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Understanding glycation kinetics of individual peptides in protein hydrolysates

Yuxi Deng, Claire I. Butré, Peter A. Wierenga*

Laboratory of Food Chemistry, Wageningen University, Postbus 17, 6708 AA, Wageningen, the Netherlands

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ABSTRACT

Protein hydrolysates contain peptides with different lengths, type (α/ε-) and number of amino groups; these properties might influence the peptide glycation kinetics during the Maillard reaction. To identify the effects of peptide properties and hydrolysate composition on glycation kinetics, the glycation kinetics of individual peptides in hydrolysates was followed using quantitative peptide analysis. α-Lactalbumin was hydrolysed and glycated with D-glucose (0–8 h, 50 °C, dry heating with 65% humidity). The hydrolysates (degree of hydrolysis 2, 4, 6, and 8%) contained 25 unique peptides, ranging from 2 to 123 AAs with 0–12 lysine(s). The glycation rate constant (k_g) and the maximum average degree of glycation ($DG\_P_{av,max}$) of peptides were independent of the hydrolysate composition. The maximum DG of α-NH_2 and ε-NH_2 groups was 12.8% and 60.0%, respectively. With this information, the $DG\_P_{av,max}$ of individual peptides [9–59% for peptides with 0–2 lysine(s)] could be predicted.

1. Introduction

The reaction kinetics of the Maillard reaction has typically been studied using pure peptides and proteins. However, protein hydrolysates are used as ingredients in many food products, e.g., hypoallergenic infant formula. During industrial processing (or storage) in the presence of reducing carbohydrates, the peptides can become glycated through the Maillard reaction. In a glycated whey protein hydrolysate, 6 times more advanced Maillard products were found compared with the glycated intact protein (Mulcahy, Park, Drake, Mulvihill, & O'Mahony, 2016). Unlike intact proteins, peptides with various amino acid (AA) sequences are present in protein hydrolysates. In addition, there are more α-amino groups (per gram of dry matter) present in the hydrolysates than in the intact proteins. This could be the cause of the observed differences in the formation of advanced Maillard products. For synthetic peptides, glycation kinetics were suggested to be influenced by the peptide length (Kim & Lee, 2009), and the type (α/ε-) and number of amino groups (Münch et al., 1999; Penndorf, Biedermann, Maurer, & Henle, 2007). It is unclear how much these peptide properties and the hydrolysate composition affect the glycation kinetics of individual peptides in hydrolysates. To answer this, this study followed the glycation kinetics of individual peptides in protein hydrolysates.

In the literature, the Maillard reaction was reported to occur more in protein hydrolysates than in intact proteins. For instance, for whey protein hydrolysate with a degree of hydrolysis (DH) of 9.3%, the level of advanced Maillard products formed were 6 times more than in glycated whey protein isolate (Mulcahy et al., 2016). However, in that study the differences in the extent of the Maillard reaction was mostly observed by the formation of brown colour, but not by the changes in the amount of available lysine (Mulcahy et al., 2016). Hence, the conclusions were based on the extent to which the secondary reactions occurred. It was unclear whether the observed difference was similar in the initial stage of the reaction, i.e., the glycation, where the reducing carbohydrates react with free amino groups of proteins or peptides. Studies with synthetic peptides showed differences between the chemical reactivity of α- and ε-amino groups. In the reaction with triazinyl dye, the reaction rate constant of the ε-amino group on lysine was ~10 times higher than that of the α-amino group (Cowley, 1996).

For the Maillard reaction, many studies observed a brown colour formation (absorbance at 420 nm) besides the loss of amino groups and/or carbohydrates (Ajandouz & Puigserver, 1999; Kwak & Lim, 2004; Lamberts, Rombouts, & Delcour, 2008; Münch et al., 1999). This means that secondary reactions already took place in the samples (Ajandouz & Puigserver, 1999; Kwak & Lim, 2004; Lamberts et al., 2008; Münch et al., 1999). For instance, the

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* Corresponding author. Tel.: +31 317483786.
E-mail address: peter.wierenga@wur.nl (P.A. Wierenga).

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browning intensities of lysine and 8 other AAs during 0–12 h of heating with D-glucose were measured (Kwak & Lim, 2004). The reaction rate constant of the ε-amino group, estimated from the browning intensities *versus* the heating time fitted with a 1st order reaction equation, was ~2.5 times higher than that of the α-amino group (Kwak & Lim, 2004). The fitted maximum browning intensity of the two types of amino groups were similar (Kwak & Lim, 2004). Due to the occurrence of secondary reactions, it is difficult to draw conclusions on the glycation reactivity of the two types of amino groups.

For studies that investigated the reaction kinetics of glycation, the substrates used were mainly pure intact proteins (Akıllıoğlu, Çelikbıçak, Salih, & Gökmen, 2017; Cardoso, Wierenga, Gruppen, & Schols, 2018; Meltretter & Pischetsrieder, 2008; Priego-Capote, Ramírez-Boo, Finamore, Gluck, & Sanchez, 2014; Yeboah, Alli, Yaylayan, Konishi, & Stefanowicz, 2000). Some of the studies applied enzymatic hydrolysis after the glycation of proteins to investigate the differences in the glycation positions (Meltretter & Pischetsrieder, 2008; Priego-Capote et al., 2014). However, since the enzymatic hydrolysis was applied after the proteins were glycated, only the ε-amino groups of the lysine residues on the peptides could be glycated. Therefore, it is difficult to draw conclusions on the glycation reactivity of the α- or ε-amino groups when a mixture of different peptides was glycated in a hydrolysate.

For synthetic peptides that do not contain lysine residues, the glycation of α-amino groups was reported to depend on the type of N-terminal AA and the peptide length. For instance, the glycation rate constants of dipeptides with histidine as the N-terminal AA were 1–4 times (depending on the C-terminal AA) higher than that of dipeptides with the other 7 AAs tested as the N-terminal AA, which all had similar rate constants (~5 s^{-1} M^{-1}) (Bai, Ueno, & Manning, 1989). The effect of peptide length was proposed to be due to the increase in pK_a of the α-amino group, caused by the carboxyl group of the C-terminal AA (Kim & Lee, 2009). This means that fewer α-amino groups were unprotonated at the pH during glycation (pH 7.8) (Kim & Lee, 2009). The rate constants of di- and tri-glycine, calculated from 1st order reaction equation of the percentages of unbound glucose versus heating time, were ~2 times higher than that of glycine (Kim & Lee, 2009). Since protonation is a very fast equilibrium, the shift in pKa is not expected to affect the extent of reaction. However, the final percentage of unbound glucose in glycine solution was ~25% higher than that in di- or tri-glycine solutions (Kim & Lee, 2009).

The influence of this shift in pKa on glycation was also discussed in synthetic peptides that contained lysines (Venkatraman, Aggarwal, & Balaram, 2001). A shift in pKa was observed for lysines in 78 proteins, ranging from 5.7 to 12.1 (Grimsley, Scholtz, & Pace, 2009). This shift in pKa was attributed to the surrounding AAs of lysine residues, i.e., neighbouring AAs. For example, the extent of glycation of synthetic peptides that had aspartic acid as a neighbouring AA (44 or 60% glycated, depending on the position of aspartic acid) was lower than that of peptides surrounded by neutral AAs (76% glycated) (Venkatraman et al., 2001). Besides the charge of surrounding AAs, the hydrophobicity was also reported to affect the glycation (Mennella, Visciano, Napolitano, Del Castillo, & Fogliano, 2006). For dipeptides with lysine as the N-terminal AA, if the C-terminal AA was hydrophobic, the dipeptides were fully glycated after 30 min (Mennella et al., 2006). When other AAs were used as the C-terminal AA, 30–90% of the peptides remained non-glycated, with an exception of arginine as the C-terminal AA, which 10% of the dipeptide remained non-glycated (Mennella et al., 2006).

In this study, to identify the influences of peptide properties on the glycation kinetics of peptides in protein hydrolysates, the glycation of individual peptides in hydrolysates was followed. Hydrolysates that contained peptides with different properties, i.e., peptide length, type (α/ε-) and number of amino groups in the peptide, and different relative abundances, were prepared and glycated under identical conditions.

2. Materials and methods

2.1. Materials

α-Lactalbumin was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). Approximately 72% of it was in apo form and the rest was in holo form, based on results from circular dichroism (Deng, Wierenga, Schols, Sforza, & Gruppen, 2017). *Bacillus licheniformis* protease (BLP) (batch NS-37005) was obtained from Novozymes (Bagsvaerd, Denmark). BLP is specific for glutamic and aspartic acids residues (Butré, Sforza, Gruppen, & Wierenga, 2014a). The BLP powder was partly insoluble, and was purified as described previously (Butré, Wierenga, & Gruppen, 2014c). The suspension of BLP was centrifuged (10 min, 4000 ×*g*, 25 °C) and the supernatant was dialysed (cut-off 12–14 kDa) against 150 mM NaCl solution, then against demineralised water, and then freeze-dried. The freeze-dried powder had a protein content of ~60% (w/w) based on the Dumas results (N × 5.93, based on the AA composition of the protein as described in Uniprot accession code P80057, www.uniprot.org). The enzyme activity was 3.9 AU mg^{-1} min^{-1}, as determined using the azocasein assay (Akpinar & Penner, 2001). The purity of BLP was 100% based on the UV_{280} peak area determined using the PDA detector attached to the ultra-performance liquid chromatography (UPLC) system and was 92% based on the UV_{214} peak area (A_{214}). In the UV_{214} chromatogram, two peaks were found, of which the main peak (78% of total A_{214}) was identified to be the BLP (23.6 kDa) and the minor peak (14% of total A_{214}) was the pro-peptide (6.9 kDa). All other chemicals were of analytical grade and purchased from Sigma or Merck.

2.2. Preparation of apo α-LA

Ethylenediaminetetraacetic acid (EDTA) was used to chelate the calcium ions attached to the α-lactalbumin molecules. EDTA was added to 1 L of 10% (w/v) protein solution at an α-lactalbumin to EDTA ratio of 1:5 (mol mol^{-1}), and the solution was stirred overnight at 4 °C. To remove EDTA, the solution was ultra-filtered with 7 L of Millipore water over a UFP-10-C-5 membrane (cut-off 10 kDa, AG Technologies Corp., Tokyo, Japan). The prepared apo α-lactalbumin (further referred to as α-LA) was freeze-dried and stored at −20 °C before further analysis. The protein content was ~90% (w/w) based on the Dumas method (N × 6.25; based on the AA composition as described in Uniprot accession code P00711). Of the total protein content, ~90% was α-LA, based on the A_{214} determined using the PDA detector attached to the UPLC system, and the remaining 10% consisted of β-lactoglobulin and bovine serum albumin. It has been previously published that the extent and rate of glycation were similar for α-lactalbumin and β-lactoglobulin (Cardoso et al., 2018). In this article, the focus was on the glycation kinetics of peptides generated from α-LA.

2.3. Production of α-LA hydrolysates with different degrees of hydrolysis

The enzymatic hydrolysis was performed as described elsewhere (Butré, Sforza, Gruppen, & Wierenga, 2014b). The α-LA was dissolved in 20 mL Millipore water at a concentration of 1% (w/v). The solution was adjusted to pH 8.0 and equilibrated for 0.5 h at 37 °C in a pH-stat device (Metrohm, Herisau, Switzerland). The BLP was dissolved (10 mg powder mL^{-1}) in Millipore water and added to the equilibrated protein solution to reach an enzyme/substrate ratio of 1:100 (w/w) (Butré et al., 2014b). The hydrolysis was performed at 37 °C using a pH-stat device with 0.2 M NaOH to keep the pH constant. The degree of hydrolysis (DH_{stat}) was calculated using the equation published previously (Adler-Nissen, 1986). The hydrolysis was stopped at DH 2, 4, 6 and 8% (labelled DH2-DH8) by adjusting the pH to 2 using 5 M HCl (Butré et al., 2014c). The pH of the hydrolysate solution was re-adjusted to 8.0 after ≥10 min of inactivation. As a control experiment, 1% α-LA solution was incubated in the absence of BLP. The added volume of NaOH from the control experiment was subtracted from the added volume of NaOH at all time points during hydrolysis, to compensate for the consumption of NaOH due to the dissolution of CO_2. The samples were stored at −20 °C.

2.4. *Glycation of intact and hydrolysed α-LA*

The glycation of intact and hydrolysed α-LA was performed as described previously (Deng et al., 2017). To a 5 mL protein (or hydrolysate) solution at 1% (w/v), 0.05 mL of a 1 M sodium phosphate buffer (pH 8.0) was added to reach a molarity of phosphate buffer of 10 mM, with a negligible change in protein concentration. D-Glucose was dissolved in 10 mM sodium phosphate buffer (pH 8.0) at concentrations of 3.5, 3.8, 4.4, 4.9 and 5.5 mg mL^{-1}. Five mL of each D-glucose solution was mixed with 5 mL of the hydrolysate solution (1%, w/v) with a DH_{stat} of 0, 2, 4, 6 and 8%, respectively, to reach a molar ratio of free amino groups to reducing ends of 1:2. The concentration of free amino groups in each hydrolysate was calculated based on the number of lysine residues (12 in α-LA) and N-termini per original protein molecule. The number of N-termini is equal to $1+DH_{stat}/100 \times 122$ (122 is the number of peptide bonds in α-LA). The mixtures were freeze-dried, followed by incubation in a dry state at 50 °C under 65% relative humidity for 0, 2, 4, 6 and 8 h in a humidity control chamber (HCP108, Memmert, Schwabach, Germany). The glycated samples were labelled $DH2\_G_{0\text{-}8}$-$DH8\_G_{0\text{-}8}$. The non-hydrolysed α-LA (DH0) was glycated for 0–24 h ($DH0\_G_{0\text{-}24}$) and the reaction was performed in duplicate. As a control, the hydrolysates were also incubated without D-glucose ($DH0\_{}_{8}$-$DH8\_{}_{8}$) at 50 °C under 65% relative humidity for 8 h. All samples were freeze-dried and stored at −20 °C.

2.5. o-*phthaldialdehyde method*

The total number of free amino groups of the samples ($DH0\_G_{0\text{-}8}$-$DH8\_G_{0\text{-}8}$) was determined using the *o*-phthaldialdehyde (OPA) method (Butré, Wierenga, & Gruppen, 2012). The samples were dissolved at a concentration of 0.5% (w/v) in a 2% (w/v) SDS solution. Five microlitres of sample solution was added to 300 μL of OPA reagent and incubated for 10 min. The experiments were performed in a 96-well plate using Tecan Infinite F500 (Tecan, Männedorf, Switzerland) to obtain the UV_{340} absorption of the samples. The measurements were performed in triplicate. A calibration curve was made with leucine. The protein concentration of each sample was determined by the Dumas method. The total degree of glycation (DG_T_{OPA}) of the hydrolysates was calculated using equation (1), adapted from an equation published previously (Delahaije, Wierenga, Giuseppin, & Gruppen, 2014):

$$DG\_T_{OPA}\,(\%) = \frac{(\#NH_{2,u} - \#NH_{2,g})}{\#NH_{2,u}} \times 100 \qquad (1)$$

where $\#NH_{2,u}$ and $\#NH_{2,g}$ were the number of free amino groups per original protein molecule in the untreated and glycated hydrolysates, respectively, calculated from the concentration of free amino groups, corrected for the concentration of total proteinaceous matter.

2.6. *Total protein content*

The protein content was determined using a Flash EA 1112 NC Analyser (Thermo Fisher Scientific Inc, Waltham, MA, USA). The nitrogen-protein conversion factor of 6.25 for non-hydrolysed α-LA was used, calculated from the AA composition of α-LA (Uniprot accession code P00711). Nitrogen-protein conversion factors of 6.27, 6.29, 6.31 and 6.33 were used for hydrolysates with a DH_{stat} of 2, 4, 6 and 8%, respectively, based on the number of water molecules introduced to the peptides during hydrolysis.

2.7. *Reversed phase ultra-high performance liquid chromatography*

The samples were analysed on an H class Acquity UPLC® system (Waters, Milford, MA, USA) equipped with a BEH C18 column (1.7 μm, 2.1 × 100 mm, Waters). An Acquity PDA® detector (Waters) was attached to the ultra-high performance liquid chromatography (UPLC) system. The eluents and elution gradient were the same as described previously (Butré et al., 2014b). Eluent A was 1% (v/v) acetonitrile (ACN) containing 0.1% (v/v) trifluoreacetic acid (TFA) in Millipore water and eluent B was 100% ACN containing 0.1% (v/v) TFA. Samples were incubated for 2 h with 100 mM dithiothreitol (DTT) in 50 mM Tris–HCl buffer at pH 8.0 to reduce the disulphide bridges, diluted to a protein concentration of 0.1% (w/v powder) and centrifuged (10 min, 14,000 ×g, 20 °C) before injection (4 μL). The gradient was as follows: 0–2 min isocratic on 3% B; 2–10 min linear gradient from 3% to 22% B; 10–16 min linear gradient 22–30% B; 16–19 min linear gradient 30–100% B; 19–24 min isocratic on 100% B; 24–26 min linear gradient 100–3% B and 26–30 min isocratic on 3% B. The flow rate was 350 μL min^{-1}. The UV absorbance at 214 nm was monitored for the absolute quantification of peptide concentrations (as described below).

2.8. *Electrospray ionisation time of flight mass spectrometry*

The mass spectra of the samples were measured using an online SYNAPT G2-Si high definition mass spectrometry (Waters) coupled to the RP-UPLC system. Sodium iodide was used for the MS calibration. The MS and MS/MS (MSe) data were collected using the methods developed previously (Butré et al., 2014b). The MSe method was not dedicated to optimise the signal of fragments of each specific peptide, but to reach an efficient detection of fragments of all peptides in hydrolysates. Online lock mass (angiotensin II, $[M+2H]^{2+}$: 523.7751) was acquired. Based on the differences in the measured and theoretical lock mass, corrections were applied on *m/z* (mass-to-charge ratio) of the peptides during measurements. The detection was under positive ion mode. The capillary voltage was set to 3 kV and the source temperature was set at 120 °C. The sample cone was operated at 35 V. The desolvation gas (250 °C, 800 L h^{-1}) and cone gas (200 L h^{-1}) was nitrogen. The trap gas was set at 1.5 mL min^{-1}. MS and MS/MS were performed between *m/z* 100 and 3000 with a scan time of 0.3 s. The trap collision energy was set at 6 V in single MS mode and ranged from 20 to 30 V in MSe mode. The transfer collision energy was 4 V in MS mode and switched between 4 and 10 V MSe mode. The data were analysed manually using MassLynx software v4.1 (Waters).

2.9. *Peptide identification and quantification*

Because the AA sequence of α-LA is known, the peptides were manually annotated based on *m/z* values and the charge states obtained from the MS spectra. The *m/z* value and the charge state of each peptide were used as input values in an in-house developed Matlab software. The peptide identifications were performed based on comparisons between the experimental masses and the theoretical masses of possible matching α-LA peptide sequences. The mass tolerance between the theoretical and measured masses for the accepted peptide annotation was set at 100 ppm. The identification was, where possible, confirmed by identifying the b and y fragments in MS/MS spectra. Glycated peptides were identified by the extra masses of n dehydrated glucose(s) (+n × 162 Da) on top of the mass of original peptides. The concentration of each peptide ($C_{peptide}$) was calculated using equation (2) (Butré et al., 2014b):

$$C_{peptide}\ (\mu m) = \frac{A_{214} \cdot Q}{\varepsilon_{214} \cdot l \cdot V_{inj} \cdot k_{cell}} \tag{2}$$

All hydrolysates were injected at an equal concentration [g L^{-1}]. To compare samples, $C_{peptide}$ was re-calculated using a correction for the changed protein content in glycated hydrolysates, based on the Dumas results. A_{214} [μAU min] is the UV peak area at 214 nm, V_{inj} (μL) is the injected volume of the sample, Q (μL min^{-1}) is the flow rate and l (cm) is the path length of the UV cell, which is 1 cm according to the manufacturer. ε_{214} (M^{-1} cm^{-1}) is the molar extinction coefficient at 214 nm, calculated as described previously (Kuipers & Gruppen, 2007). The molar extinction coefficient of glycated α-LA was previously shown to be the same as the untreated α-LA by determining the UV absorbances of a dilution series of non-glycated and glycated α-LA solutions with known concentrations (based on the Dumas results) (Deng et al., 2017). Therefore, the molar extinction coefficients of the glycated and non-glycated peptides were assumed to be the same.

Due to multiple reflections by the coating of the cell, the effective path length of the light through the cell is not the same as the length specified by the producer. To correct for this effect, the cell constant of the UV detector (k_{cell}) was determined using a series of standard solutions made by β-lactoglobulin, β-casein and angiotensin II, and the approach described elsewhere (Kosters, Wierenga, de Vries, & Gruppen, 2011). The ratio between the measured and expected A_{214} was taken as the value of k_{cell}. For the UV cell used in this work, the k_{cell} was determined to be 0.78 (Deng, van der Veer, Sforza, Gruppen, & Wierenga, 2018b). The linear region of the A_{214} in the PDA detector ranges from 5×10^1 to 6×10^5 μAU min. Peptide quantification was done for peptides with a $A_{214} \geq 5 \times 10^1$ μAU min. For all hydrolysates, on average 95 ± 10% of the expected total A_{214} was found. Of the total A_{214}, on average 94 ± 3% in the samples was assigned to annotated peptides. In certain cases, a glycated peptide co-eluted with the non-glycated peptide. In such cases, the A_{214} was divided over the co-eluting peptide depending on the intensity of the total ion count of each peptide. The standard error on the peptide concentration for one hydrolysate injected in triplicate with this quantification method was previously determined to be ~6% (Butré et al., 2014b). In addition, for duplicate hydrolyses, the average standard error of the peptide concentration was ~15% (Deng, Gruppen, & Wierenga, 2018a).

2.10. Quality check of the annotation and quantification of the peptide analysis

The quality of the peptide analysis was checked by determining the amino acid (AA), peptide and molar sequence coverages of α-LA hydrolysates, calculated by equations (3)–(5), respectively (Butré et al., 2014b):

$$\textit{Amino acid sequence coverage}\ (\%) = \frac{\#\textit{unique annotated AA}}{\#AA_{protein}} \times 100\% \tag{3}$$

$$\textit{Peptide sequence coverage}\ (\%) = \frac{\#AA(\textit{annotated peptides})}{\#AA(\textit{annotated peptides}) + \#AA(\textit{missing peptides})} \times 100\% \tag{4}$$

$$\textit{Molar sequence coverage}\ (\%) = \left\{ 1 - \frac{\sqrt{\frac{\sum (C_n - C_0)^2}{(\#AA_{protein} - 1)}}}{C_0} \right\} \times 100\% \tag{5}$$

where C_n (μM) is the concentration of each individual AA (n) in the protein sequence obtained from peptide quantification; C_0 (μM) is the initial injected protein concentration and $\#AA_{peptide}$ is the number of AAs in the protein sequence (Butré et al., 2014b). The initial injected protein concentrations were determined using the Dumas method. The missing peptides are peptides that were not quantified but should be present given the annotated peptides (Butré et al., 2014b). The three parameters were determined for all non-glycated and glycated hydrolysates. To further check the quality of the analysis, the degree of hydrolysis based on peptide analysis (DH_{MS}) of each hydrolysate was calculated using equation (6):

$$DH_{MS}\ (\%) = \frac{\sum C_{i,t}}{2 \times \#\textit{peptide bonds} \times C_0} \times 100 \tag{6}$$

where #peptide bonds is the number of peptide bonds and $C_{i,t}$ (μM) is the concentration of cleavage site products formed and *i* equals the sum of concentrations of all peptides of sequence (x−y), for which $I = (x{-}1)$ or $I = y$. The $C_{i,t}$ can be calculated using equation (7):

$$C_{i,t}\ (\mu m) = \sum \left\{ C_{peptide}[x-y]_t | i = x-1 \cup i = y \right\} \tag{7}$$

2.11. Determination of the average degree of glycation of a peptide

For each peptide in the hydrolysates, the average degree of glycation (DG_P_{av}) was calculated as the percentage of average number of glycated amino groups of the total number of amino groups in that peptide (equation (8)) (Deng et al., 2017). In other words, DG_P_{av} is the weighted average of the concentration of all variants of the peptide (glycated and non-glycated):

$$DG\_Pav\ (\%) = \frac{\sum_{i=0}^{n} \frac{i \times C_{peptide,i}}{C_{peptide,tot}}}{\#NH_{2,u}} \times 100 \tag{8}$$

where $C_{peptide,i}$ is the concentration of the peptide variant with *i* glucose molecules attached, $C_{peptide,tot}$ is the sum of the concentrations of glycated and non-glycated variants and $\#NH_{2,u}$ is the total number of amino groups of the untreated peptides, which is the sum of the number of lysine residues and the N-terminal AA.

2.12. Determination of the total degree of glycation of a hydrolysate based on peptide analysis

Based on peptide analysis, the total DG (DG_T$_{MS}$) of a hydrolysate was determined using the DG_P$_{av}$ and C$_{peptide,tot}$ in the hydrolysates (equation (9)), and compared with the total DG determined from the OPA analysis (DG_T$_{OPA}$):

$$DG_{T_{MS}}(\%) = \frac{\sum\left(C_{peptide} \times DG\_P_{av} \times \#NH_{2,u}\right)}{\sum\left(C_{peptide} \times \#NH_{2,u}\right)} \tag{9}$$

where #NH$_{2,u}$ is the total number of amino groups of the untreated peptides.

2.13. Determination of the maximum average degree of glycation and the glycation rate constant of a peptide

The maximum average degree of glycation (DG_P$_{av,max}$) and glycation rate constant (k$_g$) of each peptide (during Maillard incubation) were determined by fitting the glycation curve (DG_P$_{av}$ *versus* time) of each peptide with equation (10):

$$DG\_Pav\ (\%) = DG\_P_{av,max} \times \left(1 - e^{-k_g \cdot t}\right) + DG\_P_{av,0} \tag{10}$$

where both DG_P$_{av,max}$ (%) and k$_g$ [s^{-1}] were fitting parameters. DG_P$_{av,0}$ (%) is the DG$_{av}$ of the non-heated samples (DH0_G$_0$-DH8_G$_0$), which was set at 0 because the MS intensities of the glycated peptides in these samples were within the noise of the MS spectra. The 1st order reaction equation used here was not to determine the mechanism of reaction, but to have a high R^2 (R^2 = 1 − SS$_{residual}$/SS$_{total}$, SS means the sum of squared errors) of the fit.

2.14. Non-targeted clustering of the DG_P$_{av,max}$ and k$_g$

The DG_P$_{av,max}$ and k$_g$ of peptides were fitted with a normal distribution curve. The structure of the residuals of the fit was used to identify the number of populations in the data set. Once the number of populations (k) was identified, the Matlab method k-means clustering was used to categorise the (k) populations. The k-means clustering is a partitioning method, which uses the squared Euclidean metric to determine the distances and the k-means++ algorithm for the cluster centre initialisation. This is used to group data points into populations with minimised total sum of distances.

2.15. The maximum degree of glycation of α- and ε-amino groups

The maximum degree of glycation of α-amino groups (DG$_{max,\alpha\text{-}NH2}$) was estimated based on the average value of DG_P$_{av,max}$ of the peptides that did not contain lysine. Based on the DG$_{av,max}$, the maximum degree of glycation of ε-amino group (DG$_{max,\varepsilon\text{-}NH2}$) was calculated based on equation (11):

$$DG\_P_{av,max}\ (\%) = DG_{max,\alpha-NH_2} \times \frac{1}{\#GS} + DG_{max,\varepsilon-NH_2} \times \frac{\#lysine}{\#GS} \tag{11}$$

where the #lysine and #GS refer to the number of lysine residues and glycation sites (the sum of lysines and N-terminal AAs), respectively. The heterogeneity of the glycation process was checked by determining the DG$_{max,\varepsilon\text{-}NH2}$ of the glycated variants of the peptides, calculated by dividing the DG$_{max,\varepsilon\text{-}NH2}$ by the percentage of glycated variants (DG$_{max,\varepsilon\text{-}NH2}$)/(1-%non-glycated peptide).

3. Results and discussion

3.1. Analysis of the non-glycated hydrolysates

The preparation of the α-LA hydrolysates was reproducible, as indicated by the similarity in the hydrolysis curves (DH$_{stat}$ *versus* time) for each of the four hydrolysates prepared (Fig. 1). In the (non-glycated) hydrolysates DH2_G$_0$, DH4_G$_0$, DH6_G$_0$ and DH8_G$_0$, 17, 20, 17 and 13 peptides were identified and quantified, respectively (Table 1; Supplementary material Table S1). Combining all hydrolysates, in addition to the remaining intact protein, 24 unique peptides were annotated, of which 23 were found in more than one hydrolysate. The relative abundance (in mole mole^{-1}%) of these peptides ranged from 0.1% to 24% of the total amount of peptides (Table 1). Out of the 25 unique peptides (including the intact protein), 5 peptides did not contain any lysine. The length of these peptides ranged from 4 to 24 AAs. For the peptides that contained 1 or 2 lysine(s), the peptide lengths ranged from 2 to 8 and from 10 to 42 AAs, respectively.

3.1.1. Quality check of the peptide analysis for non-glycated hydrolysates

The quality of peptide analysis was evaluated based on the average AA, peptide and molar sequence coverages, and the DH values determined based on peptide analysis (DH$_{MS}$). For all hydrolysates, the AA sequence coverage was 100% (Table 2). The peptide sequence coverages decreased from 99% to 89% with increasing DH$_{stat}$ values, and the molar sequence coverages ranged from 85% to 58%. The average peptide and molar sequence coverages were 94% and 72%, respectively. Based on previous data (Butré et al., 2014a, b; Butré, Sforza, Wierenga, & Gruppen, 2015), the thresholds of acceptance for the average peptide and molar sequence coverages were set at 80% and 70%, respectively (Deng et al., 2018a). Both average coverages in this study were above the thresholds.

To further check the quality of peptide analysis, the DH values analysed with peptide analysis (DH$_{MS}$) of each hydrolysate was calculated, and compared with the DH$_{stat}$ based on pH-stat titration (Fig. 2). Averaged over all samples, the standard error between

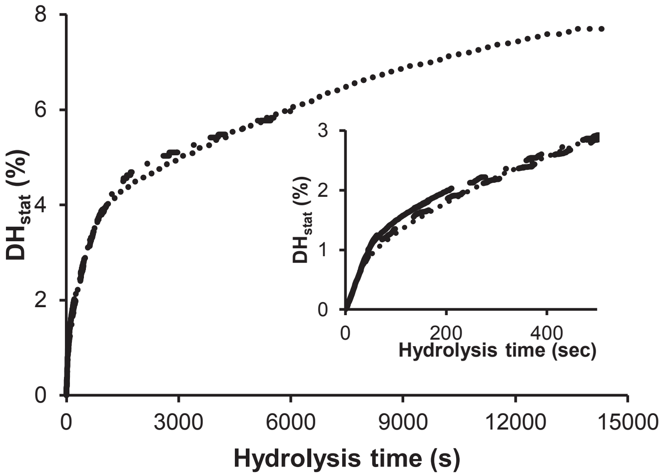

Fig. 1. Hydrolysis curves (DH$_{stat}$ *versus* time) for the preparation of α-LA hydrolysates with a DH$_{stat}$ of 2% (——), 4% (– – –), 6% (— •) or 8% (• • •). The inset shows the zoomed hydrolysis curves from 0 to 500 s.

Table 1
Number of lysine residues per peptide, peptide length and the concentration of peptides identified and quantified in non-glycated α-LA hydrolysates with a DH_{stat} of 2, 4, 6 or 8%.

Peptides	Lysine residues	Length	Concentration of peptides in hydrolysates (μM)			
			$DH2\_G_0$ (17)	$DH4\_G_0$ (20)	$DH6\_G_0$ (17)	$DH8\_G_0$ (13)
[8–11]	0	4	4.0	23.0	24.1	23.0
[117–121]	0	5	0.2	1.3	3.2	9.8
[38–46]	0	9			3.5	8.6
[38–49]	0	12		2.5	8.4	7.6
[26–49]	0	24	0.1	1.1	1.3	
[122–123]	1	2	8.6	27.7	23.0	20.2
[12–14]	1	3			2.9	7.8
[1–7]	1	7	10.9	27.1	21.5	19.0
[8–14]	1	7		0.6	1.0	
[114–121]	1	8	3.7	19.4	9.3	0.2
[114–123]	2	10	3.2	0.9	0.6	
[12–25]	2	14	0.4	4.6	29.3	37.1
[57–78]	2	22				0.5
[12–37]	2	26		0.3	3.7	0.2
[12–46]	2	35		0.6	1.7	
[12–49]	2	38	5.8	18.4	1.2	
[8–49]	2	42	5.8	1.1		
[1–49]	3	49	10.4	0.3		
[79–113]	5	35			1.5	4.7
[50–113]	6	64	3.7	19.7	21.4	15.3
[50–121]	7	72	1.7	0.5		
[50–123]	8	74	12.1	19.7		
[8–113]	8	106	1.6	0.7		
[8–121]	9	114	0.9	1.9		
[1–123]	12	123	2.3			

Table 2
The amino acid (AA), peptide and molar sequence coverages of the non-glycated and glycated α-LA hydrolysates with different DH values ($DH2\_G_{2\text{-}8}$-$DH8\_G_{2\text{-}8}$).[a]

Samples	Sequence coverage (%)		Samples	Average sequence coverage (%)	
	Peptide	Molar		Peptide	Molar
$DH2\_G_0$	99	80	$DH2\_G_{2\text{-}8}$	99 ± 0	85 ± 6
$DH4\_G_0$	94	85	$DH4\_G_{2\text{-}8}$	93 ± 0	77 ± 7
$DH6\_G_0$	92	67	$DH6\_G_{2\text{-}8}$	91 ± 2	70 ± 4
$DH8\_G_0$	89	58	$DH8\_G_{2\text{-}8}$	89 ± 0	62 ± 3
Average	94 ± 4	72 ± 12	Average	93 ± 4	73 ± 10

[a] The AA sequence coverages of all samples were 100%. The average values and standard deviations were calculated from the hydrolysates with the same DH_{stat} values but were incubated for different time.

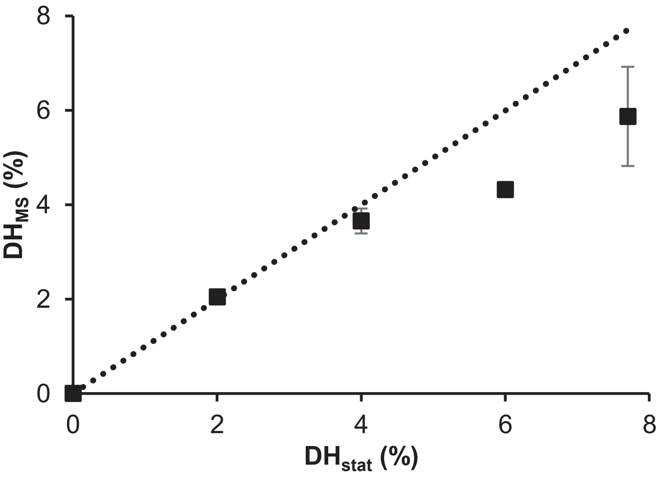

Fig. 2. Comparison of DH determined based on peptide analysis (DH_{MS}) and pH-stat titration (DH_{stat}) for α-LA hydrolysates. Each data point was the average of data for hydrolysates with the same DH_{stat} but with different Maillard incubation time (0–8 h). The dotted line (• • •) indicates the function y = x.

DH_{stat} and DH_{MS} was ~14%. This standard error was similar to previous results (Deng et al., 2018a). Based on these quality checks, the peptide analysis for non-glycated hydrolysates was sufficient to allow further analysis on the glycation on each peptide.

3.2. Analysis of the glycated α-LA hydrolysates

In the control samples $DH0\_{}_8$-$DH8\_{}_8$ (hydrolysates heated without D-glucose for 8 h), no glycated or lactosylated protein/peptides were found, confirming that non-glycated samples did not contain any glucose or lactose. The glycation of non-hydrolysed α-LA for 24 h only resulted in a DG_{MS} of 85% (Fig. 3A). Fitting the data also showed an expected plateau ($DG_{av,max}$) at 86% (using equation (10)). Since the DG was expected to be 100%, the data were also fitted using a fixed $DG_{av,max}$ of 100%. However, this fit did not describe the data well (the sum of squared residuals increased ~4 times). This was not due to aggregation (based on results from size exclusion chromatography, data not shown) or incomplete analysis of the sample in UPLC (UV area was ~100% of the expected concentration). It was also not due to side-reactions, since no unknown masses were found in the MS spectra (data not shown).

The low $DG_{av,max}$ reached was also not caused by the lack of reactants. For intact proteins as well as for hydrolysates (will be discussed later), the amount of added D-glucose was 2 times higher than the amount of free amino groups. In addition, the high-performance anion-exchange chromatography (data not shown) showed that there was still unreacted glucose (the loss of glucose was equal to the increase in DG). Apparently, the glycation process either stopped, or reached an equilibrium while there were still free reactants in the system. A plateau value for the DG_{MS} was previously also observed during the glycation of 3 different proteins with 7 different carbohydrates until 48 h of glycation. In that case, the plateau value of $DG_{av,max}$ was even more defined due to the longer incubation time (Cardoso et al., 2018). Values for $DG_{av,max}$ in that study ranged from 6% to 92% for different protein-carbohydrate combinations (Cardoso et al., 2018). From the literature, we have

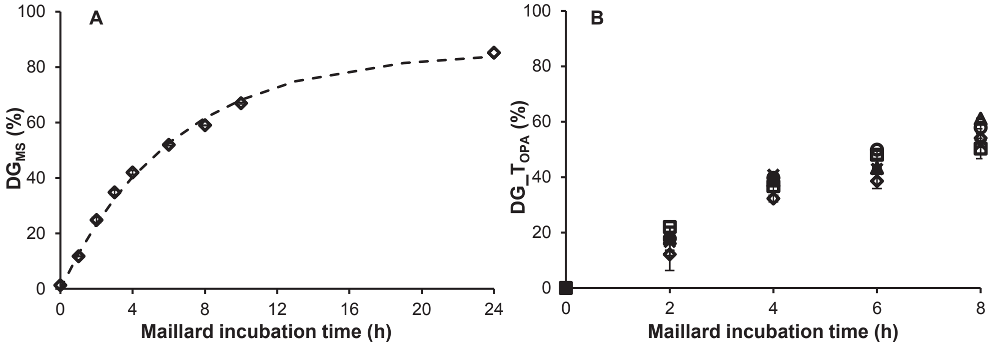

Fig. 3. (A) Average degree of glycation of non-hydrolysed α-LA as a function of Maillard incubation time. (B) Total degree of glycation analysed with OPA method (DG_T_{OPA}) versus Maillard incubation time for glycated α-LA hydrolysates with a DH_{stat} of 0% (□), 2% (◇), 4% (○), 6% (△) or 8% (×). The error bars were typically smaller than the size of the marker.

not seen clear analysis of such information, or have we observed explanations for such an effect.

The total degree of glycation of hydrolysates based on the OPA method (DG_T_{OPA}) reached ~55% after 8 h heating in the presence of D-glucose (Fig. 3B). This value was similar to the value for the non-hydrolysed α-LA (after 8 h incubation). This indicated that the free α-amino groups created by hydrolysis did not make a major contribution to the total glycation, which implies that the glycation of ε-amino group dominates the total degree of glycation.

In the glycated hydrolysates (DH2_G_{2-8}-DH8_G_{2-8}), glycated variants of all peptides were found. The glycated variants of each peptide eluted either slightly before, or at the same moment as that original peptide, illustrated for peptide α-LA [1–7] in Fig. 4A. The glycation was clear from the mass spectra (Fig. 4B). This peptide contains 1 lysine residue, meaning that glycation can occur on the ε-amino group of that lysine and/or on the α-amino group of the N-terminal AA. After 2 h incubation, 17% of this peptide had 1 glucose unit attached (*m/z* of 1012.4). The intensity ratio between the glycated and the original peptide increased over the incubation time. After 6 h, the peptide with 2 glucose units attached (*m/z* of 1174.5) was detected.

3.2.1. *Quality check of the peptide analysis for glycated hydrolysates*

Similar to the quality checks for non-glycated hydrolysates, the AA, peptide and molar sequence coverages, and the comparison between DH_{MS} and DH_{stat}, were checked for all glycated hydrolysates. All hydrolysates had an AA sequence coverage of 100%. Peptide and molar coverages were averaged over the non-glycated and glycated α-LA hydrolysates (at each DH) (Table 2). The average peptide sequence coverages were 99 ± 0, 93 ± 0, 91 ± 2 and 89 ± 0% for hydrolysates with a DH_{stat} of 2, 4, 6 and 8%, respectively, and the average molar sequence coverages were 85 ± 6, 77 ± 7, 70 ± 4 and 62 ± 3% (Supplementary material Fig. S1). Averaged over all hydrolysates, the peptide and molar sequence coverages were 93 ± 4 and 73 ± 10%, respectively, which were above the thresholds for acceptance. The DH_{MS} of each sample was comparable with the DH_{stat} (Fig. 2). In addition to the above-mentioned quality checks, for glycated hydrolysates, the concentrations of glycated and non-glycated peptides at different Maillard incubation times were compared.

Overall, there was only a minor variation in the quantified concentration (average standard error of 20%; Supplementary material Table S2) of peptides in the samples with increasing incubation time, as illustrated for DH2_G_{0-8} (average standard error of ~17%, Table 3). This is similar to the standard error in peptide concentrations determined in duplicate hydrolysates (~15%) (Deng et al., 2018a), confirming that the quality of the peptide analysis

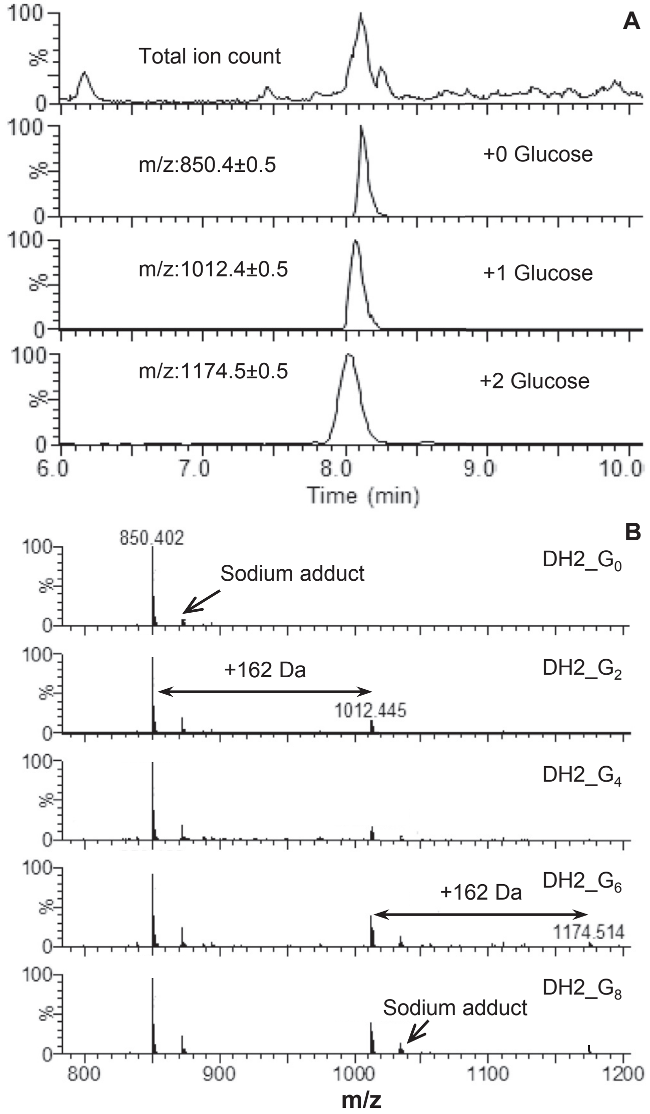

Fig. 4. (A) Total ion count and (B) mass spectra of non-glycated and glycated peptide α-LA [1–7] analysed in the RP-UPLC chromatograms of DG2_G_{0-8}, using extracted ions at *m/z* of 850.4 ± 0.5 (+0 glucose) (100% MS intensity is 3.6 × 10^4), 1012.4 ± 0.5 (+1 glucose) (100% MS intensity is 3.0 × 10^3) and 1174.5 ± 0.5 (+2 glucose) (100% MS intensity is 1.4 × 10^3).

Table 3
The total and average concentrations of peptides (glycated and non-glycated) in hydrolysates with a DH_{stat} of 2% (DH2_G_{0-8}).[a]

Peptides	Concentration of peptides in hydrolysates (μM)						
	DH2_G_0	DH2_G_2	DH2_G_4	DH2_G_6	DH2_G_8	Average	Standard error (%)
[8–11]	4.0	4.2	4.8	5.4	4.9	4.7 ± 0.6	12
[117–121]	0.2	0.2	0.2	0.3	0.3	0.2 ± 0.0	21
[26–49]	0.1	0.1	0.2	0.1	0.1	0.1 ± 0.0	35
[122–123]	8.6	12.0	14.0	12.1	15.3	12.4 ± 2.5	20
[1–7]	10.9	9.3	10.6	9.1	10.4	10.1 ± 0.8	8
[114–121]	3.7	3.3	3.7	2.9	3.0	3.3 ± 0.4	11
[114–123]	3.2	2.6	3.4	2.4	2.8	2.9 ± 0.4	15
[12–25]	0.4	0.4	0.4	0.3	0.5	0.4 ± 0.1	22
[12–49]	5.8	6.4	7.2	6.1	5.2	6.1 ± 0.8	12
[8–49]	5.8	6.6	7.6	7.1	5.7	6.5 ± 0.8	13
[1–49]	10.4	9.8	10.5	11.6	15.7	11.6 ± 2.4	20
[50–113]	3.7	4.1	5.0	4.0	3.0	4.0 ± 0.7	19
[50–121]	1.7	1.4	1.6	1.9	1.5	1.6 ± 0.2	12
[50–123]	12.1	15.0	16.6	15.2	18.0	15.4 ± 2.2	14
[8–113]	1.4	1.5	1.5	1.8	1.2	1.5 ± 0.2	14
[8–121]	0.9	1.1	1.1	1.2	0.9	1.0 ± 0.1	12
[1–123]	2.3	1.2	1.2	1.8	2.1	1.7 ± 0.5	28
						Average	17

[a] Typical standard error of peptides in duplicate hydrolysates is ~15%.

was acceptable. Furthermore, using the DG_P_{av} values, the total degrees of glycation of hydrolysates (DG_T_{MS}) were calculated, and compared with the total degrees of glycation analysed using the OPA method (DG_T_{OPA}) (Fig. 5). Averaged over all samples, the standard error between the DG_T determined using the two methods was ~13%.

3.2.2. Glycation kinetics of individual peptides in protein hydrolysates

The maximum average degree of glycation (DG_$P_{av,max}$) and the glycation rate constant (k_g) for individual peptides in all hydrolysates were determined (Table 4). The glycation of individual peptides in the hydrolysates, the glycation curves (DG_P_{av} versus time) is illustrated using peptides α-LA [38–46], α-LA [114–121] and α-LA [12–49] as examples of peptides containing 0, 1 and 2 lysine(s), respectively (Fig. 6A). The DG_P_{av} values of the peptides increased with the number of lysines in the peptides. For peptides that were present in multiple hydrolysates (e.g., α-LA [50–113]), the glycation curves in all hydrolysates were compared (Fig. 6B). Despite that, their concentrations ranged from 4.0 to 23.0 μM (Supplementary material Table S2), the changes in DG_P_{av} versus incubation time were similar. The average standard errors for DG_$P_{av,max}$ and k_g of the same peptides in different hydrolysates were both 14%. This shows that the glycation of peptides in protein hydrolysates was independent of the hydrolysate composition. Therefore, if peptides were present in several hydrolysates, the DG_P_{av} values obtained in all hydrolysates were combined to perform the fit for DG_$P_{av,max}$ and k_g (Table 4).

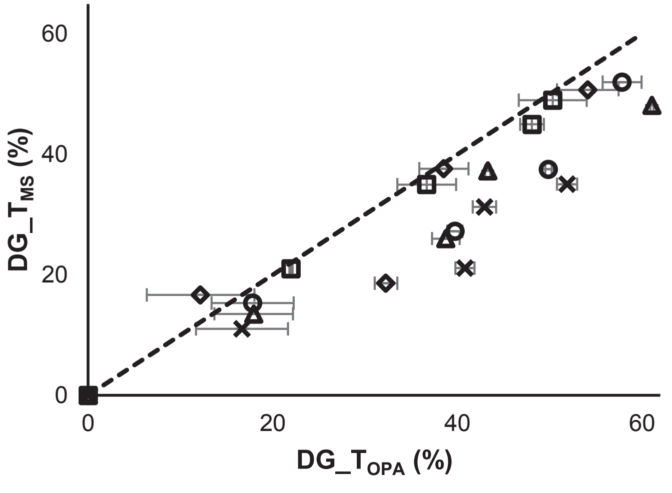

Fig. 5. Total degrees of glycation analysed with peptide analysis (DG_T_{MS}) *versus* OPA method (DG_T_{OPA}) for hydrolysates with DH_{stat} values of 0% (□), 2% (◇), 4% (○), 6% (△) or 8% (×). The dashed line (- - -) indicates the function y = x.

To identify whether there were clusters of populations of k_g and DG_$P_{av,max}$ values, the data were fitted with a normal distribution curve. For k_g and DG_$P_{av,max}$, 3 and 5 populations were identified, respectively. The data were categorised by the Matlab method k-means clustering with 3 and 5 clusters for k_g and DG_$P_{av,max}$, respectively (Table 5). The 3 k_g clusters had average values of 1.6(±0.4), 2.5(±0.2) and 5.3 (±1.1) s^{-1}, respectively. Peptides α-LA [122–123] and α-LA [12–46] had k_g values of 6.0×10^{-1} s^{-1} and 4.5×10^{-1} s^{-1}, respectively. These values were 2–3 times higher than the k_g values of the other 23 peptides (Table 4). For peptide α-LA [122–123], it could be that it is a dipeptide (KL) with leucine as the C-terminal AA and lysine as the N-terminal AA. In the literature, peptide KL has been reported to have the highest glycation rate among 11 dipeptides with different AAs as the C-terminal AA (Mennella et al., 2006). For peptide α-LA [12–46], no explanation was found for the high k_g value. For the other 23 peptides, based on the clustering results, the k_g was independent of the number of lysines (Fig. 7A) and the peptide length (Fig. 7B). The 5 DG_$P_{av,max}$ clusters had average values of 12.8(±2.6), 26.8(±3.8), 36.5(±2.1), 44.3(±2.1) and 55.3 (±2.4)%, respectively. For the DG_$P_{av,max}$ values, clearly, the clustering showed a quite good agreement (20 out of 25 peptides) with the number of lysine residues in the peptide.

3.2.3. Glycation kinetics of peptides that did not contain lysine

Similarly to the intact protein, even though the amount of added D-glucose was 2 times higher than the amount of free amino groups, the DG_$P_{av,max}$ for all peptides did not reach 100%. For peptides that did not contain lysines, i.e., peptides α-LA [8–11], α-LA [117–121], α-LA [38–46], α-LA [38–49] and α-LA [26–49], the DG_$P_{av,max}$ values were comparable: 12.8 ± 2.6% (Table 4). Because the variation in DG_$P_{av,max}$ of these peptides was small, no clear effect of the type of N-terminal AA was observed. Since these peptides only have an α-amino group as the glycation site, it means that the maximum degree of glycation for α-amino group ($DG_{max,\alpha\text{-}NH2}$) was 12.8%. It should be noted that peptide α-LA [8–11] contains an arginine (R10) within its AA sequence. The fact that the DG_$P_{av,max}$ of α-LA [8–11] was the same as the other peptides which only contained an α-amino group

Table 4

The number of lysine residues (#Lys) per peptide, peptide length, percentage of non-glycated peptide, glycation rate constant (k_g), maximum average degree of glycation ($DG\_P_{av,max}$), average number of glucose units (#Glc) per peptide, maximum degree of glycation on α- ($DG_{max,\alpha\text{-}NH2}$) and ε-amino ($DG_{max,\varepsilon\text{-}NH2}$) groups of peptides and $DG_{max,\varepsilon\text{-}NH2}$ of glycated variant of peptides, in glycated α-LA hydrolysates with a DH_{stat} of 2, 4, 6 or 8%.[a]

Peptides	#Lys	Length	k_g (10^{-1} s^{-1})						$DG\_P_{av,max}$ (%)						Avr. #Glc per peptide	% non-glycated peptide	$DG_{max,\alpha\text{-}NH2}$ (%)	$DG_{max,\varepsilon\text{-}NH2}$ (%)	$DG_{max,\varepsilon\text{-}NH2}$ of glycated variant (%
			DH2	DH4	DH6	DH8	Average	Fitted with all points	DH2	DH4	DH6	DH8	Average	Fitted with all points					
[8–11]*	0	4	2.4	2.0	2.0	1.8	2.0 ± 0.1	2.5	22.0	13.8	11.5	13.8	15.3 ± 4.6	13.0	0.13	89	13.0	–	–
[117–121]	0	5	n.d.	4.3	5.1	3.7	4.4 ± 0.7	2.7	n.d.	17.0	12.1	14.0	14.4 ± 2.5	15.9	0.16	83	15.9	–	–
[38–46]	0	9			2.6	2.8	2.7 ± 0.1	2.3			15.0	12.0	13.5 ± 2.1	14.5	0.15	87	14.5	–	–
[38–49]	0	12		1.3	1.7	1.5	1.5 ± 0.2	1.6		11.8	18.9	10.4	13.6 ± 4.5	11.5	0.12	80	11.5	–	–
[26–49]	0	24	n.d.	1.4	1.4		1.4 ± 0.0	1.1	n.d.	5.7	10.0		7.9 ± 3.0	9.1	0.09	92	9.1	–	–
[122–123]	1	2	6.1	7.3	5.1	5.8	6.1 ± 0.9	6.0	33.7	38.2	37.7	35.7	36.3 ± 2.1	36.4	0.73	36	12.8	60.2	94.1
[12–14]	1	3			2.2	3.1	2.6 ± 0.7	2.6			28.6	30.4	29.5 ± 1.3	26.9	0.54	53	12.8	46.2	98.3
[1–7]	1	7	3.5	3.6	2.6	2.8	3.1 ± 0.5	2.8	20.9	21.0	27.0	21.1	22.5 ± 3.0	23.0	0.46	64	12.8	32.2	89.4
[8–14]*	1	7		2.5	2.3		2.4 ± 0.2	2.5		31.2	27.6		29.4 ± 2.5	30.5	0.61	24	12.8	46.2	60.8
[114–121]	1	8	1.9	2.1	1.8	n.d.	2.0 ± 0.1	1.9	22.9	32.0	35.0	n.d.	30.9 ± 6.3	33.2	0.66	50	12.8	49.2	98.4
[114–123]	2	10	2.6	2.9	2.2		2.6 ± 0.4	2.3	31.6	45.0	40.0		42.5 ± 6.8	42.0	1.26	25	12.8	57.6	76.8
[12–25]	2	14	1.3	0.7	1.2	1.0	1.0 ± 0.3	0.9	34.5	42.8	35.0	32.0	36.1 ± 4.7	36.1	1.08	18	12.8	47.6	58.0
[57–78]	2	22				1.8	1.8 ± 0.0	1.8				38.6	38.6 ± 0.0	38.6	1.16	31	12.8	51.6	74.8
[12–37]	2	26		2.8	2.1	2.3	2.4 ± 0.4	2.0		39.9	46.8	42.8	43.2 ± 3.5	45.9	1.38	24	12.8	58.1	76.4
[12–46]	2	35		n.d.	4.5		4.5 ± 0.0	4.5		n.d.	35.2		35.2 ± 0.0	35.2	1.06	26	12.8	46.6	63.0
[12–49]	2	38	3.1	2.8	3.3		3.1 ± 0.2	2.6	36.5	38.2	39.9		38.2 ± 1.7	39.7	1.19	31	12.8	51.1	74.1
[8–49]*	2	42	3.0	3.5			3.3 ± 0.4	2.3	34.0	32.7			33.3 ± 0.9	36.4	1.09	37	12.8	43.6	69.2
[1–49]*	3	49	2.5	3.0			2.8 ± 0.3	2.3	40.6	42.0			41.3 ± 1.0	45.1	1.80	21	12.8	50.7	64.2
[79–113]	5	35			1.8	1.5	1.6 ± 0.2	1.5			53.8	58.6	56.2 ± 3.4	56.0	3.36	7	12.8	64.8	69.7
[50–113]	6	64	1.8	1.4	1.5	1.7	1.6 ± 0.2	1.7	48.1	64.2	64.4	58.9	58.9 ± 7.7	56.8	3.98	3	12.8	66.5	68.6
[50–121]	7	72	1.6	3.1			2.3 ± 1.1	2.6	63.0	59.5			61.3 ± 2.5	58.1	4.65	0	12.8	68.2	68.2
[50–123]	8	74	2.4	2.1			2.2 ± 0.2	2.2	45.0	55.0			50.0 ± 7.0	54.0	4.86	0	12.8	54.7	54.7
[8–113]*	8	106	1.7	1.7			1.7 ± 0.0	2.0	48.7	62.6			55.6 ± 9.9	51.9	4.67	2	12.8	60.9	62.1
[8–121]*	9	114	1.4	2.3			1.8 ± 0.6	1.9	62.7	49.5			56.1 ± 9.3	53.0	5.30	3	12.8	60.9	62.8
[1–123]*	12	123	2.9				2.9 ± 0.0	2.9	57.5				57.5 ± 0.0	57.5	7.48	0	12.8	61.3	61.3
Average																		53.9 ± 9.0	72.2 ± 13.2

[a] For peptides marked with an asterisk, arginine was not taken into account in the $\#NH_2$; n.d. indicates the glycation kinetics was not determined due to low quantity of the peptide.

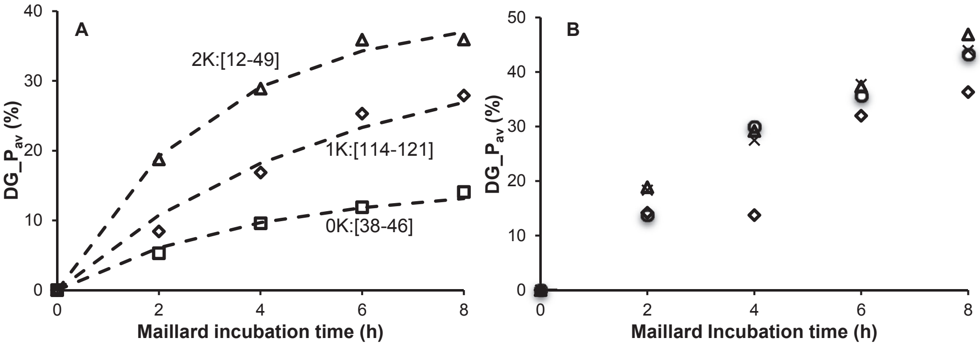

Fig. 6. (A) Glycation curves (DG_P$_{av}$ *versus* time) of peptides (□) α-LA (38–46), (◇) α-LA (114–121), and (△) α-LA (12–49) (0K, 1K, 2K: contains 0, 1 and 2 lysines, respectively) in the samples DH6_G$_{0-8}$. The dashed lines (- - -) represent the fits using a 1st order reaction equation. (B) Glycation curves of peptide α-LA (50–113) in hydrolysates with DH$_{stat}$ values of 2% (◇), 4% (○), 6% (△) or 8% (×).

Table 5
Clusters of peptides based on the glycation rate constant (k$_g$) and the maximum average degree of glycation (DG_P$_{av,max}$).

Peptides	#Lys	Length	k$_g$ (10^{-1} s^{-1})	Cluster #k$_g$	DG_P$_{av,max}$ (%)	Cluster of #DG_P$_{av,max}$
[8–11]	0	4	2.5	1	13.0	1
[117–121]	0	5	2.7	1	15.9	1
[38–46]	0	9	2.3	1	14.5	1
[38–49]	0	12	1.6	2	11.5	1
[26–49]	0	24	1.1	2	9.1	1
[122–123]	1	2	6.0	3	36.4	3
[12–14]	1	3	2.6	1	26.9	2
[1–7]	1	7	2.8	1	23.0	2
[8–14]	1	7	2.5	1	30.5	2
[114–121]	1	8	1.9	2	33.2	3
[114–123]	2	10	2.3	1	42.0	4
[12–25]	2	14	0.9	2	36.1	3
[57–78]	2	22	1.8	2	38.6	3
[12–37]	2	26	2.0	2	45.9	4
[12–46]	2	35	4.5	3	35.2	3
[12–49]	2	38	2.6	1	39.7	3
[8–49]	2	42	2.3	1	36.4	3
[1–49]	3	49	2.3	1	45.1	4
[79–113]	5	35	1.5	2	56.0	5
[50–113]	6	64	1.7	2	56.8	5
[50–121]	7	72	2.6	1	58.1	5
[50–123]	8	74	2.2	1	54.0	5
[8–113]	8	106	2.0	2	51.9	5
[8–121]	9	114	1.9	2	53.0	5
[1–123]	12	123	2.9	1	57.5	5

as the glycation site suggests that arginine did not react during the glycation process. In previous studies with free AA and intact proteins, the glycation of arginine was identified (Fenaille, Morgan, Parisod, Tabet, & Guy, 2004; Kwak & Lim, 2004). The reason that different results were obtained between the current and previous studies could be the differences in glycation conditions used.

3.2.4. *Glycation kinetics of peptides that contained lysine*

For peptides that contained lysine residues, the DG_P$_{av,max}$ ranged from 23.0% to 58.1% (Table 4). The maximum degree of glycation of ε-amino groups (DG$_{max,\varepsilon\text{-NH2}}$) in each peptide was calculated using equation (11). It was found that the DG$_{max,\varepsilon\text{-NH2}}$ averaged over peptides that contained ε-amino groups was 53.9 ± 9.0%. To check if the DG$_{max,\varepsilon\text{-NH2}}$ did not reach 100% was because of the heterogeneity of the samples, the DG$_{max,\varepsilon\text{-NH2}}$ of each peptide was also calculated after correction for the amount of remaining non-glycated peptides (Table 4). The percentage of glycated ε-amino groups of the glycated peptides was 72.2 ± 13.2%. Since this was also lower than 100%, it was concluded that all lysines in the hydrolysates were glycated to similar extents, but the glycation process either stopped before all available amino groups reacted or reached an equilibrium. This also indicated that the glycation reactivity of each lysine residue in α-LA was not significantly different from each other.

This indication was previously suggested in another article, where the bovine tryptic hydrolysis of glycated α-LA followed the theoretical scenario in which all lysines were glycated to the same extent (Deng et al., 2017). These data also showed that, on average, only 12.8% of the α-amino groups and 53.8% of the ε-amino groups were glycated. The DG$_{max,\varepsilon\text{-NH2}}$ was also obtained by fitting equation (11) to the experimental data of all peptides (Fig. 7C). The R^2 of the fit was 0.91. The DG$_{max,\varepsilon\text{-NH2}}$ was fitted to be 60.0%, which was close to the average value (53.9%) obtained from individual peptides. Because the DG$_{max,\varepsilon\text{-NH2}}$ was ~5 times higher than the DG$_{max,\alpha\text{-NH2}}$, it was concluded that the number of lysine residues of a peptide is the dominant property that determines the glycation of individual peptides in a hydrolysate. As a result, the final extents of glycation reached were similar for hydrolysates and intact proteins. This indicated that, in previous examples, when a high extent of browning intensities was shown for hydrolysates than for intact proteins (Mulcahy et al., 2016), the differences were indeed on the level of secondary reactions, but not on the glycation stage.

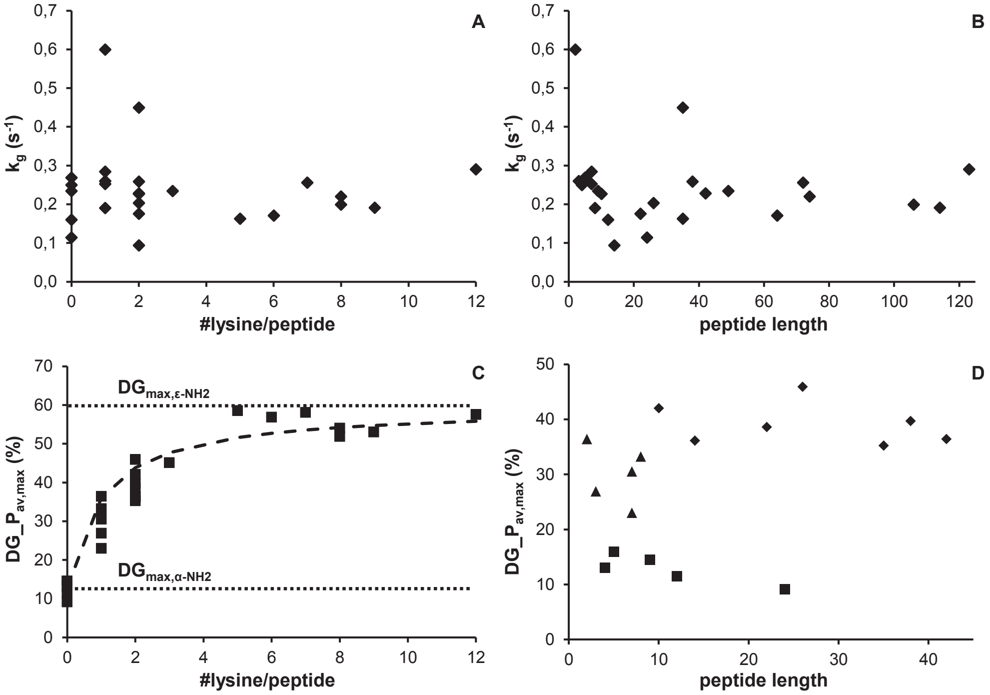

Fig. 7. (A, B) Glycation rate constant (k_g) and (C, D) DG_P$_{av,max}$ of individual peptides in α-LA hydrolysates (DH2_G$_{0-8}$-DH8_G$_{0-8}$) as a function of (A, C) the number of lysine residues per peptide (#lysine/peptide) and (B, D) the peptide length. In panel (C), the dashed line (- - -) represents the fit using equation (11). The dotted lines (• • •) represent the maximum degree of glycation on α- (DG$_{max,α\text{-}NH2}$) and ε-amino (DG$_{max,ε\text{-}NH2}$) groups. (D) DG_P$_{av,max}$ versus peptide length of peptides that contain (■) 0, (▲) 1 or (◆) 2 lysine(s).

Hydrolysates contain peptides with different numbers of lysines, meaning that the average DG_P$_{av,max}$ of these peptides would be lower than the intact proteins. However, this does not mean that the total degrees of glycation of the hydrolysates were lower than that of the intact proteins. Actually, in both hydrolysates and intact proteins, all lysines were glycated to ~60.0% and all N-termini were glycated to ~12.8%. Still, for peptides with the same number (0, 1 and 2) of lysines, there were variations in the DG_P$_{av,max}$ values. This variation was not correlated with peptide length (Fig. 7D). This means that the glycation of peptides in a hydrolysate under the experimental condition used in this study was independent of the peptide length.

4. Conclusions

In this study, using a full quantitative peptide analysis, the glycation kinetics of individual peptides in protein hydrolysates was successfully followed. The extent of glycation for all peptides did not reach 100%, meaning that the glycation process either stopped before all amino groups reacted or reached an equilibrium. The rate and extent of glycation of individual peptides in hydrolysates were independent of the hydrolysate composition. For a peptide in a hydrolysate, the number of lysine residues in the peptide is the dominant factor that determines the extent of glycation. This is because the glycation reactivity of ε-amino group (lysine) is ~5 times higher than the reactivity of α-amino group (N-terminal amino acid). The glycation rate constants were independent of the peptide length or the number of amino groups on the peptide. The outcomes of this work can be used to predict the extent of glycation of peptides in hydrolysates based on the amino acid sequences of the peptides.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2018.10.003.

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