



UNIVERSITÀ DI PARMA

ARCHIVIO DELLA RICERCA

University of Parma Research Repository

Effect of Maillard induced glycation on protein hydrolysis by lysine/arginine and non-lysine/arginine specific proteases

This is the peer reviewed version of the following article:

Original

Effect of Maillard induced glycation on protein hydrolysis by lysine/arginine and non-lysine/arginine specific proteases / Deng, Y.; Wierenga, P. A.; Schols, H. A.; Sforza, Stefano; Gruppen, H.. - In: FOOD HYDROCOLLOIDS. - ISSN 0268-005X. - 69:(2017), pp. 210-219. [[10.1016/j.foodhyd.2017.02.007](https://doi.org/10.1016/j.foodhyd.2017.02.007)]

Availability:

This version is available at: 11381/2827476 since: 2021-10-09T11:31:51Z

Publisher:

Elsevier B.V.

Published

DOI:[10.1016/j.foodhyd.2017.02.007](https://doi.org/10.1016/j.foodhyd.2017.02.007)

Terms of use:

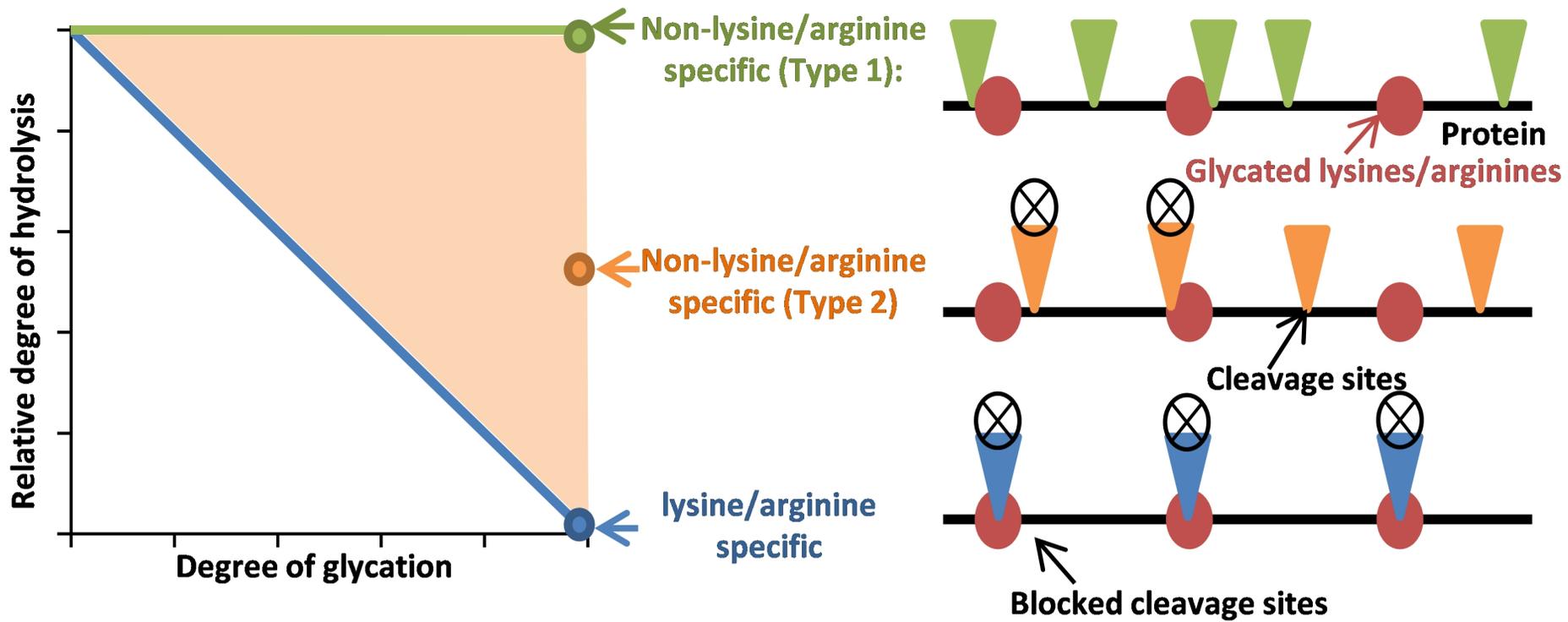
Anyone can freely access the full text of works made available as "Open Access". Works made available

Publisher copyright

note finali coverpage

(Article begins on next page)

13 August 2025



1 **Effect of Maillard induced glycation on protein hydrolysis by**
2 **lysine/arginine and non-lysine/arginine specific proteases**

3

4 Y. Deng^a, P.A. Wierenga^a, H.A. Schols^a, S. Sforza^{a,b} and H. Gruppen^{a*}

5 ^a*Laboratory of Food Chemistry, Wageningen University & Research, P.O.Box 17, 6700 AA Wageningen, The*
6 *Netherlands*

7 ^b*Department of Food Science, University of Parma, Parma, Italy*

8

9 **Corresponding author:*

10 *Telephone: +31 317483211*

11 *Email: harry.gruppen@wur.nl*

12 **Abstract:**

13 Enzymatic protein hydrolysis is sensitive to modifications of protein structure, e.g. Maillard
14 reaction. In early stages of the reaction glycation takes place, modifying the protein primary
15 structure. In later stages protein aggregation occurs. The specific effect of glycation on
16 protein hydrolysis was studied using α -lactalbumin glycated with D-glucose at 50 °C (0-10 h).
17 This resulted in proteins with different degrees of glycation (DG=0-63 %) without changes in
18 secondary, tertiary and quaternary structure. These glycated proteins were hydrolyzed by
19 lysine/arginine specific proteases (bovine and porcine trypsin) or by non-lysine/arginine
20 specific proteases (*Bacillus licheniformis* protease (BLP), α -chymotrypsin and subtilisin A). For
21 bovine and porcine trypsin, the maximal degree of hydrolysis decreased linearly with 65 %
22 from untreated to maximal glycated protein (DG=63 %). This means trypsin cannot hydrolyze
23 glycated cleavage sites. BLP and subtilisin A hydrolyses were independent of glycation, while
24 α -chymotrypsin cannot hydrolyze cleavage sites with glycated binding sites. This means for
25 non-lysine/arginine specific proteases, the effect of glycation depends on the enzyme
26 sensitivity towards modifications on binding sites. Since not all cleavage sites are efficiently
27 used by the enzymes, the extent of the effects depends on the enzyme selectivity towards
28 cleavage sites (for trypsin) or cleavage sites near glycation sites (for α -chymotrypsin).
29 Combining the results of all proteases, an equation was derived describing the effect of
30 modification of protein primary structure on the extent of hydrolysis based on the enzyme
31 specificity, selectivity and binding site sensitivity.

32

33 **Key words:** Maillard reaction; α -lactalbumin; LC-MS; protease specificity; protease selectivity;
34 binding site

35

36 **1. Introduction**

37 Enzymatic protein hydrolysis is sensitive to modifications of the protein structure, which can
38 be induced by industrial processes, e.g. Maillard reaction. In food production, the Maillard
39 reaction is sometimes applied to improve the techno-functional properties of proteins, e.g.
40 emulsion stability (Darewicz & Dziuba, 2001) and foam stability (Wierenga, Van Norél, &
41 Basheva, 2009). In other cases, the reaction is a non-desired side-effect of production and
42 storage of food products. The Maillard reaction results in glycation of lysine residues, but the
43 proteins can also undergo unfolding or aggregation. These factors may all affect protein
44 hydrolysis during digestion, as well as during industrial production of protein hydrolysates.
45 The effects may further depend on the enzymes used for the protein hydrolysis. This study
46 focusses on a quantitative understanding of the effects of glycation on enzymatic protein
47 hydrolysis.

48

49 The early stage of the Maillard reaction changes the protein primary structure. In the first
50 step of the Maillard reaction, also called glycation, the reducing end of a carbohydrate reacts
51 with a free amino group, which results in Amadori compounds. This amino group can be the
52 side chain of a lysine residue, an arginine residue or at the N-terminal amino acid in a protein.
53 For example, the glycation of α -lactalbumin with glucose at 60 °C with 65 % relative humidity
54 for 8 h resulted in a mixture of variants with 6-13 glucoses on the α -lactalbumin molecule
55 (ter Haar, Schols, & Gruppen, 2011). In perspective of protein hydrolysis, the glycation can
56 change the protein primary structure. In later stages of the reaction, secondary reactions
57 take place, which lead to protein aggregation (Chevalier, Chobert, Mollé, & Haertlé, 2001),
58 e.g. through lysine-arginine cross-links (Biemel, Reihl, Conrad, & Lederer, 2001). In addition,
59 the heating itself may result in aggregation through lysinoalanyl cross-links (Fritsch,
60 Hoffmann, & Klostermeyer, 1983) and/or non-covalent aggregation. These reactions can
61 influence the secondary, tertiary and quaternary structure of the protein. Each of these
62 structural changes can influence the susceptibility of the protein to enzymatic hydrolysis.
63 The occurrence of the secondary reactions also confuse published results of the influence of
64 glycation on protein hydrolysis. For example, one study showed that the pancreatic
65 hydrolysis rate of whey protein was decreased by glycation with dextrans (Böttger, Etzel, &
66 Lucey, 2013). Also, the hydrolysis of two infant formulas made of soy protein isolates by a
67 mixture of trypsin, chymotrypsin and intestinal peptidase showed that the sample with the

68 highest degree of glycation resulted in the lowest degree of hydrolysis (Pereyra González,
69 Naranjo, Malec, & Vigo, 2003). In contrast to these findings, other studies showed that
70 glycation has been shown to increase the extent of protein hydrolysis. In the same study,
71 when the hydrolysis of two infant formulas made of bovine milk powder were compared,
72 the sample with the highest glycation had the highest degree of hydrolysis (Pereyra González,
73 et al., 2003). Another indication of increased hydrolysis with increased glycation was
74 obtained from the comparison of the hydrolysis of ultra-high-temperature (UHT) milk
75 compared to pasteurized milk by pepsin and pancreatin. The amount of intact proteins
76 remaining after hydrolysis was lower in the UHT milk than in the pasteurized milk, despite
77 higher levels of Maillard reaction indicators (carboxymethyllysine and lactulosyllysine) in the
78 UHT milk (Wada & Lönnerdal, 2014). The inconsistent reports on the effects of Maillard
79 reaction on subsequent enzymatic hydrolysis may be in part due to the fact that not only
80 glycation, but also aggregation may have occurred in the samples. It is not clear whether the
81 dominant factor of the influence was glycation or aggregation because aggregation was also
82 reported to affect hydrolysis (Pinto, et al., 2014). One study reported that the proportion of
83 total glycated amino groups in β -casein samples was up to 40 %, while 60 % of the proteins
84 were covalently aggregated (Bhatt, et al., 2014). It was observed that the initial rate of
85 plasmin hydrolysis linearly decreased 60 % with the maximal glycated sample, which also
86 had the highest amounts of aggregates (Bhatt, et al., 2014). Another reason for the variety
87 in reported effects of glycation on hydrolysis could be that different enzymes were used.

88

89 For enzymes that are specific for lysine and arginine residues, e.g. trypsin and plasmin, it is
90 commonly assumed that the hydrolysis is negatively influenced by glycation because the
91 glycated cleavage site cannot be utilized by trypsin. As a consequence, the extent of trypsin
92 hydrolysis is expected to decrease by glycation. Most studies that annotated peptides after
93 tryptic hydrolysis of glycated proteins did not find peptides that were cleaved after glycated
94 lysine/arginine residues (Lapolla, et al., 2004; Morgan, Léonil, Mollé, & Bouhallab, 1997).
95 Only two previous studies reported cleavage peptide bonds after glycated lysines, based on
96 annotation of the formed peptides. They reported that 1 out of 19 (Moreno, Quintanilla-López,
97 Lebrón-Aguilar, Olano, & Sanz, 2008) and 3 out of 16 (Carulli, Calvano, Palmisano, &
98 Pischetsrieder, 2011) of the peptides formed during the hydrolysis were cleaved after
99 glycated lysines, respectively. A study using model peptides showed that the replacement of

100 the charged side chain of lysine by the neutral side chain of heptyline resulted in a decrease
101 of the hydrolysis rate constants by a factor of 10^2 - 10^6 (Sanborn & Hein, 1968). The
102 attachment of carbohydrate on the lysine side chain by glycation might have an even larger
103 influence because not only the charge, but also the size of the side chain of lysine is modified.

104

105 For enzymes that are not specific for lysine and arginine residues, information on the
106 influence of glycated residues proximate to the cleavage sites on protein hydrolysis has not
107 been reported. However, it has been mentioned that the binding of the enzyme towards the
108 cleavage site depends on the interaction between the catalytic environment of the enzyme
109 and the binding environment of the substrate (Schechter & Berger, 1967). According to this
110 information, the P4 to P4' positions (binding site) are the amino acids that come before and
111 after the cleavage site (P1 position) on the primary sequence (Schechter, et al., 1967). Hence,
112 if the glycation sites are on the binding site positions, it is possible that the glycation
113 influences protein hydrolysis catalyzed by non-lysine/arginine specific enzymes as well.

114

115 In addition to the above, it should be noted that during hydrolysis of native proteins, not all
116 cleavage sites are hydrolyzed at an equal rate. This means that when hydrolyzing a protein
117 by an enzyme, the final DH might not reach the theoretical maximum. It has been shown for
118 instance that bovine trypsin has different hydrolysis rates towards the various lysine and
119 arginine residues within the native β -lactoglobulin and β -casein (Cheison, Lai, Leeb, & Kulozik,
120 2011; Vorob'ev, Dalgarrondo, Chobert, & Haertlé, 2000). Recently, the relative hydrolysis
121 rate constants of the various cleavage sites on a protein were determined quantitatively and
122 defined as selectivity (Claire I Butré, Sforza, Gruppen, & Wierenga, 2014). It was shown that
123 6 out of 26 of the cleavage sites in β -lactoglobulin were not cleaved by *Bacillus licheniformis*
124 protease (BLP) and 9 cleavage sites were responsible for 95 % of the total hydrolysis rate
125 constant (Claire I Butré, et al., 2014). This means that the glycation of some sites might have
126 a larger effect on the total hydrolysis than the glycation of other sites.

127

128 In the present study, the effects of glycation on enzymatic hydrolysis were studied using α -
129 lactalbumin and D-glucose. The hydrolysis was performed using lysine/arginine specific
130 enzymes (i.e. bovine and porcine trypsin) and non-lysine/arginine specific enzymes (i.e. BLP,
131 α -chymotrypsin and subtilisin A). The aim is to determine whether altering protein primary

132 structure by glycation influences the protein hydrolysis catalyzed by enzymes with various
133 specificities and the quantitative relationship between the degree of glycation and degree of
134 hydrolysis.

135

136 **2. Materials and methods**

137 **2.1 Materials**

138 α -Lactalbumin (α -LA) was obtained from Davisco Foods International Inc. (Le Sueur, MN,
139 USA). The monoisotopic mass of the protein is 14,178 Da, as found in ExPASy PeptideMass
140 (P00711) (http://web.expasy.org/peptide_mass). Mass spectrometry (MS) analysis showed
141 that there were no naturally glycosylated variants of the protein present in the sample. Of
142 the α -LA powder, the total protein content is ~92.8 % (w/w), as determined using Dumas
143 method ($N \times 6.25$, based on the amino acid composition of the α -LA as described in Uniprot
144 P00711, www.uniprot.org). Of the total protein content, ~90 % is α -LA, based on the
145 proportion of α -LA UV_{214} peak area of the total UV_{214} area using reversed phase ultra-high
146 performance liquid chromatography (RP-UHPLC) (Claire. I. Butré, Sforza, Wierenga, &
147 Gruppen, 2015). The other proteins present were β -lactoglobulin and bovine serum albumin
148 according to MS results (data not shown). The α -LA was free of lactose and glucose, as
149 determined by high performance anion exchange chromatography (HPAEC, data not shown).
150 This was also confirmed by the fact that no glycation by glucose or lactose was found after
151 heating the protein for 10 h at 50 °C with 65 % relative humidity (determined using MS, data
152 not shown). Based on the results from circular dichroism (Heijnis, Wierenga, Van Berkel, &
153 Gruppen, 2010), ~72 % of the α -LA was in the apo form (data not shown).

154

155 Bovine trypsin (EC 3.4.21.4, Sigma-Aldrich, St. Louis, MO, USA) was treated with tosyl-
156 phenylalanyl-chloromethyl ketone (TPCK) to inactivate any chymotrypsin activity present
157 (chymotrypsin activity is ≤ 0.1 N-benzoyl-L-tyrosine-ethyl-ester (BTEE) units/mg protein).
158 Porcine trypsin (EC 3.4.21.4, Sigma-Aldrich) was also treated with TPCK and the
159 chymotrypsin activity is ≤ 1 BTEE units/mg protein. Both bovine and porcine trypsin powders
160 are essentially salt-free lyophilized. According to the manufacturer, the activities of bovine
161 trypsin and porcine trypsin were $\geq 10,000$ and 13,000-20,000 benzoyl-L-arginine-ethyl-ester
162 (BAEE) units/mg protein, respectively. The protein content of the bovine trypsin preparation

163 is ~80 % as based on Dumas results (N×5.97, based on the amino acid composition of the
164 protein as described in Uniprot P00760), from which 100 % of the protein is bovine trypsin
165 (based on UV₂₈₀ peak area from RP-UHPLC). Based on the UV₂₁₄ peak area, 93.4 % is the
166 intact enzyme and the remaining 6.6 % were identified using MS as autolysis products of
167 bovine trypsin (data not shown). The protein content of porcine trypsin is ~86 % as based on
168 Dumas results (N×5.84, based on the amino acid composition of the protein as described in
169 Uniprot P00761), from which 100 % of the protein is trypsin (based on UV₂₈₀ peak area from
170 RP-UHPLC). Based on the UV₂₁₄ peak area, 92.6 % is the intact enzyme and the remaining 7.4 %
171 were identified as autolysis products (data not shown). *Bacillus licheniformis* protease (BLP)
172 (batch NS-37005) was obtained from Novozymes (Bagsvaerd, Denmark). The BLP powder
173 was partly insoluble. Therefore, the suspension was centrifuged (10 min, 4000×g, 25 °C) and
174 the supernatant was subsequently dialyzed (cut-off 12-14 kDa) against 150 mM sodium
175 chloride solution and subsequently demineralized water. The freeze dried BLP powder
176 contained 60 % (w/w) protein (N×5.93, based on the amino acid composition of the protein
177 as described in Uniprot P80057) and an activity of 3.9 AU mg⁻¹ min⁻¹ determined by the
178 azocasein assay (Akpinar & Penner, 2001). The purity of BLP was determined to be 100 %
179 from the UV₂₈₀ peak area coupled with RP-UHPLC and 92 % from the UV₂₁₄ peak area. In the
180 UV₂₁₄ chromatogram, two peaks were found, of which the main peak (78 % of total peak
181 area) was identified using MS to be BLP (23.6 kDa) and the minor peak (14 % of total peak
182 area) was the pro-peptide (6.9 kDa) (Claire I. Butré, Wierenga, & Gruppen, 2014).

183

184 α-Chymotrypsin (EC 3.4.21.1, Sigma-Aldrich) was treated with N-p-tosyl-L-phenylalanine
185 chloromethyl ketone (TPCK) to inactive any trypsin activity present. The α-chymotrypsin is
186 essentially a salt-free lyophilized powder, which had ≥40 BTEE units/mg protein. The protein
187 content of α-chymotrypsin is ~86 % based on Dumas results (N×5.99, based on the amino
188 acid composition of the protein as described in Uniprot P00766), from which 100 % of the
189 protein is α-chymotrypsin (based on UV₂₈₀ and UV₂₁₄ peak area from RP-UHPLC). Subtilisin A
190 (EC 3.4.21.62, Sigma-Aldrich) is essentially salt-free lyophilized powder with a protein
191 content of ~86 % as based on Dumas results (N×5.97, based on the amino acid composition
192 of the protein as described in Uniprot P00780). Since subtilisin A did not bind to the RP
193 column, sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to confirm
194 that impurities in the subtilisin A were neglectable (data not shown).

195 The cleavage sites of these enzymes and the glycation sites in α -lactalbumin are indicated in
196 **figure 1**. All other chemicals were of analytical grade and purchased from Sigma or Merck.

197

198 **2.2 Methods**

199 **2.2.1 Glycation of α -lactalbumin (α -LA)**

200 Glycation of α -LA was performed essentially as described previously (ter Haar, et al., 2011).
201 α -LA (10 mg powder/mL) and D-glucose (3.5 mg/mL) were each dissolved separately in 10
202 mM sodium phosphate buffer pH 8.0. These two solutions were mixed to reach a molar ratio
203 of total free amino groups (12 lysines, 1 arginine and the N-terminal): sugar reducing ends of
204 1:2 and subsequently freeze dried (in 30 mL batches). The freeze dried powder was
205 incubated in a humidity control chamber HCP108 (Mettler, Schwabach, Germany) at 50 °C
206 with 65 % relative humidity for 0, 1, 2, 3, 4, 6, 8 and 10 h and labelled as AG₀ -AG₁₀. Samples
207 were immediately cooled down and stored at -20 °C before dialysis. To remove the buffer
208 salts and free glucose, the samples were dialyzed (cut-off 12-14 kDa) against demineralized
209 water for ≥ 72 h at 4 °C, freeze dried again and then stored at -20 °C. The concentration of
210 remaining glucose in the sample was ≤ 0.001 g glucose/g protein based on HPAEC analysis
211 (data now shown). As control for effects of heating, an α -LA solution mixed with 10 mM
212 sodium phosphate buffer pH 8.0 without glucose was made. This sample was freeze-dried
213 and heated the same way as described above for 10 h (labelled as A₁₀). The UV₂₈₀ extinction
214 coefficients of untreated α -LA and AG₁₀ were determined to be $1.4 \pm 0.02 \cdot 10^{-4} \text{ L} \cdot \text{M}^{-1} \cdot \text{cm}^{-1}$ by
215 measuring the UV₂₈₀ of a dilution series of a protein solution with a known concentration
216 (based on Dumas results). The value described in Uniprot (P00711) is $1.4 \cdot 10^{-4} \text{ L} \cdot \text{M}^{-1} \cdot \text{cm}^{-1}$. This
217 means that the glycation does not have an influence on the extinction coefficient of the
218 protein. Therefore, $1.4 \cdot 10^{-4} \text{ L} \cdot \text{M}^{-1} \cdot \text{cm}^{-1}$ was used to calculate the concentration of the protein
219 for all experiments at UV₂₈₀. All samples were made in duplicate. The standard deviations in
220 the results of characterization of glycated α -LA represent the differences between the
221 separately glycated duplicate samples.

222 **2.2.2 Size exclusion chromatography (SEC)**

223 SEC was performed on an ÄKTA micro system (GE Healthcare, Uppsala, Sweden) equipped
224 with a Superdex 75 HR 10/300 column (GE Healthcare) as described previously (Claire I.
225 Butré, Wierenga, & Gruppen, 2012). The experiments were performed at 20 °C with 10 mM
226 sodium phosphate buffer pH 8.0 containing 150 mM NaCl (filtered over a 0.22 μm

227 membrane) at a flow rate of 800 $\mu\text{L}/\text{min}$. The untreated $\alpha\text{-LA}$, $\text{AG}_0\text{-AG}_{10}$ and A_{10} were
228 dissolved in the eluent (with a protein concentration of 0.2 % (w/v) based on UV_{280}). The
229 eluate (50 μL) was monitored at 280 nm. The average standard deviation of the duplicates
230 for all samples was $\sim 1\%$.

231 2.2.3 Circular Dichroism (CD)

232 CD spectra were measured using a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) as
233 described elsewhere (Delahaije, Wierenga, Giuseppin, & Gruppen, 2014). The native and
234 glycated proteins were dissolved in 10 mM sodium phosphate buffer at pH 8.0 at protein
235 concentrations of 0.01 % (w/v) and 0.1 % (w/v) (based on UV_{280}) for far and near UV-CD,
236 respectively. The measurements were performed at 37 $^\circ\text{C}$ in quartz cuvettes with an optical
237 path length of 1 mm. The secondary structure of the samples was measured using far UV-CD
238 recorded from 190 to 260 nm. The tertiary structure of the samples was measured using
239 near UV-CD recorded from 250 to 350 nm. All spectra reported are the average of ten
240 spectra.

241 2.2.4 o-Phtaldialdehyde method (OPA)

242 The amount of free amino groups was determined using the OPA method (Claire I. Butré, et
243 al., 2012). The native and glycated proteins were dissolved ($7 \cdot 10^{-4}$ M) in 2 % (w/v) sodium
244 dodecyl sulfate solution. 5 μL of the protein solution was added to 300 μL of OPA reagent
245 solution and incubated for 10 min. The experiments were performed in triplicate with a 96
246 well plate using Tecan Infinite F500 (Tecan, Männedorf, Switzerland) to measure UV_{340}
247 absorption of the samples. To calculate the number of free amino groups, a calibration curve
248 was made using leucine. The protein concentration of each sample was determined by UV_{280} .
249 The average degree of glycation (DG_{av}) is defined as the average percentage of blocked
250 amino groups per protein, which was calculated from equation 1 (Delahaije, et al., 2014):

$$251 \quad \text{DG}_{\text{av,OPA}}(\%) = (\#\text{NH}_{2,\text{untreated}} - \#\text{NH}_{2,\text{sample}}) / \#\text{NH}_{2,\text{untreated}} \times 100 \% \quad (1)$$

252 where $\#\text{NH}_{2,\text{untreated}}$ is the total number of free amino groups per untreated $\alpha\text{-LA}$ molecule
253 and $\#\text{NH}_{2,\text{sample}}$ is the number of free amino groups per protein molecule after glycation.

253 2.2.5 Reversed phase ultra-high performance liquid chromatography electron spray 254 ionization time of flight mass spectrometry (RP-UHPLC-ESI-Q-TOF-MS)

255 The untreated and glycated $\alpha\text{-LA}$ were analyzed on an H class Acquity UPLC system (Waters,
256 Milford, MA, USA) equipped with a C4-reversed phase column (UPLC BEH C4 1.7 μm ,
257 2.1 \times 100 mm, Waters) coupled to an Acquity UPLC[®] PDA detector (Waters). Eluent A was

258 Millipore water containing 1 % (v/v) acetonitrile (ACN) and 0.1 % (v/v) trifluoroacetic acid
 259 (TFA). Eluent B was ACN containing 0.1 % (v/v) TFA. The gradient profile was performed
 260 using the following steps: from 0 to 2 min isocratic 10 % B, from 2 to 12 min linear gradient
 261 from 10 % to 75 % B, from 12 to 15 min linear gradient from 75 % to 100 % B, from 15 to 20
 262 min isocratic at 100 % B, then re-equilibration to the initial conditions. Flow rate was 350
 263 $\mu\text{L}/\text{min}$. Samples were diluted into eluent A (1 mg/mL) and centrifuged (10 minutes, $4,000 \times$
 264 g, $20\text{ }^\circ\text{C}$) before injection. The injection volume was 5 μL . The PDA detector was operated at
 265 a sampling rate of 40 points/second in the range 200-400 nm with a 1.2 nm resolution. The
 266 molecular masses of untreated α -LA, AG₀-AG₁₀ and A₁₀ were determined with an online
 267 Synapt G2-Si high definition mass spectrometer (Waters). The MS was calibrated with
 268 sodium iodide. The capillary voltage was set to 3 kV with the source operation in positive ion
 269 mode and the source temperature at $150\text{ }^\circ\text{C}$. The sample cone was operated at 30 V.
 270 Nitrogen was used as desolvation gas ($500\text{ }^\circ\text{C}$, 800 L/h) and cone gas (200 L/h). MS were
 271 collected between m/z 150-4000 with a scan time of 0.3 seconds. Data were acquired by
 272 MassLynx software v4.1 (Waters). The mass spectra were deconvoluted and centered to bar
 273 spectra. To compare the MS data with OPA results, the DG_{av} was calculated from equation 2,

$$DG_{av,MS}(\%) = \sum_{i=0}^n \frac{i \times \text{total ion count of variant } i}{\text{total ion count of all variants}} \bigg/ \#NH_{2,untreated} \times 100\% \quad (2)$$

274 where variant i indicates the protein molecule with i number of glucose molecules attached
 275 and $\#NH_{2,untreated}$ is the total number of free amino groups per untreated α -LA molecule.

276 2.2.6 High performance size exclusion chromatography (HPAEC)

277 The amounts of glucose and lactose in the samples were analyzed by HPAEC using an ICS
 278 5000 (Thermo Scientific, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (2 mm
 279 i.d. \times 250 mm) in combination with a Carbo Pac guard column (2 mm i.d. \times 50 mm) and
 280 pulsed amperometric detection (PAD). The measurements were performed as described
 281 elsewhere (Van Gool, et al., 2011).

282 2.2.7 Enzymatic hydrolysis

283 The untreated and glycosylated α -LA variants were dissolved in 10 mL Millipore water at a
 284 protein concentration of 1 mg/mL (based on UV_{280}). The protein solutions were preheated
 285 for 30 minutes at 37°C and then adjusted to pH 8.0 before addition of the enzyme. During
 286 the hydrolysis (2 hours at 37°C), the pH was kept constant with 0.02 M NaOH using a pH-stat
 287 (Metrohm, Herisau, Switzerland) (Claire I. Butré, et al., 2012). All enzymes were dissolved (10

288 mg powder/mL) in Millipore water and added to the protein solution. The overall hydrolysis
 289 rate constant k_{hydr} was obtained by fitting equation 3 to the curve of DH versus time t,

$$DH(\%) = DH_{max}^{enzyme} - DH_{max}^{enzyme} / (1 + k_{hydr} \times t \times DH_{max}^{enzyme}) \quad (3)$$

290 in which k_{hydr} and DH_{max}^{enzyme} are fitting parameters for overall hydrolysis rate and maximum
 291 DH, respectively. It was assumed that the enzymatic hydrolysis follows the second order
 292 reaction kinetics. Parameter $k_{hydr} \times DH_{max}^{enzyme}$ was used to compare hydrolysis rates of the
 293 glycosylated proteins in order to rule out the influence of on DH_{max}^{enzyme} the k_{hydr} . To obtain similar
 294 value of $k_{hydr} \times DH_{max}^{enzyme}$ (s^{-1}) for untreated α -LA, an enzyme substrate ratio of 1:25, 1:38, 1:25,
 295 1:13 and 1:23 (w/w protein) was used by adding 50 μ L bovine trypsin, 30 μ L porcine trypsin,
 296 67 μ L BLP, 90 μ L α -chymotrypsin or 50 μ L subtilisin A solution in 10 mL protein solution,
 297 respectively. The enzyme was added to the substrate solution within 5 minutes after
 298 dissolving in water in order to prevent autolysis. The experimental degree of hydrolysis (DH)
 299 was calculated using equation 4 (Adler-Nissen, 1986),

$$DH(\%) = V_b \times N_b \times \frac{1}{\alpha} \times \frac{1}{m_p} \times \frac{1}{h_{tot}} \times 100 \% \quad (4)$$

300 where V_b is the volume of NaOH added in L; N_b is the normality of NaOH; α is the average
 301 degree of dissociation of the α -NH group ($1/\alpha=1.3$ at 37°C and pH 8.0) (Claire I. Butré, et al.,
 302 2014); m_p is the mass of protein in the solution in g; h_{tot} is the total number of moles of
 303 peptide bonds per gram protein substrate ($8.6 \cdot 10^{-3}$ mmol/g for α -LA). The h_{tot} is calculated
 304 based on the number of peptide bonds per α -LA molecule and the molecular mass
 305 mentioned in Uniprot (P00711). A blank experiment was performed by incubating the
 306 untreated α -LA in 10 mL Millipore water at a protein concentration of 1 mg/mL (based on
 307 UV_{280}) with no enzyme for 2 hours. The added value of NaOH from the blank experiment was
 308 subtracted from all time points in every hydrolysis.

309

310 **3. Results and discussion**

311 **3.1 Characterization of glycosylated α -LA**

312 In the untreated α -LA as well as in the controls (AG_0 and A_{10}), 13 ± 1 amino groups per protein
 313 were determined by OPA measurements (no further data shown). This is equal to the sum of
 314 the number of lysines (12) plus the N-terminal in the α -LA sequence. Upon heat treatment in
 315 the presence of glucose, the $DG_{av,OPA}$ as determined by the OPA method, increased from 0 %

316 (AG₀) to 53 % (AG₁₀) (**Figure 2**). The degree of glycation was also quantified using the mass
 317 spectra (DG_{av,MS}) (**Figure 3**). For the blank sample containing glucose and protein but without
 318 heating (AG₀), the mass spectrum showed that ~20 % of α-LA molecules had one attached
 319 glucose molecule (resulting in a molecular mass of 14,340 Da) (**Figure 3**). This corresponds to
 320 an average degree of glycation (DG_{av,MS}) of 1.5 % (**Figure 2**). The DG_{av,MS} calculated using MS
 321 increased from 1.5 % (AG₀) to 63.0 % (AG₁₀) (**Figure 2**). Since OPA and MS results showed
 322 similar values for glycation and MS method had lower standard deviations, MS results were
 323 further used to present the DG_{av}. In addition, the MS method visualized the distribution of
 324 masses of each sample, providing the information on the heterogeneity of the sample. It is
 325 important to note that even the mass distribution does not represent the complete
 326 heterogeneity of the sample because there are also variants of glycated proteins with the
 327 same molecular mass. These variants have the same number of glucose moieties attached
 328 but at different locations within the protein. The number of possible molecules with a
 329 certain number of glycated residues can be calculated from equation 5:

$$N = \frac{\#total\ glycation\ sites!}{(\#total\ glycation\ sites - \#glycated\ sites)! \times \#glycated\ sites!} \quad (5)$$

330 where N is the number of possible molecules with the same number of glucose attached and
 331 n! represents the n factorial (i.e. n!=1*2*3*... *n). For α-LA as many as 3,432 possible
 332 variants can be formed when 7 out of 14 possible glycation sites are modified. As a
 333 consequence, for the case of AG₈, in which 11 variants of α-LA-glucose conjugates were
 334 identified by MS with 3 to 13 glucoses attached, there were actually 16,277 ($\sum_{i=3}^{13} \frac{14!}{(14-i)! \times i!}$
 335 =16,277, i=#glycated sites) possible modified protein molecules.

336
 337 Using CD, it was shown that at 37 °C the secondary and tertiary structure of all samples (AG₀-
 338 AG₁₀ and A₁₀) were similar to that of the untreated α-LA (**Figures 4A** and **4B**, respectively). In
 339 addition, it was confirmed by SEC that in all samples (untreated α-LA, AG₀-AG₁₀ and A₁₀), the
 340 majority (≥90 %) of α-LA was present as monomeric proteins (based on the UV₂₈₀ peak area
 341 of the monomer as percentage of the total UV₂₈₀ peak area) (**Figure 5**). This means that the
 342 changes observed in hydrolysis can indeed be attributed to the modification of protein
 343 primary structure, rather than other effects of the modification or treatment.

344 3.2 Effect of glycation on protein enzymatic hydrolysis

345 3.2.1 Enzymatic hydrolysis of untreated α -LA by all enzymes

346 For each enzyme and substrate combination, the theoretical maximal DH that can be
347 reached during hydrolysis was calculated from equation 6.

$$DH_{max,theo}^{enzyme} (\%) = \frac{\#cleavage\ sites}{\#peptide\ bonds} \times 100\ \% \quad (6)$$

348 For trypsin (specific for lysine and arginine), BLP (specific for glutamic acid and aspartic acid)
349 and α -chymotrypsin (specific for leucine, phenylalanine and tyrosine) hydrolysis of α -LA, the
350 theoretical maximal degrees of hydrolysis are 10.7, 16.4 and 17.2 %, respectively (**Figure 1**).
351 For subtilisin A, the maximal theoretical degree of hydrolysis is 100 % since it is an a-specific
352 enzyme. The experimental DH values of untreated α -LA hydrolyzed by bovine trypsin, BLP
353 and α -chymotrypsin are 3.6, 11.2 and 10.2 %, respectively, which are much lower than the
354 $DH_{max,theo}^{enzyme}$ values. It is concluded that not all cleavage sites were efficiently hydrolyzed by
355 these enzymes. Based on the fitting parameter $DH_{max,untreated}^{enzyme}$ of untreated α -LA (derived from
356 equation 3), the percentage of cleavage sites that were efficiently used by the enzyme, or in
357 other words the percentage of high selectivity sites (HSS) of the enzyme, was estimated from
358 equation 7.

$$HSS_{enzyme}^{substrate} (\%) = \frac{DH_{max,untreated}^{enzyme}}{DH_{max,theo}^{enzyme}} \times 100\ \% \quad (7)$$

359 For porcine trypsin (PT), the fitting parameter $DH_{max,untreated}^{PT}$ of untreated α -LA is 10.7 %
360 (derived from equation 3), which is the same as the $DH_{max,theo}^{PT}$ (10.7 %) (equation 6).
361 Therefore, the $HSS_{PT}^{\alpha-LA}$ (%) is ~100 %. The $DH_{max,untreated}^{BT}$ of α -LA by bovine trypsin is one
362 third of the $DH_{max,untreated}^{PT}$ by porcine trypsin. A higher HSS for porcine trypsin compared to
363 bovine trypsin was expected because porcine trypsin has been reported to have less missed
364 cleavages during protein hydrolysis than bovine trypsin (Walmsley, et al., 2013), which
365 means that porcine trypsin can hydrolyze the cleavage sites more efficiently.

366 3.2.2 Theoretical dependency of protein hydrolysis with modifications on primary 367 structure

368 Based on the abovementioned results from the model made with α -LA and glucose, we
369 derived equation 8 to describe the theoretical dependence of the degree of hydrolysis on
370 the modifications of protein primary structure:

$$DH_{max}^{enzyme}(\%) = DH_{max,untreated}^{enzyme} - \frac{m \times n \times q}{\#peptide\ bonds} \quad (8)$$

371 where m ($0 \leq m \leq 100$ %) represents the percentage of cleavage sites that have a
 372 modification on at least one of the binding site positions (P4 to P4'). The value of m
 373 depends on the enzyme specificity and the primary sequence of the substrate (**Figure 1**).
 374 Taking one cleavage site D63 (underlined) for BLP as an example, WCKDDQNP (P4 to P4')
 375 is the binding site of D63. K62 is the glycation site that is on one of the binding site
 376 positions (P2) of D63. Parameter n ($0 \leq n \leq 1$) represents the binding site dependency
 377 coefficient. The value of $n=0$ when the interaction between the enzyme and the
 378 cleavage site is not influenced by modifications on the binding site; $n=1$ when the
 379 enzyme cannot hydrolyze cleavage sites with modifications on the binding site.
 380 Parameter q is the number of high selectivity sites that can be influenced by the
 381 modifications. In the case of glycation, the way in which q depends on the average
 382 degree of glycation (DG_{av}) can take three forms:

383 A) The glycation takes place equally at all cleavage sites (EQ) (equation 9);

$$q_{EQ} = (DG_{av} \times \#glycation\ sites - \frac{1}{\#glycation\ sites}) \times HSS_{enzyme}^{substrate} \quad (9)$$

384 B) The glycation takes place first at the high selectivity sites (HSS) (equation 10);

$$q_{HSS} = \begin{cases} DG_{av} \times \#glycation\ sites - \frac{1}{\#glycation\ sites} & DG_{av} < HSS_{enzyme}^{substrate} \\ HSS_{enzyme}^{substrate} \times \#glycation\ sites - \frac{1}{\#glycation\ sites} & DG_{av} \geq HSS_{enzyme}^{substrate} \end{cases} \quad (10)$$

385 C) The glycation takes place first at the low selectivity sites (LSS) (equation 11);

$$q_{LSS} = \begin{cases} 0 & DG_{av} < 100 - HSS_{enzyme}^{substrate} \\ DG_{av} \times \#glycation\ sites - \frac{1}{\#glycation\ sites} & DG_{av} \geq 100 - HSS_{enzyme}^{substrate} \end{cases} \quad (11)$$

386 The glycation of N-terminal amino group is corrected in all cases because the glycation on
 387 the N-terminal amino groups does not influence the extent of hydrolysis.

388 3.2.3 Effect of glycation on hydrolysis catalyzed by non-lysine/arginine specific 389 enzymes

390 For BLP and subtilisin A hydrolyses, the experimental DH after 2 h hydrolysis for the
 391 untreated and glycated samples were 11.2 (± 0.2) % and 28.0 (± 0.5) %, respectively (**Figure**
 392 **6A**). The fitting parameter DH_{max}^{BLP} and $DH_{max}^{subtilisin}$ of all samples were 11.7 (± 0.2) % and 30.0

393 (± 0.7) %, respectively (**Figure 7A**). These results showed that the extent of hydrolysis of BLP
394 and subtilisin A is independent of glycation. This was expected because these two enzymes
395 are reported to be not specific for lysine and arginine. In addition, the above mentioned
396 results further indicate that there were no other changes in the protein structure except
397 glycation. For BLP hydrolysis, it showed that even with 12 out of 20 cleavage sites ($m=60$ %
398 in equation 8) (**Figure 1**), the hydrolysis was not influenced by glycation. In the case of
399 subtilisin A, $m=100$ % since subtilisin A is an α -specific enzyme. Similar as for BLP, the
400 hydrolysis of subtilisin A was completely independent of the primary structural modification
401 of the protein. It is concluded that BLP and subtilisin A can hydrolyze cleavage sites with
402 modified residues on the binding site. Thus, the binding site dependency coefficient n equals
403 to 0 in the equation 8 for BLP and subtilisin A hydrolyses.

404

405 For α -chymotrypsin hydrolysis, the experimental DH after 2 h hydrolysis decreased from 10.2
406 to 6.9 % (32 %) with DG_{av} increasing from 0-63 % (**Figures 6B**). The fitting parameter
407 $DH_{max}^{\text{chymotrypsin}}$ also decreased by 32 % (from 10.1 % to 6.9 %) with increasing DG_{av} (**Figures 7A**).
408 Even though the specificity of α -chymotrypsin is not lysine and arginine, the extent of
409 hydrolysis was negatively influenced by glycation. Because there are no other changes in
410 protein structure except glycation, the binding site dependency coefficient n of α -
411 chymotrypsin in equation 8 is not 0. There are 12 out of 21 ($m=57$ %) cleavage sites of α -
412 chymotrypsin that have glycation sites on the binding site (**Figure 1**). If the binding site
413 dependency coefficient n is equal to 1, the theoretical scenario of $DH_{max}^{\text{chymotrypsin}}$ on DG_{av} was
414 calculated using equations 8 to 11 (**Figure 7A**). The dependency of fitting parameter
415 $DH_{max}^{\text{chymotrypsin}}$ of the chymotrypsin hydrolyses on DG_{av} follows the theoretical scenario that
416 the glycation takes place to all sites equally on a protein. These results showed that once the
417 cleavage sites of α -chymotrypsin have undergone modification on the binding site, the
418 enzyme does not hydrolyze the cleavage sites anymore.

419 **3.2.4 Effect of glycation on hydrolysis catalyzed by lysine/arginine specific enzymes**

420 In contrast to hydrolysis catalyzed by non-lysine/arginine specific enzymes, the obtained
421 experimental DH values after 2 h hydrolysis using bovine trypsin decreased from 3.6 ± 0.1 %
422 to 1.2 ± 0.1 % (65 % decrease) with DG_{av} increasing from 0 to 63 % (**Figure 6C**). The fitting
423 parameter DH_{max}^{BT} (derived from equation 3) decreased linearly ($R^2=0.97$) from 3.5 ± 0.1 % to

424 1.2±0.1 % (66 % decrease) with increasing DG_{av} (**Figures 6C and 7B**). For porcine trypsin, the
 425 obtained experimental DH after 2 h hydrolysis decreased from 10.2±0.5 % to 3.6±0.3 % (65 %
 426 decrease) with DG_{av} increasing from 0 to 63 % (**Figures 6D**). The fitting parameter DH_{\max}^{PT}
 427 (derived from equation 3) also decreased linearly ($R^2=0.96$) from 10.7±0.3 % to 3.7±0.2 % (65 %
 428 decrease) with increasing DG_{av} (**Figures 7B**). Because the bovine and porcine trypsin are
 429 specific for lysine and arginine, all modified lysines are automatically part of the binding site
 430 ($m=100$ % in equation 8). The theoretical scenario of $DH_{\max}^{BT/PT}$ on DG_{av} was calculated using
 431 equation 8 to 11, using the binding site dependency coefficient $n=1$ (**Figure 7B**). The
 432 dependence of fitting parameter $DH_{\max}^{BT/PT}$ of the bovine and porcine trypsin hydrolysis on
 433 DG_{av} follows the theoretical scenario when the glycation takes place equally on all glycation
 434 sites, which showed the same behavior as α -chymotrypsin hydrolysis. These results indicate
 435 that all amino groups on the protein had equal probability to be glycated. Because all the
 436 cleavage sites are hydrolyzed efficiently in the case of porcine trypsin hydrolyses ($HSS_{PT}^{\alpha-LA}$
 437 =100 %), the decrease of DH_{\max}^{PT} of porcine trypsin matches exactly with the increase of the
 438 DG_{av} (**Figure 7B**). The above mentioned results of bovine and porcine trypsin showed that
 439 lysine/arginine specific enzymes cannot hydrolyze glycated cleavage sites ($m=100$ % and $n=1$
 440 in equation 8). The values of fitting parameters DH_{\max} , HSS , m and n for hydrolysis of α -LA by
 441 all tested enzymes were summarized in **table 1**.

442 3.2.5 Comparison of the effect of glycation on hydrolysis catalyzed by various enzymes

443 To compare the influence of glycation among all enzymes, the DH_{\max}^{enzyme} values were
 444 normalized as relative degree of hydrolysis ($DH_{\max}^{enzyme} / DH_{\max,untreated}^{enzyme}$) (**Figure 7C**), to allow fair
 445 comparison of the effects of glycation on hydrolysis among different enzymes. It is clear that
 446 the bovine and porcine trypsin hydrolyses have the exact same dependency on glycation.
 447 Therefore, the only differences between the two enzymes are the number of high selectivity
 448 sites ($HSS_{enzyme}^{substrate}$). Both the bovine and porcine trypsin hydrolysis follow the theoretical
 449 scenario when $m=100$ % and $n=1$ in equation 8. This is the maximal influence of primary
 450 structure modification of a protein on the enzymatic hydrolysis. For all three other enzymes,
 451 since the $0 \leq m \leq 100$ % and $0 \leq n \leq 1$, it was expected that the effect of glycation on the
 452 hydrolysis would be smaller than the effect on hydrolysis of bovine and porcine trypsin. For
 453 BLP and subtilisin A, since the hydrolyses are independent of glycation and $m=60$ % and 100
 454 %, respectively, the n for both enzymes needs to be 0. This means that for all proteins, BLP

455 and subtilisin hydrolyses would be independent of glycation. For α -chymotrypsin, $n=1$ but m
456 can be from 0 to 100 % for different substrates, which means that the effect of glycation on
457 α -chymotrypsin hydrolysis depends on the protein amino acid sequence. For both
458 lysine/arginine specific and non-lysine/arginine specific enzymes, the overall hydrolysis rate
459 constants ($k_{\text{hydr}} \cdot \text{DH}_{\text{max}}$) were independent of glycation (**Figure 7D**). It is concluded that only
460 the extent of hydrolysis was influenced by glycation, but not the rate of the hydrolysis.

461

462 **4. Conclusion**

463 The modifications of protein primary structure by Maillard induced glycation can
464 quantitatively affect the extent of protein enzymatic hydrolysis, even in the absence of
465 changes in secondary, tertiary or quaternary structure of the proteins. The effect of glycation
466 on hydrolysis depended on the proteases used. Lysine/arginine specific proteases, e.g.
467 bovine and porcine trypsin, cannot hydrolyze glycated cleavage sites. For non-lysine/arginine
468 specific proteases, e.g. BLP and subtilisin A, glycation does not affect the extent of hydrolysis,
469 while α -chymotrypsin cannot hydrolyze cleavage sites with glycated binding sites. Since not
470 all cleavage sites are used by the enzymes, the extent of the effects depends on the enzyme
471 selectivity towards cleavage sites (for trypsin) or cleavage sites near glycation sites (for α -
472 chymotrypsin). Combining the results of all proteases, an equation was derived to describe
473 the effect of modification of protein primary structure on the extent of hydrolysis based on
474 the enzyme specificity, selectivity and binding site sensitivity. With these outcomes, an
475 estimation can be obtained on the effect of the modification (glycation) of protein primary
476 structure on the extent of hydrolysis during *in vitro*, e.g. production of commercial
477 hydrolysates and *in vivo* hydrolysis, e.g. digestion of food proteins.

478

479 **Acknowledgement**

480 This work is supported by NanoNextNL, a micro and nanotechnology consortium of the
481 government of the Netherlands and 130 partners. This work has also been financially
482 supported by FrieslandCampina, The Netherlands

483 **References**

- 484 Adler-Nissen, J. (1986). *Enzymatic hydrolysis of food proteins*. London, UK: Elsevier Applied
485 Science Publishers.
- 486 Akpinar, O., & Penner, M. H. (2001). Peptidase activity assays using protein substrates. In
487 *Current Protocols in Food Analytical Chemistry*. Corvallis, OR, USA: John Wiley & Sons.
- 488 Bhatt, H., Cucheval, A., Coker, C., Patel, H., Carr, A., & Bennett, R. (2014). Effect of
489 lactosylation on plasmin-induced hydrolysis of β -casein. *International Dairy Journal*, 38(2),
490 213-218.
- 491 Biemel, K. M., Reihl, O., Conrad, J., & Lederer, M. O. (2001). Formation pathways for lysine-
492 arginine cross-links derived from hexoses and pentoses by Maillard processes: unraveling
493 the structure of a pentosidine precursor. *Journal of Biological Chemistry*, 276(26), 23405-
494 23412.
- 495 Böttger, F. H., Etzel, M. R., & Lucey, J. A. (2013). *In vitro* infant digestion of whey protein-
496 dextran glycates. *Food Digestion*, 4(2), 76-84.
- 497 Butré, C. I., Sforza, S., Gruppen, H., & Wierenga, P. A. (2014). Introducing enzyme selectivity:
498 a quantitative parameter to describe enzymatic protein hydrolysis. *Analytical and*
499 *Bioanalytical Chemistry*, 406(24), 5827-5841.
- 500 Butré, C. I., Sforza, S., Wierenga, P. A., & Gruppen, H. (2015). Determination of the influence
501 of the pH of hydrolysis on enzyme selectivity of *Bacillus licheniformis* protease towards whey
502 protein isolate. *International Dairy Journal*, 44, 44-53.
- 503 Butré, C. I., Wierenga, P. A., & Gruppen, H. (2012). Effects of ionic strength on the enzymatic
504 hydrolysis of diluted and concentrated whey protein isolate. *Journal of Agricultural and Food*
505 *Chemistry*, 60(22), 5644-5651.
- 506 Butré, C. I., Wierenga, P. A., & Gruppen, H. (2014). Influence of water availability on the
507 enzymatic hydrolysis of proteins. *Process Biochemistry*, 49, 1903-1912.
- 508 Carulli, S., Calvano, C. D., Palmisano, F., & Pischetsrieder, M. (2011). MALDI-TOF MS
509 characterization of glycation products of whey proteins in a glucose/galactose model system
510 and lactose-free milk. *Journal of Agricultural and Food Chemistry*, 59(5), 1793-1803.
- 511 Cheison, S. C., Lai, M. Y., Leeb, E., & Kulozik, U. (2011). Hydrolysis of β -lactoglobulin by
512 trypsin under acidic pH and analysis of the hydrolysates with MALDI-TOF-MS/MS. *Food*
513 *Chemistry*, 125(4), 1241-1248.

514 Chevalier, F., Chobert, J. M., Mollé, D., & Haertlé, T. (2001). Maillard glycation of β -
515 lactoglobulin with several sugars: comparative study of the properties of the obtained
516 polymers and of the substituted sites. *Lait*, 81(5), 651-666.

517 Darewicz, M., & Dziuba, J. (2001). The effect of glycosylation on emulsifying and structural
518 properties of bovine β -casein. *Food/Nahrung*, 45(1), 15-20.

519 Delahaije, R. J. B. M., Wierenga, P. A., Giuseppin, M. L. F., & Gruppen, H. (2014). Improved
520 emulsion stability by succinylation of patatin is caused by partial unfolding rather than
521 charge effects. *Journal of Colloid and Interface Science*, 430(0), 69-77.

522 Fritsch, R., Hoffmann, H., & Klostermeyer, H. (1983). Formation of lysinoalanine during heat
523 treatment of milk. *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, 176(5), 341-345.

524 Heijnis, W. H., Wierenga, P. A., Van Berkel, W. J. H., & Gruppen, H. (2010). Directing the
525 oligomer size distribution of peroxidase-mediated cross-linked bovine α -lactalbumin. *Journal*
526 *of Agricultural and Food Chemistry*, 58(9), 5692-5697.

527 Lapolla, A., Fedele, D., Reitano, R., Aricò, N. C., Seraglia, R., Traldi, P., Marotta, E., & Tonani, R.
528 (2004). Enzymatic digestion and mass spectrometry in the study of advanced glycation end
529 products/peptides. *Journal of the American Society for Mass Spectrometry*, 15(4), 496-509.

530 Moreno, F. J., Quintanilla-López, J. E., Lebrón-Aguilar, R., Olano, A., & Sanz, M. L. (2008).
531 Mass spectrometric characterization of glycated β -lactoglobulin peptides derived from
532 galacto-oligosaccharides surviving the *in vitro* gastrointestinal digestion. *Journal of the*
533 *American Society for Mass Spectrometry*, 19(7), 927-937.

534 Morgan, F., Léonil, J., Mollé, D., & Bouhallab, S. (1997). Nonenzymatic lactosylation of bovine
535 β -lactoglobulin under mild heat treatment leads to structural heterogeneity of the
536 glycoforms. *Biochemical and Biophysical Research Communications*, 236(2), 413-417.

537 Pereyra Gonzáles, A. S., Naranjo, G. B., Malec, L. S., & Vigo, M. S. (2003). Available lysine,
538 protein digestibility and lactulose in commercial infant formulas. *International Dairy Journal*,
539 13(2-3), 95-99.

540 Pinto, M. S., Léonil, J., Henry, G., Cauty, C., Carvalho, A. F., & Bouhallab, S. (2014). Heating
541 and glycation of β -lactoglobulin and β -casein: aggregation and *in vitro* digestion. *Food*
542 *Research International*, 55, 70-76.

543 Sanborn, B. M., & Hein, G. E. (1968). The interaction of trypsin with neutral substrates and
544 modifiers. *Biochemistry*, 7(10), 3616-3624.

545 Schechter, I., & Berger, A. (1967). On the size of the active site in proteases. I. Papain.
546 *Biochemical and Biophysical Research Communications*, 27(2), 157-162.

547 ter Haar, R., Schols, H. A., & Gruppen, H. (2011). Effect of saccharide structure and size on
548 the degree of substitution and product dispersity of α -lactalbumin glycosylated via the Maillard
549 reaction. *Journal of Agricultural and Food Chemistry*, 59(17), 9378-9385.

550 Van Gool, M. P., Vancsó, I., Schols, H. A., Toth, K., Szakacs, G., & Gruppen, H. (2011).
551 Screening for distinct xylan degrading enzymes in complex shake flask fermentation
552 supernatants. *Bioresource Technology*, 102(10), 6039-6047.

553 Vorob'ev, M. M., Dalgalarondo, M., Chobert, J. M., & Haertlé, T. (2000). Kinetics of β -casein
554 hydrolysis by wild-type and engineered trypsin. *Biopolymers*, 54(5), 355-364.

555 Wada, Y., & Lönnerdal, B. (2014). Effects of different industrial heating processes of milk on
556 site-specific protein modifications and their relationship to *in vitro* and *in vivo* digestibility.
557 *Journal of Agricultural and Food Chemistry*, 62(18), 4175-4185.

558 Walmsley, S. J., Rudnick, P. A., Liang, Y., Dong, Q., Stein, S. E., & Nesvizhskii, A. I. (2013).
559 Comprehensive analysis of protein digestion using six trypsins reveals the origin of trypsin as
560 a significant source of variability in proteomics(). *Journal of Proteome Research*, 12(12),
561 5666-5680.

562 Wierenga, P. A., Van Norél, L., & Basheva, E. S. (2009). Reconsidering the importance of
563 interfacial properties in foam stability. *Colloids and Surfaces A: Physicochemical and*
564 *Engineering Aspects*, 344(1-3), 72-78.

566 **Figure legends**

567 **Table 1:** the maximal degree of hydrolysis (DH_{max}), the percentage of high selectivity sites
568 (HSS), the percentage of cleavage sites that have a modification on at least one of the
569 binding site positions (m) and the binding site dependency coefficient (n) for hydrolysis
570 of α -lactalbumin by all tested enzymes from the fit of equations 2, 7, 8 and 8,
571 respectively.

572 **Figure 1:** Amino acid sequence of α -lactalbumin. (∇) indicates BLP cleavage sites (DE)
573 without glycation sites on the binding site (P4 to P4' positions). (\blacktriangledown) indicates BLP cleavage
574 sites with glycation sites on the binding site. (\diamond) indicates α -chymotrypsin cleavage sites (FLY)
575 without glycation sites on the binding site. (\blacklozenge) indicates α -chymotrypsin cleavage sites with
576 glycation sites on the binding site. Amino acids in bold are glycation sites as well as trypsin
577 cleavage sites.

578 **Figure 2:** Average degrees of glycation of α -lactalbumin heated with glucose at 50 °C with 65 %
579 relative humidity over 0-10 h Maillard incubation time. (\square) OPA results ($DG_{av,OPA}$) and (\blacklozenge) MS
580 results ($DG_{av,MS}$).

581 **Figure 3:** Deconvoluted mass spectra of α -lactalbumin heated with glucose at 50 °C with 65 %
582 relative humidity over 0-10 h.

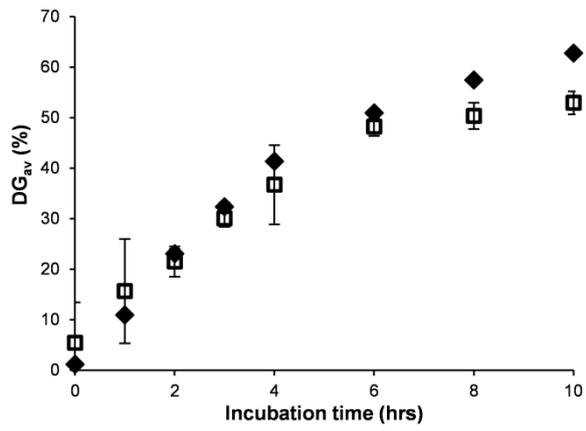
583 **Figure 4:** (A) Far UV-CD spectra and (B) near UV-CD of untreated α -lactalbumin and α -
584 lactalbumin heated with glucose at 50 °C with 65 % relative humidity over 0-10 h, since there
585 was no significant difference between the spectra, the line is the average of spectra over all
586 samples. Error bars are shown to illustrate the standard deviation.

587 **Figure 5:** Size exclusion elution pattern of untreated α -lactalbumin and α -lactalbumin heated
588 with glucose over 0-10 h and the inset shows the percentage of monomeric proteins. The
589 dashed line (-----) in the inset represents the percentage of monomeric protein in A_{10} .

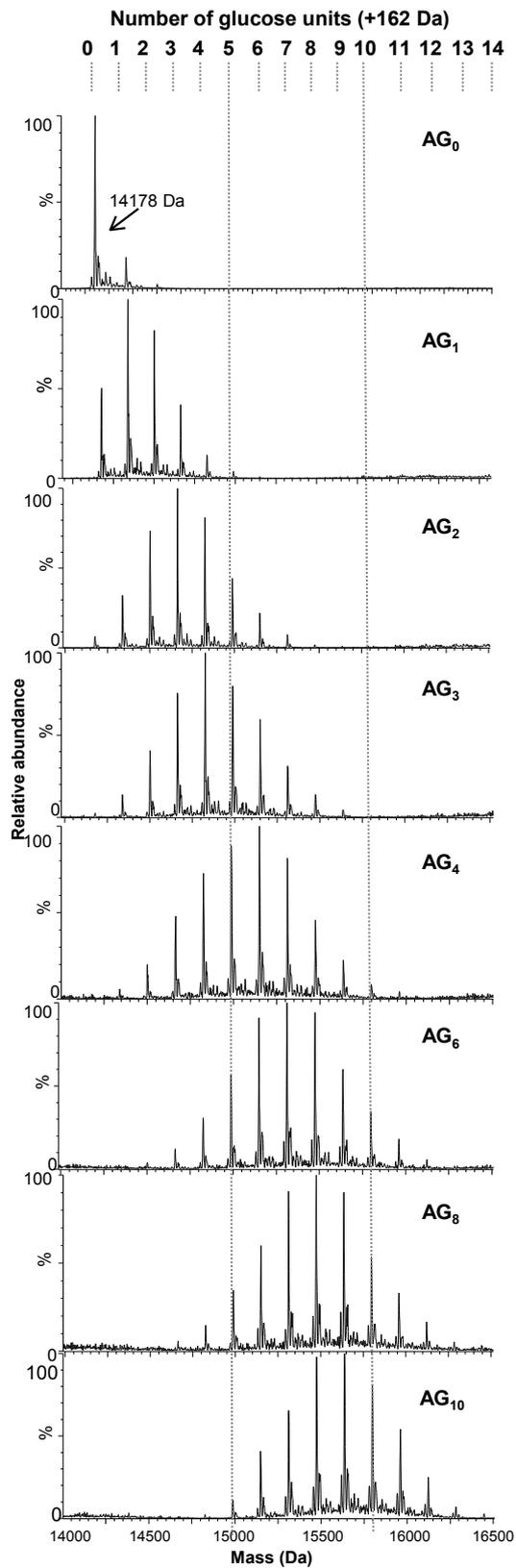
590 **Figure 6:** (A) BLP and subtilisin A, (B) α -chymotrypsin, (C) bovine trypsin and (D) porcine
591 trypsin hydrolysis curves of untreated α -lactalbumin and α -lactalbumin heated with glucose
592 over 0-10 h. In figure (A), error bars are shown to illustrate the standard deviations of
593 hydrolysis curves of all samples. The averaged standard errors in panel (A) for subtilisin A
594 and BLP were 4.2 and 2.3 %, respectively.

595 **Figure 7:** Correlation between DH_{max}^{enzyme} and DG_{av} of untreated α -lactalbumin and α -
596 lactalbumin heated with glucose over 0-10 h hydrolyzed by (A) non-lysine/arginine specific

597 enzymes: (◆) BLP, (▲) α -chymotrypsin and (◇) subtilisin A, or by (B) lysine/arginine specific
 598 enzymes: (□) bovine trypsin and (■) porcine trypsin. (C) indicates the relative DH_{max}^{enzyme} versus
 599 DG_{av} and (D) indicates the overall hydrolysis rate constant $k_{hydr} \cdot DH_{max}$ versus DG_{av} . Lines
 600 indicate: (-----) $DH_{max,EQ}^{enzyme} / DH_{max,untreated}^{enzyme}$ (%) for bovine and porcine trypsin, (—·) $DH_{max,EQ}^{chymotrypsin}$
 601 (%), (·····) $DH_{max,EQ}^{PT}$ (%) and (—··) $DH_{max,EQ}^{BT}$ (%). For bovine trypsin, $DH_{max,LSS}^{BT}$ (%) (—) and
 602 $DH_{max,HSS}^{BT}$ (%) (—) illustrate the scenarios where glycation occurs firstly at low and high
 603 selectivity sites, respectively.



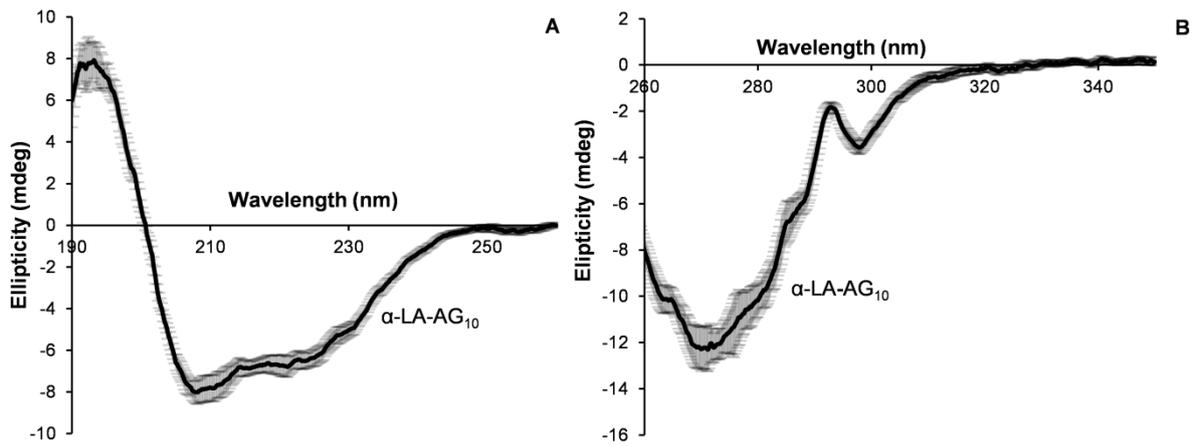
611
 612 **Figure 2:** Average degrees of glycation of α -lactalbumin heated with glucose at 50 °C with 65 %
 613 relative humidity over 0-10 h Maillard incubation time. (□) OPA results (DG_{av,OPA}) and (◆) MS
 614 results (DG_{av,MS}).



615

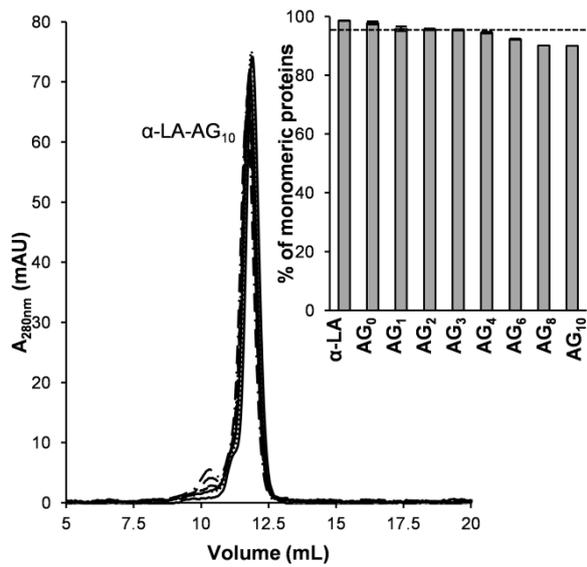
616 **Figure 3:** Deconvoluted mass spectra of α -lactalbumin heated with glucose at 50 °C with 65 %

617 relative humidity over 0-10 h.



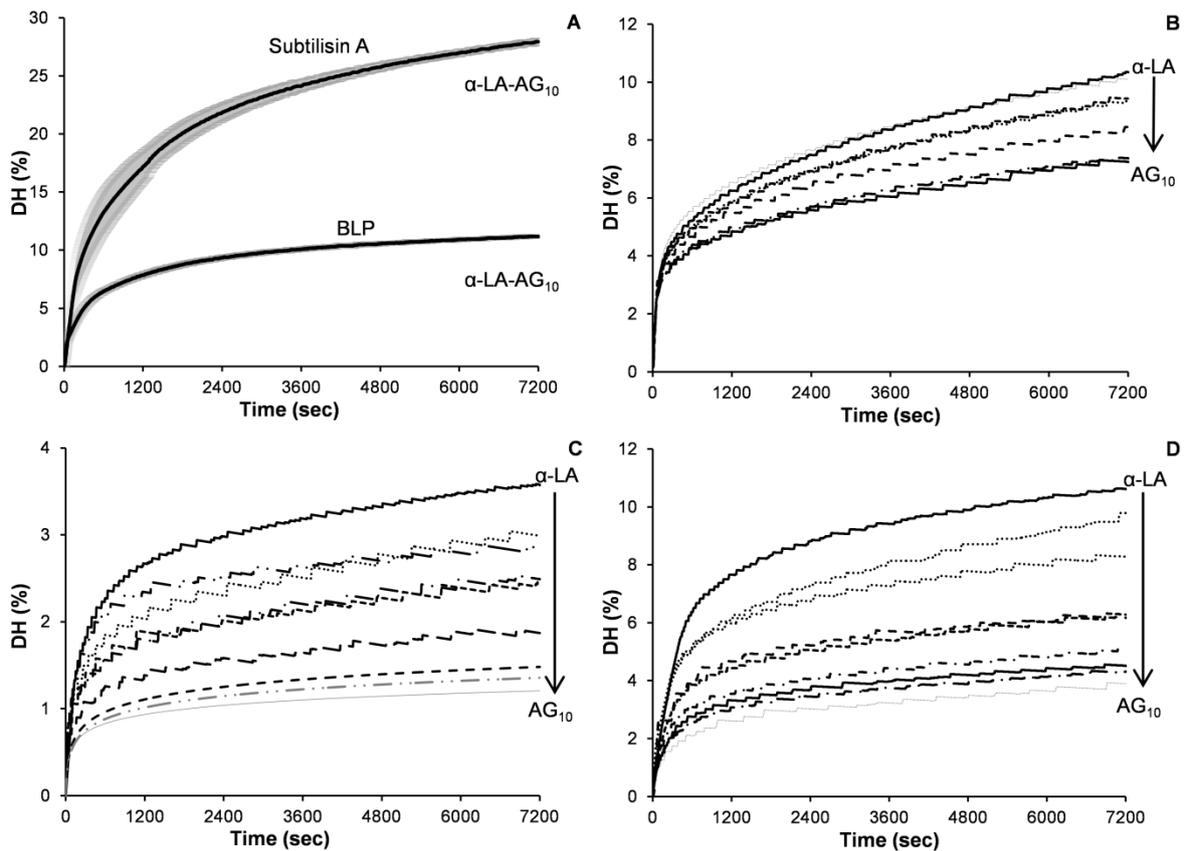
618

619 **Figure 4:** (A) Far UV-CD spectra and (B) near UV-CD of untreated α -lactalbumin and α -
 620 lactalbumin heated with glucose at 50 °C with 65 % relative humidity over 0-10 h, since there
 621 was no significant difference between the spectra, the line is the average of spectra over all
 622 samples. Error bars are shown to illustrate the standard deviation.



623

624 **Figure 5:** Size exclusion elution pattern of untreated α -lactalbumin and α -lactalbumin heated
 625 with glucose over 0-10 hand the inset shows the percentage of monomeric proteins. The
 626 dashed line (-----) in the inset represents the percentage of monomeric protein in A₁₀.

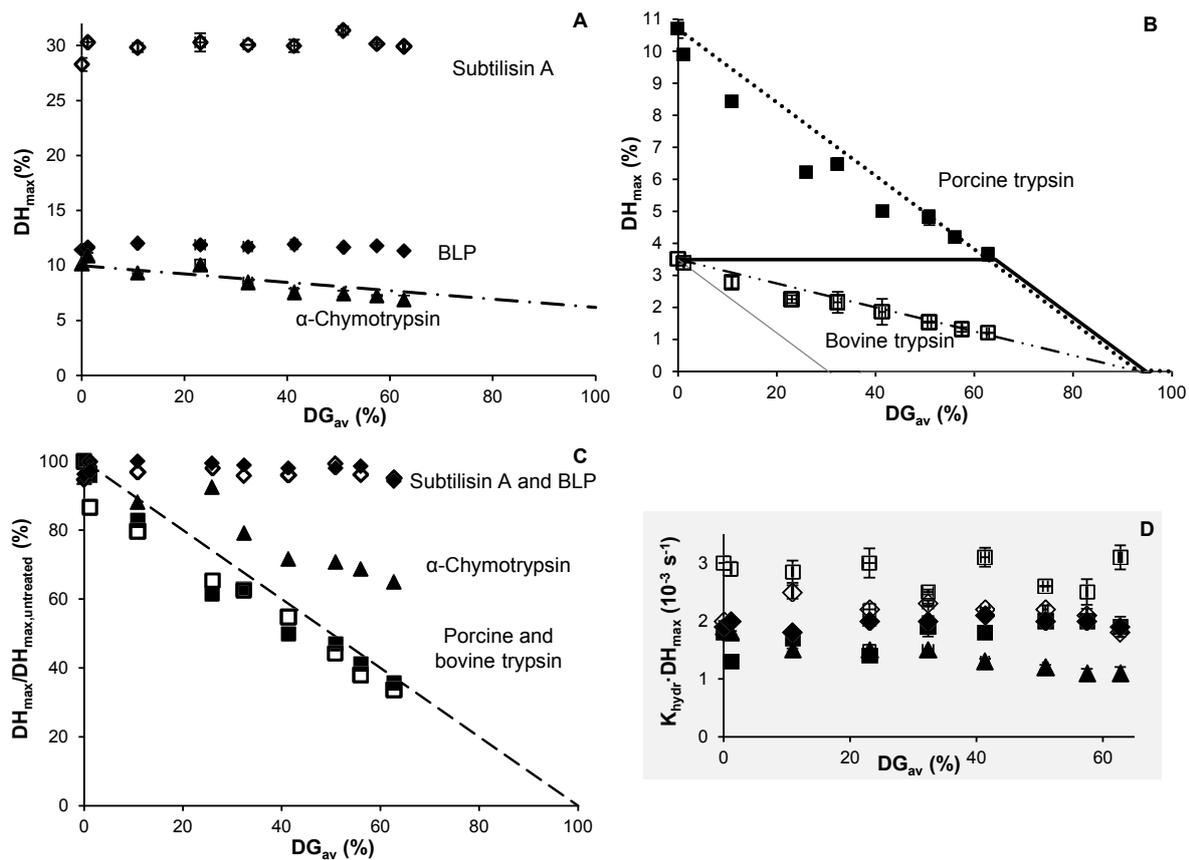


627

628 **Figure 6:** (A) BLP and subtilisin A, (B) α -chymotrypsin, (C) bovine trypsin and (D) porcine
 629 trypsin hydrolysis curves of untreated α -lactalbumin and α -lactalbumin heated with glucose
 630 over 0-10 h. In figure (A), error bars are shown to illustrate the standard deviations of
 631 hydrolysis curves of all samples. The averaged standard errors in panel (A) for subtilisin A
 632 and BLP were 4.2 and 2.3 %, respectively.

633 **Table 1:** the maximal degree of hydrolysis (DH_{max}), the percentage of high selectivity sites
 634 (HSS), the percentage of cleavage sites that have a modification on at least one of the
 635 binding site positions (m) and the binding site dependency coefficient (n) for hydrolysis
 636 of α -lactalbumin by all tested enzymes from the fit of equations 2, 7, 8 and 8,
 637 respectively.

Enzyme	DH_{max} (%)	HSS (%)	m (%)	n
Bovine trypsin	3.5	33	100	1
Porcine trypsin	10.7	100	100	1
BLP	11.7	71	60	0
Subtilisin A	30.0	30	100	0
α-Chymotrypsin	10.1	59	57	1



639
 640 **Figure 7:** Correlation between DH_{max}^{enzyme} and DG_{av} of untreated α -lactalbumin and α -
 641 lactalbumin heated with glucose over 0-10 h hydrolyzed by (A) non-lysine/arginine specific
 642 enzymes: (\blacklozenge) BLP, (\blacktriangle) α -chymotrypsin and (\blacklozenge) subtilisin A, or by (B) lysine/arginine specific
 643 enzymes: (\square) bovine trypsin and (\blacksquare) porcine trypsin. (C) indicates the relative DH_{max}^{enzyme} versus
 644 DG_{av} and (D) indicates the overall hydrolysis rate constant $k_{hydr} \cdot DH_{max}$ versus DG_{av} . Lines
 645 indicate: (-----) $DH_{max,EQ}^{enzyme} / DH_{max,untreated}^{enzyme}$ (%) for bovine and porcine trypsin, (—·) $DH_{max,EQ}^{chymotrypsin}$
 646 (%), (·····) $DH_{max,EQ}^{PT}$ (%) and (—·) $DH_{max,EQ}^{BT}$ (%). For bovine trypsin, $DH_{max,LSS}^{BT}$ (%) (—) and
 647 $DH_{max,HSS}^{BT}$ (%) (—) illustrate the scenarios where glycation occurs firstly at low and high
 648 selectivity sites, respectively.

Highlights:

- Glycated proteins (GP) without aggregation were produced under mild conditions.
- Glycated sites (GS) are not used by specific (Lys/Arg) enzymes.
- Non-Lys/Arg specific enzymes can be sensitive to presence of GS on binding site.
- Extent of the effects depends on the number of high selectivity cleavage sites.
- Hydrolysis of GP is described by enzyme specificity, sensitivity and selectivity.