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Identification of Disulfide Bonds in Wheat Gluten Proteins by Means of Mass Spectrometry/Electron Transfer Dissociation

Elena Lutz, Herbert Wieser, and Peter Koehler*

German Research Center for Food Chemistry, Lise-Meitner-Strasse 34, D-85354 Freising, Germany

ABSTRACT: Disulfide bonds within gluten proteins play a key role in the breadmaking performance of wheat flour. In the present study, disulfide bonds of wheat gluten proteins were identified by using a new liquid chromatography−mass spectrometry (LC-MS) technique with alternating electron transfer dissociation (ETD)/collision-induced dissociation (CID). Wheat flour was partially hydrolyzed with thermolysin (pH 6.5, 37 °C, 16 h), and the digest was subjected to LC-MS with alternating ETD/CID fragmentation. Whereas CID provided peptide fragments with intact disulfide bonds, cleavage of disulfide bonds was preferred over peptide backbone fragmentations in ETD. The simultaneous observation of disulfide-linked and disulfide-cleaved peptide ions in the mass spectra not only provided distinct interpretation with high confidence but also simplified the conventional approach for determination of disulfide bonds, which often requires two separate experiments with and without chemical reduction. By application of the new method 14 cystine peptides were identified. Eight peptides confirmed previously established disulfide bonds within gluten proteins, and the other six cystine peptides were identified for the first time. One of the newly identified cystine peptides represented a "head-to-tail" cross-link between high molecular weight glutenin subunits. This type of cross-link, which has been postulated as an integral part of glutenin models published previously, has now been proven experimentally for the first time. From the six remaining cystine peptides interchain disulfide bonds between α-gliadins, γ-gliadins, and low molecular weight glutenin subunits were established.

KEYWORDS: *wheat flour, gluten proteins, cystine peptides, disulfide bonds, mass spectrometry*

INTRODUCTION

An optimal mixture of gliadins and glutenins is important in the breadmaking process, because together they form gluten, a continuous protein network that gives wheat dough its unique viscoelasticity and gas-holding capacity after the mixing of flour with water. Gluten proteins comprise several hundred components; according to their solubility in aqueous alcohols they can be subdivided into soluble gliadins and insoluble glutenins.[1] Their functions during dough preparation are divergent: hydrated gliadins have little elasticity and are less cohesive than glutenins; they contribute mainly to the viscosity and extensibility of wheat dough. In contrast, hydrated glutenins are both cohesive and elastic and are responsible for dough strength and elasticity. On the basis of the amino acid sequences, amino acid compositions, and molecular weights (MW), gliadins can be classified into four different types: α-, γ-, ω5-, and ω1,2-gliadins.[2] Glutenins are polymeric proteins consisting of both high molecular weight subunits (HMW-GS) and low molecular weight subunits (LMW-GS).[3] The MW of individual gluten protein types (subunits) ranges from about 25000 to 85000.

Disulfide bonds play a key role in determining the properties of gluten proteins.[4] They link two cysteine residues, either within a single protein (intrachain) or between proteins (interchain). Most gliadins are monomeric and contain either no cysteine (ω5- and ω1,2-gliadins) or intrachain disulfide bonds (α- and γ-gliadins). With a few exceptions, α-gliadins contain six and γ-gliadins, eight, cysteine residues located in the C-terminal domains and forming three and four homologous intrachain disulfide bonds.[5−7] A minor portion of gliadins is present in the oligomeric HMW gliadin fraction[8,9] or in the polymeric glutenin fraction.[10,11] It is assumed that these gliadins have an odd number of cysteine residues caused by point mutation of the amino acid sequences and are consequently linked to other proteins by an interchain disulfide bond. Exemplarily, γ-gliadins have been shown to be involved in an interchain disulfide bond with LMW-GS.[12]

Due to their polymeric nature the MW of the glutenins ranges from about 500 000 to >10 million.[3] Both HMW- and LMW-GS form intra- as well as interchain disulfide bonds, the latter being responsible for polymerization. LMW-GS contain eight cysteine residues; six of them are in positions homologous to those of α- and γ-gliadins and, therefore, have been suggested to be linked via intrachain disulfide bonds.[6,12,13] Two further cysteine residues unique to LMW-GS are involved in interchain disulfide bonds to other LMW-GS and to y-type HMW-GS.[12,13] x-Type HMW-GS include four or five and y-type HMW-GS seven cysteine residues, respectively The two cysteine residues of x-type subunits located next to the N terminus were found to be linked by an intrachain bond.[12] At present, interchain cross-links have been identified only for adjacent cysteine residues of y-type HMW-GS, which are connected in parallel to corresponding cysteine residues of another y-type HMW-GS, and for an additional cysteine residue, which is disulfide-linked to LMW-GS.[13,14]

A number of glutenin models postulate HMW-GS being linked end-to-end, possibly head-to-tail, by interchain disulfide

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bonds to form a linear backbone of glutenin polymers. The only experimental evidence for this has been provided by a study of Kasarda and co-workers,[15] showing that the C-terminal part of an x-type HMW-GS is linked to the N-terminal part of a y-type HMW-GS, although the precise position of cysteine residues involved in this cross-link could not be identified.

The methods to identify disulfide bonds in gluten proteins applied in the past included fractionation of gluten proteins, enzymatic digestion of the proteins, extensive purification of the resulting peptide mixture, sophisticated identification of cystine peptides by differential chromatography prior to and after reduction of disulfide bonds, and isolation and Edman sequencing of cystine and cysteine peptides.[5,6,12−14] This technique is time-consuming and labor-intensive, and minor disulfide bonds have not been identified so far. Thus, the limiting factor for the identification of disulfide bonds of HMW-GS is probably due to the fact that this protein type is a minor fraction of total gluten (≈10%) and even of the glutenin fraction (≈30%). Therefore, more selective and more sensitive methods are required to identify disulfide bonds that have not been detected yet.

Recently, Wu et al.[16] introduced mass spectrometry (MS) as a method for the determination of disulfide cross-links using online liquid chromatography (LC)-MS with electron transfer dissociation (ETD). Disulfide-linked peptide ions were identified by collision-induced dissociation (CID) and ETD fragmentation, and the disulfide-cleaved or partially cleaved peptide ions were characterized in a subsequent MS^3 step (Figure 1). This method has been used to investigate

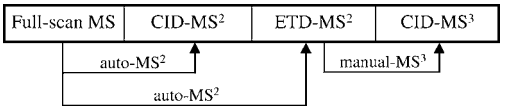

Figure 1. LC-MS method to identify disulfide bonds in wheat gluten proteins: data acquisition scheme with CID-MS^2, ETD-MS^2, and CID-MS^3 steps.[16]

recombinant therapeutic proteins; however, it has not been applied to gluten proteins to date.

The aim of this study was, therefore, to introduce the method of Wu et al.[16] into cereal chemistry to identify disulfide bonds of gluten proteins in wheat flour by analyzing enzymatic protein digests by LC-MS with alternating CID and ETD fragmentation. The method was tested by the identification of already known disulfide bonds and then applied for disulfide cross-links that have not been described up to now; that is, "head-to-tail" cross-links between HMW-GS were the focus of this study.

MATERIALS AND METHODS

Wheat Flour. Wheat kernels of the German cultivar 'Akteur' (harvest 2009) were milled into flour with a Quadrumat Junior Mill (Brabender, Duisburg, Germany). The flour was sifted through a 0.2 mm screen. The nitrogen content was determined by using the method of Dumas on an FP-328 nitrogen analyzer (Leco, Kirchheim, Germany). A conversion factor of 5.7 was used to calculate the protein content from the nitrogen content. Moisture and ash contents of the flour were determined according to ICC Standards 110/1 and 103/1, respectively.[17,18] The flour contained 15.6% moisture, 10.7% protein, and 0.46% ash.

Chemicals. The quality of all chemicals was "pro analysi" (p.a.) or stated otherwise. Sodium azide, methanol (LiChrosolv), hydrochloric acid (32%, w/w), ammonia solution (25%, w/w), trichloroacetic acid, glacial acetic acid, tris (hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), Coomassie Brillant Blue R-250, acetonitrile (LiChrosolv), and formic acid (98−100%) were from Merck (Darmstadt, Germany). Calcium chloride hexahydrate, thermolysin (from *Bacillus thermoproteolyticus rokko*, activity = 66.7 U/mg protein), sodium hydroxide (≥98%), and trifluoroacetic acid (≥98%) were from Sigma-Aldrich (Steinheim, Germany). 3-(*N*-Morpholino)-propanesulfonic acid (MOPS) was provided by AppliChem (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), serva blue G250, phenol red, and dithioerythritol (DTE) were from Serva (Heidelberg, Germany). The water used was deionized by a Millipore-O Milli-Q purification system.

SDS−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was carried out according to the method of Kasarda and co-workers[19] with a NuPAGE 10%-Bis-Tris [bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane-HCl] single gradient gel, 1.0 mm × 10 well (Invitrogen, Carlsbad, CA, USA). The running buffer was MOPS (50 mmol/L MOPS, 50 mmol/L Tris, 3.5 mmol/L SDS, 1 mmol/L EDTA) containing DTE (5 mmol/L) as reducing agent added to the inside chamber. Flour (23 mg) was extracted with 1 mL of extraction buffer (293.3 mmol/L sucrose, 246.4 mmol/L Tris, 69.4 mmol/L SDS, 0.51 mmol/L EDTA, 0.22 mmol/L serva blue G250, 0.177 mmol/L phenol red, 0.105 mmol/L hydrochloric acid) for 24 h under reducing contitions (DTE, 50 mmol/L). The suspended flour was then heated for 10 min to 60 °C while shaking and then centrifuged at 2400*g* for 5 min at 20 °C. Seven proteins with different MW (myosin 200 000; β-galactosidase 116 000; bovine serum albumin 68 000; ovalbumin 43 000; carbonic anhydrase 29 000; trypsin inhibitor (soybean) 20 000 lysozyme (chicken egg white) 14 000) were used as markers. The sample volumes on the gel were between 5 and 10 μL per slot. Run time was 55 min at 50 V and 230 mA. After the electrophoretic run, proteins were fixed for 30 min in trichloroacetic acid (12%, w/v). Proteins were stained for 30 min in Coomassie Brilliant Blue R-250 according to the method of Neuhoff et al.[20] Gels were first destained twice with methanol/water/acetic acid (50:40:10, v/v/v) for 15 min and then overnight with water/methanol/acetic acid (80:10:10, v/v/v).[19]

Enzymatic Digestion of Flour with Thermolysin. Wheat flour (100 mg) was suspended in a solution (1 mL) containing calcium chloride (4 mmol/L), sodium azide (7 mmol/L), and thermolysin (0.1 mg); the ratio of enzyme to flour protein was 1:100 (w/w). The pH was adjusted to 6.5 with sodium hydroxide (0.1 mL/L).[14] During hydrolysis the pH changed not more than 0.2 unit. Samples were magnetically stirred at 37 °C, and after 16 h, the reaction was stopped by lowering the pH to 2.0 with hydrochloric acid (1 mol/L). The digest was centrifuged at 9600*g* for 20 min at 4 °C. The supernatant was collected and used for further purification.

Solid Phase Extraction (SPE). The supernatant after thermolytic digestion was purified by SPE on Strata-X-C devices (Phenomenex, Aschaffenburg, Germany). SPE cartridges were subsequently preconditioned with 1 mL of each methanol and trifluoroacetic acid (0.1%, v/v). The digest (1 mL) was applied, and the cartridge was washed with methanol (50%, v/v; 5 × 1 mL). Afterward, peptides were eluted with ammonia solution (1 mL; 2 mol/L) and freeze-dried.

Strategy for Identifying Cystine Peptides. *Known Cystine Peptides.* For identifiying cystine peptides already described in the literature,[5,6,12,21] the molecular mass of each parent peptide was calculated. Calculated masses of single-, double-, and triple-charged precursor ions with an isolation width of $m/z = m/z_{(ion)} \pm 2$ were then included in the MS program to select specific precursor ions for CID and ETD fragmentation in each run. Resulting b-/y- (CID) and c-/z- (ETD) fragment ions were then assigned to the known peptide masses. In ETD spectra, the mass of at least one single peptide fragment formed by disulfide bond cleavage was required to be present as a major fragment ion.

Unknown Cystine Peptides. For identiying unknown cystine peptides, a protein database search was first carried out to gather information about amino acid sequences of gluten proteins and possible point mutations in the neighborhood of cysteine residues

[PIR-database (http://pir.georgetown.edu); keywords: triticum, aegilops, gliadin, glutenin]. On the basis of available amino acid sequences and using the cleavage sites of the peptidase thermolysin, a target mass list of potential cysteine peptides was created. In the first MS step full-scan spectra were acquired. Precursor ions for CID and ETD fragmentation were automatically isolated ("auto MS") starting with the most intense ion of the full MS scan. In the MS-MS spectra obtained by ETD major fragment ions were identified on the basis of the following criteria: the m/z value could be assigned to the target mass list, and the sum of both fragment ions corresponded to the single charged parent ion in the spectrum. If only one major fragment ion in the ETD spectrum could be unambiguously assigned to the target list, the m/z value of the corresponding second peptide fragment was calculated by subtracting the m/z value of the major fragment ion from the m/z value of the single charged parent peptide. Matches were then confirmed by the corresponding CID spectra and subsequent MS^3 steps of the single peptides (see below).

LC-MS with Alternating ETD/CID Fragmentation. LC-MS experiments were performed on an HCT-Ultra PTM ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) with alternating CID/ETD detection coupled with an UltiMate 3000 HPLC (Dionex, Idstein, Germany) system equipped with a Synergi Fusion-RP C_{18} column (8 nm pore and 4 μm particle size, 2 × 250 mm, 35 °C). The mass spectrometer contained a spherical ion trap with an electrospray ionization (ESI) interface running in the positive mode. The ESI parameters were as follows: capillary voltage, −4000 V; capillary exit voltage, 136.5 V; and skimmer voltage, 40 V. Nitrogen was used as drying (flow rate = 8 L/min, 325 °C) and nebulizing gas (pressure = 0.2 MPa). Fluoranthene radical anions for ETD fragmentation were generated in an additional chemical ionization source filled with fluoranthene and methane gas.[22]

The mobile phase for LC separation was (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in acetonitrile. The following gradient was used: (i) isocratic for 5 min at 0% B for sample loading; (ii) linear from 0 to 20% B over 30 min; (iii) linear from 20 to 90% B over 2 min; (iv) isocratic at 90% B for 5 min; (v) linear from 90 to 0% B in 2 min; (vi) isocratic at 0% B for 16 min. The flow rate was 0.2 mL/min. Two microliters of the purified and lyophilized thermolytic digest dissolved in 0.5 mL of formic acid (0.1%, v/v) was injected. As shown in Figure 1 an auto-MS^2 mode was used for the alternating CID/ETD-MS^2 fragmentation, and a manual-MS^3 mode was applied for disulfide-cleaved peptides in a following step. After a full-scan MS spectrum from m/z 150 to 3000 had been acquired (smart target value 3×10^5 ions or a maximum acquisition time 100 ms), CID-MS^2 (fragmentation amplitude of 1 V) and ETD-MS^2 [ion current control (ICC) > 6×10^5 counts] scan steps were performed on the same precursor ion. For the known cystine peptides, an acquisition mode including masses of the precursor ions with an isolation width of $m/z = m/z_{(ion)} \pm 2$ was used to select precursor ions for CID and ETD fragmentation. For unknown cystine peptides, precursor ions for CID and ETD were isolated using the acquisition mode with an isolation width of $m/z = m/z_{(ion)} \pm 4$. An additional MS^3 step by CID with a fragmentation amplitude of 1 V was performed on the prior ETD spectrum after isolation of the ion of the disulfide-cleaved peptide (isolation width $m/z = m/z_{(ion)} \pm 2$) (Figure 1). For this, an additional LC-MS run was required, and the minimum intensity of the precursor ions in the ETD-MS^2 spectrum was 10^5 counts.

RESULTS AND DISCUSSION

Sample Selection and Preparation. In contrast to previous studies, in which gliadin and glutenin fractions isolated from gluten were investigated,[5,6,12−14] white flour from the German winter wheat cultivar 'Akteur' (harvest 2009) was used as the starting material in this study. 'Akteur' is one of the German wheat cultivars with the best baking performance. SDS-PAGE of the flour (Figure 2) showed that this cultivar had the HMW-GS composition Ax1, Dx5, Bx7, By9, and Dy10.[23] The combination of HMW-GS 5 and 10 is usually associated

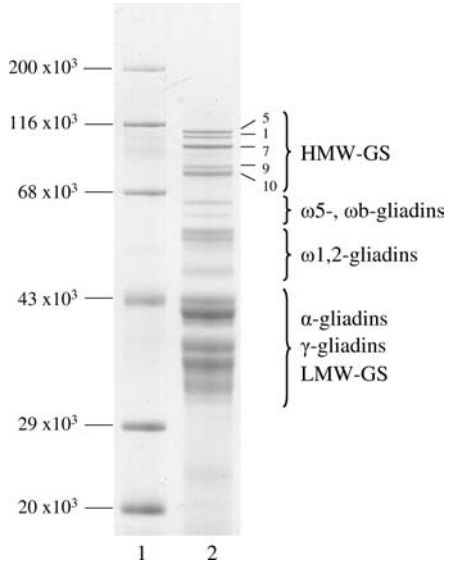

Figure 2. SDS-PAGE of reduced flour protein of the German wheat cultivar 'Akteur' [lane 1, protein marker (see Materials and Methods); lane 2, flour proteins].

with high baking performance. In particular, the presence of an extra cysteine residue in HMW-GS 5 as compared to HMW-GS 2 is thought to be responsible for the good technological properties of wheat cultivars containing this HMW-GS because it enables improved polymerization as compared to cultivars with the HMW-GS combination 2 and 12.[3,4]

In the first step of the sample workup flour proteins were partially hydrolyzed with thermolysin, which is known to cleave peptide bonds preferentially before hydrophobic amino acids such as leucine, isoleucine, phenylalanine, valine, methionine, and alanine.[24] However, peptide bonds involving the amino groups of tyrosine, glycine, threonine, and serine residues were also found to be susceptible to hydrolysis to a minor extent.[24] In previous studies thermolysin had been shown to be suitable for the partial hydrolysis and subsequent identification of disulfide bonds in gluten proteins[5,6,12−14] because it effectively transferred gluten proteins to soluble peptides, which had suitable molecular masses for MS analysis. Other peptidases such as trypsin and chymotrypsin transferred only a low portion of the gluten proteins into soluble peptides, and, in the case of trypsin, large peptides were generated, which were not suitable for structure determination. Despite the reported pH optimum between pH 7.0 and 9.0[25] partial hydrolysis was carried out at pH 6.5 to minimize thiol−disulfide bond interchange, which might occur at higher pH values. At pH 6.5 sufficient activity was still present to enable extensive hydrolysis of the proteins. In previous studies[12] it has been shown that appropriate peptide mixtures were obtained with an enzyme to substrate ratio of 1:100 and 16 h of incubation at 37 °C. The buffer solution contained sodium azide to prevent microorganisms from growing during incubation and calcium chloride to slow self-inactivation of the enzyme. After 16 h, the hydrolysis was stopped by inactivating the enzyme by lowering the pH to 2.0. Peptides in the supernatant of the digest were purified by SPE on Strata-X-C SPE devices. This is a mixed stationary phase using strong cation exchange and nonpolar functional groups for separation. After preconditioning with methanol and 0.1% TFA, sulfonyl residues of the SPE cartridges were present in the anionic form. Positively charged peptides in the acidic sample

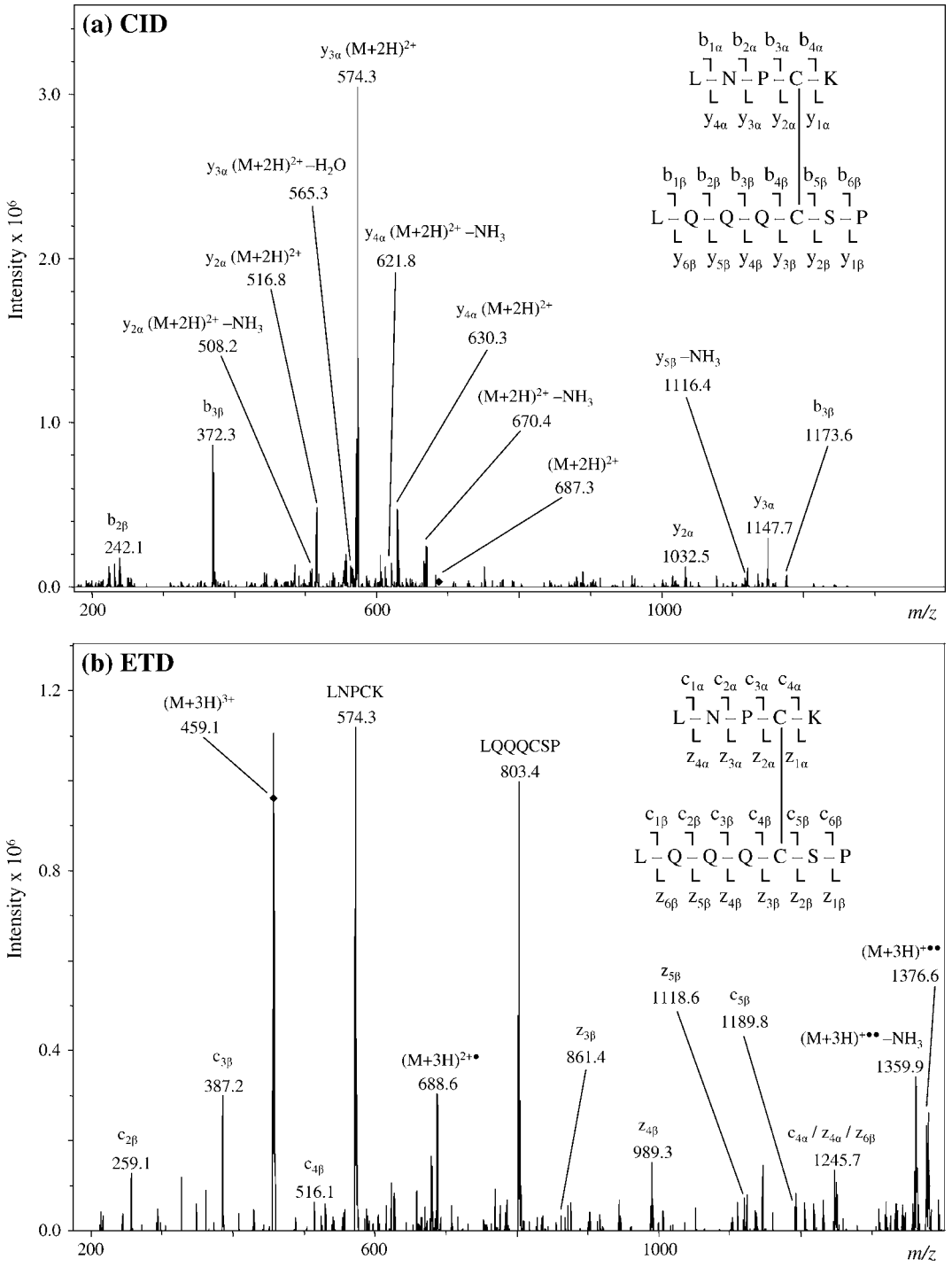

Figure 3. LC-MS^2 spectra of peptide VIII: (a) CID spectrum of the precursor ion with m/z 687.3 $(M + 2H)^{2+}$; (b) ETD spectrum of the precursor ion with m/z 459.1 $(M + 3H)^{3+}$.

solution subsequently linked to the resin by ionic and hydrophobic interactions. Washing with 50% methanol removed impurities such as neutral salts. Peptides were then eluted from the SPE resin with ammonia solution (2 mol/L). The purified peptide mixture was then subjected to LC-MS to selectively identify cystine peptides.

Method for Identifying Cystine Peptides. Cystine peptides were identified and their structures were determined by means of MS using a combination of fragmentation techniques, namely ETD and classical CID (Figure 1). After LC separation peptides entered the MS and after electrospray ionization, full-scan spectra were acquired, and the charge state of the peptide ions was assigned. Then, double- or triple-charged ions were selected and subjected to fragmentation by alternating CID and ETD. In ETD multiple positively charged precursor ions reacted with radical anions from fluoranthene. The resulting fragmentation provided fragments that were different from those obtained by CID. Whereas in CID cleavage of the peptide bond produced b- and y-type fragment ions, ETD fragmented at the N–C_α bond, generating even-electron c-type and odd-electron z•-type product ions.[26] Furthermore, in CID the disulfide bond remained intact,

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Table 1. Identified Cystine Peptides from Gluten Proteins[a]

Cystine peptide	Peptide fragments	Amino acid sequence[a]	Cysteine residue[b]	Gluten protein type	Reference
I	I-1	SRCQA	C^w	α-Gli	(5)
	I-2	VYIPPYCT	C^z	α-Gli	
II	II-1	LQQCKP	C^d	γ-Gli	(6)
	II-2	IWPQSDCQ	C^e	γ-Gli	
III	III-1	LQC	C^w	γ-Gli	(12)
	III-2	VSPDCST	C^z	γ-Gli	
IV	IV-1	LQC	C^w	γ-Gli	(6)
	IV-2	VYVPPECS	C^z	γ-Gli	
V	V-1	SQQPFPQQPQQPCPQTQQPQQ	C^{b*}	γ-Gli	(12)
	V-2	LGQC	C^x	LMW	
VI	VI-1	FSQQQPCSQQQQQP	C^{b*}	LMW	(12)
	VI-2	LGQC	C^x	LMW	
VII	VII-1	LQQQCSP	C^d	LMW	(12)
	VII-2	SCH	C^e	LMW	
VIII	VIII-1	LNPCK	C^c	LMW	(21)
	VIII-2	LQQQCSP	C^d	LMW	
IX	IX-1	VTCPQQ	C^e	HMW 5	-
	IX-2	LPAMCR	C^z	HMW 1 / HMW 5 / HMW 7	
X	X-1	IPEQSRCQ	C^w	α-Gli	-
	X-2	LQQCNP	C^d	γ-Gli	
XI	XI-1	VYIPPYCSTT	C^z	α-Gli	-
	XI-2	LGQC	C^x	LMW	
XII	XII-1	LQC	C^w	γ-Gli	-
	XII-2	FSQQQPCSQQQQQP	C^{b*}	LMW	
XIII	XIII-1	LGQC	C^x	LMW	-
	XIII-2	MCN	C^y	α-Gli / γ-Gli / LMW	
XIV	XIV-1	LQQQCSP	C^d	LMW	-
	XIV-2	MCN	C^y	α-Gli / γ-Gli / LMW	

[a] [a] One-letter-code of the amino acids. [b] Nomenclature of cysteine residues according to Koehler et al.[8]

whereas disulfide bond cleavages were preferred over peptide backbone fragmentations in ETD. This opened the possibility to examine the peptides with intact (CID spectrum) as well as with cleaved disulfide bond (ETD spectrum) and, thus, to get information about the presence of a disulfide bond and the amino acid sequence of the peptide. Additional sequence information was available by subjecting disulfide-cleaved peptide ions from ETD to a third dissociation step (CID-MS^3)[16] (Figure 1).

Figure 3 illustrates the identification of cystine peptides by using one of the gluten peptides (peptide VIII) as an example. The structure of this peptide is given in Table 1. LC-MS analysis provided full-scan spectra (not shown), in which the most intense ions were automatically selected for alternating CID and ETD fragmentations ("auto-MS^2"). The mass accuracy of the ion trap MS used in this study was $m/z \pm 0.5$; thus, in a few cases the charge state was not assigned correctly. In the resulting CID and ETD spectra relevant peptide ions were assigned according to the following principles. If positively charged fragments of the N-terminal part of the peptides were obtained, these were indexed as a, b, c, etc. If the charge remained on the C-terminal fragments, x-, y-, and z-fragments were obtained. The number of amino acid residues present in the fragment ions was indicated by numbers, for example, 1, 2, 3, etc. Furthermore, the indices α and β referred to the respective cysteine peptide ions corresponding to the parent cystine peptide. In the CID spectrum (Figure 3a) only fragments containing a disulfide bond were obtained, and the fragment ions preferably belonged to the b- and y-series. In contrast, the ETD spectrum (Figure 3b) also provided fragments without a disulfide bond, showing the signals of the two cysteine peptides α and β, namely LNPCK (m/z 574.3) and LQQQCSP (m/z 803.4). With this type of fragmentation mainly ions from the c- and z-series were obtained. To obtain additional sequence information the α- and β-ions from the ETD spectrum (Figure 3b) were selected manually and subjected to CID-MS^3. An additional LC-MS run was required for this experiment, and a minimal intensity of $\geq 10^5$ counts for the α- and β-ions was necessary, which was not the case for all peptides. The CID-MS^3 spectra of the disulfide-cleaved α- and β-peptides LNPCK and LQQQCSP (Figure 4) provided valuable information about the amino acid sequences. In this way 14 cystine peptides were identified and sequenced. The structures are given in Table 1.

Assignment of Cystine Peptides to Gluten Protein Types. Seven cystine peptides that had been identified in thermolytic hydrolysates of wheat gluten proteins in previous studies[5,6,12−14] and one additional cystine peptide, identified after high-pressure and temperature treatments,[21] were also found in the present study and confirmed postulated disulfide bonds (Table 1). Peptide I contained fragments from α-gliadins and represented an intrachain disulfide bond between cysteine residues C^w and C^z (nomenclature of cysteine residues according to Koehler et al.[12]). Peptides II, III, and IV were examples for two intrachain cross-links between cysteines C^d and C^e as well as C^w and C^z of γ-gliadins, respectively. Peptide V represented an interchain cross-link between C^{b*} and C^x of a γ-gliadin and a LMW-GS. Cystine peptide VI contained an interchain cross-link between C^{b*} and C^x of two LMW-GS. Peptide VII confirmed the intrachain disulfide bond between

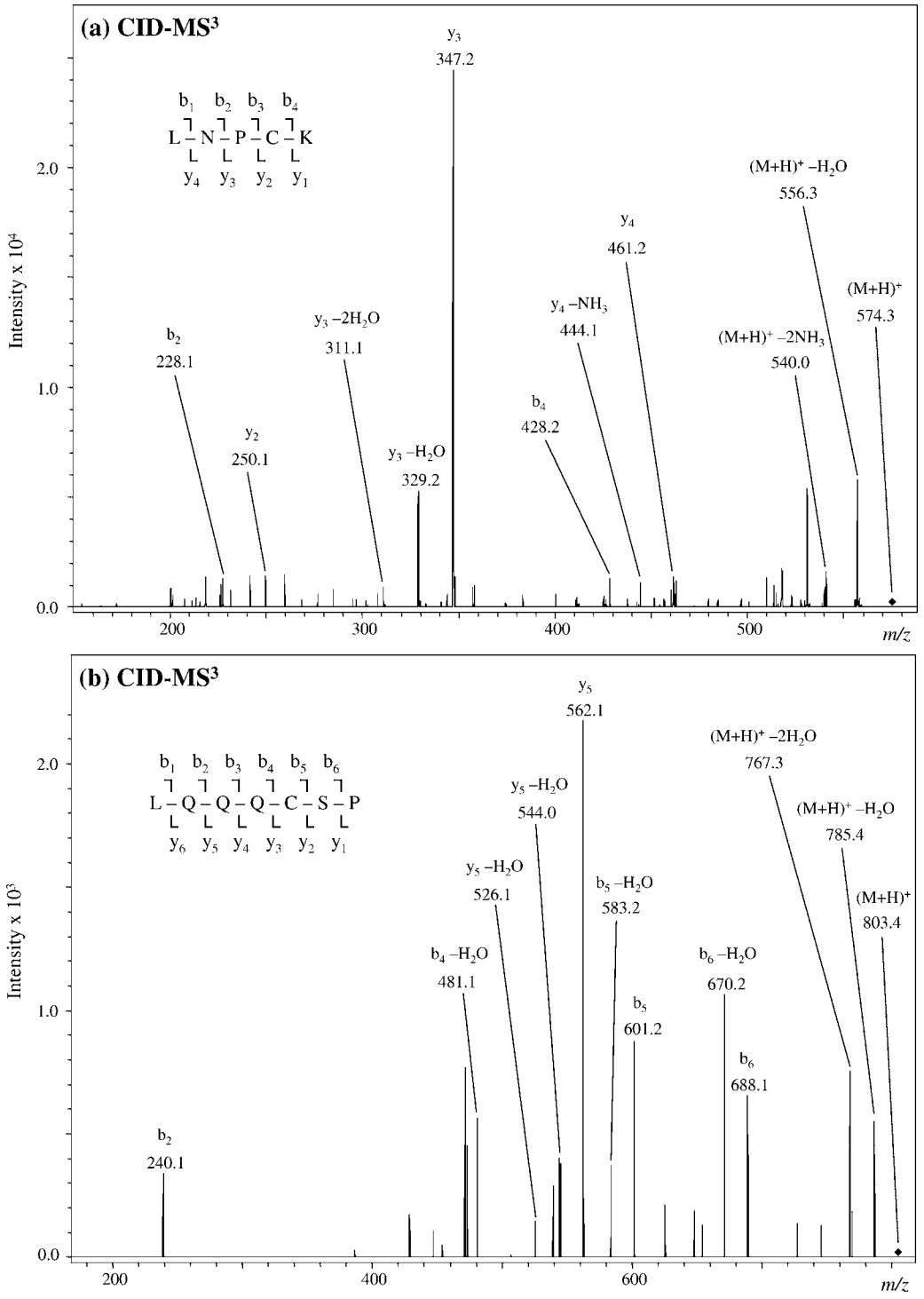

Figure 4. LC-MS^3 spectra of the peptide ions of LNPCK and LQQQCSP from the ETD spectrum displayed in Figure 3: (a) fragments obtained from the precursor ion of peptide LNPCK with m/z 574.3 $(M + H)^+$; (b) fragments obtained from the precursor ion of peptide LQQQCSP with m/z 803.4 $(M + H)^+$.

cysteine residues C^d and C^e of LMW-GS. Figure 5 illustrates that these peptides fitted into a "disulfide map" of gluten proteins, which had been obtained as a result of previous studies.[5,6,12−14] Finally, peptide VIII contained fragments from LMW-GS and represented either an intra- or interchain disulfide bond between cysteine residues C^c and C^d.[21]

Table 1 shows six further cystine peptides from gluten proteins, which were identified for the first time. Although it was not possible to unequivocally assign peptide IX to known amino acid sequences of gluten proteins, this peptide considerably contributes to understanding gluten structure. The disulfide bond involves the cysteine residue C^e unique to HMW-GS Dx5 and C^z, which is present in the C-teminal domain of all x-type HMW-GS (Table 2). The disulfide bond between these two cysteine residues is assumed to be an interchain rather than an intrachain link due to the polymeric nature of glutenins. This disulfide bond has been postulated as integral part in several glutenin models (see, e.g., refs 3 and 27)

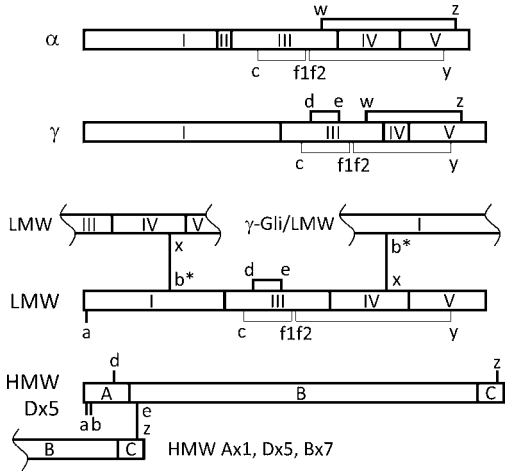

Figure 5. Matching of disulfide bonds determined in this study with the commonly accepted disulfide structure of gluten proteins. Broad lines represent disulfide bonds that have been confirmed; thin lines represent disulfide bonds that have not been detected in this study.

Table 2. Covalent Structure and Origin of Peptide IX from HMW-GS

		87		97		107
HMW-GS Dx5 (*29*)		… L L K R Y Y P S V T C P Q Q V S Y Y P G Q …				
IX	IX-1			V T C P Q Q		
	IX-2			L P A M C R		
		787		797		807
HMW-GS Ax1 (*30*)		… Q Q L A A Q L P A M C R L E G G D A L L A …				
		805		815		825
HMW-GS Dx5 (*29*)		… Q Q L A A Q L P A M C R L E G G D A L S A …				
		748		758		768
HMW-GS Bx7 (*31*)		… Q Q L A A Q L P A M C R L E G S D A L S T …				

and represents a head-to-tail cross-link between HMW subunits as illustrated in Figure 5. Evidence for this type of interchain HMW-GS cross-link has already been obtained by Tao et al.[15] In this work a high MW, SDS-insoluble glutenin fraction was digested with endoproteinase Lys-C. Several peptides with MW close to those of intact HMW-GS were obtained. N-Terminal sequencing indicated that the N-terminal domain of HMW-GS Dy10 was linked to the C-terminal part of HMW-GS Bx17 in a head-to-tail orientation. Due to the high MW of the peptides, the N-terminal cysteine residue of HMW-GS Dy10 involved in this cross-link could not be determined. The result of the present study not only confirmed the existence of head-to-tail HMW-HMW-GS disulfide bonds in wheat gluten but also specified the cysteine residues involved in this type of cross-link.

Assignment of the amino acid sequences of peptides X, XI, and XII (Table 1) showed that they all contained interchain disulfide bonds. Peptide X contained amino acid sequences from α- and γ-gliadins, which were linked by a disulfide bond via cysteine residues C^w (α-gliadins) and C^d (γ-gliadins), respectively (Table 3), whereas in peptide XI cysteine residues C^z of an α-gliadin and C^x of a LMW-GS were connected (Table 4). The disulfide bond of peptide XII was formed between C^w and C^{b*} of γ-gliadins and LMW-GS, respectively (Table 5). Peptide XIII represented an interchain disulfide bond between cysteine residue C^x of LMW-GS and cysteine residue C^y of either α- or γ-gliadins or LMW-GS (Table 6). It was not possible to determine the origin of C^y as the amino acid sequence MCN is present in α- and γ-gliadins as well as in

Table 3. Covalent Structure and Origin of Peptide X from α- and γ-Gliadins

		156		166		176
α-Gliadin A212 (*28*)		… Q L W Q I P E Q S R C Q A I H N V V H A I …				
X	X-1			I P E Q S R C Q		
	X-2			L Q Q C N P		
		150		160		170
γ-Gliadin pW1020 (*32*)		… N P C K N F L L Q Q C N H V S L V S S L V …				
		135		145		155
γ-Gliadin pTag1436 (*33*)		… N P C K N F L L Q Q C K P V S L V S S L W …				

Table 4. Covalent Structure and Origin of Peptide XI from α-Gliadins and LMW-GS

		250		260		270
α-Gliadin A1235 (*28*)		… A M C N V Y I P P Y C S T T I A P F G I F …				
XI	XI-1			V Y I P P Y C S T T		
	XI-2			L G Q C		
		256		266		276
LMW-GS LP1211 (*34*)		… Q Q Q Q P Q Q L G Q C V S Q P L Q Q L Q Q …				

Table 5. Covalent Structure and Origin of Peptide XII from γ-Gliadins and LMW-GS

		189		199		209
γ-Gliadin pW1020 (*32*)		… Q L A Q I P Q Q L Q C A A I H S V A H S I …				
XII	XII-1			L Q C		
	XII-2			F S Q Q Q P C S Q Q Q Q Q P		
		56		66		76
LMW-GS GluB3-2 (*35*)		… Q P Q P F S Q Q Q P C S Q Q Q Q Q P L S Q …				

Table 6. Covalent Structure and Origin of Peptide XIII from LMW-GS and α-Gliadins or γ-Gliadins or LMW-GS

		256		266		276
LMW-GS LP1211 (*34*)		… Q Q Q Q P Q Q L G Q C V S Q P L Q Q L Q Q …				
XIII	XIII-1			L G Q C		
	XIII-2			M C N		
		242		252		262
α-Gliadin A212 (*28*)		… N L A L Q T L P A M C N V Y I P P Y C T I …				
		248		258		268
γ-Gliadin pW1020 (*32*)		… S L V L K T L P T M C N V Y V P P D C S T …				
		299		309		319
LMW-GS LP1211 (*34*)		… S I A L R T L P T M C N V N V P L Y E T T …				

LMW-GS. Peptide XIV was similar to peptide XIII with the cysteine residue C^y being connected to C^d of a LMW-GS (Table 7) via an interchain disulfide bond.

Significance of the Identified Disulfide Bonds for Gluten Structure. Cystine peptides I–VIII do not need to be further discussed because they confirm disulfide bonds of gluten proteins determined already in previous studies.[5,6,12−14,21] However, not all of the known disulfide bonds

Table 7. Covalent Structure and Origin of Peptide XIV from LMW-GS and α-Gliadins or γ-Gliadins or LMW-GS

		125		135		145
LMW-GS LMWG-1D1 (*36*)		… N P C K V F L Q Q Q C S P V A M P Q R L A …				
XIV	XIV-1			L Q Q Q C S P		
	XIV-2			M C N		
		242		252		262
α-Gliadin A212 (*28*)		… N L A L Q T L P A M C N V Y I P P Y C T I …				
		248		258		268
γ-Gliadin pW1020 (*32*)		… S L V L K T L P T M C N V Y V P P D C S T …				
		299		309		319
LMW-GS LP1211 (*34*)		… S I A L R T L P T M C N V N V P L Y E T T …				

of gluten proteins have been identified by the new method (Figure 5). In particular, disulfide bonds of α- and γ-gliadins as well as LMW-GS, in which the adjacent cysteine residues C^{f1} and C^{f2} were involved, could not be established. This type of cystine peptide, which is composed of three fragments linked via two disulfide bonds, was obviously difficult to detect. The same was also the case for cysteine residues C^{c1} and C^{c2} of y-type HMW-GS. This is currently still the main limitation of the new method. On the other hand, the new approach was capable of identifying a head-to-tail cross-link between x-type HMW-GS for the first time (peptide IX). There is no doubt that this contributes to a better understanding of the structure of the polymeric glutenins (Figure 5). The further five cystine peptides X–XIV were identified for the first time and contained amino acid sequences from different gluten protein types (α-, γ-gliadins, LMW-GS), all representing interchain disulfide bonds. From peptides X, XI, XII, and possibly XIII and XIV it can be assumed that the corresponding α- and γ-gliadins did not occur in the monomeric but in the oligomeric HMW gliadin or polymeric glutenin fractions[8−11] linked by an interchain disulfide bond. A closer look into the primary structure of gliadins is necessary to explain these findings. With a few exceptions, α-gliadins contain six and γ-gliadins, eight, cysteine residues. Individual (= isolated) proteins of these types have been shown to contain three and four homologous intrachain disulfide bonds.[5−7] However, in ethanol-soluble gliadin up to 30% oligomeric proteins, the so-called HMW gliadins, are present,[9] which might contain medium MW proteins composed of disulfide-linked aggregates between gliadins and between gliadins and GS as well as between GS. Thus, aggregates containing exclusively gliadins are likely to be present but probably with a content below 30%. Moreover ω-, α-, and γ-type gliadins have been identified in the glutenin fraction[10,11] This might be caused by gliadin subtypes with odd numbers of cysteine residues due to point mutations of individual amino acids (e.g., serine → cysteine) as described by Okita et al.[28] Thus, the newly identified interchain cross-links between gliadins (cystine peptide X) may be caused by gliadins with an odd number of cysteine residues. Furthermore, in gluten or in flour, proteins with an odd number of cysteine residues may act as chain terminators and get linked to the glutenin polymer.[3] This type of disulfide cross-link has also been found in this study (cystine peptides XI–XIV). The orientation of the newly detected disulfide bonds in α- and γ-gliadins as well as LMW-GS differs from the structures published so far (Figure 5). Published data[12] and also peptide V suggest that additional cysteine residues in γ-gliadins are possible and that they participate in interchain disulfide bonding. From the data obtained in this study it can be assumed that an odd number of cysteine residues in specific gliadin subtypes might alter the common intrachain disulfide structure and would allow cysteine residues usually involved in intrachain disulfide bonds being available for interchain cross-links. However, additional experiments, for example, with HMW gliadin, would be necessary to address this problem.

Concluding Remarks. The results revealed that it is possible to identify disulfide bonds in partially hydrolyzed gluten proteins by LC-MS with alternating CID/ETD fragmentation and subsequent CID-MS^3 fragmentation of isolated fragment ions from ETD. The simultaneous observation of disulfide-linked and disulfide-cleaved peptide ions in the mass spectra after CID/ETD fragmentation opens the possibility to get information about disulfide bonds as well as amino acid sequences. Moreover, sample preparation in the new approach is easier and faster as compared to conventional approaches for the identification of disulfide bonds, which often require extensive purification and multiple chromatographic steps.[5,6,12−14] A disadvantage of the new method is that it requires information about the amino acid sequence in the neighborhood of the relevant cysteine residues. Furthermore, in future studies sample workup will be modified as the presence of 2 mol/L ammonia during SPE could possibly promote thiol–disulfide interchange reactions. Omitting this step or blocking of free thiols before enzymatic digestion would possibly help in minimizing artifact formation. The identified peptides are the basis for further investigations, in which cystine peptide concentrations in flour, dough, and bread will be determined. In this way, changes of disulfide bonds due to mixing or heat treatment during breadmaking can be monitored. These studies are currently underway.

AUTHOR INFORMATION

Corresponding Author

*Phone: +49 8161 712928. Fax: +49 8161 712970. E-mail: peter.koehler@tum.de.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Osborne, T. B.; Harris, I. F. The chemistry of the proteins of the wheat kernel. II. Preparation of proteins in sufficient amount for hydrolysis. *Z. Anal. Chem.* **1907**, *46*, 749–756.

(2) Wieser, H. Relation between gliadin structure and coeliac toxicity. *Acta. Paediatr.* **1996**, *412*, 3–9.

(3) Wieser, H.; Bushuk, W.; MacRitchie, F. The polymeric glutenins. In *Gliadin und Glutenin. The Unique Balance of Wheat Quality*; Wrigley, C. W., Békés, F., Bushuk, W., Eds.; American Association of Cereal Chemists: St. Paul, MN, 2006; pp 213–240.

(4) Shewry, P. R.; Tatham, A. S. Disulphide bonds in wheat gluten proteins. *J. Cereal Sci.* **1997**, *25*, 207–227.

(5) Müller, S.; Wieser, H. The location of disulphide bonds in α-type gliadins. *J. Cereal Sci.* **1995**, *22*, 21–27.

(6) Müller, S.; Wieser, H. The location of disulphide bonds in monomeric γ-type gliadins. *J. Cereal Sci.* **1997**, *26*, 169–176.

(7) Grosch, W.; Wieser, H. Redox reactions in wheat dough as affected by ascorbic acid. *J. Cereal Sci.* **1999**, *29*, 1–16.

(8) Huebner, F. R.; Bietz, J. A. Improved chromatographic separation and characterization of ethanol-soluble wheat proteins. *Cereal Chem.* **1993**, *70*, 506–511.

(9) van Eckert, R.; Berghofer, E.; Ciclitira, P. J.; Chirdo, F.; Denery-Papini, S.; Ellis, H. J.; Ferranti, P.; Goodwin, P.; Immer, U.; Mamone, G.; Mendez, E.; Mothes, T.; Novalin, S.; Osman, A.; Rumbo, M.; Stern, M.; Thorell, L.; Whim, A.; Wieser, H. Towards a new gliadin reference material – isolation and characterisation. *J. Cereal Sci.* **2006**, *43*, 331–341.

(10) Wieser, H.; Seilmeier, W.; Belitz, H. D. Characterization of ethanol-soluble extractable reduced subunits of glutenin separated by reversed-phase high-performance liquid-chromatography. *J. Cereal Sci.* **1990**, *12*, 63–71.

(11) Lew, E.J.-L.; Kuzmicky, D. D.; Kasarda, D. D. Characterization of low molecular weight glutenin subunits by reversed-phase high-performance liquid-chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and N-terminal amino acid sequencing. *Cereal Chem.* **1992**, *69*, 508–515.

(12) Köhler, P.; Belitz, H.-D.; Wieser, H. Disulphide bonds in wheat gluten: further cystine peptides from high molecular weight (HMW) and low molecular weight (LMW) subunits of glutenin and from γ-gliadin. *Z. Lebensm.-Unters. Forsch.* **1993**, *196*, 239–247.

(13) Keck, B.; Koehler, P.; Wieser, H. Disulfide bonds in wheat gluten. Cystine peptides derived from gluten proteins following peptic and thermolytic digestion. *Z. Lebensm.-Unters. Forsch.* **1995**, *200*, 432−439.
(14) Köhler, P.; Belitz, H.-D.; Wieser, H. Disulphide bonds in wheat gluten: isolation of a cystine peptide from glutenin. *Z. Lebensm.-Unters. Forsch.* **1991**, *192*, 234−239.
(15) Tao, H. B.; Adalsteins, A. E.; Kasarda, D. D. Intermolecular disulfide bonds link specific high-molecular-weight glutenin subunits in wheat endosperm. *Biochim. Biophys. Acta* **1992**, *1159*, 13−21.
(16) Wu, S.-L.; Jiang, H.; Lu, Q.; Dai, S.; Hancock, W. S.; Karger, B. L. Mass spectrometric determination of disulfide linkages in recombinant therapeutic proteins using online LC-MS with electron-transfer dissociation. *Anal. Chem.* **2009**, *81*, 112−122.
(17) International Association for Cereal Science and Technology. Method 110/1. In *ICCs Standards*, 7th suppl. ed.; Moritz Schaefer: Detmold, Germany, 2004.
(18) International Association for Cereal Science and Technology. Method 104/1. In *ICCs Standards*, 7th suppl. ed.; Moritz Schaefer: Detmold, Germany, 2004.
(19) Kasarda, D. D.; Woodard, K. M.; Adalsteins, A. E. Resolution of high-molecular-weight glutenin subunits by a new SDS-PAGE system incorporating a neutral pH buffer. *Cereal Chem.* **1998**, *75*, 70−71.
(20) Neuhoff, V.; Arold, N.; Taube, D.; Ehrhardt, W. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **1988**, *9*, 255−262.
(21) Kieffer, R.; Schurer, F.; Köhler, P.; Wieser, H. Effect of hydrostatic pressure and temperature on the chemical and functional properties of wheat gluten: studies on gluten, gliadin and glutenin. *J. Cereal Sci.* **2007**, *45*, 285−292.
(22) Syka, J. E.; Coon, J. J.; Schroeder, M. J.; Shabanowitz, J.; Hunt, D. F. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9528−9533.
(23) Payne, P. I.; Nightingale, M. A.; Krattinger, A. F.; Holt, L. M. The relationship between HMW glutenin subunit composition and the bread-making quality of British-grown wheat varieties. *J. Sci. Food Agric.* **1987**, *40*, 51−65.
(24) Matsubara, H. Purification and assay of thermolysin. In *Methods in Enzymology*; Perlmann, G. E., Lorand, L., Eds.; Academic Press: London, U.K., 1970; Vol. *XIX*, pp 642−651.
(25) Morihara, K.; Tsuzuki, H.; Oka, T. Comparison of the specificities of various neutral proteinases from microorganisms. *Arch. Biochem. Biophys.* **1968**, *123*, 572−588.
(26) Coon, J. J. Collisions or electrons? Protein sequence analysis in the 21st century. *Anal. Chem.* **2009**, *81*, 3208−3215.
(27) Graveland, A.; Bosveld, P.; Lichtendonk, W. J.; Marseille, J. P.; Moonen, J. H. E.; Scheepstra, A. A model for the molecular structure of the glutenins from wheat flour. *J. Cereal Sci.* **1985**, *3*, 1−16.
(28) Okita, T. W.; Cheesbrough, V.; Reeves, C. D. Evolution and heterogeneity of the α-/β-type and γ-type gliadin DNA sequences. *J. Biol. Chem.* **1985**, *260*, 8203−8213.
(29) Anderson, O. D.; Greene, F. C.; Yip, R. E.; Halford, N. G.; Shewry, P. R.; Malpica-Romero, J. M. Nucleotide sequences of the two high-molecular-weight glutenin genes from the D-genome of a hexaploid bread wheat, *Triticum aestivum* L. cv Cheyenne. *Nucleic Acids Res.* **1989**, *17*, 461−462.
(30) Halford, N. G.; Field, J. M.; Blair, H.; Urwin, P.; Moore, K.; Robert, L.; Thompson, R.; Flavell, R. B.; Tatham, A. S.; Shewry, P. R. Analysis of HMW glutenin subunits encoded by chromosome 1A of bread wheat (*Triticum aestivum* L.) indicates quantitative effects on grain quality. *Theor. Appl. Genet.* **1992**, *83*, 373−378.
(31) Anderson, O. D.; Greene, F. C. The characterization and comparative analysis of high-molecular-weight glutenin genes from genomes A and B of a hexaploid bread wheat. *Theor. Appl. Genet.* **1989**, *77*, 689−700.
(32) Scheets, K.; Hedgcoth, C. Nucleotide sequence of a γ gliadin gene: comparisons with other γ gliadin sequences show the structure of γ gliadin genes and the general primary structure of γ gliadins. *Plant Sci.* **1988**, *57*, 141−150.
(33) Bartels, D.; Altosaar, I.; Harberd, N. P.; Barker, R. F.; Thompson, R. D. Molecular analysis of γ-gliadin gene families at the complex Gli-1 locus of bread wheat (*T. aestivum* L.). *Theor. Appl. Genet.* **1986**, *72*, 845−853.
(34) Pitts, E. G.; Rafalski, J. A.; Hedgcoth, C. Nucleotide sequence and encoded amino acid sequence of a genomic gene region for a low molecular weight glutenin. *Nucleic Acids Res.* **1988**, *16*, 11376.
(35) Database Uni Prot KB/TREMBL (http://pir.georgetown.edu), code B2Y2Q7.
(36) Colot, V.; Bartels, D.; Thompson, R.; Flavell, R. Molecular characterization of an active wheat LMW glutenin gene and its relation to other wheat and barley prolamin genes. *Mol. Gen. Genet.* **1989**, *216*, 81−90.