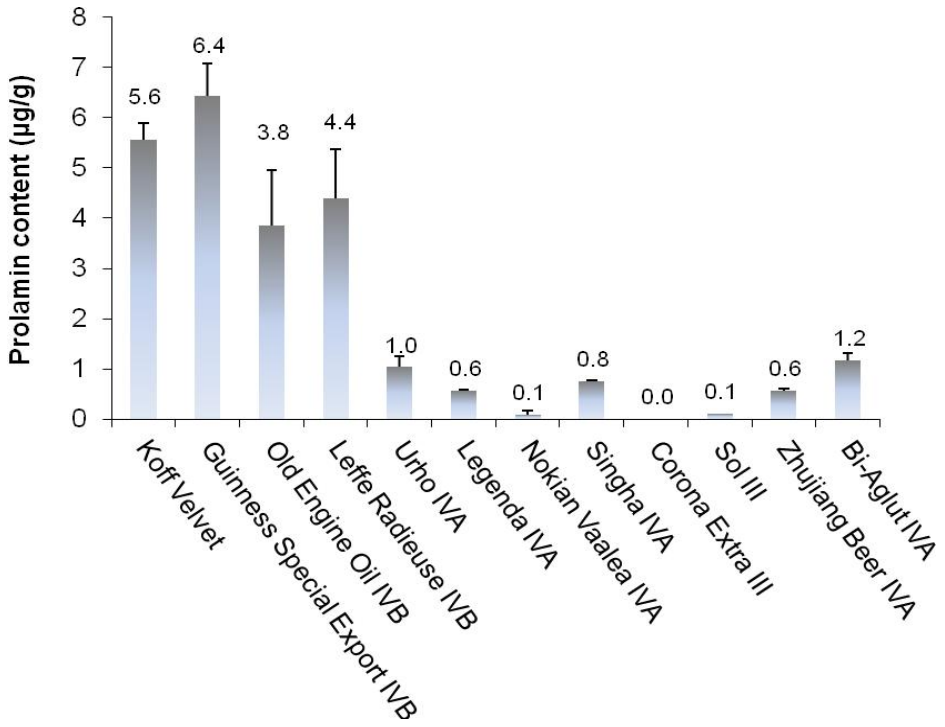

Figure 7. Hordein levels in different lager beers when a standard made of barley malt was used in a sandwich ω-gliadin ELISA. Error bars indicate the standard deviation of the mean of three repeats.

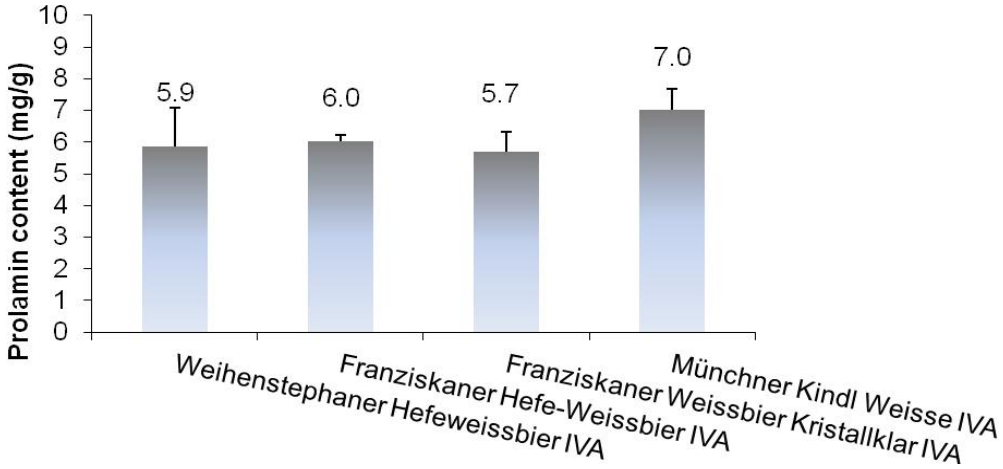

Figure 8. Prolamin contents of the wheat beers by a sandwich ω-gliadin ELISA. Error bars indicate the standard deviation of the mean of three repeats.

The hordein compositions of selected beer samples were studied with SDS-PAGE (**I:** Figure 1). Western blotting analysis with a polyclonal anti-gliadin antibody showed that some barley prolamins remained. Most of them were from 38 000 to 40 000 g/mol in size (**I:** Figure 2,3). For one wheat beer sample that was included in the study, gliadins reacted much more strongly with the antibody than hordeins. Neither distinct separation nor identification of the wheat proteins could be made.

5.2 Determination of barley prolamins in oats (II)

We prepared contaminated oat samples by mixing barley meal with oat meal. Oat meal was milled from oat grains that were carefully checked for contamination. The R5 antibody overreacted substantially in the presence of barley prolamins when the samples were analysed with the sandwich R5 ELISA. The gluten levels obtained with the R5 ELISA were much higher than the levels calculated on the basis of the protein content in the barley meal. By preparing our own reference material by extracting hordeins from pearled barley, we were able to develop a new standard curve (Figure 9). The linear area of the hordein standard curve was obtained at lower concentrations than the linear area of the gliadin standard, which explains the higher results for the samples containing barley. The detected hordein levels using the new curve were very close to the expected values (**II:** Figure 3).

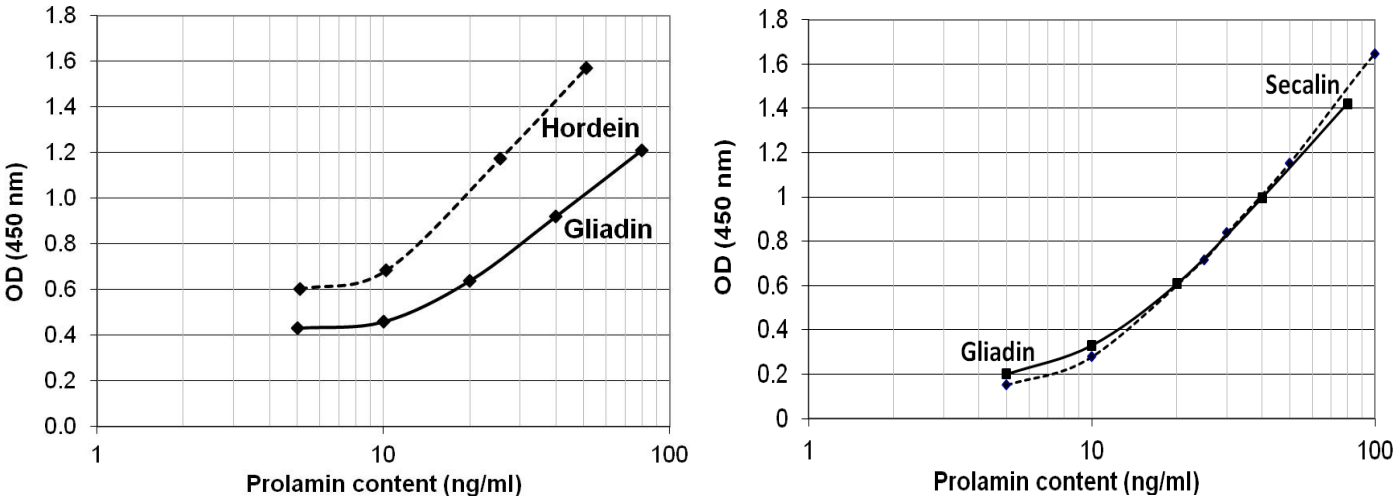

Figure 9. Comparison of the standard curves for hordein and secalin with the gliadin standard curve of the sandwich R5 ELISA.

Due to the different reactivity of hordeins and gliadins with the R5 antibody, we tested how the R5 antibody reacts with secalins and whether gluten contents in samples containing rye as a contaminant would be as high as with barley (Figure 9). Secalins were observed to behave in a very similar manner to the gliadin standard of the assay. Therefore, there is no need for a specific standard for samples containing rye.

We also tested whether the analysis results were higher because the prolamin-detecting antibodies recognize oat prolamins (Figure 10). As expected, the polyclonal anti-gliadin antibody reacted strongly with avenins, which is explained by the polyclonal character of the antibody. The ω-gliadin and the R5 antibodies recognized avenins in the SDS and 60% ethanol extracts. A very low recognition of avenin was seen in the cocktail extract. As the protein contents were not set to the same level before electrophoretic analysis, due to the extraction protocols four times more proteins were present in the 60% ethanol extract than in the cocktail extract. Both of the monoclonal antibodies were able to recognise oat avenins. However, since the recognition was relatively weak, it does not by itself explain the unexpectedly high results obtained for samples containing barley.

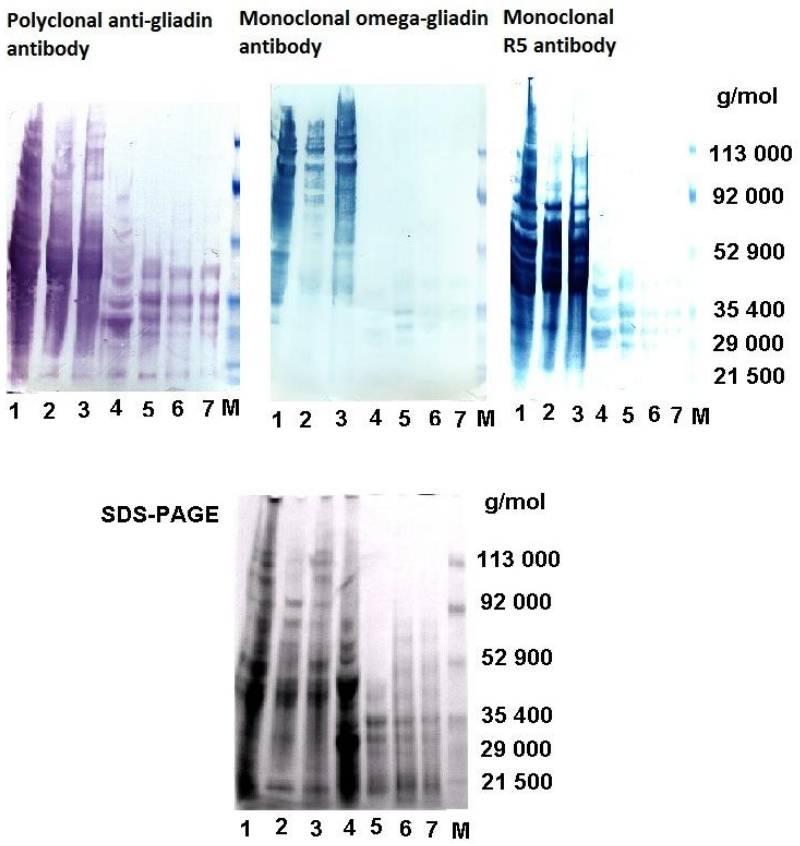

Figure 10. Sodium dodecyl sulphate-polyacryl amide gel electrophoresis (SDS-PAGE) and Western blot analysis of wheat and oat samples. Samples 1–3 were wheat samples extracted with the SDS buffer (1), 60% v/v ethanol (2) and the cocktail solution (3). Samples 4–7 were oat samples extracted with the SDS buffer (4), 60% v/v ethanol (5) and the cocktail solution (6–7). M is a molecular mass marker. A polyclonal rabbit anti-gliadin antibody, a monoclonal ω-gliadin antibody and a monoclonal R5 antibody were used in immunodetection. The SDS-PAGE gel was stained with Coomassie Brilliant Blue R-250.

5.3 Determination of hydrolysed rye prolamins (III)

We studied the extensity of hydrolysis of rye prolamins in different sourdough systems. Native rye meal and germinated rye meal were used to assess the effect of increasing amounts of endogenous enzymes. Four different starter organisms were used in combination with chemical controls. The pH decreased during sourdough fermentation from 6.2 (sourdough prepared from native rye meal) and 5.6 (sourdough prepared from germinated rye meal) to pH 3.7. The chemical sourdoughs had a constant pH ranging from 3.4 to 3.6. The sourdough samples were studied by Western blotting with the R5 antibody, and by the sandwich and competitive R5 tests.

In Western blotting analysis, the R5 antibody detected all the major secalins from unfermented rye meal (**III:** Figure 7). The fermentation of native rye meals modified the secalin bands by distributing

them into several bands, and smaller protein bands appeared. The analysis demonstrated extensive prolamin hydrolysis in the sourdough fermentation of germinated rye. All of the major secalin groups disappeared, leaving only a faint protein band of about 70 000 g/mol (**III:** Figure 7).

The sandwich and competitive R5 ELISA tests were used to quantify the residual prolamins in sourdough samples. The results indicated that the prolamins were hydrolysed very efficiently in sourdough systems with lactic acid bacteria fermentation, but also in chemical controls. In sourdoughs made of germinated rye meal, more than 99.5% of the original rye prolamins were hydrolyzed. The prolamin concentrations ranged from 240 to 480 ppm when determined by sandwich R5 ELISA and from 280 to 430 ppm with competitive R5 ELISA (**III:** Table 1).

The residual proteins after hydrolysis in acidic conditions were further analysed using immunoprecipitation with the R5 antibody. In addition to rye sample, wheat and barley malt samples were included. SDS-PAGE separation was performed for the samples before and after immunoprecipitation (Figure 11). It was observed from the gel that the prolamins had been degraded and proteins with smaller molecular weights had appeared. The R5 antibody was able to recognise residual wheat and rye prolamins of about 20 000–40 000 g/mol in size. Considerably more prolamins were recognised in the barley extract. Their mass range was about 20 000–60 000 g/mol; however, low binding was also visible in the mass range about 70 000–150 000 g/mol.

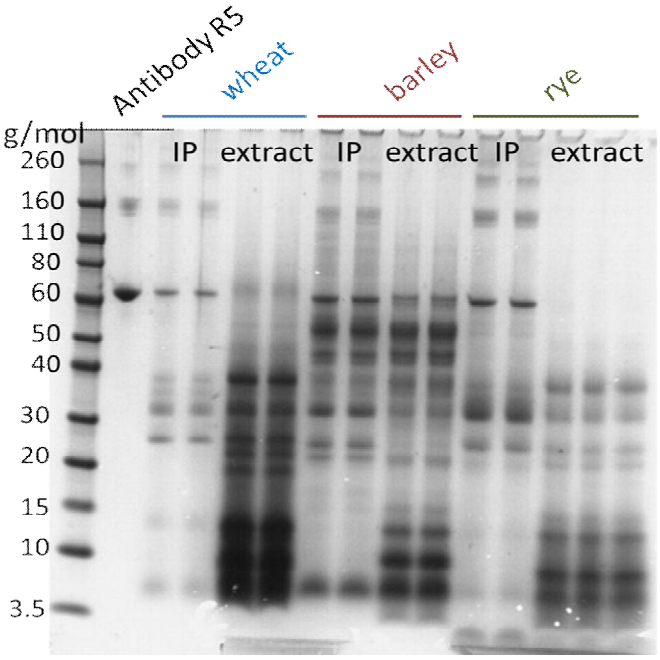

Figure 11. Sodium dodecyl sulphate-polyacryl amide gel electrophoretic (SDS-PAGE) separation before (extract) and after immunoprecipitation (IP) of residual prolamins of wheat, barley and rye degraded in acidic conditions. The protein bands of the antibody R5 are visible in the lane after the molecular mass standard.

5.4 Optimizing the extraction (IV)

The optimal conditions for the extraction of prolamins were investigated by comparing different concentrations of aqueous ethanol, 1-propanol and 2-propanol, out of which 40% 1-propanol was determined to be the most efficient. The effect of temperature during extraction was tested by performing the extraction at RT (21 ºC), 50 ºC or at 60 ºC (Figure 12). Heating the samples above RT significantly increased the extractability; however, no significant difference was observed between 50 ºC and 60 ºC. The use of DTT during extraction increased the amount of extractable prolamins (Figure 12, **IV:** Figure 1). Higher concentrations of DTT increased the yield, but were considered incompatible with immunological analysis. An extraction solution containing 1% of DTT was observed to be sufficient.

The extraction efficiency of 40% 1-propanol with 1% DTT was compared with the efficiency of extraction with the cocktail solution (Figure 13, **IV:** Table 2). No significant differences were detected except when extracting prolamins from the wheat bread sample, in which 40% 1-propanol was superior.

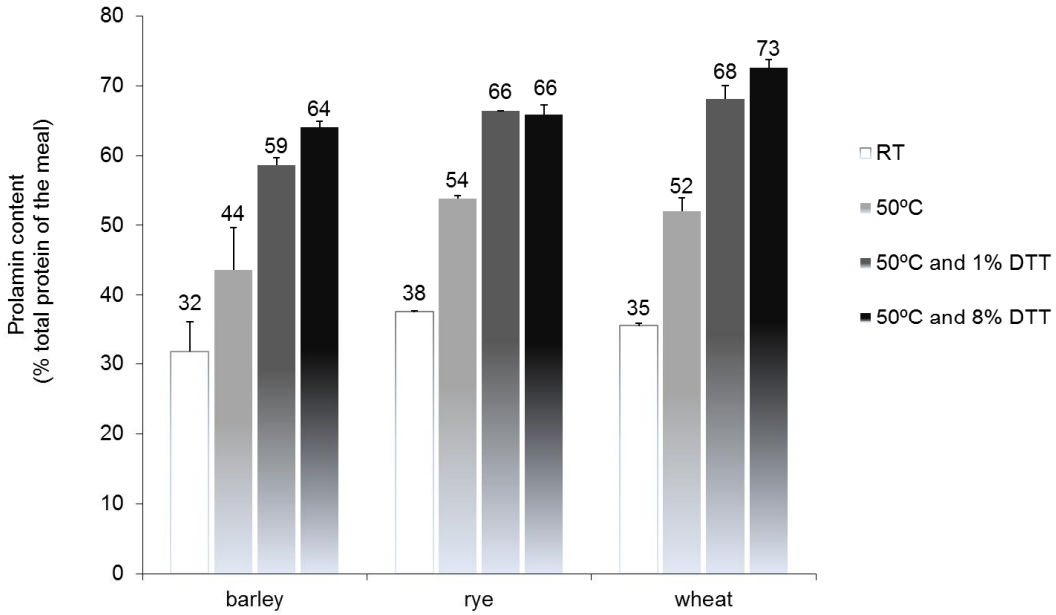

Figure 12. Extracted prolamin content of barley, rye and wheat at RT and 50 ºC with different levels of dithiothreitol. The samples were extracted with a 1:10 ratio of 40% 1-propanol for 20 min. Error bars indicate the standard deviation of the mean of two repeats.

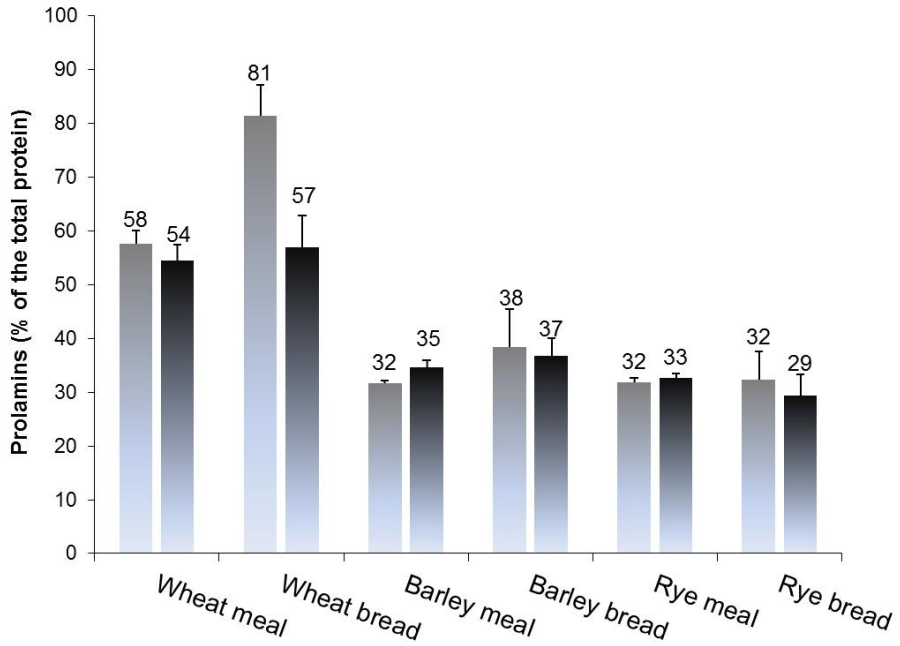

Figure 13. Prolamin contents of wheat, barley and rye meals and bread extracted using 40% 1-propanol with 1% dithiothreitol (gray) or with the cocktail solution (black). Protein contents were analysed by the Lowry method. Error bars indicate the standard deviation of the mean of three repeats.

Gel electrophoresis of the Osborne fractions of wheat, barley and rye showed that a large number of wheat prolamins required reduction before becoming soluble (**IV:** Figure 3). However, all of the prolamin groups of wheat, including a proportion of the HMW glutenin subunits, were present in the alcohol extract, even without reduction. The C-hordeins and a proportion of the B-hordeins were dissolved in aqueous 1-propanol without reduction, whereas the remaining polymeric B-hordeins and D-hordeins required reduction or even SDS buffer before solubilization. Rye prolamins were mainly extracted with aqueous 1-propanol without reduction, although more of the γ-75 and HMW secalins were extracted after reduction.

The prolamin fractions of wheat, barley and rye were also characterized with a lab-on-chip technique. Unique electrophoregrams were obtained for each cereal, which enabled their distinction from one another; however, the peaks migrated at the same molecular weights (Figure 14). Therefore, this technique cannot be used to identify cereals from a mixture. The protein patterns were clearly different from the patterns in SDS-PAGE electrophoresis and could not be identified (Figure 14).

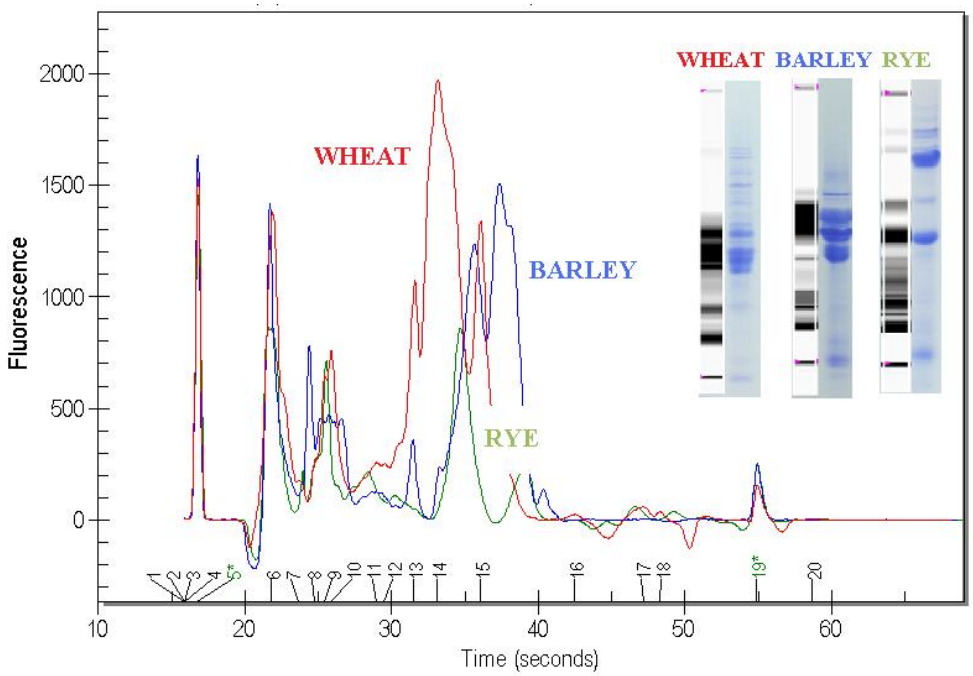

Figure 14. Electrophoregrams of wheat, barley and rye prolamins produced by an automated electrophoresis system. The protein patterns were compared with SDS-PAGE separation (blue lane upper right corner). Samples were from sequential extraction and extracted with 40% 1-propanol at 50 ºC without reducing.

We characterized the affinities of prolamin-specific antibodies that are used in gluten analysis against the prolamin groups that were soluble in 40% 1-propanol (**VI:** Figure 3). The polyclonal anti-gliadin antibody recognized all of the prolamin groups of wheat, barley and rye and some of the water and salt soluble proteins. The ω-gliadin antibody strongly detected ω-gliadins and HMW glutenin subunits, HMW and γ-75 secalins, and C- and D-hordeins, whereas a weak response was obtained with B-hordeins and ω-secalins. The R5 antibody reacted strongly with α-, γ-gliadins and LMW glutenins, with all of the secalin fractions, and with B-hordeins. It also recognized ω-gliadins and HMW glutenin subunits and C-hordeins, but less intensively. In addition, the antibody R5 reacted with some of the water and salt-soluble proteins of wheat and rye.

5.5 Determination of deamidated gluten (V)

The competitive ELISA methods based on the antibodies R5 and G12 recognized the synthetic peptides with varying intensities, whereas neither of the tested sandwich ELISA methods were able to recognize them. The concentration of the peptides ranged from 24% to 50% when quantified by competitive R5 ELISA and compared with the original amount of the peptide weighed into the sample solution (**V:** Table 2). The G12 antibody only recognized the peptide with 33 amino acids, which it was originally raised against. The ability of antibodies to recognize the deamidated peptides was considerably decreased. The relative intensity obtained for the peptides, which modelled tranglutaminase

deamidation, was only 4–8% compared to the intensity obtained for the intact peptides (**V:** Figure 1). The random deamidation reduced the intensities down to the level of 13–54% from the intensity of the intact peptide.

In addition to the peptides, native and acid-deamidated gluten proteins were analysed by the ELISA methods. The ELISA methods based on ω-gliadin and A1-G12 failed to recognize the deamidated gluten, whereas their response to the vital gluten was as expected (**V:** Figure 2). The sandwich and competitive R5 ELISA methods also failed to recognize deamidated gluten in the same concentrations as with the vital gluten (Figure 15). However, when the protein content of samples containing deamidated gluten was increased approximately 600 times, they were measurable with sandwich R5 ELISA (**V:** Figure 3). Competitive R5 ELISA needed about 125 times higher protein contents for deamidated gluten samples than for the vital gluten (**V:** Figure 4).

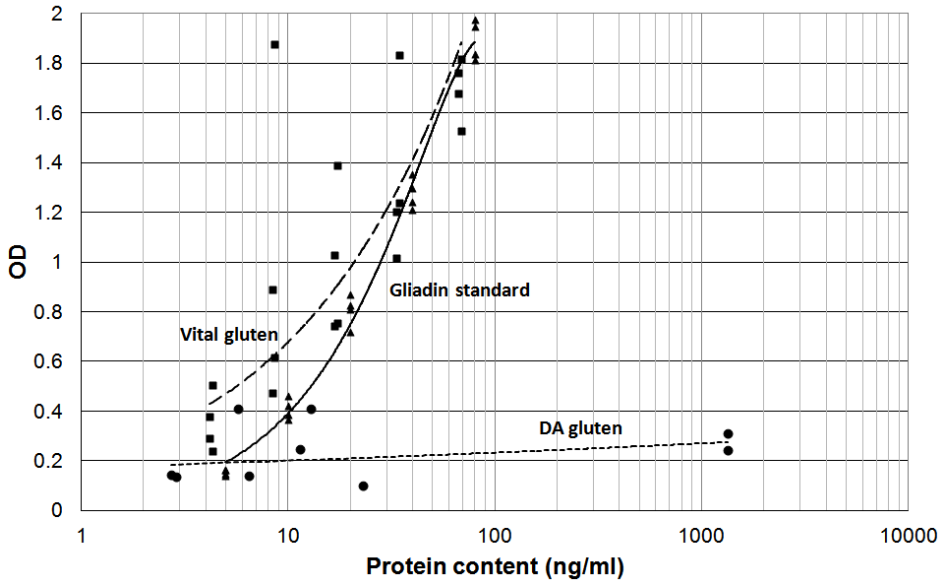

Figure 15. Comparative reactivity of a vital and deamidated gluten by sandwich R5 ELISA.

6 DISCUSSION

6.1 Prolamins in beers

At the time of starting this thesis, the main obstacle to barley prolamin analysis was that they could not be detected with the recommended gluten analysis assay (ω-gliadin ELISA by Skerritt). Only about 4–8% of barley prolamins could be detected with antibody directed against the ω-gliadin fraction of wheat, and quantitative results for hordeins could not be obtained. Our aim was to develop a better standard for barley prolamins that could more accurately measure the hordein content even at low levels. Unfortunately, this was not very simple, including problems in getting stable standard curves. The discrepancy of the results could be due to the inconsistent reactivity of ω-gliadin antibody with hordeins. Nevertheless, an approximately twelve times higher concentration of hordeins was needed to obtain the same absorbances as for gliadins. At the same time, Malmheden Yman and Kruse (2005) extracted barley hordein to prepare a hordein standard for beer analysis. They separated hordein from barley flours instead of barley malt, as in our studies. They used the standard with ELISA based on ω-gliadin antibody and noted that their results were in accordance with those obtained with competitive R5 ELISA. At that time, competitive R5 ELISA was under development and was only available to those people involved with the PWG. However, their results, together with our findings, support the use of hordein standard for samples containing barley. Since beer does not contain similar proteins to those of native barley, a barley malt standard could, in our opinion, offer better standard material to quantify prolamins in beer. Differences between malts may, however, cause differences between malt standards.

With the SDS-PAGE analysis combined with Western blotting, we noticed the presence of hordeins in lager beers. The protein content of beer is about 5–7 g/l (Gorinstein et al. 1999). These proteins are mostly polypeptides that have originated from degraded barley proteins. Beer polypeptides, especially those with a high proline content, i.e. hordeins, contribute to the haze formation and foam stabilization of beer (Siebert 1999). The level of hordeins decreases considerably during the process, and only 0.11% of the original hordeins have been determined to end up in the final beer (Dostálek et al. 2006). This resembles the hordein levels measured in our beer samples. Most of the hordeins are precipitated in the brewer's spent grain, and only minor amounts end up in the final beer. Aggregated B- and D-hordeins hydrolyse during malting into smaller fragments, which increases their solubility (Celus et al. 2006). We noticed some proteins left mainly in the same molecular weight area as B-hordeins, although they have been shown to be completely hydrolysed by the end of brewing in a previous study by Sheehan and Skerritt (1997). However, protein Z also has a similar molecular weight of 40 000 g/mol. Protein Z is a major protein in beer and contains glutamine-rich repetitive sequences that resemble the prolamin storage proteins of wheat (Ostergaard et al. 2000). Consequently, there is a possibility that protein Z is detected by prolamin-specific antibodies, although it does not belong to the group of proteins that are harmful to people with coeliac disease. Wheat beers, on the other hand, contained high levels of prolamins, which clearly excludes their use in a gluten-free diet. It seems that wheat prolamins become easily soluble after hydrolysis, whereas barley prolamins tend to precipitate.

Although, the usage of a barley standard improved the detection of hordeins by the ELISA method, it is likely that sandwich ELISAs are unable to detect all of the hordein residues from beer, apparently due to the lack of multiple binding sites on hydrolysed peptides. The gluten levels have been noted to be considerably smaller with the sandwich than with the competitive methods (Dostálek et al. 2006). The gluten levels determined from the beers were relatively small; however, we were unable to compare them with a competitive method. The competitive R5 ELISA is currently used for the detection of barley prolamins in beers. However, the method has not been tested by international ring trials, which would be important before the method could be taken in wider use. Moreover, the method in its present form gives the results in peptide concentrations, and conversion to the gluten level is only an estimate. New methods based on the detection of harmful peptides, such as 33mer and other sequences in α-gliadin, have entered the market (Spaenij-Dekking et al. 2004, Morón et al. 2008b). These methods could be suitable to detect hordeins from beers. It can be questioned, however, whether all harmful peptides are detected with these methods. The antibodies used in the assays have high specificities for certain sequences, and some of the harmful peptides may therefore go undetected. Furthermore, the suitability of the methods has not yet been tested with beer samples.

Another approach to improve the gluten analysis of beers is the development of standards that would match the hydrolysed sample proteins better than the native gliadin standards. Our attempt to create a barley malt standard was a step towards that. Gessendorfer et al. (2009) had a similar approach and developed a new reference material by enzymatically hydrolysing prolamins from wheat, rye, and barley. Although, their approach was similar to ours, their reference material contained prolamins from all harmful cereals. A reference material consisting of hydrolysed prolamins could be more suitable for the immunochemical quantitation of similarily hydrolysed prolamins in beer and syrups than standard containing native proteins. However, differences in the degree of hydrolysis affect the affinity of antibodies. This problem might be resolved by using the same controlled hydrolysis for sample proteins as has been used for standard proteins before the analysis. Nevertheless, thorough testing and validation is needed before any new reference material can be used in ELISAs. Currently, the hydrolysed reference material of Gessendorfer et al. (2009) is being tested in the competitive R5 ELISA, and if shown suitable, it could solve the problems in detecting gluten in beers. The new standard could also provide more consistent results for different batches of beers, which has been a major problem to date. The inconsistency of the results has unnecessarily reduced the number of beers with a gluten-free label, since manufacturers do not want to take a risk of one batch being over the limit of 20 ppm of gluten, which has been set in the Codex Alimentarius for gluten-free products.

6.2 Barley contamination in oats

When oats were allowed for people with coeliac disease, concern was raised about potential contamination by harmful cereals. Oats, as well as other gluten-free cereals, can easily be contaminated during cultivation, storage, milling, transportation or processing of food. When oat products have been studied, unexpectedly high levels of contamination have been found (Gélinas et al. 2008, Hernando et al. 2008). Of the analysed oat samples, only 10% contained less than 3 ppm of gluten, 10% between 45–200 ppm and 80% between 200–8000 ppm (Hernando et al. 2006). These high results were

obtained with sandwich ELISA based on the R5 antibody. This antibody is able to detect barley prolamins, which is a significant improvement over the ω-gliadin antibody. However, the contamination levels in oats of up to 15 000 ppm (Mujico et al. 2005) raised doubts over whether the reported values were accurate. These levels would mean the presence of about 20% of barley flour in the tested sample.

We demonstrated that the R5 antibody overreacts with oat samples contaminated with barley meal, causing unrealistic results when measuring gluten levels. We prepared contaminated oat samples by mixing barley meal with oat meal that was milled from oat grains that were carefully checked to ensure the absence of foreign grains. The gluten levels obtained with R5 ELISA were considerably higher than the levels calculated on the basis of the protein content of barley meal. Because of these results, we were back in the situation where barley prolamins could not be accurately measured by gluten analysis assays. We decided to use the same approach as with beer samples, and prepared our own reference material by extracting hordeins from pearled barley. We developed a standard curve with this hordein standard and analyzed the samples again using this new curve. The detected hordein levels were now very close to the expected values. This supported the view that the standard should very closely resemble the analyte in order to obtain reliable results. Unfortunately, in practice this might be impossible. Commercial gluten-free samples can contain any of the harmful cereals and very often contain a combination of them (Mujico et al. 2005). Because of this, it would be impossible to choose the appropriate standard for each material. Instead, some sort of combination standard could be more accurate.

Another possible explanation for the high gluten contents recorded in oat samples contaminated by barley is that they could be caused by oat prolamins. Western blot analysis of oat samples with the R5 and the ω-gliadin antibodies revealed that both of the antibodies were able to recognize oat prolamins. This was also confirmed by Comino et al. (2011). It was questioned whether prolamin-specific antibodies react with oat prolamins, increasing the response. However, the response was very weak compared to the antibody reaction with wheat prolamins, and it had only a minor contribution to the determination of the total prolamin.

6.3 Hydrolysis of prolamins in sourdoughs

In Finland and in many parts of Eastern Europe, rye products constitute a considerable part of the diet. Rye bread is often one of the products that people who have been diagnosed in adulthood with coeliac disease long for. A gluten-free diet is often deficient in minerals and dietary fibre due to the high levels of gluten-free starch used in the products. Many of the gluten-free cereals or pseudocereals could add to the nutritive value, but unfortunately they often have an unappealing taste. Rye, on the other hand, would be a good source of fibre and minerals and have a desirable flavour if it did not contain proteins harmful to people with coeliac disease. Rye could be used as part of gluten-free baking if the harmful proteins were hydrolyzed to such low levels that they would have no remaining immunoactivity.

Although extensive prolamin hydrolysis has also been observed with wheat prolamins (DiCagno et al. 2004, Loponen et al. 2007, Rizello et al. 2007), rye prolamins are the ones that seem to be the most

efficiently hydrolysed during fermentation (Loponen et al. 2011). This characteristic offers a possibility to use rye in gluten-free products to produce the desired taste and flavour. Another characteristic that favours rye over wheat is that rye prolamins do not have such an important role as wheat prolamins in structure formation during baking. Rye prolamins are not able to form a gluten network similarly to wheat prolamins; therefore, hydrolysis of rye prolamins does not affect the baking qualities of rye. In wheat, a similar situation would destroy the baking qualities by hydrolysing the gluten network, and therefore prohibit wheat baking. The structure formation in rye baking is mainly based on pentosans, mostly arabinoxylans, and their ability to bind water and retain gas. The amount of soluble arabinoxylans increases in rye sourdoughs due to cereal hemicellulases (Boskov Hansen et al. 2002). Because of this, rye sourdough could potentially be used in gluten-free baking processes, which are often based on structure-forming carbohydrate polymers.

We were able to reduce the prolamin content of rye meals by 99.5% from the original level under sourdough conditions by using endogenous rye enzymes. Cereal grains naturally contain several enzymes that hydrolyze proteins. They are important for the grain to develop and eventually germinate. The main function of prolamins is to store nitrogen and carbon for germination; therefore, it can be expected that grain contains endogenous tools to break down these proteins to provide material for the seedling to grow. Resting grain contains some aspartic proteinases and carboxypeptidases, but when germination starts the amount and number of enzymes increases significantly. The main proteolytic activity of germinating rye grain is due to aspartic and cysteine proteinases (Brijs et al. 2002), but carboxypeptidases and serine and metallo proteinases also have an important role in the process. Therefore, we used germinated rye in sourdough systems to enhance the degradation of rye prolamins. However, even after extensive hydrolysis, the prolamin levels still exceeded the maximum levels for gluten-free products and could not be considered safe as such. It is, nevertheless, possible to use small amounts of sourdough in gluten-free baking. We added rye sourdough to gluten-free oat flour and a brownish color and rye flavour appeared in the bread, improving its quality.

The prolamin contents of the sourdough samples were determined with the sandwich and competitive R5 ELISA methods. The results obtained by the sandwich method were not multiplied by two and the results obtained with the competitive method were divided by 250, as advised by the manufacturer of the assay, to obtain the prolamin content. Since most of rye prolamins are soluble in aqueous alcohols and the sourdough process further increases their solubility, it can be assumed that the majority of rye prolamins are in the sample solution after extraction and are detected. Furthermore, the results of study **IV** (Figure 3) demonstrate that the R5 antibody was able to recognize all the secalin subgroups. Therefore, there was no need to multiply the secalin concentrations and the prolamin concentration of sourdoughs was equal to the total gluten content. Although the rye prolamins were successfully degraded to very low levels in sourdough systems, some of the prolamins remained and were detected by the R5 antibody. The experiments were carried out to identify the residual proteins or peptides. These proteins were separated after hydrolysis by immunoprecipitation using the R5 antibody as a capturing antibody. The residual proteins from acidic barley and wheat malt autolysates, in addition to rye proteins, were analysed and specific proteins with highly similar molecular weights were detected from the extracts. It would be interesting to examine more in detail the residual prolamins that are able to resist hydrolysis.

It is difficult to estimate whether extensive hydrolysis reduces or even abolishes the immunoactivity of peptides. Hydrolysing proteins in complex food systems cause the formation of varying amounts of different peptides, and the conditions may be difficult to optimize so that the final peptide composition is always the same. Testing of the harmfulness of peptides has difficulties, since T-cell tests have varying results and *in vivo* testing is expensive and difficult to perform. Fortunately, an animal model in which mice are used to estimate the harmfulness of different prolamin peptides has been developed (Freitag et al. 2009). The method was found to be suitable for studies on coeliac disease-causing peptides and hydrolysed proteins (Freitag et al. 2011).

6.4 Effect of extraction on gluten analysis

Gluten analysis methods, including sample preparation, have been developed for wheat, probably because wheat is used more widely in diets compared to rye and barley. In addition, wheat starch is frequently used as a basis for gluten-free products. It is therefore important to be able to detect and quantify residual wheat prolamins from gluten-free products. Recently, oats were permitted in a gluten-free diet if their purity from harmful cereal proteins could be ensured. Contamination found in oats has been caused as often by barley as by wheat (Gélinas et al. 2008), which makes it more important for the analysis method to detect barley prolamins as accurately as wheat prolamins. The prolamins of wheat, barley and rye have differences in the solubilities of their subgroups that need to be considered before quantitative analysis. While wheat contains high amounts of insoluble prolamins, rye prolamins are almost completely soluble in aqueous alcohols. Certain subgroups of barley prolamins demand strong reduction and disaggregation before they are solubilized (Shewry et al. 1980, Celus et al. 2006). Because of these differences and the importance of accurate detection of barley and rye prolamins, we studied and compared different extraction protocols in order to determine the extraction conditions in which all of the prolamin subgroups would be extracted. We considered it important for the extraction method to be suitable for processed samples, not just for flours.

We found that prolamins of wheat, barley and rye are most efficiently extracted with 40% 1-propanol with 1% DTT at 50 °C. This method was more efficient in the extraction of barley and rye prolamins than the most commonly recommended extraction protocol with 60% ethanol. The difference between these two alcohols was not so evident with wheat prolamins, as was also observed in the study of Wieser et al. (1994), in which the most efficient extraction of gliadins was obtained with either 60% ethanol or 50% 1-propanol. However, prolamins of rye and barley are different, and aqueous propanol extracts more prolamins from the samples than aqueous ethanol, which has also been noted in several previous studies (Lauriére et al. 1976, Shewry et al. 1980, Shewry et al. 1983). In particular, prolamins with a polymeric nature, such as B-hordeins, seem to prefer propanolic solutions (Shewry et al. 1980), and significant amounts of polymeric prolamins have been reported to co-extract with 1-propanol (Fu and Sapirstein 1996, Bean et al. 1998). Charbonnier et al. (1981), however, considered ethanol to be the most efficient extractant with respect to purity. Shewry et al. (1983) also noted the presence of proteins other than secalins in propanol extraction. Neither SDS-PAGE nor automated electrophoresis, which were used in the present study, revealed any significant levels of proteins other than prolamins in the meal samples. However, we were unable to identify the prolamin subgroups by automated electrophoresis due to the highly dissimilar patterns compared to those in SDS-PAGE.

The importance of reduction and an elevated temperature in the efficient extraction of prolamins was shown in present study. Monomeric prolamins were readily soluble at RT without reduction, but polymeric prolamins needed reduction and heating before they were solubilized. This is in accordance with previous studies on wheat and barley prolamins (Shewry et al. 1980, Byers et al. 1983, Fu and Sapirstein 1996, Howard et al. 1996). The reduction is needed to break the disulphide bonds between the proteins to solubilize them in alcoholic solutions. The location of disulphide bonds has a strong influence on the reduction efficiency. The bonds between the proteins break at lower reducing agent concentrations; however, the bonds inside the proteins need much higher concentrations of reducing agent (Lavelli et al. 1996). Despite reduction, it may still be impossible to get all the proteins into solution, because they can exist in large aggregates or be attached to other polymers such as starch or lipids. The SDS buffer was also noted to extract residual prolamins from the meal samples after alcoholic extractions in our study (**IV**: Figure 3). In addition, heat treatment lowers the solubility of proteins. Heating causes the unfolding of proteins, exposing sulphydryl groups that are able to form new bonds. These new bonds cause conformational changes in prolamin proteins, resulting in even larger protein complexes with a lower solubility (Schofield et al. 1983). Lagrain et al. (2011) observed that the same gliadin levels were not extracted after heating, even with reduction, suggesting the existence of other bonds in their structure. The β-elimination reaction of cysteine, which involves the formation of dehydroalanine and free sulphydryl groups in gliadin and glutenin, has been suggested to be a possible additional cross-linker between the proteins (Rombouts et al. 2010). The cross-link is generated between dehydroalanine and cysteine or lysine; however, this reaction needs alkaline pH conditions to occur. Isopeptide bonds between lysine and glutamine or asparagine have also been found after heat treatment (Rombouts et al. 2011).

In addition to the use of reduction, the efficiency of the extraction is increased by elevating the temperature. We noted in our study that elevating the extraction temperature from RT to 50 °C significantly increased the yield. However, we did not notice any improvement in the efficiency by elevating the temperature to 60 °C, which has been used in previous studies (van den Broeck et al. 2009). The amount of prolamins extracted might also be increased by adding more extraction steps. Bean et al. (1998) observed that when wheat prolamins were extracted three times for 5 min with 50% 1-propanol, only negligible amounts of protein were left in the third fraction, indicating the high efficiency of the first two repeats. We considered whether it would be more convenient to have only one extraction step before analysis, since then it would be simpler and faster to perform. Although if only 5 min extraction times are used, as in the study of Bean et al. (1998), the total extraction time would not be much longer, more work is needed to complete multiple extraction steps instead of only one.

Although reduction is an important factor in the extraction of prolamins, reducing agents have been noted to interfere with the analysis methods used for gluten detection. Therefore, their usage has to be carefully considered before analysis. Reduction agents modify the protein structure, which in turn affects to the antibody-antigen reactions, leading to errors in quantitative determination (Doña et al. 2008). Sufficient dilution of the extracted samples is often necessary. We demonstrated in study **IV** that increasing the level of DTT did not produce a significant increase the prolamin yield, and good efficiency could already be obtained with 1% DTT. It should be noted, however, that when reduction is used in the extraction process, polymeric prolamins are also extracted and the total gluten fraction is

obtained in the solution. In Codex Stan 118-1979, prolamins are described as “the fraction from gluten that can be extracted by 40–70% of ethanol.” This is problematic, since the total prolamin fraction, including polymeric prolamins, is considered harmful for coeliacs, not only the alcohol-soluble fraction. To resolve this, the alcohol-soluble prolamin content is defined in Codex Stan 118-1979 as being a half of the total content, and the analysis results are therefore multiplied by two. Obviously, this is not the case when reduction is used in sample preparation and the total prolamin fraction is extracted. Multiplication by two would then lead to a doubling of the true gluten content.

Recently, some other studies on prolamin extraction have been published. New interest in the extraction of gluten proteins seems to have been raised following the development of new gluten analysis methods. Van den Broeck et al. (2009) compared the extraction protocols that are used today and developed a new optimized two-step extraction protocol. The protocol included extraction by 50% aqueous 2-propanol for 30 min at RT followed by extraction with 50% aqueous 2-propanol, 50 mM Tris-HCl (pH 7.5) containing 1% DTT for 30 min at 60 °C. When compared with the other extraction protocols, they reported obtaining more complete extraction with a higher gluten protein content, which also had higher concentrations of glutenins. Gessendorfer et al. (2010), on the other hand, compared the extraction efficiency of a buffered salt solution containing *tris*(2-carboxyethyl)phosphine (TCEP) and guanidine hydrochloride with the commercial cocktail solution and 60% ethanol. The 60% ethanol was only able to extract 37% of the prolamins from wheat bread as compared to the amount that was extractable with the cocktail solution. The protein yield was increased to 95% when 20–50 mmol/L TCEP was used in the ethanol solution. The addition of guanidine hydrochloride was observed to be essential for the quantitative extraction of prolamins. However, we did not observe any advantage in using guanidine hydrochloride, since better extraction yields were obtained with 40% 1-propanol than with the cocktail solution. On the contrary, it might have caused disturbance, lowering the gluten measurements for wheat bread, since the gluten levels were lower when extracted with the cocktail solution than with 40% 1-propanol.

Another factor affecting quantitative gluten analysis is the reactivity of the prolamin-specific antibodies with different prolamin subgroups. The results obtained with gluten assays are multiplied by two to obtain the total gluten content, because it is assumed that only a half of the prolamins are extracted. It would also be sensible to double the results if only a half of the gluten proteins were recognized by the antibody. However, our Western blot analysis showed that both the ω-gliadin and the R5 antibody recognized the polymeric glutenins, which suggests the possibility of directly quantifying prolamins without multiplication (**IV:** Figure 3), although the affinity of the R5 antibody against glutenins was shown to be low (also in Allred and Ritter 2010). In addition to inaccuracies caused by multiplication, the diverse reactions between antibodies and prolamin subgroups may cause problems in interpreting quantitative results. In accordance with a previous study by Wieser and Seilmeier (1999), which demonstrated the variability in antibody affinities against different prolamin subgroups, we also noticed clear differences between three prolamin-specific antibodies in their cross-reactivity with prolamins of wheat, barley and rye. The polyclonal anti-gliadin antibody recognised all of the prolamin groups of wheat, barley and rye, whereas the ω-gliadin antibody mainly recognized the high molecular weight proteins of wheat, barley and rye and the antibody R5 the medium molecular weight prolamin groups. The polyclonal anti-gliadin antibody and the R5 antibody recognized some of the water and salt-soluble proteins of wheat and rye, indicating the presence of prolamins in these fractions. It was noted that

moderate salt solutions can increase the solubility of prolamins. Fu et al. (1996) observed that 2% NaCl in distilled water extracted almost all gliadins and most of the glutenins. Only the ω-gliadins were not entirely solubilized. They suggested that salt induces conformational changes in the protein structure and consequently makes them more readily soluble in water. However, increasing the salt concentration reduced the solubility of higher molecular weight gliadins and glutenins (Kim and Bushuk 1995). If Osborne fractionation is used, water and salt-soluble albumins and globulins are removed before the extraction of prolamins. Based on the observations of Fu et al. (1996) and the results of our study, some of the prolamins may be removed with these fractions. However, if no pre-extraction with salt solution is performed, some albumins and globulins are solubilised with prolamins into the alcohol solution. It has been reported that albumins and globulins are extracted with 50% 1-propanol in addition to prolamins (Fu and Sapirstein 1996). However, when using an immunological method for quantification, this should not be a problem, since the prolamin-specific antibodies should not react with proteins other than prolamins. The defatting procedure that is sometimes used prior to the extraction of prolamins from certain food products, such as sausages, also influences the immunological analysis of prolamin proteins with ELISA assays of R-Biopharm and ImmunoTech, a Beckmann company (Gabrovská and Rysová 2004). The gliadin content of wheat flour after hexane extraction was close to the original sample; however, the quantitative gliadin content almost doubled after chloroform extraction and decreased almost to a half after petrol ether extraction. Rye meal defatting with water-saturated 1-butanol before extraction by 60% ethanol resulted in a 40% loss of the secalin yield (Charbonnier et al. 1981). Due to these problems, we did not use defatting for our samples. However, this was not necessary because of their low fat content.

It is a challenge to accurately quantify prolamins due to their high complexity and the sensitivity to disturbances of ELISA analysis. Nevertheless, the extraction protocol should allow the complete extraction of different prolamins from wheat, barley and rye, maintaining their immunoreactivity with detecting antibodies. The complexity of prolamins also affects antibody recognition, since some prolamin subgroups are recognized more intensively than others. This highlights the importance of a suitable standard for the analysis. The ideal situation would be where the standard is composed of the same prolamin subgroups and in similar relative proportions to those present in the sample.

6.5 Modification of gluten by deamidation

Food proteins are influenced by process treatments, including isolation, purification, modification and drying procedures, and the presence of other food constituents. Proteins may have also been hydrolysed into smaller peptides during the process, e.g. in fermentation processes and brewing. Some food products undergo extrusion or enzymatic treatments during processing, both of which influence the structure and solubility of the proteins. In addition to the changes caused by processing, proteins can be deliberately modified to improve their functional properties and their applicability in foods. All of these treatments affect the surface structure of proteins, which consequently affects antibody binding and quantitative analysis.

Deamidation significantly decreased the recognition of gluten proteins and peptides by monoclonal R5, ω-gliadin and G12 antibodies (**V:** Figure 1–4). Two types of deamidation of prolamin-derived peptides were investigated: random and specific deamidation by tTG. Random deamidation modelled the possible situation that could occur during food processing, whereas specific deamidation caused by the tTG enzyme modelled the harmful peptides with increased immunoactivity in coeliac disease. Both types significantly reduced the intensities. This was observed in our study and also in that of Kahlenberg et al. (2004) with deamidated epitopes of 33mer. In addition, Morón et al. (2008a) reported the decreased ability of G12 and A1 antibodies to recognize deamidated 33mer. In addition, deamidation of intact gluten proteins decreased or abolished the recognition of the proteins by the antibodies. This may have serious consequences if deamidated gluten proteins ended up in products that were generally considered gluten-free, e.g. milk or meat products. Since gluten is a cheap by-product of the starch industry, its usage may increase and deamidated gluten proteins may consequently be found in increasing numbers of food products. In addition, gluten is a vegetable protein, which further increases the interest its use in various foods.

Deamidation is used to increase the poor solubility of gluten proteins and improve their foam and emulsion forming cababilities. Deamidation in food processes may be induced by acidic or enzymatic treatments. Deamidation has also been shown to be an important factor in coeliac disease, since it changes prolamin peptides to forms that are recognized by HLA-DQ2 and -DQ8 molecules. This type of deamidation is caused by tTG. Deamidation by tTG increases their harmfulness to coeliac patients (Molberg et al. 1998, Sjöström et al. 1998; van de Wal et al. 1998b). It has been observed that some peptides that are not recognized in their native form may be recognized after specific deamidation of glutamine residues. For example, deamidation of wheat and maize prolamins with microbial transglutaminase increased the immunoglobulin A reactivity of coeliac disease patients, suggesting that gluten-free maize may also cause a risk to people with coeliac disease, especially after treatment with microbial transglutaminase (Cabrera-Chavez et al. 2008). It is not known, however, whether deamidation induced by acid treatment increases or reduces the harmfulness of prolamin peptides. Terreaux et al. (1998) observed an increase in the affinity of gliadin peptides to HLA molecules after chemical deamidation, whereas Berti et al. (2007) noted in their study that chemical deamidation by acid and heat treatment lowered the immunoreactivity, while tTG-catalysed deamidation increased the immunoreactivity. Therefore, they suggest that chemical deamidation could be used to lower the reactivity of residual gluten and consequently reduce the harmful effect. Deamidation by tTG is highly specific, and the locations of proline residues in the amino acid sequence determine which glutamines are deamidated (Vader et al. 2002a), whereas acidic deamidation is random and any of the glutamines may be deamidated. Because of the random nature, it is difficult to estimate the behaviour of the randomly deamidated peptides with HLA molecules. The immune response is highly specific to certain sequences, and even the smallest changes in the amino acid sequence may either abolish or create an immune response (Ellis et al. 2003). Therefore, preventing deamidation could be used as a novel therapy in coeliac disease.

Deamidation influences the accuracy of quantification by immunoassays, because it changes the protein surface structure. The antibodies used for gluten quantification are raised against proteins or peptides of native proteins, and their ability to accurately detect modified proteins can therefore substantially differ due to differing affinity between the antibody and the antigen. The modified gluten can actually be more immunoreactive compared to native gluten, and at the same time difficult to detect with current gluten analysis tools.

7 CONCLUSIONS

The main findings of the present work were that quantitative gluten analysis can be greatly improved by selecting a better standard for the analysis and using 40% 1-propanol with 1% DTT at 50 °C for the extraction of prolamins. With these procedures, the unrealistic results obtained for products containing barley can be corrected, and furthermore, no multiplication of the results is necessary to obtain the total gluten content. However, the results also showed that deamidation of gluten proteins considerably reduced the antibody reaction, which would lead to the substantial underestimation of gluten contents.

Gluten analysis methods have considerably developed since the first standard was set for gluten-free products in 1979. At that time the maximum gluten content allowed in gluten-free products was expressed in terms of the level of total nitrogen, while we now measure the actual harmful sequences from the samples. This means that a great deal of knowledge has been obtained on the pathogenesis of coeliac disease, which has been used in the development of new analysis methods. Despite the progress, there is still no accurate method to detect barley prolamins from beer that would give the total gluten content. Another still unresolved problem concerns the evaluation of contaminations in gluten-free products. The present study revealed that the R5 antibody, which is the antibody in the currently recommended method, overreacts with hordeins, leading to unrealistic results. It was shown that more realistic results are obtained if a standard that more closely resembles the sample proteins is used. However, in most cases it would be impossible to select the appropriate standard, and whether a standard that is a mixture of harmful prolamins would give more accurate results should be tested. The remaining difficulty is how to relate the obtained results to the maximum gluten levels that are set by the Codex Alimentarius. Nevertheless, we recommend the use of a hordein standard with samples containing barley.

Promising results were obtained with the rye sourdough systems, in which secalins were efficiently degraded. Both the sandwich and the competitive method of R5 gave comparable results. In our study, secalins were observed to behave in a very similar manner in sandwich R5 ELISA when compared to the gliadin standard of the assay. However, it shoud be noted that the majority of rye prolamins in sourdoughs are extracted without reduction and recognized by the R5 antibody. Consequently, the doubling of the results, as advised when performing an analysis with a sandwich method, would lead to overestimation of true secalin content.

Sample preparation is an important part of the analysis and should take into account the differences between prolamins of wheat, barley and rye. However, many of the reagents that are used to obtain efficient extraction interfere with the analysis by immunological assays. Therefore, a compromise is needed between the yield and retaining the immunoactivity of proteins. It is useless to use extraction methods that reduce protein immunoreactivity, but efficient extraction of prolamins is necessary. Our results demonstrated the superiority of 1-propanol over ethanol in the extraction of prolamins, and the use of 1-propanol is therefore recommended together with 1% DTT.

The modification of gluten proteins presents another obstacle for the analysis methods. The methods should not only recognise and accurately quantify different prolamin subgroups, but also quantify

modified prolamins. Modification by deamidation significantly lowered the affinity against gluten proteins of all of the tested antibodies. It would not be a problem if modification reduced the harmfulness of the proteins, but this not probable. In fact, the deamidation by tTG after ingestion and digestion of gluten is shown to increase their affinity to HLA molecules and consequently increase the harmful immune reaction in coeliac patients. The decrease in the detection of deamidated proteins may allow products containing high amounts of deamidated gluten be sold as gluten-free and create a potential threat to people with coeliac disease.

Although people with coeliac disease can eat gluten-free products without the fear of being exposed to high levels of harmful proteins, considerable work still needs to be done with gluten analysis methods. The recommended method based on the R5 antibody is known to detect several unharmful proteins in addition to overreaction with hordeins, which may unnecessarily reduce the variety of gluten-free products. Hopefully, new methods that are thoroughly tested with commercial food samples containing several ingredients that may affect the antibody-antigen reaction will soon be introduced for gluten analysis to guarantee the safety and wide variety of gluten-free products in the future.

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