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Improved identification of wheat gluten proteins through alkylation of cysteine residues and peptide-based mass spectrometry

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Received
2 May 2013

Accepted
9 July 2013

Published
24 July 2013

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The concentration and composition of wheat gluten proteins and the presence, concentration and location of cysteine residues therein are important for wheat flour quality. However, it is difficult to identify gluten proteins, as they are an extremely polymorphic mixture of prolamins. We here present methods for cysteine labeling of wheat prolamins with 4-vinylpyridine (4-VP) and iodoacetamide (IDAM) which, as compared to label-free analysis, substantially improve identification of cysteine-containing peptides in enzymic prolamins digests by electrospray ionization - tandem mass spectrometry. Both chymotrypsin and thermolysin yielded cysteine-containing peptides from different gluten proteins, but more proteins could be identified after chymotryptic digestion. In addition, to the best of our knowledge, we were the first to label prolamins with isotope coded affinity tags (ICAT), which are commonly used for quantitative proteomics. However, more peptides were detected after labeling gluten proteins with 4-VP and IDAM than with ICAT.

Wheat is one of the major food raw materials with a typical worldwide production of $7 \cdot 10^8$ tonnes per year¹. The wide use of wheat flour for food applications is mainly the result of the properties of its particular storage proteins. This complex mixture of similar but distinct proteins is referred to as prolamins. In the wheat kernel, prolamins are present as gliadins and glutenins. Upon mixing wheat flour with water, these proteins develop into gluten, a viscoelastic protein network. Gluten has a great impact on the quality of a whole range of cereal-based foods^{2,3}.

Prolamins are extremely polymorphic due to gene mutation into many allelic forms. Native gliadins in wheat flour are monomers and subdivided into α -, γ -, and ω -gliadins. They contain no (ω) or only (α and γ) intramolecular disulfide bonds. The glutenins occur as polymers of glutenin subunits (GS), which contain free thiol groups, intramolecular, and intermolecular disulfide bonds. The intermolecular disulfide bonds are responsible for the polymeric nature of glutenins. A distinction is made between high molecular weight (HMW) and low molecular weight (LMW) GS⁴. The latter are subdivided into typical (m-, s- or i-type) LMW-GS and gliadin-like LMW-GS, which are in fact α -, γ -, or ω -gliadins with an additional or missing cysteine residue as a result of a point mutation⁵. Common or bread wheat (*Triticum aestivum* L.) is a hexaploid species. A single bread wheat variety contains 6 genes for HMW-GS, 20 or more for LMW-GS, 29 or more for γ -gliadins, up to 150 for α -gliadins, and at least 5 for ω -gliadins. Not all of these genes are expressed^{4,6-12}. Even for wheat flour from a single wheat cultivar, the protein composition and corresponding amino acid sequences are rarely entirely known.

There are still important gaps in the knowledge on post-translational modifications leading to the disulfide structure during the synthesis of prolamins in the growing plant^{4,13}. Whether HMW- and LMW-GS separately form linear backbones and whether only few cross-links between HMW- and LMW-GS exist, remains to be confirmed¹⁴. Also, disulfide bond formation and thiol/disulfide interchange reactions between GS during dough development and bread baking are not well understood¹⁵. Even though all individual gluten proteins and their subunits probably contribute in some way to gluten functionality in food, one group is of particular interest, namely the HMW-GS¹⁶. Strategies to improve gluten and, hence, wheat quality include manipulation of the number and composition of HMW-GS by inserting additional genes encoding mutant and wild-type proteins^{17,18}. In addition, typical m-, s- or i-type LMW-GS contribute to glutenin polymers as they can form two intermolecular



SS bonds with LMW-GS or HMW-GS, while gliadin-like LMW-GS are assumed to have a negative impact on visco-elastic properties as they act as chain terminators during polymerization^{5,19}. Information on the location of cysteine residues in GS is required to understand the contribution of specific prolamins to gluten properties^{5,16}. Additionally, quantification of prolamins and their cysteine-containing peptides is useful for evaluating gluten functionality. Apart from that, there is still a demand for good, reliable and sensitive methods to unambiguously identify gluten in foods, because such proteins trigger an inflammatory reaction in celiac patients²⁰. With respect to prolamins quantification in (so-called) gluten-free products, Tanner *et al.*²¹ outlined the problems associated with solely relying on the enzyme-linked immuno sorbent assay (ELISA) and highlighted the need for development of new sensitive and selective quantitative assays such as liquid chromatography-mass spectrometry (LC-MS).

Electrospray ionization (ESI) MS and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS have contributed to assessing prolamins heterogeneity and gluten structure determination²². As such, Lutz *et al.*²³ introduced ESI-MS/MS with alternating electron transfer dissociation (ETD) and collision-induced dissociation (CID) to detect disulfide-linked prolamins peptide ions. However, this technique requires prior knowledge of the location of cysteine residues. Elucidation of the gluten network structure and evaluation of gluten functionality still require means to facilitate identification, but also quantification of prolamins and localization of their cysteine-residues. Camafeita *et al.*^{24,25} presented a system allowing quantification of small quantities of wheat gliadins using MALDI-TOF-MS. However, they acknowledged the need for a procedure to accurately quantify total gluten.

Despite the proteomic research already performed on cereal storage proteins, analysis of wheat prolamins still faces several challenges. Because of their high molecular weights and high levels of glutamine, proline and hydrophobic amino acids, gluten proteins are not extractable in the salt buffers typically used for protein characterization³. Furthermore, trypsin, the enzyme of choice in most proteomic studies because of its reliability and specificity, is not suited in the case of cereal storage proteins²⁰. Sequences of gluten proteins have few sites for tryptic hydrolysis, as they are low in arginine and lysine^{2,26}. In addition, the repetitive character of gluten protein sequences results in a substantial sequence redundancy. Finally, the low cysteine levels in gluten proteins [ca. 2.2 mol%²⁷] further complicate identification of cysteine-labeled gluten peptides, despite their importance for flour quality.

Cysteine alkylation is a successful strategy to improve the identification²⁸ and even quantification^{29,30} of cysteine residues via cysteine-containing peptides³¹. A variety of cysteine derivatizing agents is available, most often based on the reaction with iodoacetyl groups (with iodine as a good leaving group) or with double bonds (for Michael-type addition reactions)²⁹. The popular derivatizing agents iodoacetamide (IDAM) and 4-vinylpyridine (4-VP) transform cysteine residues in S-carbamidomethyl cysteine and S-β-(4-pyridylethyl) cysteine, respectively²⁸ (Figure 1). Cysteine residues can also be labeled with isotope-coded affinity tags (ICAT) and subsequently quantified (Figure 1). ICAT labeling was originally designed for quantitative analysis of complex protein mixtures³², but is now also used to determine the redox state of cysteine residues in proteins^{33–35}.

Even though literature reports on various cysteine labeling techniques for standard proteins²⁸, the lack of extractability of gluten proteins in the buffers typically used for derivatization reactions (see above) complicates cysteine labeling. For instance, when Islam *et al.*³⁶ studied the expression of wheat seed proteins in relation to chromosome deletion under the standard conditions of ICAT labeling, they identified only non-gluten proteins, almost all of them with molecular weights below 30,000. In a comparable effort to quantify highly hydrophobic membrane proteins, Ramus *et al.*³⁷ noted the

need for specialized buffers for ICAT labeling. In the specific case of gluten proteins, both the sample extraction procedure and labeling conditions, be it with IDAM, 4-VP or ICAT, remain to be optimized.

Overall, localization of cysteine residues and detection of cysteine-containing gluten peptides with MS/MS are laborious and difficult. Given the importance of gluten protein identification and the localization of cysteine residues for understanding end product quality of wheat-based food products, this paper explores the potential of cysteine labeling (4-VP, IDAM, ICAT) for identifying prolamins and quantifying cysteine-containing gluten peptides.

Results

Optimal conditions for cysteine labeling of wheat prolamins.

Gluten proteins have limited extractability in most buffers and solvents due to their extremely high level of glutamine, which forms strong hydrogen bonds, the high level of hydrophobic amino acids, and low levels of amino acids with ionizable side chains. In order to obtain complete cysteine labeling, it was of the utmost importance to maximize gluten protein extraction. Therefore, extraction conditions were optimized. An efficient extraction system for wheat storage proteins is 50% (v/v) 1-propanol containing a reducing agent with extraction at 60°C³⁸. Alkylation of cysteine with IDAM requires high pH, since cysteine reacts as thiolate anion³⁹. Complete extractability of wheat flour proteins was achieved by shaking for 30 min at 60°C in a Tris/HCl buffer [0.5 mol/L buffer (pH 8.5), diluted to 50% (v/v) with 1-propanol] containing tris-(2-carboxyethyl)phosphine (TCEP). TCEP is a reducing agent suitable for almost complete extraction of prolamins⁴⁰. The buffer was apt for labeling with either IDAM or 4-VP. Because IDAM and 4-VP are unstable and light-sensitive, solutions were prepared immediately before use and alkylation was performed in the dark. At very alkaline pH, IDAM also reacts with amino groups of lysine and histidine⁴¹. Here, over-alkylation was substantially reduced if not prevented by strictly controlling alkylation time (30 min), temperature (37°C) and pH (8.5). Amino acid analysis of alkylated wheat flour proteins confirmed that, under these conditions, all cysteine residues had reacted with IDAM with no side reactions such as N-alkylation (results not shown).

Impact of cysteine labeling on detection of cysteine-containing peptides.

Wheat prolamins were extracted under the conditions described above. Cysteine residues were then (i) reduced, (ii) reduced and labeled with IDAM or (iii) reduced and labeled with 4-VP. The protein mixtures were hydrolyzed with chymotrypsin (37°C, 24 h, pH 7.8, enzyme/substrate: 1/50) and separated on a C₁₈ reversed phase (RP) column with 0.1% (v/v) formic acid as the mobile phase. Based on comparison of the MS/MS data with theoretical peptide sequences of the wheat flour storage proteome, (i) three, (ii) twenty-four, or (iii) sixteen cysteine-containing peptides were detected, respectively (Table 1). Figure 2 shows an example of MS/MS spectra which allowed identifying the peptide YPSVTCPCQ QVSY. This peptide was identified with and without prior alkylation. The MS/MS spectra of the alkylated peptides resulted in a Mascot score exceeding 40, which indicated identity or extensive similarity (p < 0.05). The MS/MS spectrum of the reduced peptide had a Mascot score higher than 15 and was manually confirmed based on independent fragment peaks matching the theoretical peptide fragments. In the spectra of the two alkylated peptides and the non-alkylated one, especially b-fragments with a high m/z value led to successful identification. The detection of YPSVTCPCQQVSY also allowed investigating the impact of alkylation on retention time on a C₁₈ RP column. The retention time increased in the following order: 4-VP < IDAM < free thiol, which is in agreement with Jiang *et al.*⁴². Under acidic conditions, 4-VP and IDAM labeled peptides are more hydrophilic than their non-alkylated equivalents, and in the presence of formic acid 4-VP labeled peptides elute earlier than their

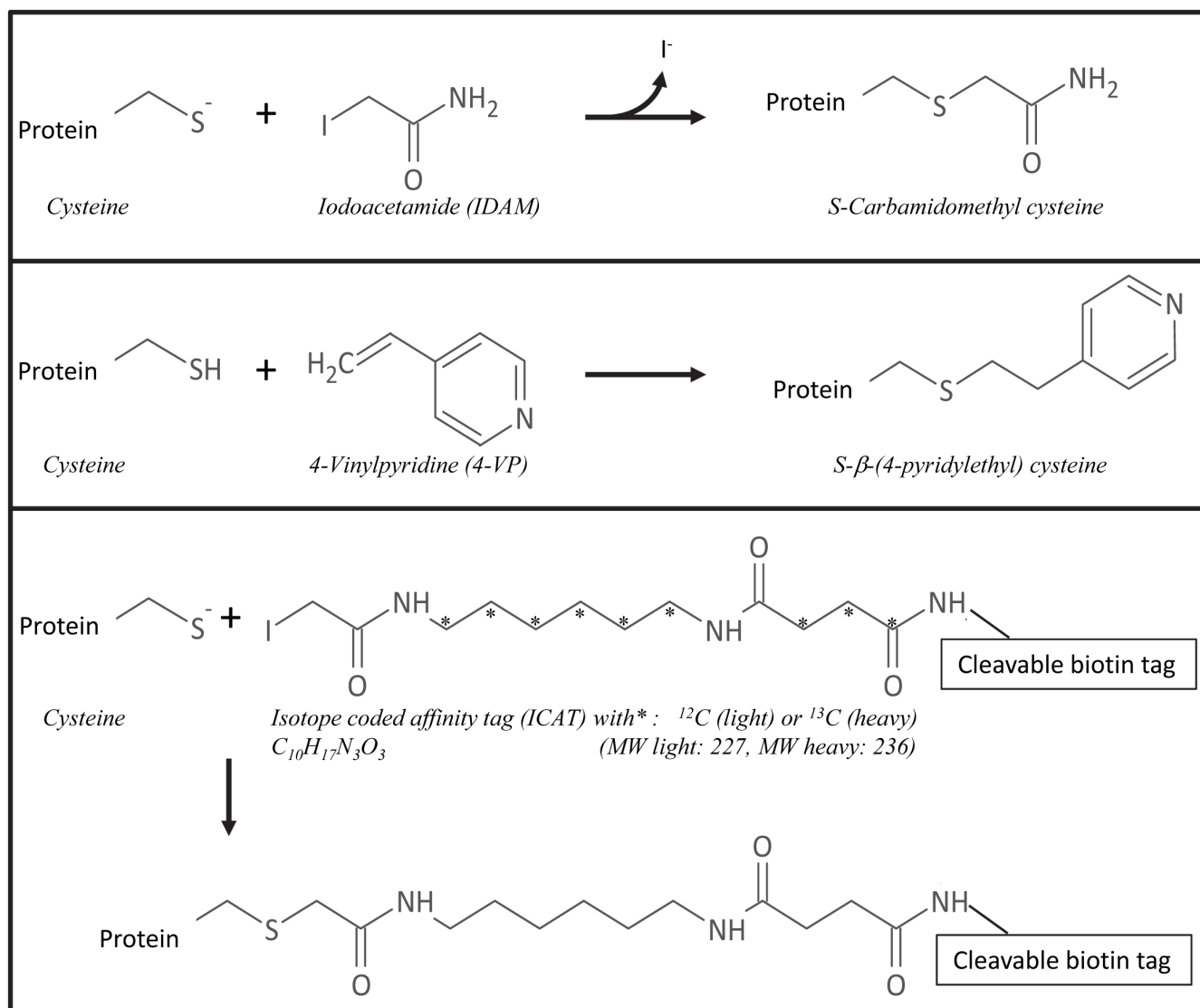


Figure 1 | Schematic overview of cysteine labeling reactions.

IDAM labeled equivalents⁴². It should be emphasized that YPSVTCPPQVSY was one of the few cysteine-containing peptides detected after reduction and no labeling. Considerably more cysteine-containing peptides were found after labeling with either IDAM or 4-VP, which can be ascribed to the prevention of thiol group oxidation when converting this functional group into stable thioethers.

Average MS/MS sequence coverages of wheat flour proteins were not significantly different after reduction (71.2%), or reduction followed by labeling with either IDAM (69.2%) or 4-VP (71.3%). Apparently, labeling of cysteine residues of wheat flour proteins specifically facilitates the detection of the labeled peptides, but has little or no impact on the overall detection of gluten peptides. With all wheat prolamins having repetitive sequences, their identification mainly depends on peptides with unique sequences in the N or C-terminal domains. Since no N-terminal wheat prolamins were detected except for those from some HMW-GS, it was investigated whether wheat prolamins could rely on detecting specific cysteine-containing peptides. For every detected cysteine-containing peptide, its occurrence in various protein types was evaluated (Table 2). The following protein types were considered: various HMW-GS [referred to by their Payne score⁴³], m-, s- and i-type LMW-GS (typical LMW-GS), α - and γ -like LMW-GS

(gliadin-like LMW-GS), and α - and γ -gliadins. For instance, the peptide YPSVTCPPQVSY is unique for HMW-GS 1Dx5, while QCEREL is found in all HMW-GS except in some HMW-GS from the B genome (1Bx7 and 1Bx17)⁴⁴. In contrast, the peptide CCQQL occurs in both in wheat prolamins and wheat albumins. One identified peptide (SQVCF) contained the additional cysteine residue specific for an α -like LMW-GS. The other detected LMW-GS peptides were only present in typical m-, s- or i-type LMW-GS (e.g. LQQQCIPVAM), or both in typical and gliadin-like LMW-GS (e.g. CSVNVPL). The detected α - and γ -gliadin peptides all contained cysteine residues involved in intramolecular cross-links in native wheat gluten. Some of them are specific for either α - or γ -gliadin (e.g. CTIAPVGF), but most of them also occur in gliadin-like LMW-GS (e.g. QQCNPVSL). Overall, peptides specific for each prolamins type, except for D-LMW-GS, were detected. D-LMW-GS or ω -bound gliadins are ω -gliadins with only one cysteine residue and they account for less than 1% of the total protein content in wheat flour⁴⁵. This makes the detection of cysteine containing D-LMW-GS peptides difficult. The chymotryptic peptides from GS are a good basis for an MS study of changes in the SS structure during glutenin synthesis in the developing wheat plant. In the context of gluten functionality, it is helpful that various prolamins were detected, especially because gliadins, LMW-GS and HMW-GS have a different



Amino acid sequence	Reduction				Alkylation with iodacetamide				Alkylation with 4-vinylpyridine			
	M _r	M/z	Score	RT (min)	M _r	M/z	Score	RT (min)	M _r	M/z	Score	RT (min)
	ALETIPAMCNVY	1380.6	691.3	24	55.1	1428.7	715.4	19	50.1			
CCQGL	707.3	708.2	44	18.4								
CCQGL	820.4	821.3	68	33.9								
CCQGLW	893.4	894.3	63	41.3								
CNVY					602.3	603.3	17	16.9				
CSTIAPF	895.4	896.4	19	45.1								
CSVNVPL					835.4	836.3	30	36.0				
CTIAPF	707.3	708.3	34	44.6	755.4	756.3	35	37.9				
CTIAPVGF	863.4	864.4	59	48.6	911.5	456.8	78	42.5				
CTIAPVGF	976.5	977.5	47	58.9	1024.5	513.3	34	51.6				
ELEACGGWQQQL					1606.8	804.3	15	23.1				
GGCSF	597.2	598.2	25	20.9								
GGCVSQPQQQL	1271.6	636.8	40	28.4	1319.6	660.9	20	25.6				
GGCVSQPQQSQQQQL	1742.8	872.4	53	27.4								
IPCMDVLI					993.5	497.9	20	51.3				
IPEGSRCAIH	1280.6	641.4	17	22.2								
IGECCSRL					993.4	497.7	68	25.9				
ILGGCNPVSL												
LQQCNPVSL					1057.5	529.7	44	39.1	1218.6	610.4	22	41.7
LQQGQIPVAM									1105.6	553.9	42	35.2
NIPCKNFL					891.4	446.8	24	17.6	1234.6	618.3	16	40.6
GCEREL					833.4	417.7	39	15.9				
GCERELQEL					1203.6	602.7	136	32.4				
GGCNPVSL					944.4	945.4	46	34.7				
GQINPCKL												
GGQIIPCMDVLI					1442.7	722.4	30	66.7	1490.8	746.4	16	60.4
GGQINPCKNIL					1354.7	678.4	28	29.2				
SQGGPCSQGGQQPL					1683.8	842.9	534	25.4	1731.8	578.4	71	23.4
SQVCF					639.3	640.2	23	32.1				
VPDPCSTINVPY					1360.6	681.3	22	46.5				
VPPECSIM									979.5	490.8	25	34.5
WQSSCH					803.3	804.3	17	43.2				
YFSVTCQGVSY	1370.6	686.3	20	45.3	1427.6	714.9	47	41.9	1475.7	738.8	42	38.8

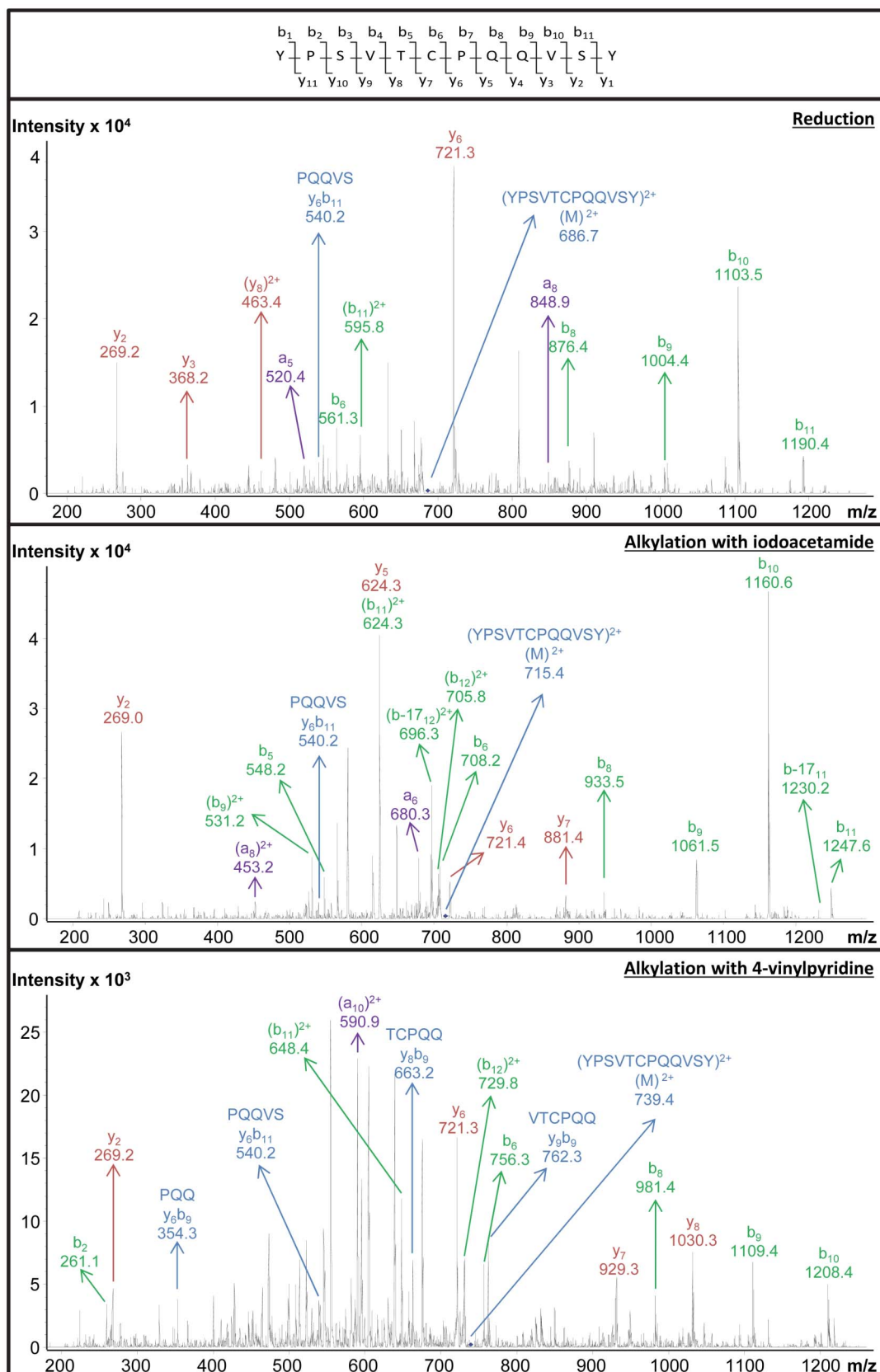


Figure 2 | CID MS/MS spectra of the cysteine-containing peptide YPSVTCPQQVSY. The peptide was found in a chymotryptic digest of a wheat (*cv.* Akteur) flour extract. Top: unlabeled (reduced). Middle: IDAM-labeled. Bottom: 4-VP-labeled.


Table 2 | Occurrence of cysteine-containing peptides from the wheat storage proteome detected by LC-MS/MS after chymotryptic digestion of wheat flour (cv. Akteur) using MS/MS

Amino acid sequence	Present in	Detected after
ALETLPAMCNVY	α -like LMW-GS, α -gliadin	Labeling with IDAM or 4-VP
CCQQL ^{1*}	By9, Dy10, m-, s-, and i-type LMW-GS, α - and γ -like LMW-GS, α - and γ -gliadins	Labeling with IDAM
CCQQLL	s-type LMW-GS, α -gliadin	Labeling with IDAM
CCQQLW	α -like LMW-GS, α -gliadin	Labeling with IDAM
CNVY*	α - and γ -like LMW-GS, α - and γ -gliadin	Labeling with 4-VP
CSTTIAPF	α -like LMW-GS, α -gliadin	Labeling with IDAM
CSVNVPL	m-, s-, and i-type LMW-GS, α - and γ -like LMW-GS	Labeling with 4-VP
CTIAPF	α -like LMW-GS, α -gliadin	Labeling with IDAM or 4-VP
CTIAPVGF	α -gliadin	Labeling with IDAM or 4-VP
CTIAPVGIF	α -like LMW-GS, α -gliadin	Labeling with IDAM or 4-VP
ELEACQQWVDQQL ²	Bx7	Labeling with 4-VP
GQCSF	m-, s- and i-type LMW-GS	Labeling with IDAM
GQCVSQPQQQL	m-, s- and i-type LMW-GS	Labeling with IDAM or 4-VP
GQCVSQPQQSQQQQL	m- and s-type LMW-GS, γ -like LMW-GS	Labeling with IDAM
IPCMDVVL	α -like LMW-GS, α -gliadin	Labeling with 4-VP
IPEQSRCQAIH	α -like LMW-GS, α -gliadin	Reduction
LGECCSRL	Puroindoline a	Labeling with IDAM
LLQQCNPVSL	γ -like LMW-GS, γ -gliadin	Labeling with 4-VP
LQQCNPVSL	γ -like LMW-GS, γ -gliadin	Labeling with IDAM or 4-VP
LQQCIPVAM	i-type LMW-GS	Labeling with 4-VP
NPCKNFL	γ -like LMW-GS, γ -gliadin	Labeling with IDAM
QCEREL ³	Ax1, Dx5, By9, Dy10	Labeling with IDAM
QCERELQEL ⁴	Dx5	Labeling with IDAM
QQCNPVSL	γ -like LMW-GS, γ -gliadin	Labeling with IDAM
QQLNPCKL	m-type LMW-GS	Reduction
QQQLIPCMDVVL	α -like LMW-GS, α -gliadin	Labeling with IDAM or 4-VP
QQQLNPCKNIL	γ -like LMW-GS, γ -gliadin	Labeling with IDAM
SQQQPCSQQQQQL	s-type LMW-GS	Labeling with IDAM or 4-VP
SQVCF	α -like LMW-GS	Labeling with IDAM
VPPDCSTINVPY	γ -like LMW-GS, γ -gliadin	Labeling with IDAM
VPPECSIM	γ -like LMW-GS, γ -gliadin	Labeling with 4-VP
WQSSCH	m-, s- and i-type LMW-GS	Labeling with IDAM
YPSVTCPQQVSY	Dx5	Reduction or labeling with IDAM or 4-VP

¹Also present in other γ -type HMW-GS.

²Also present in other Bx-type HMW-GS.

³Also present in other HMW-GS.

⁴Also present in other Dx-type HMW-GS.

*Also present in non-wheat prolamins that are toxic to celiac patients.

impact on the gluten network^{5,19}. Also in the context of celiac disease it is essential that methodologies are available for detecting all types of wheat prolamins. Focusing solely on labeled cysteine-containing peptides offers the possibility to rapidly assess gluten composition in general and that of individual proteins responsible for gluten intolerance in particular.

Chymotryptic versus thermolytic digestion. Besides chymotrypsin also thermolysin is used in bottom-up proteomics of gluten²⁰. Trypsin, the enzyme commonly used to hydrolyze proteins prior to MS analysis, was not used in this study because theoretical *in-silico* digests of gluten proteins showed that the amount of detectable cysteine-containing peptides (m/z range 200–2000) after tryptic digestion was only 15 to 30% of that after chymotryptic or thermolytic digestion due to the low abundance of lysine and arginine residues. After reduction and cysteine alkylation with IDAM, gluten proteins were digested with chymotrypsin or thermolysin, and the detection of chymotryptic and thermolytic gluten peptides was compared. More cysteine-containing gluten peptides were found after chymotryptic (24) than after thermolytic digestion (15) (Table 3). This difference was noted for all prolamins types, except for γ -gliadins and γ -like LMW-GS. Different cysteine-residues were found in both cases. Hence, to detect as many cysteine-containing peptides as possible by LC-MS, it proved advisable to perform both chymotryptic as well as thermolytic digestion. This is in line with Vensel et al.²⁶ who demonstrated that the use of multiple

enzymes increased the number of wheat proteins identified. The highest MS/MS full protein sequence coverage was obtained after thermolytic digestion, except in the case of HMW-GS (Figure 3). To conclude, for a single analysis of the entire wheat storage proteome, chymotryptic digestion is expected to provide most information.

ICAT labeling of cysteine residues from gluten proteins. Another task in proteomics is protein quantification^{46,47}. This is especially challenging in the case of gluten proteins, given their limited solubility. In this study, ICAT was used to label wheat prolamins (Figure 1). The manufacturer's protocol (Cleavable ICAT Reagent Kit for Protein Labeling) recommends dissolving the proteins in Tris/HCl buffer (0.05 mol/L, pH 8.5) containing 0.1% SDS, and subsequently labeling them in the same buffer with cleavable ICAT reagent in acetonitrile. This procedure allowed identifying cysteine-containing peptides of water-soluble proteins, such as bovine serum albumin, but it failed to do so in the case of wheat flour proteins (results not shown). Even when isolated prolamins fractions were subjected to the manufacturer's protocol, no cysteine-containing peptides were detected, probably because prolamins were not soluble under these conditions. Since derivatization reactions with IDAM and ICAT are both based on reaction of a thiol residue with an IDAM group, ICAT labeling for wheat flour proteins was optimized based on the procedure for IDAM labeling as described above. Wheat flour proteins were first reduced by shaking for 30 min at 60°C in



Table 3 | S-carbamidomethyl cysteine-containing gluten peptides, detected by LC-MS/MS after thermolytic digestion of a reduced/alkylated protein extract of wheat flour (*cv.* Akteur)

Amino acid sequence	M_r	M/z	Score	RT (min)	Present in
LCCQQ	707.3	708.3	36	11.8	α -like LMW-GS, α -gliadin
LGQC	476.2	477.2	20	10.8	m-, s- and i-type LMW-GS
LGQCS	563.2	564.2	16	11.0	m-, s- and i-type LMW-GS, γ -like LMW-GS
LKACQQ	746.4	747.3	16	22.4	Dx-type HMW-GS
LNPCKN*	744.4	373.3	18	11.9	γ -like LMW-GS, γ -gliadin
LQQCKP	772.4	773.4	16	10.8	γ -like LMW-GS, γ -gliadin
LQQCNP	758.3	759.3	28	15.5	γ -like LMW-GS, γ -gliadin
LQQQCIP	885.4	886.5	32	30.4	i-type LMW-GS
LQQQCSP	859.4	860.4	40	16.4	m-, s- and i-type LMW-GS
VRPDCST	833.4	834.4	18	11.4	α -like LMW-GS, α -gliadin
VSAKCRS	806.4	404.3	17	5.7	γ -like LMW-GS, γ -gliadin
VSPDCST	764.3	765.3	23	14.0	γ -like LMW-GS, γ -gliadin
VYIPPYCT	1011.5	1012.5	23	42.0	α -like LMW-GS, α -gliadin
VYVPPDCST	1036.5	1037.5	32	28.2	γ -like LMW-GS, γ -gliadin
VYVPECS	949.4	950.4	44	29.2	γ -like LMW-GS, γ -gliadin

*Also present in non-wheat prolamins that are toxic to celiac patients.

0.500 mol/L Tris/HCl buffer (pH 8.5) diluted to 50% (v/v) with 1-propanol and containing 0.001 mol/L reducing agent (TCEP). The buffer contained no sodium dodecyl sulfate, which would compromise the labeling reaction⁴⁸. The protein solution was subsequently labeled with either light or heavy cleavable ICAT reagent (Figure 1) in this buffer for 2 h at 37°C. This procedure is further referred to as the new protocol. When the new protocol was applied to bovine serum albumin, chymotryptic digestion produced a number of detectable cysteine-containing peptides (Table 4). The Tris/HCl buffer was thus suitable for ICAT labeling. Even though labeling under these conditions yielded many S-carbamidomethylated prolamins peptides after chymotryptic digestion (Table 1), no ICAT-labeled gluten peptides were detected by LC-MS. However, no unlabeled cysteine-containing gluten peptides were found either. Hence, it is reasonable to assume that cysteine residues were successfully labeled with ICAT in the Tris/HCl buffer, but that the technical problems had their origin in the subsequent enzymic hydrolysis or affinity chromatography. We here reasonably assumed that ICAT labeling did not affect the rate and specificity of chymotrypsin, since the enzyme acts at peptide bonds of amino

acid residues other than cysteine⁴⁹. While isolation of labeled peptides by biotin-avidin affinity chromatography significantly reduces the complexity of the peptide mixture, it sometimes suffers from non-specific binding of cysteine-free peptides and/or irreversible adsorption of some cysteine-peptides²⁹. More cysteine-containing peptides were derived from bovine serum albumin after IDAM than after ICAT labeling. Considering the lower cysteine level in prolamins [ca. 2.2 mol%²⁷] than in bovine serum albumin [6.0 mol%⁵⁰], it cannot be excluded that inefficient affinity chromatography resulted in the lack of detection of cysteine-containing peptides in the case of gluten. Therefore, samples were analyzed again, this time focusing on the peptides that had been detected after S-carbamidomethylation. Only compounds with m/z values corresponding to the doubly or triply charged ion of potential ICAT labeled peptides were fragmented. One peptide (QCERELQEL) was now detected. Figure 4 shows the corresponding MS/MS spectra. Quantification of cysteine-containing peptides by ICAT labeling of wheat storage proteins remains difficult. Alternative labeling agents include ²D-, ¹³C- and ¹⁸O-coded reagents based on 2- or 4-VP⁵¹, IDAM⁵², or iodoacetic acid⁵³. Some

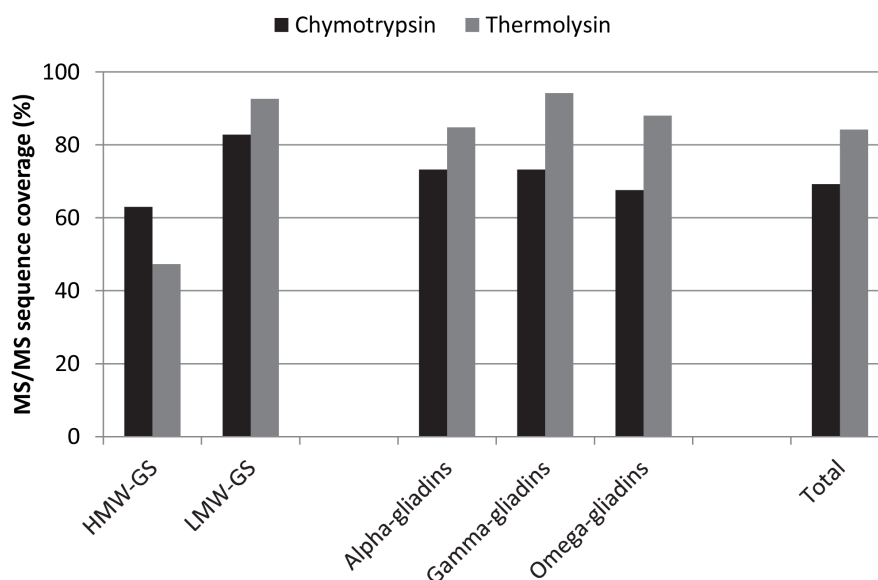


Figure 3 | Average MS/MS sequence coverages (%) of wheat gluten proteins. Wheat (*cv.* Akteur) gluten proteins were reduced, labeled with IDAM, and subsequently digested with either chymotrypsin or thermolysin.



Table 4 | ICAT-labeled cysteine-containing peptides from bovine serum albumin, detected after a single LC-MS/MS run. Cysteine residues were labeled according to the new protocol and all peptides were manually confirmed as described in the materials and methods section

Amino acid sequence	Mr	z	M/z	Score	RT (min)
AEDKDVCKNY	Light: 1410.6	2+	706.3	97	20.8
	Heavy: 1419.6		710.8	37	20.8
		3+	471.3	69	20.9
CDEF	Light: 739.3	1+	740.2	20	29.2
	Heavy: 748.3		749.3	38	29.2
				474.3	15
ECADDRADL	Light: 1233.5	2+	617.7	16	26.2
	Heavy: 1242.5		622.3	28	26.3
		3+	412.3	15	26.2
			415.3	15	26.1

of these reagents are commercially available, e.g. 4-VP-d₃, 2-VP-d₇, IDAM-d₄, iodoacetic acid-¹³C₂. Others can be readily prepared^{52,53}.

Discussion

Results in this paper show that labeling of cysteine-containing wheat prolamin with 4-VP or IDAM facilitates the detection of the peptides using LC-MS/MS with CID. The proposed methods to identify cysteine-peptides in an unknown flour mixture form a basis for further elucidating wheat prolamin structures as a result of redox-based post-translational modifications during plant development, but also during food processing which often includes heating.

Cysteine labeling for quantitative proteomics of wheat storage proteins can further be helpful to evaluate gluten functionality and to develop alternative methods to detect gluten contamination in so-called gluten-free products. For example, the current calculation of gluten content by considering it to be twice the gliadin content is invalid when assessing levels of contamination by gluten in so-called gluten-free products⁵⁴.

Labeling wheat prolamins with ICAT reagent was successful but complicated. ²H-, ¹³C- and ¹⁸O-coded reagents are probably better alternatives for quantification purposes. Further work exploring the opportunities of cysteine labeling to accurately determine gluten levels would be extremely valuable. The combination of isotopic labeling and MS is a promising tool for detection and quantification of specific cysteine-containing prolamin peptides as well as entire prolamins.

Methods

Materials. Wheat kernels of the cv. Akteur (harvest 2009) were milled into patent flour with a Bühler MLU 202 Mill (Uzwil, Switzerland) and the flour sifted through a 0.2 mm screen. The Cleavable ICAT Reagent Kit for Protein Labeling was purchased from AB Sciex (Darmstadt, Germany). All solvents, chemicals and reagents were at least of analytical grade. Water was deionized by a Millipore-O Milli-Q purification system.

Cysteine labeling. Cysteine residues of the Akteur wheat flour proteins were reduced and subsequently alkylated with IDAM, 4-VP, or ICAT. Reduction was performed by shaking 8 mg wheat flour (equivalent to 1 mg protein containing about 0.2 μmol cysteine) for 30 min at 60°C under nitrogen atmosphere in 320 μL Tris/HCl buffer (0.50 mol/L, pH 8.5), diluted to 50% (v/v) with 1-propanol and 8 μL reducing reagent (0.050 mol/L TCEP). The mixtures were centrifuged (10 min, 10 · 10³ g) and the residues discarded. Alkylation with IDAM was performed by adding 12 μL 0.50 mol/L IDAM solution to the supernatant (1 mg protein) and shaking for 30 min at 37°C in the dark. For 4-VP alkylation, 0.5 μL pure 4-VP was added and mixtures (1 mg protein) were shaken for 30 min at 60°C. ICAT labeling was performed by 120 min reaction at 37°C of ICAT reagents (1 unit which labels about 50 μmol cysteine under the present conditions) with protein (1 mg) dissolved in the denaturing buffer containing reducing reagent. Because a modified denaturing buffer was used, labeling conditions were different from those described in the manufacturer's protocol (Cleavable ICAT Reagent Kit for Protein Labeling). All labeling steps were performed under nitrogen atmosphere.

Amino acid analysis. Gluten amino acids were liberated by acid hydrolysis and separated by high-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAEC-IPAD) as described by Rombouts *et al.*²⁷. Freeze dried samples (5.0 mg protein, db) were heated (24 h, 110°C) in 1.0 mL 6.0 mol/L HCl containing 0.1% phenol and 3.0 mmol/L norleucine (as internal standard). Reaction mixtures were subsequently diluted (200-fold) in deionized water and filtered (Millex-GP, 0.22 μm, polyethersulfone; Millipore). Amino acids (injection volume 25 μL, flow rate 0.25 mL/min, 30°C) were separated on an AminoPac PA10 column (250 × 2 mm; Dionex Benelux, Amsterdam, The Netherlands) using a Dionex BioLC system (Dionex, Sunnyvale, CA, USA) equipped with Chromleon Version 6.70 software (Dionex Benelux). Gradient conditions and detection waveform were as previously described²⁷. Amino acid levels were expressed on dry matter protein (μmol/g protein) and calculated from the relative peak areas of the amino acid to norleucine. Relative standard deviations did not exceed 10%. Relative standard deviations did not exceed 10%.

Protein digestion. Prior to digestion, protein solutions were evaporated to dryness. For chymotrypsin digestion, 1.000 mL chymotrypsin solution [0.020 mg α-chymotrypsin/mL and 0.040 mol/L urea in 0.100 mol/L Tris/HCl (pH 7.8)] was added and the mixture was shaken for 24 h at 37°C. For thermolytic digestion, 1.000 mL thermolysin solution [0.100 mg thermolysin/mL and 0.020 mol/L CaCl₂ · 6H₂O in 0.200 mol/L Tris/HCl (pH 6.5)] was added and the mixture was shaken for 16 h at 37°C. Digestions were stopped by lowering the pH to 1.4 by adding 3.0 μL trifluoroacetic acid (TFA).

Purification of peptides. After reduction (followed by labeling with IDAM or 4-VP) and subsequent digestion, peptides were purified by solid phase extraction on Strata-X-C devices (Phenomenex, Aschaffenburg, Germany). Solid phase extraction cartridges were conditioned with methanol (1.0 mL) and equilibrated with 1.0 mL 0.1% TFA. The digests (1.0 mL) were loaded and the cartridges washed with 50% (v/v) methanol (5 × 1.0 mL). Peptides were eluted with 1.0 mL 2.0 mol/L ammonia, which was then evaporated. Samples with reduced, S-carbamidomethylated or S-pyridylethylated cysteine residues were dissolved in 1.0 mL 0.1% (v/v) formic acid. Samples with ICAT labeled cysteine residues were further purified by affinity chromatography. Avidin cartridges were activated with 2.0 mL elute buffer (30% (v/v) acetonitrile containing 0.4% (v/v) TFA) and 2.0 mL load buffer (pH 7.2, 0.020 mol/L sodium phosphate, 0.300 mol/L sodium chloride). The peptides, dissolved in 1.0 mL load buffer, were loaded and the cartridges washed with 0.5 mL load buffer, 1.0 mL 0.010 mol/L sodium phosphate buffer (pH 7.2) containing 0.150 mol/L sodium chloride, 1.0 mL 0.050 mol/L ammonium bicarbonate (pH 8.3) containing 20% (v/v) methanol and 1.0 mL milliQ water. Biotinylated peptides were eluted with 0.80 mL elution buffer, which was then evaporated to dryness. ICAT labeled peptides were incubated for 120 min at 37°C with 90 μL of TFA to cleave the biotin moiety. Samples were evaporated to dryness and dissolved in 50 μL 0.1% formic acid.

LC-MS/MS. LC-MS/MS experiments were performed on an HCT-Ultra PTM ion trap MS (Bruker Daltonics, Bremen, Germany) with CID, coupled with an UltiMate 3000 HPLC (Dionex, Idstein, Germany) system equipped with an Aeris PEPTIDE 3.6 μm XB-C₁₈ column (2.10 × 150 mm; Phenomenex). The MS contained a spherical ion trap with an ESI interface running in the positive mode (capillary voltage: −4000 V; capillary exit voltage: 158.5 V; skimmer voltage: 40 V). Nitrogen was used as drying (8.0 L/min, 325°C) and nebulizing gas (0.2 MPa). The mobile phases for LC separation were (A) 0.1% (v/v) formic acid and (B) 0.1% (v/v) formic acid in acetonitrile. The following gradient was used: (i) linear from 0 to 32% B over 80 min; (ii) linear from 32 to 90% B over 5 min; (iii) isocratic at 90% B for 9 min; (iv) linear from 90 to 0% B in 1 min; (v) isocratic at 0% B for 10 min. The flow rate was 0.2 mL/min, injection volume was 5 μL, and column temperature was 30°C. The scan range was *m/z* 200 to 2000 (smart target value: 200,000; maximum acquisition time: 100 ms), and CID-MS² scan steps were performed on precursor ions using the AutoMS/MS mode (fragmentation amplitude: 1.0 V; collision gas: helium).

Analysis of MS results. MS/MS data in a Mascot Generic File (*.mgf) using the Bruker Daltonics Data Analysis 3.4 software were analyzed using the MS/MS ions search module of Mascot software (Matrix Science, London, UK) based on the SwissProt database 2012 (taxonomic category: *Viridiplantae*; peptide mass tolerance: ± 0.1%; fragment mass tolerance: ± 0.5 Da; peptide charges: 1⁺, 2⁺ and 3⁺; monoisotopic ions). MS/MS data (*.mgf) were also compared with a database containing gluten sequences from literature²⁶ and the NCBI database (National Library of Medicine, Bethesda, MD, USA) using the SequenceEditor module of the Bruker Biotools software 3.2 (*m/z* range: 200–3,000; peptide and MS/MS tolerance: ± 0.5; peptide charges: 1⁺, 2⁺ and 3⁺; monoisotopic ions). Potential chymotrypsin cleavage sites were entered as follows: at the C-terminal end of Phe, His, Leu, Met, Trp and Tyr, but not before Pro. Possible thermolysin cleavage sites were entered as follows: at the N-terminal end of Val, Ile, Ala, Leu, Met and Phe, if not preceding Pro. Two missed cleavages by the enzymes were allowed. S-pyridylethyl and S-carbamidomethyl were set as fixed modifications on cysteine in the corresponding samples, and oxidation at methionine was used as variable modifications in all samples. Charge state deconvolution and deisotoping were not performed. The presence of detected peptides in wheat prolamins was evaluated by performing a blast search on the Uniprot database.

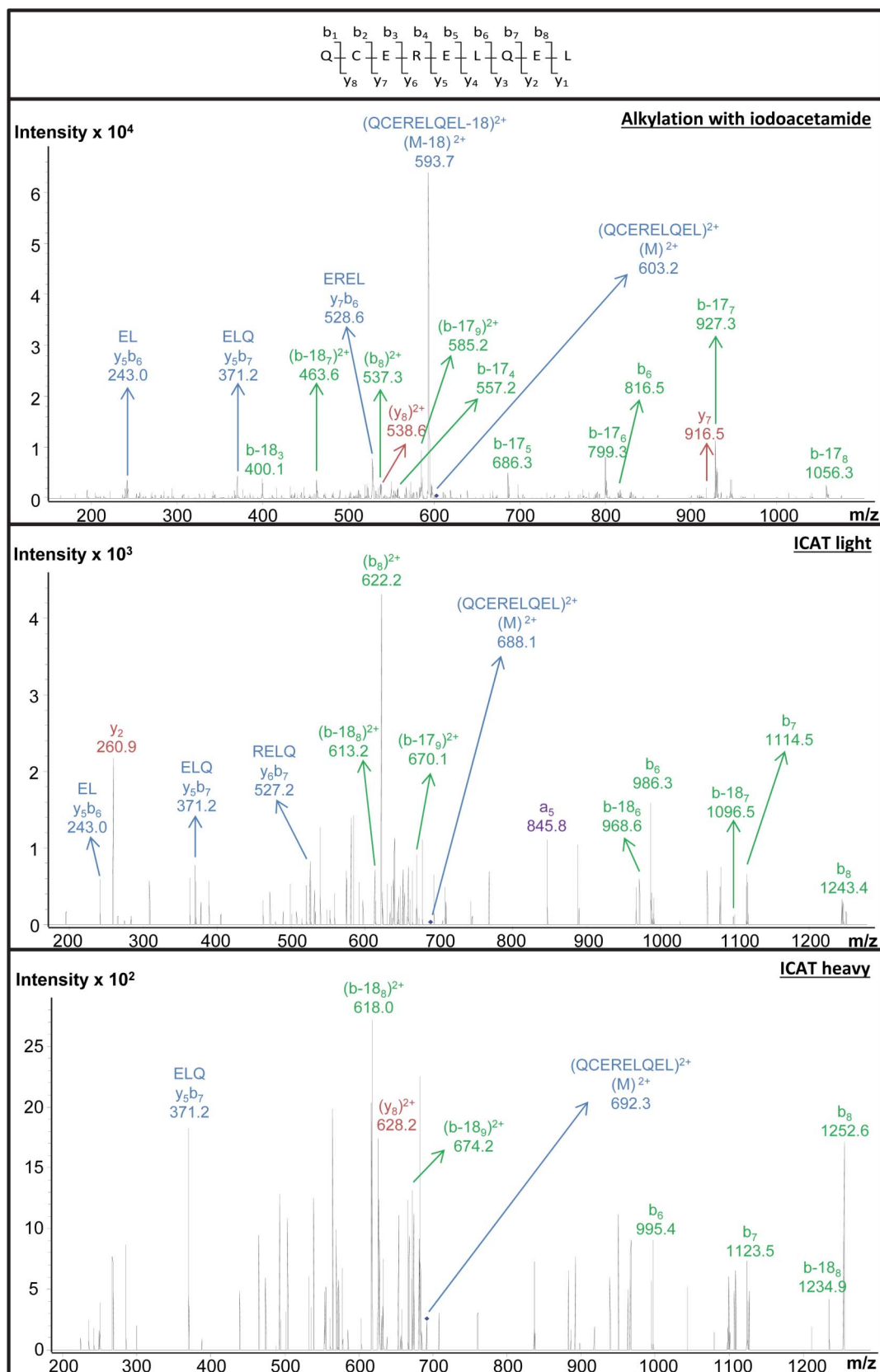


Figure 4 | CID MS/MS spectra of the cysteine-containing peptide QCERELQEL. The peptide was found in a chymotryptic digest of a wheat (*cv.* Akteur) flour extract. Top: IDAM-labeled. Middle: light ICAT-labeled. Bottom: heavy ICAT-labeled.



Criteria for peptide identification. Individual ion scores based on peptide mass fingerprints were calculated as $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Individual ion scores > 40 were considered to indicate identity or extensive similarity ($p < 0.05$). All peptide identifications with scores between 15 and 40 were manually validated, essentially following the rules of Chen *et al.*⁵⁵. For doubly or triply charged ions consisting of x amino acids, at least $x-2$ isotopically resolved independent fragment peaks were required to match the theoretical peptide fragments. Only a-, b- or y-ions, or associated peaks arising due to water or amine loss were considered as daughter 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. Only one isotopically resolved peak with an intensity higher than one third of the maximum intensity, an m/z value larger than that of the doubly/triply charged parent ion and not matching a theoretical peptide fragment, were allowed. For single charged ions, either the b-ion or y-ion series were required to confirm at least three consecutive amino acids in the peptide sequence. Large mass errors among the fragment ions and low/no intensity spectral peaks at the position of a readily cleavable proline residue caused a peptide to be rejected.

1. Faostat. *Food and Agricultural commodities production*, <faostat.fao.org> (2011). Date of access 8th of July 2013.
2. Dupont, F. M., Vensel, W. H., Tanaka, C. K., Hurkman, W. J. & Altenbach, S. B. Deciphering the complexities of the wheat flour proteome using quantitative two-dimensional electrophoresis, three proteases and tandem mass spectrometry. *Proteome Sci.* **9**, 10 (2011).
3. Delcour, J. A. *et al.* Wheat gluten functionality as a quality determinant in cereal-based food products. *Annu. Rev. Food Sci. Technol.* **3**, 469–492 (2012).
4. Wieser, H. Chemistry of gluten proteins. *Food Microbiol.* **24**, 115–119 (2007).
5. D'Ovidio, R. & Masci, S. The low-molecular-weight glutenin subunits of wheat gluten. *J. Cereal Sci.* **39**, 321–339 (2004).
6. 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.* **77**, 689–700 (1989).
7. Anderson, O. D., Gu, Y. Q., Kong, X., Lazo, G. R. & Wu, J. The wheat omega-gliadin genes: structure and EST analysis. *Funct. Integr. Genomics* **9**, 397–410 (2009).
8. DuPont, F. M., Vensel, W., Encarnacao, T., Chan, R. & Kasarda, D. D. Similarities of omega gliadins from *Triticum urartu* to those encoded on chromosome 1A of hexaploid wheat and evidence for their post-translational processing. *Theor. Appl. Genet.* **108**, 1299–1308 (2004).
9. Gianibelli, M. C., Larroque, O. R., MacRitchie, F. & Wrigley, C. W. Biochemical, genetic, and molecular characterization of wheat glutenin and its component subunits. *Cereal Chem.* **78**, 635–646 (2001).
10. Gu, Y. Q. *et al.* Genomic organization of the complex alpha-gliadin gene loci in wheat. *Theor. Appl. Genet.* **109**, 648–657 (2004).
11. Qi, P. F. *et al.* The gamma-gliadin multigene family in common wheat (*Triticum aestivum*) and its closely related species. *BMC Genomics.* **10**, 168 (2009).
12. Salentijn, E. M. *et al.* Tetraploid and hexaploid wheat varieties reveal large differences in expression of alpha-gliadins from homoeologous Gli-2 loci. *BMC Genomics.* **10**, 48 (2009).
13. Finnie, C., Sultan, A. & Grasser, K. D. From protein catalogues towards targeted proteomics approaches in cereal grains. *Phytochemistry.* **72**, 1145–1153 (2011).
14. Wieser, H., Bushuk, W. & MacRitchie, F. In *Gliadin and Glutenin. The Unique Balance of Wheat Quality* (eds Wrigley, C. W., Békés, F. & Bushuk, W.) Ch. 7, 213–240 (American Association of Cereal Chemists, 2006).
15. Naeem, H. A., Paulon, D., Irmak, S. & MacRitchie, F. Developmental and environmental effects on the assembly of glutenin polymers and the impact on grain quality of wheat. *J. Cereal Sci.* **56**, 51–57 (2012).
16. Shewry, P. R., Tatham, A. S., Barro, F., Barcelo, P. & Lazzari, P. Biotechnology of breadmaking: unraveling and manipulating the multi-protein gluten complex. *Biotechnology (N Y)*. **13**, 1185–1190 (1995).
17. Mifflin, B., Napier, J. & Shewry, P. Improving plant product quality. *Nat. Biotechnol.* **17**, 13–14 (1999).
18. Payne, P. I. Genetics of wheat storage proteins and the effect of allelic variation on bread-making quality. *Annu. Rev. Plant Phys.* **38**, 141–153 (1987).
19. Kasarda, D. D. In *Wheat is unique* (ed Pomeranz, Y.) 277–302 (American Association of Cereal Chemistry, 1989).
20. Šalplachta, J., Marchetti, M., Chmelik, J. & Allmaier, G. A new approach in proteomics of wheat gluten: combining chymotrypsin cleavage and matrix-assisted laser desorption/ionization quadrupole ion trap reflectron tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**, 2725–2728 (2005).
21. Tanner, G. J., Colgrave, M. L., Blundell, M. J., Goswami, H. P. & Howitt, C. A. Measuring hordein (gluten) in beer – A comparison of ELISA and mass spectrometry. *PLoS ONE* **8**, e56452 (2013).
22. Mamone, G., De Caro, S., Di Luccia, A., Addeo, F. & Ferranti, P. Proteomic-based analytical approach for the characterization of glutenin subunits in durum wheat. *J. Mass Spectrom.* **44**, 1709–1723 (2009).
23. Lutz, E., Wieser, H. & Koehler, P. Identification of disulfide bonds in wheat gluten proteins by means of mass spectrometry/electron transfer dissociation. *J. Agric. Food Chem.* **60**, 3708–3716 (2012).
24. Camafeita, E., Alfonso, P., Acevedo, B. & Mendez, E. Sample preparation optimization for the analysis of gliadins in food by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Mass Spectrom.* **32**, 444–449 (1997).
25. Camafeita, E. *et al.* Selective identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of different types of gluten in foods made with cereal mixtures. *J. Chromatogr. A* **823**, 299–306 (1998).
26. Vensel, W. H., Dupont, F. M., Sloane, S. & Altenbach, S. B. Effect of cleavage enzyme, search algorithm and decoy database on mass spectrometric identification of wheat gluten proteins. *Phytochemistry.* **72**, 1154–1161 (2011).
27. Rombouts, I. *et al.* Wheat gluten amino acid composition analysis by high-performance anion-exchange chromatography with integrated pulsed amperometric detection. *J. Chromatogr. B* **1216**, 5557–5562 (2009).
28. Sechi, S. & Chait, B. T. Modification of cysteine residues by alkylation. A tool in peptide mapping and protein identification. *Anal. Chem.* **70**, 5150–5158 (1998).
29. Leitner, A. & Lindner, W. Current chemical tagging strategies for proteome analysis by mass spectrometry. *J. Chromatogr. B* **813**, 1–26 (2004).
30. Tao, W. A. & Aebersold, R. Advances in quantitative proteomics via stable isotope tagging and mass spectrometry. *Curr. Opin. Biotechnol.* **14**, 110–118 (2003).
31. Paulech, J., Solis, N. & Cordwell, S. J. Characterization of reaction conditions providing rapid and specific cysteine alkylation for peptide-based mass spectrometry. *Biochim. Biophys. Acta* **1834**, 372–379 (2013).
32. Gygi, S. P. *et al.* Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **17**, 994–999 (1999).
33. Sethuraman, M. *et al.* Isotope-coded affinity tag (ICAT) approach to redox proteomics: identification and quantitation of oxidant-sensitive cysteine thiols in complex protein mixtures. *J. Proteome Res.* **3**, 1228–1233 (2004).
34. Leichert, L. I. *et al.* Quantifying changes in the thiol redox proteome upon oxidative stress in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 8197–8202 (2008).
35. Garcia-Santamarina, S. *et al.* The oxidized thiol proteome in fission yeast – optimization of an ICAT-based method to identify H₂O₂-oxidized proteins. *J. Proteomics.* **74**, 2476–2486 (2011).
36. Islam, N., Tsujimoto, H. & Hirano, H. Wheat proteomics: relationship between fine chromosome deletion and protein expression. *Proteomics* **3**, 307–316 (2003).
37. Ramus, C., Gonzalez de Peredo, A., Dahout, C., Gallagher, M. & Garin, J. An optimized strategy for ICAT quantification of membrane proteins. *Mol. Cell. Proteomics* **5**, 68–78 (2006).
38. Marchylo, B. A., Kruger, J. E. & Hatcher, D. W. Quantitative reversed-phase high-performance liquid chromatographic analysis of wheat storage proteins as a potential quality prediction tool. *J. Cereal Sci.* **9**, 113–130 (1989).
39. Lundell, N. & Schreitmuller, T. Sample preparation for peptide mapping – A pharmaceutical quality-control perspective. *Anal. Biochem.* **266**, 31–47 (1999).
40. Gessendorfer, B., Wieser, H. & Koehler, P. Optimisation of a solvent for the complete extraction of prolamins from heated foods. *J. Cereal Sci.* **52**, 331–332 (2010).
41. Boja, E. S. & Fales, H. M. Overalkylation of a protein digest with iodoacetamide. *Anal. Chem.* **73**, 3576–3582 (2001).
42. Jiang, X., Shamshurin, D., Spicer, V. & Krokhin, O. V. The effect of various S-alkylating agents on the chromatographic behavior of cysteine-containing peptides in reversed-phase chromatography. *J. Chromatogr. B* **915–916**, 57–63 (2013).
43. Payne, P. I., Nightingale, M. A., Krattiger, 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.* **40**, 51–65 (1987).
44. Shewry, P. R. *et al.* In *Wheat: Chemistry and Technology* (eds Khan, K. & Shewry, P. R.) 223–298 (AACC International, 2009).
45. Wieser, H. Comparative investigations of gluten proteins from different wheat species I. Qualitative and quantitative composition of gluten protein types. *Eur. Food Res. Technol.* **211**, 262–268 (2000).
46. Bantscheff, M., Schirle, M., Sweetman, G., Rick, J. & Kuster, B. Quantitative mass spectrometry in proteomics: a critical review. *Anal. Bioanal. Chem.* **389**, 1017–1031 (2007).
47. Kline, K. G. & Sussman, M. R. Protein quantitation using isotope-assisted mass spectrometry. *Annu. Rev. Biophys.* **39**, 291–308 (2010).
48. Smolka, M. B., Zhou, H., Purkayastha, S. & Aebersold, R. Optimization of the isotope-coded affinity tag-labeling procedure for quantitative proteome analysis. *Anal. Biochem.* **297**, 25–31 (2001).
49. Turecek, F. Mass spectrometry in coupling with affinity capture-release and isotope-coded affinity tags for quantitative protein analysis. *J. Mass Spectrom.* **37**, 1–14 (2002).
50. Hirayama, K., Akashi, S., Furuya, M. & Fukuhara, K. Rapid confirmation and revision of the primary structure of bovine serum albumin by ESIMS and Frit-FAB LC/MS. *Biochem. Biophys. Res. Commun.* **173**, 639–646 (1990).
51. Sebastiano, R., Citterio, A., Lapadula, M. & Righetti, P. G. A new deuterated alkylating agent for quantitative proteomics. *Rapid Commun. Mass Spectrom.* **17**, 2380–2386 (2003).
52. Nelson, K. J., Day, A. E., Zeng, B. B., King, S. B. & Poole, L. B. Isotope-coded, iodoacetamide-based reagent to determine individual cysteine pK(a) values by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Biochem.* **375**, 187–195 (2008).
53. Wang, S. & Kaltashov, I. A. A new strategy of using O18-labeled iodoacetic acid for mass spectrometry-based protein quantitation. *J. Am. Soc. Mass Spectr.* **23**, 1293–1297 (2012).



54. Wieser, H. & Koehler, P. Is the calculation of the gluten content by multiplying the prolamin content by a factor of 2 valid? *Eur. Food Res. Technol.* **229**, 9–13 (2009).
55. Chen, Y., Kwon, S. W., Kim, S. C. & Zhao, Y. Integrated approach for manual evaluation of peptides identified by searching protein sequence databases with tandem mass spectra. *J. Proteome Res.* **4**, 998–1005 (2005).

Acknowledgments

Ine Rombouts and Bert Lagrain wish to acknowledge the Research Foundation – Flanders (FWO, Brussels, Belgium) for their positions as postdoctoral fellows and financial support during a research stay at the German Research Centre for Food Chemistry (Freising, Germany). The authors would like to thank Ines Otte and Sami Kaviani-Nejad (German Research Center for Food Chemistry, Freising, Germany) for excellent technical assistance and help with the MS measurements.

Author contributions

Conceived and designed the experiments: I.R., B.L., M.B., P.K. Performed the experiments: B.L., M.B. Analyzed the data: I.R. Contributed reagents/materials/analysis tools: P.K., J.D. Wrote the paper: I.R.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Rombouts, I., Lagrain, B., Brunnbauer, M., Delcour, J.A. & Koehler, P. Improved identification of wheat gluten proteins through alkylation of cysteine residues and peptide-based mass spectrometry. *Sci. Rep.* **3**, 2279; DOI:10.1038/srep02279 (2013).



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