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TITLE: Determination of exposed sulphhydryl groups in heated  $\beta$ -lactoglobulin A using IAEDANS and mass spectrometry

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19 **Determination of exposed sulfhydryl groups in heated  $\beta$ -lactoglobulin A using**  
20 **IAEDANS and mass spectrometry.**

21

22

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33 **Abstract**

34 This paper takes a new approach to determining which sulfhydryl groups are exposed  
35 during the heat denaturation of bovine  $\beta$ -lactoglobulin A. The sulfhydryl groups  
36 exposed after heating were blocked with 5-((((2-  
37 iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS). The results  
38 show that IAEDANS is a suitable blocking agent and its absorbance at 336nm enabled  
39 the quantification of exposed sulfhydryl groups in a mixture of protein species by gel  
40 permeation chromatography. Combined with the specific fragmentation of bound  
41 IAEDANS by Matrix Assisted Laser Desorption Ionization (MALDI) MS/MS in  
42 negative ionization mode, this facilitated the identification of peptides that contained  
43 blocked cysteines after enzymatic digestion of the protein. During MALDI MS/MS of  
44 the peptides, in positive ionization mode, the IAEDANS molecule remained bound to  
45 the cysteines making it possible to identify exactly which cysteine had been exposed  
46 after heating. In  $\beta$ -lactoglobulin A it was found that Cysteine 66 and Cysteine 160  
47 were predominantly exposed regardless of the length of exposure to heat.

48

49

50 **Keywords:**

51  $\beta$ -lactoglobulin, free sulfhydryl, IAEDANS, MALDI mass spectrometry

## 52 **Introduction**

53  $\beta$ -Lactoglobulin ( $\beta$ -lg) makes up approximately 50 % of the whey proteins from  
54 bovine milk. It exhibits a predominant role in the functional properties of whey  
55 ingredients. Its native molecular structure is well-established [1]. Native  $\beta$ -lg is  
56 mainly formed with nine  $\beta$ -strands (labeled A to I) organized into two  $\beta$ -sheets facing  
57 each other and a C-terminal  $\alpha$ -helix. It contains five cysteines, giving rise to two  
58 intramolecular disulfide bonds (between Cys66 and Cys160, and between Cys106 and  
59 Cys119) and leaving one sulfhydryl group at position 121 (Cys121) buried in the  
60 molecule. Under physiological conditions, this latter is inaccessible for chemical  
61 reactions. Under favorable conditions (heat, pH or pressure) the globular structure of  
62  $\beta$ -lg unfolds and thereby exposing reactive groups such as sulfhydryl groups on the  
63 protein surface for further aggregation [2]. The exact molecular mechanism leading to  
64 formation of aggregates is not fully understood but disulfide interchange reactions and  
65 the formation of new intra- and intermolecular disulfide bonds was shown to be  
66 prevalent at neutral pH [3-5]. One of the first events taking place during heating is the  
67 formation of non-native monomers containing non-native sulfhydryl groups exposed  
68 on the protein surface resulting from intra-molecular sulfhydryl/disulfide exchange  
69 reactions [6]. Exposed sulfhydryl groups are further able to react with other proteins  
70 leading to the formation of dimers, oligomers and larger aggregates [3, 4, 7]. When  
71 the dimers and trimers were digested with trypsin and analyzed by mass spectrometry  
72 in MALDI and electrospray modes, it was found that several new disulfide bonds  
73 were formed: Cys121-Cys160, Cys106/119/121-Cys160, as well as an intermolecular  
74 Cys160-Cys160 [7, 8]. Cys66-Cys66 linked peptides were only found at low ionic  
75 strength [9]. Under specific system conditions (15 minutes at 80°C), it was found that  
76 35 % of the Cys160 in  $\beta$ -lg is not involved in disulfide bonds after heating, suggesting

77 that its release following sulfhydryl/disulfide exchange reactions is an important step  
78 to propagate intermolecular exchange reactions [10]. Determining which sulfhydryl  
79 groups are exposed on heating (important contributors for sulfhydryl/disulfide  
80 interchange reactions) will give a greater insight into mechanism of how the  
81 sulfhydryl/disulphide exchange reactions proceed. Additionally it will lead to a better  
82 understanding of the reactivity and functionalities of the molecular species formed on  
83 heating.

84 Mass spectrometry analysis performed on an enzymatic digest from heated proteins is  
85 a common method to identify new disulfide bonds formed on heating and draw  
86 conclusions about the chemical reactivity of sulfhydryl groups. However, a  
87 disadvantage of this experimental design is that the conditions of the enzymatic  
88 digestion, i.e. pH 8.0 for a trypsin digest, can cause disulfide reshuffling [11]. This  
89 can be overcome if the sulfhydryl groups are blocked prior to trypsin digestion to  
90 ensure that no reshuffling reactions could take place. The work described in this paper  
91 focused on cysteines which are accessible for chemical reactions (sulfhydryl groups  
92 exposed on the protein surface) after the heat-denaturation of  $\beta$ -lg rather than on those  
93 involved in disulfide linkages. We developed a method using IAEDANS (5-(((2-  
94 iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid) for labeling and  
95 identifying those sulfhydryl groups. This reagent is commonly used as a fluorescence  
96 probe [12] but for the purposes of this study we rather took advantage of its  
97 absorbance at 336 nm.

98

## 99 **Materials and Methods**

### 100 *$\beta$ -lactoglobulin purification*

101 Chemicals were generally sourced from Sigma Aldrich, France, unless stated  
102 otherwise.  $\beta$ -lg was purified from whey protein concentrate (WPC), which was  
103 prepared in-house from fresh milk (pilot plant of Moorepark Technology Ltd.) with  
104 minimal heat treatment to ensure minimal protein denaturation during processing. 25  
105 mL of a 10 % (w/w) WPC reconstituted in mobile phase was injected on a Q-  
106 Sepharose column (Amersham Biosciences). A 10 mM Tris-HCl pH 7.0 buffer (HCl  
107 from Prolabo) with a 0 to 400 mM NaCl gradient was used to elute the proteins. The  
108  $\beta$ -lg A fractions was collected, dialyzed extensively to remove salts and freeze-dried.  
109 The purity of  $\beta$ -lg A was greater than 99%.

110

111 The experimental procedures outlined in the following sections are summarized in  
112 figure 1.

### 113 ***Heat denaturation***

114 Solution of  $\beta$ -lg A was prepared in 20 mM phosphate buffer pH 7.0 containing 50  
115 mM NaCl. The concentration of  $\beta$ -lg A was determined from the absorbance of the  
116 solution at 278 nm, using the specific extinction coefficient of 0.96 L/g/cm, and the  
117 final concentration was adjusted to 5 g/L (272  $\mu$ M using 18,362 g/mol for the  
118 molecular weight of  $\beta$ -lg A) with 20 mM phosphate buffer pH 7.0 containing 50 mM  
119 NaCl. 1 mL aliquots of the protein solution were heated in a water bath at 78°C for 20,  
120 40 and 60 minutes.

121

### 122 ***Electrophoresis***

123 Polyacrylamide gel electrophoresis (SDS-PAGE) was carried according to the method  
124 of Laemmli [13]. Samples of the protein before and after heating were run under  
125 denaturing but non-reducing conditions. The electrophoresis was carried out using

126 15% polyacrylamide gels, at a constant voltage of 155V in a Mini Protean II system  
127 (Bio-Rad, Alpha Technologies, Dublin, Ireland.) Molecular weight standards (GE  
128 Healthcare UK limited) were run on the gel to allow the determination of the  
129 molecular weights of the aggregates.

130

### 131 ***Blocking of sulfhydryl groups with IAEDANS***

132 100  $\mu$ L of an IAEDANS (Invitrogen Molecular Probes, France) solution (2.26 g/L in  
133 20 mM phosphate buffer pH 7.0, 50 mM NaCl) was added to unheated and heat-  
134 treated samples (IAEDANS/exposed sulfhydryl groups molar ratio greater than 4, see  
135 below). Samples were incubated overnight at 37°C in the dark in order to ensure the  
136 complete blocking of exposed sulfhydryl groups. These conditions did not induce a  
137 progression in the aggregation process of  $\beta$ -lg. After incubation, the samples were  
138 dialyzed against phosphate buffer to remove excess IAEDANS.

139

### 140 ***High-performance gel permeation chromatography***

141 The samples were analyzed by high-performance gel permeation chromatography  
142 (HP-GPC) both before and after treatment with IAEDANS. The samples were diluted  
143 1 in 2 and applied to a TSK G3000 SWXL column (TosoHaas, Montgomeryville,  
144 PA. USA). The HPLC system consisted of a Waters 2695 separation module with a  
145 Waters 2487 dual wavelength absorbance detector, the absorbance was measured  
146 simultaneously at 280 nm and 336 nm. A 20 mM sodium phosphate, 50 mM NaCl  
147 buffer pH 7.0 at a flow rate of 0.8 mL/min was used for elution. Molecular species  
148 eluted from the column were assigned according to their molecular weight using  $\alpha$ -  
149 lactalbumin (14,4kDa), ovalbumin (45kDa) and bovine serum albumin (66 kDa) as  
150 standards.

151 .

### 152 *Ellman's Assay*

153 The Ellman's assay [14] was carried out on unheated and heated samples as well  
154 samples that were blocked with IAEDANS after heating and extensive dialysis, to  
155 determine the extent of blocking. The protein solutions were diluted ten fold in either  
156 50 mM Tris-glycine pH 8.0 (for determination of exposed sulfhydryl groups) or in 50  
157 mM Tris-glycine pH 8.0 with 8 M urea (total sulfhydryl groups, urea from Prolabo,  
158 France). 1 mL of diluted solution was placed in a cuvette and 20  $\mu$ L of 5,5'-dithio-  
159 bis(2-nitrobenzoic acid) (DTNB [Merck, France], 10 mM in the Tris-glycine buffer)  
160 was added. The absorbance of the samples was read at 412 nm. The number of  
161 sulfhydryl groups per molecule of  $\beta$ -lg was calculated from the absorbance reading  
162 using 13,600 L/mol/cm as extinction coefficient [15].

163

### 164 *Reduction and alkylation of proteins*

165 200  $\mu$ L of the  $\beta$ -lg solution, which was treated with IAEDANS and subsequently  
166 dialyzed, was diluted with 800  $\mu$ L of 50 mM Tris-HCl buffer containing 6 M urea (pH  
167 8.5) and 10  $\mu$ L of 100 mM dithiothreitol (DTT [Pharmacia Biotech, France] in 50 mM  
168 Tris-HCl buffer containing 6 M urea at pH 8.5). The reduction was carried out at 56°C  
169 for 1 hour. After the reduction 40  $\mu$ L of iodoacetamide (IAA), 2 M, was added to the  
170 solution and it was incubated at room temperature in the dark. The solutions were  
171 dialyzed against a 10 mM carbonate buffer pH 8.5 overnight to remove excess  
172 reagents.

173

174 ***Protein hydrolysis***

175 Dialyzed samples were adjusted to pH 8.0 by the addition of HCl. A stock solution of  
176 1 g/L trypsin was added to the protein solution to bring it to an enzyme to protein ratio  
177 of 1:100 (w/w). The solutions were hydrolyzed overnight at 37°C. 200 µL of  
178 hydrolyzed solution was removed and the pH was reduced below 3 with TFA in order  
179 to inactivate the enzyme. The remainder of the trypsin-hydrolyzed sample had  
180 chymotrypsin (1 g/L) added to a final enzyme to protein ratio of 1:100 (w/w). The  
181 sample was again digested overnight at 37°C and stopped by reducing the pH below 3  
182 with TFA.

183

184 ***Reverse phase chromatography***

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

193

194 ***Mass spectrometry***

195 Peptides absorbing at 336 nm were collected and concentrated to dryness in a Speed  
196 vac. After reconstitution in 5 µL of 50 % acetonitrile containing 0.1 % TFA solution,  
197 they were analyzed by a hybrid quadrupole time of flight (Q/TOF) mass spectrometer  
198 QStar XL, fitted with a MALDI<sup>TM</sup> II (Applied Biosystems/ MDS Sciex, Toronto,

199 Canada). For the MALDI experiments, typically 1  $\mu$ l of co-crystallized sample with 1  
200  $\mu$ l of  $\alpha$ -cyano-4-hydroxycinnamic acid matrix were ionized with a laser beam (337  
201 nm, 20 Hz) and peptide  $\beta$ -CN from  $\beta$ -casein (193-209) was used as a calibration  
202 standard. In each sample the more representative mono-charged ions were submitted  
203 to MS/MS fragmentation with a collision energy depending on the m/z value. Spectra  
204 were acquired in the reflector mode (in both positive and negative ionization modes)

205

## 206 **Results and Discussion**

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

### 214 ***Extent of blocking of the sulfhydryl groups exposed on heating with IAEDANS***

215 Ellman's assay was used to quantify sulfhydryl groups in  $\beta$ -lg solutions before and  
216 after reaction with IAEDANS in order to determine the extent of blocking with  
217 IAEDANS (exposed sulfhydryl groups). Unheated sample of  $\beta$ -lg gave, in the  
218 presence of urea (total sulfhydryl groups),  $0.99 \pm 0.02$  mol of sulfhydryl groups per  
219 mol of protein, which is consistent with one sulfhydryl group per  $\beta$ -lg molecule. When  
220 the assay was carried out in the absence of urea (Figure 1, Stage I),  $0.18 \pm 0.01$   
221 mol/mol of protein were obtained in accordance with previous results [16]. Hoffmann  
222 and van Mill [3] also observed a reaction between DTNB and the sulfhydryl group of  
223 native  $\beta$ -lg at pH 8. Under Ellman's conditions (pH 8), the sulfhydryl group of native

224  $\beta$ -lg is partially accessible for reaction with DTNB due to the reversible  
225 conformational change of  $\beta$ -lg molecules between pH 6 and 8.5, also referred as  
226 Tanford transition [17]. In contrast, no reaction between IAEDANS and native  $\beta$ -lg  
227 (unheated sample) took place at pH 7 indicating that at pH 7 the sulfhydryl group of  
228  $\beta$ -lg was inaccessible to IAEDANS, see later (Figure 3B). Hence, the concentration of  
229 residual native  $\beta$ -lg after heat treatment (for which the sulfhydryl group is partially  
230 accessible to DTNB but inaccessible to IAEDANS) has to be considered for the  
231 quantification of the sulfhydryl groups which have been exposed on heating.

232 After 20 minutes of heating at 78°C, the total sulfhydryl groups had decreased to  
233  $0.87\pm 0.03$  mol/mol of protein. It is presumably brought about by the oxidation of  
234 sulfhydryl groups into disulfide bonds that terminate the propagation reaction  
235 involved with  $\beta$ -lg [2]. In contrast, the concentration of sulfhydryl groups accessible  
236 to DTNB in the absence of urea increased to  $0.45\pm 0.02$  mol/mol of protein. However,  
237 DTNB can react with species having exposed sulfhydryl groups after heat treatment  
238 and the residual native  $\beta$ -lg; the sulfhydryl group of this latter being partially  
239 accessible to DTNB when placed under Ellman conditions (pH 8) due to Tanford  
240 transition [17]. After 20 min of heating at 78°C,  $29\pm 2$  % of  $\beta$ -lg molecules remained  
241 native (soluble after precipitation of denatured/aggregated proteins at pH 4.7) and are  
242 then partially accessible to DTNB. When the heated sample of  $\beta$ -lg was treated with  
243 IAEDANS (Figure 1, Stage II), dialyzed and subsequently analyzed by Ellman's  
244 assay, the results showed that accessible sulfhydryl groups for reaction with Ellman's  
245 reagent was  $0.06\pm 0.02$  mol/mol of protein, consistent with the proportion of residual  
246 native  $\beta$ -lg after 20 min of heating at 78°C. Therefore, the concentration of exposed  
247 sulfhydryl groups in heat treated  $\beta$ -lg and blocked with IAEDANS was 0.39 mol/mol  
248 of proteins ( $0.45$  mol/mol minus  $0.06$  mol/mol). The difference between the exposed

249 sulfhydryl groups and the total sulfhydryl groups showed that there are still 54 %  
250 sulfhydryl groups (0.39 mol/mol out of 0.87 mol/mol) that were not accessible to  
251 IAEDANS. They were presumably buried within denatured species and residual  
252 native  $\beta$ -lg after 20 min of heating at 78°C. The number of inaccessible sulfhydryl  
253 groups to IAEDANS decreased upon prolonged heating and reached 22 % after 60  
254 minutes at 78°C (data not shown).

255 IAEDANS binding to molecular species of  $\beta$ -lg formed upon heating was further  
256 analyzed by HP-GPC using a simultaneous detection at 280 nm and 336 nm  
257 (Figure 2). The chromatogram of the heated solutions showed the presence of native  
258  $\beta$ -lg (retention time,  $t_r = 12.4$  min) and non-native  $\beta$ -lg monomers (co-eluted with  
259 native  $\beta$ -lg) as well as non-native dimers ( $t_r = 11.5$  min), oligomers and larger  
260 aggregates ( $t_r = 7.7$  min). These molecular species had already been identified in a  
261 previous study [18] and the electrophoresis results confirm their presence (insert on  
262 Figure 2A). IAEDANS binding had little effect on the chromatogram observed at 280  
263 nm. However, at 336 nm an increased absorbance is seen for all the molecular species  
264 indicating that all molecular species had exposed sulfhydryl groups free to react with  
265 IAEDANS. Note that there had been an absorbance observed at 336 nm for the  
266 aggregates before the addition of IAEDANS, which was probably due to the  
267 aggregates scattering light. The difference of absorbance at 336 nm between the  
268 samples analyzed before and after blocking with IAEDANS only results from the  
269 exposed sulfhydryl groups that were blocked by IAEDANS (Figure 2B). The  
270 difference in the chromatographic peak area was used to calculate the proportion of  
271 exposed sulfhydryl groups for non-native monomers, non-native dimers, oligomers  
272 and aggregates (Table 1). After 20 minutes of heating at 78°C, 41.8 % of the exposed  
273 sulfhydryl groups were in the larger aggregates, 27.9 % were in the non-native

274 monomers (native  $\beta$ -lg does not react with IAEDANS) while 15 % were in both  
275 oligomers and dimers .

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

282

### 283 *Identification of the sulfhydryl groups exposed on heating*

284 Samples that were hydrolyzed with trypsin (Figure 1, Stage III) showed a high degree  
285 of digestion when analyzed by RP-HPLC (Figure 3) and crosschecking of both the  
286 primary sequence of  $\beta$ -lg and the trypsin specificity enabled us to identify individual  
287 peptides absorbing at 214 nm as reported previously [6]. In the unheated sample, no  
288 peaks were detected at 336 nm, which confirms the absence of bound IAEDANS to  
289 native  $\beta$ -lg. However, in the heated sample, three major peaks (labeled 1, 2 & 3 in  
290 Figure 3) and a number of peaks with lower intensities were detected at 336 nm.  
291 These peaks were analyzed and sequenced by tandem mass spectrometry (MS/MS).  
292 Some of the peaks contained more than one peptide. However, peptides that contained  
293 IAEDANS could be unambiguously identified by MS/MS in negative ionization  
294 mode, whereby a specific fragment from bound IAEDANS is released ( $m/z=338.9$ ),  
295 see Figure 4A. The linkage of IAEDANS to  $\beta$ -lg sulfhydryl groups probably weakens  
296 the  $C_{\beta}$ -S bond of the cysteine lateral chain favoring its cleavage and the release of S-  
297 AEDANS (5-(((2-thioacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid) when  
298 MS/MS is conducted under negative ionization mode. The negative charge density of

299 the released fragment explains its occurrence on the mass spectrum ( $m/z=338.9$ ),  
300 figure 4A. Only the peptides releasing the specific fragment at  $m/z=338.9$  are  
301 summarized in Table 2. Then, peptides blocked with IAEDANS were sequenced for  
302 identification by MS/MS in positive ionization mode. The peptides eluted at 30 min  
303 were not assigned because of the absence of specific fragment at  $m/z=338.9$  using  
304 MS/MS in negative ionization mode and the lack of consistency of their amino acid  
305 sequence determined by MS/MS in positive ionization mode. In contrast, peptides that  
306 were blocked with IAEDANS in peaks 1 and 2 were assigned to the peptide 61-69  
307 containing blocked Cys66 (peak 1) and the peptide 149-162 containing blocked  
308 Cys160 (peak 2).

309 The peptides eluted in peak 3 were identified by their mass with MS in positive  
310 ionization mode and corresponded to peptide 101-124 containing two or three blocked  
311 cysteines (Cys106, Cys119 and/or Cys121) with a  $m/z$  of 3475 and 3725, for the  
312 former the remaining cysteine being blocked by IAA. However, further  
313 characterisation by MS/MS (positive ionization mode) was not possible because the  
314 peptides were very stable and could not be completely fragmented. The lack of  
315 MS/MS data made it impossible to identify which of the three cysteines were blocked  
316 with IAEDANS in the case of the peptides blocked with two IAEDANS. Peak 4 at  
317 42 minutes could be associated to a mixture of peptides 102-124 ( $m/z=3095$  minus  
318 one  $\text{NH}_3$ ) containing one blocked cysteine at position 119 or 121, but not at position  
319 106 as confirmed by MS/MS.

320 There was a number of smaller peaks absorbing at 336 nm indicating the presence of  
321 cysteines blocked with IAEDANS. These peaks (5 to 8) were also analyzed by  
322 MALDI MS/MS and found to be due to unspecific cleavage in the peptide sequence.  
323 These cuts often occurred close to cysteine residues, indicating that the presence of

324 the blocking agent IAEDANS can make the peptide more susceptible to cleavage.  
325 Presently, it is unclear if the mechanism under these unspecific cleavages has  
326 enzymatic or chemical origins. For instance peak 5 could be unambiguously assigned  
327 to the peptide 121-124 with the Cys121 blocked by IAEDANS (Figure 4B). The  
328 parent ion of this latter is observed at  $m/z=796.3$  under positive ionization mode.  
329 Among the different fragmentations that occurred during the sequencing by MS/MS  
330 in positive ionization mode, the loss of a fragment with  $m/z=409$  confirms the  
331 occurrence of a cysteine residue blocked by IEADANS; Although it was not directly  
332 observed on the mass spectrum, figure 4B, its complementary ion fragment (RVL at  
333  $m/z=387.3$ ) was. The peak eluting at 34 minutes (peak 6) was assigned to the peptide  
334 110-124. The MS/MS data showed a good correlation for either Cys119 or Cys121  
335 being blocked by IAEDANS suggesting a mixture of both peptides under peak 6. The  
336 peak eluting at 35 minutes (peak 7) was found to be the peptide 106-124 with only  
337 Cys121 being blocked by IAEDANS. The peak eluting at 46 minutes (peak 8) had the  
338 Cys119 blocked by IAEDANS (Table 2).

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

349 cysteines. In fact, the peptides that contained Cys106/119/121 blocked by IAEDANS  
350 had its absorbance at 336 nm greatly enhanced compared to peptides having only one  
351 blocked cysteine with IAEDANS. It is therefore reasonable to conclude that Cys106  
352 was found to be the less reactive cysteine toward IAEDANS. Furthermore, when the  
353 sample was double-digested by trypsin and subsequently by chymotrypsin, in order to  
354 induce separation of Cys106 from Cys119 and Cys121, no Cys106 blocked by  
355 IAEDANS was found (data not shown). These results were obtained regardless of the  
356 heating time up to 60 minutes at 78°C (data not shown). This confirms that only small  
357 quantities of Cys106 were accessible for reaction with IAEDANS after heat-treatment  
358 of the protein. This is not unexpected as rapid sulfhydryl/disulfide interchange  
359 between Cys106-Cys119 and Cys106-Cys121 may take place upon heat-denaturation.  
360 This also supports the general concept of the mechanism of heat-denaturation of  $\beta$ -lg,  
361 whereby the released sulfhydryl group at position Cys119 or Cys121 can react further  
362 with the disulfide bond Cys66-Cys160 (intra- or intermolecular), releasing a non-  
363 native sulfhydryl group at position 66 or 160 [6, 7, 9, 10, 19]. Either way, Cys106 is  
364 likely to be involved in disulfide bonds and therefore less likely to be accessible for  
365 reaction with IAEDANS. The lower reactivity of Cys106 could be attributed to the  
366 fact that this residue is buried in the core of the  $\beta$ -lg molecule and remains  
367 inaccessible even when the protein unfolds, as already suggested by Livney and  
368 Dalglish, 2004 [20]. In contrast, other sulfhydryl groups of  $\beta$ -lg are readily exposed  
369 on the protein surface on heating and available for sulfhydryl/disulfide interchange  
370 reactions.

371

372 ***Conclusions***

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

393

#### 394 *Abbreviations used*

$\beta$ -lg	$\beta$ -lactoglobulin
Cys	Cysteine
DTNB	5,5'-dithio-bis(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

395

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- 462

463 **Figure Captions**

464

465 Figure 1: Experimental design

466

467 Figure 2: (A) GPC profile at 280nm of  $\beta$ -lactoglobulin heated for 20min at 78°C  
468 before and after blocking with IAEDANS. The gel insert has a molecular weight  
469 standard in lane 1, an unheated sample in lane 2, and samples heated for 20, 40 and 60  
470 minutes in lanes 3 to 5 respectively. (B) Chromatograms at 336nm of the same  
471 samples. Lines indicate limits for integration of areas under the curves for further  
472 calculation of exposed sulphydryl groups (Table 1).

473

474 Figure 3: RP-HPLC chromatograms of trypsin-digested sample of  $\beta$ -lactoglobulin  
475 unheated (A & B) and heated for twenty minutes at 78°C (C & D) monitored at  
476 214nm (A & C) at 336nm (B & D). Numbers are used to identify peaks of interest  
477 used in the text and in Table 2.

478

479 Figure 4: MALDI MS-MS (negative ionization mode, A and positive ionization  
480 mode, B) of the peptide  $^{124}\text{RVL}^{121}\text{C-IAEDANS}$  (parent ions at m/z 794.0384 in  
481 negative mode and m/z 796.3472 in positive mode) resulting from the unspecific cut  
482 of the peptide 101/102-124.

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485

Component	-SH Concentration ( $\mu\text{M}$ )	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

486

487 Table 1: Sulfhydryl (-SH) groups exposed in the different molecular species formed  
488 after heating a  $\beta$ -lactoglobulin sample for 20 min at 78°C. The total protein  
489 concentration was 272  $\mu\text{M}$  using 18,362 g/mol for the molecular weight of  $\beta$ -  
490 lactoglobulin. The concentration of exposed -SH groups after heat-treatment (106.5  
491  $\mu\text{M}$ ) was calculated from the results of the Ellman's assay (0.39 mol/mol of protein,  
492 see text). The percentage associated to each molecular species was determined by  
493 integration of the chromatograms in Figure 2B.

494

Peak No. (Fig 3.)	Retention time (min)	Peptides	Cys blocked by IAEDANS	Parent ions (m/z)
1	22	61-69	66	1428
2	39	149-162	160	1964
3	48	101-124	106,119 and 121 two of Cys106, 119 or 121	3725 3475
4	42	102-124	119 or 121	3095 <sup>a</sup>
5	26	121-124 <sup>b</sup>	121	796
6	34	110-124 <sup>b</sup>	119 or 121	2024
7	35	106-124 <sup>b</sup>	121	2559
8	46	...-124 <sup>c</sup>	119	2730

496 <sup>a</sup> – m/z minus one NH<sub>3</sub>

497 <sup>b</sup> – unspecific cut

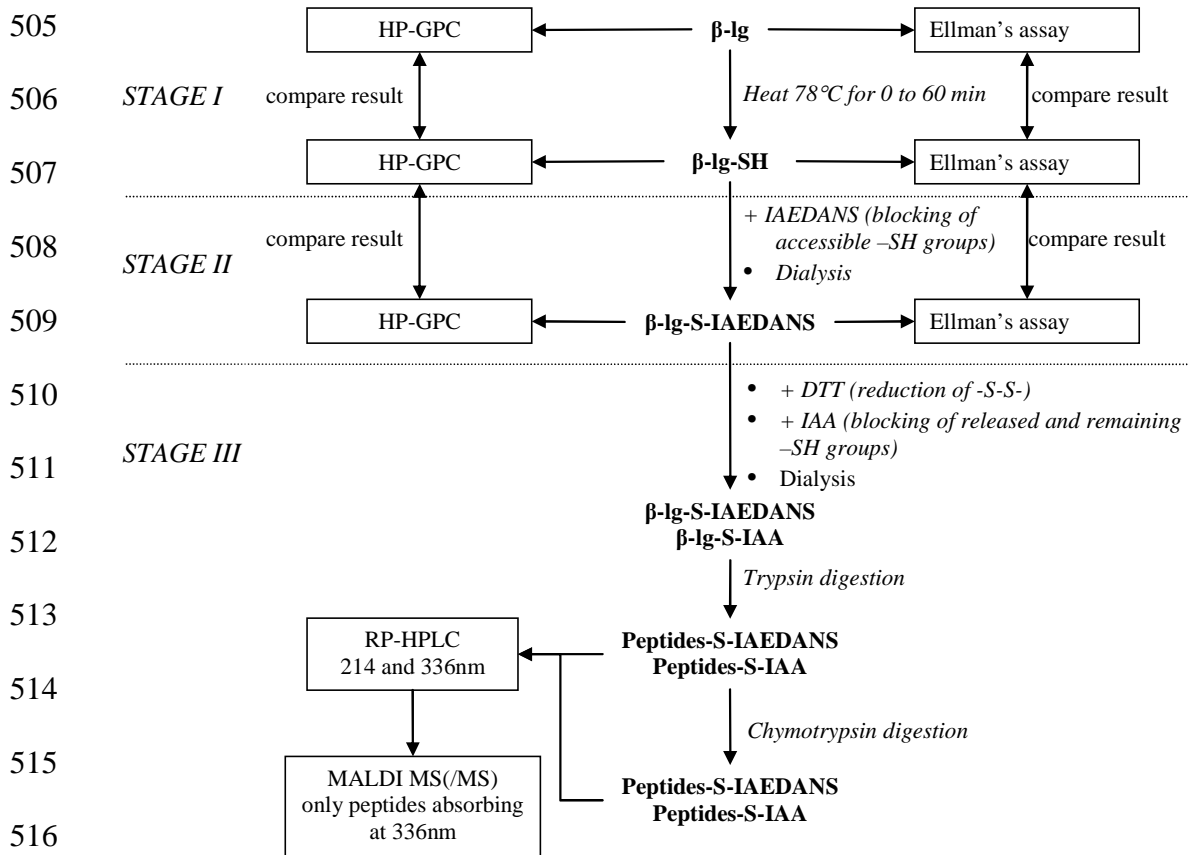
498 <sup>c</sup> – MS/MS result is unambiguous for peptide fragment 119-124 but not clear  
499 thereafter because of the absence of peptide fragments for masses higher than  
500 m/z=1463.

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502 Table 2: Summary of peptides of interest.

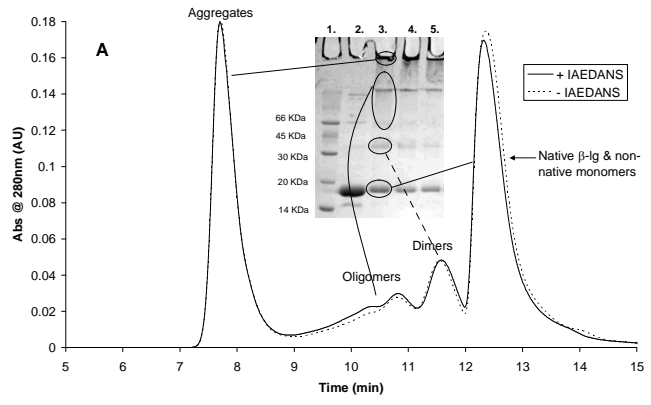
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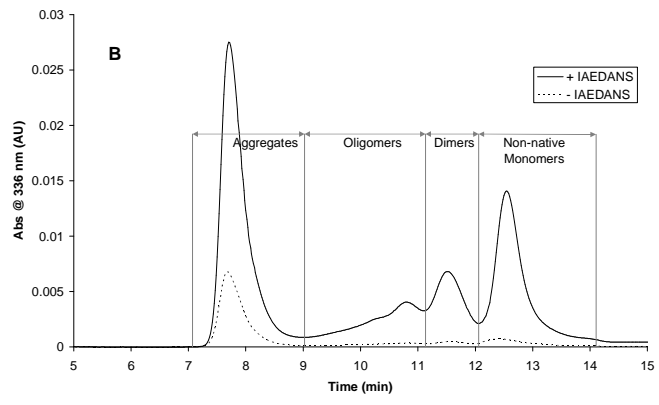


**Figure 1**

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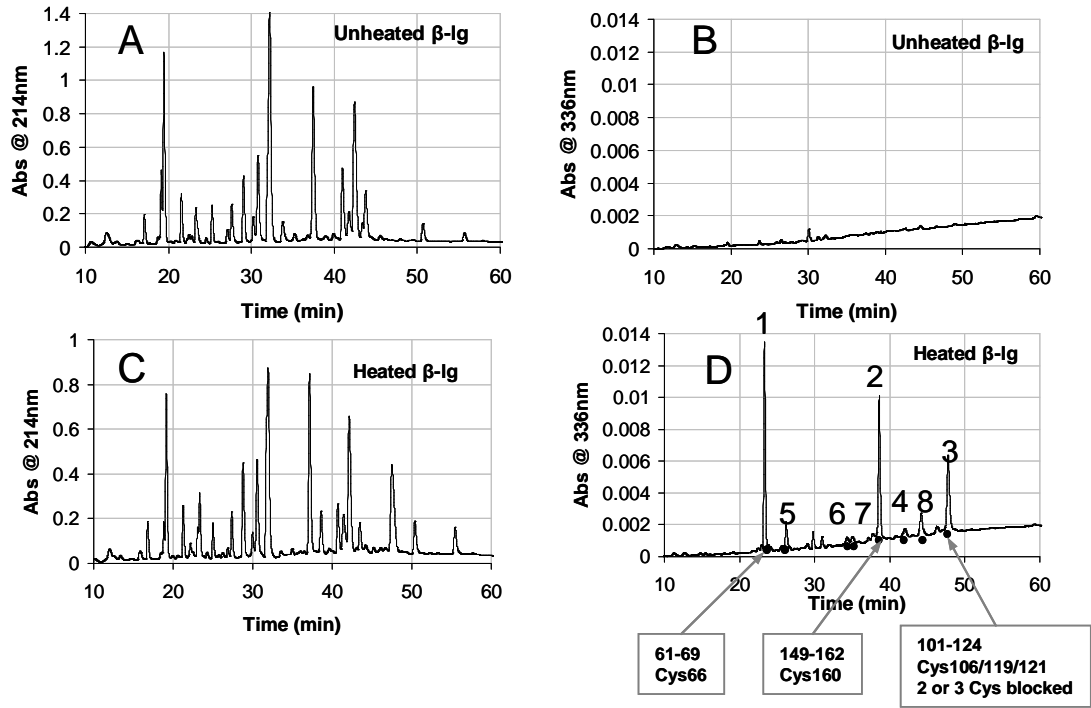


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**Figure 2**

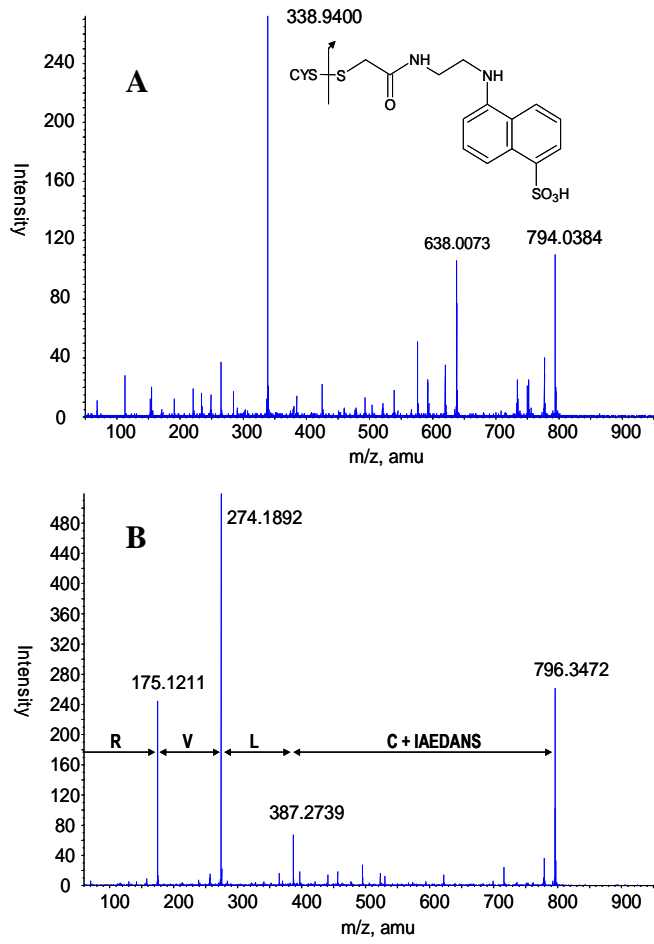


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533 *Figure 3*

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**Figure 4**