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# A Novel LC−MS Application To Investigate Oxidation of Peptides Isolated from β-Lactoglobulin

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**ABSTRACT:** Oxidation of β-lactoglobulin (β-Lg), a typical milk whey protein, was investigated by oxidizing its three tryptic peptides after separation and fractionation by preparative HPLC. Oxidation was performed with $H_2O_2$ and $Fe^{3+}$ in piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer containing ascorbic acid, by keeping the samples in an oven of +37 °C for 14 days. Changes in the oxidized peptides were then analyzed with LC−ESI-QIT-MS. The peptides chosen were Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR), Leu-Ile-Val-Thr-Gln-Thr-Met-Lys (LIVTQTMK) and Val-Leu-Val-Leu-Asp-Thr-Asp-Tyr-Lys (VLVLDTDYK), all containing amino acids of oxidative interest. Especially methionine (M) was prone to oxidize as well as dioxidize, along with tyrosine (Y), histidine (H) and/or proline (P). The ions *m/z* 854 [ALPMHIR + O], *m/z* 950 [LIVTQTMK + O] and *m/z* 966 [LIVTQTMK + 2O] are considered very promising indicators of β-Lg oxidation. Consequently, this study proposes a novel approach in peptide oxidation research through monitoring the oxidation markers identified with the LC−$MS^n$.

**KEYWORDS:** *peptide oxidation, β-lactoglobulin, LC−MS, methionine sulfoxide, methionine sulfone, dityrosine, 2-oxohistidine, semialdehyde*

## INTRODUCTION

Oxidation, as is well-known, is a major concern affecting the shelf life of foodstuffs. Reactive oxygen species are present, and metals readily catalyze the oxidation reactions, finally causing structural changes in food components. Lipids are considered the main factor in the oxidative processes. However, it is equally important to understand the distinct protein oxidation reaction mechanisms, as the significance of dietary protein including low-fat foods and diets high in protein is increasing. Oxidation of proteins may well be the major cause in deterioration of food quality in such cases. Food protein oxidation leads to loss of nutritive value through alterations in the amino acid profile, but also toxic and carcinogenic compounds have been shown to form.[1−3]

Protein oxidation analyses are still often carried out via the traditional 2,4-dinitrophenylhydrazine method of quantifying the carbonyl compounds via their DNPH derivative.[4,5] However, not all protein oxidation products are of carbonyl form, yet not all carbonyl compounds are a result of protein oxidation.[5] Thus there is a definitive need for more precise protein oxidation detection methods. A more modern and sensitive LC−MS technique has so far only been used for the specific analysis of α-aminoadipic and γ-glutamic semialdehydes as markers of protein oxidation.[6] On the peptide level, Elias et al.[7] have applied the LC−MS technique but focused on the antioxidative effects of peptides on lipids in oxidized emulsions.

Bovine β-lactoglobulin (β-Lg) consists of 162 amino acids (178 amino acids including the signal peptide part), with a sequence well-known and available, resulting in a 18.4 kDa (19.9 kDa) protein. β-Lg is a primary component of whey and a nutritionally valuable protein comprising up to 55% of the total whey proteins in bovine milk. It is thus a significant protein in any milk based food product. β-Lg is well-researched due to its nutritional value, allergenicity, mild antioxidativity, ability to bind e.g. vitamin D and palmitic acid as well as its structural similarity to human serum retinol binding protein.[8,9]

In order to better understand the amino acid level oxidation reactions typical for β-Lg, it is sensible to approach the question by oxidizing its digested peptides. Trypsin is a natural enzyme in the human gastric system with desirable analytical properties such as high reproducibility, effectivity and specificity for cleaving the peptides from the carboxyl side of lysine (K) and arginine (R), except when they are preceded by proline (P). The forming peptides can therefore be theoretically well predicted and chosen for fractioning by their preferred amino acid content.

The amino acids generally considered most prone to oxidize are methionine, cysteine, histidine, lysine, arginine and proline as well as tyrosine, tryptophan and phenylalanine.[10,11] Although information on their individual oxidation pathways is somewhat available, we consider oxidation of peptides and their analysis via LC−MS a new approach in understanding protein oxidation, beyond general antioxidative purposes.

Thus, the aim of this study was to apply LC−ESI-$MS^n$ for the oxidation investigation of a typical milk protein β-lactoglobulin. The objective was to identify oxidatively prone peptides that could be used as markers of protein oxidation.

## MATERIALS AND METHODS

**Materials.** Chromatographically purified and lyophilized β-lactoglobulin (β-Lg), variants A + B from bovine milk was purchased from Sigma Aldrich, Inc. (St. Louis, MO, USA). Sequencing grade modified trypsin was obtained from Promega Corp./BioFellows (Madison, WI, USA). Of the reagents used in the analyses ammonium

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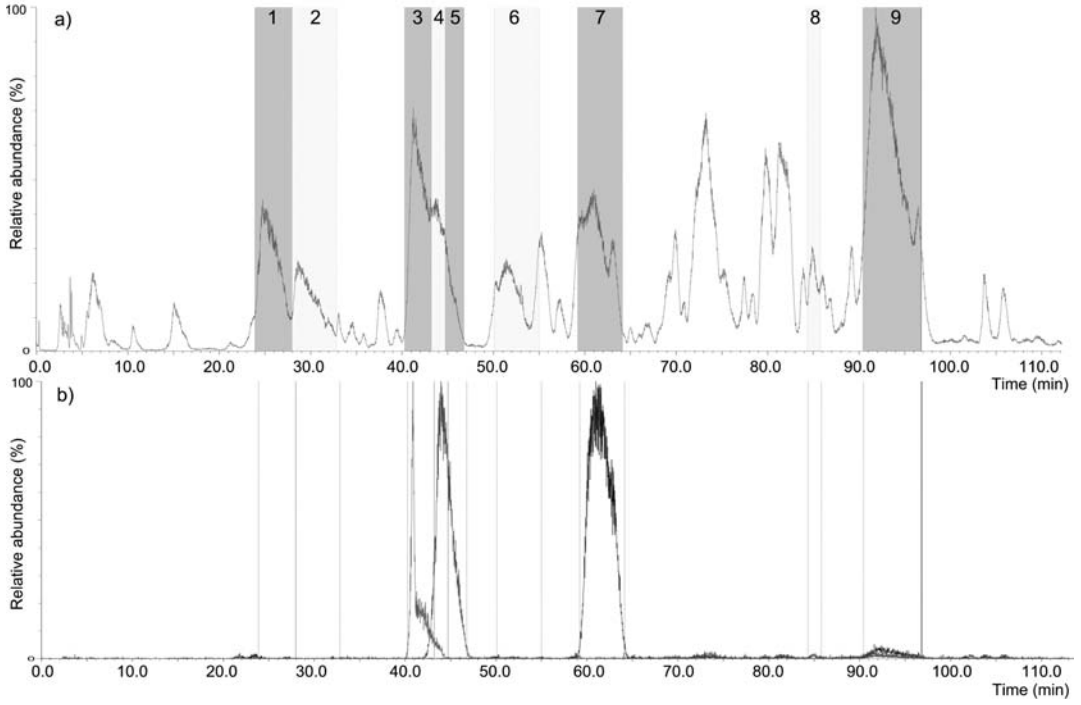


**Figure 1.** Fractionation of the chosen tryptic $\beta$-Lg peptides with prep-HPLC. (a) Total ion chromatogram of the whole digest sample; shadings represent all the fractions collected, of which fractions 3, 4, 5 and 7 were used in this study. (b) Overlaid selected ion chromatograms of $m/z$ 838 (peptide ALPMHIR, collected in fraction 3), $m/z$ 934 (LIVTQTMK, fractions 4 and 5 combined as one sample) and $m/z$ 1066 (VLVLDTDYK, collected in fraction 7).

bicarbonate, hydrogen peroxide (30% wt solution in water) and iron(III) chloride (reagent grade, 97%) were purchased from Sigma-Aldrich (Steinheim, Germany) whereas L-(+)-ascorbic acid was a product of Merck (Darmstadt, Germany) and PIPES-buffer [piperazine-1,4-bis(2-ethanesulfonic acid)] from Fluka BioChemika (Buchs, Switzerland). All other chemicals used in the analyses were supplied by J.T.Baker (Deventer, The Netherlands), Rathburn (Walkerburn, Scotland) or Sigma-Aldrich (Steinheim, Germany) in either HPLC or reagent grade. Water used was always purified first by the Milli-Q system (Millipore Corp., Bedford, MA, USA).

**Digestion and Fractionation.** The in-liquid digestion of $\beta$-lactoglobulin was performed by weighing 10 mg of the protein to an Eppendorf tube. The protein was dissolved into 1 mL of 50 mM ammonium bicarbonate ($NH_4HCO_3$, pH 8) buffer, and sequencing grade modified trypsin was added in the ratio of 1:250 w/w, e.g. 20 $\mu$L + 20 $\mu$L of 1 $\mu$g/$\mu$L freshly prepared enzyme solution. After careful stirring, the sample tube was placed in an oven of +37 °C overnight. The digestion was stopped by placing the tube in a freezer (−20 °C) for at least another night prior to further analysis at the HPLC.

Fractionation of the $\beta$-Lg peptides resulting from the tryptic digestion was performed using a Waters automated preparative HPLC system (Waters Corp., Milford, MA, USA) with a binary gradient module (Waters 2545) and an active flow splitter (in ratio 1:200) coupled with a single quadrupole mass spectrometer (Waters) and PDA (Waters 2996) detectors (the latter recorded between the wavelengths 190 and 400 nm) as well as a sample manager (Waters 2767). The system was operated with MassLynx 4.1 software (Waters). The column used was C18 Waters XBridge Prep BEH130 (5 $\mu$m, 10 × 250 mm). Separation of the peptides was achieved by gradient elution of MQ-water and acetonitrile (ACN), both of which contained 0.1% (v/v) formic acid. The flow rate was 6 mL/min throughout the run. The gradient began and was kept constant at 5% ACN for 15 min, then rose linearly to 35% ACN within 120 min. A final washing step included a fast raise up to 75% ACN for 5 min to rinse the column before balancing for the next run. A volume of 950 $\mu$L of the peptide sample was injected at a time, and fractions containing the desired peptides were collected into glass tubes according to the programmed $m/z$ value list of the fraction collector. An example of a fraction collection is illustrated in Figure 1. All fractions were transferred into plastic falcon tubes and stored frozen at −20 °C until further analysis.

Altogether 10 peptides with no missed cleavages and 9 peptides with one missed cleavage were observed matching the theoretical $\beta$-Lg peptides with $m/z$ values between 500 and 2000 (the range collected). Of these, 8 peptides were further identified with the LC−MS$^n$. The three peptides chosen (Table 1) contained at least one amino acid of oxidative interest, for example methionine, tyrosine, leucine, lysine, proline and/or arginine.[6,10] In addition they showed high abundance and good chromatographic separability from the digested samples.

**Table 1. The Tryptic $\beta$-Lg Peptides Fractionated and Oxidized in This Research**

| peptide referred[a] | $[M + H]^{1+}$ | amino acid sequence[a] |
|---|---|---|
| ALP**M**HIR$_{158-164}$ | $m/z$ 838 | Ala-Leu-Pro-**Met**-His-Ile-Arg |
| LIVTQT**M**K$_{17-24}$ | $m/z$ 934 | Leu-Ile-Val-Thr-Gln-Thr-**Met**-Lys |
| VLVLDTD**Y**K$_{108-116}$ | $m/z$ 1066 | Val-Leu-Val-Leu-Asp-Thr-Asp-**Tyr**-Lys |

[a]The amino acids expected to be most prone to oxidation are indicated in boldface.

**Oxidation of $\beta$-Lg Peptides.** Prior to the oxidation of each chosen peptide, the sample was concentrated by combining all relevant fractions from 15 digestions and fractionings. During the combining, all falcon tubes were rinsed well at least twice with ACN. The excess eluent of the combined sample was then evaporated in a rotary evaporator (Rotavapor-R, Büchi Labortechnik AG, Flawil, Switzerland), using a water bath where 30 °C was not exceeded in order not to promote any changes in the peptides.

The oxidation experiment was set up similarly to Estevez et al.,[6] by dissolving the dry, evaporated peptide sample into 15 mL of freshly made 15 mM PIPES-buffer solution (pH 6.0) that also included 0.1 mM ascorbic acid and 10 $\mu$M $FeCl_3$. The solution was then


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carefully divided into triplicates in glass vials, and fresh $H_2O_2$ solution (final concentration 1 mM) was added to each sample, along with a magnet, right before closing the caps. The samples were then stirred well, and day 0 oxidation subsamples of 200 $\mu$L each were drawn into Eppendorf tubes and frozen until analysis. The oxidation samples were then placed in an oven of +37 °C with magnet stirring constantly on. Further subsampling was carried out on days 1, 4, 7, 10 and 14, which was the last day of the oxidation experiment.

**LC–MS Analysis of the Fractionated and Oxidized $\beta$-Lg Peptides.** All unoxidized peptides as well as the subsamples of each time point were finally analyzed with Agilent 1100 HPLC consisting of a binary pump, a degasser, an automated sample manager, a column heating unit and DAD and fluorescence detectors (all by Agilent Technologies, Santa Clara, CA, USA). In addition, the HPLC was coupled with a Bruker Esquire quadrupole ion trap mass spectrometer (QIT-MS, from Bremen, Germany) using electrospray ionization (ESI) in positive mode. The column used was Waters XBridge BEH130 C18, with 3.5 $\mu$m and 2.1 × 100 mm diameters, designed for peptide separation and including a precolumn. Injection volume was 10 $\mu$L of each peptide sample. Flow rate was always kept constant at 350 $\mu$L/min and column temperature at 30 °C. Eluents were always 0.1% formic acid in water (A) and in acetonitrile (B). The so-called general gradient began from 5% B where it was first kept for 4 min, then raised linearly to 16% B within 25 min, and again raised linearly to 22% B in 6 min. Only for peptide VLVLDTDYK a slightly different gradient was used, when the initial 5% of solvent B was kept constant for only 2 min, then raised linearly to 20% B within 31 min, and again raised linearly to 26% B in 6 min. Following each gradient, a washing step was used to rinse the column with 80% of solvent B for 5 min before balancing for next injection.

The DAD detector was set to 214 nm, except for the analysis of peptide VLVLDTDYK, where also 235 and 274 nm were recorded. The fluorescence detector was always set to excite at 280 nm and emit at 350 nm.

The MS parameters were manually optimized for one of the chosen peptides, with $m/z$ ratio 838 and trialed to function for the remaining peptides as well. The parameters were set according to the following: dry temperature 300 °C, dry gas 9.0 L/min, nebulizer 70.0 psi, capillary 4500 V, end plate offset −400, trap drive 80.0, capillary exit 200.0, capillary exit offset 135.0, skimmer 1 65.0, skimmer 2 20.0, lens 1 −2.0, lens 2 −50.0, octupole 4.5, octupole Δ 2.7 and octupole rf amplitude 220.0 Vpp.

Mass spectra were recorded with the full scan mode $m/z$ range of 200–1200 for peptides ALPMHIR ($m/z$ 838) and LIVTQTMK ($m/z$ 934), but 200–2200 for VLVLDTDYK ($m/z$ 1066). In the case of the first two peptides, a higher sensitivity and repeatability were achieved by limiting a closely coeluting $\beta$-Lg peptide Thr141–Lys151 with $m/z$ ratio of 1246 out of the collection range. On the other hand, for VLVLDTDYK a possible dipeptide oxidation product was anticipated close to the high end of the $m/z$ range, which was therefore extended. In $MS^n$ mode used for the identification of each peptide and oxidation product, manual fragmentation and $m/z$ range were modified according to individual needs, but fragmentation amplitude of 1.45 was used at all times.

Within each set of samples analyzed, an in-house reference was used. Peptide TPEVDDEALEK with $m/z$ 1246 was fractionated and collected similarly to the sample peptides to be used for the reference purpose. Action limits were set at mean ± 2 × standard deviation (SD) from multiple analyses during several injection days, taking into account the integrated area of the extracted ion chromatogram of $m/z$ 1246. An aliquot of this peptide was defrosted and analyzed together with each day's set of samples. In case the in-house reference yielded a value outside the action limits, the whole set of analyzed samples was rejected. In addition, all subsamples of the same peptide were always run in the same analysis set to minimize any possible day-to-day variance.

## RESULTS AND DISCUSSION

**Oxidation of Peptide ALPMHIR.** The fractionated $\beta$-Lg peptide ALPMHIR, seen with $[M + H]^{1+}$ $m/z$ 838, proved to oxidize rapidly as only a very small amount of intact peptide could be detected on day 0. Interestingly, this peptide has also previously been characterized as a lactokinin and the most potent ACE-inhibitory peptide from $\beta$-Lg, with an $IC_{50}$ value of 43 $\mu$mol/L.[12] This peptide contained methionine (Table 1), which was considered as one of the most prone amino acid targets for oxidation. The major oxidation product observed overall was $m/z$ 854 resulting in $\Delta m$ = +16 amu. This is a characteristic mass increase in oxidation reactions by addition of an oxygen atom, as illustrated by methionine in Figure 2.

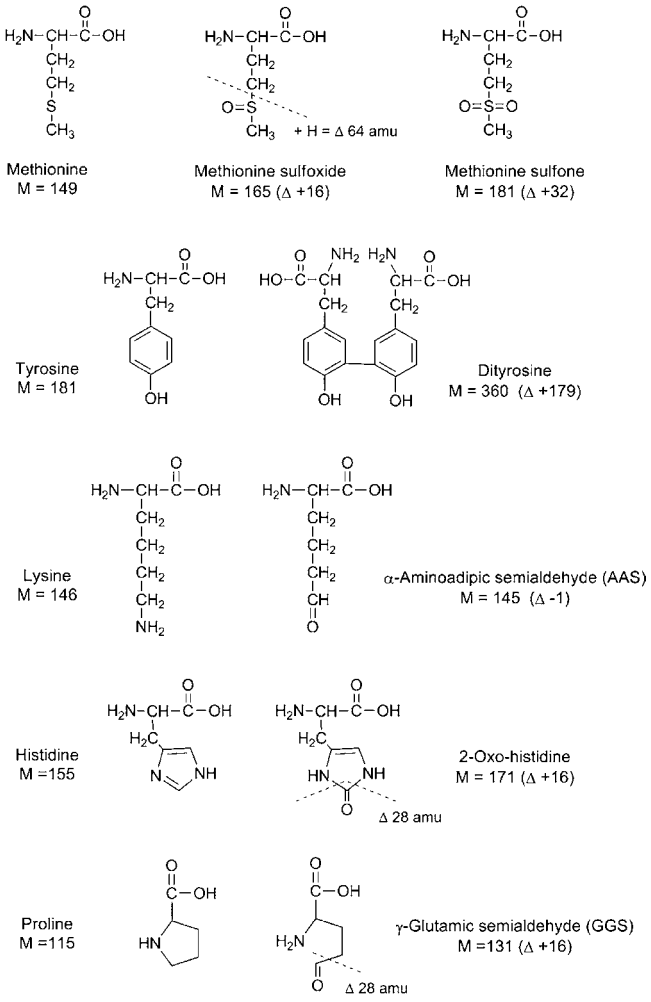


**Figure 2.** The structures of methionine, tyrosine, lysine, histidine and proline together with their most typical oxidation products and some characteristic fragmentations.

This oxidized form of the peptide, [ALPMHIR + O], was prominent already in day 0 ion chromatograms (Figure 3). However, the peak area of [ALPMHIR + O] in the ion chromatograms was notably decreasing already toward day 4, suggesting secondary oxidation reactions. Between days 4 and 14 the amount of [ALPMHIR + O] in the samples was rather level or even slightly increasing again.

What was special about the ion chromatogram peak $m/z$ 854, [ALPMHIR + O], is that it had two distinct retention times, with only 0.5 min apart, as seen in Figure 4. The first of them


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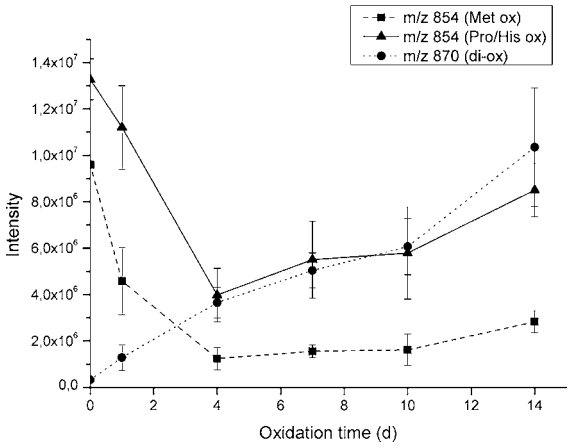


**Figure 3.** Evolution of the peptide ALPMHIR main oxidation products within time ($n$ = 3) as monitored by LC–MS. Methionine oxidation was seen with $m/z$ 854 at $t_R$ 11.5 min and another oxidation product with the same $m/z$ 854 at $t_R$ 12.0 min, both resulting from [ALPMHIR + O]. The oxidation product with $m/z$ 870 ($t_R$ 14.2 min) is the dioxidized peptide [ALPMHIR + 2O].

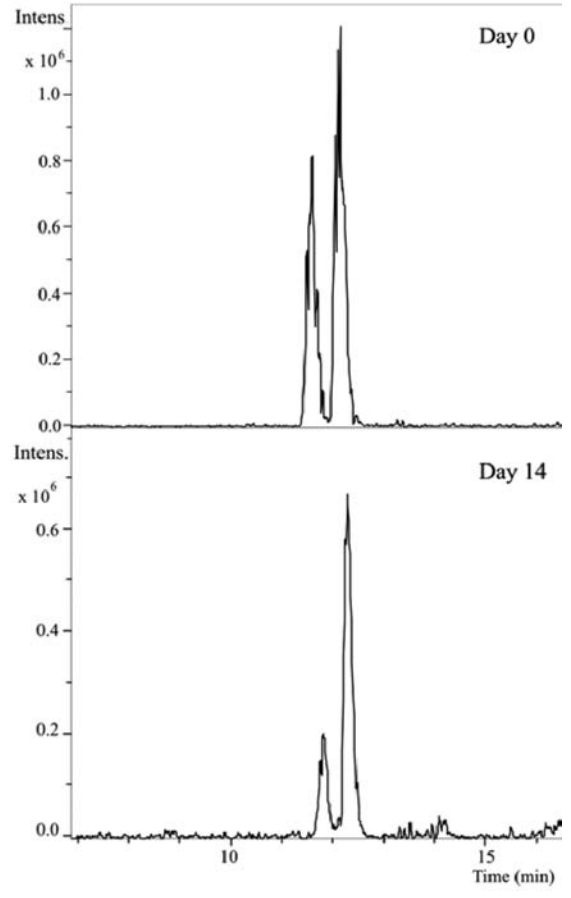


**Figure 4.** Selected ion chromatograms of $m/z$ 854, [ALPMHIR + O], from oxidation day 0 (top) and 14 (below), indicating two different oxidation products with the same $m/z$ ratio.

($t_R$ 11.5 min) underwent more rapid degradation during the time of oxidation, whereas the changes in the second ion chromatogram peak ($t_R$ 12.0 min) were not so dramatic although following the same trend (Figure 3). In addition to methionine, also proline and histidine of the amino acids present in peptide ALPMHIR are known to react with oxygen[10] and would result in a mass addition of +16 amu (final mass $m/z$ 854). Especially histidine is, in a metal catalyzed system like this, very likely to oxidize into 2-oxohistidine (Figure 2).[13]

From the MS/MS fragmentation of both the oxidized peptide [ALPMHIR + O] peaks with parent ion $m/z$ 854 (Table 2) several similarities were seen. Neutral loss of

**Table 2. MS/MS Fragmentation of Both Chromatographically Separated Peaks of $m/z$ 854 (from Oxidation Day 1)**

| $t_R$ 11.5 min[a] | | $t_R$ 12.0 min[b] | |
|---|---|---|---|
| $m/z$ | rel int | $m/z$ | rel int |
| 836 | 4.1 | 792 | 15.1 |
| 791 | 23.8 | 791 | 40.9 |
| 790 | 100.0 | 790 | 100.0 |
| 789 | 4.6 | 679 | 4.8 |
| 679 | 3.9 | 551 | 7.2 |
| 665 | 4.1 | 546 | 5.6 |
| 615 | 6.5 | 467 | 5.1 |
| 606 | 4.1 | 430 | 5.0 |
| 546 | 8.1 | 425 | 8.6 |
| 527 | 4.0 | 418 | 7.4 |
| 502 | 2.9 | 392 | 5.8 |
| 431 | 7.4 | 390 | 6.1 |
| 425 | 9.1 | 374 | 5.5 |
| 408 | 3.2 | 373 | 5.2 |
| 403 | 3.5 | 277 | 5.5 |

[a] $t_R$ 11.5 min belongs to [ALPMHIR + O] where methionine has been oxidized. [b] $t_R$ 12.0 min, [ALPMHIR + O], is another, nonconfirmed oxidation product.

Δ 18 amu from the parent ion, in this case yielding the fragment ion $m/z$ 836, is typical for any peptide, and is usually considered to be loss of a water molecule ($H_2O$). The most intense fragmentation peak of both parent ions was $m/z$ 790, which could have resulted either from a loss of Δ 64 amu from the parent ion or Δ 46 amu from the ion $m/z$ 836, after loss of water. According to Lagerwerf et al.[14] as well as Guan et al.[15] the loss of Δ 64 amu (methanesulfenic acid, $CH_3SOH$) is unique for peptides containing methionine that has oxidized into methionine sulfoxide. On the other hand, the loss of Δ 46 amu has quite recently been proven[16] a characteristic fragment of 2-oxohistidine through the combined loss of another $H_2O$ and CO. However, as this may apply to the amino acid oxidation product, it does not apply to 2-oxohistidine in a peptide. In addition to histidine, also proline is rather prone to oxidation resulting in a γ-glutamic semialdehyde (Figure 2). Unfortunately the differences in fragmentation could not be distinguished for the histidine and proline oxidation products because of not having an accurate mass MS detector.

The critical difference between the otherwise very similar fragmentation patterns of both the [ALPMHIR + O] peaks was in the isotopic peak ratios of fragment $m/z$ 790, as the $m$ + 2 characteristic for sulfur was only seen for the second eluting peak ($t_R$ 12.0 min). Therefore it was concluded that the first eluting [ALPMHIR + O] peak ($t_R$ 11.5 min) belonged to the peptide where methionine had oxidized into methionine sulfoxide, and then fragmented into $m/z$ 790 due to the loss of the sulfur containing $CH_3SOH$. In the second eluting peak ($t_R$ 12.0 min) the fragment $m/z$ 790 was due to the 2-oxohistidine breakup, leaving the sulfur containing methionine side chain intact in the peptide.

Another reaction of interest was peptide dioxidation, the addition of two oxygens, resulting in $m/z$ 870 (from $m/z$ 838 + 2 × 16 amu) and [ALPMHIR + 2O]. This peak eluted after the two before mentioned monoxidated peptide peaks, at $t_R$ 14.2 min. The [ALPMHIR + 2O] was hardly present on day 0, but was clearly increasing along time. Already on day 4 of the experiment, the amount of dioxidized [ALPMHIR + 2O] was higher than the first eluting [ALPMHIR + O] product and around the same level as the second eluting [ALPMHIR + O] product. On day 14 the [ALPMHIR + 2O] peak was the most significant oxidation product seen in the chromatograms. The amino acids in peptide ALPMHIR prone to dioxidize were proline, arginine, as well as methionine through its sulfone formation[10] as illustrated in Figure 2.

**Oxidation of Peptide LIVTQTMK.** Another peptide chosen for oxidation, LIVTQTMK with $[M + 1]^{1+}$ $m/z$ 934, also contained methionine. Thus its predicted, as well as observed, major oxidation product peak was $m/z$ 950 resulting from +16 amu [LIVTQTMK + O] through methionine sulfoxide formation (Figure 2). Unlike peptide ALPMHIR, unoxidized peptide LIVTQTMK was notably present on day 0 in most samples, and on day 1 the amount of unoxidized LIVTQTMK had only 1.3% RSD difference between the three replicates (Figure 5).

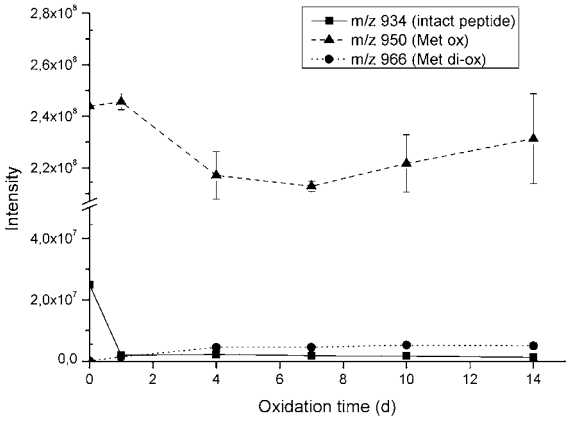


**Figure 5.** Evolution of the peptide LIVTQTMK ($m/z$ 934), its primary oxidation product [LIVTQTMK + O] ($m/z$ 950) as well as the dioxidation product's [LIVTQTMK + 2O] ($m/z$ 966) changes within time ($n$ = 3) as monitored by LC−MS.

The major oxidation product peak $m/z$ 950 was on day 0 the most dominant in one sample and less present in the other two samples. Yet, on day 1 the difference between all three replicates was only 1.2% RSD. This led to the conclusion that for some unexplained reason the initiation of the oxidation had been heterogeneous, but the first 24 h of oxidation in +37 °C seemed to have a rather unifying effect.

From day 1 onward the amount of intact peptide LIVTQTMK was slightly decreasing by approximately 30% toward the end of the oxidation experiment. In addition, also the primary oxidation product [LIVTQTMK + O] showed a slight decrease of 12% between days 1 and 4, thus indicating that a secondary oxidation had begun (Figure 5). Indeed, another notable oxidation product was observed, producing the ion $m/z$ 966 and characterized as the result of dioxidation ($m/z$ 934 + 2 × 16 amu). Very little of this [LIVTQTMK + 2O], if at all, was present on day 0, and the formation was modest during the first day of oxidation. The dioxidation was on the increase mostly between oxidation days 1 and 4, but kept on forming slowly after this as well.

All three peaks were easily separated in the chromatogram with the retention times 13.5 min ($m/z$ 950, [LIVTQTMK + O]), 16.0 min ($m/z$ 966, [LIVTQTMK + 2O]) and 22.0 min ($m/z$ 934, LIVTQTMK). Due to their feature of not reacting further and on the other hand easy detectability, both of the ions $m/z$ 950 and $m/z$ 966 were considered as very promising indicators of oxidation also in more complex oxidation models.

**Oxidation of Peptide VLVLDTDYK.** The third peptide of interest was VLVLDTDYK with $[M + 1]^{1+}$ ion of $m/z$ 1066. The unoxidized peptide peak was observed at retention time 27.0 min and with a relatively high intensity especially on day 0. Approximately 20% of that was lost during the first day of oxidation, but from there on the peptide peak seemed to diminish rather moderately (Figure 6). Within oxidation at

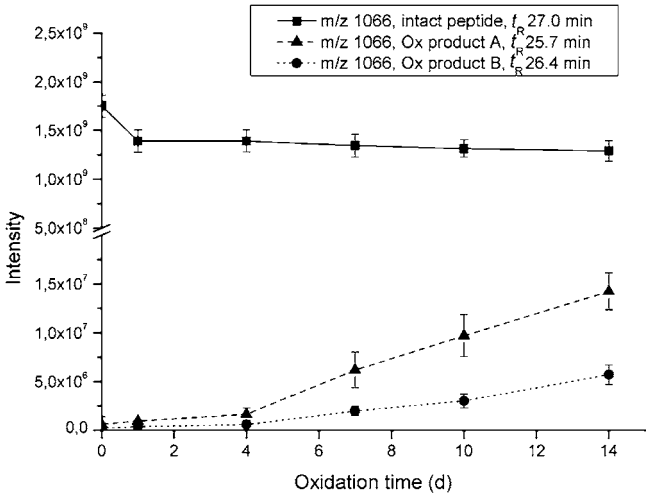


**Figure 6.** Evolution of the peptide VLVLDTDYK ($m/z$ 1066, $t_R$ 27.0 min) and the two most prominent oxidation products "A" ($m/z$ 1066, $t_R$ 25.7 min) and "B" ($m/z$ 1066, $t_R$ 26.4 min) illustrated within time ($n$ = 3) as monitored by LC−MS.

+37 °C, two new chromatographic peaks with the same $m/z$ value 1066 as the unoxidized peptide were seen rising and increasing at each oxidation time point (Figure 7a). These new peaks eluted slightly earlier, with retention times 25.7 min (A) and 26.4 min (B).

Each of the three chromatographic peaks with $m/z$ 1066 were fragmented in order to compare any differences that might have led to their chromatographic separation. The unoxidized peptide ($t_R$ 27.0 min) fragmented amino acid by amino acid, thus confirming the sequence Val-Leu-Val-Leu-Asp-Thr-Asp-Tyr-Lys (Figure 8). An identical fragmentation pattern was also recorded from the oxidation product A ($t_R$ 25.7 min). Oxidation product B ($t_R$ 26.4 min) had also several of the same peptide fragments, but unlike in the other two peaks, the dominant fragment was $m/z$ 756 whereas the most dominant fragment of the other two, $m/z$ 526, was completely missing.

Tyrosine was considered as the most likely amino acid to oxidize in peptide VLVLDTDYK, and its major oxidation product is known to be dityrosine[17] as shown in Figure 2. The dityrosine-peptide was expected to produce the ion $[M + 1]^{1+}$ $m/z$ 2131, which was however not very markedly present in the samples. On the other hand, a doubly charged ion of the

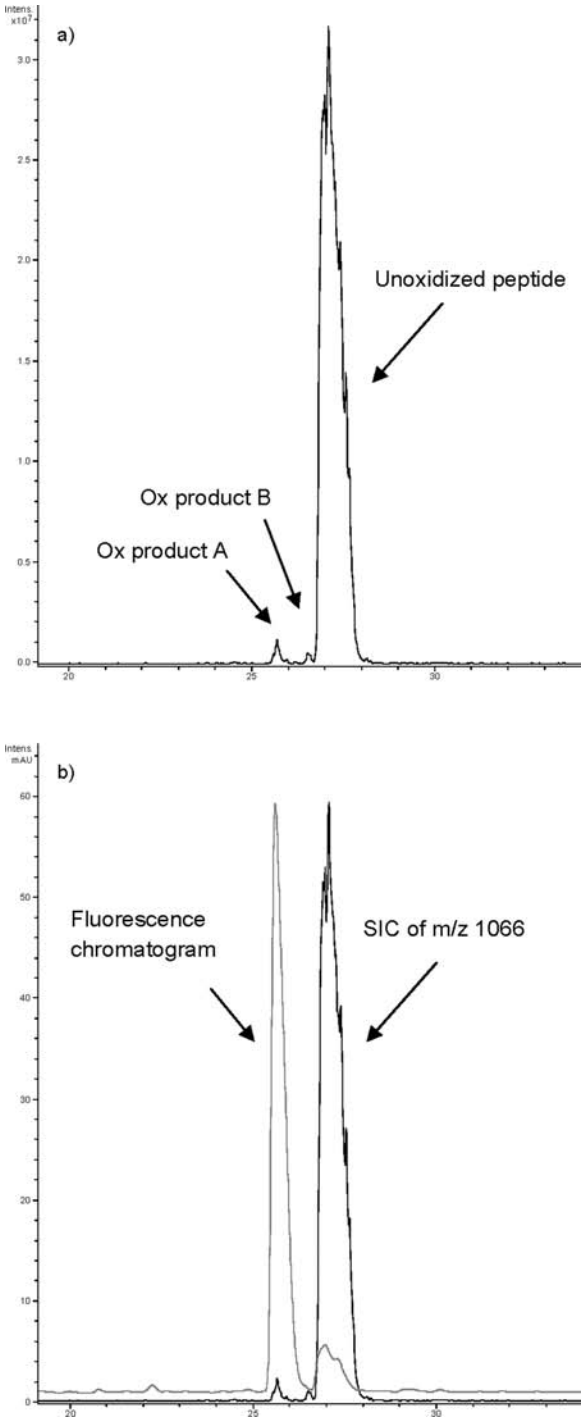


**Figure 7.** (a, top) Selected ion chromatogram of $m/z$ 1066 from day 14 of peptide VLVLDTDYK oxidation, showing the three distinct peaks with the same $m/z$ value. (b, bottom) Selected ion chromatogram of $m/z$ 1066 overlaid with fluorescence chromatogram of peptide VLVLDTDYK from oxidation day 14.

dityrosine-peptide would result in $[M + 2]^{2+}$ of $m/z$ 1066, the same as the intact peptide.

The only other amino acid likely to oxidize in peptide VLVLDTDYK was lysine.[18] Especially in metal-catalyzed oxidation, as in this experiment, aminoadipic semialdehyde is a well-known oxidation product of lysine, and would result in a $\Delta m = -1$ amu of the peptide (Figure 2). However, selected ion chromatogram of $m/z$ 1065 did not clearly stand out from the

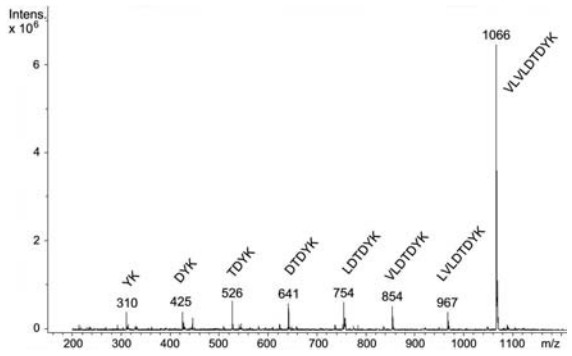


**Figure 8.** Sequence confirmed via fragmentation of unoxidized peptide VLVLDTDYK ($m/z$ 1066, $t_R$ 27 min) by LC−QIT-MS/MS.

considered isotopic peaks of the three $m/z$ 1066, so lysine oxidation was not confirmed.

Fluorescence turned out to be the key factor in identifying the three chromatographic peaks with the same $m/z$ value of 1066 (Figure 7b). Only the first eluting peak had strong fluorescence thus confirming it was the dityrosine[17] oxidation product. From Figure 7b it can be seen that even though the peak of oxidation product A was notably smaller than the intact peptide peak, its fluorescence was clearly most dominant. Oxidation product B did not possess fluorescent properties, but its growth was notable when monitored by UV on both wavelengths 274 nm (tyrosine specific) and 235 nm (for tyrosine and dityrosine). It is also possible that oxidation product B was resulting from rather slow lysine oxidation to aminoadipic semialdehyde. In the case of oxidation product B, longer oxidation time would have enabled a more reliable fragmentation examination of a more prominent chromatographic peak.

Since oxidation and dioxidation of several amino acids in $\beta$-Lg peptides were identified and could be monitored in this study, we propose this method as a novel approach in peptide oxidation studies. The ions $m/z$ 950 [LIVTQTMK + O], $m/z$ 966 [LIVTQTMK + 2O] and $m/z$ 854 [ALPMHIR + O] are considered very promising indicators of complete food protein, $\beta$-lactoglobulin, oxidation.

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### Notes

The authors declare no competing financial interest.

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