



Barley γ 3-hordein: Glycosylation at an atypical site, disulfide bridge analysis, and reactivity with IgE from patients allergic to wheat

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ARTICLE INFO

Article history:

Received 6 March 2012

Received in revised form 27 July 2012

Accepted 28 July 2012

Available online 4 August 2012

Keywords:

Disulfide bridge

Glycosylation

IgE reactivity

Mass spectrometry

Prolamin

Wheat allergy

ABSTRACT

Post translational modifications of a seed storage protein, barley γ 3-hordein, were determined using immunochemical and mass spectrometry methods. IgE reactivity towards this protein was measured using sera from patients diagnosed with allergies to wheat. N-glycosylation was found at an atypical Asn-Leu-Cys site. The observed glycan contains xylose. This indicates that at least some γ 3-hordein molecules trafficked through the Golgi apparatus. Disulfide bridges in native γ 3-hordein were almost the same as those found in wheat γ 46-gliadin, except the bridge involving the cysteine included in the glycosylation site. IgE reacted more strongly towards the recombinant than the natural γ 3-hordein protein. IgE binding to γ 3-hordein increased when the protein sample was reduced. Glycosylation and disulfide bridges therefore decrease epitope accessibility. Thus the IgE from patients sensitized to wheat cross-react with γ 3-hordein due to sequence homology with wheat allergens rather than through shared carbohydrate determinants.

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1. Introduction

Wheat and barley are major crops in western societies. Wheat and barley seed storage proteins have been thoroughly studied for many decades in order to understand their role in nutrition and food technology processes.

In cereals, the seed storage proteins belong to the prolamin family, so-called for their high proline and glutamine content. They are classified into different groups depending on their degree of polymerization, cysteine content, molecular weight, and N-terminal sequence [1]. Besides the prolamins belonging to the sulfur-poor prolamin group (wheat ω -gliadins and barley C-hordeins) or to the high molecular weight prolamin group (wheat high molecular weight glutenin subunits, and barley D-hordeins) [2], many prolamins belong to the sulfur-rich prolamin group. This latter group comprises, on one hand, the wheat low molecular weight glutenin subunits (LMW-GSs), as well as the barley B hordeins,

which are included in the gluten macro-polymers, and on the other hand, the wheat α - and γ -gliadins as well as the barley γ -hordeins, which are monomeric. The γ -hordeins are a minor group of storage protein in barley seeds which encompasses three genetically and antigenically distinct members, γ 1, γ 2, γ 3 [3,4]. The proteins within each group, and also between homologous groups from wheat and barley, share strong homologies in sequence and structure (repetitive domains, disulfide bridges).

In addition to their role in nutrition, unfortunately prolamins are responsible for adverse reactions including an intolerance, called coeliac disease, and various immunoglobulin E (IgE)-mediated allergies, such as baker's asthma [5], food allergy [6,7] and also cutaneous allergies induced by hydrolyzed wheat proteins used as additives [8]. However, despite sequence homologies, different prolamins show strong differences in IgE binding *in vitro* [9,10].

In order to analyze the specificity of IgE binding, well-characterized proteins are essential. IgE reactivity depends on the protein sequence and the presence of linear epitopes. It also depends on the protein structure which controls epitope presentation to IgE and also activates some epitopes. Protein folding which occurs partly through disulfide bridges is clearly an important element in this conformation. Other post-translational modifications such as glycosylation could lead to cross-reactive carbohydrate determinants. For instance, in baker's asthma, some IgE reactions resulted from the antibody binding to wheat

Abbreviations: CAM, carbamidomethylated; DIG, digoxigenin; IHHWP, immediate hypersensitivity to hydrolyzed wheat protein; LMW-GS, low molecular weight glutenin subunit; TFMS, trifluoromethanesulfonic acid; WDEIA, wheat-dependent exercise-induced anaphylaxis

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allergens via their protein sequence, but also from binding to carbohydrate determinants [11]. Furthermore, the wheat allergen Tri a Bd 27k is itself a glycoprotein [12] belonging to the soluble fraction of flour proteins.

In general, prolamins are considered not to be glycosylated. However, there are several reports describing prolamins glycosylation. In α -gliadins, carbohydrates have been detected, but without covalent binding [13]. Some wheat proteins belonging to both the gliadin and glutenin families [14], and γ 3-hordein [15] showed a reaction with anti-(xylose containing N-glycans) antibodies and so these proteins are thought to be glycosylated.

This study focused on γ 3-hordein (Uniprot ID P80198), a minor protein in barley seeds but with particular features. In addition to its glycosylation, γ 3-hordein was described to react with IgE from wheat allergic patients [16,17]. We aimed to characterize γ 3-hordein glycosylation and estimate its possible involvement in IgE-binding by comparing it with recombinant γ 3-hordein. In addition, the nature of the glycosylation could also give some insight into γ 3-hordein trafficking in barley endosperm cells. Indeed, γ 3-hordein is known to be involved in protein transport [18]: the absence of γ 3-hordein in the mutant Nevsky led to a dramatic change in hordein polypeptide targeting, with the hordein storage proteins being largely deposited in the lumen of the rough endoplasmic reticulum. The authors hypothesized that γ 3-hordein could maintain the prolamins in a transport competent state.

This study also examined γ 3-hordein folding via disulfide bridges. Unlike wheat LMW-GSs and barley B-hordein, γ 3-hordein is monomeric and only forms intra-molecular disulfide bridges. Thus, we analyzed disulfide bridge folding and investigated the possible involvement of this post-translational modification in IgE binding *in vitro*.

2. Material and methods

2.1. Production of recombinant γ 3-hordein

The plasmid pET101/DTopo (Invitrogen, Carlsbad, CA) containing the cloned gene [17] was introduced into the *E. coli* T7 Express strain (New England Biolabs, Beverly, MA). A 10-mL overnight pre-culture was used to inoculate 200 mL of LB medium containing ampicillin and incubated for 2 h at 37 °C. IPTG (1 mM final concentration) was then added for a 4-hour induction. Bacteria were harvested, washed with 0.9% (w/v) NaCl, and pelleted. The pellet was re-suspended in 50 mM Tris-HCl, pH 8.5, containing protease inhibitors (Roche-Diagnostics, Mannheim, Germany) and lysed using a cell disrupter (Constant Systems, Daventry, UK). Inclusion bodies were pelleted and solubilized in 50 mM Tris-HCl, pH 8.5 containing 2% (w/v) SDS. Proteins were reduced with 0.1 M DTT for 30-min and further alkylated for 30-min in the dark with 0.5 M iodoacetamide. After addition of 2 volumes of ethanol, 1-hour incubation and centrifugation, the recombinant γ 3-hordein was recovered from the supernatant by overnight precipitation with NaCl (0.5 M final concentration).

2.2. Plant material

Seeds from the barley (*Hordeum vulgare* L.) Risø 56 mutant were used in this study. This mutant was obtained from the wild cultivar Carlsberg II and lacks B-hordeins [19]. It was previously used for γ -hordein studies [20,15].

2.3. Hordein extraction and natural γ 3-hordein purification

Seeds, after removing germ and external layers, were crushed, and hordeins were extracted with 35% w/w 1-propanol in 0.1 M acetic acid [15]. After centrifugation, the proteins from the supernatant were precipitated overnight with two volumes of 7.5% (w/v) NaCl.

The pellet containing the Risø 56 hordein extract was re-suspended in Laemmli sample buffer containing 2% (w/v) SDS.

For γ 3-hordein purification, the Risø 56 hordein extract was run on SDS-PAGE and proteins were blotted on PVDF membrane according to [21]. The band corresponding to γ 3-hordein was cut out and further eluted with 50% (w/v) isopropanol, 20 mM ammonium carbonate buffer. The purified protein was lyophilized and stored at –20 °C.

2.4. Chemical de-glycosylation of natural γ 3-hordein

Chemical de-glycosylation was carried out according to [22] with slight modifications: one volume of trifluoromethanesulfonic acid (TFMS) and two volumes of anisole were mixed under a flow of argon at room temperature. 400 μ L of this solution was added to 100 μ g of dried purified natural γ 3-hordein in a 4-mL reactival (Pierce-ThermoFisher, Rockford, IL) and stirred at 0 °C for 1.5 h. To recover the proteins, three volumes of 70% (v/v) diethyl ether cooled at –70 °C were added very gently to the vial in an ice bath and stirred. Two volumes of ice-cooled 50% (v/v) pyridine were then added carefully to the solution. The resulting precipitate of pyridinium salts was re-dissolved by vortexing and the ether phase removed and discarded. The ether extraction of the aqueous phase was repeated once. Proteins were further recovered from the aqueous phase by precipitation with three volumes of acetone overnight at –20 °C.

2.5. Protein quantization, SDS-PAGE, transfer to membranes

Protein quantization was performed using Vera's protocol adapted to a microplate format [23].

SDS-PAGE was carried out under standard conditions and the proteins were transferred to PVDF membranes according to Lauriere's protocol [21]. Proteins were stained in control gels according to Neuheff et al. [24].

2.6. Immunodetection of blotted proteins

Total glycoproteins were stained directly on blots using the kit (Roche Diagnostics, Mannheim, Germany) according to [25]. This technique uses glycan periodate oxidation, conjugation with digoxigenin-succinyl- ϵ -amino-caproic acid hydrazide solution (DIG-hydrazide), and incubation with an anti-(DIG-hydrazide) antibody. The detection method used anti-rabbit-IgG alkaline-phosphatase-conjugated antibody and 4-nitro blue tetrazolium chloride solution (NBT)/5-bromo-4-chloro-3-indolylphosphate solution (BCIP) staining. Control membranes were stained with amido black 10 B [26].

Specific glycoproteins were detected using immunoblotting with rabbit polyclonal antibodies raised against carrot β -fructosidase [27] and *Cucurbita* ascorbate oxidase [28]. These antibodies recognize purified glycoproteins containing N-glycans with xylose [27], and xylose + fucose motives [28] respectively. To verify that γ 3-hordein could still be detected on the blot after chemical de-glycosylation, the membrane was incubated with a rabbit anti- γ 3-hordein antibody. In all these experiments, the detection was as described above.

2.7. In-solution proteolysis of purified natural γ 3-hordein used for glycosylation analysis

Samples (50–60 μ g purified natural γ 3-hordein) were re-solubilized in 0.08 M Tris-HCl, pH 8.5, 40% (w/v) 1-propanol. The protein was reduced with 120 mM DTT for 30 min at 60 °C, alkylated with 4-vinyl-pyridine for 3 h at 60 °C, and further precipitated overnight with 3 volumes acetone at –20 °C. The pelleted protein was then solubilized with 70% (v/v) formic acid and cleaved overnight with cyanogen bromide (CNBr) at a final concentration of 25 ng/ μ L. The samples were diluted with water and lyophilized. In order to reduce the size of fragments, the peptides were re-suspended in 0.2 M ammonium

bicarbonate, and incubated overnight at 37 °C with trypsin (from bovine pancreas, supplied by Roche Diagnostics) at an enzyme/protein ratio of 1/20. After evaporation in the speed-vac, the samples were re-solubilized with 0.1% (v/v) formic acid.

After protein solubilization with 50 mM ammonium bicarbonate, reduction with DTT as above, and further incubation with 0.1 M iodoacetamide (in 10 mM Tris-HCl pH 8) for 20 min at room temperature in the dark, the protein was digested with endoproteinase Glu-C (from *Staphylococcus aureus* strain V8, supplied by Roche Diagnostics). The proteins were then acetone-precipitated as above, re-solubilized with 50 mM ammonium bicarbonate pH 7.8 containing 0.5 M urea, and 20 mM methylamine, and further digested overnight at 25 °C, at an enzyme/protein ratio of 1/20.

2.8. In-gel proteolysis of γ 3-hordein, used for disulfide bridge determination

Several samples of Risø 56 hordein extract were submitted to SDS-PAGE. The gel was stained using Coomassie blue according to [24], and the band corresponding to γ 3-hordein was cut out for each sample. The gel pieces were washed with water, incubated with acetonitrile, and dried in the speed-vac. Free cysteines were alkylated without a reducing agent by re-hydration for 45 min at 0 °C with a solution of 0.1 M iodoacetamide in 0.1 M NH_4HCO_3 . The samples were washed further, twice with 0.1 M NH_4HCO_3 and dried twice with 100% acetonitrile. For proteolysis the samples were incubated for 1 h at 37 °C with a mixture of chymotrypsin + trypsin (Roche Diagnostics, 0.6 $\mu\text{g}/\mu\text{L}$ of each enzyme) in 25 mM NH_4HCO_3 , 5 mM CaCl_2 . Peptides were further extracted by successive incubations at 37 °C with 5% (v/v) formic acid, then 50% (v/v) acetonitrile, and then 100% acetonitrile. The supernatants were pooled and dried in the speed-vac. They were recovered at the time of injection with 1% (v/v) formic acid.

2.9. Mass spectrometry methods

High performance liquid chromatography was carried out with a Spectra System equipment (Thermo Fisher Scientific, Waltham, MA) comprising a SCM1000 vacuum membrane degasser, P4000 gradient pumps and a manual injector. 10 μL samples were loaded onto a reversed-phase BioBasic-18 column (2×150 mm, 300 Å pore size, 5 μm film thickness, Thermo Electron Corporation). The column was eluted at a flow-rate of 0.2 mL min^{-1} at 20 °C with 5% of solvent B (0.1% (v/v) formic acid in acetonitrile) in A (0.1% (v/v) formic acid in water) for 2 min and then with a linear gradient of B in A from 5 to 45% over 40 min then 45 to 95% over 5 min before re-equilibration. The column eluant was introduced in the electrospray ionization source of a Thermo Electron LCQ Deca ion-trap mass spectrometer operating in positive ion mode. Instrument parameters were capillary temperature 280 °C, capillary voltage 30 V, spray voltage 4.5 kV, sheath gas flow 80 a.u., and auxiliary gas flow 5 a.u. Mass spectra were acquired scanning from m/z 200 to 2000. Ion fragmentation was carried out using a normalized collision energy of 35 arbitrary units. Two identical chromatography-mass spectrometry runs for each sample were recorded and the data pooled to improve the number of ions recorded. Mass spectrometry data were then analyzed using BioWorks 3.1™ Thermo Scientific and MassMatrix v 1.3.0 [29] softwares.

2.10. Human sera from patients allergic to wheat products

Human sera from previous studies [8,9] which showed some reactivity with γ 3-hordein, were chosen for use in the present study. The patients suffer from different wheat-dependent allergies: dietary and cutaneous immediate hypersensitivity to hydrolyzed wheat proteins (IHHPW); sensitized to IHHPW by only cutaneous contact (two patients); atopic eczema dermatitis syndrome; food allergy; and wheat-dependent exercise-induced anaphylaxis (WDEIA). IHHPW

patients were not allergic to natural wheat proteins [8]. This study was approved by the Committee for Protection of Human Subjects in Biomedical Research of Cochin Hospital (Paris, France), and each patient gave informed consent before blood sampling.

2.11. IgE binding assays

The Risø 56 hordein extract and recombinant γ 3-hordein were run on SDS-PAGE and transferred to PVDF membrane [21]. The Risø 56 hordein extract was analyzed on a Coomassie stained gel in order to quantify the amount of γ 3-hordein it contained using Multi-Gauge software (Fujifilm, Tokyo, Japan). 6 μg of recombinant reduced-alkylated γ 3-hordein and 31 μg of Risø 56 hordein extract (non-reduced, and reduced-alkylated respectively) were loaded on the gel: this corresponded to 6 μg of natural γ 3-hordein.

Membranes were immunoblotted according to [9] with some modifications. All washes and incubations, except incubation with the goat antibody, were carried out with the following blocking solution: TBS (50 mM Tris buffer (pH 7.5), 0.15 M NaCl) containing, 2% (w/v) polyvinylpyrrolidone 40, and 0.1% (w/v) Tween 20. After blocking for 1 h, membranes were incubated overnight with patient sera (1/20 final dilution). Membranes were then washed and incubated for 1 h with goat biotinylated antibody against human IgE (Vector Laboratories, USA) at a 1/15,000 dilution in TBS containing 3% (w/v) dry milk and 0.1% (w/v) Tween 20. The membranes were washed and further incubated for 1 h with streptavidin HRP conjugate (Pierce-ThermoFisher, Rockford, IL), at 1/7500 dilution. After washes, membranes were incubated for 5 min with the SuperSignal West Dura Extended duration substrate (Pierce-ThermoFisher). IgE chemiluminescence was detected using a Fuji Las3000 camera (Fujifilm, Tokyo, Japan) and quantified using Multi-Gauge software. A control membrane was processed as above but without patient serum to establish the specificity of detection.

3. Results

3.1. Obtaining of natural and recombinant γ 3-hordein

When the non-reduced Risø 56 hordein extract was run on SDS-PAGE (Fig. 1 lane 1), a band at around 36 kDa corresponded to the well-separated γ 3-hordein. The upper bands were other hordeins as described in [3,18]. This distinct position facilitated gel slice cutting for mass spectrometry analyses. Following Western blotting, natural γ 3-hordein was either immunodetected or purified from the membrane. The reduced and alkylated purified γ 3-hordein is shown in Fig. 1, lane 3. A single band was observed showing a high level of purity. A single band was also obtained for recombinant γ 3-hordein (Fig. 1, lane 4) showing a convenient purity after a solubility-based extraction procedure followed by reduction and alkylation. Reduced and alkylated γ 3-hordein (Fig. 1 lanes 2–4) migrated more slowly in SDS-PAGE than natural and non-reduced γ 3-hordein: this was probably due to an increase in the apparent molecular diameter related to the cleavage of disulfide bridges. The identity of γ 3-hordein bands was confirmed by mass spectrometry experiments (see below).

3.2. Immunochemical evidence for γ 3-hordein glycosylation

Glycoproteins were specifically stained on membrane through glycan oxidation, conjugation with DIG-hydrazide, and detection with an anti-DIG antibody [25]. A clear signal was observed for γ 3-hordein in the reduced and alkylated Risø 56 hordein extract (Fig. 2, lane 1, with protein control in lane 2) and a weaker signal was detected for another protein migrating among the C-hordein group.

Further information about the glycan groups involved was obtained using antibodies raised against purified glycoproteins with known glycan structures. Two antibodies were used: the anti- β -fructosidase antibody recognizes a glycoprotein with a N-glycan necessarily containing

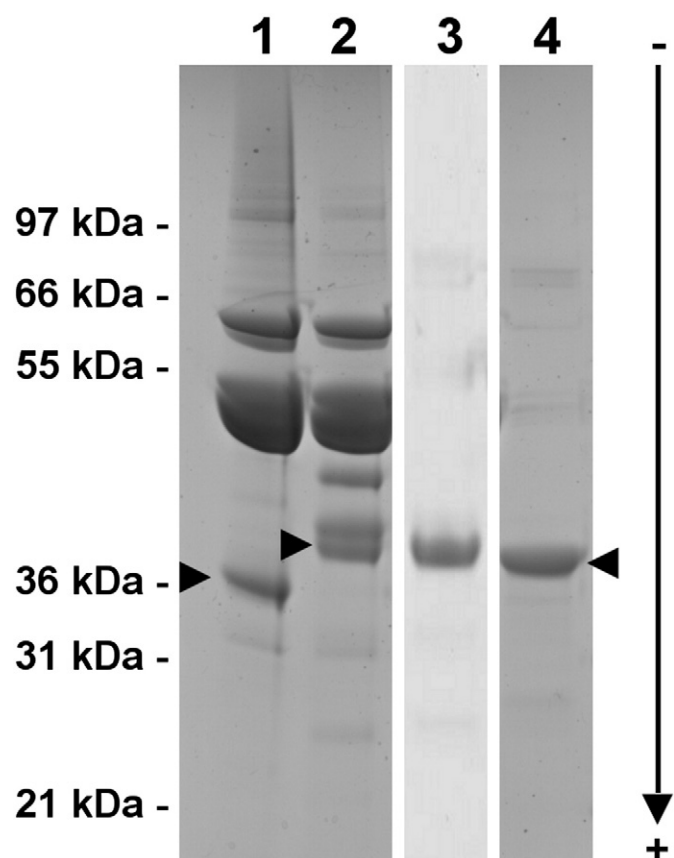


Fig. 1. SDS-PAGE of Risø 56 hordein extract, purified natural and recombinant γ 3-hordeins. Non-reduced Risø 56 hordein extract (lane 1). Reduced and alkylated Risø 56 hordein extract (lane 2). Reduced and alkylated purified γ 3-hordein (lane 3). Reduced and alkylated recombinant γ 3-hordein (lane 4). γ 3-hordein is indicated with arrow heads.

xylose [27]; the anti-ascorbate oxidase antibody recognizes a glycoprotein with both *N*-glycans containing either xylose or fucose [28]. Positive signals were obtained for both antibodies on a Western blot with purified natural γ 3-hordein (Fig. 2, lanes 3 and 5 respectively). As there is no homology between γ 3-hordein and β -fructosidase or ascorbate oxidase sequences, these results suggested that the cross-reaction was due to the glycan moiety. Chemical de-glycosylation of purified natural γ 3-hordein abolished recognition by the anti-ascorbate oxidase antibody (Fig. 2, compare lane 5 (natural γ 3-hordein) to lane 6 (de-glycosylated)). A control (Fig. 2, lane 7) carried out after de-glycosylation using a rabbit anti- γ 3-hordein antibody showed that de-glycosylation did not alter the protein structure or its immunoreactivity. Thus, lack of recognition by the anti-ascorbate oxidase antibody was only due to glycan removal.

A combination of different immunochemical techniques gave the same result, thus reducing the risk of false positive responses. Together, these data showed that γ 3-hordein is *N*-glycosylated with a glycan structure containing a xylose and perhaps a fucose. The γ 3-hordein sequence (Table 1) contains only two potential atypical *N*-glycosylation consensus sequences [30] at N₁₄₀LC₁₄₂ and N₁₇₄SC₁₇₆. The sequence N₉₃PS₁₁ is probably not glycosylated due to the Pro residue [31], and no other Asn residues were included in a tripeptide potentially able to receive a *N*-glycan. Mass spectrometry was used to confirm this glycosylation and determine the site involved.

3.3. Identification of the *N*-glycosylation site

After reduction and alkylation of the cysteine residues, the purified natural and recombinant γ 3-hordeins were subjected, in a first

step, to CNBr-hydrolysis (cleavage after methionine residues) which results preferentially in high-molecular-weight peptides (MW higher than 3000 Da). In a second step, the samples were digested with trypsin, to obtain shorter peptides containing the two potential glycosylation sites (Table 1). The peptides were separated using HPLC and analyzed with ESI LC-MS/MS. Overall there were less digestion products for natural γ 3-hordein than for the recombinant protein because enzymatic cleavage of natural γ 3-hordein was reduced compared to that of the recombinant protein, with the total ion current decreasing from 30 to 40%. Comparison of the chromatograms from natural and recombinant γ 3-hordein (Appendix A, Fig. A1) showed that several peptides were equally present in both protein digests. Accordingly, these peptides were not affected by any post-translational modification. However, some clear-cut differences were also found between the two digests. In particular, two peptides eluted at 17.80 min and 19.68 min were more abundant in the recombinant protein than in the natural protein.

The peptides were identified from their molecular mass and fragmentation pattern. Several modifications were taken into consideration: methionine was modified either as homoserine (M-30) or as homoserine lactone (M-48) following CNBr cleavage, and some peptides showed increased mass due to some adducts. High-molecular-weight peptides were identified through the distribution of the multiply charged ions in their mass spectra and after deconvolution (Appendix A, Table A1). We obtained very high coverage of the sequence (97%) of recombinant γ 3-hordein. The two peptides which were weakly represented in digested natural γ 3-hordein corresponded to P₉₃–K₁₄₃ and E₁₄₄–K₁₅₅. The significant absence of the P₉₃–K₁₄₃ peptide which contains the putative *N*-glycosylation site N₁₄₀LC₁₄₂, and the weak representation of the E₁₄₄–K₁₅₅ peptide in natural γ 3-hordein could indicate that cleavage with trypsin was less efficient at the K₁₄₃–E₁₄₄ site, perhaps due to steric modification close to the cutting site. On the other hand, there was no significant variation in the amount of the V₁₅₆–K₁₇₉ peptide, which contains N₁₇₄SC₁₇₆, in the two digests.

In another experiment, purified natural and recombinant γ 3-hordeins were reduced and alkylated and further digested with endoproteinase Glu-C (cleavage after glutamate residues). This reduced the size of the peptide containing the proposed glycosylation site N₁₄₀LC₁₄₂ (Table 1 and Appendix A, Table A1). The Q₁₃₆–E₁₄₄ peptide was clearly detected in the recombinant protein digest but was weakly recovered in the natural protein digest, consistent with a putative N₁₄₀ modification. A new peptide appeared at 25.32 min (chromatogram data not shown) displaying some evidence for the presence of a glycan moiety. In the ESI-MS of this peptide, ions appeared to have arisen from partial loss of

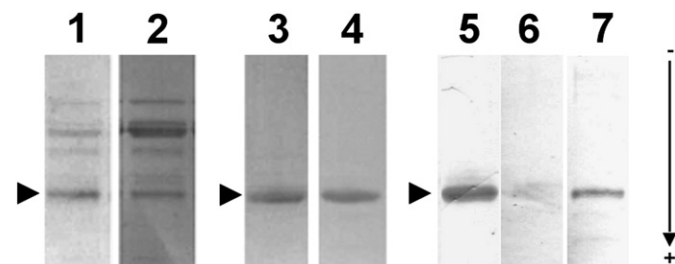


Fig. 2. Glycosylation of natural γ 3-hordein was detected on immunoblots. Glycoprotein characterization: reduced Risø 56 hordein extract, stained using an anti-DIG-hydrazide antibody after glycan oxidation and DIG-hydrazide conjugation (lane 1) and amido-black staining (lane 2) as a control. Specific detection of *N*-glycans containing xylose: purified natural γ 3-hordein stained using an anti- β -fructosidase antibody (lane 3) and amido-black staining (lane 4) as a control. Comparison between natural and de-glycosylated γ 3-hordein: purified natural γ 3-hordein (lane 5), chemically de-glycosylated γ 3-hordein (lane 6), both stained on the same membrane using an anti-ascorbate oxidase antibody. Control blot: chemically de-glycosylated purified natural γ 3-hordein stained using a rabbit anti- γ 3-hordein antibody and mouse anti-rabbit alkaline-phosphatase-conjugated (lane 7). γ 3-hordein is indicated with arrow heads.

Table 1
γ3 hordein sequence.

10	20	30	40	50	60	70
ITTTTMMQFN	SGLELERPQQ	LFPOWQPLPQ	QPPFLQOEPE	QPYPQQOPLP	QQQPFPPQQPQ	LPHQHQFPQQ
▲	▲	◇	◇	◇	◇	◇
80	90	100	110	120	130	140
LPQQQFPQQM	PLQPQQQFPQ	QMPLQPQQQP	QFPQQKPFQ	YQQPLTQQPY	PQQQPLAQQQ	PSIEEQHQLN
▲	◇	▲	▲	◇	◇	◇
150	160	170	180	190	200	210
<u>LC</u> KEFLLOQC	TLDEKVPLLQ	SVISFLRPHI	SOQNSCQLKR	QQCCQQLANI	NEQSRCPAIQ	TIVHAIVMQQ
▲	▲	◇	◇	◇	◇	◇
220	230	240	250	260	270	280
QVQQQVGHGF	VQSQLOQLGQ	GMPIQLQQQP	GQAFVLPQQQ	AQFKVVGSLV	IQTLPLMCNV	HVPPYCSPFG
◇	◇	▲	◇	◇	◇	◇
290						
SMATGSGGQ						
▲						

Underlined: potential non-conventional N-glycosylation sites. In bold the site found to be actually glycosylated. With gray bottom: Cys residues. ▲: CNBr cleavage site. ||: endoproteinase Glu-C cleavage site. ◇: chymotrypsin cleavage site. ▲: trypsin cleavage site.

carbohydrate groups (Table 2). The parent ion at $m/z = 1899$ corresponded to the peptide $Q_{136}-E_{144}$ ($[M+H]^+ = 1169$) with two N-acetyl glucosamines and two mannoses. Ions arising from the loss of two mannoses and one N-acetyl glucosamine were identified and they were also found to be the major species (mono or doubly charged) with adducts. It is known that glycans tend to produce multiple adducts in positive ion mode [32]. The ion corresponding to the intact glycopeptide was not observed and it was not possible to unambiguously assign a glycoform. Disaccharides, trisaccharides and tetrasaccharide were also detected (Table 2). These ions, which could provide some information on glycan structure, were low in intensity due to their poor ionization efficiency. Fragmentation of the major species ($[M+2H]^{2+} = 710$) led to a MS/MS spectrum showing some of the b- and y-ions typical of the MS/MS spectrum of the $Q_{136}-E_{144}$ peptide recovered in the recombinant γ3-hordein digest (Table 3).

3.4. Disulfide bridges in natural γ3-hordein

The Risø 56 hordein extract was run on SDS-PAGE and bands corresponding to natural γ3-hordein were cut out. Two samples were prepared and one was reduced and alkylated, leading to the carbamidomethylation of cysteine residues, while the other was not; both were further subjected to in-gel digestion with a mixture of chymotrypsin–trypsin. In order to identify potential free cysteine residues not involved in disulfide bridges, a third sample was incubated with iodoacetamide without prior reduction. Peptide mixtures were analyzed using tandem MS and the data were first analyzed using BioWorks 3.1™ software. After reduction and alkylation, most of the γ3-hordein sequence was recovered (data not shown). In contrast, without reduction, no peptides containing cysteine residues were identified suggesting their involvement in disulfide

bridges. Disulfide-linked peptides in tandem MS data sets were identified using the MassMatrix program [29] (Appendix A Fig A2a to A2f). Four disulfide bridges were identified with significant statistical scores (a pp or pp2 value greater than six [29]): $C_{150}-C_{176}$, $C_{196}-C_{276}$, and the two bridges involving consecutive Cys, $C_{183}-C_{268}$ and $C_{184}-C_{268}$ (Table 4). As shown in Table 4, the scores were almost equivalent for bridges $C_{183}-C_{268}$ and $C_{184}-C_{268}$. Both cysteines were also found to be carbamidomethylated (CAM) in the two dipeptides $QQC_{CAM}C_{184}-QQL-C_{268}NVHVPPY$ and $QQC_{183}C_{CAM}QQL-C_{268}NVHVPPY$, showing that one of the consecutive cysteines was free. This led to two hypotheses: heterogeneity in natural γ3-hordein folding, with a bridge involving one or the other consecutive cysteines; or spontaneous changes

Table 2

Mass spectrometric data obtained from the ESI-MS of glycosylated $Q_{136}-E_{144}$ originated from endoproteinase Glu-C digestion of natural γ-3 hordein.

Structure ^a	Mono charged ion [M+H] ⁺	Doubly charged ion [M+2H] ²⁺
Man ₂ GlcNAc ₂ -P	1899	
XylManGlcNAc ₂ -P		935
GlcNAc ₂ -P	1575	
GlcNAc-P	1372	
ManGlcNAc ₂ -P + adduct ^b	1784	
GlcNAc ₂ -P + adduct ^b	1621	
GlcNAc-P + adduct ^b	1419	710
Man ₂ GlcNAc ₂	731	
Man ₂ GlcNAc	529	
XylManGlcNAc	498	
ManGlcNAc	366	
XylMan	295	

^a P corresponds to the peptide moiety $[Q_{136}HQLNLCKE_{144}]$, GlcNAc to N-acetyl glucosamine residue, Man to mannose residue, Xyl to xylose residue.

^b Adduct corresponding to the hydroxylation of lysine and methylamine adduction.

Table 3
MS/MS data from the Q₁₃₆–E₁₄₄ peptide moiety recovered in the case of recombinant or native γ -3 hordein digested with endoproteinase Glu-C compared to the theoretical fragmentation.

Amino acid	Theoretical ionization							
	Q ₁₃₆	y8	y7	y6	y5	y4	y3	y2
	H	Q	L	N	L	C*	K	E ₁₄₄
	b1	b2	b3	b4	b5	b6	b7	b8
Theory	b ions		y ions		Recombinant γ 3 hordein		Native γ 3 hordein	
					b ions	y ions	b ions	y ions
Q	129.07							
H	266.13		1041.53		265.7			1043.3
Q	394.18		904.47		394.4	904.1		904.0
L	507.27		776.41		507.3	776.2	508.3	775.2
N	621.31		663.33		618.1	663.2		662.2
L	734.39		549.28		734.2	549.2		548.4
C*	894.46		436.20			436.0		
K	1022.55		276.14			276.1	1021.6	
E			148.04					

C* indicates that cysteine was carbamidomethylated (M = 160).

between consecutive cysteines during the extraction and analytical process.

The last potential disulfide bridge involved one of the consecutive cysteine residues and C₁₄₂ which is included in the N-glycosylation site N₁₄₀LC₁₄₂. MassMatrix takes into account the fact that a glycan moiety can be added to asparagine residues. Thus the QLNLC₁₄₂K peptide was identified with a carbamidomethylated cysteine and an asparagine residue modified with the addition of one N-acetylglucosamine (Table 4). The tripeptide NLC₁₄₂KEF–QQC₁₈₃C₁₈₄ QQL–C₂₆₈NVH containing a modified N₁₄₀ residue was also identified, but its score was not significant. C₁₄₂, near the glycosylated N₁₄₀ residue, was mainly free, but its involvement in a disulfide bridge could not be absolutely excluded.

A schematic representation of γ 3-hordein structure highlighting the observed post-translational modifications is shown in Fig. 3.

3.5. Involvement of glycosylation and disulfide bridges in IgE binding

Our aim was to determine how the observed post-translational modifications of γ 3-hordein may affect IgE binding *in vitro*. Non-reduced and reduced-alkylated Risø 56 hordein extracts, as well as recombinant reduced-alkylated γ 3-hordein were run on SDS-PAGE and transferred to membranes. After incubation with patient sera, bound IgE were detected. In this experiment IgE reactivity towards γ 3-hordein was compared under three conditions: i) the natural glycosylated protein with disulfide bridges, ii) the reduced natural glycosylated protein, and iii) the recombinant protein, without glycosylation or disulfide bridges. The same amount of γ 3-hordein was loaded in each lane. The protein samples used for IgE binding experiments were the same as those shown in Fig. 1, lanes 1, 2, 4. We tested IgE binding with a set of six sera from patients suffering from five different wheat-dependent allergies (dietary and cutaneous IHHWP, IHHWP through only cutaneous contact, atopic dermatitis, Food allergy, and WDEIA).

A similar IgE-reactivity pattern was observed for all six sera tested. The only differences observed were whether the IgE recognized other γ - or C-hordeins. Two representative examples are shown in Fig. 4 (panels A and B, with control membrane panel C). The semi-quantitative analysis of IgE binding to γ 3-hordein for the six sera is shown in Fig. 5. For each serum, IgE bound strongly to the recombinant reduced-alkylated γ 3-hordein and, to a lesser extent to the natural γ 3-hordein from the reduced-alkylated Risø 56 hordein extract. The difference between these proteins was glycosylation of the natural protein, as no other post-translational modifications were observed in mass spectrometry experiments. Thus, glycosylation decreased IgE binding to γ 3-hordein. This

binding was very weak or absent with natural γ 3-hordein from the non-reduced Risø 56 hordein extract. This suggests that disulfide bridges clearly prevented binding of IgE to γ 3-hordein. Therefore, for the studied sera, both glycosylation and disulfide bridge formation decreased epitope accessibility.

4. Discussion

Glycosylation of γ 3-hordein was demonstrated using immunochemistry and confirmed with mass spectrometry experiments: it occurred at the Asn₁₄₀LeuCys₁₄₂ site, and decreased accessibility to the closest cleavage sites for trypsin and endoproteinase Glu-C.

Although glycosylation led to highly significant differences in the chromatograms of digested recombinant and natural γ 3-hordeins, peptides reflecting an absence of glycosylation were still detected at low levels in the natural γ 3-hordein sample probably due to heterogeneity in the protein glycosylation pattern.

The mass spectra of the glycopeptide detected in the digest of natural γ 3-hordein revealed that the glycan moiety contained at least two N-acetyl glucosamines, two mannoses and one xylose. This is a relatively standard N-glycan composition in plant glycoproteins [33]. However the N-glycosylation site, Asn-X-Cys, with a cysteine residue instead of a serine or threonine residue, is more atypical but has been described previously, for example in [34–37]. Bause and Legler [38] previously showed that the free sulfhydryl group in cysteine could be involved in asparagine glycosylation. This glycosylation site is an important feature in γ 3-hordein synthesis pathway and its resulting structure because the cysteine at this position (Cys₁₄₂) cannot be involved in formation of intra-molecular disulfide bridges at the time of glycosylation. Thus it appears that the protein was glycosylated in the endoplasmic reticulum prior to disulfide bridge formation, as suggested by Miletich and Broze [34]. This glycosylation pattern also indicates that the protein trafficked through the Golgi apparatus, where the precursor oligosaccharide was modified by addition of a xylose.

To our knowledge, this study is the first detailed analysis of a N-glycosylation site on a prolamin and the first evidence that the corresponding N-glycan contains xylose. In general, prolamins from wheat, barley or rye are considered not to be glycosylated. However, several immunochemical studies did give some indication for glycosylation among gliadin, glutenin or hordein families [14,15]. In our study, a signal was also detected in the Risø 56 hordein extract, with proteins that migrated like C- and γ -hordeins. As C-hordeins

Table 4

LC-MS/MS analysis of natural γ 3-hordein disulphide bridges according to MassMatrix software version 1.3.0 [29]. Corresponding MS/MS spectra are detailed in the supplementary data (Appendix A Fig A2a to A2f).

Peptides containing cysteines	Observed MW	Charge	Δ mass	Scores			Bridge
				pp	pp2	pp _{tag}	
ISQQNSC ₁₇₆ QL LLQQC ₁₅₀ TL	1835.72	1	0.18	10.9	13.3	4.6	C ₁₅₀ -C ₁₇₆
ISQQNSC ₁₇₆ QL LLQQC ₁₅₀ TL	1836.21	2	0.31	6.5	6.4	1.7	C ₁₅₀ -C ₁₇₆
LRPHISQQNSC ₁₇₆ QL LQQC ₁₅₀ TL	2339.69	2	0.5	15.1	8.8	3.8	C ₁₅₀ -C ₁₇₆
QQC ₁₈₃ CQQL C ₂₆₈ NVH	1320.25	2	0.72	12.2	6.5	2.2	C ₁₈₃ -C ₂₆₈
QQC ₁₈₄ CQQL C ₂₆₈ NVH	1320.25	2	0.72	11.2	6.6	1.3	C ₁₈₄ -C ₂₆₈
C ₁₉₆ PAIQTIVHAIVM* C ₂₇₆ SPF	1862.97	2	1.03	5.9	7.6	6.5	C ₁₉₆ -C ₂₇₆
QQC ₁₈₃ C*QQL C ₂₆₈ NVHVPPY	1832.29	2	0.49	7.6	6.6	0.7	C ₁₈₃ -C ₂₆₈
QQC*C ₁₈₄ CQQL C ₂₆₈ NVHVPPY	1832.29	2	0.49	6.8	6.1	0.7	C ₁₈₄ -C ₂₆₈
QLN ^N LC* ₁₄₂ K	979.23	3	0.73	8.3	7.3	0.7	
N ^N LC ₁₄₂ KEF QQC ₁₈₃ C ₁₈₄ CQQL C ₂₆₈ NVH	2274.09	3	1.14	1.6	6.7	0.8	C ₁₄₂ ^a C ₁₈₃ C ₁₈₄ C ₂₆₈

The sample was natural γ 3-hordein after chymotrypsin + trypsin digestion.

M*: oxidized Met (mass + 16). C*: carbamidomethylated Cys (mass + 57).

N^N: Asn + N-acetylglucosamine (mass + 203). *The scores for the tri-peptide were not significant.

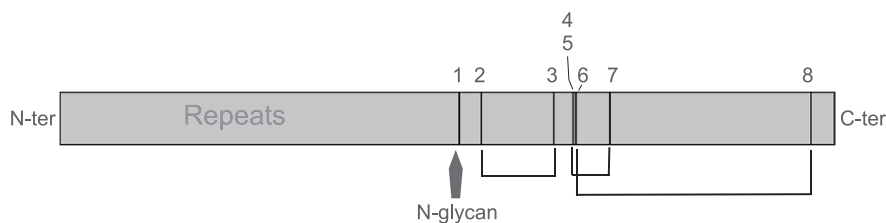


Fig. 3. A schematic diagram showing post-translational modifications of γ 3-hordein: glycosylation and disulfide bridges as deduced from our results. Numbers correspond to cysteine residues. 1: C₁₄₂, 2: C₁₅₀, 3: C₁₇₆, 4: C₁₈₃, 5: C₁₈₄, 6: C₁₉₆, 7: C₂₆₈, 8: C₂₇₆.

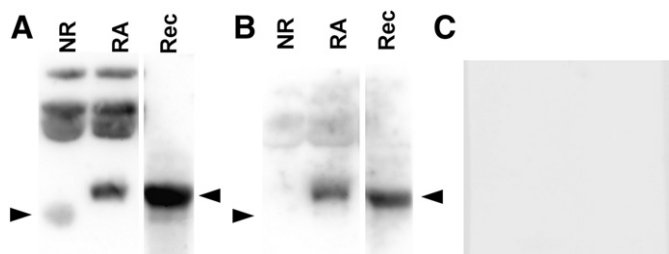


Fig. 4. IgE reactivity of two patient sera towards hordeins. Protein samples were: non-reduced Risø 56 hordein extract (NR); reduced-alkylated Risø 56 hordein extract (RA); reduced-alkylated recombinant γ 3-hordein (Rec). Chemiluminescence detection of IgE on a membrane incubated with serum no. 1, from a patient diagnosed with dietary and cutaneous IHHWP (A), on another membrane incubated with serum no. 2, from a patient diagnosed with WDEIA (B), and on a control membrane incubated without serum (C). γ 3-hordein is indicated with arrow heads.

do not contain cysteine and probably no glycosylation sites, the observed signal could correspond to glycosylation of other hordeins. Because many proteins in the prolamin family are highly homologous, other prolamins could contain the same glycosylation site as γ 3-hordein, and thus, perhaps, be glycosylated. A BLAST search of the UniProt protein sequence database using the γ 3-hordein sequence as query showed that many gliadins, glutenins, secalins or hordeins display the Asn-Pro-Cys sequence at a similar position. However, this sequence is not a glycosylation site because the proline residue was described to inhibit transfer of oligosaccharide onto the classic Asn-X-(Ser/Thr) site [39]. The Asn-Ser-Cys site, which is not glycosylated in γ 3-hordein, is also frequently found in various prolamins. The same search using translated BLAST (tblastn from the US National Library of Medicine) against the Expressed Sequence Tags database (dbEST) on the NCBI website identified some wheat sequences which are strictly equivalent to γ 3-hordein and contain the same Asn-Leu-Cys glycosylation site (for example Genbank accessions : [CJ636154](#), [CD909761](#), [CJ527721](#), [CD918887](#), [CD918888](#), [CD900609](#), [CD901853](#), [AL811346](#), [CJ527722](#), [CD908515](#), [CD902631](#), [GD186717](#), [GD186708](#), [CJ636155](#), [GD186728](#)). Thus sequence similarities suggest that some wheat prolamins could be glycosylated like γ 3-hordein.

Findings from immuno-histochemical studies also support this hypothesis. Several studies previously showed that for these storage proteins, two trafficking pathways can occur in wheat endosperm: they are transported into the vacuole through the Golgi apparatus or accumulate directly in the lumen of the endoplasmic reticulum [40].

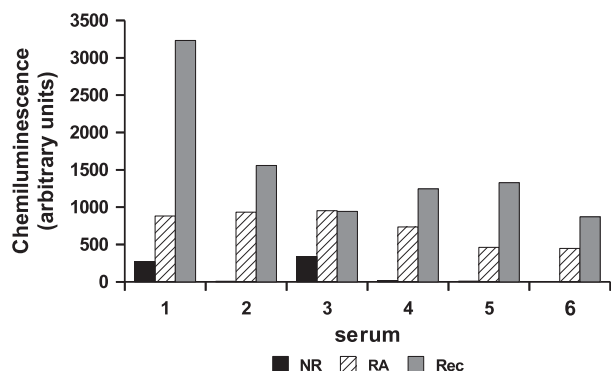


Fig. 5. IgE reactivity of patient sera towards γ 3-hordein. Semi-quantitative analysis of chemiluminescence IgE detection on membranes with non-reduced Risø 56 hordein extract (NR), reduced-alkylated Risø 56 hordein extract (RA), and reduced-alkylated recombinant γ 3-hordein (Rec). Chemiluminescence is in arbitrary units. Patients were diagnosed with various wheat allergies: dietary and cutaneous IHHWP (serum 1), WDEIA (serum 2), atopic eczema dermatitis syndrome (serum 3), food allergy (serum 4), IHHWP after only cutaneous contact (sera 5, 6).

Thus, it does appear possible, although rare, that other proteins belonging to the prolamin family have a similar glycosylation pattern to that of γ 3-hordein.

The organization of γ 3-hordein disulfide bridges observed here is very similar to data obtained for wheat γ 46-gliadin, a protein homologous to barley γ 3-hordein [41]; an overview of wheat γ 46-gliadin structure is shown in [42]. γ 3-hordein differs from γ 46-gliadin mainly due to the absence of a bridge between C₁₄₂ and the C₁₈₃ or C₁₈₄, as a result of glycosylation of the majority of γ 3-hordein molecules. In one LMW-GS showing equivalent disulfide bridge folding [43] the partners for consecutive cysteines were determined: Cys no. 4 (equivalent to C₁₈₃ in γ 3-hordein) was bound to Cys no. 1 (C₁₄₂ in γ 3-hordein) and Cys no. 5 (C₁₈₄ in γ 3-hordein) was bound to the Cys no. 7 (C₂₆₈ in γ 3-hordein). γ 3-hordein folding confirms that barley or wheat prolamins, with the same number of cysteines, share significant homology in disulfide bridge folding as shown previously [41–45].

The patients selected for the IgE-reactivity experiment are sensitized to wheat products but they were chosen because their IgE are known to react with γ 3-hordein. In general, there is no direct sensitization to barley due to the absence of contact with this cereal which is not commonly used in human food, except for beer production. As beer contains a very low concentration of barley proteins, consumers are exposed to low levels. IgE reactivity towards γ 3-hordein is therefore probably due to a cross-reaction between wheat and barley proteins resulting from sequence and structural homologies.

The patients selected suffer from a large spectrum of wheat allergies. These differ due to the route of sensitization and the various processes to which the allergens were subjected (milling, fermentation and cooking, industrial hydrolysis). In a previous study we observed that γ 3-hordein binds IgE through different epitopes in WDEIA and IHHWP [17]. Despite the diversity in patient sensitization, our results showed that exactly the same features were involved in IgE binding to γ 3-hordein in each case. The intensity of this binding was clearly related to epitope accessibility, which was favored by disulfide bridge cleavage, and the absence of *N*-glycans. Thus, while cross-reactivity between different allergens is sometimes due to carbohydrate determinants [11], it is not the case for γ 3-hordein, where the *N*-glycan seems clearly not involved in IgE binding.

5. Conclusion

In this study we fully characterized the *N*-glycosylation of a cereal storage protein for the first time. The xylose found in the *N*-glycan is evidence that γ 3-hordein trafficked through the Golgi apparatus. It raises the question of whether some equivalent proteins in wheat are also glycosylated, as suggested by previous immunochemical results. This question relates to our knowledge of the synthesis conditions of prolamins, their trafficking in endosperm, and accumulation in grain. Some prolamins are known to react with IgE in serum from wheat allergic patients but little is known about the structure of IgE-reacting epitopes. In the case of γ 3-hordein, the epitopes recognized by IgE were not carbohydrate determinants, or related to protein folding; they appear to be either linear, or depend on only very localized non-covalent folding of peptide chains.

Acknowledgements

We greatly acknowledge Lucia Eynard, a former post doctoral fellow in our group, who initiated this work and gained the first evidence for γ 3-hordein glycosylation. L. Jestin (Unité Mixte de Recherche, Institut National de la Recherche Agronomique-Université Clermont II, Amélioration et Santé des Plantes, Clermont-Ferrand, France), is greatly acknowledged for providing us with grain from the barley Risø56 mutant. Part of this work was supported by the AQS program no.

01P0622 from the French Research Ministry, and French grant ANR-PRA program no. 291.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbapap.2012.07.016>.

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Supplementary Material (for online publication)

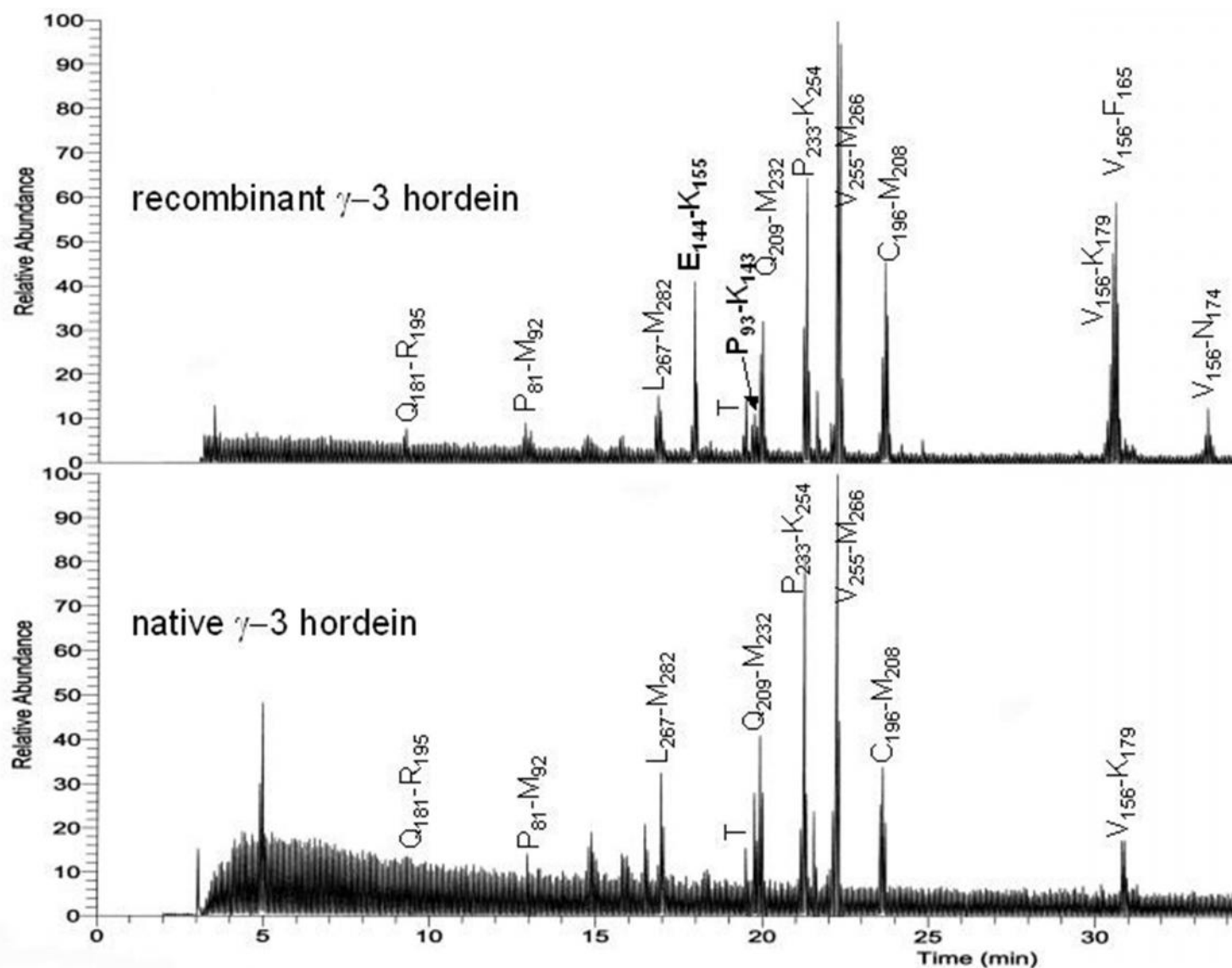


Fig. A1. LC-MS base peak chromatograms of peptides obtained from the digestion of recombinant and native γ 3 hordein with CNBr and trypsin. Peptides were recognized from their MS/MS analyse. Peptides in bold disappeared in the case of native γ 3 hordein. T was a peptide originated from trypsin autolysis.

Table A1– Summary of peptides identified with MS/MS from recombinant $\gamma 3$ hordein proteolysed with (i) CNBr, (ii) CNBr and trypsin or (iii) endoproteinase Glu-C.

Peptide position	calculated monoisotopic [M + H] ⁺ ^a (Da)	observed [M + H] ⁺ ^b (Da)	modification ^c	charge state of major ions
(i) CNBr attack (4vinyl-pyridine-alkylated natural $\gamma 3$ hordein)				
I1-M6	619.33	619.3		+ 1
P81-M92	1421.72	1421.7		+ 1, + 2
L267-M282	1912.88	1912.8	2 PE Cys	+ 1, + 2, + 3
Q209-M232	2633.32	2633.3		+ 2, + 3
Q7-M80	8872.41	8894.3	+ Na	+ 5, + 6
P233-M266	3712.06	3712.4		+ 2, + 3, + 4
P93-M208	14057.15	14122.1	6 PE Cys + 3 Na	+ 9, + 10, + 11
(ii) CNBr attack + trypsin digestion (4vinyl-pyridine-alkylated natural $\gamma 3$ hordein)				
Q181-R195	1972.91	1972.8	2 PE Cys	+ 2, + 3
P81-M92	1421.72	1439.6	HS	+ 1, + 2
L267-M282	1912.88	1930.6	2 PE Cys + HS	+ 1, + 2, + 3
E144-K155	1571.78	1571.7	1 PE Cys	+ 1, + 2
P93-K143	6139.07	6139.0	1 PE Cys	+ 4, + 5
Q209-M232	2633.32	2651.2	HS	+ 2, + 3
P233-K254	2522.35	2522.2		+ 2, + 3
V255-M266	1226.73	1244.6	HS	+ 1
C196-M208	1470.80	1488.7	1 PE Cys + HS	+ 1, + 2
V156-K179	2840.56	2840.5	1 PE Cys	+ 2, + 3
(iii) Endoproteinase Glu-C digestion (iodoacetamide-alkylated natural $\gamma 3$ hordein)				
Q136-E144	1169.57	1169.4	1 CAM Cys	+ 1, + 2
F145-E154	1266.60	1266.5	1 CAM Cys	+ 1
I1-E14	1670.77	1670.6		+ 1, + 2
K155-E192	4563.35	4563.2	3 CAM Cys	+ 3, + 4

^a theoretical monoisotopic peptide masses were predicted from SIB (Swiss Institute of Bioinformatics) proteomic tools

(http://web.expasy.org/peptide_mass) taking account cysteine residues modification and methionine modification to form homoserine lactone.

^b measured monoisotopic peptide masses indicate other modifications : methionine modified to form homoserine instead of homoserine lactone and presence of sodium adduct.

^c observed modifications were precised as cysteine residues pyridylethylated (PE Cys) or carbamidomethylated (CAM Cys), methionine modified as homoserine (HS) and presence of sodium adduct.

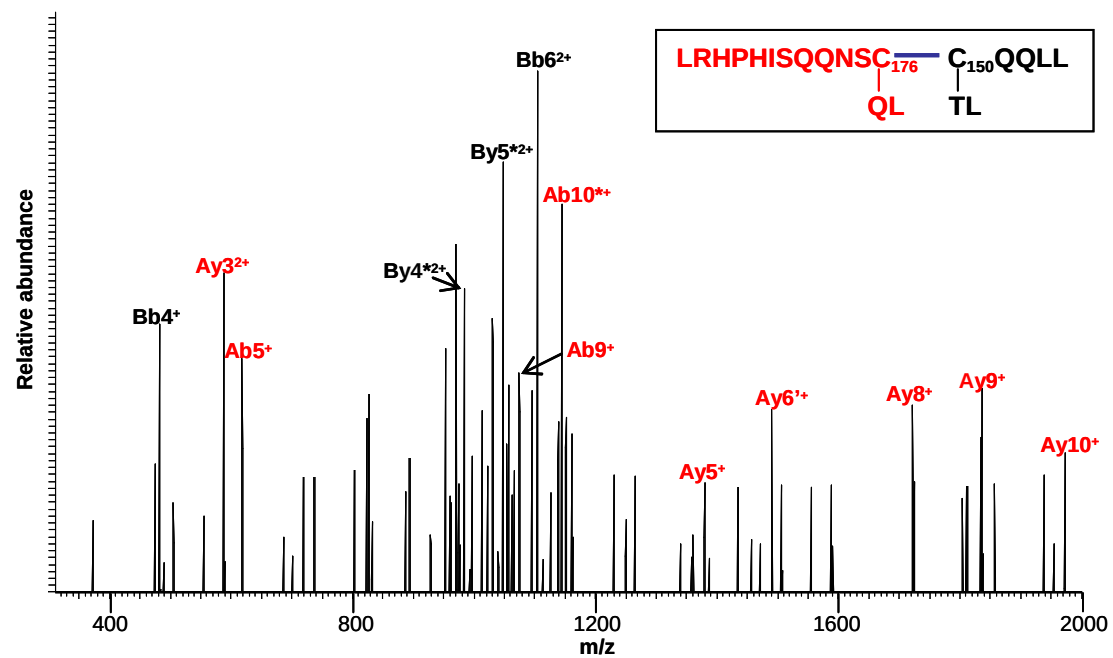


Fig. A2a1. Tandem mass spectrum of the ion $m/z = 1170.35$ ($z = 2$) corresponding to the dipeptide LRPHISQQNSCQL – LLQQCTL. Major ions are indicated as referred in the table of ions below with those coming from chain A in red and from chain B in black.

chainA :

#	b ^{'++}	b ^{*++}	b ⁺⁺	b ^{'+}	b ^{*+}	b ⁺	seq	y ^{'++}	y ^{*++}	y ⁺⁺	y ^{'+}	y ^{*+}	y ⁺	#
1	--	--	57.55	--	--	114.09	L	1161.10	1161.59	1170.10	2321.19	2322.17	2339.20	M
2	--	127.09	135.60	--	253.17	270.19	R	1104.55	1105.05	1113.56	2208.10	2209.09	2226.11	12
3	--	175.61	184.13	--	350.22	367.25	P	1026.50	1027.00	1035.51	2052.00	2052.98	2070.01	11
4	--	244.14	252.66	--	487.28	504.30	H	977.98	978.47	986.98	1954.95	1955.93	1972.96	10
5	--	300.68	309.20	--	600.36	617.39	I	909.45	909.94	918.45	1817.89	1818.87	1835.90	9
6	343.71	344.20	352.71	686.41	687.39	704.42	S	852.91	853.40	861.91	1704.80	1705.79	1722.81	8
7	407.74	408.23	416.74	814.47	815.45	832.48	Q	809.39	809.88	818.40	1617.77	1618.76	1635.78	7
8	471.77	472.26	480.77	942.53	943.51	960.54	Q	745.36	745.85	754.37	1489.71	1490.70	1507.72	6
9	528.79	529.28	537.79	1056.57	1057.55	1074.58	N	681.33	681.82	690.34	1361.66	1362.64	1379.67	5
10	572.30	572.80	581.31	1143.60	1144.59	1161.61	S	624.31	624.80	633.32	1247.61	1248.60	1265.62	4
11	1031.52	1032.01	1040.52	2062.03	2063.02	2080.04	C	580.79	581.29	589.80	1160.58	1161.56	1178.59	3
12	1095.55	1096.04	1104.55	2190.09	2191.07	2208.10	Q	121.58	122.07	130.58	242.15	243.13	260.16	2
	--	--	--	--	--	--	L	57.55	--	66.55	114.09	--	132.10	1

chainB :

#	b ^{'++}	b ^{*++}	b ⁺⁺	b ^{'+}	b ^{*+}	b ⁺	seq	y ^{'++}	y ^{*++}	y ⁺⁺	y ^{'+}	y ^{*+}	y ⁺	#
1	--	--	57.55	--	--	114.09	L	--	--	--	--	--	--	
2	--	--	114.09	--	--	227.18	L	1104.55	1105.05	1113.56	2208.10	2209.09	2226.11	6
3	--	169.61	178.12	--	338.21	355.23	Q	1048.01	1048.50	1057.02	2095.02	2096.00	2113.03	5
4	--	233.64	242.15	--	466.27	483.29	Q	983.98	984.47	992.99	1966.96	1967.94	1984.97	4
5	1045.03	1045.52	1054.03	2089.04	2090.03	2107.05	C	919.95	920.45	928.96	1838.90	1839.88	1856.91	3
6	1095.55	1096.04	1104.55	2190.09	2191.07	2208.10	T	108.07	--	117.08	215.14	--	233.15	2
	--	--	--	--	--	--	L	57.55	--	66.55	114.09	--	132.10	1

Fig. A2a2. Table of ions associated to the above tandem mass spectrum displaying their nature (y or b series, charge state, water loss). Among theoretical ions, red ions are present into the fragmentation mass spectrum. Chain A in red.

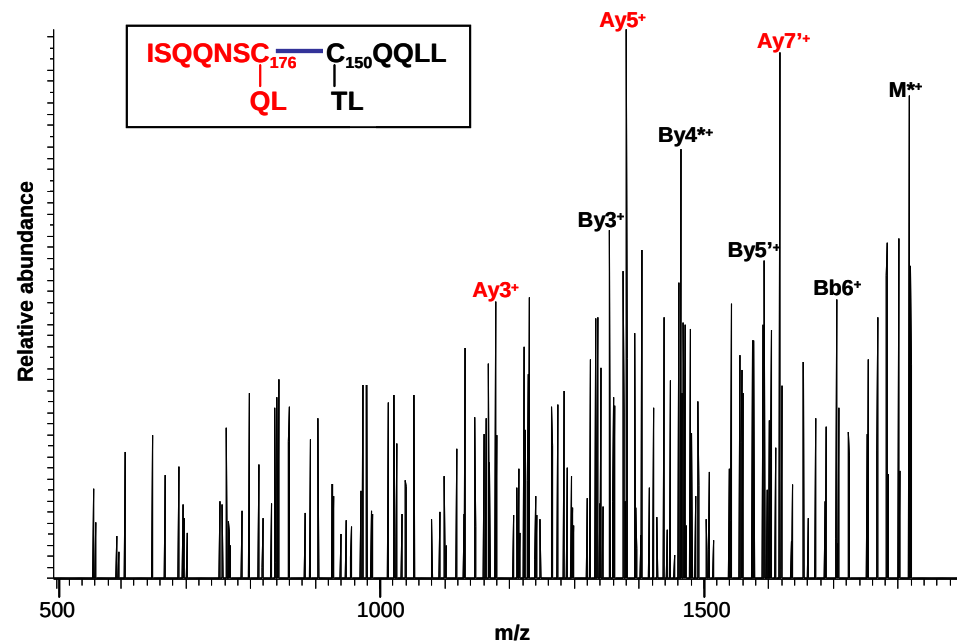


Fig. A2b1. Tandem mass spectrum of the ion $m/z = 1835.72$ ($z = 1$) corresponding to the dipeptide ISQQNSCQL – LLQQCTL. Major ions are indicated as referred in the table of ions below with those coming from chain A in red and from chain B in black.

chainA :

#	b ⁺	b ⁺⁺	b ⁺	seq	y ⁺	y ⁺⁺	y ⁺	#
1	---	---	114.09	I	1817.89	1818.87	1835.90	M
2	183.11	---	201.12	S	1704.80	1705.79	1722.81	8
3	311.17	312.16	329.18	Q	1617.77	1618.76	1635.78	7
4	439.23	440.21	457.24	Q	1489.71	1490.70	1507.72	6
5	553.27	554.26	571.28	N	1361.66	1362.64	1379.67	5
6	640.30	641.29	658.32	S	1247.61	1248.60	1265.62	4
7	1558.74	1559.72	1576.75	C	1160.58	1161.56	1178.59	3
8	1686.79	1687.78	1704.80	Q	242.15	243.13	260.16	2
	---	---	---	L	114.09	---	132.10	1

chainB :

#	b ⁺	b ⁺⁺	b ⁺	seq	y ⁺	y ⁺⁺	y ⁺	#
1	---	---	114.09	L	---	---	---	
2	---	---	227.18	L	1704.80	1705.79	1722.81	6
3	---	338.21	355.23	Q	1591.72	1592.70	1609.73	5
4	---	466.27	483.29	Q	1463.66	1464.65	1481.67	4
5	1585.75	1586.73	1603.76	C	1335.60	1336.59	1353.61	3
6	1686.79	1687.78	1704.80	T	215.14	---	233.15	2
	---	---	---	L	114.09	---	132.10	1

Fig. A2b2. Table of ions associated to the above tandem mass spectrum displaying their nature (y or b series, charge state, water loss). Among theoretical ions, red ions are present into the fragmentation mass spectrum. Chain A in red.

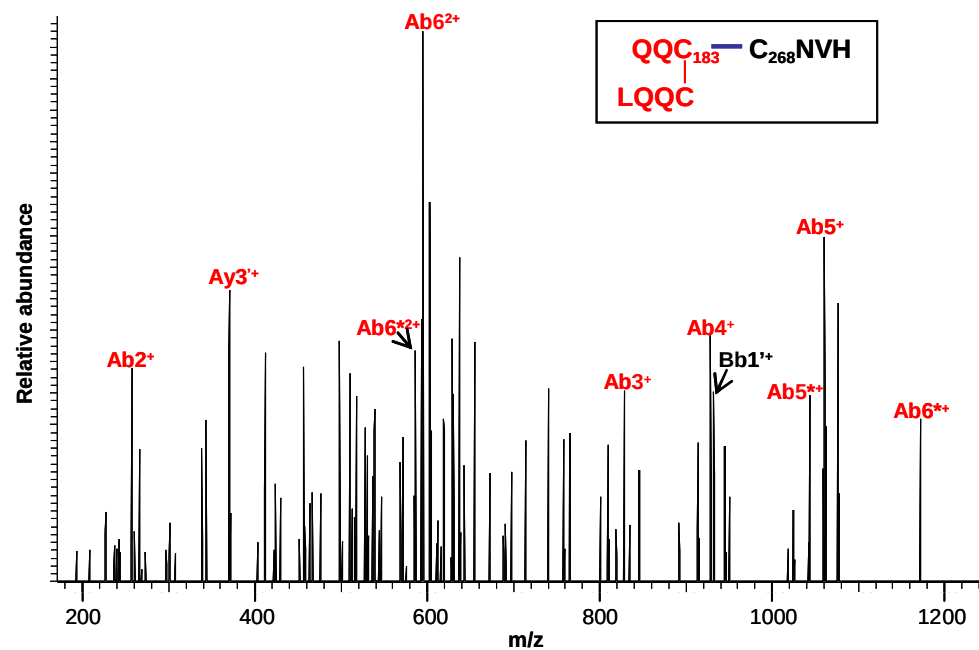


Fig. A2c1. Tandem mass spectrum of the ion $m/z = 660.63$ ($z = 2$) corresponding to the dipeptide QQCCQQL – CNVH. Major ions are indicated as referred in the table of ions below with those coming from chain A in red and from chain B in black.

chainA :

#	b ⁺⁺⁺	b ^{*++}	b ⁺⁺	b ⁺	b ^{**}	b ⁺	seq	y ⁺⁺⁺	y ^{*++}	y ⁺⁺	y ⁺	y ^{*+}	y ⁺	#
1	---	56.52	65.04	---	112.04	129.07	Q	651.26	651.75	660.27	1301.52	1302.50	1319.53	M
2	---	120.55	129.07	---	240.10	257.12	Q	587.23	587.73	596.24	1173.46	1174.44	1191.47	6
3	406.15	406.64	415.16	811.30	812.28	829.31	C	523.20	523.70	532.21	1045.40	1046.39	1063.41	5
4	457.66	458.15	466.66	914.31	915.29	932.32	C	237.11	237.60	246.12	473.22	474.20	491.23	4
5	521.69	522.18	530.69	1042.37	1043.35	1060.38	Q	185.61	186.10	194.61	370.21	371.19	388.22	3
6	585.72	586.21	594.72	1170.42	1171.41	1188.43	Q	121.58	122.07	130.58	242.15	243.13	260.16	2
	---	---	---	---	---	---	L	57.55	---	66.55	114.09	---	132.10	1

chainB :

#	b ⁺⁺⁺	b ^{*++}	b ⁺⁺	b ⁺	b ^{**}	b ⁺	seq	y ⁺⁺⁺	y ^{*++}	y ⁺⁺	y ⁺	y ^{*+}	y ⁺	#
1	467.17	467.66	476.18	933.34	934.32	951.35	C	---	---	---	---	---	---	
2	524.19	524.69	533.20	1047.38	1048.36	1065.39	N	176.09	176.58	185.10	351.18	352.16	369.19	3
3	573.73	574.22	582.73	1146.45	1147.43	1164.46	V	119.07	---	128.08	237.13	---	255.15	2
	---	---	---	---	---	---	H	69.54	---	78.54	138.07	---	156.08	1

Fig. A2c2. Table of ions associated to the above tandem mass spectrum displaying their nature (y or b series, charge state, water loss). Among theoretical ions, red ions are present into the fragmentation mass spectrum. Chain A in red.

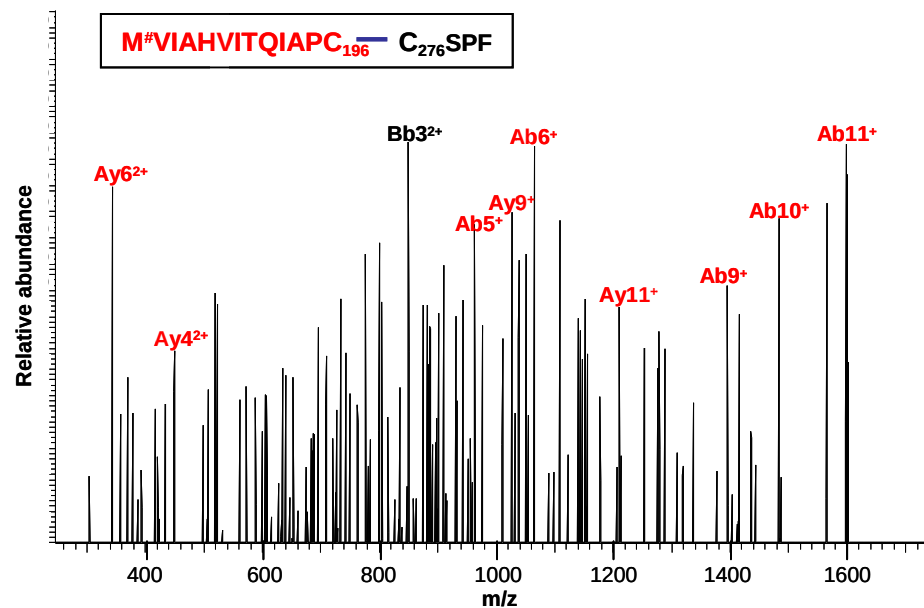


Fig. A2d1. Tandem mass spectrum of the ion $m/z = 931.97$ ($z = 2$) corresponding to the dipeptide CPAIQTIVHAIVM[#] – CSPF with oxidized methionine residue. Major ions are indicated as referred in the table of ions below with those coming from chain A in red and from chain B in black.

chainA :

#	b ⁺⁺	b ⁺⁺⁺	b ⁺⁺	b ⁺	b ⁺⁺	b ⁺	seq	y ⁺⁺	y ⁺⁺⁺	y ⁺⁺	y ⁺	y ⁺	y ⁺	#
1	268.59	--	277.59	536.16	--	554.17	C	922.45	922.94	931.45	1843.89	1844.87	1861.90	M
2	317.11	--	326.12	633.22	--	651.23	P	645.87	646.36	654.87	1290.72	1291.71	1308.73	12
3	352.63	--	361.64	704.25	--	722.26	A	597.34	597.83	606.34	1193.67	1194.66	1211.68	11
4	409.17	--	418.18	817.34	--	835.35	I	561.82	562.31	570.83	1122.63	1123.62	1140.64	10
5	473.20	473.69	482.21	945.40	946.38	963.41	Q	505.28	505.77	514.28	1009.55	1010.53	1027.56	9
6	523.73	524.22	532.73	1046.44	1047.43	1064.45	T	441.25	--	450.25	881.49	--	899.50	8
7	580.27	580.76	589.27	1159.53	1160.51	1177.54	I	390.73	--	399.73	780.44	--	798.45	7
8	629.80	630.29	638.81	1258.60	1259.58	1276.61	V	334.18	--	343.19	667.36	--	685.37	6
9	698.33	698.82	707.34	1395.65	1396.64	1413.67	H	284.65	--	293.65	568.29	--	586.30	5
10	733.85	734.34	742.85	1466.69	1467.68	1484.70	A	216.12	--	225.13	431.23	--	449.24	4
11	790.39	790.88	799.40	1579.78	1580.76	1597.79	I	180.60	--	189.61	360.20	--	378.21	3
12	839.93	840.42	848.93	1678.84	1679.83	1696.85	V	124.06	--	133.06	247.11	--	265.12	2
	--	--	--	--	--	--	M	74.52	--	83.53	148.04	--	166.05	1

chainB :

#	b ⁺⁺	b ⁺⁺⁺	b ⁺⁺	b ⁺	b ⁺⁺	b ⁺	seq	y ⁺⁺	y ⁺⁺⁺	y ⁺⁺	y ⁺	y ⁺	y ⁺	#
1	747.87	748.36	756.87	1494.73	1495.71	1512.74	C	--	--	--	--	--	--	
2	791.38	791.87	800.39	1581.76	1582.74	1599.77	S	166.58	--	175.59	332.16	--	350.17	3
3	839.91	840.40	848.91	1678.81	1679.80	1696.82	P	123.07	--	132.07	245.13	--	263.14	2
	--	--	--	--	--	--	F	74.54	--	83.55	148.08	--	166.09	1

Fig. A2d2. Table of ions associated to the above tandem mass spectrum displaying their nature (y or b series, charge state, water loss). Among theoretical ions, red ions are present into the fragmentation mass spectrum. Chain A in red.

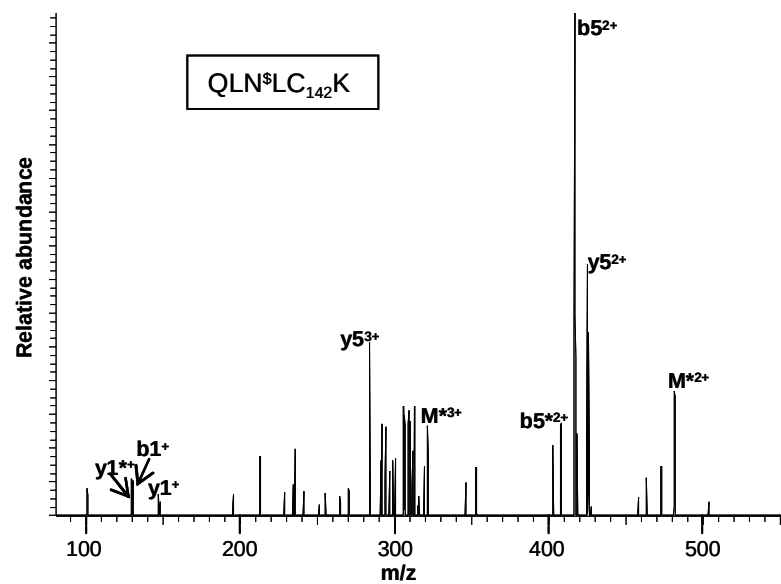


Fig. A2e1. Tandem mass spectrum of the ion $m/z = 327.08$ ($z = 3$) corresponding to the peptide QLN^SLCK with carbamidomethylated cysteine and the addition of *N*-acetylglucosamine on asparagine residue. Major ions are indicated as referred in the table of ions below.

#	b ^{'+3}	b ^{*+3}	b ⁺³	b ^{'++}	b ^{*++}	b ⁺⁺	b ^{'+}	b ^{*+}	b ⁺	seq	y ^{'+3}	y ^{*+3}	y ⁺³	y ^{'++}	y ^{*++}	y ⁺⁺	y ^{'+}	y ^{*+}	y ⁺	#
1	—	38.02	43.69	—	56.52	65.04	—	112.04	129.07	Q	320.83	321.16	326.84	480.74	481.24	489.75	960.48	961.47	978.49	M
2	—	75.71	81.39	—	113.07	121.58	—	225.12	242.15	L	278.15	278.47	284.15	416.72	417.21	425.72	832.42	833.41	850.43	5
3	—	181.42	187.10	—	271.63	280.14	—	542.25	559.27	N	240.45	240.78	246.45	360.17	360.67	369.18	719.34	720.32	737.35	4
4	—	219.11	224.79	—	328.17	336.68	—	655.33	672.36	L	134.74	135.07	140.75	201.61	202.10	210.62	402.22	403.20	420.23	3
5	—	272.46	278.13	—	408.18	416.70	—	815.36	832.39	C	97.05	97.38	103.05	145.07	145.56	154.08	289.13	290.12	307.14	2
	—	—	—	—	—	—	—	—	—	K	43.71	44.03	49.71	65.05	65.55	74.06	129.10	130.09	147.11	1

Fig. A2e2. Table of ions associated to the above tandem mass spectrum displaying their nature (y or b series, charge state, water loss). Among theoretical ions, red ions are present into the fragmentation mass spectrum.

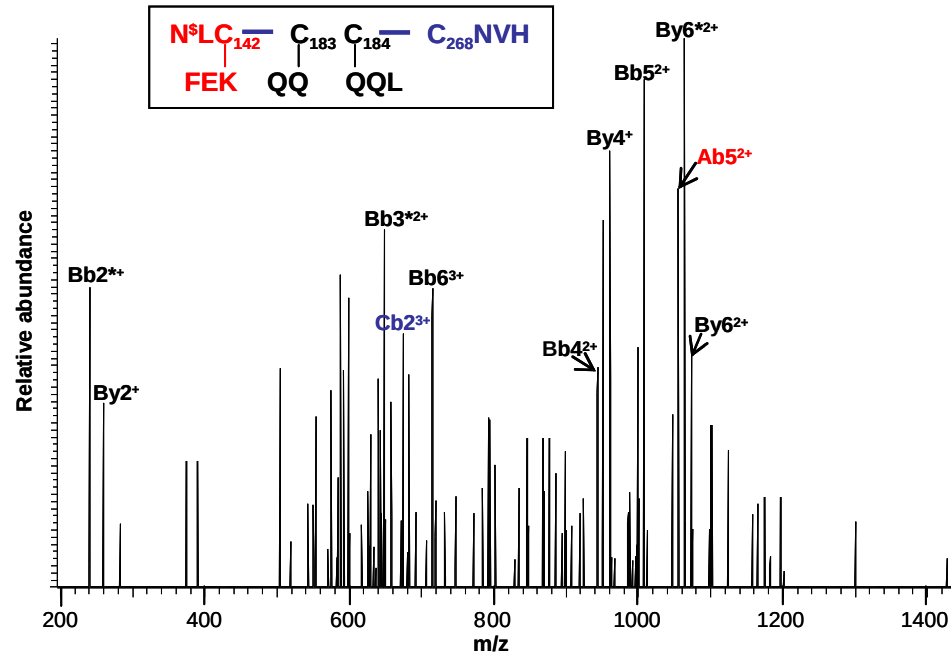


Fig. A2f1. Tandem mass spectrum of the ion $m/z = 758.70$ ($z = 3$) corresponding to the tripeptide N^SLCKEF – QQCCQQL - CNVH with the addition of *N*-acetylglucosamine on asparagine residue N₁₄₀. Major ions are indicated as referred in the table of ions below with those coming from chain A in red, from chain B in black and, from chain C in blue.

chain A

#	b ^{'+3}	b ^{*+3}	b ⁺³	b ^{'++}	b ^{*++}	b ⁺⁺	b ^{'+}	b ^{*+}	b ⁺	seq	y ^{'+3}	y ^{*+3}	y ⁺³	y ^{'++}	y ^{*++}	y ⁺⁺	y ^{'+}	y ^{*+}	y ⁺	#
1	--	--	106.71	--	--	159.57	--	--	318.13	N	752.32	752.64	758.32	1127.97	1128.46	1136.98	2254.93	2255.92	2272.95	M
2	--	--	144.41	--	--	216.11	--	--	431.21	L	646.61	646.94	652.61	969.41	969.90	978.42	1937.81	1938.80	1955.82	5
3	611.58	611.91	617.58	916.86	917.35	925.87	1832.72	1833.70	1850.73	C	608.91	609.24	614.92	912.87	913.36	921.87	1824.73	1825.71	1842.74	4
4	654.28	654.60	660.28	980.91	981.40	989.92	1960.81	1961.80	1978.82	K	135.74	136.07	141.75	203.11	203.60	212.12	405.21	406.20	423.22	3
5	697.29	697.62	703.29	1045.43	1045.92	1054.44	2089.86	2090.84	2107.87	E	93.04	--	99.05	139.06	--	148.07	277.12	--	295.13	2
	--	--	--	--	--	--	--	--	--	F	50.03	--	56.03	74.54	--	83.55	148.08	--	166.09	1

chainB :

#	b ^{'+3}	b ^{*+3}	b ⁺³	b ^{'++}	b ^{*++}	b ⁺⁺	b ^{'+}	b ^{*+}	b ⁺	seq	y ^{'+3}	y ^{*+3}	y ⁺³	y ^{'++}	y ^{*++}	y ⁺⁺	y ^{'+}	y ^{*+}	y ⁺	#
1	--	38.02	43.69	--	56.52	65.04	--	112.04	129.07	Q	--	--	--	--	--	--	--	--	--	
2	--	80.70	86.38	--	120.55	129.07	--	240.10	257.12	Q	709.6 3	709.9 6	715.6 3	1063.9 4	1064.4 3	1072.9 5	2126.8 8	2127.8 6	2144.8 9	6
3	432.5 2	432.8 5	438.5 2	648.27	648.77	657.28	1295.5 4	1296.5 2	1313.5 5	C	666.9 4	667.2 7	672.9 5	999.91	1000.4 0	1008.9 2	1998.8 2	1999.8 0	2016.8 3	5
4	623.2 5	623.5 7	629.2 5	934.37	934.86	943.37	1867.7 2	1868.7 1	1885.7 3	C	314.8 0	315.1 3	320.8 1	471.70	472.19	480.70	942.39	943.38	960.40	4
5	665.9 3	666.2 6	671.9 4	998.39	998.89	1007.4 0	1995.7 8	1996.7 7	2013.7 9	Q	124.0 7	124.4 0	130.0 8	185.61	186.10	194.61	370.21	371.19	388.22	3
6	708.6 2	708.9 5	714.6 2	1062.4 2	1062.9 2	1071.4 3	2123.8 4	2124.8 2	2141.8 5	Q	81.39	81.72	87.39	121.58	122.07	130.58	242.15	243.13	260.16	2
	--	--	--	--	--	--	--	--	--	L	38.70	--	44.71	57.55	--	66.55	114.09	--	132.10	1

chainC :

#	b ^{'+3}	b ^{*+3}	b ⁺³	b ^{'++}	b ^{*++}	b ⁺⁺	b ^{'+}	b ^{*+}	b ⁺	seq	y ^{'+3}	y ^{*+3}	y ⁺³	y ^{'++}	y ^{*++}	y ⁺⁺	y ^{'+}	y ^{*+}	y ⁺	#
1	629.59	629.92	635.59	943.88	944.37	952.89	1886.75	1887.74	1904.76	C	--	--	--	--	--	--	--	--	--	
2	667.60	667.93	673.61	1000.90	1001.39	1009.91	2000.80	2001.78	2018.81	N	117.73	118.06	123.73	176.09	176.58	185.10	351.18	352.16	369.19	3
3	700.63	700.95	706.63	1050.44	1050.93	1059.44	2099.87	2100.85	2117.88	V	79.72	--	85.72	119.07	--	128.08	237.13	--	255.15	2
	--	--	--	--	--	--	--	--	--	H	46.69	--	52.70	69.54	--	78.54	138.07	--	156.08	1

Fig. A2f2. Table of ions associated to the above tandem mass spectrum displaying their nature (y or b series, charge state, water loss). Among theoretical ions, red ions are present into the fragmentation mass spectrum. Chain A in red and chain C in blue.