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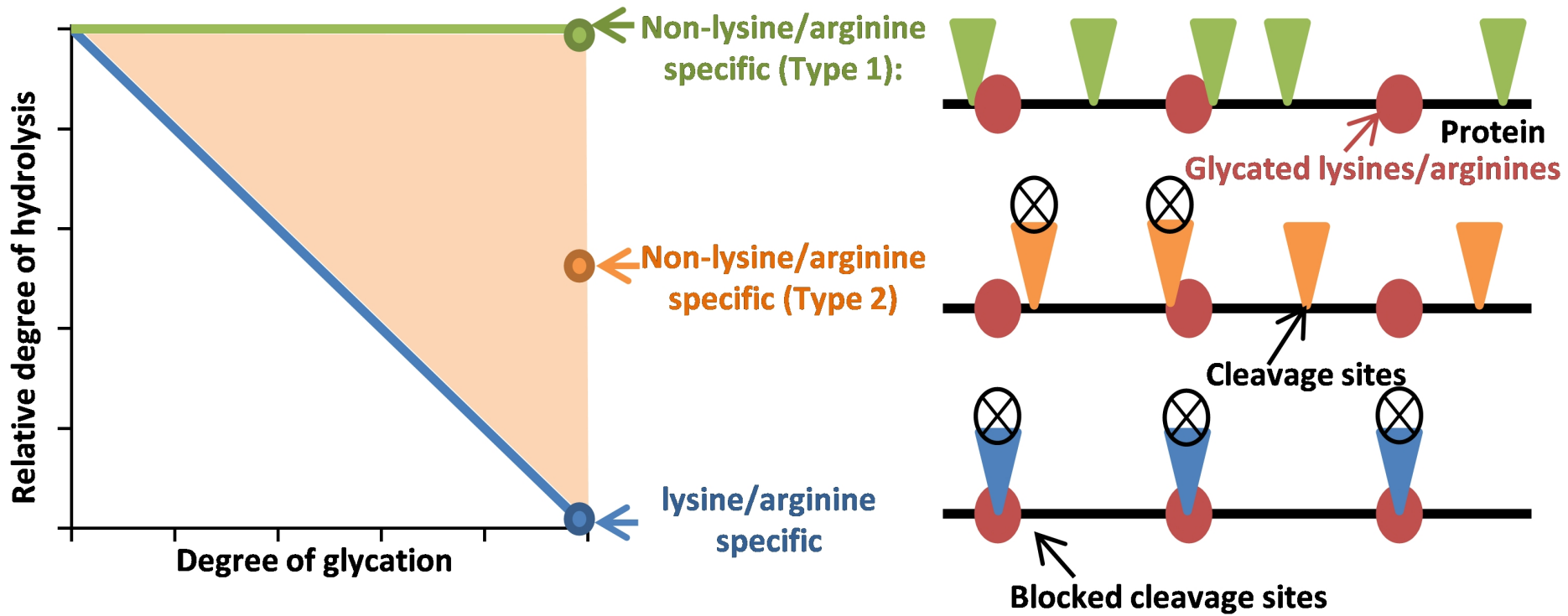
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Relative degree of hydrolysis

Degree of glycation

Non-lysine/arginine specific (Type 1):

Non-lysine/arginine specific (Type 2)

lysine/arginine specific

Protein

Glycated lysines/arginines

Cleavage sites

Blocked cleavage sites

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

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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

1. Introduction

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

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

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 Gonzáles,

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 lactulosylysine) 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 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, Dalgalarrondo, 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

2. Materials and methods

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×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 ℃ 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 ≥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 UV$_{280}$ peak area from RP-UHPLC). Based on the UV$_{214}$ 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 UV$_{280}$ peak area from

170   RP-UHPLC). Based on the UV$_{214}$ 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 UV$_{280}$ peak area coupled with RP-UHPLC and 92 % from the UV$_{214}$ peak area. In the

180   UV$_{214}$ 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 UV$_{280}$ and UV$_{214}$ 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 (Memmert, Schwabach, Germany) at 50 ℃

206 with 65 % relative humidity for 0, 1, 2, 3, 4, 6, 8 and 10 h and labelled as AG_0 -AG_{10}. Samples

207 were immediately cooled down and stored at -20 ℃ 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 ℃, freeze dried again and then stored at -20 ℃. 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 A_{10}). The UV_{280} extinction

214 coefficients of untreated α-LA and AG_{10} were determined to be $1.4 \pm 0.02 \cdot 10^{-4}$ $L \cdot M^{-1} \cdot cm^{-1}$ by

215 measuring the UV_{280} of a dilution series of a protein solution with a known concentration

216 (based on Dumas results). The value described in Uniprot (P00711) is $1.4 \cdot 10^{-4}$ $L \cdot M^{-1} \cdot cm^{-1}$. This

217 means that the glycation does not have an influence on the extinction coefficient of the

218 protein. Therefore, $1.4 \cdot 10^{-4}$ $L \cdot M^{-1} \cdot cm^{-1}$ was used to calculate the concentration of the protein

219 for all experiments at UV_{280}. 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 ℃ 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 μL/min. The untreated α-LA, AG_0-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 ~1 %.

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 °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.

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):

$$DG_{av,OPA}(\%) = (\#NH_{2,untreated} - \#NH_{2,sample})/\#NH_{2,untreated} \times 100 \ \% \tag{1}$$

251   where $\#NH_{2,untreated}$ is the total number of free amino groups per untreated α-LA molecule

252   and $\#NH_{2,sample}$ is the number of free amino groups per protein molecule after glycation.

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

255   The untreated and glycated α-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   2.1×100 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) trifluoreacetic 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 μL/min. Samples were diluted into eluent A (1 mg/mL) and centrifuged (10 minutes, 4,000 ×

264 g, 20 °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, AG_0-AG_{10} and A_{10} 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 °C. The sample cone was operated at 30 V.

270 Nitrogen was used as desolvation gas (500 °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,

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

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.

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. × 250 mm) in combination with a Carbo Pac guard column (2 mm i.d. × 50 mm) and

280 pulsed amperometric detection (PAD). The measurements were performed as described

281 elsewhere (Van Gool, et al., 2011).

2.2.7 Enzymatic hydrolysis

283 The untreated and glycated 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,

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

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 glycated 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 μL bovine trypsin, 30 μL porcine trypsin,

296 67 μL BLP, 90 μL α-chymotrypsin or 50 μL 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),

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

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/α=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 (8.6·10^{-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 glycated 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 %

11

(AG$_0$) to 53 % (AG$_{10}$) (**Figure 2**). The degree of glycation was also quantified using the mass spectra (DG$_{av,MS}$) (**Figure 3**). For the blank sample containing glucose and protein but without heating (AG$_0$), the mass spectrum showed that ~20 % of α-LA molecules had one attached glucose molecule (resulting in a molecular mass of 14,340 Da) (**Figure 3**). This corresponds to an average degree of glycation (DG$_{av,MS}$) of 1.5 % (**Figure 2**). The DG$_{av,MS}$ calculated using MS increased from 1.5 % (AG$_0$) to 63.0 % (AG$_{10}$) (**Figure 2**). Since OPA and MS results showed similar values for glycation and MS method had lower standard deviations, MS results were further used to present the DG$_{av}$. In addition, the MS method visualized the distribution of masses of each sample, providing the information on the heterogeneity of the sample. It is important to note that even the mass distribution does not represent the complete heterogeneity of the sample because there are also variants of glycated proteins with the same molecular mass. These variants have the same number of glucose moieties attached but at different locations within the protein. The number of possible molecules with a certain number of glycated residues can be calculated from equation 5:

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

(5)

where N is the number of possible molecules with the same number of glucose attached and n! represents the n factorial (i.e. n!=1*2*3*…*n). For α-LA as many as 3,432 possible variants can be formed when 7 out of 14 possible glycation sites are modified. As a consequence, for the case of AG$_8$, in which 11 variants of α-LA-glucose conjugates were identified by MS with 3 to 13 glucoses attached, there were actually 16,277 ($\sum_{i=3}^{13} \frac{14!}{(14-i)! \times i!}$ =16,277, *i*=#glycated sites) possible modified protein molecules.

Using CD, it was shown that at 37 °C the secondary and tertiary structure of all samples (AG$_0$-AG$_{10}$ and A$_{10}$) were similar to that of the untreated α-LA (**Figures 4A** and **4B**, respectively). In addition, it was confirmed by SEC that in all samples (untreated α-LA, AG$_0$-AG$_{10}$ and A$_{10}$), the majority (≥90 %) of α-LA was present as monomeric proteins (based on the UV$_{280}$ peak area of the monomer as percentage of the total UV$_{280}$ peak area) (**Figure 5**). This means that the changes observed in hydrolysis can indeed be attributed to the modification of protein primary structure, rather than other effects of the modification or treatment.

3.2 Effect of glycation on protein enzymatic hydrolysis

3.2.1 Enzymatic hydrolysis of untreated α-LA by all enzymes

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

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

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

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

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

3.2.2 Theoretical dependency of protein hydrolysis with modifications on primary structure

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

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

371 where m (0≤m≤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, WCK<u>D</u>DQNP (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≤n≤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);

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

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

$$q_{HSS} = \begin{cases} DG_{av} \times \#glycation\ sites - \dfrac{1}{\#glycation\ sites} & DG_{av} < HSS_{enzyme}^{substrate} \\[2em] HSS_{enzyme}^{substrate} \times \#glycation\ sites - \dfrac{1}{\#glycation\ sites} & DG_{av} \geq HSS_{enzyme}^{substrate} \end{cases} \tag{10}$$

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

$$q_{LSS} = \begin{cases} 0 & DG_{av} < 100 - HSS_{enzyme}^{substrate} \\[2em] DG_{av} \times \#glycation\ sites - \dfrac{1}{\#glycation\ sites} & DG_{av} \geq 100 - HSS_{enzyme}^{substrate} \end{cases} \tag{11}$$

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 a-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 $DH_{max}^{chymotrypsin}$ 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 $DH_{max}^{chymotrypsin}$ on DG_{av} was

414 calculated using equations 8 to 11 (**Figure 7A**). The dependency of fitting parameter

415 $DH_{max}^{chymotrypsin}$ 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

15

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≤*m*≤100 % and 0≤*n*≤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

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

4. Conclusion

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

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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). (▼) indicates BLP cleavage

574   sites with glycation sites on the binding site. (◇) indicates α-chymotrypsin cleavage sites (FLY)

575   without glycation sites on the binding site. (♦) 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. (▫) OPA results (DG$_{av,OPA}$) and (♦) 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.
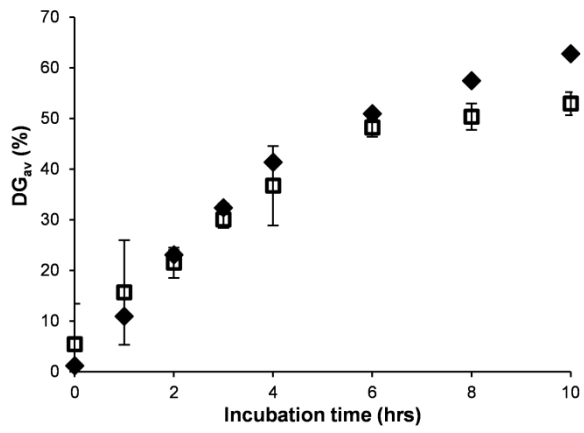
583   **Figure 4:** (A) Far UV-CD spectra and (B) near UV-CD of untreated α-lactalbumin and α-

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 hand the inset shows the percentage of monomeric proteins. The

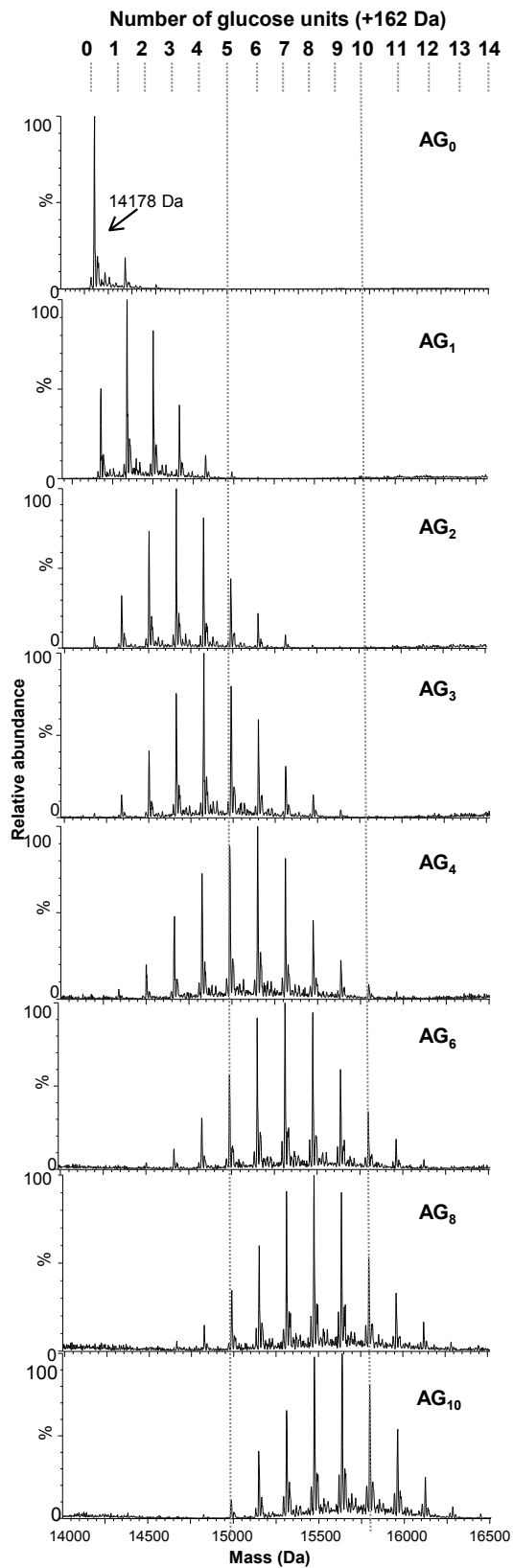589   dashed line (------) in the inset represents the precentage of monomeric protein in A$_{10}$.

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 $\mathrm{DH}_{max}^{enzyme}$ 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

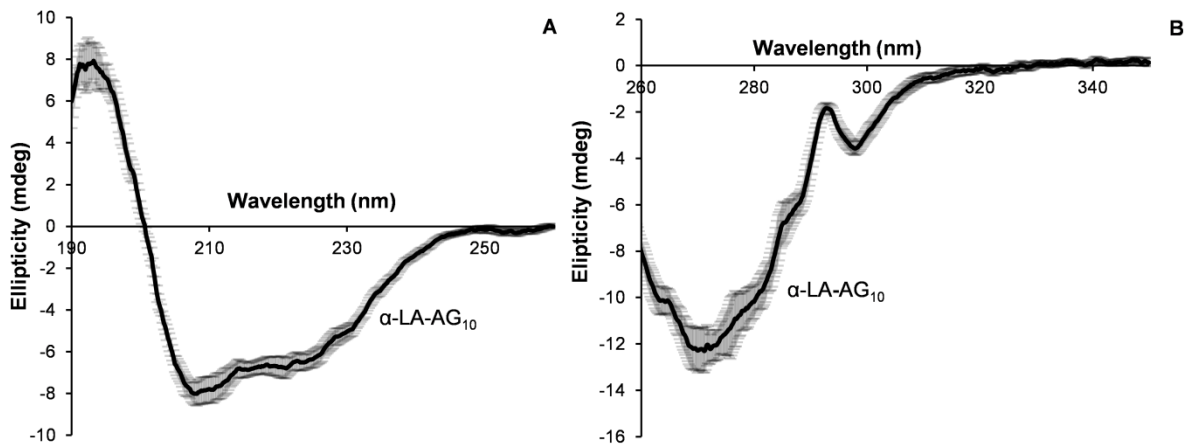603   selectivity sites, respectively.

```
  ▽  ◇       ▼   ♦  ▼♦  ▼♦        ♦        ◇  ▽        ◇       ◇▽                  ▽    ▽◇  ◇◇                  ▼▼
EQLTKCEVFRELKDLKGYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNKIWCKDDQNPHS
```

```
              ▼   ♦♦▼▼▼▽◇   ▽▽             ♦▼        ◇    ♦        ♦   ▼  ♦▼      ♦  ▼ ♦
SNICNISCDKFLDDDLTDDIMCVKKILDKVGINYWLAHKALCSEKLDQWLCEKL
```

Figure 1: Amino acid sequence of α-lactalbumin. (▽) indicates BLP cleavage sites (DE) without glycation sites on the binding site (P4 to P4' positions), (▼) indicates BLP cleavage sites with glycation sites on the binding site. (◇) indicates α-chymotrypsin cleavage sites (FLY) without glycation sites on the binding site. (♦) indicates α-chymotrypsin cleavage sites with glycation sites on the binding site. Amino acids in bold are glycation sites as well as trypsin cleavage sites.

23

611

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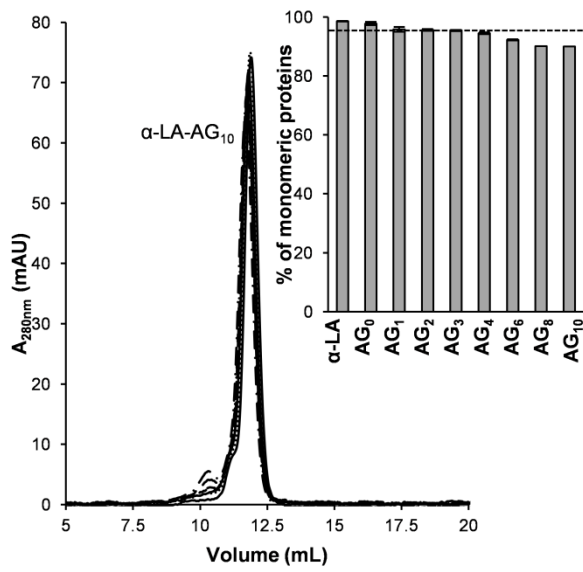results ($DG_{av,MS}$).

Number of glucose units (+162 Da)

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

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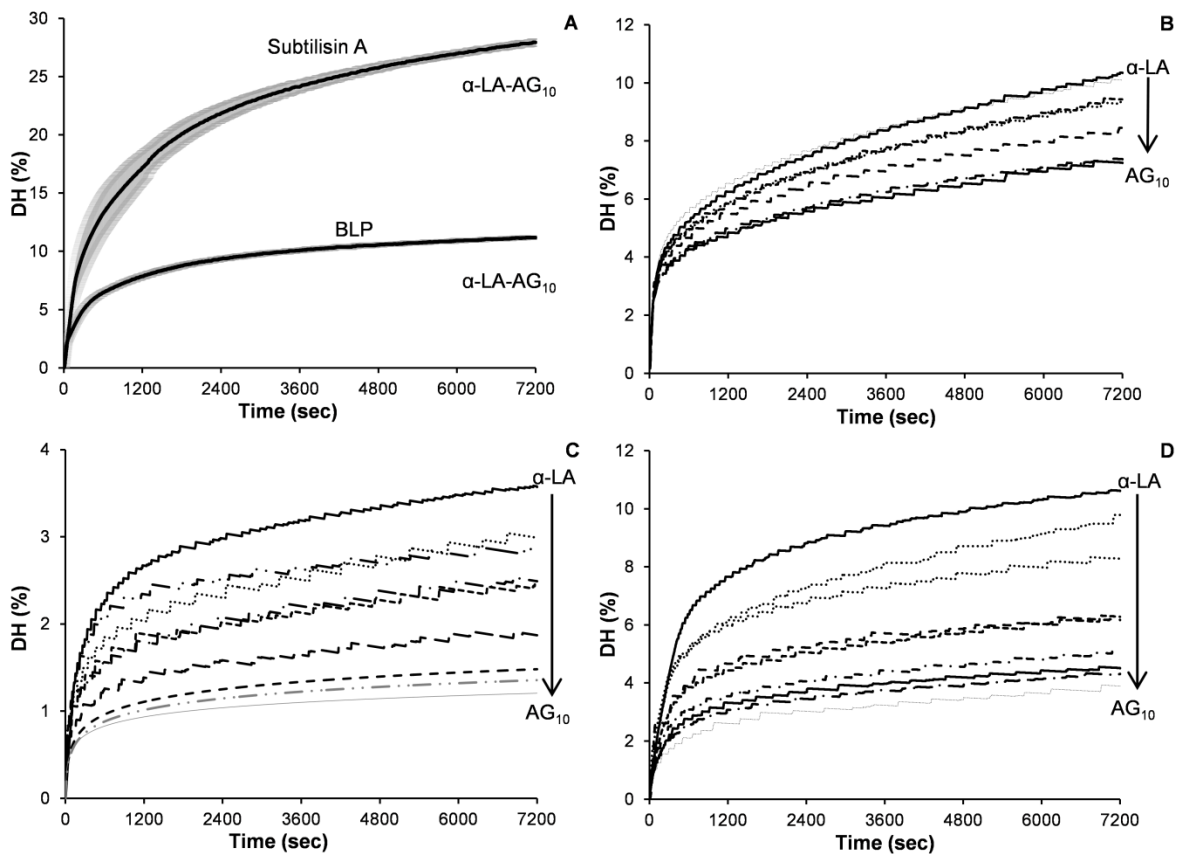
617   relative humidity over 0-10 h.

618

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

623

Figure 5: Size exclusion elution pattern of untreated α-lactalbumin and α-lactalbumin heated with glucose over 0-10 hand the inset shows the percentage of monomeric proteins. The dashed line (------) in the inset represents the precentage of monomeric protein in A_{10}.
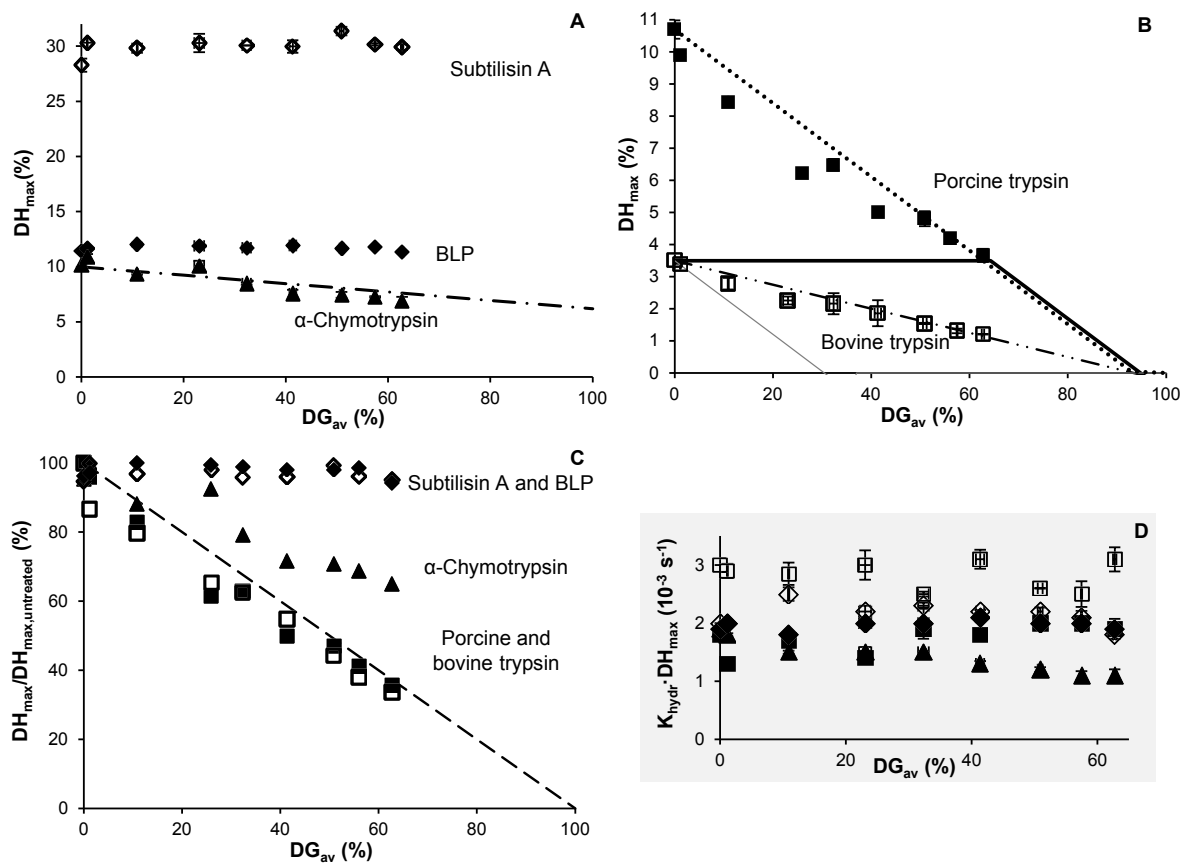
Figure 6: (A) BLP and subtilisin A, (B) α-chymotrypsin, (C) bovine trypsin and (D) porcine trypsin hydrolysis curves of untreated α-lactalbumin and α-lactalbumin heated with glucose over 0-10 h. In figure (A), error bars are shown to illustrate the standard deviations of hydrolysis curves of all samples. The averaged standard errors in panel (A) for subtilisin A 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

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

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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.