JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Exposed Sulfhydryl Groups in Heated β-Lactoglobulin A Using IAEDANS and Mass Spectrometry

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This paper takes a new approach to determining which sulfhydryl groups are exposed during the heat denaturation of bovine β-lactoglobulin A. The sulfhydryl groups exposed after heating were blocked with 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS). The results show that IAEDANS is a suitable blocking agent, and its absorbance at 336 nm enabled the quantification of exposed sulfhydryl groups in a mixture of protein species by gel permeation chromatography. Combined with the specific fragmentation of bound IAEDANS by matrix-assisted laser desorption ionization (MALDI) MS/MS in negative ionization mode, this facilitated the identification of peptides that contained blocked cysteines after enzymatic digestion of the protein. During MALDI MS/MS of the peptides, in positive ionization mode, the IAEDANS molecule remained bound to the cysteines, making it possible to identify exactly which cysteine had been exposed after heating. In β-lactoglobulin A it was found that cysteine 66 and cysteine 160 were predominantly exposed regardless of the length of exposure to heat.

KEYWORDS: β-Lactoglobulin; free sulfhydryl; IAEDANS; MALDI mass spectrometry

INTRODUCTION

β-Lactoglobulin (β-lg) makes up approximately 50% of the whey proteins from bovine milk. It exhibits a predominant role in the functional properties of whey ingredients. Its native molecular structure is well-established (*1*). Native β-lg is mainly formed with nine β-strands (labeled A−I) organized into two β-sheets facing each other and a C-terminal α-helix. It contains five cysteines, giving rise to two intramolecular disulfide bonds (between Cys66 and Cys160 and between Cys106 and Cys119) and leaving one sulfhydryl group at position 121 (Cys121) buried in the molecule. Under physiological conditions, the latter is inaccessible for chemical reactions. Under favorable conditions (heat, pH, or pressure) the globular structure of β-lg unfolds, thereby exposing reactive groups such as sulfhydryl groups on the protein surface for further aggregation (*2*). The exact molecular mechanism leading to formation of aggregates is not fully understood, but disulfide interchange reactions and the formation of new intra- and intermolecular disulfide bonds were shown to be prevalent at neutral pH (*3*−*5*). One of the first events taking place during heating is the formation of non-native monomers containing non-native sulfhydryl groups exposed on the protein surface resulting from intramolecular sulfhydryl/disulfide exchange reactions (*6*). Exposed sulfhydryl groups are further able to react with other proteins, leading to the formation of dimers, oligomers, and larger aggregates (*3*, *4*, *7*). When the dimers and trimers were digested with trypsin and analyzed by mass spectrometry in MALDI and electrospray modes, it was found that several new disulfide bonds were formed: Cys121−Cys160 and Cys106/119/121−Cys160 as well as an intermolecular Cys160−Cys160 (*7*, *8*). Cys66−Cys66-linked peptides were found only at low ionic strength (*9*). Under specific system conditions (15 min at 80 °C), it was found that 35% of the Cys160 in β-lg is not involved in disulfide bonds after heating, suggesting that its release following sulfhydryl/disulfide exchange reactions is an important step to propagate intermolecular exchange reactions (*10*). Determining which sulfhydryl groups are exposed on heating (important contributors for sulfhydryl/disulfide interchange reactions) will give a greater insight into the mechanism of how the sulfhydryl/disulfide exchange reactions proceed. Additionally, it will lead to a better understanding of the reactivity and functionalities of the molecular species formed on heating.

Mass spectrometry analysis performed on an enzymatic digest from heated proteins is a common method to identify new disulfide bonds formed on heating and to draw conclusions about the chemical reactivity of sulfhydryl groups. However, a disadvantage of this experimental design is that the conditions

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10.1021/jf070397r CCC: $37.00
Published on Web 07/25/2007

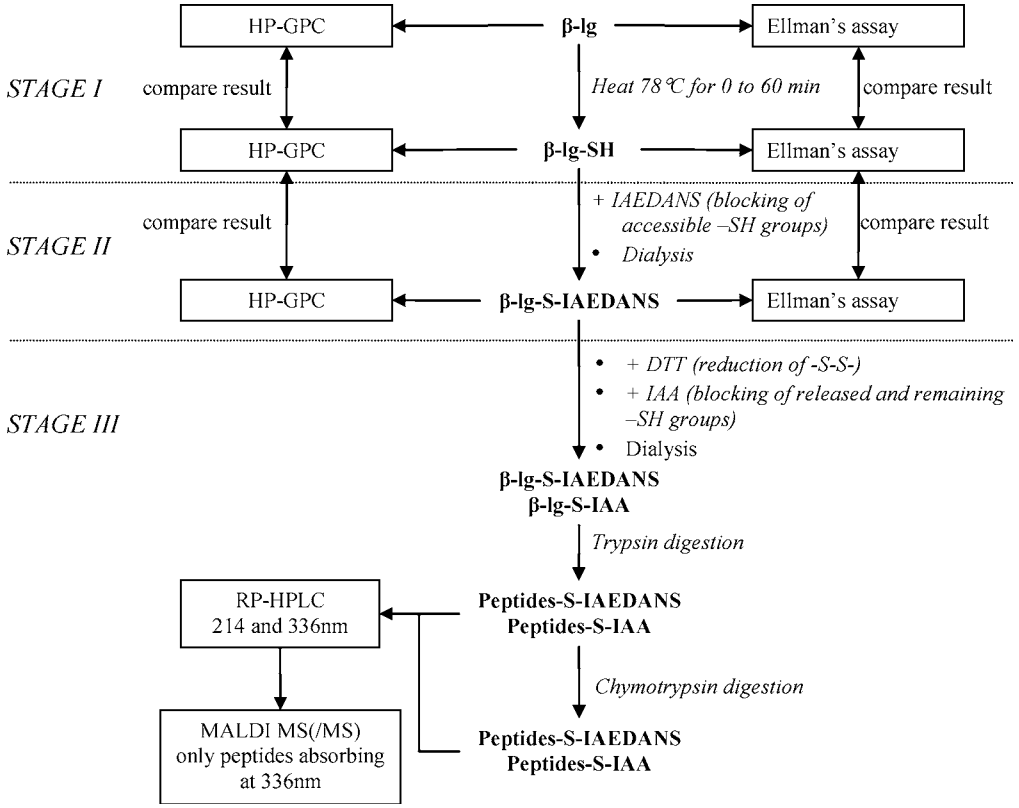

Figure 1. Experimental design.

of the enzymatic digestion, that is, pH 8.0 for a trypsin digest, can cause disulfide reshuffling (*11*). This can be overcome if the sulfhydryl groups are blocked prior to trypsin digestion to ensure that no reshuffling reactions could take place. The work described in this paper focused on cysteines which are accessible for chemical reactions (sulfhydryl groups exposed on the protein surface) after the heat denaturation of β-lg rather than on those involved in disulfide linkages. We developed a method using 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS) for labeling and identifying those sulfhydryl groups. This reagent is commonly used as a fluorescence probe (*12*), but for the purposes of this study we instead took advantage of its absorbance at 336 nm.

MATERIALS AND METHODS

***β*-Lactoglobulin Purification.** Chemicals were generally sourced from Sigma Aldrich, France, unless stated otherwise. β-Lg was purified from whey protein concentrate (WPC), which was prepared in-house from fresh milk (pilot plant of Moorepark Technology Ltd.) with minimal heat treatment to ensure minimal protein denaturation during processing. Twenty-five milliliters of a 10% (w/w) WPC reconstituted in mobile phase was injected on a Q-Sepharose column (Amersham Biosciences). A 10 mM Tris-HCl, pH 7.0, buffer (HCl from Prolabo) with a 0−400 mM NaCl gradient was used to elute the proteins. The β-lg A fractions was collected, dialyzed extensively to remove salts, and freeze-dried. The purity of β-lg A was >99%.

The experimental procedures outlined in the following sections are summarized in **Figure 1**.

Heat Denaturation. A solution of β-lg A was prepared in 20 mM phosphate buffer, pH 7.0, containing 50 mM NaCl. The concentration of β-lg A was determined from the absorbance of the solution at 278 nm, using the specific extinction coefficient of 0.96 L/g/cm, and the final concentration was adjusted to 5 g/L (272 μM using 18362 g/mol for the molecular mass of β-lg A) with 20 mM phosphate buffer, pH 7.0, containing 50 mM NaCl. One milliliter aliquots of the protein solution were heated in a water bath at 78 °C for 20, 40, and 60 min.

Electrophoresis. Polyacrylamide gel electrophoresis (SDS-PAGE) was carried according to the method of Laemmli (*13*). Samples of the protein before and after heating were run under denaturing but nonreducing conditions. The electrophoresis was carried out using 15% polyacrylamide gels, at a constant voltage of 155 V in a Mini Protean II system (Bio-Rad, Alpha Technologies, Dublin, Ireland.) Molecular weight standards (GE Healthcare U.K. Ltd.) were run on the gel to allow the determination of the molecular weights of the aggregates.

Blocking of Sulfhydryl Groups with IAEDANS. One hundred microliters of an IAEDANS (Invitrogen Molecular Probes, France) solution (2.26 g/L in 20 mM phosphate buffer, pH 7.0, 50 mM NaCl) was added to unheated and heat-treated samples (IAEDANS/exposed sulfhydryl groups molar ratio greater than 4, see below). Samples were incubated overnight at 37 °C in the dark to ensure the complete blocking of exposed sulfhydryl groups. These conditions did not induce a progression in the aggregation process of β-lg. After incubation, the samples were dialyzed against phosphate buffer to remove excess IAEDANS.

High-Performance Gel Permeation Chromatography (HP-GPC). The samples were analyzed by HP-GPC both before and after treatment with IAEDANS. The samples were diluted 1 in 2 and applied to a TSK G3000 SWXL column (TosoHaas, Montogomeryville, PA). The HPLC system consisted of a Waters 2695 separation module with a Waters 2487 dual-wavelength absorbance detector, and the absorbance was measured simultaneously at 280 and 336 nm. A 20 mM sodium phosphate, 50 mM NaCl buffer, pH 7.0, at a flow rate of 0.8 mL/min was used for elution. Molecular species eluted from the column were assigned according to their molecular mass using α-lactalbumin (14.4 kDa), ovalbumin (45 kDa), and bovine serum albumin (66 kDa) as standards.

Ellman's Assay. Ellman's assay (*14*) was carried out on unheated and heated samples as well samples that were blocked with IAEDANS after heating and extensive dialysis, to determine the extent of blocking. The protein solutions were diluted 10-fold either in 50 mM Tris-glycine, pH 8.0 (for determination of exposed sulfhydryl groups), or in 50 mM Tris-glycine, pH 8.0, with 8 M urea (total sulfhydryl groups, urea from Prolabo, France). One milliliter of diluted solution was placed in a cuvette, and 20 μL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Merck,

France; 10 mM in the Tris-glycine buffer) was added. The absorbance of the samples was read at 412 nm. The number of sulfhydryl groups per molecule of β-lg was calculated from the absorbance reading using 13600 L/mol/cm as extinction coefficient (*15*).

Reduction and Alkylation of Proteins. Two hundred microliters of the β-lg solution, which was treated with IAEDANS and subsequently dialyzed, was diluted with 800 μL of 50 mM Tris-HCl buffer containing 6 M urea, pH 8.5, and 10 μL of 100 mM dithiothreitol (DTT; Pharmacia Biotech, France; in 50 mM Tris-HCl buffer containing 6 M urea at pH 8.5). The reduction was carried out at 56 °C for 1 h. After the reduction, 40 μL of iodoacetamide (IAA), 2 M, was added to the solution, and it was incubated at room temperature in the dark. The solutions were dialyzed against a 10 mM carbonate buffer, pH 8.5, overnight to remove excess reagents.

Protein Hydrolysis. Dialyzed samples were adjusted to pH 8.0 by the addition of HCl. A stock solution of 1 g/L trypsin was added to the protein solution to bring it to an enzyme to protein ratio of 1:100 (w/w). The solutions were hydrolyzed overnight at 37 °C. Two hundred microliters of hydrolyzed solution was removed, and the pH was reduced below 3 with TFA to inactivate the enzyme. The remainder of the trypsin-hydrolyzed sample had chymotrypsin (1 g/L) added to a final enzyme to protein ratio of 1:100 (w/w). The sample was again digested overnight at 37 °C and stopped by reducing the pH below 3 with TFA.

Reverse Phase Chromatography. Both samples (trypsin and trypsin/chymotrypsin digested) were separated on a reverse phase Symmetry C18 column (2.1 × 150 mm; Waters, Milford, MA). The column was equilibrated with solvent A [0.106% (v/v) trifluoroacetic acid in Milli-Q water] and eluted with a linear gradient of 3−60% solvent B (0.1% (v/v) trifluoroacetic acid in 4:1 (v/v) acetonitrile/Milli-Q water) over 60 min. RP-HPLC separations were achieved at 40 °C at a flow rate of 250 μL/min (acetonitrile from Prolabo, France). Peptides were detected simultaneously at 214 and 336 nm using the Waters 2487 dual-wavelength absorbance detector.

Mass Spectrometry. Peptides absorbing at 336 nm were collected and concentrated to dryness in a Speed vac. After reconstitution in 5 μL of 50% acetonitrile containing 0.1% TFA solution, they were analyzed by a hybrid quadrupole time-of-flight (Q/TOF) mass spectrometer QStar XL, fitted with a MALDI II (Applied Biosystems/ MDS Sciex, Toronto, Canada). For the MALDI experiments, typically 1 μL of cocrystallized sample with 1 μL of α-cyano-4-hydroxycinnamic acid matrix were ionized with a laser beam (337 nm, 20 Hz) and peptide β-CN from β-casein (193−209) was used as a calibration standard. In each sample the more representative monocharged ions were submitted to MS/MS fragmentation with a collision energy depending on the *m*/*z* value. Spectra were acquired in the reflector mode (in both positive and negative ionization modes).

RESULTS AND DISCUSSION

Figure 1 shows a diagram of the main steps of the experimental design used for this work. In the following sections the sulfhydryl groups are referred to as *exposed sulfhydryls*, for those being exposed on the surface of β-lg molecules and accessible for reaction with IAEDANS (the concentration of exposed sulfhydryl groups was determined with DTNB in the absence of urea before and after the reaction of β-lg molecules with IAEDANS), or *total sulfhydryls*, for those able to bind DTNB in the presence of urea.

Extent of Blocking of the Sulfhydryl Groups Exposed on Heating with IAEDANS. Ellman's assay was used to quantify sulfhydryl groups in β-lg solutions before and after reaction with IAEDANS to determine the extent of blocking with IAEDANS (exposed sulfhydryl groups). An unheated sample of β-lg gave, in the presence of urea (total sulfhydryl groups), 0.99 ± 0.02 mol of sulfhydryl groups per mole of protein, which is consistent with one sulfhydryl group per β-lg molecule. When the assay was carried out in the absence of urea (**Figure 1**, stage I), 0.18 ± 0.01 mol/mol of protein was obtained in accordance

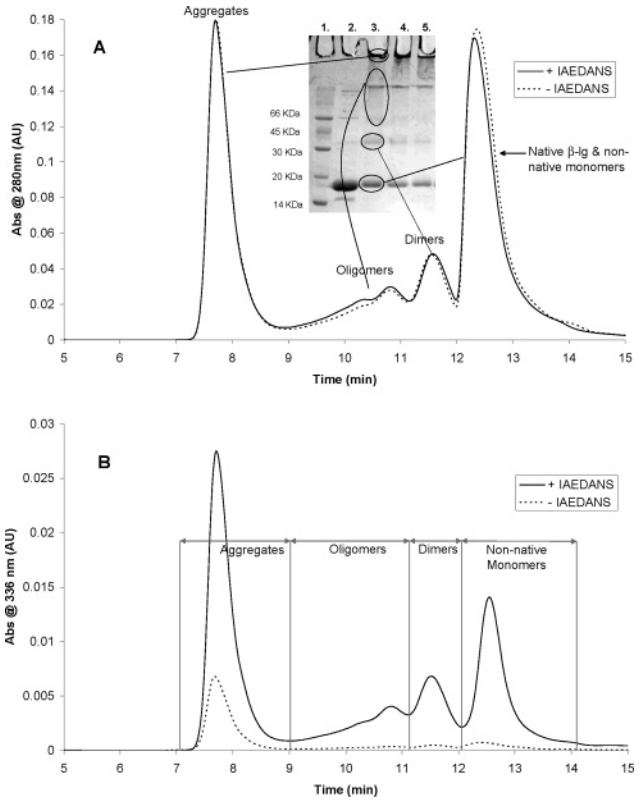

Figure 2. (**A**) GPC profile at 280 nm of β-lactoglobulin heated for 20 min at 78 °C before and after blocking with IAEDANS. The gel insert has a molecular weight standard in lane 1, an unheated sample in lane 2, and samples heated for 20, 40, and 60 min in lanes 3, 4, and 5, respectively. (**B**) Chromatograms at 336 nm of the same samples. Lines indicate limits for integration of areas under the curves for further calculation of exposed sulfhydryl groups (**Table 1**).

with previous results (*16*). Hoffmann and van Mill (*3*) also observed a reaction between DTNB and the sulfhydryl group of native β-lg at pH 8. Under Ellman's conditions (pH 8), the sulfhydryl group of native β-lg is partially accessible for reaction with DTNB due to the reversible conformational change of β-lg molecules between pH 6 and 8.5, also referred to as Tanford transition (*17*). In contrast, no reaction between IAEDANS and native β-lg (unheated sample) took place at pH 7, indicating that at pH 7 the sulfhydryl group of β-lg was inaccessible to IAEDANS, (see later) (**Figure 3B**). Hence, the concentration of residual native β-lg after heat treatment (for which the sulfhydryl group is partially accessible to DTNB but inaccessible to IAEDANS) has to be considered for the quantification of the sulfhydryl groups that have been exposed on heating.

After 20 min of heating at 78 °C, the total sulfhydryl groups had decreased to 0.87 ± 0.03 mol/mol of protein. It is presumably brought about by the oxidation of sulfhydryl groups into disulfide bonds that terminate the propagation reaction involved with β-lg (*2*). In contrast, the concentration of sulfhydryl groups accessible to DTNB in the absence of urea increased to 0.45 ± 0.02 mol/mol of protein. However, DTNB can react with species having exposed sulfhydryl groups after heat treatment and the residual native β-lg, the sulfhydryl group of the latter being partially accessible to DTNB when placed under Ellman conditions (pH 8) due to Tanford transition (*17*). After 20 min of heating at 78 °C, 29 ± 2% of β-lg molecules remain native (soluble after precipitation of denatured/aggregated proteins at pH 4.7) and are then partially accessible to DTNB. When the heated sample of β-lg was treated with

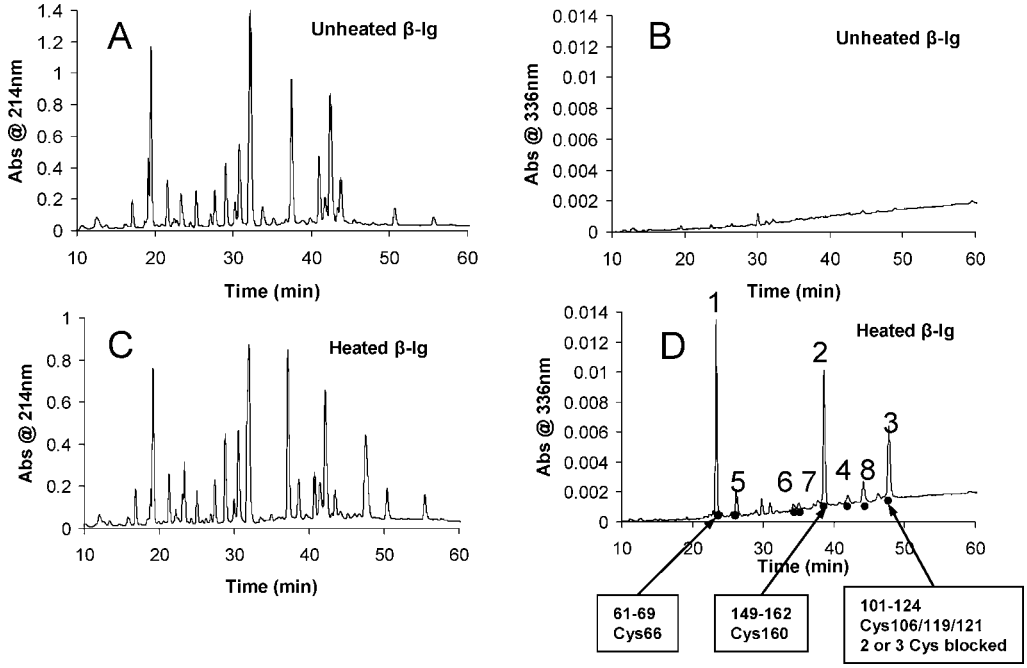

Figure 3. RP-HPLC chromatograms of trypsin-digested sample of β-lactoglobulin unheated (**A**, **B**) and heated for 20 min at 78 °C (**C**, **D**) monitored at 214 nm (**A**, **C**) at 336 nm (**B**, **D**). Numbers are used to identify peaks of interest used in the text and in **Table 2**.

IAEDANS (**Figure 1**, stage II), dialyzed, and subsequently analyzed by Ellman's assay, the results showed that the amount of accessible sulfhydryl groups for reaction with Ellman's reagent was 0.06 ± 0.02 mol/mol of protein, consistent with the proportion of residual native β-lg after 20 min of heating at 78 °C. Therefore, the concentration of exposed sulfhydryl groups in heat-treated β-lg and blocked with IAEDANS was 0.39 mol/mol of proteins (0.45 minus 0.06 mol/mol). The difference between the exposed sulfhydryl groups and the total sulfhydryl groups showed that there are still 54% sulfhydryl groups (0.39 of 0.87 mol/mol) that were not accessible to IAEDANS. They were presumably buried within denatured species and residual native β-lg after 20 min of heating at 78 °C. The number of sulfhydryl groups inaccessible to IAEDANS decreased upon prolonged heating and reached 22% after 60 min at 78 °C (data not shown).

IAEDANS binding to molecular species of β-lg formed upon heating was further analyzed by HP-GPC using simultaneous detection at 280 and 336 nm (**Figure 2**). The chromatogram of the heated solutions showed the presence of native β-lg (retention time, $t_r = 12.4$ min) and non-native β-lg monomers (coeluted with native β-lg) as well as non-native dimers ($t_r = 11.5$ min), oligomers, and larger aggregates ($t_r = 7.7$ min). These molecular species had already been identified in a previous study (*18*), and the electrophoresis results confirm their presence (inset in **Figure 2A**). IAEDANS binding had little effect on the chromatogram observed at 280 nm. However, at 336 nm an increased absorbance is seen for all of the molecular species, indicating that all molecular species had exposed sulfhydryl groups free to react with IAEDANS. Note that there had been an absorbance observed at 336 nm for the aggregates before the addition of IAEDANS, which was probably due to the aggregates scattering light. The difference of absorbance at 336 nm between the samples analyzed before and after blocking with IAEDANS results only from the exposed sulfhydryl groups that were blocked by IAEDANS (**Figure 2B**). The difference in the chromatographic peak area was used to calculate the proportion of exposed sulfhydryl groups for non-native monomers, non-native dimers, oligomers, and aggregates (**Table 1**).

Table 1. Sulfhydryl (–SH) Groups Exposed in the Different Molecular Species Formed after Heating of a β-Lactoglobulin Sample for 20 min at 78 °C[a]

component	–SH concentration (μM)	distribution (%)
aggregates	44.5	41.8
oligomers	16.5	15.5
dimers	15.8	14.8
monomers	29.7	27.9
total	106.5	100

[a] The total protein concentration was 272 μM using 18362 g/mol for the molecular mass of β-lactoglobulin. The concentration of exposed –SH groups after heat treatment (106.5 μM) was calculated from the results of the Ellman assay (0.39 mol/mol of protein, see text). The percentage associated with each molecular species was determined by integration of the chromatograms in **Figure 2B**.

After 20 min of heating at 78 °C, 41.8% of the exposed sulfhydryl groups were in the larger aggregates and 27.9% were in the non-native monomers (native β-lg does not react with IAEDANS), whereas 15% were in both oligomers and dimers.

As shown in this paper, coupling the global quantification of the sulfhydryl groups by Ellman's method with the HP-GPC method presented here can offer an interesting tool to estimate the proportion of exposed sulfhydryl groups on various molecular species included in the same sample. In addition, using IAEDANS enabled the quantification of only the non-native monomers of β-lg having exposed sulfhydryl groups after heating.

Identification of Sulfhydryl Groups Exposed on Heating. Samples that were hydrolyzed with trypsin (**Figure 1**, stage III) showed a high degree of digestion when analyzed by RP-HPLC (**Figure 3**), and cross-checking of both the primary sequence of β-lg and the trypsin specificity enabled us to identify individual peptides absorbing at 214 nm as reported previously (*6*). In the unheated sample, no peaks were detected at 336 nm, which confirms the absence of bound IAEDANS to native β-lg. However, in the heated sample, three major peaks (labeled 1−3 in **Figure 3**) and a number of peaks with lower intensities were detected at 336 nm. These peaks were analyzed and sequenced

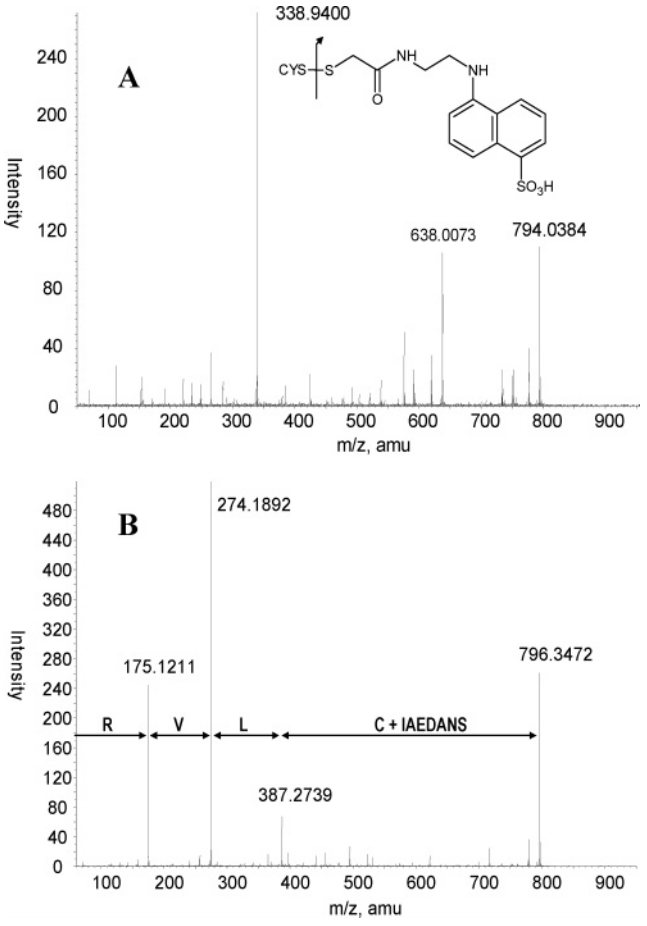

Figure 4. MALDI MS/MS (negative ionization mode, **A**; and positive ionization mode, **B**) of the peptide ^{124}RVL^{121}C-IAEDANS (parent ions at *m*/*z* 794.0384 in negative mode and *m*/*z* 796.3472 in positive mode) resulting from the unspecific cut of the peptide 101/102–124.

by tandem mass spectrometry (MS/MS). Some of the peaks contained more than one peptide. However, peptides that contained IAEDANS could be unambiguously identified by MS/MS in negative ionization mode, whereby a specific fragment from bound IAEDANS is released (*m*/*z* 338.9) (see **Figure 4A**). The linkage of IAEDANS to β-lg sulfhydryl groups probably weakens the C_β−S bond of the cysteine lateral chain, favoring its cleavage and the release of 5-((((2-thioacetyl)amino)ethyl)-amino)naphthalene-1-sulfonic acid (S-AEDANS) when MS/MS is conducted under negative ionization mode. The negative charge density of the released fragment explains its occurrence on the mass spectrum (*m*/*z* 338.9) (**Figure 4A**). Only the peptides releasing the specific fragment at *m*/*z* 338.9 are summarized in **Table 2**. Then, peptides blocked with IAEDANS were sequenced for identification by MS/MS in positive ionization mode. The peptides eluted at 30 min were not assigned because of the absence of a specific fragment at *m*/*z* 338.9 using MS/MS in negative ionization mode and the lack of consistency of their amino acid sequence determined by MS/MS in positive ionization mode. In contrast, peptides that were blocked with IAEDANS in peaks 1 and 2 were assigned to the peptide 61−69 containing blocked Cys66 (peak 1) and the peptide 149−162 containing blocked Cys160 (peak 2).

The peptides eluted in peak 3 were identified by their mass with MS in positive ionization mode and corresponded to peptide 101−124 containing two or three blocked cysteines (Cys106, Cys119, and/or Cys121) with a *m*/*z* of 3475 and 3725, for the former the remaining cysteine being blocked by IAA.

Table 2. Summary of Peptides of Interest

peak (**Figure 3**)	retention time (min)	peptide	Cys blocked by IAEDANS	parent ion (*m*/*z*)
1	22	61–69	66	1428
2	39	149–162	160	1964
3	48	101–124	106, 119, and 121	3725
			two of Cys106, 119, or 121	3475
4	42	102–124	119 or 121	3095[a]
5	26	121–124[b]	121	796
6	34	110–124[b]	119 or 121	2024
7	35	106–124[b]	121	2559
8	46	...–124[c]	119	2730

[a] *m*/*z* minus one NH_3. [b] Unspecific cut. [c] MS/MS result is unambiguous for peptide fragment 119–124 but not clear thereafter because of the absence of peptide fragments for masses higher than *m*/*z*1463.

However, further characterization by MS/MS (positive ionization mode) was not possible because the peptides were very stable and could not be completely fragmented. The lack of MS/MS data made it impossible to identify which of the three cysteines were blocked with IAEDANS in the case of the peptides blocked with two IAEDANS. Peak 4 at 42 min could be associated with a mixture of peptides 102−124 (*m*/*z* 3095 minus one NH_3) containing one blocked cysteine at position 119 or 121, but not at position 106 as confirmed by MS/MS.

There were a number of smaller peaks absorbing at 336 nm, indicating the presence of cysteines blocked with IAEDANS. These peaks (5−8) were also analyzed by MALDI MS/MS and found to be due to unspecific cleavage in the peptide sequence. These cuts often occurred close to cysteine residues, indicating that the presence of the blocking agent IAEDANS can make the peptide more susceptible to cleavage. Presently, it is unclear if the mechanism under these unspecific cleavages has enzymatic or chemical origins. For instance, peak 5 could be unambiguously assigned to the peptide 121−124 with the Cys121 blocked by IAEDANS (**Figure 4B**). The parent ion of the latter is observed at *m*/*z* 796.3 under positive ionization mode. Among the different fragmentations that occurred during the sequencing by MS/MS in positive ionization mode, the loss of a fragment with *m*/*z* 409 confirms the occurrence of a cysteine residue blocked by IEADANS. Although it was not directly observed on the mass spectrum (**Figure 4B**), its complementary ion fragment (RVL at *m*/*z* 387.3) was. The peak eluting at 34 min (peak 6) was assigned to the peptide 110−124. The MS/MS data showed a good correlation for either Cys119 or Cys121 being blocked by IAEDANS, suggesting a mixture of both peptides under peak 6. The peak eluting at 35 min (peak 7) was found to be the peptide 106−124 with only Cys121 being blocked by IAEDANS. The peak eluting at 46 min (peak 8) had the Cys119 blocked by IAEDANS (**Table 2**).

From the total area under the peaks in the chromatograms at 336 nm (**Figure 3D**), including the peaks due to unspecific cuts, we can conclude that the various cysteines exposed on the protein surface after heat treatment are not evenly distributed. This probably depends on the different reactivity of each cysteine and the accessibility of the disulfide bonds for sulfhydryl/disulfide interchange reactions. It is not excluded that the conformation the protein adopts under the conditions of heat treatment (temperature, pH, ionic strength, etc.) determines the proportion of exposed cysteines. Varying these conditions could modify the proportionality. Under the conditions used in the present study, Cys66 is blocked in greatest amounts followed by Cys160. Blocked Cys106 can be found in only peak 3 and accounts for only one of three cysteines. In fact, the peptide

that contained Cys106/119/121 blocked by IAEDANS had its absorbance at 336 nm greatly enhanced compared to peptides having only one blocked cysteine with IAEDANS. It is therefore reasonable to conclude that Cys106 was found to be the less reactive cysteine toward IAEDANS. Furthermore, when the sample was double-digested by trypsin and subsequently by chymotrypsin, in order to induce separation of Cys106 from Cys119 and Cys121, no Cys106 blocked by IAEDANS was found (data not shown). These results were obtained regardless of the heating time up to 60 min at 78 °C (data not shown). This confirms that only small quantities of Cys106 were accessible for reaction with IAEDANS after heat treatment of the protein. This is not unexpected as rapid sulfhydryl/disulfide interchange between Cys106−Cys119 and Cys106−Cys121 may take place upon heat denaturation. This also supports the general concept of the mechanism of heat denaturation of β-lg, whereby the released sulfhydryl group at position Cys119 or Cys121 can react further with the disulfide bond Cys66−Cys160 (intra- or intermolecular), releasing a non-native sulfhydryl group at position 66 or 160 (*6*, *7*, *9*, *10*, *19*). Either way, Cys106 is likely to be involved in disulfide bonds and therefore less likely to be accessible for reaction with IAEDANS. The lower reactivity of Cys106 could be attributed to the fact that this residue is buried in the core of the β-lg molecule and remains inaccessible even when the protein unfolds, as already suggested by Livney and Dalgleish (*20*). In contrast, other sulfhydryl groups of β-lg are readily exposed on the protein surface upon heating and are available for sulfhydryl/disulfide interchange reactions.

Conclusions. Determining the sulfhydryl groups exposed on protein surfaces (accessible for chemical reactions) is of great interest for understanding and controlling their functionalities (aggregation properties, antioxidant properties, etc.). Results presented in this study constitute a new methodology for reaching this goal. In the present study, we combined specific sulfhydryl-blocking reagents and MALDI-TOF mass spectrometry to rapidly identify the exposed cysteine groups on β-lactoglobulin molecules following heating. By its specific absorbance at 336 nm, IAEDANS allows a rapid detection of peptides of interest and consequently their rapid identification combining negative and positive ionization modes. The results reveal some differences in the chemical reactivity of the five cysteine residues of β-lactoglobulin and complete the finding of others on the reactivity and the role of the various cysteine residues. Cys106 seems to be always involved in different disulfide bonds, whereas the other four cysteine residues reacted with IAEDANS, indicating that they were sometimes generated in free sulfhydryl form following sulfhydryl/disulfide exchange reactions. In addition to the role of Cys160 as a major player in sulfhydryl/disulfide exchange reaction (*10*), results obtained highlight the prevalent occurrence of Cys66 as an exposed cysteine under our conditions of heat treatment. For more complex system (mixture of proteins, proteins with high cysteine content such as bovine serum albumin, etc.) this approach would make analysis simpler as blocked cysteine can be rapidly identified.

ABBREVIATIONS USED

β-1g, β-lactoglobulin; Cys, cysteine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; HP-GPC, high-performance gel permeation chromatography; IAA, iodoacetamide; IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino)-naphthalene-1-sulfonic acid; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; RP-HPLC, reverse phase high-performance liquid chromatography; SH, sulfhydryl groups; S−S, disulfide bonds; t_r, retention time; Tris, tris(hydroxymethyl)aminomethane; WPC, whey protein concentrate.

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Received for review February 12, 2007. Revised manuscript received May 21, 2007. Accepted June 1, 2007. Travel costs associated with exchanges of scientists between the authors' laboratories were co-funded by the Enterprise Ireland/French Ministry for Foreign Affairs Ulysses Exchange Research Program. The cost of project work was supported by the Food Institutional Research Measure (FIRM) of the National Development Plan 2000-2006 (Ireland). J.J.K. was funded by the Teagasc Walsh Fellowship Scheme.

JF070397R