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Isolation and proteinchemical characterization of HMW-gliadins from wheat flour

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List of amino acids

Amino acid	Three letter code	One letter code
alanine	Ala	A
arginine	Arg	R
asparagine	Asn	N
aspartate	Asp	D
cysteine	Cys	C
glutamate	Glu	E
glutamine	Gln	Q
glycine	Gly	G
histidine	His	H
isoleucine	Ile	I
leucine	Leu	L
lysine	Lys	K
methionine	Met	M
phenylalanine	Phe	F
proline	Pro	P
serine	Ser	S
threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
valine	Val	V

List of abbreviations

Abbreviation	Meaning
APCI	athmospheric pressure chemical ionization
B.C.	before Christ
CI	chemical ionization
CID	collision induced dissociation
CSH	cysteine
CSSC	cystine
DTE	dithioerythritol
DTT	dithiothreitol
ESI	electrospray ionization
ETD	electron transfer dissociation
FAB	fast atom bombardment
Fig.	figure
GMP	glutenin macropolymer
GPC	gel permeation chromatography
GS	glutenin subunit(s)
GSH	glutathione (reduced)
GSSG	glutathione (oxidized)
HGL	high-molecular-weight gliadins
HMW	high-molecular-weight
IR	infrared
kV	kilovolt
L.	Linné
LC	liquid chromatography
LMW	low-molecular-weight
m/z	mass/charge ratio
MALDI	matrix-assisted laser desorption/ionization
MRM	multiple reaction monitoring

List of abbreviations

MS	mass spectrometry/mass spectrometer
MW	molecular weight
n	number
NEMI	N-ethylmaleinimide
pH	pH-value
pp.	pages
PWG	Prolamin Working Group
RP	reversed-phase
RP-HPLC	reversed-phase high-performance liquid chromatography
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SEM	secondary electron multiplier
SH	thiol
SIM	single ion monitoring
SRM	selected reaction monitoring
ssp.	subspecies
T.	Triticum
Tab.	table
TIC	total ion current
TOF	time-of-flight
TRIS	Tris(hydroxymethyl)aminomethane
UV	ultraviolet

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1. Introduction

1.1 Cereals

Different species of cereals have been used as forage or food plants for a long time. First evidence of cultivation, which had initially been done with barley (*Hordeum vulgare*), einkorn (*Triticum monococcum*) and emmer (*Triticum dicoccon*), has been dated about 11000 B.C and was located in Mesopotamia. The different species started to spread from there gradually via the western part of Asia all over Europe. Spelt (*Triticum spelta*) and wheat (*Triticum aestivum*) were developed from the wild forms (einkorn and emmer) and numerous other wild grass and cereal species by selective cross-breeding and selection of the seeds. Initially, oats (*Avena sativa*) and rye (*Secale cereale*) were considered as weeds in the cultivation of barley and wheat. However, their extensive resistance also resulted in the cultivation of these two species. The cultivation of rice (*Oryza sativa*) and corn (*Zea mays*) started about 5000 years ago. Rice plays an important role mainly in Asia, corn in Central and South America and sorghum (*Panicum miliaceum*) in subtropical and tropical regions of Africa and Asia. All of the named cereal species are assigned to the botanical grass family (*Poaceae*), just like their original forms and other wild grasses (Belitz et al. 2001).

1.2 Wheat

Wheat is one of the oldest cultivated plants worldwide. Numerous different species developed in the course of time. Among all wheat species, common wheat plays the most important role today regarding crop area and production quantity. It is one of the most important cereals on earth besides corn and rice and is used for the production of bread and numerous other food products. Additionally, durum wheat (*T. durum*) is of economic importance, because it is the raw material for pasta production. Wheat is classified into three groups, the diploids, the tetraploids and the hexaploids. This classification goes back to the breeding of different wheat species in the course of time. Initially, the diploid genome BB of a wild grass species (*Aegilops speltoides*) was crossbred with the genome AA of einkorn (diploid). The outcome of this was the genome AABB of emmer (tetraploid). The additional crossbreeding of this genome with the genome DD of another wild grass species (*Aegilops tauschii*) led to spelt (*T. aestivum ssp. spelta*), which represents the first hexaploid form of wheat and is still

cultivated today. However, the hybridization to AABBDD is supposed to have happened spontaneously, not by breeding (Feuillet et al. 2008). Common wheat belongs to the hexaploid and durum wheat to the tetraploid class (Franke 1989).

During the last 15 years the amount of wheat harvested worldwide increased by about 20 % (from 586 to 729 million tons) (source: FAOStat, 2016). This is not very remarkable considering the outstanding role of wheat as bread cereal and the rising world population. The main constituents of wheat grains are carbohydrates (70 %), proteins (10-13 %), lipids (2 %) and minerals (1.8 %) (Souci et al. 2008), but also processing of wheat grains into flour affects the composition. The content of vitamins, minerals and fiber rises with increasing extraction rate during milling, because these substances mainly occur in the external layers of the wheat grain. In the industrialized countries the consumption of bread covers 50 % of the carbohydrates, 30 % of the proteins and 50-60 % of the B-vitamins required for human nutrition and is essential for the supply with minerals and trace elements (Belitz et al. 2001). Morphologically, the wheat grain is divided into pericarp, episperm, aleurone layer, kernel and germ. Kernel and aleurone layer together are called endosperm. The endosperm makes up about 85 % of the wheat grain and most of the proteins are located there.

1.3 Wheat proteins

In 1906, T. B. Osborne has developed a method to separate wheat proteins into different subfractions according to solubility (Osborne 1906). Albumins, globulins and gliadins can be extracted consecutively by using water, a buffered solution of sodium chloride and aqueous alcohol, while the glutenins remain in the residue and can only be extracted with a diluted base (used by Osborne 1906) or a reducing agent in aqueous alcohol (used by later working groups, e.g. Wieser et al. 1998). Gliadins and glutenins together form the so-called gluten, the wheat glue, which is decisive for the baking quality of wheat flour. The ratio of gliadins to glutenins is between 1.5:1 and 3.1:1 (Wieser and Koehler 2009). Albumins and globulins, which are non-gluten proteins, mainly occur in the outer layers of the wheat grain and mostly consist of metabolic and protective proteins. Some gliadins are also soluble in water and can, therefore, be found in the albumin fraction (Wieser et al. 1998). Thus, a combined extraction of the albumins and globulins with aqueous salt solution within the

Osborne fractionation has been developed in more recent studies (Wieser et al. 1998) to prevent part of the gliadins from being dissolved. These non-gluten proteins, which are about 15-20 % (albumins about 11 %, globulins about 5 %) of the total flour protein, are nutritionally more valuable than gluten proteins due to their amino acid composition with higher amounts of the essential amino acid lysine (Wrigley and Bietz 1988), but play a minor role in breadmaking.

1.4 Gluten proteins

Upon addition of water, wheat flour forms a cohesive, viscoelastic dough. The formed protein network gives excellent gas holding capacity to the dough, making it possible to obtain a leavened bread with an elastic crumb and a crispy crust. The two main components of wheat gluten proteins are the gliadins and the glutenins. The optimal ratio of gliadins to glutenins is important for the properties of gluten (Wieser et al. 1994). It is possible to obtain the gluten from dough by washing out the starch with water or salt solution (Beccari 1745, translated by Bailey 1941). The amount of wet gluten isolated with this method is about 30 % of the flour weight. The dry gluten consists of about 90 % protein (mainly gliadins and glutenins), 8 % lipids and 2 % carbohydrates (Belitz et al. 2001). The diversity of gluten proteins is enormous; there are several hundred components, partly monomeric, partly aggregated. The latter are connected by covalent and non-covalent interactions (Wieser et al. 1991). Generally, reduced gluten protein components are divided into a low-molecular- (LMW-), a medium-molecular- (MMW-) and a high-molecular-weight (HMW-) group. The HMW-glutenin subunits (GS) with a molecular weight (MW) range of 67000-88000 belong to the HMW-group, the ω_5- and $\omega_{1,2}$-gliadins (MW 44000-55000 and 34000-44000, respectively) to the MMW-group and the LMW-GS, the γ- and α-gliadins (MW 32000-39000, 31000-35000 and 28000-35000, respectively) to the LMW-group (Krause et al. 1988, Wieser et al. 1991, Lagrain et al. 2013, Seilmeier et al. 2001, Mendez et al. 1998). The MW range of unreduced gluten proteins is 3×10^4 to 3×10^7. Due to their different properties, the various protein fractions and components have divergent functions in dough making. While gliadins are essential for viscosity and extensibility, glutenins play a key role for elasticity and strength of the dough (Khatkar et al. 1995). Disulfide bonds, but also non-covalent bonds like hydrophobic interactions, hydrogen bonds or electrostatic ionic bonds contribute to these properties. Repetitive amino

acid sequences and an extreme amino acid composition (more than 50 mol-% of the total amount are proline and glutamine) are characteristic for gluten proteins, but their physiological value is low due to their low amounts of essential amino acids like methionine (0.0-1.6 mol-%), threonine (0.4-3.8 mol-%) and lysine (0.2-1.1 mol-%) (Tab. 1).

Table 1: Amino acid composition [mol-%][a] of gliadin types and glutenin subunits of the wheat cultivar Rektor (Wieser et al. 1991)

	HMW-GS[b]	ω_5-gliadins	$\omega_{1,2}$-gliadins	LMW-GS[b]	α-gliadins	γ-gliadins
Asx	0.7-0.9	0.3-0.5	0.5-1.3	0.7-1.5	2.7-3.3	1.9-4.0
Thr	3.2-3.8	0.4-0.6	0.8-2.3	1.8-2.9	1.5-2.3	1.6-2.4
Ser	6.4-8.4	2.6-3.3	5.8-6.3	7.7-9.5	5.3-6.6	4.9-6.8
Glx	35.9-37.0	55.4-56.0	42.5-44.9	38.0-41.9	35.8-40.4	34.2-39.1
Pro	11.2-12.8	19.7-19.8	24.8-27.4	14.0-16.2	15.0-16.6	15.8-18.4
Gly	18.2-19.8	0.6-0.8	0.9-2.1	2.3-3.2	1.9-3.9	2.0-3.0
Ala	2.9-3.5	0.2-0.3	0.3-1.3	1.7-2.3	2.6-4.1	2.8-3.5
Cys	0.6-1.3	0.0	0.0	1.9-2.6	1.9-2.2	2.2-2.8
Val	1.6-2.7	0.3	0.6-1.4	3.8-5.3	4.2-4.9	4.4-5.4
Met	0.1-0.3	0.0	0.0-0.3	1.2-1.6	0.4-0.9	1.2-1.6
Ile	0.7-1.1	4.3-4.7	1.9-3.5	3.6-4.4	3.6-4.6	4.0-4.6
Leu	3.1-4.3	2.7-3.3	3.9-5.3	5.3-7.5	6.5-8.7	6.4-7.3
Tyr	5.1-6.4	0.6-0.7	0.8-1.5	0.9-1.9	2.3-3.2	0.6-1.4
Phe	0.2-0.5	9.0-9.5	7.6-8.1	3.8-5.5	2.9-3.9	4.7-5.6
His	0.8-1.9	1.3-1.4	0.6-1.1	1.3-1.8	1.4-2.8	1.1-1.5
Lys	0.7-1.1	0.4-0.5	0.3-0.6	0.2-0.6	0.2-0.6	0.4-0.9
Arg	1.6-2.1	0.5-0.6	0.5-1.4	1.5-2.1	1.7-2.9	1.2-2.9

[a] without tryptophan
[b] HMW: high molecular weight; LMW: low molecular weight; GS: glutenin subunit

1.4.1 Gliadins

Gliadins are mainly present as monomers in flour and gluten and are generally classified in four different subclasses or types, the ω_5-, $\omega_{1,2}$-, α- and γ-gliadins (Bietz and Wall 1972, 1973, 1980, Wieser 1996). The N-terminal sequences and the different MWs are the criteria for differentiation. The MW range of gliadins is between

28000 and 55000. The amino acid sequences of gliadins are dominated by proline and glutamine, while the physiologically important amino acid lysine plays a minor role (Tab. 1). The ω-gliadins have the highest amounts of proline, glutamine and phenylalanine. About 80 % of the ω-gliadins consist of these three amino acids (Shewry et al. 1986). The ω-gliadins are called „poor in sulfur“, because they lack cysteine and methionine. The α- and γ-gliadins are called rich in sulfur (about 2-4 % cysteine and methionine) and the cysteine residues form intramolecular disulfide bonds (Shewry et al. 1986).The γ-gliadins usually have eight and the α-gliadins six cysteine residues (Köhler et al. 1993, Wieser 2003). The gliadins are soluble in aqueous alcohols and dilute acids. The total amount of gliadins is slightly higher than 50 % of the wheat proteins. The α-gliadins, which have the lowest MW, are the quantitatively dominating group, followed by the γ-gliadins. The different types can be separated easily by reversed-phase high-performance liquid chromatography (RP-HPLC) in their unreduced forms. The separation order is ω_5-, $\omega_{1,2}$-, α- and γ-gliadins (Bietz 1983, 1985) according to hydrophobity.

Today, mainly hexaploid common wheat (*Triticum aestivum* L.) is used for breadmaking. This species has 42 chromosomes. They belong to three different genomes (A, B and D), which consist of seven pairs of chromosomes each. Each pair of chromosomes has a long and a short arm (Payne 1987). Genetic studies on gliadins by Wrigley and Shepherd (1973) revealed that all gliadin sequences are encoded on wheat chromosomes 1A, 1B, 1D and 6A, 6B 6D. While the genes of the ω-gliadins and most of the γ-gliadins (Gli-1-genes) are located on the short arms of the chromosomes 1 (Gli-A1, Gli-B1, Gli-D1) very close to each other, the three loci Gli-A2, Gli-B2, Gli-D2, which can be found on the short arms of the chromosomes 6A, 6B and 6D, code for α-gliadins (Payne et al. 1984).

1.4.2 Glutenins

In contrast to the gliadins, the glutenins form disulfide-linked polymers (Huebner and Wall 1976, Kieffer and Belitz 1981, Werbeck and Belitz 1988, 1993, Werbeck et al. 1989). They are divided into the HMW- (MW 67500-88000), the LMW- (MW 32000-39000) GS and the ω_b-gliadins. Both intramolecular and intermolecular disulfide bonds can be found within the glutenin fraction, of which the latter allow the formation of protein aggregates with MWs from about 100000 up to ten million (Graveland et al.

1982, 1985). Additionally, the so-called ω_b-gliadins occur within the glutenin fraction. Although they biochemically belong to the gliadin fraction, they cannot be extracted with aqueous alcohols due to intermolecular disulfide bonds with GS. In terms of the amino acid composition, HMW-GS are different to the other gluten protein types. They have a lower amount of proline, but the amounts of glycine (18.2-19.8 mol-%) and tyrosine (5.1-6.4 mol-%) are relatively high.The LMW-GS are similar to the α- and γ-gliadins (Tab. 1).

The major part of the glutenins (about 60 %) is composed of LMW-GS followed by HMW-GS (30 %). The rest (10 %) can be assigned to gliadins with an odd number of cysteine residues in their amino acid sequence (ω_b-gliadins). The nomenclature of HMW-GS is based on the work of Payne et al. (1981). In their initial studies, HMW-GS were designated numbers from 1 to 12 with HMW-GS 1 to 7 belonging to the x-type and 8 to 12 to the y-type. In contrast to the gliadins, glutenins are not soluble in aqueous alcohols. When a reducing agent such as dithiothreitol (DTT) is added, the glutenins can be dissolved in aqueous alcohols as GS. Often, disaggregating reagents such as urea or SDS are added together with DTT to further improve the solubility. If urea or SDS solutions without reducing agent are used for extraction, only a part of the glutenins is dissolved. The part that is not soluble in SDS-solution is called glutenin macropolymer (GMP) (Graveland et al. 1982) and is mainly built up of HMW- and LMW-GS. The amount of GMP is strongly related to the dough strength and the bread volume (Weegels et al. 1996), which is why it is important for the quality of wheat flours. Its content is about 20-40 mg/g flour (Müller et al. 2016).

Analogous to α- and γ-gliadins, the genes coding for LMW-GS are located on the short arms of the wheat chromosomes 1A, 1B and 1D. In general, the LMW-GS are very similar to the α- and γ-gliadins considering the MW, the number and position of the cysteine residues, the intramolecular disulfide bonds and the amino acid composition. Each wheat cultivar has about 9 to 16 different LMW-GS, which are subdivided into B-, C- and D-types due to their different mobility in SDS-PAGE. The HMW-GS of wheat are encoded on loci Glu-A1, Glu-B1 and Glu-D1, located on the long arms of chromosomes 1A, 1B and 1D (Payne et al. 1984, Payne et al. 1987). Both types of HMW-GS are coded on each locus, an x-type of higher MW and a y-type of lower MW (Payne et al. 1981). Based on the genetic information, six different HMW-GS in each wheat cultivar would be expected. However, some of these genes

do not express proteins. The genes of the x-type HMW-GS on the Glu-1A-locus and of the y-type HMW-GS on the Glu-1B-locus are not always expressed and the gene of the y-type HMW-GS on the Glu-A1-locus is generally not expressed. Thus, the number of HMW-GS in wheat cultivars investigated so far varies between three and five.

The ratio of HMW- and LMW-GS is influenced by both genotype and environment and affects numerous end-use properties of wheat. The amount of nitrogen (N), which is available to the plant during growth, significantly affects the amounts of all protein types and also the crude protein content, which is highly correlated with the baking quality of wheat. Wieser and Seilmeier (1998) showed that the quantities of albumins and globulins were not influenced by different levels of N-fertilization. In contrast, the effect on the storage proteins, especially the gliadins, was enormous. The gliadin/glutenin ratio (1.4.3) was increased by fertilization. The gluten protein types present in higher concentrations (LMW-GS, α- and γ-gliadins) were more strongly influenced than the others occurring in lower contents. The x-type HMW-GS showed higher increases induced by fertilization than the y-type. Also, a dependence on the cultivar was found confirming the influence of the genotype on the effectivity of fertilization. All in all, the effect of N-fertilization was stronger in the more hydrophilic proteins than in the more hydrophobic ones (Wieser and Seilmeier 1998). The fertilization with Sulphur (S), however, had a weaker influence on the crude protein content. A lack of S caused a significant increase of ω-gliadins, which are low in S. S-deficiency during growth increased the ratio of HMW- to LMW-GS (MacRitchie and Gupta 1993, Wieser et al. 2004). This relative proportion of the GS also influences the molecular weight of the polymers. The higher the molecular weight of the polymers, the more HMW-GS were present in comparison to the LMW-GS (Larroque et al. 1997). Additionally, the gliadin/glutenin-ratio increased under S-deficiency (Wieser et al. 2004). The molecular weight distribution of the gluten proteins is correlated with the baking quality of wheat cultivars. Singh and MacRitchie (2001) postulated that only polymers of a certain size have an influence on the elasticity of the protein network. Experiments with different wheat cultivars showed that genotypes with a higher ratio of HMW-GS to LMW-GS have a higher gluten strength (Gupta 1992). The x-type predominates within HMW-GS compared to the y-type; their ratio is specific for each cultivar and is typically between 1.4 and 3.2 (Wieser and Kieffer 2001).

The climate during plant growth plays an important role in the formation of gluten proteins. Extreme frost or heat have a decisive effect on the baking quality. While average temperatures below 30 °C increased dough firmness, warmer average temperatures led to softer doughs. Reasons for that are changes in the polymer/monomer-ratio, a decrease in the amount of GMP and an increase of the gliadin/glutenin-ratio (MacRitchie and Lafindra 1997, Southan and MacRitchie 1999).

HMW-GS are commonly linked to the baking quality of wheat cultivars due to their contribution to gluten formation. The presence or absence of certain types as well as their concentration are associated with good or poor baking properties. The allele pair Dx5+Dy10 is considered to be best for breadmaking, while the pairs Bx6+By8 and Dx2+Dy12 are rated worst (Payne et al. 1987). The x-types (seven in total) of the HMW-GS are more important for good baking quality than the y-types (five in total). The highest contributions are ascribed to subunit Dx5, which has an additional cysteine residue, and subunit Bx7, which occurs most frequently in wheat (Wieser and Zimmermann 2000). The higher concentration of GMP found in wheat cultivars with subunits Dx5+Dy10 in comparison to cultivars with subunits Dx2+Dy12 confirmed the strong correlation of the GMP content, the composition and the concentration of HMW-GS with dough elasticity and bread volume (Moonen et al. 1982, Southan and MacRitchie 1999, Kelfkens and Lichtendonk 2000, Müller et al. 2016). Because of the importance of the different subtypes of the HMW-GS for the baking quality, an evaluation system was developed for wheat cultivars by Payne et al. (1987), which has been called “Payne-Score”.

The influence of LMW-GS on the rheological properties of wheat doughs is still unclear. The main reasons are that the separation of individual subunits has not been successful and the similarity of LMW-GS to gliadins is too high. Studies by Payne and Corfield (1979) showed that the relative proportion of GS to each other changes with decreasing molecular weight of the glutenin. The lower the MW of the glutenin polymers, the more subunits with smaller MW are present in the polymers compared to subunits with higher MWs. This indicates that LMW-GS have a stronger influence on the extensibility and not on the elasticity of the dough (Cornish et al. 2006). In general, the MW distribution of the glutenins is considered to be an important indicator of the baking quality of a wheat cultivar. However, this distribution mostly

depends on the disulfide bonds. These are influenced by several genetic, environmental or processing factors (Wieser et al. 2006).

1.4.3 Gliadin/glutenin-ratio

Other factors important for the baking quality are the amounts of gliadins and glutenins, the resulting gliadin/glutenin-ratio and the total amount of gluten in wheat flour. A balanced ratio of both protein fractions is essential for wheat dough with good viscoelastic properties, because gliadins and glutenins fulfil different functions in the dough (Belton 1999, Singh and MacRitchie 2001, Wieser and Kieffer 2001). The gliadins serve as “plasticizers” or “solvents” for the glutenins (Wieser and Kieffer 2001). Doughs with an excess of gliadins are therefore viscous and soft, while an excess of glutenins leads to doughs with too much firmness and strength. The typical ratio of gliadins to glutenins in common wheat is between 1.5 and 3.1 (Wieser and Köhler 2009). As already mentioned earlier (1.4.1 and 1.4.2) the gliadin/glutenin-ratio can be influenced by genetic and environmental effects such as fertilization, soil or climate, which then directly affect the baking quality of wheat cultivars.

1.4.4 HMW-gliadins (HGL)

A small part of the gliadin fraction consists of oligomers with subunits linked by intermolecular disulfide bonds. These have been named differently by various working groups. Terms such as HMW-gliadins (HGL), aggregated gliadins or ethanol-soluble glutenins have been defined (Beckwith et al. 1966; Bietz and Wall 1980; Shewry et al. 1983; Huebner and Bietz 1993). The diversity of descriptions shows that these proteins have properties of both gliadins (solubility in aqueous alcohols) and glutenins (subunits linked by intermolecular disulfide bonds). Studies by Bietz and Wall (1980), Shewry et al. (1983) and Tatham et al. (1987) indicated that the amino acid sequences of HGL are similar to S-rich prolamins like α- and γ-gliadins, while they showed clear differences to those of HMW-GS or ω-gliadins. Later studies on the N-terminal amino acid sequences showed that α- and γ-gliadins, but also LMW-GS are part of HGL (Huebner and Bietz 1993). The secondary structure of HGL was described to be 34-37 % α-helix, 18-24 % β-sheet and 39-48 % β-turn and random coil. Studies on the amino acid composition showed high amounts of

glutamic acid and glutamine (35-38 mol-%), proline (15-17 mol-%) and cysteine (2-3 mol-%) (Tatham et al. 1987). This composition is very similar to those of S-rich prolamins like α- and γ-gliadins. Wieser (1994) revealed that the influence of HGL on the rheological properites of wheat doughs is more similar to those of total gliadins than to total glutenins. The percentage of HGL in total gliadins depends on the wheat cultivar. Experiments with analytical gel permeation chromatography (GPC) revealed amounts between 13 and 20 % of total gliadins (Wieser 1994). It was postulated that modified α- and γ-gliadins with an odd number of cysteine residues in their amino acid sequence act as so-called terminators and stop the polymerization of glutenins (Kasarda 1989), which could be an indicator for the formation pathway of HGL. Köhler et al. (1993) identified an intermolecular disulfide bond between a γ-gliadin and a LMW-GS in the glutenin fraction, which matches this theory. However, it is still unclear, if HGL naturally occur in the wheat grain, or, if they are an artefact formed during protein extraction caused by thiol-disulfide exchange reactions. All in all, HGL are poorly characterized compared to the other components of wheat gluten.

1.4.5 Positions of cysteine residues in gluten protein types

All amino acid sequences of the gluten protein types are divided into characteristic domains (Fig. 1). Despite its comparatively low amount of about 2 % of the total composition of amino acids, cysteine plays an essential role for the structure and functionality of gluten (Wieser 2003, Grosch and Wieser 1999). The cysteine residues of gluten protein types shown in Fig. 1 are named according to Köhler et al. (1993) in this thesis.

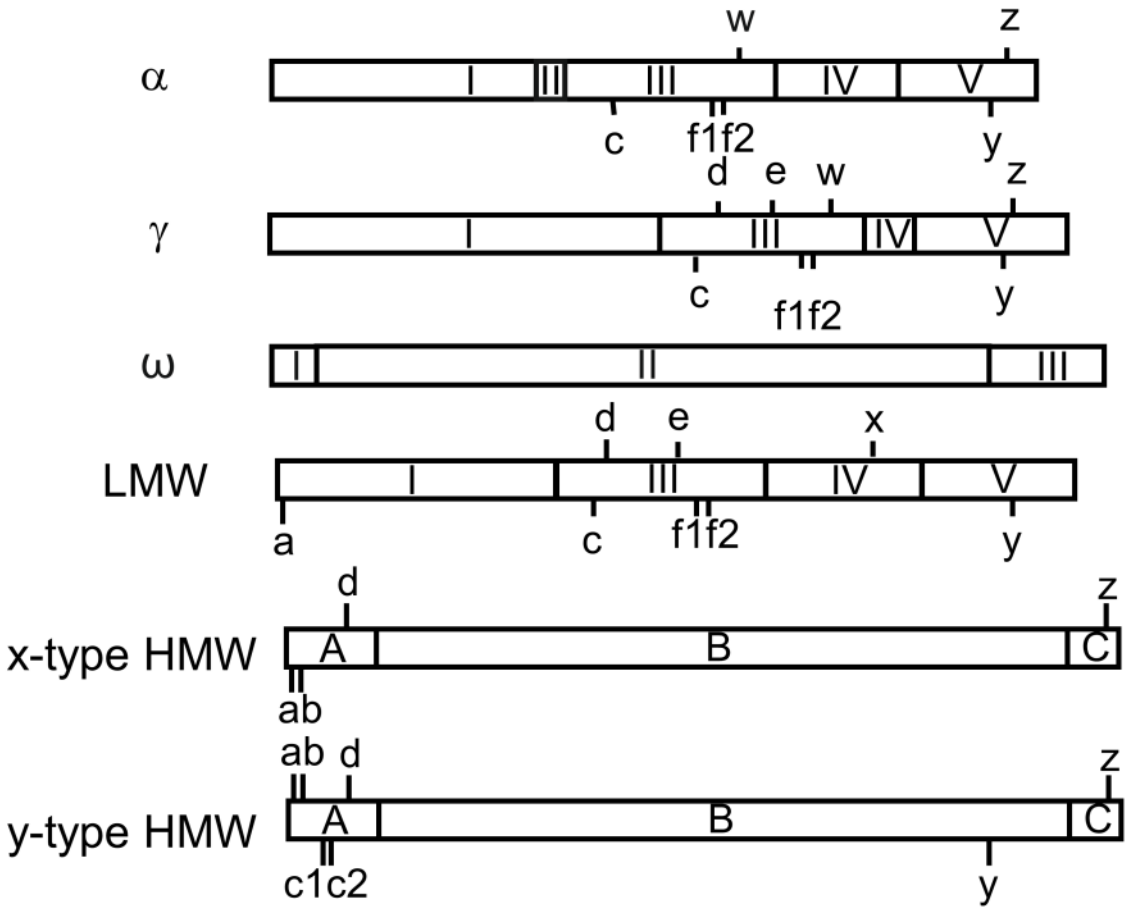

Figure 1: Schematic representation of sequence domains and positions of cysteine residues of gluten protein types according to Wieser and Grosch (1999) and Wieser (2007); α: α-gliadin, γ: γ-gliadin, ω: ω-gliadin, LMW: low-molecular-weight, HMW: high-molecular-weight

The α- and γ-gliadins as well as LMW-GS are encoded on the same parts of the wheat genome (1.4.1 and 1.4.2) and also have structural similarities (Fig. 1). The α-gliadins are divided into five domains according to Kasarda et al. (1984) and the first two domains account for about 40-50 % of the primary structure of this protein type. Domain I is the N-terminal domain, which is poor in cysteine and has high amounts of glutamine, proline and phenylalanine. It is characterized by repetitive sequences like for example QPQPFPPQQPYP, while domain II only consists of glutamine. The C-terminal part is rich in cysteine and is composed of the domains III, IV and V, of which III and V are homologous (Köhler et al. 1995). The differences of these domains compared to domains I and II are that they have less glutamine and proline, but higher amounts of amino acids with an S-atom in their side chain, mainly cysteine (Wieser 2001), and that they do not have repetitive sequences. The γ-gliadins also have an N-terminal domain poor in cysteine, and a C-terminal domain rich in cysteine (Kasarda et al. 1984). Other characteristic features of the N-terminal part of the

sequence are also repetitive sequences, like for example PQQPFPQ. Domain II of the α-gliadins, which consists of poly-glutamine, is missing. The LMW-GS are divided into four domains analogous to the γ-gliadins and also have an N-terminal domain poor in cysteine (domain I) as well as a C-terminal domain rich in cysteine (domains III, IV and V) (Kasarda et al. 1984). Just like in α- and γ-gliadins, the N-terminal part of the sequence (domain I) is characterized by high amounts of glutamine, proline and phenylalanine as well as repetitive sequences like Q_nPPFS with n = 2-10. Another aspect the LMW-GS have in common with α- and γ-gliadins are high amounts of amino acids with an S-atom in their side chain (for example all cysteine residues of the sequence) in the three C-terminal domains III, IV and V (Wieser 2001).

Usually, α-gliadins contain six cysteine residues, all of them are located in the C-terminal part of the sequence (Kasarda et al. 1984, Anderson et al. 1984, Reeves and Okita 1987) (Fig. 1). The cysteine residues C^c, C^{f1}, C^{f2} and C^w are located in domain III, the residues C^y and C^z in domain V. Additionally, a modified sequence of α-gliadins probably generated by point mutation has been published by Okita et al. (1985). In this sequence an additional cysteine residue can be found in domain IV. The γ-gliadins typically contain eight cysteine residues, which are located in the C-terminal part of the sequence in analogy to the α-gliadins. Six of them (C^c, C^d, C^e, C^{f1}, C^{f2} and C^w) are present in domain III and two (C^y and C^z) in domain V (Scheets et al. 1985, Bartels et al. 1986, Rafalski 1986). Furthermore, two genetic modifications with cysteine replacing serine either in domain I (Scheets and Hedgcoth 1988) or in domain III have been described (Shewry and Tatham 1997). Similar to the γ-gliadins the LMW-GS usually have eight cysteine residues. All of them are also located in the C-terminal section of the sequence with the exception of cysteine residue C^a occurring in domain I. Six of these cysteine residues (C^c, C^d, C^e, C^{f1}, C^{f2} and C^y) can be found at the same positions as in the amino acid sequence of γ-gliadins. This emphasizes the strong genetic relationship of these two gluten protein types. The eighth cysteine residue is located in domain IV (Okita 1984, Okita et al. 1985, Colot et al. 1989, Lew et al. 1992). Genetic variations have been found, which either have an additional cysteine residue in domain III (Pitts et al. 1988) or lack the cysteine residue C^a.

The ω-gliadins are commonly classified into ω_5- (molecular weight approximately 50000) and $\omega_{1,2}$-gliadins (molecular weight approximately 40000) (Wieser 2007), which have similar structures. In large parts they consist of repetitive sequences (e.g. PQQPFPQQ) (Wieser 2007) and have no cysteine in their sequences (Wieser 2007). One exception is a modified sequence identified by König et al. (2010) with a cysteine residue at the C-terminal end of the amino acid chain generated by point mutation (Fig. 2). This protein type is called ω_b-gliadins

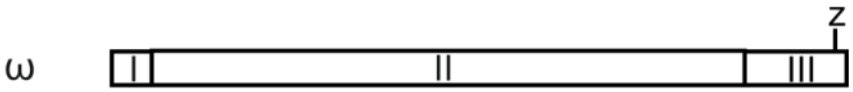

Figure 2: Schematic representation of sequence domains of ω-gliadins. A small portion of ω-gliadins contains the additional cysteine residue c^z and is called ω_b-gliadins

Both x- and y-types of HMW-GS can be subdivided into three domains, the N-terminal domain A, the central domain B consisting of repetitive sequences and the C-terminal domain C (Shewry et al. 1986, Halford et al. 1987, 1992, Anderson et al. 1989, Anderson and Greene 1989) (Fig. 1). In domain A that consists of 80-105 amino acid residues, no repetitive sequences are present. The repetitive domain B is composed of significantly more amino acids (480-700) (Shewry et al. 1992). After that, the relatively short domain C with 42 amino acids completes the sequence (Shewry et al. 1992). The repetitive sequence QQPGQG is the main building block of domain B. It is occasionally interrupted and filled by repetitive insertions like YYPTSP, QQG or QPG. The most common amino acids in domain B are glutamine, proline and glycine. In contrast, domains A and C include a lot of amino acids with charged side chains like glutamine, asparagine, arginine and lysine. The x- and the y-type of the HMW-GS are distinguished by variations in the domains A and B. Usually four cysteine residues can be found in the sequence of the x-type HMW-GS, three of them (C^a, C^b, C^d) in domain A and one (C^z) in domain C (Sugiyama et al. 1985, Anderson and Greene 1989, Halford et al. 1992). Subunit 1Dx5 is an exception, because it has an additional cysteine residue (C^e), which is located at the beginning of domain B (Anderson et al. 1989). The seven cysteine residues typical of y-type HMW-GS are usually located in domain A (C^a, C^b, C^{c1}, C^{c2}, C^d), but also in the domains B (C^y) and C (C^z) (Halford et al. 1987, Anderson et al. 1989).

1.4.6 Disulfide bonds of wheat gluten

Cysteine plays a minor role in wheat flour protein with an amount of about 5 %, of which 0.5 % are low-molecular-weight thiols and 4.5 % are protein-bound cysteine residues (Wieser et al. 1998). The major part of cysteine is present as cystine and forms either intramolecular disulfide bonds between cysteine residues located in one protein chain or intermolecular bonds between cysteine residues of two different amino acid chains. The formation of disulfide bonds occurs just after synthesis of the protein chains in the lumen of the endoplasmatic reticulum and affects protein functionality by influencing their folding (Kasarda 1999). Possible variations of formation during processing are thiol-disulfide exchange reactions or oxidation (Grosch and Wieser 1999). The latter is favored when using oxidation reagents, like for example potassium iodate or potassium bromate, while thiol-disulfide exchange reactions can be induced by free thiol groups present as thiolate like for example in glutathione due to neutral or alkaline pH or high temperatures (Lagrain et al. 2006).

The intramolecular disulfide bonds of both α- and γ-gliadins have been fully characterized by different working groups (Fig. 3) (Müller and Wieser 1995, 1997; Köhler et al. 1997). Three crosslinks between the cysteine residues C^{c}/C^{f1}, C^{f2}/C^{y} and C^{w}/C^{z} are present in both protein types, but γ-gliadins form an additional disulfide bond between the cysteine residues C^{d}/C^{e}. Due to the additional cysteine residue c^{z}, ω_b-gliadins form intermolecular disulfide bonds to GS and, therefore, belong to the glutenin fraction (König et al. 2010). The so-called γ-bound gliadins are comparable. They are modified γ-gliadins with the additional cysteine residue C^{b*} that is able to form an intermolecular disulfide bond with LMW-GS (Köhler et al. 1993). The cysteine residues C^{b*} and C^{x} of LMW-GS could be responsible for chain termination during the polymerization of the GS during gluten formation.

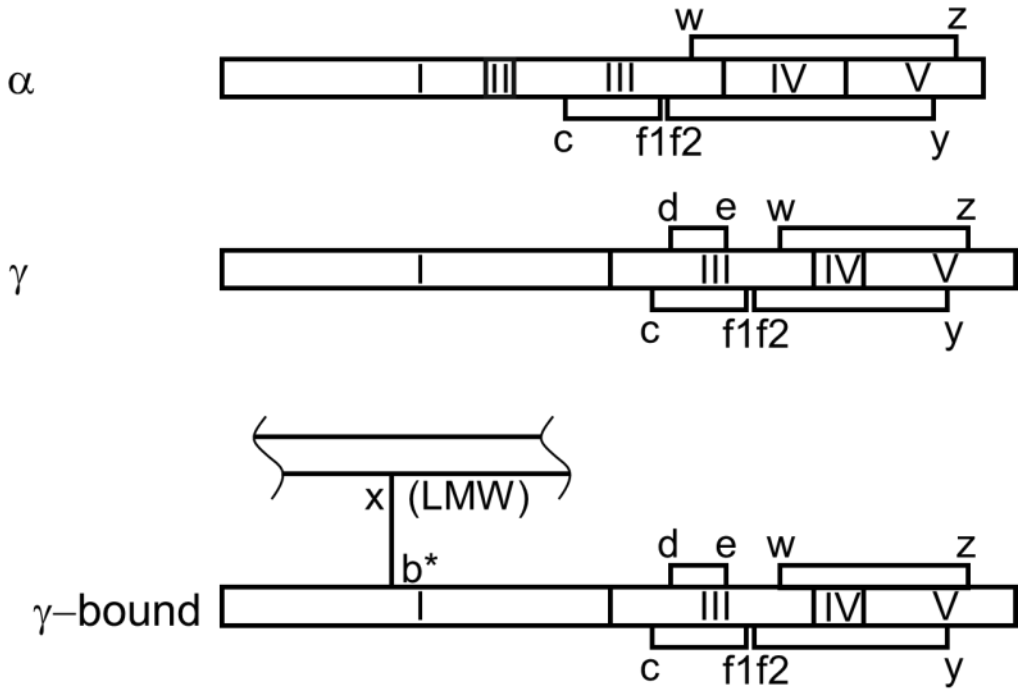

Figure 3: Schematic representation of disulfide bonds of gliadins according to Grosch and Wieser (1999) and Köhler et al. (1993); α: α-gliadins; γ: γ-gliadins; γ-bound: γ-gliadin linked with LMW-GS

Contrary to the gliadins, both intramolecular and intermolecular disulfide bonds are present in LMW- and HMW-GS, which occur as polymers in their native state (Shewry and Tatham 1997). As mentioned before (1.4.5) six of the eight cysteine residues of LMW-GS are homologous to those of the α- and γ-gliadins. These six cysteine residues are considered to form three intramolecular bonds C^{c}/C^{f1}, C^{f2}/C^{y} and C^{w}/C^{z} (Fig. 4) (Köhler et al. 1993, Keck et al. 1995, Müller and Wieser 1997). The cysteine residue C^{a} has been postulated to form intermolecular disulfide bonds, but an experimental proof is missing up to now (Köhler et al. 1993). The cysteine residues C^{b*} and C^{x}, which are usually missing in the monomeric α- and γ-gliadins, are thought to be responsible for the aggregative nature of LMW-GS. Disulfide bonds of these two cysteine residues either with the γ-gliadins (C^{b*}/ C^{b*}) or with other LMW-GS (C^{b*}/ C^{b*}; C^{b*}/ C^{x}) have been found (Köhler et al. 1993, 1997). Additionally, C^{x} is able to form a disulfide bond with the cysteine residue C^{y} of y-type HMW-GS. This crosslink has been identified by Keck et al. (1995) for the first time. The cysteine residue C^{b*} was found by Köhler et al. (1993) in a position previously unknown for SH-groups in the amino acid sequence of LMW-GS. It appears to be no standard position of an SH-group within LMW-GS.

Just like LMW-GS, HMW-GS usually do not occur as monomers in their native state. Therefore, it can be assumed that they also form both intramolecular and intermolecular disulfide bonds. However, due to their low abundance, not all crosslinks involving these proteins have been found so far. Evidence for an intramolecular bond between the cysteine residues C^a and C^b in domain A of the x-type as well as two neighboring intermolecular disulfide bonds (C^{c1}/C^{c1} and C^{c2}/C^{c2}) in domain A of the y-type has been found by Köhler et al. (1991, 1993). Lutz et al. (2012) identified a putatively intermolecular disulfide bond involving the cysteine residues C^e (unique to HMW-GS Dx5) and C^z (present in all x-type HMW-GS) (Fig. 4).

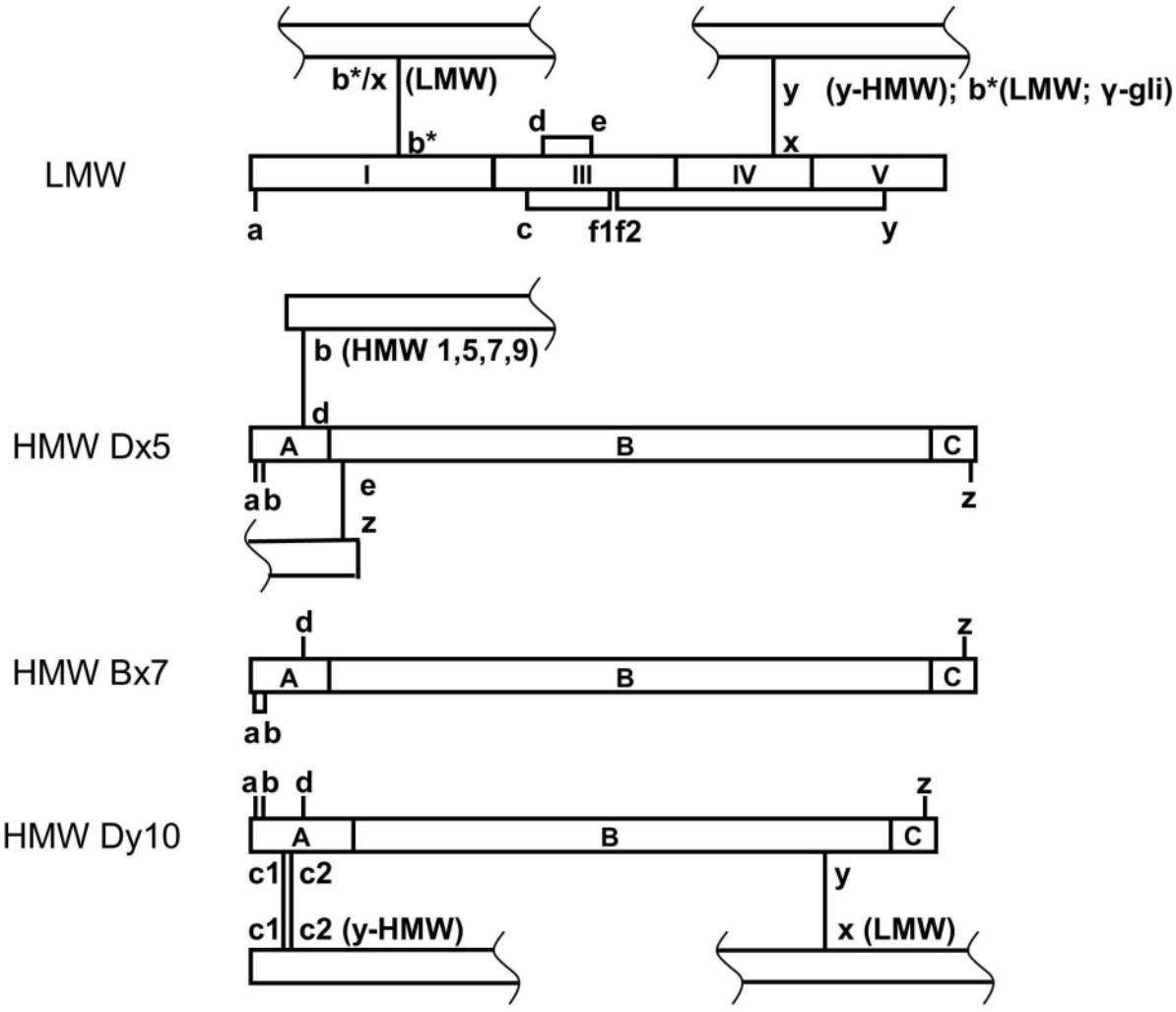

Figure 4: Schematic representation of disulfide bonds of LMW- and HMW-GS according to Lutz et al. (2012) and Grosch and Wieser (1999). Disulfide bonds of free cysteine residues have not been identified yet

This working group also identified intermolecular disulfide bonds between α- and γ-gliadins (C^w/C^d), α-gliadins and LMW-GS (C^z/C^x), γ-gliadins and LMW-GS (C^w/C^{b*})

and LMW-GS and α-, γ-gliadins or LMW-GS (C^x/C^y; C^d/C^y) respectively, which had not been detected so far (Lutz et al. 2012). However, more work will be neccessary to get more insight into the disulfide structure of wheat gluten. For example, a disulfide bond involving ω-gliadins has not been found so far, although a sequence containing a single cysteine residue is available (http://www.uniprot.org/ uniprot/B6ETS0, viewed 10.06.17) and was experimentally confirmed (König et al. 2010).

The disulfide bonds are formed after protein synthesis with assistance of the enzyme protein-disulfide-isomerase in the lumen of the endoplasmatic reticulum and intramolecular bonds are formed faster than intermolecular ones (Shewry et al. 1995, Shewry 1999). Some cysteine residues are not included in this disulfide formation and exist as free thiol groups (Wieser et al. 1998). Numerous investigations about redox reactions occuring during milling, dough preparation and baking and about the influence of redox reagents on the properties of dough and baking show the distinct influence of the disulfide structure on the baking quality (Joye et al. 2009). The addition of reducing agents like glutathione or cysteine leads to softer, more extensible doughs (Grosch and Wieser 1999) and causes a strong decrease of protein aggregation (Lawrence and Payne 1983, Werbeck and Belitz 1988). It can be assumed that intermolecular disulfide bonds are cleaved more easily than intramolecular ones, because the oligomeric HMW-GS have been detected in partially reduced glutenins (Lawrence and Payne 1983). The use of oxidizing agents, like for example potassium bromate, can strengthen the dough (Hahn et al. 1996). This reagent also makes it possible to slowly repolymerize LMW- and HMW-GS as shown by reoxidation experiments with isolated GS (Antes and Wieser 2001). The MW of the reoxidized protein polymers generated in these experiments can increase to several million. Reoxidation experiments with reduced gliadins showed that the formation of intramolecular disulfide bonds was preferred, while intermolecular ones were preferred with GS (Beckwith et al. 1965, Beckwith and Wall 1966). The addition of reoxidized, aggregated HMW-GS to flour (Schropp et al. 1995, 1996) caused stronger doughs. This matched with results found in work done by Seilmeier et al. (1991) and Wieser et al. (1994), who showed the positive influence of HMW-GS on the resistance to extension of wheat doughs. Reoxidation experiments with single HMW-GS done by Shani et al. (1992) showed that both x- and y-type HMW-GS form oligomers, but the x-type aggregated more rapidly than the y-type.

1.4.7 Influence of disulfide bonds on gluten structure

As already mentioned before (1.4.6), disulfide bonds play an essential role for the structure of gluten proteins. Based on experimentally determined disulfide bonds of proteins belonging to the LMW group of gluten proteins (α-, γ-gliadins, LMW-GS), Müller and Wieser (1997) postulated two-dimensional models of these protein types (Fig. 5-7). All of these three models have the disulfide bonds C^{c}/C^{f1} and C^{f2}/C^{y} of the two neighboring cysteine residues C^{f1} and C^{f2} in common. These crosslinks result in two so-called disulfide loops, one in domain III and one between domains III and V as starting and ending points. The loop between domains III and V is further subdivided in α- and γ-gliadins by the disulfide bond C^{w}/C^{z}, which causes an additional loop between these two sequence sections. This second loop is not present in LMW-GS. γ-Gliadins and LMW-GS have another loop in domain III in common, caused by the disulfide bond C^{d}/C^{e}, which does not exist in α-gliadins. Therefore, α-gliadins only have two small loops (AB and C) and one large loop (D) (Fig. 5). The loop C^{d}/C^{e} already mentioned earlier in γ-gliadins and LMW-GS is important for protein stabilization. γ-Gliadins form three small (A, B and C) as well as one large ring (D) caused by their four intramolecular disulfide bonds (Fig. 6). LMW-GS are characterized by one large (CD) and two small loops (A and B) due to the missing bond C^{w}/C^{z} (Fig. 7).

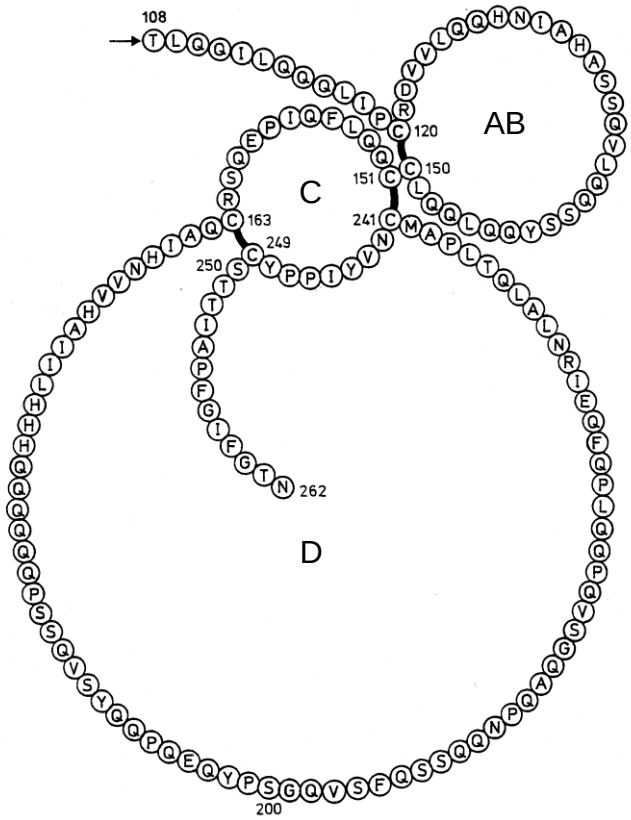

Figure 5: Schematic presentation of the structure of sequence domains III-V of α-gliadins according to Müller and Wieser (1997). Loops created by disulfide bonds are named AB, C and D. C^{c}: Pos. 120; C^{f1}: Pos. 150; C^{f2}: Pos. 151; C^{w}: Pos. 163; C^{y}: Pos. 241; C^{z}: Pos. 249

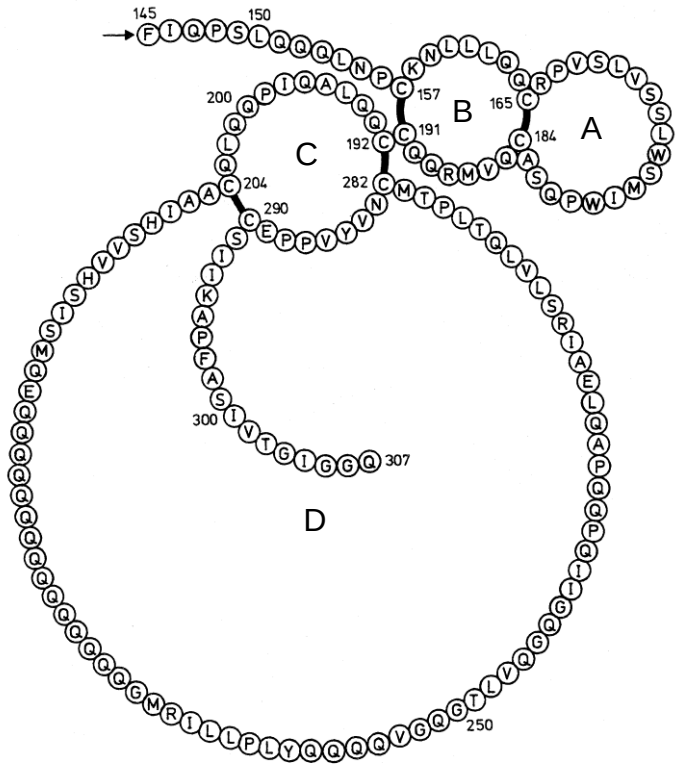

Figure 6: Schematic presentation of the structure of sequence domains III-V of γ -gliadins according to Müller and Wieser (1997). Loops created by disulfide bonds are named A, B, C and D. C^{c}: Pos. 157; C^{d}: Pos. 165; C^{e}: Pos. 184; C^{f1}: Pos. 191; C^{f2}: Pos. 192; C^{w}: Pos. 204; C^{y}: Pos. 282; C^{z}: Pos. 290

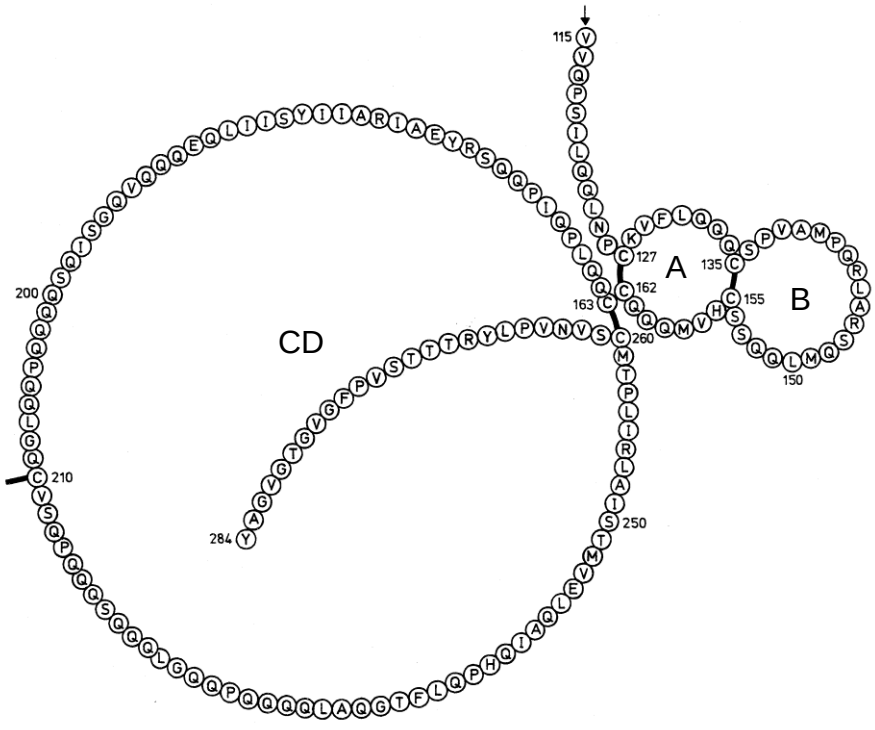

Figure 7: Schematic presentation of the structure of sequence domains III-V of LMW-GS according to Müller and Wieser (1997). Loops created by disulfide bonds are

named A, B and CD. C^c: Pos. 127; C^d: Pos. 135; C^e: Pos. 155; C^{f1}: Pos. 162; C^{f2}: Pos. 163; C^x: Pos. 210; C^y: Pos. 260

The structures of glutenins are hard to determine due to their numerous intra- and intermolecular disulfide bonds, their very high molecular weight, their poor solubility and the unknown contribution of non-covalent interactions to the structure. Due to the lack of experimental evidence, several models of the glutenins have been postulated, which are based either on linear or on highly branched glutenin polymers.

While Ewart (1968, 1972, 1977, 1979) suggested a linear polymer with random linkages of HMW- and LMW-GS, Graveland et al. (1985) postulated branched structures. The HMW-GS form the backbone of the structure. One y-type is always linked with two x-type subunits by head-to-tail crosslinks. The LMW-GS are linked to the y-type HMW-GS as branches or side chains. All of the following models are based on this model. Kasarda (1989) considered the ratio of HMW- and LMW-GS in his model (10-20 % and 80-90 %, respectively) and also assumed that the subunits are connected by intermolecular disulfide bonds. The structure Kasarda (1989) postulated is more branched and random than that published by Graveland et al.(1985), because the polymerization of subunits proceeds until a protein subunit with only one free thiol group stops it. The existance of oligomeric HMW-GS (Shewry et al. 1992), however, is not explained by this model. After more knowledge about the positions of disulfide bonds of gluten proteins had been gained (Köhler et al. 1993, Keck et al. 1995, Müller and Wieser 1995, 1997, Wieser and Müller 1996, 2000), another model based on the considerations of Graveland et al. (1985) was developed by Wieser et al. (2006) (Fig. 8). Like in the previous models, HMW-GS linked by intermolecular disulfide bonds form the backbone. The new aspect of this model is that the backbone is branched by polymeric LMW-GS (Lindsay and Skerritt 2000). Additionally, based on the quantitative composition of the glutenins (LMW-/HMW-GS ≈ 2/1 and x-type/y-type HMW-GS ≈ 2.5/1) and the molecular weight of the GS, a molecule-double-unit – built up by two x-type and four y-type HMW-GS – of glutenins was postulated as the backbone and not a single protein chain as it had been postulated in all previous models. A side-chain of about 30 LMW-GS branches off the central y-type HMW-GS and an x-type HMW-GS is linked to both of its ends. Another part of the model is the assumption that HMW-GS Dx5 forms an additional intermolecular disulfide bond in the N-terminal domain (Köhler et al. 1997). These building blocks have a molecular weight of about 1,500,000 and polymerize by

intermolecular disulfide bonds between x-type HMW-GS forming large polymeric protein aggregates. The interruption of this polymerization, which happens more often within LMW-GS than HMW-GS, is caused by gliadins with an odd number of cysteine residues in their sequence, low-molecular-weight thiols like glutathione or cysteine or other proteins with a single free thiol group (Hüttner and Wieser 2001a, 2001b). In all of these models, disulfide bonds play an essential role. This underlines their importance not only for the structure but also for for the function and properties of gluten proteins.

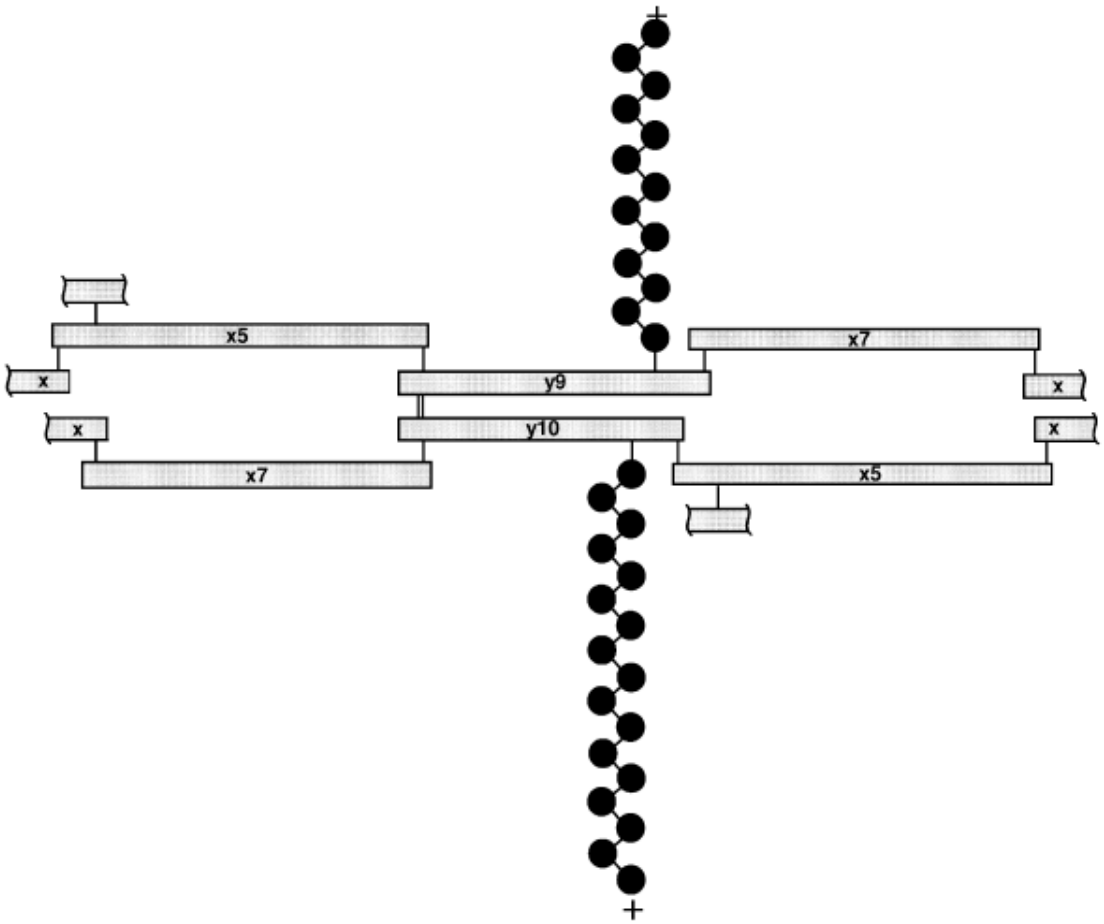

Figure 8: Model of of the backbone of the glutenin polymer formed by a double unit composed of LMW- and HMW-GS and connected by interchain disulfide bonds (Wieser 2007, adapted from Wieser et al. 2006). x, x-type HMW-GS; y, y-typeHMW-GS; ●, LMW-GS; +, chain terminator

1.4.8 Changes of disulfide bonds of wheat gluten induced by processing

During the baking process, numerous changes occur in the disulfide structure of gluten proteins. These changes provide an essential contribution to the properties of

bread crumb and crust. For example, air is included into the flour-water-mixture during kneading (Scanlon and Zghal 2001). A part of the disulfide bonds is cleaved by the influence of mechanic forces, which enables the formation of a continuous, viscoelastic gluten network (Singh and MacRitchie 2001). This cleavage affects about 20 % of the disulfide bonds provided that the dough gets in contact with air for at least ten minutes. These 20 % are reduced to thiol groups, which are oxidized back to disulfide bonds during fermentation (mellowness) (Graveland et al. 1980). Wieser (2003) postulated that thiol-disulfide exchange reactions between LMW-GS, glutathione, cysteine and other proteins occur during this process, which is neccessary for an optimal rearrangement of the partly reduced gluten proteins. The structure of gluten proteins caused by disulfide bonds gives properties to gliadins and glutenins, which are indispensable for the dough structure. Polymeric glutenins form a continuous network due to their intermolecular disulfide bonds, which is responsible for the elasticity and strength of the doughs. The monomeric gliadins with their intramolecular bonds act as „plasticizer“ for the glutenin network and are responsible for viscosity and extensibility of the doughs. Thus, it is obvious that the optimal ratio of gliadins and glutenins is essential for good dough and baking properties (Wieser et al. 1994) (1.4.3).

Nothing happens to the disulfide structure during the baking process until a temperature of about 40 °C is reached (Lefebvre et al. 2000). The gluten proteins start to unfold at about 45 °C, which leads to an increased exposion of hydrophobic groups and a resulting lower solubility of the proteins (Guerrieri et al. 1996). Gluten loses its functional properties at about 75 °C. The solubility decreases even more, because the size of the molecule aggregates grows (Booth et al. 1980, Schofield et al. 1983). The reasons can be both oxidation of free thiol groups (Singh and MacRitchie 2004) and an intensified thiol-disulfide exchange of all gluten protein types except the cysteine-free ω-gliadins (Schofield et al. 1983, Lagrain et al. 2005). This was confirmed by Wieser et al. (1998), who found clear differences between the solubility of gluten protein types of wheat bread and the corresponding flour under reducing and non-reducing conditions. The solubility of the gliadins in 60 % ethanol under non-reducing conditions was substantially lower in bread compared to the flour. The α- and γ-gliadins were more affected than the cysteine-free ω-gliadins. No differences were found under reducing conditions. At 80 °C and a moisture content of 20 %, the number of free thiol groups within the gluten proteins sank and a rise of the

number of disulfide bonds and an increased oxidation rate of thiol groups was observed (Weegels et al. 1994). Singh and MacRitchie (2004) investigated the temperature dependence of the polymerization by means of aqueous dispersions of gluten proteins. They verified that polymerization of the glutenins occured only at temperatures below 100 °C and that a decrease of alcohol-soluble gliadins combined with an increase of alcohol-insoluble glutenins happened at temperatures above 120 °C, which they adjusted using an autoclave. This points to a co-polymerization of gliadins and glutenins at these temperatures. Just like Weegels et al. (1994a, 1994b) postulated, the polymerization of the glutenins below 100 °C involves the oxidation of thiol groups. At higher temperatures, thiol-disulfide exchange reactions seem to be involved in the incorporation of gliadins (Singh and MacRitchie 2004) into the glutenins leading to structural changes (Morel et al. 2002). Lagrain et al. (2011) proved that these changes in the structure are mainly caused by β-elimination reactions within intramolecular disulfide bonds. These reactions support the formation of dehydroalanine and free thiol groups, which are then involved in thiol-disulfide exchange reactions with other SS-groups of the gliadins. This leads to a fast formation of intermolecular disulfide bonds and, therefore, to a polymerization of the gliadins. The addition of thiol-blocking reagents at temperatures below 100 °C decreased the cross-linking of gliadins and glutenins due to the lower concentration of free thiols, while the addition of reducing agents increased cross-linking. This also points out the high importance of free thiol groups, the disulfide structure of gluten proteins and thiol-disulfide exchange reactions for the baking process (Lagrain et al. 2005, 2006, 2007a, 2007b, 2008a, 2008b, 2008c). Increased pressure affects the disulfide structure similar to heating (Kieffer et al. 2007). Increasing pressure and temperature caused a decrease of the solubility of gliadins in 60 % ethanol. This was caused by a rearrangement of disulfide bonds between gliadins and glutenins. Intramolecular bonds are converted to intermolecular ones, which leads to an incorporation of α- and γ-gliadins into glutenin aggregates. This was proven by verifying two intermolecular disulfide bonds, which are not present in native gluten. They consisted of two peptides each from LMW-GS and γ-gliadins.

1.4.9 Glutathione and Cysteine

The oxidized form of cysteine called cystine was isolated for the first time from bladder stones by W. H. Wollaston in 1810 and from horn by L. Mörner in 1899 (Belitz et al. 2001). Glutathione was discovered in 1888 by de-Rey-Pailhade, who isolated it from baker's yeast (Meister 1988). At first, it was supposed that its structure was a dipeptide consisting of glutamic acid and cysteine. Later investigations by Hunter and Eagles (1927) and Hopkins (1929) revealed its true structure (Fig. 9): Glutathione is a tripeptide formed by the amino acids glutamic acid, cysteine and glycine. Glutamic acid forms a peptide bond with cysteine via its γ-carboxy group.

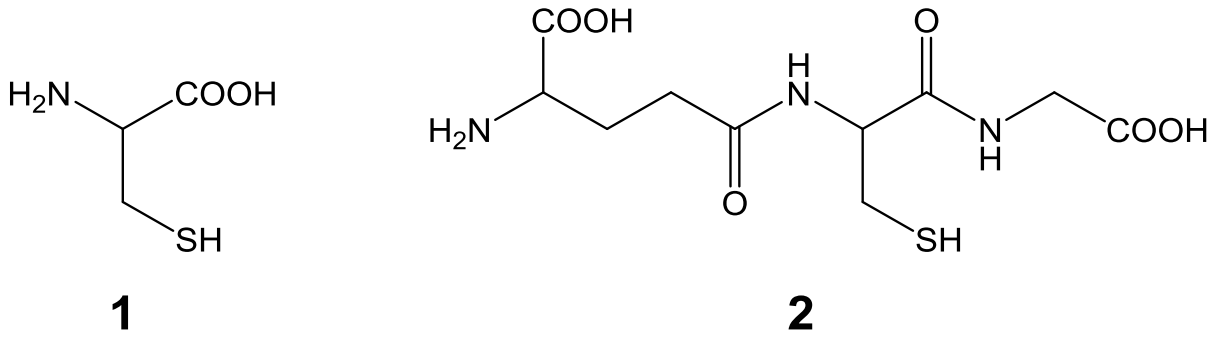

Figure 9: Structures of (**1**) cysteine and (**2**) glutathione (Reinbold 2010)

Both glutathione and cysteine affect the rheology of wheat doughs. This was shown in studies done by Ziegler (1940) and Swanson and Andrews (1943; 1944; 1945). Even additions of nanomolar concentrations (160-490 nmol/g flour of glutathione and 500-2390 nmol/g flour of cysteine, respectively) provoked changes in the dough properties. The addition of either substance made the doughs softer and the volume of the pastries decreased. A higher extensibility of the doughs and a lower resistance to extension was also observed after addition of either substance (Frater et al. 1961, Villegas et al. 1963). This effect is caused by thiol-disulfide exchange reactions of both thiols with the disulfide bonds of the gluten proteins (1.4.6) leading to depolymerization. This induces weakening of the gluten network and, therefore, softening of the dough (Sarwin et al. 1993). The underlying reactions are depicted in Fig. 10. Both the reduced and the oxidized forms of the thiols can be reaction partners of the proteins.

Depolymerization/termination:

protein-SS-protein + GSH → protein-SSG + protein-SH

protein-SS-protein + CSH → protein-SSC + protein-SH

protein-SH + protein-SH → protein-S-S-protein

protein-SS-protein + GSSG → protein-SSG + GSH

protein-SH + GSH → protein-SSG

protein-SS-protein + CSSC → protein-SSC + CSH

protein-SH + CSH → protein-SSC

Polymerization:

protein-SS-protein + protein-SH → protein-S-S-protein-S-S-protein-SH

Figure 10: Thiol-disulfide exchange reactions between gluten proteins and glutathione (GSH)/cysteine (CSH). GSSG: oxidized glutathione; CSSC: cystine

Studies by Koehler et al. (2003) revealed that especially the cysteine residues C^{b*} and C^{x} of LMW-GS, which are thought to have a key role in glutenin polymerization, take part in these reactions.

Glutathione was identified in wheat flour for the first time by Tkachuk (1969), who extracted glutathione traced with [C^{14}]-iodoacetic acid from a flour extract and proved its existence after several purification steps with ion exchangers by amino acid analysis of the traced derivative. The presence of the constituting amino acids, the presence of N-terminal glutamic acid and conformities of IR-, MS- and UV-spectra with those of S-carboxymethylglutathione confirmed the structure of the iodoacetic acid derivative of glutathione. Cysteinylglycine and γ-glutamylcysteine were identified by the same experimental approach (Tkachuk 1970, Tkachuk and Mellisch 1977). The presence of free cysteine in wheat flour was only proven in 1988 by amino acid analysis of a wheat flour extract gained by dialysis (Ewart 1988).

The functional effects of these thiols endogenous to wheat flour were then investigated more closely and yielded very different results. Coventry et al. (1972) determined total amounts of glutathione in different wheat cultivars and the rheological properties of the resulting doughs and concluded that there is a high

correlation between the amount of glutathione and the weakening of the resulting doughs. Li et al. (2004) investigated the amounts of glutathione and cysteine, their oxidized forms as well as two intermediates of the glutathione synthesis pathway (γ-L-glutamyl-L-cysteine and L-cysteinyl-L-glycine) in a total of 36 wheat flours. Additionally, they analyzed crude protein contents, rheological properties and baking performance of the flours. In contrast to Coventry et al. (1972), no sigificant correlations were found between the concentrations of LMW thiols and the flour parameters analyzed. The addition of ascorbic acid to wheat dough antagonizes dough softening caused by glutathione. Ascorbic acid is first oxidized to dehydroascorbic acid, which is then used by glutathione dehydrogenase as cosubstrate to oxidize glutathione to glutathione disulfide. Glutathione may either be naturally present in the flour or may have been generated by thiol-disulfide exchange reactions (Grosch and Wieser 1999).

1.4.10 Blocking of free thiol groups

Glutathione and cysteine react in very similar ways, which is mainly due to their very reactive thiol group. This group is especially susceptible to redox reactions and nucleophilic substitutions (Wonisch and Schaur 2001). The thiol group is oxidized to the disulfide particularly under alkaline conditions within a few hours up to a few days (Reinbold 2010). Depending on the analytical experiment, it may be necessary to protect this sensitive group. This is most commonly achieved by alkylation. This reaction is called ‘blocking’ in this thesis. Suitable reagents are, for example, iodoacetamide (**3**), iodoacetic acid (**4**) (Reinbold 2010), 4-vinylpyridine (**5**) (Rombouts et al. 2013), 2-vinylpyridine (**6**) and N-ethylmaleinimide (NEMI) (**7**) (Griffith 1980) (structures are shown in Fig. 11).

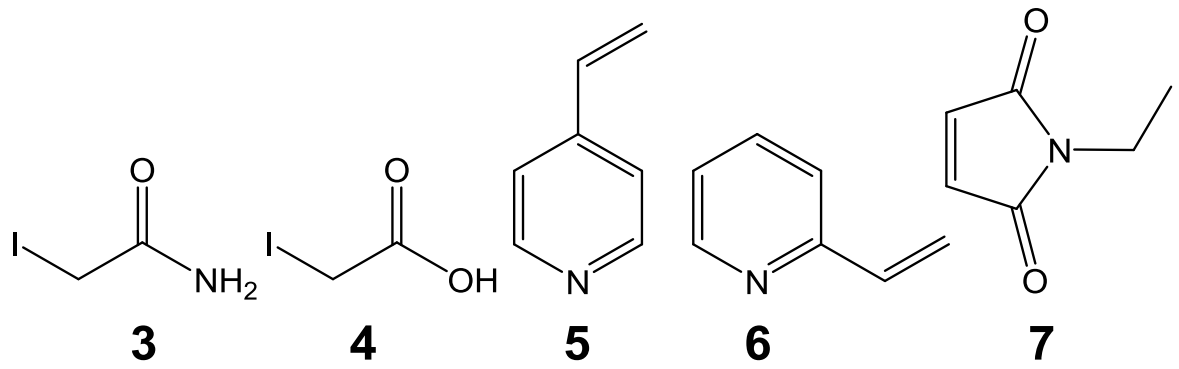

Figure 11: Structures of reagents for blocking of thiol groups. (**3**) iodoacetamide, (**4**) iodoacetic acid, (**5**) 4-vinylpyridine, (**6**) 2-vinylpyridine and NEMI (**7**).

Thiol group blocking reactions are usually nucleophilic substitutions or nucleophilic additions. Fig. 12 shows a nucleophilic addition, in which the thiol group of glutathione is blocked by NEMI (**7** in Fig. 11). On the one hand, this blocking reaction makes it possible to isolate thiol group-containing molecules as stable thioether derivatives and, on the other hand, to suppress side reactions during sample work-up. Both options make analytical experiments with thiol group-containg compounds, for example in wheat flour, much easier than without blocking reagent.

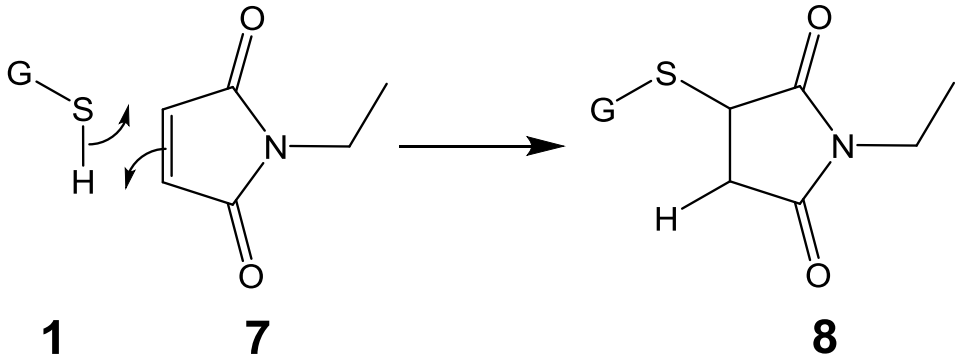

Figure 12: Alkylation of the free thiol group of glutathione with NEMI

1.4.11 Chemical analysis of gluten proteins

Usually a pre-separation or an isolation, respectively, is necessary to analyze the gluten proteins. This can be done by certain extraction methods, precipitation methods or gel chromatography (size exclusion chromatography) (Osborne 1906, Jones et al. 1959, Bushuk and Wrigley 1971, Simmonds and Wrigley 1972, Bietz et al. 1973, Orth and Bushuk 1973, Arakawa and Yonezawa 1975, Huebner and Wall 1976, Payne and Corfield 1979, Kieffer and Belitz 1981, Bottomley et al. 1982,

Hamada et al. 1982, Byers et al. 1983). The method most commonly used today is the so-called Osborne-fractionation (Osborne 1906), in which the flour proteins are separated according to solubility into four different fractions (albumins, globulins, gliadins and glutenins). Wheat flour is extracted with water first, which separates the albumins from the rest of the flour. After that the residue is treated with an aqueous sodium chloride solution, which is buffered at a pH-value of 7.6. The globulins can be found in the salt solution, while the gliadins and the glutenins are still in the residue. The gliadins are then extracted with 60 to 70 % ethanol in water. In the historic reports, the glutenins were gained by extracting the residue with absolute alcohol and then with ether. After that the solvents were removed and the glutenins were dissolved in 0.2 % potassium hydroxide. Today, the albumin and globulin fractions may be isolated as a combined fraction by extracting the flour directly with a buffered salt solution. The glutenins are obtained as a solution of GS by extracting the residue of the gliadin extraction under reducing, disaggregating conditions (1-propanol (50 %), TRIS-HCl (0.1 mol/L), urea (2 mol/L), DTE (1 %)) (Fig. 13). The current procedure is referred to as modified Osborne fractionation in this thesis. The concentrations of the Osborne fractions can be quantitated gravimetrically or by RP-HPLC (see below).

A more detailed but qualitative separation of the flour proteins can be achieved by electrophoretic methods like one- or two-dimensional sodium dodecylsulfate-polyacrylamide-gel electrophoresis (SDS-PAGE). After reduction, individual gliadin types and GS are obtained (Payne et al. 1981, Holt et al. 1981, Moonen et al. 1982, Jackson et al. 1983, Krause et al. 1988, Lew et al. 1992). By SDS-PAGE, the proteins are separated according to their molecular size, and if reference proteins are used, the molecular weight can be estimated. The method can only be used for analytical purposes and not for protein preparation.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is another well-established analytical method for the separation of gluten proteins into their types and subunits (Bietz 1983, 1985, Wieser et al. 1987, Seilmeier et al. 1987, Freedman et al. 1988, Wieser et al. 1990, 1990, Lew et al. 1992). Proteins are separated according to surface hydrophobicity. The hydrophilic ω_5-gliadins are eluted first within the gliadin fraction, followed by $\omega_{1,2}$-gliadins, α-gliadins and, finally, γ-gliadins, which are the most hydrophobic gliadin type. Within the reduced glutenin

fraction, the glutenin-bound ω_b-gliadins are followed by HMW-GS and LMW-GS (Wieser 1987, 1989).

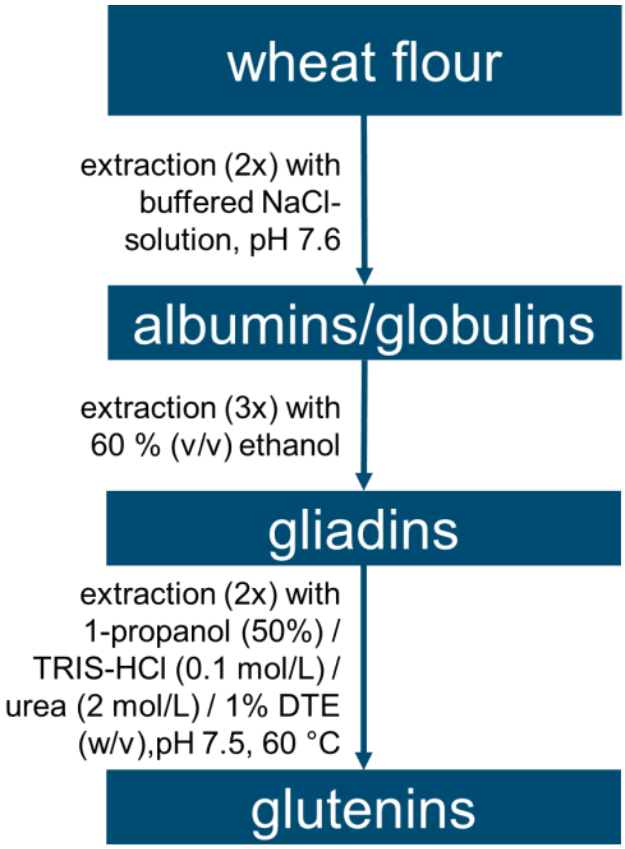

Figure 13: Scheme of the modified Osborne-fractionation according to Wieser et al. (1998).

In contrast to SDS-PAGE, a quantitation of the different protein types and subunits is possible with RP-HPLC. This can be achieved by external calibration with the so-called Prolamin Working Group (PWG-) gliadin (van Eckert et al. 2006), a gliadin preparation with a defined protein content and composition. The HPLC peak area at 210 nm is highly correlated to the amount of protein (Marchylo et al. 1989). RP-HPLC is used for numerous applications - analytical or preparative - such as differentiation of wheat cultivars, preparation of single gluten protein types, determination of correlations between protein composition and rheological properties and baking properties of wheat flours or studies on how environmental conditions affect the protein composition (Bietz 1983, 1985, Burnouf and Bietz 1984, Huebner and Bietz 1987, Seilmeier et al. 1988, Wieser et al. 1990a, 1990b).

Disulfide bonds of gliadins and glutenins have first been identified using gluten isolated from wheat flour (Köhler et al. 1991, 1993, Müller and Wieser 1995, 1997

and Keck et al. 1995). For this pupose, gluten was washed out from dough with water, before gliadins and glutenins were separated by extracting the gluten with aqueous ethanol. Studies on disulfide bonds of gluten proteins of wheat flour have been carried out by Lutz et al. (2012). Proteins were partially hydrolzed with trypsin and/or thermolysin and pre-separated according to molecular size by gel chromatography followed by differential chromatography by means of RP-HPLC (Fig. 14).

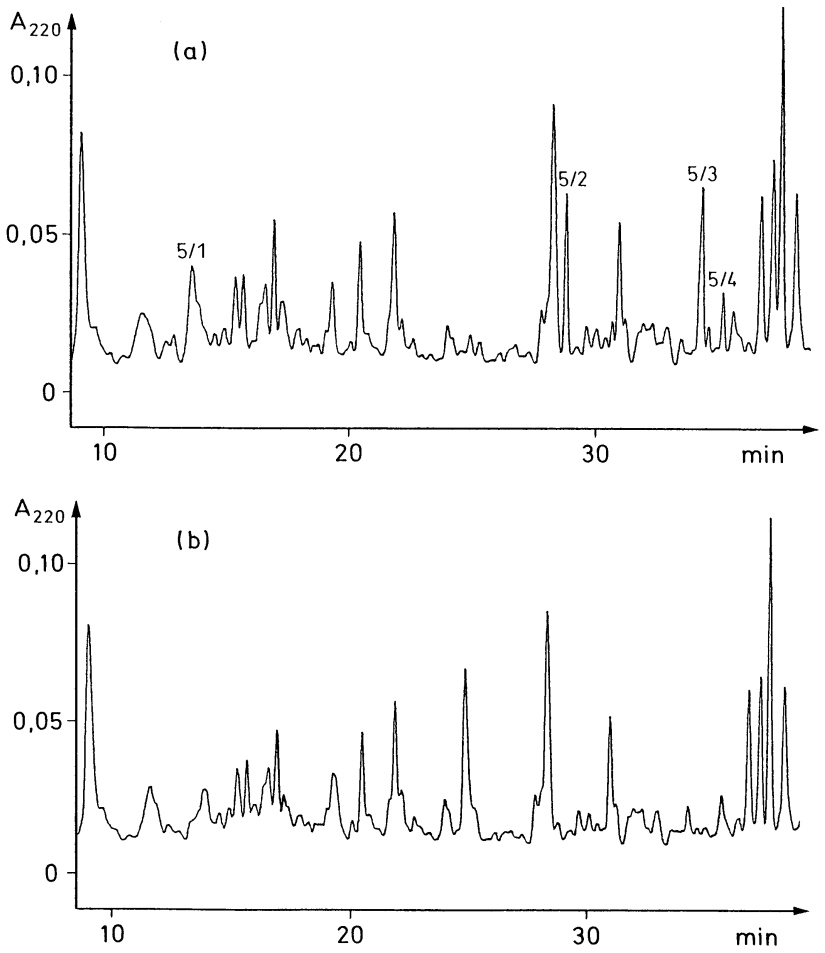

Figure 14: Differential chromatography of peptide mixtures by RP-HPLC (a) before and (b) after reduction of disulfide bonds (Köhler et al. 1991). Four cysteine peptides (5/1; 5/2; 5/3 and 5/4) are marked in chromatogram (a).

Differential chromatography implies that the protein fractions collected after gel chromatography were separated by RP-HPLC before and after treatment with a reducing agent. The fragments of the cystine peptides had different retention times after reduction compared to the intact cystine peptides before reduction in the corresponding RP-HPLC chromatograms (Henschen 1986). Thus, peptides visible in the chromatogram of the unreduced sample that did not appear in the chromatogram

of the reduced sample were supposed to be cystine peptides. These peptides were collected, reduced and alkylated (Henschen 1986, Friedman 1970). After isolation by RP-HPLC, the derivatized cysteine peptides were sequenced by automated Edman-degradation. The last step was the assignment of the amino acid sequences of the cysteine peptides to sequences of gluten proteins. The reason for this “off-line” analytical strategy was that a direct detection of the cystine peptides with UV-light was not possible, because only the peptide bond is UV-active at 210-220 nm and not the disulfide bond. More recent methods used liquid chromatography/mass spectrometry (LC-MS) to identify cystine peptides in complex mixtures (see 1.5.5).

1.5 Mass spectrometry

1.5.1 Principle

MS has become one of the most important analytical methods for protein analysis during the last years, especially when it comes to identification and structural analysis. An MS consists of an ion source, a mass analyzer and a detector. The ion source creates a spray of gaseous ions from the sample, which are then separated in the mass analyzer according to the mass/charge ratio (*m/z*) followed by analysis in a detector that creates the signals (Fig. 15). The spectrum shows, which ions are present and how abundant they are. The MS is connected to a computer containing specific software that allows the operator to analyze and interprete the data from the spectrum and to visualize the results in graphical form. Each component of an MS can be constructed in numerous different ways to achieve specific functions (Fig. 15) that can be combined in several ways. Prior to MS, the samples are often separated by means of liquid chromatography to separate the individual compounds and also to remove substances that could disturb the MS detection (Remane and Herzschuh 1977, Hesse et al. 1984, Budzikiewicz and Schäfer 2005, Gross 2013). After separation, the eluate is transferred into a spray that is desolvatized in a heatable interface.

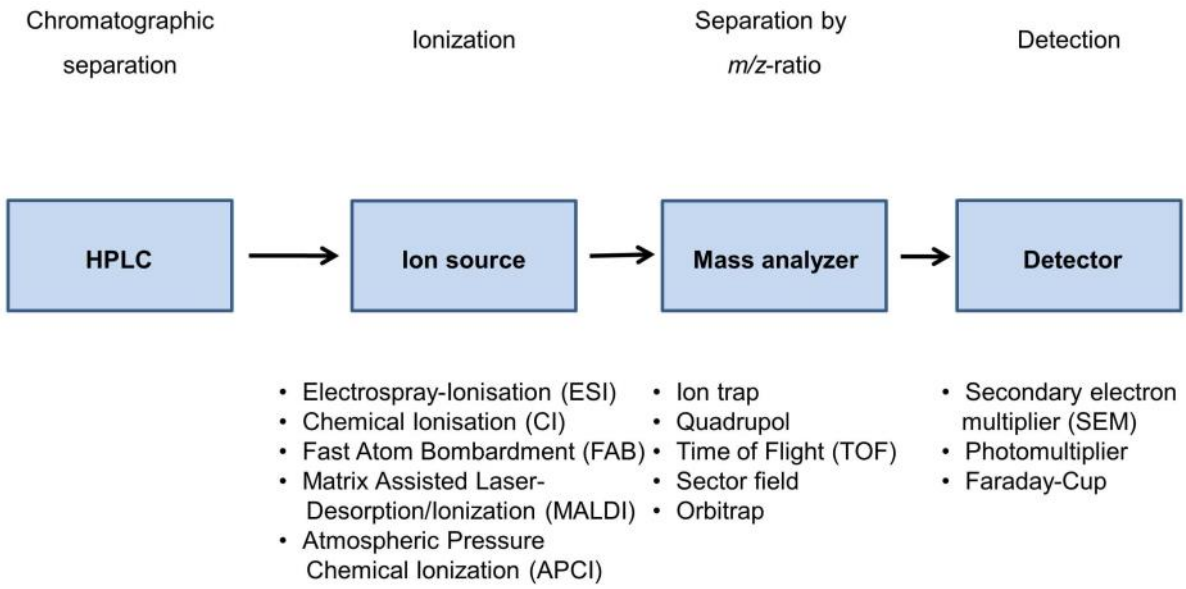

Figure 15: Schematic overview of LC-MS and mass spectrometric techniques

1.5.1.1 Ion source

During electrospray-ionization (ESI) the sample is sprayed into a fine aerosol through an electrically charged capillary (Fenn et al. 1989) at the outlet of the HPLC system. Organic solvents like, for example, acetonitrile or methanol, and aqueous acids, like for example, 0.01-0.1 % formic acid or acetic acid, are typically used as liquid phase. ESI has many advantages like high sensitivity, good traceability of molecules with a high molecular weight, good ionization ability for many analytes, good suitability for polar analytes due to low thermic stress or the possibility to connect the MS with a HPLC. It has comparatively few disadvantages, like for example the requirement for samples to be liquid or high costs due to high gas consumption. Thus, ESI is one of the most common ionization methods. Chemical ionization (CI) is very similar to ESI.

In the CI mode, an ionization gas such as argon, helium or nitrogen, is present in the ion source in excess, is ionized by an electron beam and then transfers its charges onto the analyte molecules. Almost no excess energy is transferred to the sample molecule, which suppresses fragmentation of the analyte and promotes the predominant formation of one ion type that is suitable as precursor ion. Therefore, little structural information is generated using CI, which is why it is mainly used to determine the molecular weight of molecules, which can be ionized easily. However, CI is not suitable for thermally instable analytes, because all samples have to be

converted to the gaseous state for ionization. Nevertheless, it is a very gentle method for molecules fulfilling these requirements.

During fast atom bombardment (FAB), high-energy atoms or ions are fired at the analytes in order to achieve their ionization. Prior to that, the sample molecules are brought onto a carrier material together with a matrix such as glycerine or 3-nitrobenzylchloride. The method works without increased temperature and is, therefore, seen as relatively gentle. Similar to CI, mainly precursor ions and few product ions are observed, which is why FAB is also usually used for the determination of the molecular weight of the analytes. Especially thermally unstable molecules are ionized with this method. Another advantage of the method is that both positively and negatively charged ions can be generated. The spectra of those ions are often different and can complement each other.

During matrix-assisted laser desorption/ionization (MALDI), the analytes are first spotted onto a special target plate together with a matrix that is compatible with the properties of the analytes (for example sinapic acid for proteins). Special techniques such as the dried-droplet method are used for sample application. The desorption/ionization is done by bombardment with a laser beam, whose wavelength is the same as the absorption maximum of the matrix molecule used. The matrix is ionized and transfers its charge to the analyte molecules. MALDI allows the measurement of intact molecule ions making it the most effective ionization type for large molecules such as proteins. Usually MALDI is combined with a time-of-flight (TOF) mass analyzer.

Atmospheric pressure chemical ionization (APCI) is similar to ESI and CI. The analyte solution is nebulized at the end of a capillary in a nitrogen flow and the resulting spray is led over a ceramic surface heated to 300-400 °C that vaporizes the solvent. The resulting gas is converted into a plasma by generating a high voltage (about 5 kV) with a needle-shaped electrode (corona-needle). Ions are generated from the solvent molecules in the plasma, which then ionize the analytes. APCI is mainly used when the analytes under investigation can not or only poorly be ionized by ESI or CI. In addition to the ionization methods described here, numerous other methods (for example ionization by inductive coupled plasma, field ionization, photo ionization) exist, which, however, play a smaller role especially in proteomics

(Remane and Herzschuh 1977, Hesse et al. 1984, Budzikiewicz and Schäfer 2005, Gross 2013).

1.5.1.2 Mass analyzer

The ion trap enables an effective retention (“trapping”) of ions using suitable electric and magnetic fields. It is possible to either „freeze“ ions with a certain *m/z* or to retain all ions in the trap and only release ions with a certain *m/z* by targeted changes in the electric and magnetic fields. There are numerous different variants and functional principles. The most common one is the so-called quadrupol-trap or Paul-trap, in which a temporally changing electromagnetic field in a quadrupol, is used. A huge advantage of an ion trap MS is the ability to directly select ions for further fragmentation, which makes this type of MS very valuable for peptide and protein analysis. Using a quadrupol MS, the ions are first accelerated in a static electrical field. After that, they drift along an axis between four parallel bar electrodes (quadrupol). The mass-to-charge selection takes place in the alternating field of these four electrodes, so that only ions with a certain *m/z*-ratio can pass the field. This selection depends on the ion mass and its charge, the radius and the interspace of the quadrupol bars, the strength of the direct voltage and the frequency and the amplitude of the invested alternating voltage applied. The resolution (ability to distinguish two peaks of slightly different *m/z*-ratio) of such an MS is variable across its measuring range. The higher the ion mass, the higher the resolution. Usually the resolution is adjusted in such a way that two ions with a mass difference of one can be separated from each other over the whole measurement range, which corresponds to the so-called unity resolution. Advantages of the quadrupol are, for example, its simple handling, short scan times, ease of calibration as well as its very high sensitivity and selectivity. Two disadvantages of a quadrupol are that its sensitivity declines with increasing ion masses and that it has a fixed resolution. Nevertheless, this mass analyzer is one of the most popular ones and frequently used in practice.

Another very common mass analyzer in proteomics is the so-called TOF. A cloud of charged particles is accelerated in an electric field and released into a flight tube, which is free of any external charges. The separation of the ions is again achieved due to their *m/z*-ratio. Heavy particles have a longer flight time in the tube compared

to lightweight ones. In order to counterbalance possible differences in the time of entrance or the kinetic energy of the ions, these analyzers work with a so-called reflector electrode, which reverses the flight direction of the ions in the tube. Ions with higher energy penetrate the field of this electrode further compared to ions with a lower energy. The resolution can be improved by a factor of 10 (from 1:1000 to 1:10000) with the use of an electrode like this. This class of analyzers is mainly combined with MALDI and usually provides the basis for the determination of the molecular weights of individual analytes in a mixture. TOF analyzers are suitable to detect ions with molecular weights of up to 100,000.

The sector field MS works with two condensator plates, which are slightly curved and held at a constant potential of direct current. The plates focus the velocity, energy and direction of the ions, which means that ions with the same *m/z*-ratio, but different kinetic energies are brought to the same energy level. The separation by different *m/z*-ratios is done in a magnetic sector field, which is positioned before or after an electric sector field depending on the geometry of the analyzer. The different geometries have different advantages and disadvantages regarding the resolution or the requirement of time or sample material (Remane and Herzschuh 1977, Hesse et al. 1984, Budzikiewicz and Schäfer 2005, Gross 2013).

The selectivity of detection can be increased by using tandem MS (MS/MS). In this method, certain precursor ions are fragmented and the product ions are analyzed. The mass analysis is performed in two steps. At first suitable ions of the analyte, the so-called precursor ions are isolated in a first mass analyzer. Then the precursor ions are transferred into a so-called collision cell, which contains gas molecules of an inert gas, like for example helium or N. Within this collision cell the ions are fragmented and the generated product ions are transferred to another mass analyzer for the second mass analysis. The two steps can be separated by means of time (tandem-in-time) or space (tandem-in-space). Many combinations of mass analyzers are known, like for example triple quadrupol, QqTOF (quadrupol quadrupol time of flight), TOF-TOF or TRAP-orbitrap. The Triple-Quadrupol-MS works with the so-called tandem-in-space principle. The two mass analyzers and the collision cell are spatially separated. The first quadrupol is set to select the precursor ions of interest. These are fragmented in the second quadrupol by collision with inert gas molecules and the resulting product ions are measured in the third quadrupol. The ion trap is an

example for the tandem-in-time mode. This mode of operation is described in detail in chapter 1.5.3.

An MS can be used in several different scanning modes. Selected ion monitoring (SIM) is an MS scanning mode, in which only a limited mass-to-charge ratio range is transmitted/detected by the instrument, as opposed to the full spectrum range. This mode of operation typically results in significantly increased sensitivity. Selected reaction monitoring (SRM) is a method used in tandem MS, in which an ion of a particular mass is selected in the first stage of a tandem MS and an ion product of a fragmentation reaction of the precursor ion is selected in the second MS stage for detection. Multiple reaction monitoring (MRM) is an application of SRM to multiple product ions from one or more precursor ions. (source: http://mass-spec.lsu.edu/msterms/ index.php/; viewed 14.06.2017)

1.5.1.3 Detector

One of the most common detectors in MS is the so-called secondary electron multiplier (SEM). Electrons are generated from the ion current coming from the mass analyzer by means of a so-called conversion diode. The polarity of the diode is contrary to that of the ions. The potential of this diode is relatively high and can be up to several kilovolts. The generated electrons are accelerated to a second diode, where more electrons are created. A serial connection of several accelerating diodes generates an electron cascade, which in turn results in an electric signal. In devices equipped with a quadrupol, the ions have to be accelerated again before they enter the detector. In devices equipped with a magnetic field, the detector is integrated directly into the vacuum. Advantages of the SEM are its high linearity (up to six dimensions), its high factor of amplification (10^4-10^8), its short time of activation (in a range of only nanoseconds) and last, but not least, its high robustness and reliability. Disadvantages are its relatively short life span, its gradually decreasing sensitivity and its low sensitivity to ions with high molecular weights.

A photomultiplier has a functional principle similar to the SEM. The only difference is that the electrons hitting the conversion diode create a burst of photons by striking a phosphorescent screen. These photons are then multiplied and measured. The Faraday-collector or Faraday-cup is used to measure the absolute quantity of ion

currents or electrical currents. A metallic cup held at a constant potential, is brought into the current, which has to be measured, for this purpose. The charges of the collected ions are counterbalanced by electrons, which flow into or out of the cup through a very high ohmic resistance. The geometrical form and the so-called suppressor electrodes prevent the reflected ions or generated secondary electrons from leaving the cup. This enables the measurement of the absolute value of the ion current or the electron current, respectively, which is an advantage of this detector. In contrast to SEM, the sensitivity is also constant over time and does not depend on the mass. The Faraday-cup also has a high robustness and reliability. Disadvantages in comparison to the SEM are lower sensitivities and very long reaction times (Remane and Herzschuh 1977, Hesse et al. 1984, Budzikiewicz and Schäfer 2005, Gross 2013).

1.5.2 Analysis of low-molecular-weight thiols by means of MS

From the year 2000 on, the first methods for the quantitation of glutathione in different matrices by means of LC-MS were developed in order to raise the selectivity compared to the methods used before. Camera et al. (2001) established an LC-MS-method in the selected ion monitoring (SIM) mode with thiosalicylic acid as internal standard. The thiol group of glutathione and the internal standard, respectively, were protected by alkylation with NEMI and the analytes were separated by using a nucleosil column and measured by ESI-MS. This method was used to analyze glutathione in peripheric mononuclear blood cells. The application of selected reaction monitoring (SRM) extensively improved the selectivity (Loughlin et al. 2001). In this method, iodoacetic acid was used to block the thiol group and the quantitation of glutathione was done by external calibration. LC-MS was also used by further working groups in order to quantitate glutathione and cysteine in oxidized and reduced form as well as several other related thiols in cellular tissue samples taken from rats and mice (Guan et al. 2003, Bouligand et al. 2006). Both Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) (Guan et al. 2003) and iodoacetic acid (Bouligand et al. 2006) were applied to block free thiol groups. An internal standard was used as already described by Camera et al. (2001). However, they used glutathione ethyl ester instead of thiosalicylic acid. Both methods described so far were indeed very selective and specific for glutathione and cysteine detection, but mistakes in the

quantitation were discovered due to matrix interferences and resulting ion suppressions in the ESI source. These ion suppressions resulted in wrong values, when external calibration or classical internal standards were used. Therefore, isotopically labeled internal standards, which eliminated these problems, were used from that time on (Rellán-Álvarez et al. 2006, Zhu et al. 2008). In 2006, an LC-APCI-MS-method was developed to analyze cysteine, in which underivatized amino acids were separated by a RP-column and measured in the SIM-mode (Özcan and Senyuva, 2006). However, due to the susceptibility of the thiol group to oxidation, only the total amount of cysteine plus cystine in the sample could be determined. In order to quantitate the exact amounts of glutathione and cysteine, a derivatization of the thiol group was necessary, which was achieved by using iodoacetic acid (Li et al. 2004, 2004) or Ellman's reagent (Guan et al. 2003). A stable isotope dilution assay (SIDA) for the quantitation of glutathione and related compounds in wheat flour was developed by Reinbold et al. (2010). For this purpose, a known amount of an isotopically labeled standard of glutathione and cysteine was added to the wheat flour samples and the sample preparation, which consisted of an S-alkylation step with iodoacetic acid and an N-acylation step with dansyl chloride, was done with both standard and analyte together in one reaction vessel. After purification, the samples were measured using an LC-Triple Quadrupol MS. The quantification of glutathione and cysteine was done by comparing the ratios of internal standard and analyte in the investigated samples. The method was calibrated by preparing mixtures of the unlabeled and labeled analytes in different ratios that were analyzed by LC-MS/MS. The absolute concentrations of the standard solutions were determined by RP-HPLC at 210 nm using commercial glutathione and cysteine with defined purity.The biggest advantage of SIDA is the compensation of analyte losses during sample preparation, because the ratio of analyte and isotopically labelled standard stays the same during all steps of the preparation due to their high chemical similarity.

1.5.3 Analysis of cysteine peptides by means of MS

As already mentioned earlier (1.4.9, 1.5.2) the analysis of cysteine-containing compounds is very difficult due to the reactivity of its thiol group and the susceptibility of this group to oxidation. In order to inhibit side reactions of this functional group, it has to be derivatized to enable proper analysis of cysteine peptides. For this

purpose, the blocking reagents shown in Fig. 11 can be used. ESI-ion trap-MS has become the most common MS-method for the analysis of peptides. The specificity of the detection can be improved, if tandem MS (MS/MS) is used (principle in 1.5.1.2). This method was used, for example, by Rombouts et al. (2013), who identified cysteine peptides in gluten proteins after reduction of the disulfide bonds and alkylation of the cysteine residues with iodoacetamide, 4-vinylpyridine or isotope coded affinity tags (ICAT).

In contrast to triple quadrupol MS (see 1.5.1.2), ion traps operate in the so-called tandem-in-time mode to generate product ions. After all ions have been collected in the trap during a first time interval, only the ions with a specific *m/z*-ratio stay in the trap during a second time interval. These ions are then fragmented in a third time interval using voltage with high frequency and the resulting product ions are detected in a fourth time interval. If the MS runs in the SIM mode (no fragmentation of the selected precursor ion) individual peptides can be detected with high sensitivity. However, a noticeable increase of the selectivity can be achieved, when the MRM-mode is used. The sample solutions should be free from buffers, salts and other reagents, if the ESI is used, because these substances disturb the ionization process by suppressing the formation of free analyte ions. Buffers and salts usually dissociate in aqueous solutions and can, therefore, act as competitors of the ions. Strong ion signals can be found during detection, if salts and reagents are present in the sample, because they may form aggregates, clusters and uncharged ion pairs with the analytes. The only way to get meaningful results is to keep the samples free of disturbing substances. A commonly used way to achieve this, is RP-HPLC, which can be connected directly with the ESI source. The signal in the UV-chromatogram of the RP-HPLC and the equivalent mass in the total ion current (TIC) of the MS both appear at the characteristic retention time of the analyte (Remane and Herzschuh 1977, Hesse et al. 1984, Budzikiewicz and Schäfer 2005, Gross 2013). It has to be mentioned that the analysis of cysteine peptides provides no information about disulfide bonds, because reduced proteins or peptides have to be analyzed.

1.5.4 Identification of cystine peptides by means of MS

Several methods have already been developed in order to identify cystine peptides by means of MS. Among others, there is a method based on FAB-MS, which is

suitable to identify ions with high masses due to its high resolution. Protein hydrolyzates are analysed by FAB-MS once with and once without reduction of disulfide bonds (Morris and Pucci 1985). A method based on MALDI-TOF-MS and ESI-MS was used by Mhatre et al. (1999) for the investigation of disulfide bonds in monoclonal antibodies. Cystine peptides were identified once with intact and then with cleaved disulfide bond before and after reduction with DTT. The signal of the disulfide peptide disappeared after reduction and was replaced by two different signals that represented one of the cysteine peptides each. For the discrimination between free and disulfide-bonded cysteine residues in a protein, two different thiol blocking reagents have to be used, one before and the other after reduction of the disulfide bonds. Vasicek et al. (2009) used alkylation reagents like iodoacetamide (IDAM), 2-chloroethyldimethylamine (DML) and 3-acrylamidopropyl-trimethylammoniumchloride (APTA). Seiwert et al. (2008) used N-(2-ferroceneethyl)maleimide (FEM) before reduction of disulfide bonds and ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA) after reduction of disulfide bonds for analyzing free thiol groups and disulfide bonds of α-lactoglobulin and β-lactoglobulin A and B.

1.5.5 Alternating CID/ETD to identify cystine petides

Among others, the working groups of Syka et al. (2004), Coon et al. (2004), Wu et al. (2009) and Coon (2009) explored the combination of CID and electron transfer dissociation (ETD) fragmentation for the analysis of cystine peptides. In addition to the ESI-source, a second source using CI is present, in which radicalic anions for example from anthracene or fluoroanthene are generated. These react with multiply charged precursor peptide ions during ETD fragmentation, which leads to different fragments compared to CID fragmentation. c-Fragments with an even and z-fragments with an odd number of electrons are generated by cleavage of the N-C_α-bond (Coon, 2009) (Fig. 16, 18). In contrast, the cleavage during CID fragmentation occurs at the peptide bonds and b- and y-fragments are formed (Fig. 17, 18).

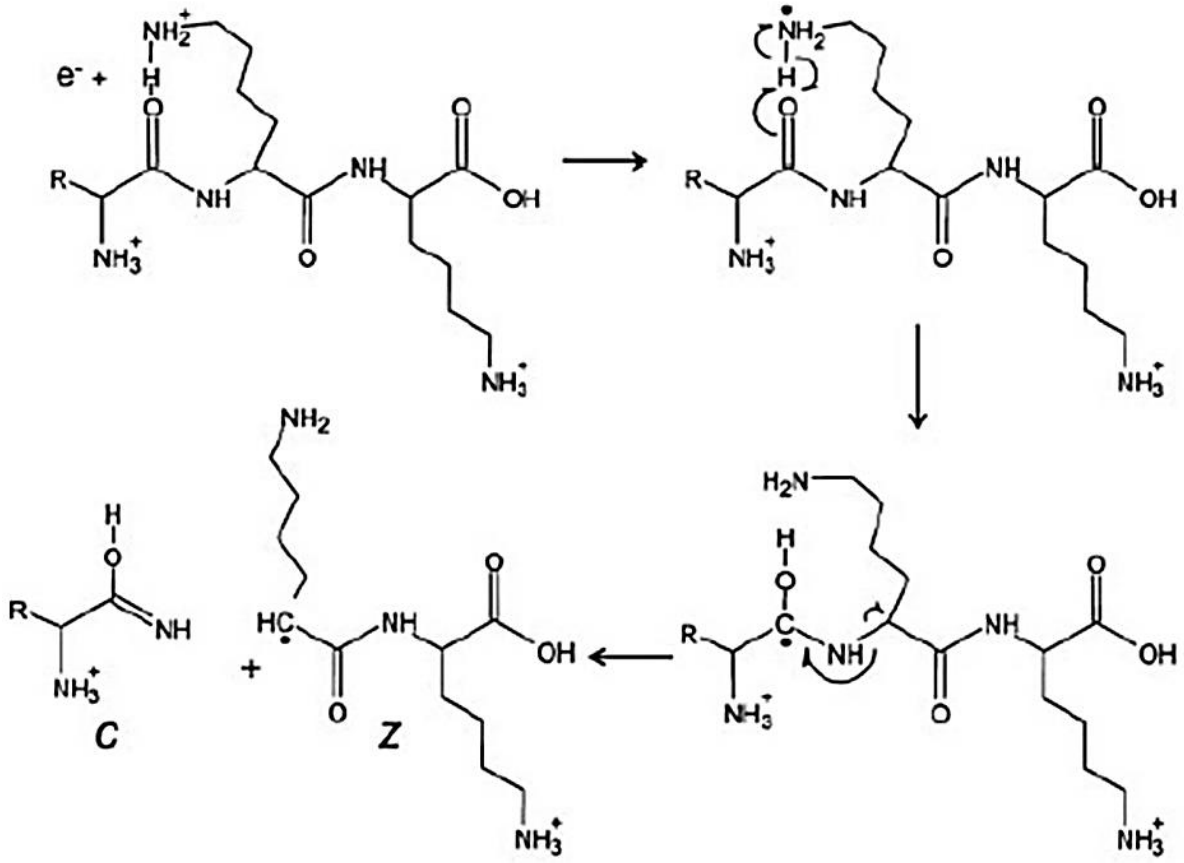

Figure 16: c- and z-fragments generated by ETD-fragmentation of a peptide ion (Syka et al. 2004)

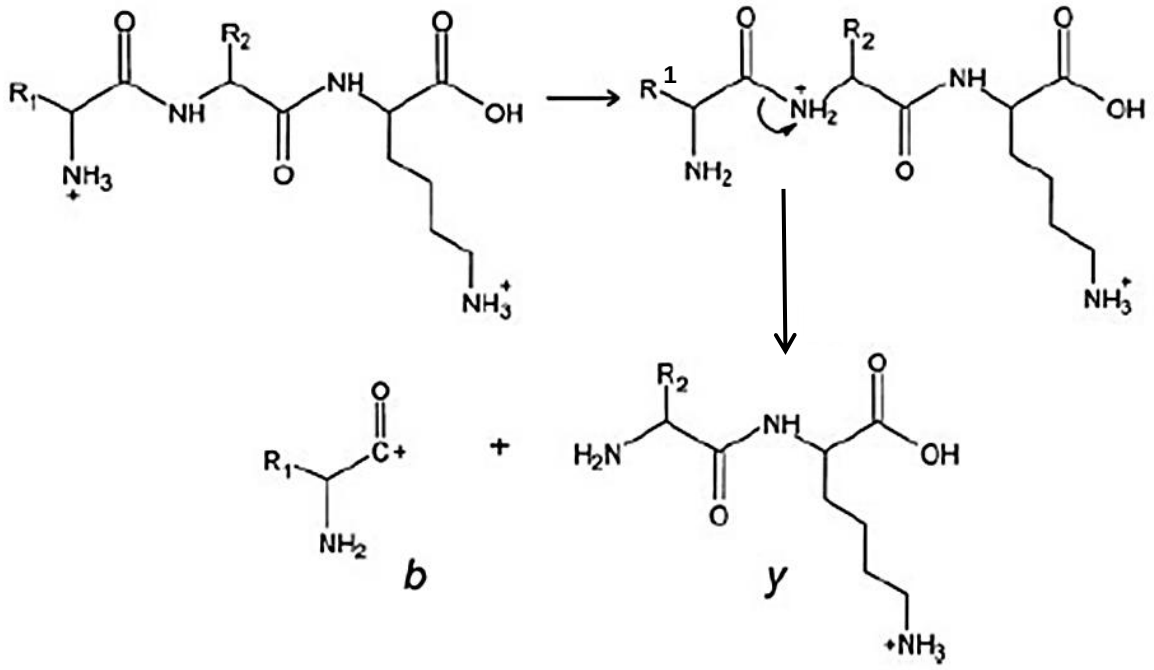

Figure 17: b- and y-fragments generated by CID-fragmentation of a peptide ion (Syka et al. 2004)

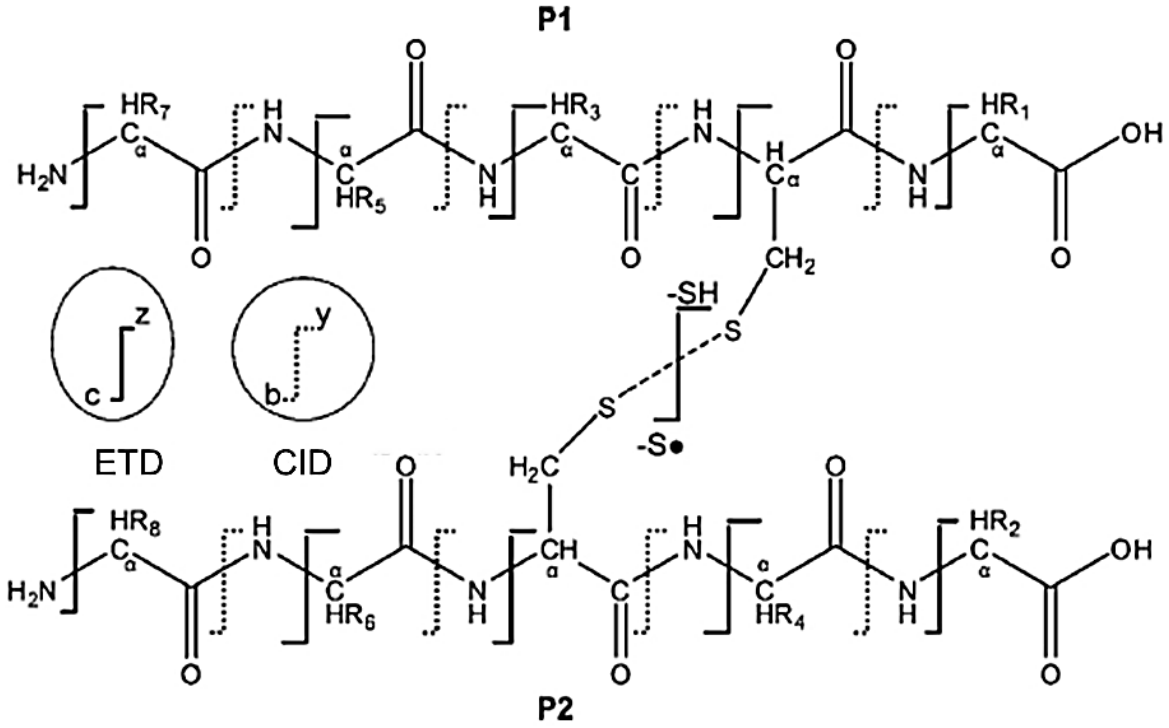

Figure 18: Cleavage sites of a disulfide peptide caused by CID and ETD fragmentation, respectively (Wu et al. 2009)

The method was used, for example, by Wu et al. (2009) for the identification of disulfide bonds in therapeutical peptides and by Syka et al. (2004) for angiotensine and a mixture of ten synthetic peptides. Coon (2009) discovered that a combination of both fragmentation methods was most effective for the analysis of protein and peptide mixtures. He was able to verify that ETD fragmentation was more suitable for posttranslational modifications and larger molecules. This technique was also used very successfully by Lutz et al. (2012) and Lutz (2013) for the analysis of disulfide bonds and disulfide structures in gluten proteins (see for example Fig. 19).

Lutz et al. (2012) used MS with alternating CID/ETD-fragmentation to identify the cystine peptides of gluten proteins directly after RP-HPLC separation. An advantage of this method is that no derivatization of cysteine residues and no reduction of cysteine peptides are necessary and the sample can be analyzed by LC-MS right after enzymatic hydrolysis. The different fragmentation methods make it possible to identify cystine peptides both with intact (CID) and cleaved disulfide bond (ETD) (Fig. 19).

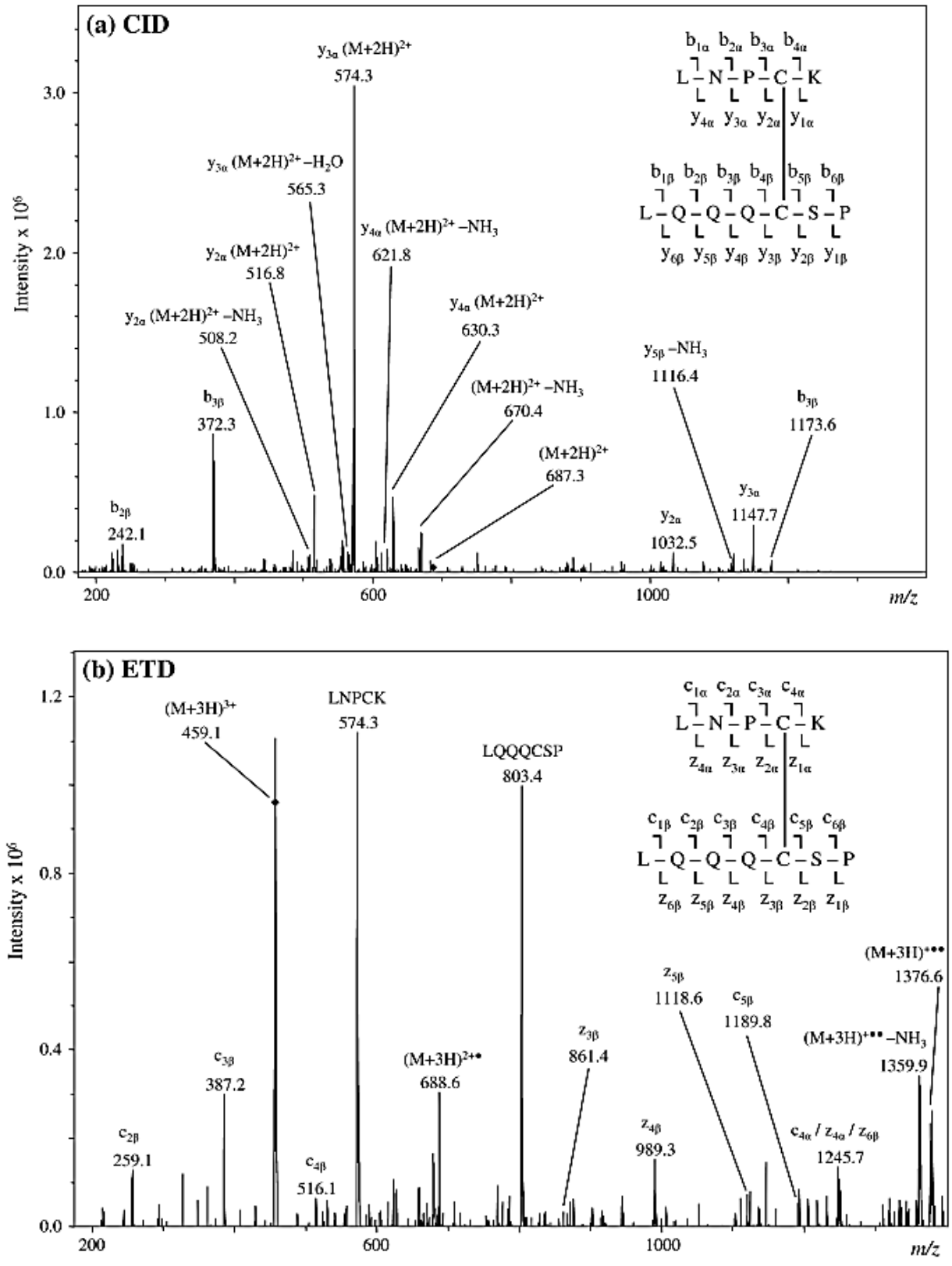

Figure 19: MS/MS-spectra of a disulfide peptide from LMW-GS, obtained by (a) collision induced dissociation (CID) of the precursor ion with *m/z* 687.3 and charge state +2 and (b) by electron transfer dissociation (ETD) of the precursor ion with *m/z* 459,1 and charge state +3 (Lutz et al. 2012)

2. Objectives of the thesis

It is known that HGL consist of oligomeric proteins. Earlier studies revealed that α- and γ-gliadins as well as LMW-GS are part of HGL. It is assumed that GS and gliadin types are connected by intermolecular disulfide bonds and that gliadins with an odd number of cysteine residues terminate the oligomers referred to as HGL. However, it is unknown so far, how many individual proteins are combined and how they are connected via disulfide bonds. Additionally, there is no information on the functionality of these oligomers in wheat flour.

Since thiol-disulfide exchange reactions between gluten proteins can occur in aqueous media, it can be expected that these reactions would happen during the Osborne extraction of wheat flour. However, the influence of free thiol groups during the extraction of gluten proteins on the content of Osborne fractions in general and on the content and the structure of HGL in particular is not known so far.

Therefore, one objective of the present study was to determine the influence of free thiol groups on the distribution of the Osborne fractions and the content of HGL. For this purpose, NEMI should be used to block free thiol groups in the first step of the Osborne fractionation. RP-HPLC and analytical gel permeation high-performance liquid chromatography (GP-HPLC) were deemed suitable to quantitate the Osborne fractions and HGL. Osborne fractionation without addition of NEMI should be carried out as a control experiment and the results of both extraction variants were compared. To be able to generalize the results, a set of 12 wheat flours from different cultivars and cultivation years should be analyzed.

Another objective of the present study was to develop a suitable isolation method for HGL and to characterize this protein fraction extensively. As described before, HGL samples from one wheat cultivar (Akteur, harvested in 2011) obtained without and with NEMI added during protein extraction should be investigated. For the isolation, a method based on preparative GP-HPLC had to be developed. In the first step of the characterization of HGL, the types and concentrations of the subunits of the oligomers should be determined by RP-HPLC after reduction of disulfide bonds, by densitometry, by analytical GP-HPLC and by N-terminal sequence analysis. After that, the involvement of disulfide bonds in the structure of HGL had to be determined

by SDS-PAGE (one-dimensional under non-reducing and reducing conditions as well as two-dimensional) and the participation of low-molecular-weight thiols (glutathione and cysteine) should be identified by spectrophotometric analysis with Ellman's reagent and LC-MS/MS. Another task was to determine the molecular size range as well as the average molecular weight of HGL by analytical GP-HPLC. Furthermore, the presence of gliadins or GS with an odd number of cysteine residues should be established by analyzing specific cysteine peptides in enzymatic hydrolyzates with ESI-ion-trap MS. Finally, the most important task was to get an insight into the disulfide structure of HGL using a proteomics approach involving ESI-ion trap MS of enzymatic hydrolyzates with only CID and alternating CID/ETD fragmentation to specifically detect cystine peptides from HGL.

3. Results

3.1 Isolation and characterization of HMW-gliadins from wheat flour

The aim of the first part of the work was to develop an isolation method for HGL followed by a complete proteinchemical characterization of the protein components which are involved in the formation of HGL. Markus Schmid carried out all experimental work, wrote the draft of the manuscript and postulated a hypothesis for the formation of HGL.

Wheat flour (cultivar Akteur, harvest year 2011) was extracted by means of a modified Osborne-fractionation. The fraction soluble in 60 % ethanol (total gliadins) was separated by GP-HPLC and three subfractions were obtained. Subfraction 1 represented 21.5 % of total gliadins and consisted of oligomeric gliadins (HGL). Subfraction 2 (15.2 %) contained ω_5-gliadins and in subfraction 3 (63.3 %) monomeric $\omega_{1,2}$-, α- and γ-gliadins were present. Two-dimensional SDS-PAGE revealed that intermolecular disulfide bonds are present in HGL. The molecular weight range, the average molecular weight and the degree of polymerization of HGL determined by analytical GP-HPLC were 66000 to 680000, about 530000 and 13 (calculated by dividing the average MW of a HGL molecule by the average MW of a subunit), respectively. Then, HGL was reduced and separated into four subfractions by preparative RP-HPLC. SDS-PAGE and semiquantitative N-terminal sequence analysis showed that HGL consists of all gliadin and glutenin subtypes. In total, the following percental amounts were determined: 48 % LMW-GS, 18 % γ-gliadins, 13% α-gliadins, 9% $\omega_{1,2}$-gliadins, 8% HMW-GS and 4% ω_5-gliadins. Based on the results, a hypothesis for the formation of HGL was postulated: The polymerization of the glutenins via intermolecular disulfide bonds is stopped by so-called terminators. These can be low-molecular-weight thiols like glutathione and cysteine, but also gliadins with an odd number of cysteine residues in their amino acid sequence due to point mutations. The oligomers, which are generated by these terminators and soluble in 60 % ethanol, are called HGL.

e-Xtra*

Isolation and Characterization of High-Molecular-Weight (HMW) Gliadins from Wheat Flour

Markus Schmid, Herbert Wieser, and Peter Koehler†

Cereal Chem. 93(6):536–542

ABSTRACT

The aim of this study was to isolate high-molecular-weight (HMW) gliadins from wheat flour and to characterize the protein components that contribute to HMW gliadins. Wheat flour Akteur was extracted with a modified Osborne procedure, and the fraction soluble in 60% ethanol (total gliadins) was separated by gel-permeation HPLC, yielding three fractions, GP1–GP3. GP1 (21.5%) consisted of oligomeric HMW gliadins, GP2 (15.2%) of ω5-gliadins, and GP3 (63.3%) of ω1,2-, α-, and γ-gliadins. Two-dimensional SDS-PAGE of HMW gliadins showed that interchain disulfide bonds were present in HMW gliadins. The molecular mass distribution of HMW gliadins determined by gel-permeation HPLC was in a range from 66,000 to 680,000 with an average degree of polymerization of 13. Reduced HMW gliadins were further separated by preparative reversed-phase HPLC into four subfractions (RP1, RP2, RP3, and RP4), which were characterized by SDS-PAGE and semiquantitative N-terminal sequencing. HMW gliadins of the wheat flour Akteur contained all types of gluten proteins: 48% low-molecular-weight glutenin subunits, 18% γ-gliadins, 13% α-gliadins, 9% ω1,2-gliadins, 8% HMW glutenin subunits, and 4% ω5-gliadins. We postulate that the existence of HMW gliadins can be explained by the presence of terminators, which interrupt the polymerization of glutenin subunits during biosynthesis and lead to polymers of limited size (oligomers) that are still soluble in aqueous ethanol.

Wheat grains and flour contain hundreds of different proteins. Traditionally, they have been grouped into four fractions according to their different solubility, the so-called Osborne fractions (Osborne 1924): albumins, soluble in water and salt solutions; globulins, insoluble in water but soluble in salt solutions; gliadins, soluble in aqueous alcohols (e.g., 60–70% ethanol) without reduction of disulfide bonds; and glutenins, soluble in aqueous alcohols only after reduction of disulfide bonds. Gliadins and glutenins are the storage proteins of wheat grains. They occur exclusively in the starchy endosperm and amount up to 70–80% of total grain proteins. The gliadin fraction contains mainly monomeric proteins, which can be classified into four types according to different amino acid sequences: ω5-, ω1,2-, α-, and γ-gliadins (Wieser 2007). Their relative molecular masses (M_rs) range from around 28,000 to 55,000. Disulfide bonds are either absent (ω-gliadins) or present as intrachain crosslinks (α- and γ-gliadins). The glutenin fraction comprises polymeric proteins linked by interchain disulfide bonds, with M_rs from about 500,000 to more than 10 million (Wrigley 1996), but distinct M_rs are not available. The reduction of disulfide bonds results in the formation of glutenin subunits (GS), which can be structurally differentiated into high-molecular-weight (HMW-) GS and low-molecular-weight (LMW-) GS with M_rs of 67,000–88,000 and 32,000–35,000, respectively (Shewry and Tatham 1990; Wieser 2007).

A minor portion of the gliadin fraction contains alcohol-soluble oligomeric proteins linked by interchain disulfide bonds. This subfraction has been called HMW gliadins (HGL), aggregated gliadins, or ethanol-soluble glutenins (Beckwith et al. 1966; Bietz and Wall 1980; Shewry et al. 1983; Huebner and Bietz 1993). In the present article, the oligomeric fraction is named HMW gliadins, because these proteins occur in the gliadin fraction and form the greatest protein subclass within this fraction with respect to molecular size. The M_r distribution and approximate ratios of the monomeric, oligomeric, and polymeric wheat proteins are schematically shown in Figure 1. As indicated by the different terms, the oligomeric fraction may be defined as gliadins (soluble in aqueous alcohols) or glutenins (linked by interchain disulfide bonds). Accordingly, the ratio of gliadins to glutenins may be 2:1 in the first case and 1:1 in the second case. Interestingly, the contribution of HGL to the rheological properties of dough and gluten was found to be similar to that of total gliadins and not to that of total glutenins (Wieser et al. 1994b). The proportion of HGL within the gliadin fraction is dependent on the wheat cultivar; investigation of nine international cultivars with analytical gel-permeation chromatography (GPC) revealed proportions between 13 and 20% (Wieser et al. 1994b). N-Terminal sequencing indicated that α- and γ-gliadins as well as LMW-GS can be found in HGL (Huebner and Bietz 1993). It was concluded that, most likely, modified α- and γ-gliadins with an odd number of cysteine residues would act as terminators and stop the polymerization of glutenins (Kasarda 1989). Altogether, HGL is, by far, chemically less characterized in comparison with gliadin and glutenin fractions. Therefore, the aim of this study was to elucidate the qualitative and quantitative composition of HGL in more detail.

MATERIALS AND METHODS

Chemicals. The purity of the chemicals was analytical grade unless stated otherwise. Formic acid (98–100%), Coomassie Brilliant Blue, dipotassium hydrogen phosphate, disodium hydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), glacial acetic acid, ethanol (LiChrosolv), glycerol, potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium dodecyl sulfate (SDS), phenol red, 1-propanol, 2-propanol (LiChrosolv), sucrose, hydrochloric acid (25%), trichloroacetic acid, tris(hydroxymethyl)-aminoethane (TRIS), urea, sodium dihydrogen phosphate, sodium borohydride, and boric acid were purchased from VWR Merck (Darmstadt, Germany). Acetonitrile (LC-MS LiChrosolv), methanol (LiChrosolv), tetrahydrofuran (Chromasolv Plus), trifluoroacetic acid (TFA), 5,5′-dithiobis(2-nitro-benzoic acid) (DTNB), and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). 3-Morpholinopropanesulfonic acid (MOPS; buffer quality) and protein marker III were purchased from Applichem (Darmstadt, Germany). Dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), and Servablue G 250 were purchased from Serva Electrophoresis (Heidelberg, Germany). Acetonitrile/water mix (sequencer grade), Biobrene reagent, *n*-butyl chloride, ethyl acetate (sequencer grade), *N*-methylpiperidine/water/methanol, 5% v/v phenyl isothiocyanate in *n*-heptane, premix

*The *e*-Xtra logo stands for "electronic extra" and indicates that a supplementary figure is published online.

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buffer concentrate, standard solution for N-terminal sequencing, TFA (100%), and TFA (25% v/v, in water) were purchased from Life Technologies (Darmstadt, Germany). Dialysis tubing (size 6, IntDia 27/32″, molecular weight cut-off 12,000–14,000) was purchased from Medicell International (London, U.K.).

Preparation of Total Gliadins and HGL. Grains of the wheat cultivar Akteur (harvest year 2011) were milled into white flour with a Quadrumat Junior II mill (Brabender, Duisburg, Germany) and sieved through a 0.2 mm mesh sieve. The obtained flour contained 15.6% moisture and 0.43% ash. For the preparation of the gliadin fraction, flour (50 g) was sequentially extracted with a buffered salt solution (2 × 200 mL; 0.4M NaCl plus 0.067M Na_2HPO_4/KH_2PO_4, pH 7.6) to remove albumins and globulins. Gliadins were then extracted with 60% v/v ethanol (3 × 200 mL). Each extraction step was performed by homogenization with a homogenizer (Ultra Turrax T25D; IKA, Staufen, Germany) for 10 min at room temperature (RT, ≈20°C). Each suspension was centrifuged for 20 min at 4,600 × *g* and RT by means of a laboratory centrifuge (Heraeus Multifuge 3L-R, Thermo Fisher Scientific, Dreieich, Germany). The supernatants of the albumin/globulin extracts were discarded and those of the gliadin extracts were combined, concentrated with a rotary evaporator (Rotavapor RII, Büchi, Essen, Germany), and dialyzed for three days against acetic acid (0.01M) and for one day against deionized water. The dialyzed solutions were then lyophilized.

For the preparation of HGL, lyophilized total gliadins (100 mg) were dissolved in 60% v/v ethanol (4 mL), filtered through a 0.45 µm membrane, and separated by preparative gel-permeation high-performance liquid chromatography (GP-HPLC) under the following conditions: instrument, Jasco HPLC (Jasco, Gross-Umstadt, Germany); column, BioSep-SEC-s3000 (21.2 × 300 mm; separation range for globular proteins 5,000–700,000; Phenomenex, Aschaffenburg, Germany); elution solvent, acetonitrile (50% v/v)/TFA (0.1% v/v); flow rate, 1.5 mL/min; temperature, RT; injection volume, 900 µL; detection, 280 nm; fraction collector, model 201 (Gilson International, Limburg-Offheim, Germany). Separated fractions were collected from several runs and lyophilized.

Protein Analysis. The crude protein content (N × 5.7, calibration with EDTA) was determined according to the Dumas method with an FP-328 combustion instrument (TruSpec, Leco, Kirchheim, Germany). For the quantitation of Osborne fractions and gluten protein types, flour (100 mg) was extracted subsequently with a salt solution, 60% v/v aqueous ethanol, and 50% aqueous 1-propanol under reducing and disaggregating conditions to obtain albumin/globulin, gliadin, and glutenin fractions, respectively (Wieser et al. 1998). Aliquots of these extracts were analyzed by reversed-phase (RP-) HPLC on a Thermo instrument (Thermo Fisher Scientific) with a C_{18} silica gel column (Dionex Acclaim 300, 2.1 × 150 mm, 3 µm, 30 nm; Thermo Fisher) at 60°C. Elution solvent A was 0.1% v/v TFA, and solvent B was 0.1% v/v TFA in acetonitrile. The solvent gradient was linear 0–10 min, 20 to 60% B (albumin/globulin) or linear 0–16 min, 30 to 52% B (gliadins, glutenins). The injection volume was 10 µL of each extract (2 mL). The flow rate was 0.3 mL/min, and the detection wavelength was 210 nm. Prolamin Working Group (PWG) gliadin (van Eckert et al. 2006) was dissolved in 60% ethanol (2.5 mg/mL), and 5, 10, and 20 µL were injected for external calibration. All quantitative determinations were made in triplicate.

Analytical GP-HPLC of the gliadin extract (2 mL) was carried out with a Thermo instrument (see earlier). The column was a BioSep SEC s3000 (4.6 × 300 mm; separation range for globular proteins 5,000–700,000; Phenomenex, Aschaffenburg, Germany) used at RT. The elution solvent was 50% v/v acetonitrile/0.1% v/v TFA. The injection volume was 10 µL, and the flow rate was 0.3 mL/min. External calibration for protein quantitation was done with PWG gliadin (see earlier). The M_r calibration was achieved by means of the following marker proteins: thyroglobulin (M_r = 680,000), bovine serum albumin (66,000), thermolysin (34,500), and cytochrome C (12,500). All quantitative determinations were made in triplicate.

One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was carried out according to the method of Lagrain et al. (2012) on a homogenous NuPAGE 10% polyacrylamide-Bis-TRIS-HCl gel at pH 6.4 (1.0 mm × 10 well; Invitrogen, Carlsbad, CA, U.S.A.) using a MOPS-TRIS running buffer (5 mM DTT, 50 mM MOPS, 50 mM TRIS, 3.5 mM SDS, 1 mM EDTA, pH 7.7). Total gliadins, HGL, or HGL subfractions (3 mg each) were mixed with 1.0 mL of sample buffer (0.154 g of DTT, 4.0 g of sucrose, 1.194 g of TRIS, 0.8 g of SDS, 0.006 g of EDTA, 0.0075 g of Serva blue G250, 2.5 mg of phenol red, and 4.23 mL of 1.0M HCl dissolved and filled to 20 mL with water), heated at 60°C for 10 min, and centrifuged (5,000 × *g*, 5 min). The supernatant was collected, and 10 or 20 µL was applied to the slots. The running time of electrophoresis was 40 min at 200 V and 115 mA. The gel was then fixed for 30 min in 12% w/v trichloroacetic acid and stained for 30 min with Coomassie Brilliant Blue R-250. The gels were destained by immersing in washing solution I (methanol/glacial acetic acid/water, 50/10/40 v/v/v) for 15 min and in washing solution II (methanol/glacial acetic acid/water, 10/10/80 v/v/v) overnight. Two-dimensional (2D) SDS-PAGE was performed to compare total gliadins and HGL. The first dimension experiment was performed as described earlier but without addition of DTT to the running and sample buffers (nonreducing SDS-PAGE). After the first run, one strip of the gel margin was cut off and put into a modified MOPS buffer containing the reducing agent DTT (50 mM) for 15 min. After reduction was completed, the single strip was put into another gel cartridge, and electrophoresis was carried out as described earlier except that DTT was added to the MOPS running buffer (5 mM).

N-Terminal sequence analysis of HGL subfractions was carried out by automated Edman degradation by means of a Procise 492 protein sequencer (Applied Biosystems, Weiterstadt, Germany) running in the pulsed-liquid mode. The amount of protein applied onto the sequencer was between 50 and 100 pmol. The delivered phenylthiohydantoin (PTH) amino acids were identified and quantitated by RP-HPLC on a Spheri 5 PTH column (2.1 × 220 mm, 55°C, Applied Biosystems) using a PTH amino acid standard mixture (40 pmol each, Life Technologies, Darmstadt, Germany) for calibration.

Quantitation of Accessible Thiol and Disulfide Groups. Accessible thiol (SH) groups were determined spectrophotometrically with Ellman's reagent according to the methods of Sarwin et al. (1993) and Lagrain et al. (2011). HGL (1.0 mg) was suspended in a solvent (900 µL) containing sodium phosphate (0.05M, pH 6.5), SDS (2.0% w/v), urea (3.0M), and EDTA (0.001M). The mixture was shaken for 60 min at RT, and then a DTNB solution (100 µL;

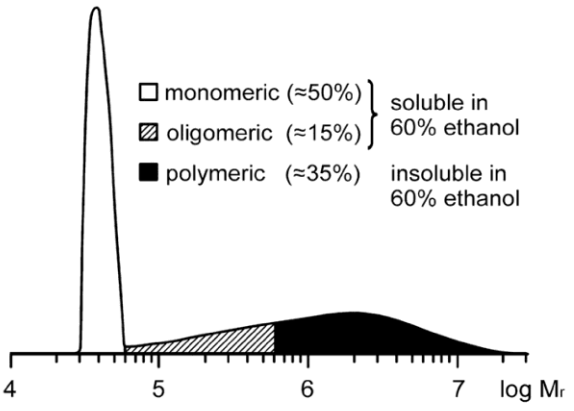

Fig. 1. Molecular mass distribution of gluten proteins.

0.1% w/v in sample buffer) was added and the mixture was further shaken. After exactly 45 min, the mixture was centrifuged (5 min, 11,000 × *g*, 20°C), and the absorbance of the supernatant was determined at 412 nm (path length = 1 cm). Two samples containing either HGL without DTNB or DTNB without HGL were used for background correction. Absorbance values were converted to levels of free SH groups (µmol of SH/g of protein) by using a calibration curve generated by glutathione. Six determinations were made.

Disulfide (SS) groups were determined as described for SH groups (see earlier) but after reduction with sodium borohydride (six determinations). HGL (1.0 mg) was mixed with sodium borohydride solution (200 µL; 2.5% w/v) and shaken for 60 min at 50°C. Then, excess sodium borohydride was degraded by mixing with HCl (100 µL; 1.0M) for 30 min at RT. Then, free SH groups were quantitated as described earlier. Samples either containing HGL plus sodium borohydride and without DTNB or containing DTNB without HGL and sodium borohydride were used for background correction. Glutathione disulfide was used for external calibration.

Quantitation of Protein-Bound Cysteine and Glutathione. The concentrations of protein-bound cysteine and glutathione were determined by a stable isotope dilution assay using liquid chromatography–tandem mass spectrometry (LC-MS^2) as described by Reinbold et al. (2008, 2014). For the reduction of disulfide bonds, HGL (2 mg) was suspended in an aqueous solution (200 µL) of TCEP (1 mg). Solutions of the internal standards (20 µL each; [$^{13}C_3$, ^{15}N] glutathione [49.8 mM] and [$^{13}C_3$, ^{15}N] cysteine [180.5 mM] dissolved in 0.1% v/v formic acid) were added, and the mixture was stirred for 30 min at RT in the dark. Then, a solution (600 µL) of iodoacetic acid (0.02M) in a boric acid/lithium hydroxide buffer (0.5M, pH 8.5) was added and stirred (30 min, RT in the dark). Afterward, dansyl chloride (500 µL; 3.7 mM dissolved in acetonitrile) was added, and the mixture was again stirred for 1 h at RT in the dark. After addition of dichloromethane (500 µL), the mixture was shaken and centrifuged (4,600 × *g*, 20 min, RT). The aqueous supernatant was filtered and centrifuged in Vivaspin centrifugal concentrators (cut-off < 3,000, 13,000 × *g*, RT) overnight. LC-MS^2 of the permeate (10 µL) was performed under the following conditions: instrument, TSQ Quantum Discovery system coupled with a Surveyor HPLC system (Thermo Finnigan, Dreieich, Germany); column, Hydro RP C_{18} (2 × 150 mm, 4 µm, 8 nm); elution solvents, 0.1% v/v formic acid in water (A) and 0.1% v/v formic acid in acetonitrile (B); gradient, linear, at 0 min, 0% B, at 25 min, 100% B; flow rate, 0.2 mL/min. The mass spectrometer was operated in electrospray ionization positive mode (capillary temperature, 290°C; sheath gas pressure, 30 arbitrary units; auxiliary gas pressure, 10 arbitrary units; spray voltage, 4 kV; capillary offset, 35 V; collision cell pressure, 67 Pa; scan time, 200 ms). The analysis of MS data files was performed with Xcalibur software (Thermo Finnigan). Triplicate determinations were made.

Densitometry. Reference preparations of HMW-GS and LMW-GS as well as of α- and ω1,2-gliadins (0.58, 0.54, 0.55, and 0.46 mg, respectively) isolated by RP-HPLC from wheat flour Akteur (kindly donated by Barbara Lexhaller) were mixed with 0.125 mL of reducing SDS-PAGE sample buffer (see earlier), heated at 60°C for 10 min, and centrifuged (5,000 × *g*, 5 min). HGL (2.5 mg/mL, dissolved in 50% aqueous 1-propanol) was reduced by adding DTT (50 mM), heated (60°C for 30 min), and separated into subfractions RP2 and RP3 by analytical RP-HPLC with the system described earlier. The samples were split into two portions with identical volume, evaporated to dryness, redissolved in 20 µL of reducing SDS-PAGE sample buffer (see earlier), heated, and centrifuged. The supernatant was collected, and 10 µL was applied to the SDS-PAGE slots. One-dimensional SDS-PAGE under reducing conditions was performed as described earlier. The gels were scanned (grayscale .tif files, 300 dpi), and the bitmaps were converted into *x*/*y*-diagrams with ImageJ freeware (National Institute of Mental Health, Bethesda, MD, U.S.A.). The peaks corresponding to HMW-GS, LMW-GS, α-gliadins, and ω1,2-gliadins were integrated. From the peak areas of the protein types present in fractions RP2 and RP3 and the areas of the one-point calibrations of the reference proteins, the amounts of the different protein types in the two fractions were obtained. Combined with the results of the determination of the protein composition of HGL by RP-HPLC, the percentage of each protein type in HGL was calculated. Duplicate determinations were made.

RESULTS AND DISCUSSION

Composition of Flour Proteins. Grains of wheat Akteur were milled into white flour (ash content 0.43% in dry mass) by means of a roller mill. The crude protein content (N × 5.7) of the flour (15.6% moisture) was 99 mg/g. The contents of Osborne fractions and gluten protein types determined by a combined extraction/RP-HPLC procedure (Wieser et al. 1998) are shown in Table I. The total content of Osborne fractions was 87.5 mg/g, which corresponded to 88% of total crude protein. As is common for wheat flour, the gliadin fraction (51.4 mg/g) was predominant, followed by the glutenin fraction (23.0 mg/g) and the albumin/globulin fraction (13.1 mg/g). In agreement with previous studies on different wheat cultivars (Wieser and Kieffer 2001), α- and γ-gliadins as well as LMW-GS (15.4–25.2 mg/g) were major components, and ω5- and ω1,2-gliadins as well as HMW-GS (4.7–6.1 mg/g) were minor components. Glutenin-bound ωb-gliadins (0.9 mg/g) were present in traces.

Characterization of Total Gliadins. The two-dimensional (2D) separation of total gliadins by SDS-PAGE is shown in Figure 2A. No reducing agent was present in the first dimension. The proteins were distributed in a M_r range from around 200,000 to 30,000. In the second dimension, a reducing agent was added, and the proteins obtained in the first dimension were separated again. The migration of monomeric proteins was not affected by the reduction

TABLE I
Contents (mg/g) of Osborne Fractions and Gluten Protein Types in Wheat Flour Akteur[a]

Method	Fraction	Proteins			
RP-HPLC	Osborne	ALGL	GLIA	GLUT	Total
		13.1 ± 0.6	51.4 ± 0.4	23.0 ± 0.6	87.5
RP-HPLC	GLIA	ω5	ω1,2	α	γ
		4.7 ± 0.1	6.1 ± 0.1	25.2 ± 0.2	15.4 ± 0.2
RP-HPLC	GLUT	ωb	HMW-GS	LMW-GS	
		0.9 ± 0.0	4.8 ± 0.2	17.3 ± 0.4	
GP-HPLC	GLIA	GLIA	HMW-GLIA	ω5	ω1,2, α, γ
		50.6 ± 0.7	10.9 ± 0.2	7.7 ± 0.2	32.0 ± 0.4

[a] Mean values of triplicate determinations ± SD. RP = reversed phase; GP = gel permeation; ALGL = albumins and globulins; GLIA = gliadins; GLUT = glutenins; ω5, ω1,2, α, and γ = gliadins; GS = glutenin subunits; and ωb = glutenin bound ω-gliadins.

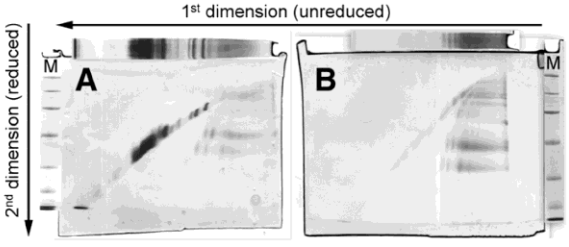

Fig. 2. Two-dimensional SDS-PAGE of total gliadins (**A**) and HMW gliadins (**B**). M = Marker proteins with the following molecular weights: myosin, 200,000; β-galactosidase, 116,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carboanhydrase, 29,000; trypsin inhibitor, 20,000; lysozyme, 14,400; and aprotinine, 6,800.

of disulfide bonds, and they migrated to the diagonal of the gel. In the case of proteins that were disulfide-linked oligomers, monomeric subunits were released; they migrated further into the gel, causing spots located below the diagonal line. Thus, 2D SDS-PAGE confirmed that interchain disulfide bonds contributed to the formation of oligomeric proteins present in total gliadins.

Total gliadins were additionally separated by analytical GP-HPLC (Fig. 3). The content of total gliadins determined by GP-HPLC (50.6 mg/g) was in agreement with that determined by RP-HPLC (Table I). GP-HPLC allowed the separation into three subfractions (GP1–GP3) according to different molecular sizes. The eluates of these subfractions were collected from several runs and analyzed by analytical RP-HPLC (Fig. 4). Although the subfractions were overlapping in their elution patterns, the chromatograms demonstrated that GP1 consisted of oligomers (HGL); its elution pattern did not show any distinct peak but only a broad elution profile. The major portion of GP2 consisted of monomeric ω5-gliadins. The remaining GP3 contained monomeric ω1,2-, α-, and γ-gliadins. The content of GP1 in flour was 10.9 mg/g (21.5% of total gliadins), that of GP2 was 7.7 mg/g (15.2%), and that of GP3 was 32.0 mg/g (63.3%) (Table I). The comparison of HGL proportions resulting from previous studies on nine international cultivars (13.2–20.3%) (Wieser et al. 1994b) revealed that Akteur was a wheat cultivar with a very high proportion (21.5%).

The M_r distribution of HGL (GP1) was estimated by calibrating the GPC column with marker proteins. The results demonstrated that HGL was roughly eluted in a range from 680,000 to 66,000, with the peak maximum at 529,000 ± 33,568. Thus, a number of HGL components had much higher M_rs than estimated by Huebner and Bietz (1993) (80,000–250,000).

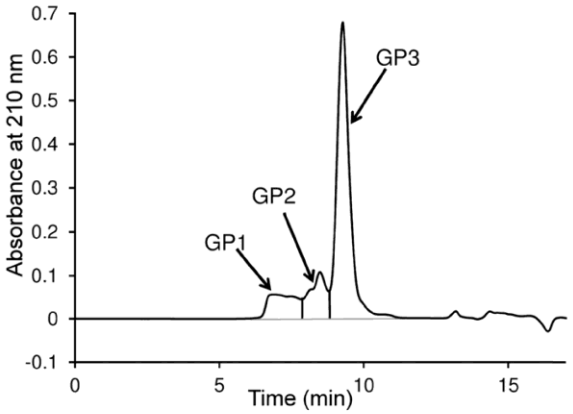

Fig. 3. Analytical gel-permeation HPLC of the gliadin fraction. Subfractions GP1, GP2, and GP3 corresponded to oligomeric HMW gliadins, monomeric ω5-gliadins, and monomeric ω1,2-, α-, and γ-gliadins, respectively.

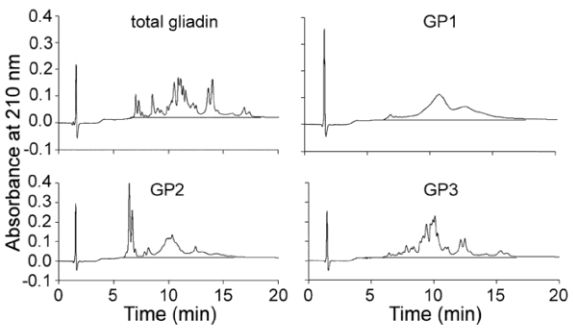

Fig. 4. Analytical reversed-phase HPLC of total gliadins and the subfractions GP1, GP2, and GP3.

Preparation of HGL. For the preparation of HGL, 50 g of flour was exhaustively extracted with a buffered salt solution to remove albumins and globulins and then extracted with 60% aqueous ethanol to obtain total gliadins, which were dialyzed to remove residual buffer salts and lyophilized. Ethanol (60% v/v) was used as the extraction solvent for gliadins, because systematic studies (Wieser et al. 1994a) revealed that this ethanol concentration provides the highest yield of gliadins. The dried fraction was preparatively separated by GP-HPLC using a column with increased length and diameter (Supplementary Fig. 1). GP1 was collected by numerous runs and lyophilized. Altogether, 18 g of total gliadins yielded 2.58 g of HGL (14.3%, based on total gliadins).

Characterization of HGL. 2D SDS-PAGE of the HGL preparation is shown in Figure 2B. The first dimension showed a protein smear with M_rs above 80,000, obviously corresponding to HGL, and protein bands with M_rs from around 80,000 to 30,000, mainly corresponding to monomeric proteins. The second dimension (after reduction of disulfide bonds) revealed that most reduced proteins were located in a M_r range below 43,000, but proteins with higher M_rs (HMW-GS) were also visible.

For further identification and quantitation of the protein components of the oligomers, HGL was reduced, and the resulting monomers were separated by preparative RP-HPLC (Fig. 5). The measurement of absorbance areas, which are highly correlated with the protein content (Wieser et al. 1998), resulted in the following proportions: 4.1% RP1, 16.4% RP2, 61.1% RP3, and 18.4% RP4 (Table II). It is known from previous studies (e.g., Wieser et al. 1990; Koenig et al. 2015) that ω5-gliadins are eluted in the range of

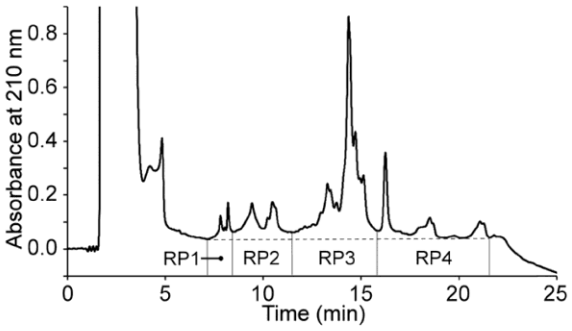

Fig. 5. Analytical reversed-phase HPLC of reduced HMW gliadins. The four subfractions RP1–RP4 are indicated.

TABLE II
Percentage of Gluten Protein Types in HMW Gliadins of Wheat Flour Akteur[a]

Subfraction	Protein Type	Percentage (%)
RP1	ω5-GLIA	4.1 ± 0.7[b]
RP2	Σ	16.4 ± 4.7[b]
	HMW-GS	7.7 ± 2.2[c]
	ω1,2-GLIA	8.7 ± 2.4[c]
RP3	Σ	61.1 ± 1.0[b]
	LMW-GS	Ø 48.2 (49.5 ± 0.8[c]/47.0 ± 0.8[d])
	α-GLIA	Ø 12.9 (11.6 ± 0.2[c]/14.1 ± 0.2[d])
RP4	γ-GLIA	18.4 ± 6.1[b]

[a] Mean values of triplicate determinations ± SD. GLIA = gliadins; and GS = glutenin subunits.
[b] Determined by reversed-phase HPLC.
[c] Estimated by semiquantitative SDS-PAGE.
[d] Estimated by semiquantitative N-terminal sequencing.

RP1, ω1,2-gliadins and HMW-GS in the range of RP2, LMW-GS and α-gliadins in the range of RP3, and γ-gliadins in the range of RP4. The eluates of the subfractions RP1–RP4 were collected, lyophilized, and analyzed by N-terminal sequencing and one-dimensional SDS-PAGE. The N-terminal sequences (nine steps) were determined by Edman degradation. Table III shows typical N-terminal sequences (nine steps) of representative gluten protein types from the literature. Sequences found in the present work are displayed in bold. Accordingly, ω5-gliadins were identified in subfraction RP1 and ω1,2-gliadin in subfraction RP2. HMW-GS could not be detected in subfraction RP2, although SDS-PAGE clearly demonstrated that this protein type was present in RP2. Obviously, N-terminal glutamic acid (E) was converted to pyroglutamic acid, which prevented N-terminal sequencing. As already shown by Lew et al. (1992), subfraction RP3 contained both the *m*- and *s*-types of LMW-GS as well as α-gliadins. In agreement with previous studies (Wieser et al. 1990), subfraction RP4 consisted of γ-gliadins.

Usually, Edman degradation is carried out as a qualitative method, that is, the identity of N-terminal amino acid sequences can be determined step by step. The experience from previous studies (Seilmeier et al. 2001), however, indicated that Edman degradation can also be used for semiquantitative purposes by quantitation of liberated single PTH amino acids. Exceptions are those amino acid residues in the sequence the quantitation of which is affected by side reactions during Edman degradation, for example, serine, cysteine, and arginine. Moreover, the first step may be critical for quantitation, in particular, when only small amounts of protein are available for analysis.

Semiquantitative N-terminal sequencing was used to determine the ratio of LMW-GS and α-gliadins in fraction RP3. Corresponding to the qualitative results (Table III), steps 3, 7, and 9 appeared to be most suitable for quantitation. Accordingly, the yields of single PTH amino acids indicated mass ratios of 84:16 (step 3), 83:17 (step 7), and 77:23 (step 9), with an average ratio of 81:19 (Table IV). Additionally, semiquantitative SDS-PAGE (Fig. 6) showed an average mass ratio of LMW-GS to α-gliadins of 77:23, which corresponded quite well to the results of the N-terminal sequence analysis. A ratio of 47:53 was determined for HMW-GS and ω1,2-gliadins in fraction RP2. The combined results of RP-HPLC, N-terminal sequence analysis, and SDS-PAGE are shown in Table II. Altogether, HGL of wheat flour Akteur consisted of

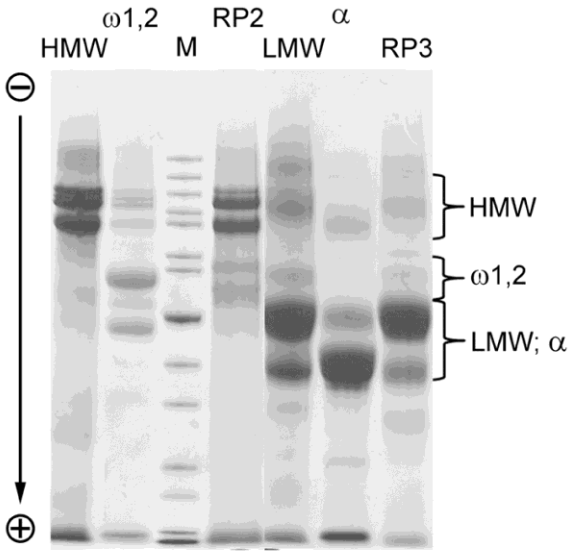

Fig. 6. SDS-PAGE of reversed-phase HPLC subfractions RP2 and RP3 for the densitometric quantitation of protein types after calibration with protein references. M indicates marker proteins (unstained protein ladder, Thermo Scientific, cat. no. 26614); ω1,2 and α are gliadins; and HMW and LMW are glutenin subunits.

TABLE III
N-Terminal Amino Acid Sequences of Proteins Present in Reversed-Phase HPLC Subfractions RP1–RP4 of HMW Gliadins of Wheat Flour Akteur[a]

		Sequence Step									
Fraction	**Protein Type**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Literature**
RP1	ω5	S	**R**	**L**	**L** Q	**S**	**P**	**R**	**G**	**K**	Seilmeier et al. (2001)
RP2	ω1,2	A	R	E	L	**N**	**P**	S	**N**	K	Seilmeier et al. (2001)
		K	E	**L**	**Q**	**S**	**P**	S	Q	N	
	HMW-GS	E	G	E	A	S	G E R	Q	L	Q	Shewry et al. (1992)
RP3	LMW-GS	**M**	**E**	**T**	S	C	**I**	**P**	**G**	**L**	Lew et al. (1992)
		S	**H**	**I**	**P**	**G**	**L**	**E**	**R**	**P**	
	α	**V**	**R**	**V**	**P**	**V**	**P**	**Q**	**L**	**Q**	Kasarda et al. (1984)
RP4	γ	N	**M**	**Q**	**V**	**D**	**P**	**S**	**G**	**Q**	Rafalski (1986)

[a] Amino acid residues detected in the present paper are shown in bold. ω5, ω1,2, α, and γ = gliadins; and GS = glutenin subunits.

TABLE IV
Semiquantitative Determination of Amino Acids and Proteins in Reversed-Phase HPLC Subfraction RP3 of HMW Gliadins of Wheat Flour Akteur by N-Terminal Sequencing[a]

			Sequence Step			
Fraction	**Protein Type**	**Amount/Ratio**	**3**	**7**	**9**	**Average Ratio**
RP3	LMW-GS		**T** **I**	**P** **E**	**L** **P**	
		pmol	328 ± 151	278 ± 138	163 ± 90	
	α		**V**	**Q**	**Q**	
		pmol	61 ± 4	57 ± 72	50 ± 37	
	Ratio	LMW-GS/α	84:16	83:17	77:23	∅ 81:19

[a] Amino acid residues detected in the present paper are shown in bold. GS = glutenin subunits; and α = gliadins.

TABLE V
Calculation of Structural Features of HMW Gliadins of Wheat Flour Akteur from Compositional Data[a]

Protein Type	Conc. (mg/g)	M_r (g/mol)	Conc. (µmol/g)	Cys Residues	Cys Conc. (µmol/g)	M_r Contrib./Subunit (g/mol)
ω5	41	55,000	0.7	1	0.7	2,255
ω1,2	87	50,000	1.7	1	1.7	4,350
HMW-GS	77	75,000	1.0	5	5.1	5,775
α	129	30,000	4.3	6	25.8	3,870
LMW-GS	482	35,000	13.8	8	110.2	16,870
γ	184	35,000	5.3	8	42.1	6,440
Total	1,000		26.8		185.6	39,560

[a] Conc. = concentration per gram of HMW gliadins; M_r = average molar mass; Cys residues = number of cysteine residues per protein subunit; Cys conc. = molar cysteine concentration; M_r contrib./subunit = contribution of protein subunit to average molar mass of a random HMW gliadin subunit; ω5, ω1,2, α, and γ = gliadins; and GS = glutenin subunits.

48% LMW-GS, 18% γ-gliadins, 13% α-gliadins, 9% ω1,2-gliadins, 8% HMW-GS, and 4% ω5-gliadins.

Concentration of S-Containing Groups and Compounds. The determination by means of the Ellman method showed that no accessible thiol groups were present in HGL. SS groups were quantitated with Ellman's reagent after a reducing step. The content of SS groups was found to be 71 ± 13 µmol per gram of protein. The concentrations of glutathione and cysteine, disulfide-linked to the proteins of HGL and potential terminators of polymerization, were determined after a reducing step by means of a stable isotope dilution assay with LC-MS[2] and isotopically labeled references. The results revealed contents of 240 ± 86 nmol of glutathione and 404 ± 8 nmol of cysteine per gram of HGL protein. The molar concentrations of the two compounds (in total 0.644 µmol/g) were in the range of the molar concentration of ω5-gliadins (Table V); however, the mass concentrations (glutathione, 74 ± 26 µg/g; cysteine, 49 ± 1 µg/g) were far below 1% of HGL and, therefore, have not been incorporated into Table II.

Considerations for the Structure of HGL. *Degree of Polymerization.* The composition of HGL from wheat cultivar Akteur shown in Table II was used to calculate the M_r of a random subunit of HGL. Assumptions for the average M_r and number of cysteine residues of the protein types are given in Table V. An average M_r of 39,560 of a random subunit of HGL was obtained. From the M_r range of HGL, determined by GP-HPLC (66,000–680,000) and the M_r of a random HGL subunit, the degree of polymerization of HGL between 2 and 17 was calculated. The average degree of polymerization calculated from the maximum of the GP-HPLC peak of HGL (529,000) was 13.

Concentration of SS Groups. From the protein composition of HGL an average cysteine concentration of 186 µmol per gram of protein was obtained (Table V). Because no free SH groups were present, all cysteine residues had to be present as disulfides, corresponding to 93 µmol of SS groups per gram of protein. These values corresponded to the experimentally determined concentration of SS groups of 71 µmol per gram of protein.

Distribution of Chain Terminators. From the M_r of a random HGL subunit (39,560) and the average degree of polymerization (13), a M_r of 514,000 for an average HGL molecule was calculated. Termination on both ends of the molecule corresponded to 2 mol of SS groups per mole of HGL. Consequently, 1 g of HGL contained 3.9 µmol of SS bonds in which terminators were involved. The comparison of these amounts with the total concentrations of glutathione plus cysteine (0.644 µmol/g) showed that 16.5% of the terminator positions were occupied by LMW thiols. Thus, proteins with an odd number of cysteine restudies were located in 83.5% of the terminator positions.

CONCLUSIONS

The existence of HGL can be explained by the presence of terminators, which interrupt the polymerization of glutenin subunits during biosynthesis and lead to polymers of limited size (oligomers) that are still soluble in aqueous ethanol. Thus, HGL could be seen as a kind of "by-product" of glutenin formation. Glutathione, cysteine, and gliadins with an odd number of cysteine residues may act as terminators. Examples of gliadins with an odd number of cysteine residues are described in protein sequence databases (e.g., www.uniprot.org/uniprot/K7WV57; www.uniprot.org/uniprot/B6UKQ2). We postulate that the high content of LMW-GS in HGL suggests that this protein type serves as the "core" of HGL. HMW-GS might also be partly involved. Gliadins and LMW thiols are likely to terminate the polymerization. Further work on the location of disulfide bonds in HGL and on HGL contents of different wheat cultivars as affected by the presence of a thiol blocking reagent during isolation is in progress.

The results of this study have been obtained using flour of the wheat cultivar Akteur. We assume that HGL from other wheat cultivars contains the same protein types as cultivar Akteur but with quantitative differences and, thus, also differences in the average molecular mass. Therefore, further studies on the composition of HGL from other wheat cultivars than Akteur as well as studies on the effect of the extraction solvent on the content and composition of HGL are currently underway.

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3.2 Alkylation of free thiol groups during extraction – effects on the Osborne fractions of wheat flour

The aim of the second part of the work was to study, how thiol group alkylation during the Osborne fractionation affects the qualitative and quantitative composition of the fractions, in particular the content of HGL. Furthermore, the differences in the composition and structure of HGL extracted in the presence (HGL-N) and absence (HGL-0) of the thiol-alkylating reagent NEMI should be elucidated. Markus Schmid carried out all experimental work, wrote the draft of the manuscript and significantly contributed to the revised version.

Twelve wheat samples from different cultivars and harvest years were extracted by a modified Osborne fractionation with and without addition of the thiol blocking reagent NEMI. To block free thiol groups, NEMI was added to the salt solution during the first step of the fractionation. Then, HGL-0 and HGL-N were separated by GP- and RP-HPLC and the protein compostions determined. A set of twelve wheat flours from different cultivars and harvest years was used to make sure that differences of the protein compositions were not specific for a single wheat cultivar or crop year. Additionally, HGL-0 and HGL-N preparations were isolated on a larger scale (ca. 230 mg) and proteinchemically compared. The content of gliadin tended to decrease after addition of NEMI, while the content of glutenin increased. The decrease of gliadins was predominantely caused by a reduced amount of α-gliadin and the increase of glutenins could be observed in all protein types, namely ω_b-gliadins, HMW- and LMW-GS. These changes lead to a decline of the gliadin/glutenin ratio in HGL-N. After addition of NEMI significantly more HGL could be extracted from most of the flour samples. It was assumed that all of these changes were results of thiol-disulfide exchange reactions, which were inhibited by NEMI in the extraction solution. The comparison of the two HGL preparations (HGL-0 and HGL-N) showed that the percental amount of all involved subunits except LMW-GS and α-gliadins did not change significantly. However, a higher amount of LMW-GS and a lower amount of α-gliadins was observed in HGL-N. Other differences were the higher amounts of total thiols (86 µmol/g protein and 71 µmol/g protein, respectively) and cysteine (0.734 µmol/g protein and 0.404 µmol/g protein, respectively) as well as the lower amount of glutathione (0.146 µmol/g protein and 0.240 µmol/g protein, respectively)

in HGL-N compared to HGL-0. The average size of a HGL oligomer also changed, when NEMI was present during extraction. It declined from about 530000 to about 470000, i.e., HGL-N was about 2 subunits shorter on average than HGL-0.

e-Xtra*

Alkylation of Free Thiol Groups During Extraction: Effects on the Osborne Fractions of Wheat Flour

Markus Schmid, Herbert Wieser, and Peter Koehler†

ABSTRACT

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Wheat proteins are classified according to solubility into the so-called Osborne fractions. Because wheat flour contains both free thiol and disulfide groups, thiol–disulfide interchange reactions are possible during extraction. Osborne fractionation of 12 different wheat flour samples was performed in the presence of *N*-ethylmaleinimide (NEMI) to alkylate free thiol groups and without addition of NEMI (control). The addition of NEMI during extraction tended to decrease the content of gliadins (predominantly α-gliadins) and caused an increase of the content of glutenins in most flour samples. Thus, alkylation of free thiol groups during extraction led to a decline of the gliadin/glutenin ratio from 2 (control) to approximately 1.5 (NEMI). NEMI and control gliadins were separated by gel-permeation HPLC into an oligomeric subfraction (high-molecular-weight [HMW] gliadins) and two monomeric subfractions. In most flours (8 of 12), the addition of NEMI led to a significant increase of the content of HMW gliadins. HMW gliadins from cultivar Akteur wheat were preparatively isolated from NEMI and control gliadins and characterized by HPLC, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and N-terminal sequencing. HMW gliadin isolated in the presence of NEMI had a significantly higher content of low-molecular-weight glutenin subunits and disulfide-bound cysteine as well as a lower content of α-gliadins and disulfide-bound glutathione compared with the control.

Osborne (1924) introduced a solubility-based classification of wheat proteins that utilized different solvents for the sequential extraction of flour, providing the fractions albumins, globulins, gliadins (GLIA), and glutenins (GLUT). Albumins are defined as the fraction soluble in water, globulins can be dissolved in dilute salt solution, and GLIA can be extracted with aqueous alcohols. Residual proteins correspond to the GLUT fraction, which can be dissolved as GLUT subunits (GS) in aqueous alcohols under reducing and disaggregating conditions. Albumins and globulins mainly consist of metabolic proteins, whereas GLIA and GLUT are storage proteins that form a gluten network upon mixing of flour with water. Some GLIA, in particular ω-types, are soluble in water to a small extent but not in dilute salt solutions (Wieser et al. 1998). Thus, in the classical Osborne fractionation, the albumin fraction contains small amounts of GLIA. Therefore, the modified Osborne procedure used in our laboratory starts the extraction of wheat flour with dilute salt solution providing a joint albumin/globulin (ALGL) fraction devoid of GLIA (Wieser et al. 1998).

The contents of the Osborne fractions in wheat flour are typically in a range of 16–34% ALGL, 48–62% GLIA, and 15–31% GLUT (Koenig et al. 2015). According to amino acid sequences, GLIA have been classified into four different types: α-, γ-, ω5-, and ω1,2-GLIA. GLUT mainly consist of low-molecular-weight (LMW) and high-molecular-weight (HMW) GS as well as small amounts of GLUT-bound ω-GLIA (ωb-GLIA). Complete amino acid sequences of many gluten protein types (e.g., accession numbers P10388, P93791, or Q1WA39) (UniProt Knowledge Base, www.uniprot.org) as well as quantitative data on the composition of the GLIA and GLUT fractions (Wieser et al. 2014) are available to date.

The Osborne fractionation does not provide a clear-cut separation of wheat proteins according to their structural classification. For example, the GLIA fraction mainly contains monomeric proteins, but a small part consists of oligomeric proteins also soluble in aqueous alcohols. We have used the term HMW GLIA (HGL) for this fraction (Schmid et al. 2016). Contents of 6–37% of total GLIA, depending on cultivar and extraction method (Beckwith et al. 1966; Huebner and Bietz 1993), have been reported for HGL. In-depth analysis of the composition of HGL from cultivar Akteur wheat flour showed that the molecular mass distribution of the oligomers was in a range of 66,000–680,000, with an average degree of polymerization of 13 (Schmid et al. 2016). All gluten protein types were present in HGL, with contents of 48% LMW-GS, 18% γ-GLIA, 13% α-GLIA, 9% ω1,2-GLIA, 8% HMW-GS, and 4% ω5-GLIA. It was hypothesized that the formation of HGL can be explained by the presence of terminators that interrupt the thiol- or disulfide (SS)-mediated polymerization of GLUT subunits during biosynthesis and lead to oligomers that are still soluble in aqueous ethanol.

However, a different interpretation for the presence of HGL would also be possible. It has been shown that wheat flours contain free thiol groups in concentrations of 10–200 μmol/g of flour (Grosch and Wieser 1999), which are able to induce structural changes of gluten proteins by promoting thiol–SS interchange reactions when they are mixed or heated in the presence of water (Lagrain et al. 2008b). In particular, LMW thiols such as glutathione and cysteine induce a depolymerization of polymeric GLUT (Grosch and Wieser 1999). Therefore, HGL could also be regarded as reaction products formed during mixing of dough. Because Osborne fractionation can be considered as a kind of "mixing" of flour, comparable reactions as during dough formation can be assumed. Consequently, alkylation of free thiol groups (e.g., by *N*-ethylmaleinimide [NEMI] or iodoacetamide) during the Osborne fractionation would inhibit thiol–SS interchange reactions (Ewart 1990; Schurer et al. 2007). However, the effect of free thiol alkylation on the distribution and composition of the protein fractions during the Osborne fractionation has not yet been studied.

Therefore, the aim of this study was to elucidate the qualitative and quantitative influence of thiol group alkylation during the Osborne fractionation in a selection of 12 wheat samples from different cultivars and harvest years on the resulting protein fractions and GLIA and GLUT types, as well as to study the differences in the composition and structure of HGL extracted in the presence and absence of the thiol-alkylating reagent NEMI.

MATERIALS AND METHODS

Chemicals. The purity of the chemicals was analytical grade or stated otherwise. In addition to the chemicals used by Schmid et al. (2016), NEMI was from Applichem GmbH (Darmstadt, Germany).

Wheat Flours. Twelve wheat grain samples of nine cultivars (Table I) were obtained from different German breeders. Grain

*The *e*-Xtra logo stands for "electronic extra" and indicates that one supplementary figure is published online.

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samples of cultivar Akteur wheat were from four different harvest years (2010, 2011, 2013, and 2015); all other samples were from single harvest years. Grains were milled into white flour on the same day by means of a laboratory roller mill (Quadrumat Junior II, Brabender, Duisburg, Germany) and passed through a 0.2 mm sieve. The flours were stored for at least 2 weeks at room temperature (approximately 20°C) before use. Analytical data of the flours were 9.0–13.2% crude protein content, 11.9–15.5% moisture content, and 0.41–0.64% (db) ash content (Table I).

Standard Determinations. The crude protein content (N × 5.7, calibration with ethylenediaminetetraacetic acid) was determined by the method of Dumas according to ICC method 167/1 (ICC 1978) with a TruSpec nitrogen analyzer (Leco, Kirchheim, Germany). An infrared balance (MA35, Sartorius, Goettingen, Germany) was used to determine the moisture contents. Flour (3.5 g) was heated to 100°C until the residual weight was constant (6–8 min). The ash contents of the flours were determined according to ICC method 104/1. All quantitative determinations were made in triplicate.

Modified Osborne Fractionation and Chromatographic Separation of Fractions. Modified Osborne fractionation was carried out as described by Schmid et al. (2016) without NEMI (control) and additionally by adding NEMI (0.6 mg/g of flour; 4.8 µmol/g of flour) to the solvent of the first extraction step of the ALGL fraction. The corresponding fractions obtained are marked with the extension "-0" for the control fractions and "-N" for the NEMI fractions in this article. ALGL-0, ALGL-N, GLIA-0, GLIA-N, GLUT-0, and GLUT-N fractions were separated and quantitated by reversed-phase (RP)-HPLC and the GLIA-0 and GLIA-N fractions additionally by gel-permeation (GP)-HPLC, as described by Schmid et al. (2016). All quantitative determinations were made in triplicate.

Preparation of GLIA-0, GLIA-N, HGL-0, and HGL-N. GLIA-0 and GLIA-N were isolated from 50 g of flour of Akteur wheat (harvest year 2011), as described by Schmid et al. (2016). A buffered salt solution (2 × 200 mL; 0.4M NaCl plus 0.067M Na_2HPO_4/KH_2PO_4, pH 7.6) that contained either no NEMI or NEMI in a concentration of 0.6 mg/g of flour (4.8 µmol/g of flour) to alkylate free thiol groups was used in the first extraction step of the ALGL fraction. After ALGL-0 and ALGL-N had been removed, GLIA-0 and GLIA-N, respectively, were extracted with 60% (v/v) ethanol (3 × 200 mL). The supernatants of the ALGL-0 and ALGL-N extractions were discarded, and those of each GLIA extraction (GLIA-0 and GLIA-N) were combined, concentrated in a rotary evaporator (Rotavapor RII, Büchi, Essen, Germany), and dialyzed for three days against acetic acid (0.01M) and one day against deionized water. The dialyzed solutions were then lyophilized.

GLIA-0 and GLIA-N (100 mg each) of Akteur wheat flour (harvest year 2011) were dissolved in 60% (v/v) ethanol (4 mL), filtered through a 0.45 µm membrane, and separated by preparative GP-HPLC, as described by Schmid et al. (2016). HGL-0 and HGL-N fractions (Fig. 1, fraction GP1) were collected from several runs and lyophilized.

Isolation of HGL Subfractions for N-Terminal Sequencing and SDS-PAGE/Densitometry. Subfractions of HGL-0 and HGL-N from Akteur wheat (harvest year 2011) were isolated by RP-HPLC under the following conditions: instrument, Thermo HPLC with a SpectraSystem UV 1000 detector (Thermo Fisher Scientific, Dreieich, Germany); column, Dionex Acclaim 300 (C_{18}, 3 µm, 30 nm, 2.1 × 150 mm, Thermo Fisher Scientific) mobile phase, solvent A was water/trifluoroacetic acid (TFA) (999/1, v/v) and solvent B was acetonitrile/TFA (999/1, v/v); gradient, linear 0–16 min, 30–52% B; flow rate, 0.3 mL/min; temperature, 60°C; injection volume, 50 µL for preparation and 10 µL for quantitation (c = 2.5 mg/mL); detection, 210 nm; and run time, 30 min. HGL-0 and HGL-N samples were dissolved in GLUT extraction solution (c = 2.5 mg/mL; 50% [v/v] 1-propanol, 2M urea, 0.05M Tris-HCl [pH 7.5], and 5% [m/v] dithiothreitol) and heated to 60°C for 10 min. Four subfractions (RP1–RP4 of HGL-0 and HGL-N, respectively) (Supplementary Fig. 1) were collected from several preparative runs, lyophilized, and stored at −24°C until analysis by N-terminal sequencing and SDS-PAGE/densitometry. Chromatograms were also used for quantitation of subfractions RP1-0 to RP4-0 and RP1-N to RP4-N. For external calibration, reference GLIA (c = 2.5 mg/mL) from the Working Group on Prolamin Analysis and Toxicity

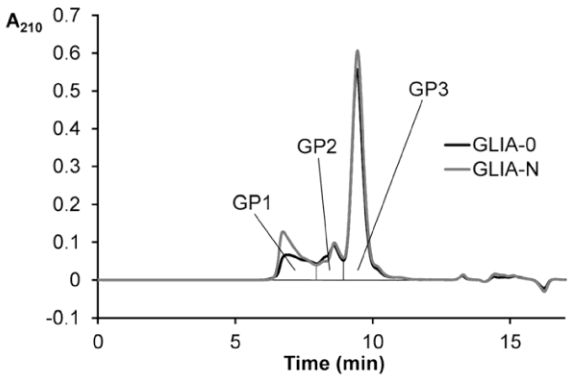

Fig. 1. Analytical gel-permeation HPLC of gliadins (GLIA) from Akteur wheat (harvest year 2011) isolated in the absence (GLIA-0) and the presence (GLIA-N) of *N*-ethylmaleinimide during modified Osborne fractionation. Subfractions GP1, GP2, and GP3 corresponded to oligomeric high-molecular-weight gliadins, monomeric ω5-GLIA, and monomeric ω1,2-, α-, and γ-GLIA, respectively.

TABLE I
Characterization of Wheat Flours[y]

Wheat Cultivar (Quality)[z]	Harvest Year	Crude Protein (%)	Ash (%, db)	Moisture (%)
Akteur (E)	2010	11.9 ± 0.0	0.61 ± 0.1	11.9 ± 0.1
Akteur (E)	2011	10.2 ± 0.1	0.43 ± 0.0	15.5 ± 0.1
Akteur (E)	2013	13.2 ± 0.1	0.58 ± 0.0	14.4 ± 0.1
Akteur (E)	2015	12.7 ± 0.3	0.64 ± 0.1	13.5 ± 0.0
Event (E)	2011	11.4 ± 0.1	0.46 ± 0.1	13.4 ± 0.1
Genius (E)	2011	11.5 ± 0.0	0.55 ± 0.1	15.1 ± 0.1
Julius (A)	2013	9.6 ± 0.1	0.49 ± 0.0	13.6 ± 0.1
Mulan (B)	2011	9.7 ± 0.1	0.51 ± 0.0	12.8 ± 0.1
Pamier (A)	2013	11.8 ± 0.3	0.46 ± 0.1	13.4 ± 0.1
Tommi (A)	2013	11.0 ± 0.1	0.43 ± 0.1	14.0 ± 0.1
Tuerkis (A)	2011	9.7 ± 0.1	0.54 ± 0.1	14.5 ± 0.1
Winnetou (C)	2013	9.0 ± 0.1	0.41 ± 0.0	14.9 ± 0.1

[y] Mean values of triplicate determinations ± standard deviations.
[z] Wheat quality according to the German classification system: E = elite; A = high; B = bread; and C = cookie.

(PWG-gliadin) (van Eckert et al. 2006) was dissolved in 60% ethanol (v/v), injected (5, 10, 15, and 20 µL), and analyzed. At least three determinations were made for quantitation.

Analytical Characterization of GLIA and HGL. GLIA-0 and GLIA-N as well as HGL-0 and HGL-N of Akteur wheat (harvest year 2011) were analytically compared by means of the methods described by Schmid et al. (2016). The following methods were used: 1) one-dimensional SDS-PAGE of GLIA-0 and GLIA-N, 2) two-dimensional (2D)-SDS-PAGE, 3) N-terminal sequence analysis of amino acid residues 1–9 of reduced HGL-0 and HGL-N subfractions RP2 and RP3, 4) SDS-PAGE/densitometry of reduced HGL-0 and HGL-N subfractions RP1–RP4 after calibration with corresponding protein references, 5) quantitation of thiol and SS groups of HGL-0 and HGL-N, and 6) quantitation of protein-bound glutathione and cysteine of HGL-0 and HGL-N by a stable isotope dilution assay with liquid chromatography tandem mass spectrometry.

Statistical Analysis. Statistical evaluation of the data was carried out with Sigma Plot 12.0 software (Systat, San Jose, CA, U.S.A.). Mean values and standard deviations were calculated. Additionally, unpaired two-way *t* tests were applied. Levels of $P \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

RP- and GP-HPLC of Protein Fractions. The contents of Osborne fractions and gluten protein types of the 12 wheat flours (Table I), extracted without NEMI or with NEMI added at the beginning of the ALGL extraction and determined by a combined extraction/RP-HPLC procedure (Wieser et al. 1998; Schmid et al. 2016), are shown in Table II. In general, the percentage of total extractable protein (Σ in Table II) was approximately 80% of the crude protein content (Table I). This is slightly higher than the values of 68–73% reported by Koenig et al. (2015) for different wheat species. In most cases (Akteur harvest years 2013 and 2015 were exceptions), the addition of NEMI did not influence the content of total extractable proteins. The same was true for the ALGL fractions. The total amount of extracted gluten proteins (GLIA + GLUT) was 61–93 mg/g for flours extracted in the presence of NEMI and 63–100 mg/g in flours extracted without NEMI. This corresponds to published contents of gluten proteins (68–94 mg/g) of wheat flours (Thanhaeuser et al. 2014). It appears that NEMI had no overall effect on the extractability of gluten proteins. The GLIA-0 and GLIA-N fractions (41.1–71.0 mg/g) dominated in all samples, and there was a general trend that the content of GLIA-N was lower compared with GLIA-0. Inversely, the concentration of total GLUT-N (11.5–32.8 mg/g) was significantly higher than GLUT-0 in 8 of 12 flours. These changes also influenced the GLIA/GLUT ratio, which declined in 7 of 12 samples when NEMI was added. Because GLIA act as plasticizers in gluten, a low GLIA/GLUT ratio is related to doughs with low extensibility and high resistance to extension. Experimental evidence for the strengthening effect of NEMI on wheat dough had already been obtained in the 1960s by Frater et al. (1960). The effect of NEMI is comparable to ascorbic acid, which leads to a decrease of free glutathione in dough and inhibits depolymerization of GLUT, thus yielding stronger dough (Grosch and Wieser 1999). The comparison of the GLIA-0 and GLIA-N protein types showed that the contents of ω5-, ω1,2-, and γ-GLIA were not affected by NEMI. α-GLIA were responsible for the decrease of GLIA-N compared with GLIA-0 because the content of this protein type significantly decreased in most samples when NEMI was used during extraction. This can be explained by the work of Lagrain et al. (2008a) on heating of gluten model systems. They showed that the activation energy of α-GLIA for thiol-SS interchange (110 kJ/mol) is lower than that of γ-GLIA (147 kJ/mol). This means that α-GLIA reacts faster than γ-GLIA, in particular at low temperature. The concurrent increase of GLUT-N compared with GLUT-0 was caused by all GLUT types. The highest increase was observed for LMW-GS-N.

GLIA-0 and GLIA-N from the modified Osborne fractionation were also separated by analytical GP-HPLC. A typical chromatogram obtained with GLIA-0 and GLIA-N of Akteur wheat (harvest

TABLE II
Concentration of Osborne Fractions, Gluten Protein Types, Gluten, Extractable Protein, and Gliadin/Glutenin Ratio of Wheat Flours Extracted in the Absence or Presence of *N*-Ethylmaleinimide[y]

		Concentration (mg/g of Flour)											
Flour, Year	Ext[z]	ALGL	ω5	ω1,2	α	γ	GLIA	ωb	HMW	LMW	GLUT	Gluten	Σ	Ratio
Akteur 2010	A	12.9a	3.2a	3.2a	23.8a	14.6a	44.8a	0.4a	3.8a	17.8a	22.0a	66.8	79.7	2.0a
	B	13.2a	3.1b	3.0a	20.2b	14.8a	41.1a	0.5a	4.1a	19.5b	24.1b	65.2	78.4	1.7b
Akteur 2011	A	8.5a	4.0a	4.6a	26.9a	13.7a	49.2a	0.5a	4.5a	17.6a	22.6a	71.8	80.3	2.2a
	B	7.2a	4.0a	4.0a	21.9b	15.3a	45.2a	0.6a	5.2a	21.6b	27.4b	72.6	79.8	1.7a
Akteur 2013	A	7.2a	6.8a	8.5a	36.2a	19.5a	71.0a	0.7a	6.7a	21.4a	28.8a	99.8	107.0	2.5a
	B	9.5a	6.1b	6.0b	29.9a	17.9a	59.9a	0.8a	7.1a	24.9b	32.8b	92.7	102.2	1.8b
Akteur 2015	A	14.6a	5.0a	6.6a	33.3a	21.1a	66.0a	0.4a	5.0a	18.7a	24.1a	90.1	104.7	2.7a
	B	14.0a	4.8a	5.6b	27.9b	20.5a	58.8b	0.5a	5.6a	21.7a	27.8b	86.6	100.6	2.1b
Event 2011	A	11.9a	5.0a	5.3a	27.5a	19.0a	56.8a	0.5a	5.4a	16.4a	22.3a	79.1	91.0	2.6a
	B	11.5b	4.8a	4.9a	23.3a	19.0a	52.0a	0.6a	5.8a	19.6a	26.0b	78.0	89.5	2.0b
Genius 2011	A	10.1a	4.9a	5.8a	33.7a	18.3a	62.7a	0.4a	4.1a	14.2a	18.7a	81.4	91.5	3.4a
	B	9.6a	4.6b	5.4b	30.5a	17.8a	58.3b	0.5a	5.0a	16.4b	21.9b	80.2	89.8	2.7b
Julius 2013	A	10.2a	3.2a	3.5a	29.4a	15.3a	51.4a	0.4a	3.4a	14.0a	17.8a	69.2	79.4	2.9a
	B	10.1a	3.2a	3.8a	25.4b	16.5b	48.9a	0.5a	4.0b	17.0b	21.5b	70.4	80.5	2.3b
Mulan 2011	A	8.0a	2.1a	3.0a	26.6a	16.4a	48.1a	0.4a	3.7a	12.2a	16.3a	64.4	72.4	3.0a
	B	9.3a	2.1a	2.9a	23.2a	16.8a	45.0a	0.3b	3.9b	13.4b	17.6b	62.6	71.9	2.6a
Pamier 2013	A	9.2a	1.1a	12.7a	28.8a	19.6a	62.2a	0.3a	5.7a	12.0a	18.0a	80.2	89.4	3.5a
	B	10.0a	1.0b	12.2a	25.0b	18.7a	56.9b	0.3a	5.4b	13.9a	19.6a	76.5	86.5	2.9b
Tommi 2013	A	11.8a	4.7a	3.6a	30.3a	16.4a	55.0a	0.7a	4.3a	19.1a	24.1a	79.1	90.9	2.3a
	B	11.1b	4.6a	3.9a	26.8a	16.7a	52.0a	0.5a	4.2b	19.8a	24.5a	76.5	87.6	2.1a
Tuerkis 2011	A	12.2a	2.4a	2.6a	27.9a	16.5a	49.4a	0.4a	3.3a	15.4a	19.1a	68.5	80.7	2.6a
	B	12.3a	2.5a	2.5a	23.5b	17.7a	46.2a	0.4a	3.5a	17.5a	21.4a	67.6	79.9	2.2a
Winnetou 2013	A	12.2a	1.1a	9.2a	25.6a	15.5a	51.4a	0.3a	3.1a	8.1a	11.5a	62.9	75.1	4.5a
	B	12.5a	0.9b	8.9a	23.7a	15.6a	49.1a	0.3a	3.3a	8.3a	11.9a	61.0	73.5	4.1b

[y] Mean values of triplicate determinations; average relative standard variation: ±3.7%. Different lowercase letters indicate statistically significant differences between extraction methods A and B, respectively (*t* test, $P \leq 0.05$). ALGL = albumins/globulins; GLIA = gliadins; GLUT = glutenins; ω5, ω1,2, α, and γ = gliadin types; HMW and LMW = high- and low-molecular-weight glutenin subunits, respectively; ωb = glutenin-bound ω-gliadins; Gluten = gliadins + glutenins; Σ = total extractable protein; and Ratio = GLIA/GLUT ratio.

[z] Extraction: A = no addition; and B = NEMI added in the first extraction step of ALGL.

year 2011) is shown in Figure 1. The chromatograms showed three subfractions (GP1–GP3) of different average molecular weight (M_r) ranges. It was known from earlier studies on GLIA-0 that GP1 contained HGL, GP2 contained ω_5-GLIA, and GP3 contained other GLIA types such as ω1,2-, α-, and γ-GLIA (Schmid et al. 2016). The area of the HGL-N peak was considerably higher compared with the peak of HGL-0. Integration of the chromatograms of all wheat cultivars provided quantitative data for GLIA-0, GLIA-N, HGL-0, and HGL-N. In general, GP- and RP-HPLC provided comparable concentrations of total GLIA-0 and total GLIA-N, respectively (Table II, Fig. 2). The concentrations of HGL-N were significantly higher than the corresponding contents of HGL-0 in 8 of 12 samples (Fig. 2). Regarding the monomeric subfractions GP2 and GP3 of GLIA-0 and GLIA-N, they were lower in the latter samples (data not shown).

The different wheat cultivars used in this study showed different responses to NEMI treatment. Depending on the baking quality, there was a trend that alkylation of free thiol groups had a stronger effect on all cultivars with very good baking quality (Akteur, Event, and Genius) (Table I). For example, the increase of the GLUT and the HGL fractions obtained in the presence of NEMI was significant in all cultivars with the E level of quality (Table II, Fig. 2). This showed that, in general, NEMI preserved a higher polymerization state of proteins. Cultivars with lower baking quality showed the same trend, but the differences were partly not significant. From this, it could be assumed that differences of NEMI and control samples between ethanol-soluble (monomeric and oligomeric) and ethanol-insoluble gluten proteins were smaller in these cultivars compared with the E-level cultivars. It can be assumed that, in normal breadmaking applications (without NEMI), the depolymerization of gluten proteins during mixing and, thus, the impact on dough and gluten properties would be more pronounced in cultivars with very good baking quality.

The M_r distribution of HGL-N and HGL-0 (Fig. 1, fraction GP1) was estimated by calibrating the GP-HPLC column with marker proteins. HGL-N was eluted in a M_r range of 66,000–680,000, identical to HGL-0. Thus, some protein components of both HGL-N and HGL-0 had a much higher M_r than that reported by Huebner and Bietz (1993) (80,000–250,000). The maximum peak height of HGL-0 indicated an M_r of approximately 530,000, whereas HGL-N showed a maximum at an approximate M_r of 472,000. However, this difference was not significant.

Isolation of HGL-N. Osborne fractionation of 50 g of flour (Akteur, harvest year 2011) in the presence of NEMI provided 1.9 g of GLIA-N (2.2.%) after lyophilization. The dried fraction was preparatively separated by GP-HPLC by using a column with increased width. HGL-N (Fig. 1, fraction GP1) was collected by numerous runs and lyophilized. Altogether, 1.9 g of GLIA-N yielded 0.23 g of HGL-N (12.1%, based on GLIA-N). In our previous study, 18 g of GLIA-0 from Akteur wheat (harvest year 2011) yielded 2.58 g of HGL-0 (14.3%, based on GLIA-0) (Schmid et al. 2016). The gravimetric yields of HGL differed from the quantitative data determined by analytical GP-HPLC (Fig. 1) because of losses of some HGL-N material during lyophilization.

2D-SDS-PAGE. The gel of the 2D-SDS-PAGE of GLIA-N from Akteur wheat (harvest year 2011) is shown in Figure 3A. No reducing agent was present in the first dimension, and the M_r of GLIA-N was approximately 30,000–200,000. Larger proteins of M_r up to 600,000 were also present but had not been able to enter the gel under nonreducing conditions. In the second dimension, in which a reducing agent was added, the migration of monomeric proteins was not affected by the reduction of SS bonds, and they migrated to the diagonal of the gel. SS-linked oligomers were cleaved and released monomeric subunits that migrated further into the gel, causing spots located below the diagonal. This experiment showed that a part of GLIA-N consisted of oligomeric proteins linked by interchain SS bonds, here referred to as HGL-N. 2D-SDS-PAGE of GLIA-0 (no NEMI added) yielded the same result (Schmid et al. 2016).

2D-SDS-PAGE of the HGL-N preparation is shown in Figure 3B. The results were basically identical with HGL-0 (Schmid et al. 2016). In the first dimension, a smear of oligomers with M_r beyond 80,000 and two protein bands of M_r in the range of 50,000–60,000 were present. The latter were most likely ω5-GLIA from fraction GP2 (Fig. 1) that became part of HGL-N because of overlap of the fractions. In the second dimension, most proteins migrated to an M_r range below 43,000. However, proteins with higher M_r, possibly corresponding to HMW-GS, were also present. Compared with the gel of GLIA-N, it was obvious that HGL-N contained only a very small portion of monomeric proteins, most likely ω5-GLIA (see above).

Quantitation of Protein Subunits of HGL. HGL-0 and HGL-N were reduced to quantitate the protein subunits that were involved in HGL formation. Separation by RP-HPLC showed four subfractions, RP1–RP4. A previous study with HGL-0 had shown that RP1 contained ω5-GLIA, RP2 consisted of ω1,2-GLIA and HMW-GS, RP3 included LMW-GS and α-GLIA, and RP4 contained γ-GLIA (Schmid et al. 2016). The RP-HPLC absorbance

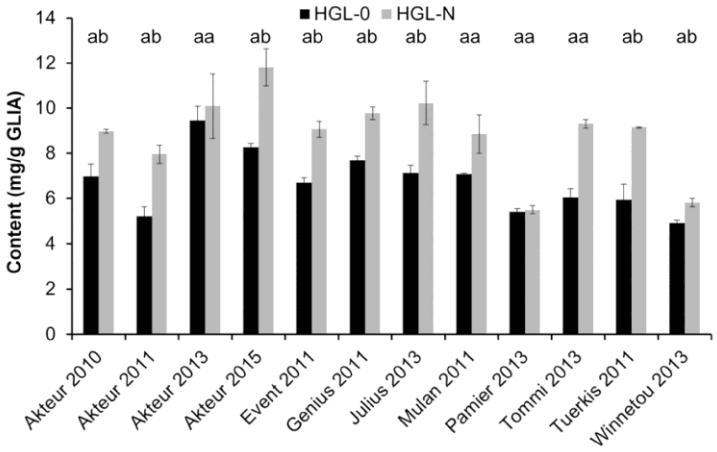

Fig. 2. Concentration of high-molecular-weight gliadins (HGL) in 12 wheat flours isolated in the absence (HGL-0) and the presence (HGL-N) of *N*-ethylmaleinimide during modified Osborne fractionation. Gliadins were separated by analytical gel-permeation HPLC. HGL-0 and HGL-N were eluted as subfraction GP1 shown in Figure 1. Mean values of triplicate determinations ± standard deviation are given. Different lowercase letters indicate significant differences within each column pair (*t* test; $P \leq 0.05$).

areas, highly correlated with the protein contents (Wieser et al. 1998), were comparable in the subfractions from both extraction alternatives (Table III). Subfraction RP3 was most abundant, followed by RP4, RP2, and RP1.

All subfractions of HGL-0 and HGL-N obtained by RP-HPLC were collected, lyophilized, and analyzed by N-terminal sequence analysis (Edman degradation) and one-dimensional SDS-PAGE. Typical N-terminal sequences (nine steps) of representative gluten protein types from literature are shown in Table IV. Sequences found in both HGL-0 and HGL-N are displayed in bold, sequences found only in HGL-0 are displayed in italics, and sequences found only in HGL-N are underlined. The experiment clearly verified the presence of ω5-GLIA in RP1, ω1,2-GLIA in RP2, LMW-GS and α-GLIA in RP3, and γ-GLIA in RP4. It was known from a previous

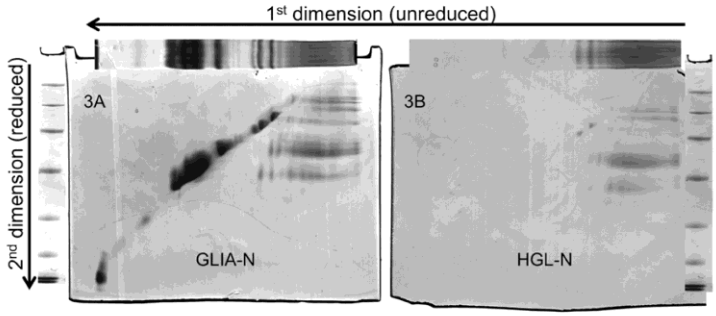

Fig. 3. Two-dimensional SDS-PAGE of gliadin (GLIA-N) (**A**) and high-molecular-weight gliadins (HGL-N) (**B**) from Akteur wheat (harvest year 2011) isolated in the presence of *N*-ethylmaleinimide during modified Osborne fractionation. At the left and right sides are marker proteins for molecular weight (myosin, 200,000; β-galactosidase, 116,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carboanhydrase, 29,000; trypsin inhibitor, 20,000; lysozyme, 14,400; and aprotinine, 6,800).

TABLE III
Proportions of Gluten Protein Types in High-Molecular-Weight Gliadins (HGL) from Akteur Wheat (Harvest Year 2011) Isolated in the Absence (HGL-0) and Presence (HGL-N) of *N*-Ethylmaleinimide During Modified Osborne Fractionation[w]

		HGL (%)	
HGL	**Protein Type**	**HGL-0**	**HGL-N**
RP1	ω5-GLIA	4.1 ± 0.6a[x]	3.8 ± 0.6a[x]
RP2	Σ	16.4 ± 4.1a[x]	12.3 ± 2.4a[x]
	HMW-GS	7.7 ± 1.9a[y]	5.6 ± 1.1a[y]
	ω1,2	8.7 ± 2.1a[y]	6.7 ± 1.3a[y]
RP3	Σ	61.1 ± 1.0a[x]	67.2 ± 1.3b[x]
	LMW-GS	47.0 ± 0.7a[y], 49.5 ± 0.7a[z]	52.7 ± 1.0b[y], 58.8 ± 1.2b[z]
	α-GLIA	14.1 ± 0.2a[y], 11.6 ± 0.2a[z]	14.5 ± 0.3b[y], 8.4 ± 0.2b[z]
RP4	γ-GLIA	18.4 ± 5.3a[x]	16.7 ± 3.0a[x]

[w] Mean values of triplicate determinations ± standard deviations. Different lowercase letters indicate statistically significant differences between corresponding values of HGL-0 and HGL-N (*t* test, $P \leq 0.05$). Data for HGL-0 taken from Schmid et al. (2016). RP1–RP4 = subfractions isolated by reversed-phase (RP) HPLC of reduced HGL; GLIA = gliadins; ω5, ω1,2, α, and γ = gliadin types; HMW-GS and LMW-GS = high- and low-molecular-weight glutenin subunits, respectively; and Σ = total protein contained in this fraction.
[x] Determined by RP-HPLC.
[y] Semiquantitative data from densitometry of sodium dodecyl sulfate gel.
[z] Semiquantitative data from N-terminal sequencing.

TABLE IV
N-Terminal Sequence Analysis of Proteins Present in Subfractions RP1–RP4 of High-Molecular-Weight Gliadins (HGL) from Akteur Wheat (Harvest Year 2011) Isolated in the Absence (HGL-0) and Presence (HGL-N) of *N*-Ethylmaleinimide During Modified Osborne Fractionation[z]

		Amino Acid Residue in Step									
HGL	**Protein**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Literature**
RP1	ω5	S	*R*	*L*	**L**	**S**	**P**	*R*	**G**	*K*	Seilmeier et al. (2001)
	…	…	…	…	Q	…	…	…	…	…	…
RP2	ω1,2	A	R	E	L	**N**	**P**	S	**N**	K	Seilmeier et al. (2001)
	…	K	E	**L**	*Q*	*S*	**P**	S	Q	N	…
	HMW-GS	E	G	E	A	S	G	Q	L	Q	Shewry et al. (1992)
	…	…	…	…	…	…	E	…	…	…	…
	…	…	…	…	…	…	R	…	…	…	…
RP3	LMW-GS	**M**	**E**	**T**	S	C	**I**	**P**	**G**	**L**	Lew et al. (1992)
	…	**S**	**H**	**I**	**P**	**G**	**L**	**E**	**R**	**P**	…
	α	**V**	**R**	**V**	**P**	**V**	**P**	**Q**	**L**	**Q**	Kasarda et al. (1984)
RP4	γ	N	*M*	**Q**	*V*	*D*	**P**	*S*	**G**	**Q**	Rafalski (1986)

[z] Sequences found in both HGL-0 and HGL-N are displayed in bold, sequences found only in HGL-0 are displayed in italics, and sequences found only in HGL-N are underlined. RP1–RP4 = subfractions isolated by reversed-phase HPLC of reduced HGL; ω5, ω1,2, α, and γ = gliadin types; and HMW-GS and LMW-GS = high- and low-molecular-weight glutenin subunits, respectively.

study that HMW-GS present in subfraction RP2 from HGL-0 were not detected in the Edman degradation because of blocked N-terminal glutamic acid (Schmid et al. 2016). SDS-PAGE of subfractions RP2 and RP3 from HGL-N (Fig. 3) confirmed that these subfractions contained the same protein subunits as the corresponding subfractions from HGL-0: HMW-GS and ω1,2-GLIA were present in RP2 and RP3 contained LMW-GS and α-GLIA.

Steps 3, 7, and 9 from the Edman degradation were chosen for the semiquantitative determination of the ratio of LMW-GS and α-GLIA in the subfractions RP3 from both HGL-0 and HGL-N (Table V). The LMW-GS/α-GLIA ratios of RP3 were 81/19 for HGL-0 and 88/12 for HGL-N. Densitometric analysis of the SDS-PAGE gels of RP3 (Fig. 4, Table III) gave comparable ratios of 78/22 for HGL-N and 77/23 for HGL-0 (Table III). For RP2, densitometry gave an HMW-GS/ω1,2-GLIA ratio of 46/54 for HGL-N and 47/53 for HGL-0 (Fig. 4, Table III). The combined results of RP-HPLC, N-terminal sequence analysis, and SDS-PAGE provided the proximate compositions of HGL-0 and HGL-N (Table III). The calculation of statistical significances showed that HGL-N contained higher contents of LMW-GS, whereas α-GLIA were enriched in HGL-0. All other differences of the contents were not significant between HGL-0 and HGL-N.

Concentration of SS Groups, SS-Bound Glutathione, and Cysteine of HGL. The Ellman method gave no detectable thiol groups in both HGL-0 and HGL-N. The concentration of SS groups in both HGL samples was not significantly different (Table VI). However, the contents of SS-bound glutathione and cysteine were different. The content of glutathione was significantly lower in HGL-N than in HGL-0, whereas SS-bound cysteine was almost twice as high in HGL-N compared with HGL-0. This is an indication for thiol–SS interchange reactions during the modified Osborne fractionation, if thiol groups are not alkylated by a thiol reagent such as NEMI. The composition of HGL-N can be regarded as the situation in the flour because thiol–SS interchange reactions were inhibited at the beginning of the Osborne extraction. Because glutathione is considered more reactive than cysteine (Sarwin et al. 1993) and is also more abundant in wheat flour (Grosch and Wieser 1999; Koehler 2003), it can be assumed that it replaces cysteine during extraction, resulting in HGL-0 enriched in glutathione compared with HGL-N.

Considerations for the Structure of HGL. *Degree of Polymerization.* The composition of HGL-N shown in Table III was used to calculate the M_r of a random subunit of HGL-N. Mean values of densitometric and N-terminal sequence data for LMW-GS and α-GLIA were taken as the basis for the calculation. The results based on assumptions for the average M_r and number of cysteine residues of the protein types are presented in Table VII. Data for HGL-0 reported by Schmid et al. (2016) are shown for comparison. The average M_r of a random subunit of HGL-N was 38,435, compared with 39,560 for HGL-0. From the M_r range of HGL-N and HGL-0 determined by GP-HPLC (both 66,000–680,000) and the M_r of a random subunit, the degree of polymerization of HGL-N was

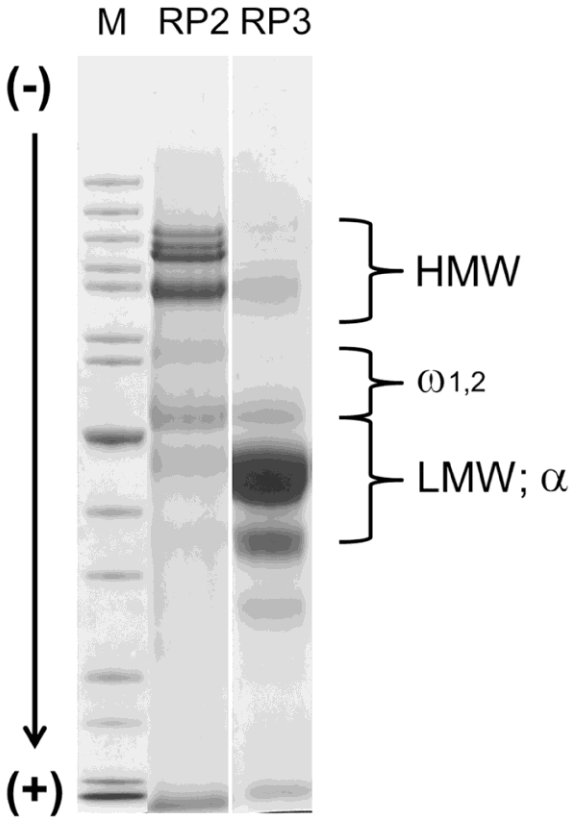

Fig. 4. SDS-PAGE of subfractions RP2 and RP3 of high-molecular-weight (HMW) gliadins from Akteur wheat (harvest year 2011) isolated in the presence of *N*-ethylmaleinimide during modified Osborne fractionation used for the semiquantitative densitometric quantitation of protein types after calibration with protein references. LMW = low molecular weight; and M = marker proteins (Unstained Protein Ladder, Thermo Scientific catalog number 26614).

TABLE V
Semiquantitative Determination of Amino Acids and Proteins in Subfraction RP3 of High-Molecular-Weight Gliadins (HGL) from Akteur Wheat (Harvest Year 2011) Isolated in the Absence (HGL-0) and Presence (HGL-N) of *N*-Ethylmaleinimide During Modified Osborne Fractionation by N-Terminal Sequencing[y]

Subfraction RP3[z]	Protein	Amino Acid Residue in Step			Average
		3	7	9	
HGL-0	LMW-GS	T	P	L	…
	…	I	E	P	…
	pmol	328 ± 151	278 ± 138	163 ± 90	…
	α	V	Q	Q	…
	pmol	61 ± 4	57 ± 72	50 ± 37	…
	LMW-GS/α ratio	84/16	83/17	77/23	81/19
HGL-N	LMW-GS	T	P	L	…
	…	I	E	P	…
	pmol	285 ± 170	137 ± 47	111 ± 62	…
	α	V	Q	Q	…
	pmol	54 ± 13	9 ± 12	20 ± 4	…
	LMW-GS/α ratio	84/16	94/6	85/15	88/12

[y] Only Edman degradation steps 3, 7, and 9 were suitable for quantitative analysis. LMW-GS = low-molecular-weight glutenin subunit; and α = gliadin.
[z] Isolated by reversed-phase HPLC of reduced HGL. Data for HGL-0 taken from Schmid et al. (2016).

between 2 and 18; for HGL-0, it was between 2 and 17. The average degree of polymerization calculated from the maximum of the GP-HPLC peak (472,000 and 530,000) was 12 and 13 for HGL-N and HGL-0, respectively. Because the difference between the GP-HPLC peak maxima was not significant, it can be assumed that HGL-0 and HGL-N were comparable with respect to molecular size.

Concentration of SS Groups. From the protein composition of HGL-N, an average content of protein-bound cysteine of 194 µmol/g of protein was calculated, compared with 186 µmol/g for HGL-0 (Table VII). Because no free thiol groups were present, all protein-bound cysteine residues had to be present as SS, corresponding to SS groups at 97 and 93 µmol/g of protein, respectively. These values correlated to the determined concentration of SS groups of 86 and 71 µmol/g of protein for HGL-N and HGL-0, respectively (Table VI). This also corresponds to the composition of HGL-N and HGL-0, because the former contains less sulfur-poor protein types (ω5-GLIA and HMW-GS) and more sulfur-rich LMW-GS compared with the latter (Table III).

Distribution of Chain Terminators. From the M_r of a random HGL-N subunit (38,435) and the average degree of polymerization (12), an M_r of 461,000 for an average HGL-N molecule was calculated. Termination on both ends of the molecule corresponded to SS groups at 2 mol/mol of HGL-N, respectively. Consequently, 1 g of HGL-N contained 4.3 µmol of SS bonds, in which terminators were involved. The comparison of these amounts with the total concentrations of glutathione plus cysteine (0.88 µmol/g) (Table VI) showed that 20.5% of the terminator positions were occupied by LMW thiols. Thus, proteins with an odd number of cysteine residues were located in 79.5% of the terminator positions of HGL-N. For HGL-0, the corresponding values were 16.5% of terminator positions linked to LMW thiols and 83.5% linked to proteins with an odd number of cysteine residues.

Possible Effect of NEMI on the Solubility of Gluten Proteins. Based on data from the literature (Broersen et al. 2006), it could also be postulated that the effects of NEMI on the formation and the content of HGL might be the result of a direct effect of the formed thioether derivatives on the solubility of gluten proteins. In our study, only the GLUT fraction was found to contain free thiol groups. Following the above hypothesis, the alkylation of free thiol groups with NEMI would improve the solubility of parts of the GLUT fraction in 60% ethanol, causing an increase of the concentration of the HGL fraction and a concurrent decease of the GLUT content. However, as shown in Figure 2 and Table II, the content of both GLUT and HGL increased upon addition of NEMI. Consequently, the pool for building blocks of HGL was the GLIA fraction, which decreased in the presence of NEMI. From these considerations, it appears logical that the differential solubility of protein fractions as affected by NEMI was owing to thiol–SS interchange and not to a direct effect of NEMI on protein solubility.

TABLE VI
Concentration of Disulfide (SS) Groups, SS-Bound Glutathione, and SS-Bound Cysteine in High-Molecular-Weight Gliadins (HGL) from Akteur Wheat (Harvest Year 2011) as Affected by the Absence (HGL-0) and Presence (HGL-N) of *N*-Ethylmaleinimide During Modified Osborne Fractionation

	Concentration (µmol/g of Protein)[x]	
Compound	**HGL-0**	**HGL-N**
SS groups[y]	71 ± 13a	86 ± 12a
Glutathione[z]	0.240 ± 0.086a	0.146 ± 0.004b
Cysteine[z]	0.404 ± 0.008a	0.734 ± 0.020b

[x] Different lowercase letters indicate statistically significant differences within the same line (*t* test, $P \leq 0.05$). Data for HGL-0 taken from Schmid et al. (2016).
[y] Mean values of six determinations ± standard deviations.
[z] Mean values of two determinations ± standard deviations.

CONCLUSIONS

The extraction of wheat proteins from flour by modified Osborne fractionation in the presence of a thiol-alkylating reagent affects the distribution of the resulting protein fractions. The fact that this was the case in a selection of 12 flours from different wheat cultivars and different harvest years points out that this might be a general effect for wheat flour. Alkylation of free thiol groups during fractionation causes a decrease of the GLIA and an increase of the GLUT content as well as an increase of oligomeric HGL. Based on the concentration of SS groups, it appears that LMW thiol compounds such as glutathione and cysteine as well as GLIA with an odd number of cysteine residues are part of HGL. They might act either as terminators during oligomerization or as thiol–SS reagents, leading to a degradation of existing polymers. HGL-N is obviously preformed in the endosperm, and mixing of dough converts it into HGL-0 by dynamic processes of oligomer degradation and oligomer reformation from polymers. This points out that HGL-N is structurally different from HGL-0. Therefore, we postulate that the state of wheat proteins in the flour cannot be investigated if free thiol groups are present during Osborne extraction because they induce thiol–SS interchange reactions. Further structural studies of SS bonds in HGL-N and HGL-0 should answer the questions of how protein subunits are linked, if the linkages occur randomly or specifically, and which proteins act as terminators.

TABLE VII
Calculation of Structural Features of High-Molecular-Weight Gliadins (HGL) from Akteur Wheat (Harvest Year 2011) Isolated in the Absence (HGL-0) and Presence (HGL-N) of *N*-Ethylmaleinimide During Modified Osborne Fractionation from Compositional Data[z]

Sample	Protein	Conc. (mg/g)	M_r (g/mol)	Conc. (µmol/g)	Cys Res. (*n*)	Cys Conc. (µmol/g)	M_r/Subunit (g)
HGL-0	ω5	41	55,000	0.7	1	0.7	2,255
HGL-N	…	38	…	0.7	…	0.7	2,090
HGL-0	ω1,2	87	50,000	1.7	1	1.7	4,350
HGL-N	…	67	…	1.3	…	1.3	3,350
HGL-0	HMW-GS	77	75,000	1.0	5	5.1	5,775
HGL-N	…	56	…	0.7	…	3.7	4,200
HGL-0	α	129	30,000	4.3	6	25.8	3,870
HGL-N	…	114	…	3.8	…	22.8	3,420
HGL-0	LMW-GS	482	35,000	13.8	8	110.2	16,870
HGL-N	…	558	…	15.9	…	127.5	19,530
HGL-0	γ	184	35,000	5.3	8	42.1	6,440
HGL-N	…	167	…	4.8	…	38.2	5,845
HGL-0	Total	1,000	…	26.8	…	185.6	39,560
HGL-N	…	1,000	…	27.2	…	194.2	38,435

[z] Conc. = concentration per gram of HGL; M_r = average molecular weight; Cys Res. = number of protein-bound cysteine residues per protein subunit; Cys Conc. = molar cysteine concentration; M_r/Subunit = contribution of protein subunit to average M_r of a random HGL subunit; ω5, ω1,2, α, and γ = gliadins; HMW-GS and LMW-GS = high- and low-molecular-weight glutenin subunits, respectively. Data for HGL-0 taken from Schmid et al. (2016).

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3.3 Disulfide structures of high-molecular-weight (HMW-) gliadins as affected by terminators

The aim of the third part of the study was to reveal the position of disulfide bonds within HGL and to identify possible changes affected by extraction. HGL-N and HGL-0 preparations were used to track changes caused by thiol-disulfide exchange reactions. Markus Schmid carried out all experimental work, wrote the draft of the manuscript and significantly contributed to the revised version. He set up an 'in-silico' database of gluten proteins with an odd number of cysteine residues and generated a list of all possible cysteine and cystine peptides thereof, which was decisive for the subsequent identification of disulfide bonds from MS data.

HGL-0 and HGL-N were hydrolyzed with thermolysin and the resulting peptides separated into nine fractions by GPC and analyzed by LC-MS with both CID and alternating CID/ETD fragmentation. The identification of cysteine peptides implied a combination of differential chromatography/MS (analysis of MS spectra from unreduced and reduced peptide mixtures), database search and comparison of the results with a list of all possible cysteine peptides from gliadins with an odd number of cysteine residues. Furthermore, it was found that CID and ETD differently cleaved the polypeptide backbone of the peptides. Cleavage sites next to amino acids occuring frequently in gluten proteins (for example G, P, or L) were preferred in CID, thus providing more product ions as compared to ETD. A high number of various disulfide bonds in both HGL-0 and HGL-N were found. Besides several disulfide bonds already described in the literature, numerous disulfide bonds were identified for the first time. Amongst others, a new head-to-tail link between HMW-GS was established, which could represent a standard link of the glutenins. Several cysteine peptides originated from proteins with point mutations that were potential chain terminatiors of HGL. Those cysteine residues detected frequently can be assumed to play a key role in the formation of both glutenins and HGL. The comparison of disulfide bonds of HGL-0 and HGL-N showed that thiol-disulfide exchange reactions occur during extraction if thiol groups are not blocked. HGL are the third, now well-defined gluten protein fraction besides monomeric gliadins and polymeric glutenins. They make up around 10 – 15 % of total gluten proteins, are soluble in aqueous

alcohols without reduction of disulfide bonds and have molecular masses between 70 000 and 700 000.

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Disulphide structure of high-molecular-weight (HMW-) gliadins as affected by terminators

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ABSTRACT

This study aimed at elucidating SS-bonds of HMW-gliadins (HGL) from wheat with the focus on terminators of glutenin polymerisation. HGL from wheat flour extracts non-treated or treated with the S-alkylation reagent N-ethylmaleinimide (NEMI) were compared. HGL from wheat flour Akteur were isolated, hydrolysed with thermolysin and the resulting peptides pre-separated by gel permeation chromatography and analysed by liquid chromatography/mass-spectrometry using alternating electron transfer dissociation/collision-induced dissociation. Altogether, 22 and 28 SS-peptides from samples without and with NEMI treatment, respectively, were identified. Twenty-six peptides included standard SS-bonds of α- and γ-gliadins, high-molecular-weight and low-molecular-weight glutenin subunits. Eleven SS-bonds were identified for the first time. Fifteen peptides unique to HGL contained cysteine residues from gliadins with an odd number of cysteines (ω5-, α- and γ-gliadins). Thus, gliadins with an odd number of cysteines, glutathione and cysteine had acted as terminators of glutenin polymerisation. Decisive differences between samples without and with NEMI treatment were not obvious showing that the termination of polymerisation was already completed in the flour. The two HGL samples, however, were different in the majority of ten peptides that included disulphide-linked low-molecular-weight (LMW) thiols such as glutathione and cysteine with the former being enriched in the non-treated HGL-sample.

1. Introduction

The storage (gluten) proteins, present in the starchy endosperm of wheat grains, play an important role in determining the unique baking quality of the flour. When flour is mixed with water, gluten proteins form a continuous network that gives dough viscoelasticity and gas-holding capacity and enables the development of bread with high volume and regular-pored elastic crumb. Wheat flour contains hundreds of gluten proteins, which occur either as monomers (gliadins) or, linked by intermolecular disulphide (SS-) bonds, as oligomers (HMW-gliadins, HGL) and polymers (glutenins) (Wrigley and Bietz, 1988). According to homologies in the amino acid sequences, gluten proteins can be classified into ω_5-, $\omega_{1,2}$-, α- and γ-gliadin types and into low-molecular-weight glutenin subunits (LMW-GS) and high-molecular-weight glutenin subunits (HMW-GS), the latter occurring as x- and y-subtypes (Wieser, 2007). Apart from the unique amino acid sequences, SS-bonds decisively contribute to the structural and functional properties of gluten proteins (Wieser, 2012). SS-bonds exist both intramolecularly (formed between two cysteine residues within the same protein chain) and intermolecularly (formed between two cysteine residues of different protein chains). Most of the SS-bonds of monomeric gliadins and polymeric glutenins have been determined during the last decades (reviewed by Shewry and Tatham (1997), Grosch and Wieser (1999, 2007)). Most ω-gliadins lack cysteine residues and are, therefore, not involved in any SS-

Abbreviations: CID, collision-induced dissociation; EIC, extracted ion chromatogram; ESI, electrospray ionisation; ETD, electron transfer dissociation; GP-, gel-permeation; GPC, gel-permeation chromatography; GS, glutenin subunit; HGL-0, high-molecular-weight gliadins extracted without alkylating reagent; HGL-N, high-molecular-weight gliadins extracted in the presence of N-ethylmaleinimide; HMW, high-molecular-weight; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LMW, low-molecular-weight; MRM, multiple reaction monitoring; MS, mass spectrometry; NEMI, N-ethylmaleinimide; RP, reversed-phase; RT, room temperature; SS, disulphide; TCEP, tris(2-carboxethyl)phosphine; TFA, trifluoroacetic acid; TRIS, tris(hydroxymethyl)-aminoethane.

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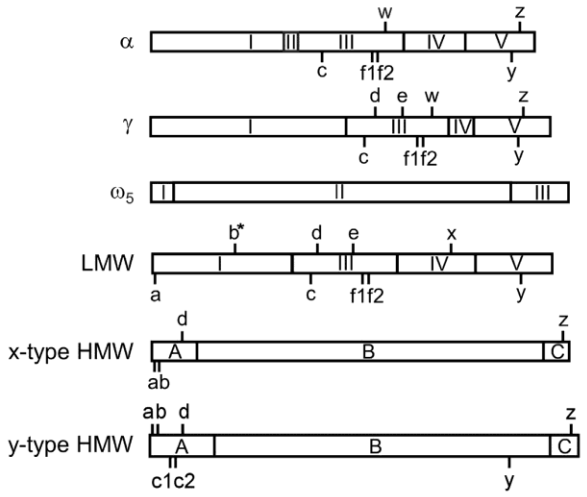

Fig. 1. Standard positions of cysteine residues of gluten proteins (nomenclature according to Köhler et al., 1993).

bonding. Standard positions of cysteine residues in gluten protein types and subunits are shown in Fig. 1. α-Gliadins possess six cysteine residues that form three intramolecular SS-bonds between residues C^c and C^{f1}, C^{f2} and C^y and C^w and C^z. Eight cysteine residues are present in γ-gliadins; six of them form SS-bonds homologous to α-gliadins. The remaining two residues (C^d, C^e) are linked intramolecularly. Polymeric glutenins contain both intra- and intermolecular SS-bonds, the latter being responsible for the polymeric state. LMW-GS include the SS-bonds C^c–C^{f1}, C^d–C^e and C^{f2}–C^y, homologous to α- and γ-gliadins and, therefore, they have been proposed to be intramolecular. Unique to LMW-GS are cysteine residues occurring in the sequence sections I (C^a or C^{b*}) and IV (C^x). They form SS-bonds to C^{b*} or C^x of a different LMW-GS and to C^y of a y-type HMW-GS. Further intermolecular SS-bonds are formed between C^e of x-type HMW-GS Dx5 and C^z occurring in all HMW-GS ("head-to-tail" cross-link) and between two y-type HMW-GS formed by neighbored cysteine residues C^{c1} and C^{c2}. The link between C^a and C^b of x-type HMW-GS Bx7 was proposed to be intramolecular (Köhler et al., 1997).

SS-bonds of HGL have not been determined up to now. Due to their oligomeric nature, it was postulated that the polymerisation of glutenins is interrupted by so-called terminators such as modified gliadins with an odd number of cysteine residues or LMW thiols such as glutathione or cysteine (Schmid et al., 2016a). A database search (UniProtKB/SwissProt, European Nucleotide Database) showed that gluten proteins with an odd number of cysteine residues occur more often than we expected. Altogether, 128 sequences of gluten proteins with an odd number of cysteine residues were found, including 42 sequences of α-gliadins, 19 of γ-gliadins, 3 of ω-gliadins, 22 of LMW- and 42 of HMW-GS (Suppl. Table S1). The aim of the present study was, therefore, to elucidate SS-bonds of HGL particularly focussed on possible terminators. HGL preparations, produced without and with N-maleinimide (NEMI) treatment (HGL-0, HGL-N) of the flour, were compared, because previous investigations demonstrated that blocking of thiol groups by S-alkylation with NEMI significantly influenced the amount and composition of HGL (Schmid et al., 2016b).

2. Materials and methods

2.1. Chemicals

The quality of the chemicals was analytical grade or stated otherwise. VWR Merck, Darmstadt, Germany: sodium chloride, disodium hydrogenphosphate, potassium dihydrogenphosphate, urea, calcium chloride hexahydrate, ammonia (25%), 1-propanol, hydrochloric acid (25%), tris(hydroxymethyl)-aminoethane (TRIS), formic acid (98–100%), disodium hydrogenphosphate, glacial acetic acid, ethanol (LiChrosolv). Sigma-Aldrich Chemie GmbH, Steinheim, Germany: acetonitrile (LC-MS LiChrosolv), methanol (LiChrosolv), trifluoroacetic acid (TFA), tris(2-carboxyethyl)phosphine (TCEP), thermolysin and NEMI. Medicell International Ltd, London, U.K.: dialysis tubings (size 6, IntDia 27/32″, molecular weight cut-off 12,000–14,000).

2.2. Preparation of total gliadins and HGL

Grains of the wheat cultivar (cv.) Akteur (harvest year 2011) were milled into white flour using a Quadrumat Junior II mill (Brabender, Duisburg, Germany) and sieved through a 0.2 mm mesh sieve. The flour contained 15.6% moisture, 9.9% crude protein and 0.43% ash. For the preparation of the gliadin fraction, flour (50 g) was sequentially extracted with a buffered salt solution (2 × 200 mL; 0.4 mol/L NaCl plus 0.067 mol/L Na_2HPO_4/KH_2PO_4, pH 7.6) to remove albumins and globulins. For the alkylation of free thiol groups in a second assay, NEMI (0.6 mg/g flour; 4.8 μmol/g flour) was added to the sample, before the first step of extraction of albumins and globulins was initiated. The corresponding fractions obtained are marked with the extension "-0" for the control fractions and "-N" for the NEMI fractions in this publication. Gliadins were then extracted with 60% (v/v) ethanol (3 × 200 mL). Each extraction step was performed by homogenisation with an Ultra Turrax T25D (IKA, Staufen, Germany) for 10 min at room temperature (RT ≈ 20 °C). Each suspension was centrifuged for 20 min at 4600×*g* and RT by means of a laboratory centrifuge (Heraeus Multifuge 3L-R, Thermo Fisher Scientific, Dreieich, Germany). The supernatants of the albumin/globulin extracts were discarded and those of the gliadin extracts were combined, concentrated with a rotary evaporator (Rotavapor RII, Büchi, Essen, Germany), dialysed for 3 days against acetic acid (0.01 mol/L) and one day against deionised water. The dialysed solutions were then lyophilised providing gliadins-0 and gliadins-N.

For the preparation of HGL, gliadins-0 or gliadins-N (100 mg) were dissolved in 60% (v/v) ethanol (4 mL), filtered through a 0.45 μm membrane and separated by preparative gel permeation high-performance liquid chromatography (GP-HPLC) under the following conditions: instrument, Jasco HPLC (Jasco, Gross-Umstadt, Germany); column, BioSep-SEC-s3000 (21.2 × 300 mm; separation range for globular proteins 5000–700,000; Phenomenex, Aschaffenburg, Germany); elution solvent, acetonitrile (50%, v/v)/TFA (0.1%, v/v); flow rate, 1.5 mL/min; temperature, RT; injection volume, 900 μL; detection, 280 nm; fraction collector, Model 201 (Gilson International, Limburg-Offheim, Germany) (Schmid et al., 2016a). Three fractions were obtained, the first fraction containing HGL-0 or HGL-N and fractions 2 and 3 containing monomeric gliadins. The fractions were collected from several runs and lyophilised. The HGL-0 and HGL-N fractions were used for further experiments.

2.3. Thermolytic digestion of HGL

For the preparation of SS-peptides, HGL-0 and HGL-N were digested with thermolysin according to previous studies on SS-

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bonds of gluten proteins (Köhler et al., 1991, 1993; Keck et al., 1995; Müller and Wieser, 1995, 1997; Lutz et al., 2012). The partial hydrolysis was carried out at pH 6.5 (somewhat lower than the optimum between pH 7.0 and 9.0) to minimise thiol/disulphide interchange. For the determination of the optimal time for the hydrolysis, 5 mg of HGL-0 or HGL-N were mixed with 0.5 mg thermolysin and 5 mL buffer solution (0.02 mol/l $CaCl_2 \bullet 6H_2O$ in 0.2 mol/L Tris/HCl; pH 6.5). Two assays were performed. The first assay was stirred at 37 °C for 8 h, an aliquot of 200 μL was taken every 30 min and 3 μL of TFA were added (→pH 2.0) to stop the digestion. The second approach was stirred at 37 °C for 1 h, an aliquot of 200 μL was taken every 5 min and the digestion was stopped. One sample taken at the start and one sample without enzyme were used as controls. All samples were filtrated and analysed by analytical reversed-phase (RP-) HPLC. The measurement system was a Thermo instrument (Thermo Fisher Scientific, Dreieich, Germany) equipped with an EC 250/3 Nucleosil 100-5 C_{18}-column (3 × 250 mm, particle size 5 μm, pore size 10 nm; Macherey Nagel, Düren, Germany) at RT. The flow rate was 0.7 mL/min, the mobile phases were (A) 0.1% (v/v) TFA and (B) 0.1% (v/v) TFA in acetonitrile and the gradient used was as follows: (i) isocratic at 0% B for 3.1 min; (ii) linear to 34.5% B in 9.9 min; (iii) linear to 38% B in 4 min; (iv) linear to 90% B in 1 min; (v) isocratic at 90% B for 5 min; (vi) linear to 0% B in 0.1 min; isocratic at 0% B for 9.9 min. The injection volume was 20 μL and detection wavelength was at 210 nm. With this system, the optimal time for hydrolysis was determined to be 40 min.

The main experiment was performed by adding 0.3 mg thermolysin and 1 mL buffer solution (0.02 mol/l $CaCl_2 \bullet 6H_2O$ in 0.2 mol/L Tris/HCl; pH 6.5) to 3 mg HGL-0 or HGL-N and shaking for 40 min at 37 °C. Digestions were stopped by adding 3 μL of TFA.

2.4. Gel permeation HPLC

The thermolytic digests of HGL-0 and HGL-N were separated into nine peptide fractions by preparative GP-HPLC (Fig. 2). The system used was a Jasco instrument (Jasco, Groβ-Umstadt, Germany) equipped with a BioBasic SEC 60-column (separation range 100–6000, 7.8 × 300 mm, particle size 5 μm, pore size 6 nm; Thermo Fisher Scientific, Dreieich, Germany) at RT. The flow rate was 0.5 mL/min, the mobile phases were (A) 0.1% (v/v) TFA and (B) 0.1% (v/v) TFA in acetonitrile and the gradient was isocratic at 20% B for 35 min per run. The injection volume was 100 μL and the detection wavelength was 230 nm. The fraction collector used was a FC-2088-30 (Jasco, Groβ-Umstadt, Germany) and nine subfractions were collected in numerous runs. These subfractions were divided in two equal parts. One part was treated with TCEP (5 mg/mL) and stirred at RT for 30 min in the dark to reduce SS-bonds. After that, all samples were evaporated to dryness, redissolved in 300 μL 0.1% formic acid, filtrated and used for MS analysis.

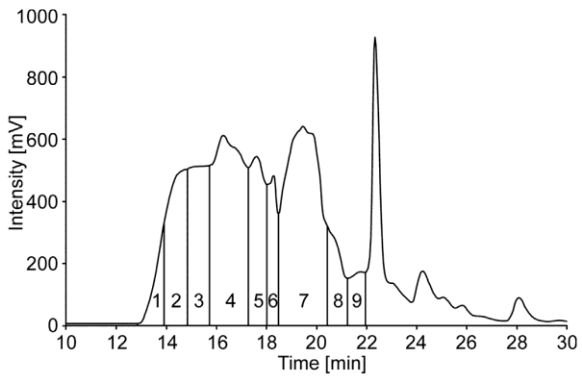

Fig. 2. Preparative gel permeation chromatography of the thermolytic digest of HGL-0. Nine subfractions were collected.

2.5. Liquid chromatography-mass spectrometry (LC-MS) of HGL peptides

The experiments were performed on a HCT-Ultra PTM ion trap MS system (Bruker Daltonics, Bremen, Germany) with alternating collision-induced dissociation (CID)/electron transfer dissociation (ETD) or CID only, coupled with an UltiMate 3000 HPLC system (Dionex, Idstein, Germany) equipped with an Aeris PEPTIDE 3.6 μ XB-C_{18} column (2.1 × 150 mm, particle size 3.6 μm, pore size 10 nm; Phenomenex). The solvent for LC was (A) 0.1% (v/v) formic acid and (B) 0.1% (v/v) formic acid in acetonitrile. The following gradient was used: (i) isocratic at 0% B for 5 min, (ii) linear to 50% B in 40 min; (iii) linear to 90% B in 10 min; (iv) isocratic at 90% B for 5 min; linear to 0% B in 2 min; (v) isocratic at 0% B for 15 min. The flow rate was 0.2 mL/min, injection volume was 15 μL and the column temperature was 30 °C. The MS had a spherical ion trap with an electrospray ionisation (ESI) interface running in the standard enhanced positive mode, and in ETD also a chemical ionisation (CI) ion source was used (methane and fluoroanthene). For ESI, the capillary voltage was −4000 V, the capillary exit voltage 145 V and the skimmer voltage 40 V. Nitrogen was used as drying (8.0 L/min, 325 °C) and nebulising gas (0.2 MPa). The scan range of the MS was *m/z* 150–3000 (smart target value: 300,000; maximum acquisition time: 100 ms), MS^2 scan steps were performed on the same precursor ion using the AutoMS^2 mode in CID (fragmentation amplitude: 0.4 V; collision gas: helium; threshold abs.: 2500 counts; threshold rel.: 5% of precursor intensity) and ETD (ion current control > 6×10^5 counts).

Mass spectra were processed and evaluated using the Bruker Daltonics Data Analysis 3.4 software. Peptide subfractions from GP-HPLC were analysed by LC-MS/CID without reduction and after reduction of SS-bonds. Base peak ion chromatograms containing MS and MS^2 data were obtained. In this mode, SS-peptides provided only signals in the chromatograms of the unreduced samples. In the chromatograms of the reduced samples initial SS-peptides were present as cysteine peptides. Unreduced peptide subfractions were also analysed by LC-MS with alternating CID/ETD detection providing CID-MS^2 and ETD-MS^2 spectra. ETD fragmented at the N-C_α bond, generating even-electron c-type and odd-electron z-type product ions, whereas cleavage of the peptide bond produced b- and y-type fragment ions in CID (Coon, 2009). Cleavage of SS-bonds was preferred over peptide bond fragmentations in ETD, whereas SS-bonds remained intact in CID. Indices α and β referred to the respective cysteine peptide ions corresponding to the parent cystine peptide (Lutz et al., 2012).

A *m/z* list of potential SS-peptides was generated before experimental MS work was started. For this purpose, only sequences of gliadins and glutenins with an odd number of cysteine residues (ω5-, α- and γ-gliadins, HMW-GS, LMW-GS) were taken from the UniProtKB/Swiss-Prot database (http://www.uniprot.org/) in order to focus the search on possible terminators (128 proteins; Suppl. Table S1). This selection was further narrowed down by taking only those gluten protein sequences that had been identified in HGL-0 by N-terminal sequence analysis in a previous study (Schmid et al., 2016a) (12 proteins; Suppl. Table S2). Next, the selected protein sequences were digested *in silico* using the PeptideMass tool of ExPasy (http://web.expasy.org/peptide_mass/) using thermolysin as peptidase and one missed cleavage to predict the cleavage products. This resulted in a pool of 92 cysteine-

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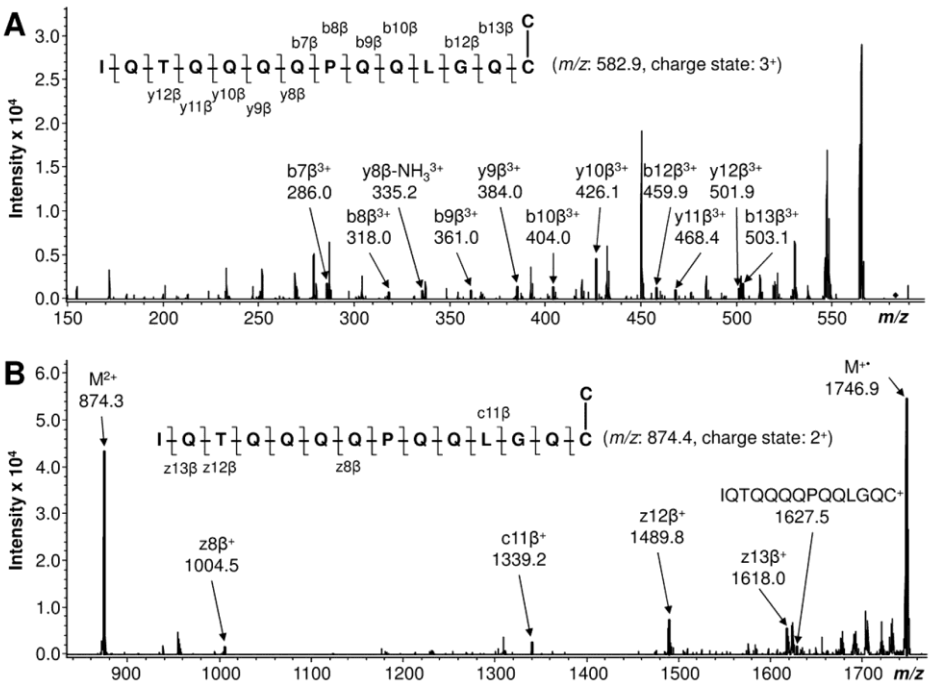

Fig. 3. CID-and CID-/ETD spectra of SS-peptides. (A) CID-spectrum and (B) alternating CID-/ETD spectra of SS-peptide XXII of HGL-N (intermolecular linkage between C^x of LMW-GS and cysteine; nomenclature of ions according to Lutz et al., 2012).

containing peptides (Suppl. Table S3). All peptides from this pool and glutathione and cysteine as potential terminators were manually combined to form 502 SS-peptides that contained all possible combinations of cysteine residues of the selected gliadins, glutenins, cysteine and glutathione with *m/z*-values of the charge states 1^+, 2^+, and 3^+ (Suppl. Table S4). The 'ProteinProspector' (http://prospector.ucsf.edu/prospector/mshome.htm; University of California, San Francisco, CA, USA) was used to predict b- and y-ions of the SS-peptides that could be expected from MS^2 (CID) experiments. The lists of the theoretical MS and MS^2 signals were compared with the experimental mass spectra. If mass conformities were found in the MS spectra, MS^2 spectra were searched for the b- and y-ions of the SS-peptide belonging to this *m/z*.

This was done in three different ways. First, the extracted ion chromatograms (EIC) of both the precursor ion and the b- and y-product ions were created and compared. If all EIC traces showed a peak at the same retention time, this peak was considered a potential SS-peptide. For confirmation, an additional selective multiple reaction monitoring (MRM) MS experiment was carried out, in which only the previously identified MS and MS^2 traces were monitored. All peptide identifications were validated manually, essentially following the rules of Chen et al. (2005). Briefly, at least five isotopically resolved independent product peaks were required to match the theoretical peptide products. Only b- or y-ions or associated peaks arising due to water or amine loss were considered as product ions of a peptide, and either the b- or y-ion series were required to confirm at least three consecutive amino acids in the peptide sequence. The signal intensity of product peaks used for these consecutive amino acids had to be at least 10% of the peak with the highest intensity in the sample. Mass deviations larger than ±0.5 among the product ions were not allowed. If ETD-MS^2 experiments had been made, the spectra were searched for c- and z-product ions of the SS-peptides and also of the corresponding SH-containing fragment peptides generated by the cleavage of the SS-bond. In the second method, also referred to as 'difference chromatography', the CID-MS and MS^2 data of peaks that were present in the chromatogram of the unreduced sample but not in the reduced sample were included in an additional MRM experiment as described above. Method three was checking the MS^2 data directly for products of SS-peptides by comparing with MS^2 signals from the theoretical mass list.

3. Results

3.1. Preparation of HGL-0 and HGL-N

HGL-0 and HGL-N isolated according to Schmid et al. (2016a) were composed of 48.3/55.8% LMW-GS, 18.4/16.7% γ-gliadins, 12.8/11.4% α-gliadins, 8.7/6.7% $\omega_{1,2}$-gliadins, 7.7/5.6% HMW-GS and 4.1/3.8% ω_5-gliadins, respectively (Schmid et al., 2016b). All five HMW-GS, present in flour (Ax1, Dx5, Bx7, By9, Dy10), were also found in both HGL preparations. The contents of protein-bound glutathione and cysteine were 240/146 nmol/g and 404/734 nmol/g, respectively.

3.2. Isolation and analysis of SS-peptides

Partial hydrolysates of HGL-0 and HGL-N with thermolysin were separated by preparative GP-HPLC. Fig. 2 shows the chromatogram of the digest from HGL-0. The digest of HGL-N had a closely related elution profile. Each of the peptide fractions was analysed by means of LC-MS/MS with CID fragmentation and with alternating CID/ETD fragmentation using an ion-trap MS. As an example, the identification of peptide XXII of HGL-N based on the CID and ETD spectra is

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Table 1
Disulphide Bonds of HMW-Gliadins without NEMI treatment (HGL-0).

SS-peptide	GP-HPLC	Peptide fragments	Amino acid sequence[1]	MS method[2]	Cysteine residue[3]	Protein type[4]	Reference[5]
I	Fr. 2	I - 1 I - 2 I - 3	LQQQLIPC LLQELCCQH MCN	CID only	C^c C^{f1} C^{f2} C^y	α α α	[1]
II	Fr. 3	II - 1 II – 2	VC[6] AMCR	Both	**C^x** C^z	α HMW	–
III	Fr. 4	III - 1 III - 2	LQQCKP ILPPSDCQV	CID only	C^d C^e	γ γ	[2]
IV	Fr. 3	IV - 1 IV - 2	MQQQ**Y**CQQL LRTLPNMCN	Both	C^{f2} C^y	γ γ	–
V	Fr. 1	V - 1 V - 2 V - 3	LNPCK MQQQCCQQL MCNVNVP	CID/ETD	C^c C^{f1} C^{f2} C^y	γ γ γ	[2]
VI	Fr. 5	VI - 1 VI - 2	LRTLPNMCN LGQC	Both	C^y C^x	γ LMW	[3]
VII	Fr. 3	VII - 1 VII - 2	LRTLPNMCN VTCPQQ	CID only	C^y C^e	γ HMW	–
VIII	Fr. 4	VIII - 1 VIII - 2	ISI**C**G MCNVY	Both	**C^z** C^y	ω α/γ	–
IX	Fr. 3	IX - 1 IX - 2	I**C**GL MCNVY	Both	**C^z** C^y	ω α/γ	–
X	Fr. 6	X - 1 X - 2	ISI**C**G IQTQQQQPQQLGQC	CID only	**C^z** C^x	ω LMW	–
XI	Fr. 1	XI - 1 XI - 2 XI - 3	I**C**G LQCERELQELQERELKACQQ LRDISPECHP	CID/ETD	**C^z** C^a C^b C^d	ω HMW LMW	–
XII	Fr. 3	XII - 1 XII - 2	LQQQCSP LQQSSCH	Both	C^d C^e	LMW LMW	[4]
XIII	Fr. 2	XIII - 1 XIII - 2	FLQQQCSP LQQSSCH	CID/ETD	C^d C^e	LMW LMW	[4]
XIV	Fr. 1	XIV - 1 XIV - 2	LGQCVSQPQQQSQQQ MCN	CID only	C^x C^y	LMW α/γ/LMW	[3]
XV	Fr. 3	XV - 1 XV - 2	VTCPQQ MCR	Both	C^e C^z	HMW HMW	[3]
XVI	Fr. 5	XVI - 1 XVI - 2	ACQQV MDQQLRDISPECHP	Both	C^b C^d	HMW HMW	[5]
XVII	Fr. 4	XVII - 1 XVII - 2	LQCERELQELQERELK MCR	Both	C^a C^z	HMW HMW	–
XVIII	Fr. 3	XVIII - 1 XVIII - 2	LKRYYPSVTCPQQ LGQC	Both	C^e C^x	HMW LMW	–
XIX	Fr. 3/4	XIX - 1 XIX - 2	MCRLEGGDA MCN	Both	C^z C^y	HMW α/γ/LMW	–
XX	Fr. 2	XX - 1 XX - 2	ECG I**C**G	CID only	C **C^z**	GSH ω	–
XXI	Fr. 5	XXI - 1 XXI - 2	ECG MCRLEGGDA	Both	C C^z	GSH HMW	–
XXII	Fr. 4	XXII - 1 XXII - 2	ECG AMCR	Both	C C^z	GSH HMW	–

[1]One-letter-code of amino acids; point mutations in bold. ECG: Glutathione (γ-L-glutamyl-L-cysteinyl-glycine).
[2]CID only: Peptide found only with CID fragmentation; CID/ETD; peptide found only with alternating CID/ETD fragmentation; both: peptide found in both modes.
[3]Nomenclature according to Köhler et al. (1993).
[4]α, α-gliadin; γ, γ-gliadin; ω, ω-gliadin; LMW, low-molecular-weight glutenin subunits; HMW, high-molecular-weight glutenin subunits.
[5][1] Müller and Wieser (1995); [2] Müller and Wieser (1997); [3] Lutz et al. (2012); [4] Köhler et al. (1993); [5] Keck et al. (1995).
[6]Uniprot no. K7WV57.

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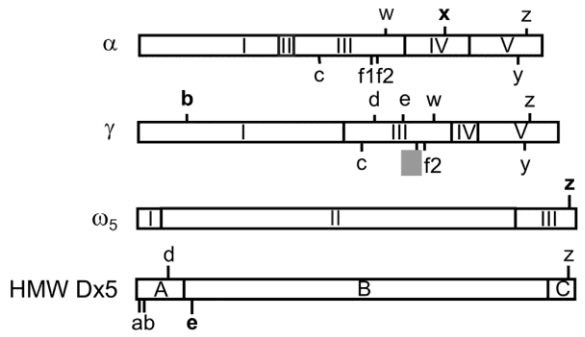

Fig. 4. Standard positions of cysteine residues and point mutations (additional cysteine in bold, missing cysteine in grey) of gluten proteins found in HGL-0 and/or HGL-N (nomenclature according to Köhler et al., 1993).

shown in Fig. 3A and B. CID spectra of further SS-peptides (VII of HGL-0, II of HGL-N, XXIII of HGL-N) are given in Suppl. Figs. S1–S3.

3.3. SS-bonds in HGL-0

Altogether 22 SS-peptides (I–XXII) were identified in HGL-0 (Table 1). The assignment of the amino acid sequences of the corresponding fragment peptides to gluten protein types demonstrated that nine SS-peptides represented links within the same type, ten SS-bonds were formed between different types and three peptides indicated links of glutathione to HMW-GS and to ω-gliadin, respectively. As expected, standard SS-bonds, formed by conserved cysteine residues (Shewry and Tatham, 1997) and formerly detected in monomeric gliadins and polymeric glutenins, were also present in HGL-0. They included links of α-gliadin (peptide I, C^c–$C^{f1}C^{f2}$–C^y) and γ-gliadin (peptide III, C^d–C^e; peptide V, C^c–$C^{f1}C^{f2}$–C^y), which were previously found to be intramolecular (Müller et al., 1995, 1997). Furthermore, the SS-links of LMW-GS in peptides XII and XIII (C^d–C^e), identified by Köhler et al. (1993) in the glutenin fraction, were proposed to be intramolecular because of homologies to monomeric α- and γ-gliadins (Müller and Wieser, 1997). The SS-bond of peptide XV (C^e–C^z) including C^e, unique to HMW-GS Dx5, and C^z, present in the C-terminal domain of all known HMW-GS, was already found by Lutz et al. (2012). This bond had been suggested to be an intermolecular rather than an intramolecular link due to steric reasons and the polymeric nature of glutenins, and might represent a head-to-tail cross-link between HMW-GS Dx5 and another HMW-GS (Lutz et al., 2012). Peptide XVI of HMW-GS contained an SS-bond between C^b and C^d that might indicate an intramolecular link of HMW-GS Dx5 (Köhler et al., 1997).

Thirteen SS-peptides included links that were identified for the first time. Another example of a possible head-to-tail cross-link of HMW-GS Dx5 was represented by peptide XVII, in which C^a and C^z formed an SS-bond. Although not found in previous work, this bond could be a standard intermolecular link of HMW-GS Dx5. Differing from standard SS-structures and possibly typical of HGL-0 were links of HMW-GS Dx5 (C^e) with C^y of γ-gliadins (peptide VII) and with C^x of LMW-GS (peptide XVIII), links of HMW-GS (C^z) with C^y of α-, γ-gliadins or LMW-GS (peptide XIX) and with glutathione (peptides XXI and XXII). In these cases C^a, C^e and C^z, cysteine residues proposed to be involved in the polymerisation of HMW-GS, were linked to gliadins, LMW-GS and glutathione, and thus, polymerisation might be stopped by these SS-bond combinations. Six SS-peptides showed point mutations of standard amino acid sequences. These point mutations (additional cysteine residues in α-gliadins and ω-gliadins and exchange of cysteine against tyrosine in γ-gliadins, Fig. 4) cause an odd number of cysteine residues in the protein sequences and lead to the termination of the polymerisation. Major contributor to changed SS-structures was the residue C^z of modified ω5-gliadins. This residue was involved in links to C^x of LMW-GS (peptide X) and C^a of HMW-GS Dx5 (peptide XI), again two cysteine residues capable of polymerisation. Furthermore, C^z of ω5-gliadins was linked to C^y of α- or γ-gliadins (peptides VIII and IX) and to glutathione (peptide XX). In peptide IV, the standard cysteine residue C^{f1} was missing and a SS-peptide containing C^c, usually linked to C^{f1}, could not be found. In this case, C^c might directly act as a terminator of polymerisation. An alternative would be that C^c was linked to C^{f2} instead of C^{f1} and then C^y was involved as terminator indicated by the SS-bond between C^y of γ-gliadins and C^e of HMW-GS 5 (peptide VII) and C^x of LMW-GS (peptide VI), respectively. Another terminator was a modified α-gliadin with an additional cysteine residue (C^x) in domain IV (Fig. 4). C^x of modified α-gliadins was linked to C^z of HMW-GS (peptide II). Altogether, the results demonstrated that HGL-0 contained a number of intramolecular and intermolecular SS-bonds previously found in gliadins and glutenins. However, typical of HGL-0 was the presence of terminators (proteins with an odd number of cysteine residues and glutathione) that might play a key role in terminating glutenin polymerisation and thus, in the formation of HGL-0. Obviously, cysteine residues C^a, C^e and C^z of HMW-GS Dx5, probably important contributors to glutenin polymerisation, are mostly affected by terminators such as modified gliadins and glutathione.

3.4. SS-bonds in HGL-N

Twenty-eight SS-peptides were identified in HGL-N that were isolated from flour after free thiol groups had been alkylated with NEMI. Nine peptides contained SS-bonds between the same protein type, twelve peptides included links between different protein types and seven peptides consisted of glutathione or cysteine that were linked to different protein types. Standard SS-bonds, also found previously, could be assigned to α-gliadins (peptide I, C^w–C^z), γ-gliadins (peptides VI - X, C^d–C^e, C^w–C^z) and HMW-GS (peptides XVII, XVIII, C^e–C^z). Modified SS-peptides, also found in HGL-0, included SS-bonds within γ-gliadin (peptide XI, C^{f2}–C^y), between α- or γ-gliadins and HMW-GS (peptide V, C^x–C^z; peptides XX and XXI, C^y–C^z), between ω5-gliadins and LMW-GS (peptide XIII, C^z–C^x) and between LMW-GS and α- or γ-gliadins (peptides XV and XVI, C^y–C^x). Links between HMW-GS Dx5 and ω5-gliadins (peptide XIV, C^z–C^z), between HMW-GS Dx5 and α- or γ-gliadins (peptide XIX, C^e–C^y) and between HMW-GS and γ-gliadins (peptide XII, C^z–C^b), between α- and ω5-gliadins (peptide II, C^x–C^z) and between α-gliadins and LMW-GS (peptides III and IV, C^x–C^x) were detected only in HGL-N. One explanation could be that these peptides also occurred in HGL-0, but were not detected there. It is, however, remarkable that they all included terminating cysteine residues (C^x of α-gliadins, C^b of γ-gliadins and C^z of ω5-gliadins, Fig. 4). It is possible that the corresponding SS-bonds were particularly sensitive to a thiol/disulphide interchange, initiated by free thiol groups at the beginning of the Osborne fractionation, so that they were only present in HGL-N. Additionally, not only SS-bonds to glutathione (peptide XXVII, C^x of α-gliadins; peptide XXVIII, C^z of ω5-gliadins) were identified, but also SS-bonds to cysteine existed (peptides XXII and XXIII, C^x of LMW-GS; peptides XXIV, XXV and XXVI, C^z of HMW-GS). The fact that SS-peptides containing cysteine as one fragment were found only in HGL-N could be explained with the higher reactivity of glutathione in comparison to cysteine. This obviously led to the depletion of cysteine during the aqueous extraction of flour in the absence of NEMI by thiol/disulphide interchange leading to HGL-0 devoid of cysteine but enriched in

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Table 2
Disulphide Bonds of HMW-Gliadins with NEMI treatment (HGL-N).

SS-peptide	GP-HPLC	Peptide fragments	Amino acid sequence[1]	MS method[2]	Cysteine residue[3]	Protein type[4]	Reference[5]
I	Fr. 2	I - 1 I - 2	LWQIPEQSQCQA VYIPPYCTIAP	CID only	C^{w} C^{z}	α α	[1]
II	Fr. 3	II - 1 II - 2	V**C**FQQSQQQYPSGQGS I**C**G	CID only	$\mathbf{C}^{x}$ $\mathbf{C}^{z}$	α ω	–
III	Fr. 3	III - 1 III – 2	V$\mathbf{C}^{6}$ IQTQQQQPQQLGQC	Both	$\mathbf{C}^{x}$ C^{x}	α LMW	–
IV	Fr. 2	IV - 1 IV - 2	LSQV$\mathbf{C}^{6}$ LGQCVSQPQQQSQQQ	Both	$\mathbf{C}^{x}$ C^{x}	α LMW	–
V	Fr. 4	V - 1 V - 2	V$\mathbf{C}^{6}$ MCRLEGGDA	Both	$\mathbf{C}^{x}$ C^{z}	α HMW	–
VI	Fr. 3	VI - 1 VI - 2	LLQQCKP ILPPSDCQV	Both	C^{d} C^{e}	γ γ	[2]
VII	Fr. 4	VII - 1 VII - 2	LLQQCDP ILPRSDCQ	Both	C^{d} C^{e}	γ γ	[2]
VIII	Fr. 4	VIII - 1 VIII - 2	LQQCDP ILPRSDCQV	CID only	C^{d} C^{e}	γ γ	[2]
IX	Fr. 3	IX - 1 IX - 2	LLQQCDP ILPRSDCQV	CID/ETD	C^{d} C^{e}	γ γ	[2]
X	Fr. 3	X - 1 X - 2	LQC VRPDCST	CID only	C^{w} C^{z}	γ γ	[3]
XI	Fr. 4	XI - 1 XI - 2	MQQQ**Y**CQQ MCNVY	CID/ETD	C^{f2} C^{y}	γ γ	–
XII	Fr. 3	XII - 1 XII - 2	F**C**QQPQRTIPQPHQT MCRLEGGDA	Both	$\mathbf{C}^{b}$ C^{z}	γ HMW	–
XIII	Fr. 4	XIII - 1 XIII - 2	I**C**GL IQTQQQQPQQLGQC	Both	$\mathbf{C}^{z}$ C^{x}	ω LMW	–
XIV	Fr. 4	XIV - 1 XIV - 2	I**C**GL MCRLEGGDA	Both	$\mathbf{C}^{z}$ C^{z}	ω HMW	–
XV	Fr. 3	XV - 1 XV - 2	LGQCVSQPQQQSQQQ MCNVY	CID only	C^{x} C^{y}	LMW α/γ	[4]
XVI	Fr. 1	XVI - 1 XVI - 2	LGQC MCNVY	Both	C^{x} C^{y}	LMW α/γ	[4]
XVII	Fr. 2	XVII - 1 XVII - 2	LKRYYPSVTCPQQ AMCR	Both	C^{e} C^{z}	HMW HMW	[4]
XVIII	Fr. 4	XVIII - 1 XVIII - 2	VTCPQQ MCRLEGGDA	CID only	C^{e} C^{x}	HMW HMW	[4]
XIX	Fr. 3	XIX - 1 XIX - 2	VTCPQQ MCNVY	CID only	C^{e} C^{y}	HMW α/γ	–
XX	Fr. 5	XX - 1 XX - 2	MCR MCNVY	Both	C^{z} C^{y}	HMW α/β	–
XXI	Fr. 3	XXI - 1 XXI - 2	MCRLEGGDA MCNVY	Both	C^{z} C^{y}	HMW α/γ	–
XXII	Fr. 6	XXII - 1 XXII - 2	C IQTQQQQPQQLGQC	Both	C C^{a}	CSH LMW	–
XXIII	Fr. 6	XXIII - 1 XXIII - 2	C LGQC	CID only	C C^{x}	CSH LMW	–
XXIV	Fr. 1	XXIV - 1 XXIV - 2	C MCRLEGGDA	Both	C C^{z}	CSH HMW	–

(*continued on next page*)

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Table 2 (*continued*)

SS-peptide	GP-HPLC	Peptide fragments	Amino acid sequence[1]	MS method[2]	Cysteine residue[3]	Protein type[4]	Reference[5]
XXV	Fr. 3	XXV - 1 XXV - 2	C | AMCR	CID only	C C^z	CSH HMW	–
XXVI	Fr. 4	XXVI - 1 XXVI - 2	C | MCR	CID only	C C^z	CSH HMW	–
XXVII	Fr. 4	XXVII - 1 XXVII - 2	ECG | LSQV**C**	Both	C **C^x**	GSH α	–
XVIII	Fr. 4	XVIII - 1 XVIII - 2	ECG | I**C**G	CID only	C **C^z**	GSH ω	–

[1]One-letter-code of amino acids; point mutations in bold. ECG: Glutathione (γ-L-glutamyl-L-cysteinyl-glycine).
[2]CID only: Peptide found only with CID fragmentation; CID/ETD; peptide found only with alternating CID/ETD fragmentation; both: peptide found in both modes.
[3]Nomenclature according to Köhler et al. (1993).
[4]α, α-gliadin; γ, γ-gliadin; ω, ω-gliadin; LMW, low-molecular-weight glutenin subunits; HMW, high-molecular-weight glutenin subunits.
[5][1] Müller and Wieser (1995); [2] Müller and Wieser (1997); [3] Köhler et al. (1993); [4] Lutz et al. (2012).
[6]Uniprot no. K7WV57.

glutathione as compared to HGL-N (Sarwin et al., 1993; Schmid et al., 2016b).

4. Discussion

The results have clearly shown that the polymerisation of glutenin subunits can be stopped by terminators such as modified gliadins with an odd number of cysteine residues and LMW thiols. As previously shown (Schmid et al., 2016a), the resulting oligomeric fraction called HGL is the third, now well-defined gluten protein fraction besides monomeric gliadins and polymeric glutenins. They make up around 10–15% of total gluten proteins, are soluble in aqueous alcohols without reduction of SS-bonds and have molecular masses between 70,000 and 700,000. The SS-peptides identified in HGL confirmed standard SS-bonds in α- and γ-gliadins and LMW-GS and may help to complete information about SS-bonds of HMW-GS 5. Cysteine residues C^a, C^e and C^z of HMW-GS Dx5, mostly affected by terminators, are likely to be involved in intermolecular SS-bonds. SS-bonds between C^b and C^d (peptides XI and XVI, Table 1) are probably intramolecular, because an intramolecular SS-bond between C^a and C^b has been excluded for steric reasons (Köhler et al., 1997) and more than three intermolecular links within the same HMW-GS are quite implausible (Kasarda, 1999). C^e is unique to HMW-GS Dx5 and its presence has often been associated with good breadmaking quality of wheat cultivars. The background could be that three intermolecular bonds provided by HMW-GS Dx5 compared to two intermolecular bonds provided by other HMW-GS strengthen the gluten network.

Most combinations of SS-bonds and most SS-peptides are present in both HGL-0 and HGL-N. This is plausible, because SS-bond formation starts rapidly after synthesis within the lumen of the endoplasmatic reticulum as integral part of protein folding, in which intramolecular SS-bonds form more rapidly than intermolecular bonds (Kasarda, 1999; Shewry and Tatham, 1997). In the current study, HGL-N was the fraction representing oligomeric gluten proteins in wheat flour. Upon mixing of flour into a dough, binding pattern and degree of polymerisation are changing (Kuktaite et al., 2004). As Osborne fractionation can be considered as a kind of "mixing" of flour, comparable reactions as during dough formation can be assumed. Thus, HGL-0 could be regarded as the fraction representing oligomeric gluten proteins in wheat dough induced by processing. Thiol/disulphide interchange reactions that occur at the beginning of the Osborne extraction procedure may play a supporting role in forming SS-structures, in particular with regards to LMW thiols by preferred incorporation of glutathione and depletion of cysteine. The high number of SS-peptides with terminating fragments in HGL-N, however, indicates that the termination of glutenin polymerisation by proteins with an odd number of cysteine residues may be almost completed in the mature grain. This is also supported by the fact that approximately 80% of the terminator positions have been postulated to be occupied by proteins with an odd number of cysteine residues and only 20% by LMW thiols (Schmid et al., 2016a,b).

In previous studies, thermolysin has been shown to be the enzyme of choice for the elucidation of disulphide bonds in gluten proteins (Köhler et al., 1993, 1997; Lutz et al., 2012; Müller et al., 1995, 1997; Müller and Wieser, 1997). This enzyme leads in part to very small peptides, e.g. II-1 and XI-1 (Table 1) and V-1 and XXVI (Table 2) where there is a chance that these peptides originate from other proteins than the assigned ones. In consequence, the method of identification very much relies on the previous purification procedures and that the gluten fractions in wheat are indeed purely made of gluten proteins. To minimise unclear assignments, special attention to the appropriate preparation of HGL has been paid in this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcs.2016.12.007.

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4. General discussion

This study had several objectives, which were investigated step by step. First of all, a suitable isolation method for HGL was developed providing both high purity and high yield. This HGL preparation was completely characterized by proteinchemical techniques to elucidate its composition and structure, of which little had been known before. For this purpose, the monomers involved in HGL, the types of disulfide bonds linking them and the average molecular weight of HGL were determined. Furthermore, the influence of thiol-disulfide exchange reactions during the Osborne extraction of wheat flour proteins on the absolute amounts and the composition of gluten fractions and types in general and on HGL in particular was investigated. For this purpose, the thiol blocking reagent NEMI was added in the first step of the Osborne extraction yielding HGL-N. The control HGL-0 was obtained without addition of NEMI. Additionally, disulfide bonds within HGL were determined and differences between HGL-0 and HGL-N enabled the postulation of a hypothesis on the formation of HGL.

4.1 Isolation and characterization of untreated HMW-gliadin (HGL-0) and the influence of thiol-disulfide exchange reactions during extraction on HGL

Total gliadins were isolated by a modified Osborne fractionation of wheat flour on a preparative scale. A system based on preparative GP-HPLC was developed that allowed the isolation of HGL in amounts of 2.58 g (HGL-0 from 18 g of total gliadins) and 0.23 g (HGL-N from 1.9 g of total gliadins) and purities adequate for further characterization. Analytical GP-HPLC was used to assess quantitative data.

All gliadin types and GS were present in HGL-0, but in considerably different concentrations. The main components were LMW-GS (48 %) followed by γ-gliadins (18 %), α-gliadins (13 %), $\omega_{1,2}$-gliadins (9 %), HMW-GS (8 %) and ω_5-gliadins (4 %). The presence of α- and γ-gliadins as well as LMW-GS identified by N-terminal sequence analysis had already been described by Huebner and Bietz (1993), the participation of the other gluten protein types, however, was shown for the first time in the present study.

Two-dimensional SDS-PAGE confirmed that intermolecular disulfide bonds linked the monomers. Another novel finding was the involvement of low-molecular-weight thiols (glutathione and cysteine) and of specific gliadins with an odd number of cysteine residues in the structure of HGL-0. Due to the enormous variability of combinations of the constituting protein types and subunits, the molecular weight determination of HGL provided a range from about 66000 to 680000, which corresponds to a number of monomers between 2 and 20. The average molecular weight was about 530000. This means that HGL is considerably larger than it had been assumed in earlier studies (Huebner and Bietz 1993, Kasarda 1989, Bietz and Wall 1980) that reported molecular weights of 80000 to 250000 and 100000 to 125000, respectively.

Thiol-disulfide exchange reactions during the extraction had a significant influence on the composition of HGL. The composition of the monomers involved in HGL almost stayed the same with and without NEMI. Like in HGL-0 all gliadin types and GS were also found in HGL-N, some of them with different contents, for example LMW-GS and α-gliadins (Tab. 2). All other subtypes did not change in content and were linked by intermolecular disulfide bonds in both HGL preparations. The high percental amounts of α-, γ- and $\omega_{1,2}$-gliadins (Tab. 2) found in both preparations indicate that these gluten protein subtypes do not only act as terminators in HGL, but are also included in the chain formation of the molecules (Fig. 20).

Table 2: Proportions of gluten protein types in HMW-gliadins (HGL) isolated in the absence (HGL-0) and the presence (HGL-N) of NEMI during modified Osborne fractionation

Protein type	**Percentage[a] (%) of HGL-0**	**Percentage[a] (%) of HGL-N**
ω_5-gliadins	4.1^{A}	3.8^{A}
$\omega_{1,2}$-gliadins	8.7^{A}	6.7^{A}
α-gliadins	11.6^{A}	8.4^{B}
γ-gliadins	18.4^{A}	16.7^{A}
HMW-GS	7.7^{A}	5.6^{A}
LMW-GS	49.5^{A}	58.8^{B}

[a] Different capital letters indicate statistically significant differences between corresponding values of HGL-0 and HGL-N (t-test, P≤0.05)

Several features of the HGL preparations changed, when free thiol groups were blocked during extraction. First of all the total concentration of thiol groups after reduction was higher in HGL-N compared to HGL-0. Furthermore, the concentrations of glutathione and cysteine (HGL-0/HGL-N: 240/146 nmol/g flour and 404/734 nmol/g flour, respectively) were different. When it comes to the molecular size of HGL, no significant differences were present. The molecular weight range was identical in both extraction alternatives (66000-680000), but the average molecular size tended to be lower in HGL-N (470000 compared to 530000). This means that an average HGL-N molecule was about 2 monomers shorter than a HGL-0 molecule.

These differences can be explained with either thiol-disulfide exchange reactions or with reactions during oxidative polymerization. Kasarda (1989) already assumed that certain gluten protein components with an odd number of cysteine residues are able to terminate the oxidative polymerization of GS by being included into the polymers. Alternatively, already existing large polymers can be depolymerized by a thiol-disulfide exchange reaction with proteins/peptides with single free cysteine residues. Both reactions described can provide oligomers of limited size that are soluble in 60 % ethanol (Fig. 10).

Gliadins with an extra cysteine residue exist due to a point mutation in the amino acid sequence, and low-molecular-weight thiols are endogenous to wheat flour. Both glutathione and cysteine were found to be present in HGL in this study. Cysteine is often replaced by glutathione because of the higher reactivity of glutathione compared to cysteine (Grosch and Wieser 1999). Both thiols also seem to be replaced frequently by gliadins with an odd number of cysteine residues in their amino acid sequence, since more monomeric gliadins and less low-molecular-weight thiols were found in HGL-0 and its average molecular size was about two monomers higher compared to HGL-N (Fig. 20).

The polymerization of glutenins usually is caused by the oxidative formation of disulfide bonds between two thiol groups from two different proteins or by thiol-disulfide exchange reactions. While the oxidation is preferred during biosynthesis of gluten proteins, thiol-disulfide exchange mostly happens during processing. If a gliadin or GS is replaced by a low-molecular-weight thiol like glutathione or cysteine during the thiol-disulfide exchange in HGL, the molecular weight declines. If a low-molecular-weight thiol is replaced by a monomer with an odd number of cysteine

residues, the molecular mass of the HGL molecule is increasing. HGL-N seems to represent the status the oligomers have in their native state. HGL-N is obviously pre-formed in the endosperm and dough mixing or the extraction of wheat proteins converts it to HGL-0 due to oligomer degradation and reformation, which shows the structural differences observed in this study. It seems that the native state of the wheat flour proteins can only be investigated, if free thiol groups are blocked during the extractions, since the structural changes caused by thiol-disulfide exchange reactions are significant.

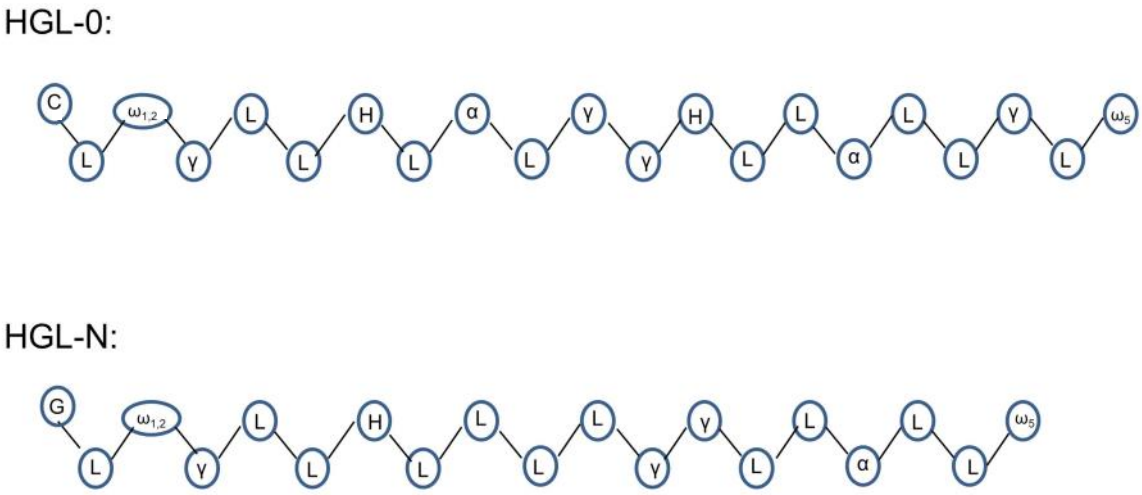

Figure 20: Schematic display of average molecules of HGL-0 and HGL-N concluded from the results of this work (L: LMW-GS; H: HMW-GS; α: α-gliadin; γ: γ-gliadin; ω_5: ω_5-gliadin; $\omega_{1,2}$: $\omega_{1,2}$-gliadin; G: glutathione; C: cysteine; -- : disulfide bond)

4.2 Influence of thiol-disulfide exchange reactions during extraction on wheat flour protein fractions

The Osborne fractionation of flour proteins starts with the addition of an aqueous salt solution to the flour. Thiol-disulfide exchange reactions can occur as soon as wheat flour is mixed with water or heated (Lagrain et al. 2007) and, therefore, these reactions cannot be excluded during the Osborne fractionation. At the beginning of this study, there was no information, if and to what extent these reactions affect the composition of gluten protein fractions, types and subunits. Hence, an Osborne fractionation with and without addition of NEMI was carried out to answer this question. Twelve flour samples were used to be able to generalize the findings. Numerous changes in the content and the composition of single gluten protein types

were found when NEMI was used. While the amount of albumins and globulins did not change in all twelve different wheat flours investigated, a decrease of gliadins (3 of 12 statistically significant) and an increase of glutenins (8 of 12 statistically significant) occurred in all samples, when NEMI was added. These changes were mainly due to a lower content of α-gliadins and a higher content of LMW-GS. The shift of protein types between fractions also influenced the gliadin/glutenin ratio that declined in most cases (7 of 12 statistically significant), when free thiol groups were blocked. Since gliadins act as „plasticizers“ in wheat dough (Wieser and Kieffer 2001) a rheological influence can be expected, when free thiol groups are blocked during dough formation, since doughs are made from flour and water. The doughs would become firmer and less extensible, just like it happens, when ascorbic acid is used (Wieser and Grosch, 2001). The amount of extractable HGL was affected when NEMI was used during the Osborne fractionation, i.e., in the same flour more HGL-N was quantified as compared to HGL-0. In two thirds of the samples, this difference was significant.

The influence of HGL on the rheological properties of dough is not known, but earlier studies (Wieser et al. 1994) showed that the effect of this protein fraction on wheat doughs is more similar to the effect of total gliadins compared to total glutenins, i.e. HGL can be expected to act as plasticizer rather than as a dough strengthener.

4.3 Disulfide structure of HGL-0

In HGL-0, which had been extracted without the addition of NEMI, eight disulfide bonds were confirmed, which had already been identified in earlier studies (Lutz et al. 2012; Köhler et al. 1993, Keck et al. 1995, Müller et al. 1995, 1997) and are typical of gliadins and glutenins. Among others, disulfide bonds of α- and γ-gliadins (C^a – $C^{f1}C^{f2}$ – C^y and C^a – $C^{f1}C^{f2}$ – C^y, C^d – C^e, respectively) were detected (Fig. 3) and also a possible head-to-tail crosslink between HMW-GS Dx5 and HMW-GS 1,5 or 7 (C^e – C^z) (Lutz et al. 2012). Thirteen disulfide bonds were identified for the first time. One of them is a typical link of the glutenins and represents another head-to-tail crosslink between two HMW-GS (C^a – C^z). All other disulfide bonds (11 in total, for example between glutathione and C^z of HMW-GS Dx5 or C^x of LMW-GS and C^z of ω-gliadins) are different from the standard disulfide crosslinks of gluten proteins and could, therefore, be specific and characteristic for HGL-0. Numerous peptides were

identified that contained sequences with point mutations, in which cysteine substituted another amino acid residue. This introduced an odd number of cysteine residues into the gluten protein and, therefore, the ability to terminate glutenin polymerization. Variants of both α- and ω-gliadins with an additional cysteine residue were detected, whereas the loss of one cysteine residue generated a γ-gliadin with seven cysteine residues. In HGL, several cysteine residues of GS, which are usually involved in glutenin polymers, were found to be linked with peptides including cysteine residues from sequence variants with point mutations suggesting a termination of glutenin polymerization by these proteins. Three cysteine residues originating from HMW- (C^e and C^z) and LMW-GS (C^x) were abundant and included in several peptides indicating that these cysteine residues might play a key role both in glutenin polymerization and termination. Additionally, several peptides linked to glutathione were detected, but the involvement of cysteine in the disulfide structure of HGL-0 could not be established.

4.4 Disulfide structure of HGL-N

In HGL-N, 18 different disulfide bonds were detected in total. Four disulfide bonds can be considered as „standard crosslinks“ of gliadins and glutenins and had already been identified in earlier studies (Köhler et al. 1993; Müller und Wieser 1995, 1997; Lutz et al. 2012). Two of them originated from γ-gliadins (C^d – C^e, C^w – C^z), one from α-gliadins (C^w – C^z) and one from HMW-GS (C^e – C^z) (Fig. 3 and 4). The remaining fourteen disulfide bonds (for example between cysteine and C^z of HMW-GS Dx5 or C^x of LMW-GS and C^z of ω-gliadin) identified in these studies had not been described so far. Again, ten cysteine peptides were detected, which only existed due to point mutations and would therefore lead to chain termination during glutenin polymerization as soon as they are incorporated into the glutenin structure. Besides point mutations, which generated an additional cysteine residue in α-, γ- and ω-gliadins, one mutation was found, which led to the loss of a cysteine residue yielding a γ-gliadin with only seven cysteine residues instead of eight. Cystine peptides containing sequences with point mutations often contained cysteine residues of HMW- and LMW-GS, which are usually involved in large glutenin polymers. It can, therefore, be assumed that the polymers are degraded by thiol-disulfide exchange due to incorporation of gliadins with modified sequences. The resulting

macromolecules are then substancially smaller than “regular” glutenin polymers. The same effect is caused by glutathione and cysteine, which were present in several peptides of HGL-N. Both thiols are able to depolymerize large polymers or to terminate the formation of glutenin polymers.

4.5 Similarities and differences in the disulfide structures of HGL-0 and HGL-N

In total 21 different disulfide bonds were identified in HGL-0, while 18 disulfide bonds were found in HGL-N. In both cases, many known disulfide bonds typical of gliadins and glutenins were present (HGL-0/HGL-N: 9/4 disulfide bonds). It was remarkable that the adjacent disulfide bonds of the α- and γ-gliadins (C^{c} – $C^{f1}C^{f2}$ – C^{y}) could only be detected in HGL-0. The head-to-tail crosslink between cysteine residues C^{e} and C^{z} of HMW-GS, which had already been described by Lutz et al. (2012), was present in both HGL-0 and HGL-N. However, a novel head-to-tail crosslink of cysteine residues C^{a} and C^{z} of HMW-GS was only identified in HGL-0. A common observation in both extraction alternatives are the numerous point mutations, one each in α- and ω-gliadins (one additional cysteine) and one in γ-gliadins (one cysteine less) were found in both HGL-0 and HGL-N. Additionally, a disulfide peptide from a γ-gliadin with a point mutation leading to an additional cysteine residue, was identified in HGL-N. Disulfide bonds between two different gluten protein types found in this study were similar, but not very numerous. In both cases, cysteine residues of LMW- and HMW-GS, which are usually involved in intermolecular disulfide bonds between GS, are linked with cysteine residues, which either only exist due to point mutations or can be found in low-molecular-weight thiols. Thus, it can be supposed that a termination of the glutenin formation has taken place both in HGL-0 and in HGL-N. One remarkable difference is that cysteine could only be detected in HGL-N as part of disulfide bonds, while cystine peptides with glutathione occurred in both preparations.

A high number of the cysteine peptides (HGL-0: 13/22; HGL-N: 12/28) found in the two extraction alternatives were only present in one of the two preparations. The explanation for the differences observed is complex and it can only be speculated, where they come from. On the one hand, it is possible that the differences are caused by the blocking of free thiol groups. This would mean that thiol-disulfide exchange reactions during the extraction of gluten proteins have a substantial

influence on the disulfide structure of HGL-0. It is eye-catching that all of the peptides found only in HGL-N contained point mutated cysteine peptides. This could indicate that the disulfide bonds corresponding to the peptides found are sensitive to thiol-disulfide exchange reactions, which would support the explanation mentioned above. On the other hand, a multitude of possible disulfide connections exists in HGL and it cannot be expected with absolute certainty that the same peptides are always found in every experiment. This would mean that it is coincidence, which peptides are found, and that the disulfide structures are not influenced by thiol-disulfide exchange.

The following points can be postulated with high confidence: Numerous peptides, which are indicators for disulfide bonds unknown to date, can be found in both HGL preparations. This shows that HGL have different disulfide structures compared to the monomeric gliadins and also compared to the glutenins. HGL appear to be a unique and independent group within the gluten proteins. Disulfide-bound cysteine was only detected in HGL-N. Considering the higher reactivity of glutathione compared to cysteine (Grosch and Wieser 1999), this result particularly indicates that at least when it comes to the low-molecular-weight thiols a thiol-disulfide exchange during the Osborne extraction takes place. With regard to the other differences found, this would further indicate that the respective cystine peptides were not detected because of coincidence or experimental conditions, but because of the thiol blocking itself.

4.6 Formation of HGL

Since disulfide bonds that appear to be typical of HGL were detected in both extraction alternatives, it can be assumed that HGL-0 are not an artefact of polymeric glutenins formed during the Osborne extraction of gluten proteins. HGL-N is formed during grain filling in the developing endosperm and already exists before processing. During Osborne extraction, HGL-N is converted into HGL-0 by thiol-disulfide exchange reactions. The formation in the endoperm seems to be tightly connected with the oxidative polymerization of GS to glutenins. Glutenins are polymers with molecular weights of up to several million (Huebner and Wall 1976, Kieffer and Belitz 1981, Werbeck and Belitz 1988, 1993, Werbeck et al. 1989, Graveland et al. 1982, 1985). Kasarda (1989) already assumed that certain gluten protein components with an odd number of cysteine residues are able to terminate

this oxidative polymerization after they have been included into the polymers. Terminators lack a second, free cysteine residue and the polymerization is interrupted. This can happen by incorporation of gliadins with a point mutated amino acid sequence, but also by low-molecular-weight thiols. Both types of terminators were found in this study. This chain termination also causes the formation of oligomeric molecules (= small polymers), which are soluble in aqueous alcohol. These molecules, which are to a certain extent side products of glutenin polymerization, are called HGL. In conclusion, the oxidative formation of disulfide bonds plays an important role in the formation of HGL-N, while thiol-disulfide exchange reactions are more important in the formation of HGL-0.

4.7 Detection of cysteine peptides

The methods used for the identification of cysteine and cystine peptides in this thesis worked quite well, although some improvement is possible in the future. Using thermolysin for enzymatic digestion of HGL was the best way to generate cystine and cysteine peptides, since thermolysin has no disufide bonds itself. Therefore, no cysteine peptides from the peptidase used for hydrolysis occurred. Since the number of different peptides in the thermolytic digest of HGL was quite high, the detection of cysteine peptides was difficult. A way to improve the identification process in future work could be to use a more specific peptidase to generate less and larger peptides. However, there is an optimal size of peptides for MS-detection. Peptides containing more than 20 amino acids usually don’t perform well in the MS with regard to signal abundance and sequence identification. Therefore, the selection of the proper peptidase is always a compromise. Using preparative GPC to gain different peptide subfractions and to lower the number of peptides in the protein solution was a suitable way to obtain clear results. In this thesis, only the first six out of ten GPC fractions contained cysteine peptides. Thus, the method for pre-separation could be improved by finding a stationary phase or an optimized gradient to get a longer retention range of the peptides, so that more fractions would be obtained with less peptides present in a particular fraction. This would improve the identification of relevant peptides in each fraction. The identification process of peptides by LC-MS/MS used in this thesis is well established and has been used by several working groups for cysteine and cystine peptide identification. Using two-dimensional LC with a separation on two columns before subjecting the eluate to MS would certainly result

in a better separation and a more precise identification of peptides. Nowadays, MS devices with a higher mass resolution are available compared to the ion trap instrument used in this thesis. It can be speculated that MS with very high mass resolution would be suitable to detect sulfur-containing peptides on the basis of the isotopic pattern of sulfur even without fragmentation by MS/MS. Peptide candidates identified in this way could be subjected to MS/MS in a second step thus providing sequence information in the same run.

5. Future research

Future research on HGL can be divided into several different aspects. The preparative isolation of HGL should be optimized further considering the high amount of material needed for later experiments. The rheological properties of HGL have been described to be similar to those of total gliadins (Wieser et al. 1994), but experimental evidence is missing to date. Thus, it is necessary to experimentally determine the functional effects of HGL. For example, the rheology of wheat doughs could be influenced by selective changes of the composition of HGL, since the qualitative and quantitative composition of these oligomeric proteins is known now. If modifications are detected, which promise a higher baking quality and better properties of wheat doughs, it may be possible to incorporate those modifications selectively in wheat cultivars in the native state by selective breeding.

In the present study, the characterization of HGL was only carried out for wheat flour of the cultivar Akteur harvested in 2011. In order to confirm the results and to find possible correlations between different properties of HGL and baking quality, it would be advisable to perform the experiments done in this study also with other wheat cultivars from other harvest years. It can be assumed that the point mutations found here are by far not the only ones that occur in gluten proteins. A detailed analysis of the disulfide structure of the gluten proteins of different wheat cultivars can also contribute to elucidating the complete disulfide structure of all HMW- and LMW-GS, which have not been totally clarified so far. As mentioned earlier in the discussion (see 4.6), not all known disulfide bonds were found in the present study, which is, however, not astonishing in view of the huge variety of possible crosslinks. Future studies could contribute to a complete clarification of the disulfide structure of gluten proteins.

The experiments, in which free thiol groups were blocked during the extraction of gluten proteins, revealed the influence of free thiol groups on the composition and structure of HGL. These experiments should be expanded, too. Even regarding earlier results in gluten protein chemistry it would be important to find out, if the changes in the qualitative and quantitative composition of gluten proteins during Osborne fractionation by thiol-disulfide exchange reactions always occur to this

extent, or, if it is still valid that the results gained after Osborne fractionation of gluten proteins show the status of the proteins in native wheat flour.

All of the aspects mentioned above show that there are still a lot of questions within this topic, which have to be investigated. The present studies represent the basis for future work.

6. Summary

Due to their different solubility during the Osborne fractionation, wheat flour proteins are classified into the water/salt-soluble albumins/globulins, the alcohol-soluble gliadins and the glutenins, which can be rendered alcohol-soluble under reducing, disaggregating conditions. About 20 % of the gliadin fraction consists of oligomers, which are called high-molecular-weight gliadins (HGL). At the beginning of this study, there was little information about the proteins contributing to HGL as well as about the disulfide bonds connecting these protein subunits. As wheat flour contains free thiol groups, for example from low-molecular-weight thiols like glutathione and cysteine or from gliadins with an odd number of cysteine residues, thiol-disulfide interchange reactions during the Osborne fractionation can be expected. However, there was no experimental evidence if such reactions really occur and if they affect the composition of the Osborne fractions and HGL. Therefore, the aims of this study were (i) to determine the influence of free thiol groups on the distribution of the Osborne fractions and the content of HGL in wheat flour and (ii) to develop a suitable isolation method for HGL and to characterize this protein fraction extensively including the identification and localization of the disulfide bonds linking the protein subunits contributing to HGL.

The quantitation of the gluten protein fractions of twelve different wheat flours after modified Osborne fractionation with and without addition of N-ethylmaleinimide (NEMI) as thiol blocking reagent showed clear differences in the composition of the protein fractions and types. After blocking free thiol groups, less gliadins and more glutenins were extracted, which led to a decrease of the gliadin/glutenin ratio. The decrease of gliadins was mainly caused by a reduction of α-gliadins, while the increase of glutenins was mainly due to low-molecular-weight (LMW-) glutenin subunits (GS). Additionally, an increase of the content of HGL was observed in all flours, if NEMI was present during extraction. Thus, thiol-disulfide exchange reactions during Osborne fractionation influenced the wheat flour proteins and, in particular, the composition of gluten protein fractions. The influence of thiol-disulfide exchange also affected the composition of HGL. The total content of disulfide-bound thiols was higher, when the extraction had been carried out with NEMI. More specifically, the amount of glutathione was lower and the amount of cysteine higher in HGL after

blocking thiol groups (HGL-N) as compared to the control without addition of NEMI (HGL-0). The types and composition of the monomers were mainly identical in both HGL-preparations, but the average molecular size of HGL changed. While it was about 530,000 in HGL-0, an average HGL-N molecule had a size of only 470,000 making it about 2 monomers shorter. It appears that glutathione and cysteine are substituted by gliadins with an odd number of cysteine residues via thiol-disulfide exchange when no thiol blocking reagent is present during extraction leading longer HGL-molecules.

The gliadin fraction of wheat flour from the cultivar “Akteur” was obtained by a modified Osborne fractionation and HGL-0 and HGL-N were obtained in the gram-range after separation from monomeric gliadins by preparative gel-permeation (GP-) high performance liquid chromatography (HPLC). The determination of the types and composition of the constituting protein subunits by N-terminal sequence analysis, densitometry and reversed-phase (RP-) HPLC showed that all gliadin and glutenin types were involved in HGL. LMW-GS and γ-gliadins had the highest contents. HGL were then digested with thermolysin and the resulting peptides separated into nine fractions by GP-HPLC and analysed by liquid chromatography/mass spectrometry (LC-MS) using alternating collision-induced dissociation/electron-transfer dissociation (CID/ETD). Altogether, 22 and 28 cystine peptides from the HGL-0 and HGl-N samples, respectively, were identified. Twenty-six peptides included standard disulfide bonds of α- and γ-gliadins, high-molecular-weight (HMW-) and LMW-GS. Eleven disulfide bonds were identified for the first time. Fifteen peptides unique to HGL contained cysteine residues from gliadins with an odd number of cysteine residues (ω5-, α- and γ-gliadins). Thus, gliadins with an odd number of cysteine residues, glutathione and cysteine had acted as terminators of oxidative glutenin polymerization or, in the case of HGL-0, of thiol-disulfide exchange reactions. Decisive differences between samples without and with NEMI treatment were not obvious and it appeared that the termination of polymerization was already completed in the flour. The two HGL samples, however, were different in the majority of ten peptides that included disulfide-linked LMW-thiols such as glutathione and cysteine with the former being enriched in the non-treated HGL sample. An interesting observation was made regarding the cleavage sites with the two different fragmentation modes CID and ETD. Amino acids most common in gluten proteins (e.g., Q, L, or P) were found next to cleavage sites more frequently in CID compared

to ETD. They seemed to be preferred in CID, which explains why more fragments were found in CID compared to ETD.

All in all, the study has shown that HGL consisting of disulfide-linked oligomeric gluten protein types and subunits, are the third, now well-defined gluten protein fraction besides monomeric gliadins and polymeric glutenins. They make up around 10 – 15 % of total gluten proteins, are soluble in aqueous alcohols without reduction of disulfide bonds and have molecular masses between 70,000 and 700,000.

7. Zusammenfassung

Aufgrund ihrer unterschiedlichen Löslichkeit während der Osborne-Fraktionierung werden die Weizenmehlproteine in die wasser-/salzlöslichen Albumine/Globuline, die alkohollöslichen Gliadine und die Glutenine, die unter reduzierenden, disaggregierenden Bedingungen alkohollöslich gemacht werden können, eingeteilt. Ungefähr 20 % der Gliadinfraktion bestehen aus Oligomeren, die HMW-Gliadine (HGL) genannt werden. Zu Beginn dieser Arbeit gab es nur wenige Informationen über die Proteine, die zum Aufbau der HGL beitragen, und über die Disulfidbrücken, die diese Proteinuntereinheiten zusammenhalten. Da Weizenmehl über freie Thiolgruppen verfügt, z. B. niedermolekulare Thiole wie Glutathion und Cystein oder Gliadine mit einer ungeraden Anzahl an Cysteinresten, sind Thiol-Disulfidaustauschreaktionen während der Osborne-Fraktionierung zu erwarten. Allerdings gab es keine experimentellen Beweise, ob derartige Reaktionen tatsächlich auftreten, und, ob sie die Zusammensetzung der Osborne-Fraktionen und der HGL beeinflussen. Daher waren die Ziele dieser Arbeit zum Einen, den Einfluss von freien Thiolgruppen auf die Verteilung der Osborne-Fraktionen und den Gehalt der HGL in Weizenmehl zu ermitteln und zum Anderen eine geeignete Isolierungsmethode für die HGL zu entwickeln und diese Proteinfraktion ausführlich zu charakterisieren einschließlich der Identifizierung und Lokalisierung der Disulfidbindungen, die die Proteinuntereinheiten zusammenfügen, die in HGL enthalten sind.

Die Quantifizierung der Glutenproteinfraktionen von zwölf verschiedenen Weizenmehlen nach der modifizierten Osborne-Fraktionierung mit und ohne Zusatz von N-Ethylmaleinimid (NEMI) als Thiolgruppenblockierungsreagenz zeigte deutliche Unterschiede in der Zusammensetzung der Proteinfraktionen und –typen. Nach der Blockierung freier Thiolgruppen wurden weniger Gliadine und mehr Glutenine extrahiert, was zu einer Abnahme des Gliadin/Glutenin-Verhältnisses führte. Die Abnahme der Gliadine wurde vornehmlich durch eine Reduzierung der α-Gliadine hervorgerufen, während die Zunahme der Glutenine hauptsächlich durch einen Anstieg der Low-molecular-weight (LMW) Gluteninuntereinheiten (GU) bedingt war. Zusätzlich wurde eine Zunahme des Gehalts an HGL in allen Mehlen verzeichnet, falls NEMI während der Extraktion anwesend war. Demnach beeinflussten Thiol-

Disulfidaustauschreaktionen während der Osborne-Fraktionierung die Weizenmehlproteine und besonders die Zusammensetzung der Glutenproteinfraktionen. Der Einfluss der Thiol-Disulfidaustauschreaktionen beeiflusste auch die Zusammensetzung der HGL. Der Gesamtgehalt der disulfidgebundenen Thiole war höher, wenn die Extraktion in Gegenwart von NEMI durchgeführt wurde. Genauer gesagt war der Gehalt an Glutathion niedriger und der Gehalt an Cystein im Vergleich zu der Kontrolle ohne Zusatz von NEMI (HGL-0) in HGL nach der Blockierung der Thiolgruppen (HGL-N) höher. Die Typen und die Zusammensetzung der Monomeren waren in beiden HGL-Präparationen größtenteils identisch, aber das durchschnittliche Molekulargewicht der HGL veränderte sich. Während es bei HGL-0 ungefähr 530000 betrug, hatte ein durchschnittliches Molekül in HGL-N nur ein Molekulargewicht von 470000, d. h. es bestand aus ungefähr zwei Monomeren weniger. Glutathion und Cystein scheinen aufgrund der Thiol-Disulfidaustauschreaktionen durch Gliadine mit einer ungeraden Anzahl an Cysteinresten ausgetauscht zu werden, falls kein Thiolblockierungsreagenz während der Extraktion genutzt wird, was zu längeren Molekülen führen könnte.

Die Gliadinfraktion aus Weizenmehl der Sorte “Akteur” wurde durch eine modifizierte Osborne-Fraktionierung erhalten und HGL-0 und HGL-N wurden durch Abtrennung von den Monomeren mittels präparativer Gelpermeationschromatographie (GP-HPLC) im Grammmaßstab gewonnen. Die Bestimmung der Proteintypen und Zusammensetzung der Proteinuntereinheiten in HGL durch N-terminale Sequenzanalyse, Densitometrie und Reversed-phase-high-performance-liquid-chromatography (RP-HPLC) zeigte, dass alle Gliadin- und Glutenintypen in den HGL enthalten waren. LMW-GU und γ-Gliadine hatten die höchsten Gehalte. Die HGL wurden dann mit Thermolysin enzymatisch verdaut, die daraus resultierenden Peptide wurden durch GP-HPLC in neun Fraktionen aufgetrennt und durch Flüssigchromatographie gekoppelt mit Massenspektrometrie (LC-MS) unter Nutzung von alternierender Collision-induced dissociation/Electron-transfer dissociation (CID/ETD) analysiert. Insgesamt wurden 22 bzw. 28 Cystinpeptide in den HGL-0- bzw. HGL-N-Präparaten identifiziert. Sechsundzwanzig Peptide enthielten bereits bekannte Standard-Disulfidbindungen von α- und γ-Gliadinen, High-molecular-weight (HMW-) und LMW-GU. Elf Disulfidbindungen wurden zum ersten Mal identifiziert. Fünfzehn Peptide, welche nur in HGL zu finden waren, enthielten Cysteinreste aus Gliadinen mit einer ungeraden Anzahl an Cysteinresten (ω5-, α- und γ-Gliadine).

Demnach hatten Gliadine mit einer ungeraden Anzahl an Cysteinresten, Glutathion und Cystein als Terminatoren der oxidativen Gluteninpolymerisation oder – im Fall von HGL-0 – der Thiol/Disulfidaustauschreaktionen fungiert. Entscheidende Unterschiede zwischen den Disulfidbindungen von HGL-0 und HGL-N wurden nicht gefunden, und es schien, dass die Polymerisation bereits im Mehl abgeschlossen worden war. Allerdings unterschieden sich die beiden HGL-Proben in zehn Peptiden, die disulfidverknüpfte niedermolekulare Thiole wie Glutathion und Cystein enthielten, wobei die Peptide mit Glutathion in den nicht behandelten HGL-0 angereichert waren. Hinsichtlich der Spaltungsstellen bei den zwei verschiedenen MS-Fragmentierungsmodi CID und ETD wurde eine interessante Beobachtung gemacht. Aminosäuren, die in Glutenproteinen sehr häufig vorkommen (z.B. Q, L oder P), wurden häufiger neben Spaltungsstellen bei der CID gefunden als bei der ETD. Sie schienen bei der CID bevorzugt zu sein, was erklärt, warum mehr Fragmente bei der CID gefunden wurden als bei der ETD.

Insgesamt hat diese Arbeit gezeigt, dass HGL, welche aus disulfidverknüpften oligomeren Glutenproteintypen und -untereinheiten bestehen, die dritte, nun genau definierte Glutenproteinfraktion neben den monomeren Gliadinen und den polymeren Gluteninen sind. Sie machen ungefähr 10 – 15 % der gesamten Glutenproteine aus, sind ohne Reduktion der Disulfidbindungen in wässrigen Alkoholen löslich und haben Molekülmassen zwischen ungefähr 70000 und 700000.

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