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Effect of Saccharide Structure and Size on the Degree of Substitution and Product Dispersity of α-Lactalbumin Glycated via the Maillard Reaction

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ABSTRACT: The course of the Maillard reaction between α-lactalbumin and various mono- and oligosaccharides in the solid state was studied using UPLC−ESI-TOF-MS. Individual reaction products were monitored for their degree of substitution per protein molecule (DSP). The Maillard reaction rate depended on the saccharide type and decreased when the saccharide size increased. Conjugation with charged saccharides was hindered when a specific average DSP was reached, probably resulting from electrostatic repulsion. The DSP varied between 0 and 15, and the standard deviation of the average DSP, which is a measure for product dispersity, increased to 1.9. Similar experiments were performed with a dipeptide. Relative reaction rates in these experiments were 1 for glucose, 0.28 for maltose, and 0.16 for maltotriose. Comparison of the results obtained using α-lactalbumin and the dipeptide made clear that the Maillard reaction rate is determined by a number of factors, including saccharide reactivity and lysine accessibility.

KEYWORDS: nonenzymatic browning, maltooligosaccharides, rhamnose, arabinose, galacturonic acid, protein functionality, glycation

1. INTRODUCTION

The Maillard reaction, also referred to as nonenzymatic browning, is an important reaction in relation to food quality, because the color, flavor, and nutritional value of the food product are affected.[1] The reaction is initiated by heating a dry mixture or a solution of proteins and reducing saccharides. Amadori rearrangements cause the amino groups in the protein to link to the reducing end of the saccharide.[2] The reactivity of different types of saccharides and the properties of the resulting glycated proteins have been the subject of a number of studies. A generally accepted view states that the reaction rate under given conditions is related to the proportion of the reducing sugar present in the open chain form: the higher this proportion, the faster and more intense the browning.[1] The open-chain proportion of a saccharide in water depends on the type of saccharide and temperature, as estimated for a series of monomeric saccharides.[3] The reactivity of a number of monomers in the Maillard reaction has been tested in solution using a shrimp protein hydrolysate and resulted in the following reactivity order: fructose ≈ glucose < arabinose < xylose < ribose,[4] which is in line with data obtained in solution using β-lactoglobulin.[5] This confirms that pentoses are, in general, more reactive than hexoses, as supported by a number of studies (ref 4 and references therein). Furthermore, the influence of the chain length on oligosaccharide reactivity using ovalbumin was examined in the dry state using a range of maltooligosaccharides, where the reactivity decreased when the chain length increased.[6] For galacturonic acid-containing saccharides, oligomers also showed a lower reactivity in the Maillard reaction compared to monomers.[6]

The degree of glycation of proteins after the Maillard reaction is often estimated by using techniques such as gel electrophoresis, analysis of the number of free amino groups through colorimetric essays, and MALDI-TOF-MS.[2,7,8] These techniques can be laborious and/or may include time-consuming necessary purification steps. Furthermore, they only provide information on the average level of modification, and no information about the product dispersity is obtained. In the past few years, the application of ESI- and MALDI-based MS techniques for the analysis of glycated proteins has become feasible.[9] The detailed structure information obtained by a number of these techniques will lead to insight into the relation between incubation time and conditions on the one hand and product composition and functionality on the other hand. In this paper, the application of a fast ESI-TOF-MS-based protocol for intact glycated protein analysis is discussed by using glycated α-lactalbumin. This technique is applied to determine the influence of the type and size of the saccharide on the Maillard reaction rate. A small lysine-containing peptide is applied to determine the reactivity of both the protein/peptide and different saccharides in the Maillard reaction. The relevance of detailed product analysis for controlling and understanding the Maillard reaction is discussed.

2. MATERIALS AND METHODS

2.1. Materials. α-Lactalbumin was obtained as a commercial powder (BioPURE, Davisco Foods International Inc., Le Sueur, MN) containing 95% (w/w) protein, 90% (w/w) of which was α-lactalbumin.[10] Trigalacturonic acid (T7407), glucose (G7528), maltotriose (M8378), maltopentaose (M8128), maltoheptaose (M7753), sodium iodide, and cesium iodide were purchased from Sigma-Aldrich (St. Louis, MO). Maltose (monohydrate) was from Merck (Darmstadt, Germany), maltooctaose from Carbosynth Ltd. (Compton, U.K.), galacturonic acid

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from Fluka Biochemica (Buchs, Switzerland), and 3-(2-furyl)acryloyl-Ala-Lys-OH (FAAL, M-1350) from Bachem (Bubendorf, Switzerland). The water used was in all cases purified by using a Milli-Q Gradient A10 system (Millipore Corp., Billerica, MA). UPLC-grade eluents were purchased from Biosolve B.V. (Valkenswaard, The Netherlands).

2.2. Methods. *2.2.1. Synthesis of Peptide−Saccharide Conjugates.* For coupling of various saccharides to the peptide 3-(2-furyl)-acryloyl-Ala-Lys-OH (FAAL) via the Maillard reaction in the dry state, 1 mL of a 3 mg/mL (8.9×10^{-6} mol) FAAL solution was added to 1.78×10^{-5} mol of (solid) saccharide (molar ratio FAAL:saccharide = 1:2) in a plastic reaction tube. Prior to this, the pH of the FAAL solution had been set to 8.0 using 0.1% ammonia. The mixtures were subsequently freeze-dried. Samples were incubated in a desiccator at 60 °C for given time intervals varying from 1 to 192 h depending on the saccharide nature at a relative humidity of 65%. The latter was reached by equilibrating the desiccator with a saturated $NaNO_2$ solution, as described previously.[7,11] Per time interval, a separate sample was incubated. Samples were then stored at −20 °C.

2.2.2. Analysis of the Remaining FAAL and FAAL Conjugates. For analysis, 12 mL of water was added to FAAL samples after incubation, leading to a concentration of 0.25 mg of FAAL/mL (based on the initial quantity present). Two microliter volumes of these solutions were injected into a Waters Acquity UPLC system (Waters Corp., Milford, MA) consisting of an autosampler, pump, column oven (40 °C), and PDA detector. The system was equipped with a Waters Acquity BEH 130 C18 PST column (2.1 × 150 mm) preceded by a Waters Vanguard BEH 300 C18 guard column, both with a particle size of 1.7 μm. Compounds were eluted at a flow rate of 250 μL/min by using a gradient obtained by mixing eluent A (water containing 1% (v/v) acetonitrile and 0.03% (v/v) TFA) with eluent B (acetonitrile containing 0.03% (v/v) TFA). Gradient description (steps are linear unless stated otherwise): 0−2 min, 97−90% A; 2−4 min, 90−75% A; 4−9 min, 75−0% A; 9−11 min, isocratic 0% A; 11−13 min, 0−97% A; 13−16 min, isocratic 97% A. Eluting compounds were detected using the PDA detector, which was constantly scanning between 200 and 400 nm. The signal at 304 nm was used for quantification of nonconjugated FAAL by means of a calibration curve (0−0.3 mg/mL). In the linear part of the time vs % FAAL modified curve, the reaction rate for each saccharide was determined, which was defined as the percentage of the initial amount of FAAL reacting per hour.

For FAAL conjugate identification, eluting compounds were subsequently directed to an in-line Waters Synapt ESI-TOF mass spectrometer, equipped with a Z-spray electrospray ionization (ESI) source. The capillary voltage was set at 3.0 kV and the source temperature at 120 °C. Nitrogen was used as the desolvation gas (350 °C, 400 L/h) and cone gas (50 L/h). MS data were acquired in the positive V-mode between m/z 50− and m/z 2000 in the continuum mode at 0.5 s/scan. The TOF detector was calibrated daily using a solution prepared by mixing sodium iodide (0.4 g) and cesium iodide (23 mg) in 200 mL of water/2-propanol (50:50, v/v). PDA and MS data were acquired and processed using MassLynx software (Waters Corp.).

2.2.3. Synthesis of α-Lactalbumin−Saccharide Conjugates. Mono- and oligosaccharides were covalently linked to α-lactalbumin using the Maillard reaction in the dry state. A 10 mg mass of α-lactalbumin (8.47×10^{-6} mol of lysine residues) was mixed with 1.69×10^{-5} mol of reducing saccharide in 2 mL of water to reach a molar ratio Lys:reducing ends = 1:2. The pH of the protein and saccharide solutions was set to 8.0 using 0.1% ammonia (if necessary). Solutions were subsequently freeze-dried. Samples were incubated and stored using the conditions mentioned in section 2.2.1.

2.2.4. Analysis of α-Lactalbumin−Saccharide Conjugates. Glycated proteins were analyzed using the UPLC−Synapt ESI-TOF-MS system mentioned in section 2.2.2. To facilitate protein analysis, the UPLC system was equipped with a MassPREP Micro desalting column and precolumn tubing from a UPLC Intact Mass Analysis Application Kit (Waters Corp.). A column temperature of 80 °C was applied. A 2 μL volume of a 1 mg of protein/mL solution was injected. Conditions as mentioned in the instruction sheet belonging to this kit were used for compound elution and column regeneration (>2 min.). Because of their complexity, the gradient and flow rates used are provided in Table 1.

Table 1. Eluents, Gradient, and Flow Rates Applied for Elution of Intact Proteins and Regeneration of the Column[a]

time (min)	% A	% B	flow rate (mL/min)
0.0	95	5	0.5
0.5	95	5	0.5
0.51	95	5	0.2
2.0	10	90	0.2
2.1	95	5	0.5
2.7	10	90	0.5
2.8	95	5	0.5
3.4	10	90	0.5
3.5	95	5	0.5
4.0	95	5	0.5

[a] All gradient steps were performed linearly. A = 0.1% (v/v) formic acid in water, and B = 0.1% (v/v) formic acid in acetonitrile.

After elution, compounds were directed to the in-line Synapt ESI-TOF-MS system. In each run, eluent from the first 0.5 min was directed to waste to prevent the MS instrument from influx of disturbing compounds. The Synapt ESI-TOF MS instrument was run in the positive V-mode at a capillary voltage of 3 kV with a source temperature of 120 °C. Nitrogen was used as the desolvation gas (800 L/h, 350 °C) and cone gas (50 L/h). Data were acquired between m/z 500 and m/z 4000 in the continuum mode with a scan time of 0.5 s. In the elution time range of the (modified) protein, about 70 MS scans were combined into 1 m/z spectrum. The MaxEnt1 option within the MassLynx software (Waters Corp.) was used for subsequent deconvolution of these spectra. From the intensities of the peaks for the various products present in a spectrum, the average degree of substitution per protein molecule (DSP) and the accompanying standard deviation were calculated. The latter was used as a value for product dispersity and was indicated as the "product dispersity index". In the DSP range indicated, the DSPs representing less than 2% of the total product spectrum were not included. In the linear part of the time vs average DSP curve, the reaction rate for each saccharide was determined, which was defined as the moles of saccharide linked per mole of protein per hour.

3. RESULTS AND DISCUSSION

3.1. Maillard Reaction Rate of Saccharides toward α-Lactalbumin. To determine the course of the Maillard reaction, α-lactalbumin was incubated with saccharides of various types and sizes. After incubation for various time intervals, samples were analyzed using UPLC−ESI-TOF-MS. In Figures 1 and 2, the original and deconvoluted spectra of α-lactalbumin, modified using glucose and maltotriose, are shown.

Figure 1 shows the combined m/z spectra as acquired. Charge states of the protein ranging from 5+ to 15+ were generated during the ioznization, resulting in a range of peaks visible in spectrum A for the same compound. The clear spectra indicate that the in-line protein separation has been successful, because coeluting salts or saccharide molecules would have disturbed protein ionization. After software-assisted deconvolution of the spectrum in Figure 1A, the mass spectrum depicted in Figure 2A is obtained. A major peak appears at 14178 Da, which corresponds

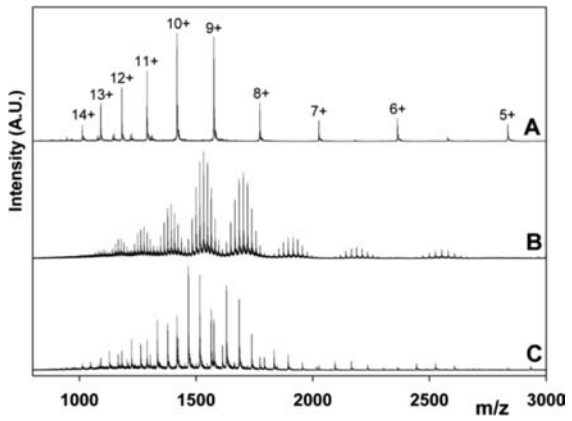

Figure 1. Combined m/z spectra (±70 scans/spectrum) after UPLC−ESI-TOF-MS analysis of α-lactalbumin incubated (A) without sugar, (B) with glucose for 4 h, and (C) with maltotriose for 4 h. Charge states are indicated in spectrum A. Intensities are scaled to the highest peak.

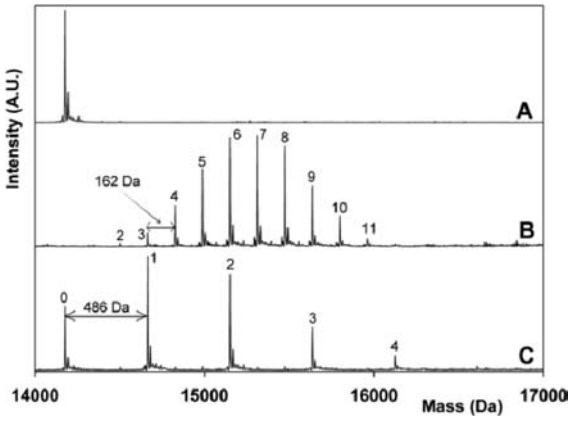

Figure 2. Deconvoluted UPLC−ESI-TOF mass spectra of α-lactalbumin incubated (A) without saccharide, (B) with glucose for 4 h, and (C) with maltotriose for 4 h. Spectra are based on the m/z spectra presented in Figure 1 and are scaled to the highest peak. The DSP is indicated.

with the expected mass of α-lactalbumin. Spectra B in Figures 1 and 2 represent α-lactalbumin after a 4 h incubation with glucose. Again, several charge states can be observed in the m/z spectrum, and multiple peaks are observed per charge state. This indicates the presence of a range of products, as visible in the deconvoluted mass spectrum. In Figure 2, this product dispersity is clearly illustrated. Covalent coupling of a glucose moiety to the protein via the Maillard reaction, accompanied by loss of a water molecule, results in a mass increase of 162 Da per anhydroglucose moiety incorporated. α-Lactalbumin molecules with 2−11 glucose molecules attached are present after 4 h of incubation, with the main products carrying 6 and 7 glucose moieties. By summing up the intensities of all of the products within one spectrum, the weighted average DSP can be calculated to be 6.8. The same average DSP could have been determined by free $-NH_2$ analysis, but this method does not provide information about product dispersity. Furthermore, analysis of free $-NH_2$ groups may be hampered by changes in the protein conformation, as reported for glycated β-lactoglobulin analysis.[12] For α-lactalbumin incubated with maltotriose (spectrum C in Figure 2)

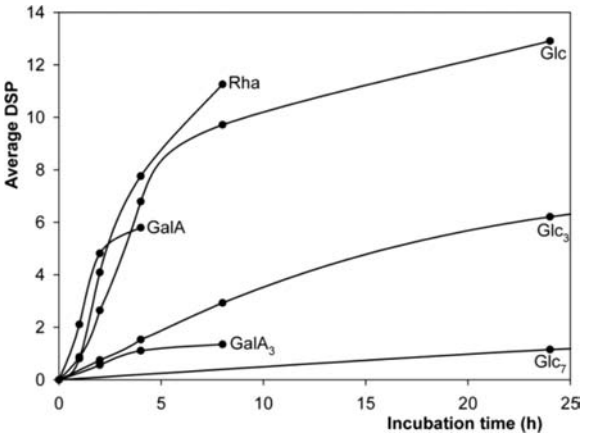

Figure 3. Overview of the average DSP resulting from the Maillard reaction between α-lactalbumin and different saccharides and incubation times. Data are based on UPLC−ESI-TOF-MS analysis. $GalA_3$ = trigalacturonic acid, Glc_3 = maltotriose, and Glc_7 = maltoheptaose.

the average DSP after 4 h of incubation was 1.5, and a range of products with different DSPs were again present. Generation of this mass spectrum (Figure 2C) shows that the software is able to deconvolute an m/z spectrum with overlapping clusters of multiply charged peaks (Figure 1C). The method presented is, altogether, a fast and accurate high-throughput tool for glycated protein analysis. Similar procedures have been developed based on LC−ESI-ion trap-MS, where detailed views on the glycation of β-lactoglobulin with glucose, lactose, and galactose were obtained.[13−15] Since both saccharide type and size are known to influence the Maillard reaction rate, DSP calculations were made for a range of saccharides and incubation times. The results are presented in Figure 3. Additionally, the DSP range, the product dispersity index, and the reaction rate were determined. All data are summarized in Table 2.

3.1.1. Reaction Rates in the Initial Phase. In Table 2, initial reaction rates are provided, which have been calculated from the linear parts of the curves. When the glucose mono- and oligomers are compared, a clear decrease of the initial reaction rate is observed with increasing oligomer size (Table 2). This trend is in line with results obtained using ovalbumin,[6] on the basis of free $-NH_2$ analysis. The reaction rate observed using α-lactalbumin was, however, much higher compared to that of ovalbumin. This is probably a result of the difference in protein type and the difference in incubation conditions used by Aoki et al. (pH 7.5, 50 °C, 65% RH) compared to our conditions (pH 8.0, 60 °C, 65% RH). Especially the temperature can be expected to have a major influence, as the open-ring proportion tends to increase when the temperature increases.[3] The relatively high saccharide concentration used by Aoki et al. (molar ratio lysine:reducing ends ≈ 1:6) did not counterbalance the effect of the lower temperature.

As shown in Table 2, the initial reaction rate observed for rhamnose is about 65% higher than observed for glucose. This result, obtained in solid-state conditions, differs from results obtained with β-lactoglobulin in solution, where similar reaction rates for glucose and rhamnose were found.[5,12] An explanation for the higher reaction rate for rhamnose compared to glucose may be the proportion of rhamnose present in the open-ring form, which is 3 times higher than for glucose as studied earlier in solution.[3] It is, however, not clear to which extent the data concerning open-ring

Table 2. Overview of the Weighted Average DSP, the Product Dispersity Index, and the DSP Range after Incubation of α-Lactalbumin with Several Saccharides for Several Time Intervals, Together with the Reaction Rate[a]

saccharide type	incubation time (h)	DSP range (2% lower limit)	weighted average DSP	product dispersity index	initial reaction rate (mol of saccharide/mol of protein/h)
glucose	1	0−3	0.9	0.9	
	2	0−6	2.7	1.4	
	4	3−10	6.8	1.8	2.0
	8	6−13	9.7	1.7	
	24	10−15	12.9	1.3	
maltotriose	2	0−3	0.8	0.8	
	4	0−4	1.5	1.1	
	8	0−6	2.9	1.4	0.4
	24	3−9	6.2	1.6	
	57	5−11	8.0	1.7	
maltoheptaose	24	0−4	1.2	1.0	
	33	0−4	1.3	1.1	
	57	0−5	1.9	1.2	0.05
	120	0−5	2.9	1.4	
	192	1−7	3.7	1.7	
rhamnose	1	0−3	0.8	0.8	
	2	1−7	4.1	1.5	3.3
	4	4−11	7.8	1.8	
	8	8−14	11.3	1.7	
arabinose	1	0−4	1.4	1.1	
	2	2−9	6.0	1.7	4.6
galacturonic acid	1	0−5	2.1	1.2	
	2	2−8	4.8	1.7	2.7
	4	2−9	5.8	1.9	
trigalacturonic acid	2	0−2	0.6	0.7	
	4	0−4	1.1	1.0	0.3
	8	0−4	1.4	1.1	

[a] The product dispersity index is defined as the weighted standard deviation of the DSP. The initial reaction rate is defined as the average number of moles of saccharide linked per mole of protein molecules per hour in the linear part of the curve in Figure 3.

proportions apply to the solid-state conditions used in this research.

Arabinose was also incubated and showed an initial reaction rate more than twice as high as that of glucose in the first 2 h of incubation. The high reaction rate can be explained on the basis of previous observations, where arabinose was found to be in the open-ring form to a >10 times higher extent than glucose,[3] thus increasing the reaction rate. This is in line with other comparative studies, some of which were performed in the liquid state.[4,5,12,16]

Galacturonic acid is about 35% more reactive than glucose, and a fast development of a brown color was observed. However, galactose and glucose showed a similar reaction rate in previous research when β-lactoglobulin was used.[12] The higher reactivity of galacturonic acid compared to glucose may be explained by an effect of the incorporation of the carboxyl group on the open-ring proportion. Trigalacturonic acid shows a reaction rate that is similar to or even slightly lower than the rate observed for maltotriose, which is contradictory to galacturonic acid being more reactive than glucose. The high charge load of trigalacturonic acid may play a role in this, because it may lead to electrostatic repulsion, as will be discussed later.

3.1.2. Reaction Rates after the Initial Phase. Depending on the type of saccharide, changes in the reaction rate can be observed in Figure 3 when a specific average DSP has been reached. For rhamnose and glucose, the flattening of the curves is probably caused by the decreasing number of free amino groups present. The reaction rates for maltotriose and maltoheptaose also decrease when the reaction proceeds, as can be deduced from the values in Table 2. This could be related to the decreasing availability of easily accessible lysine moieties, which might become an issue for more bulky oligosaccharides. A flattening of the galacturonic and trigalacturonic acid curves at an average DSP of ∼6 and ∼1.5, respectively, is observed, and this occurs at lower average DSP values than observed for, e.g., glucose and maltotriose. This "early" flattening is probably caused by electrostatic repulsion as a result of the negatively charged groups present in galacturonic acid and/or in the protein. The coupling of a (tri)galacturonic acid molecule to a given lysine moiety may disturb the diffusion of (tri)galacturonic acid to other lysine moieties in the vicinity. The 3D structure of the protein[17] shows that at least five lysine moieties are located in clusters. Other amino acids which can carry a negative charge are aspartic acid and glutamic acid. When these amino acids are considered within the 3D structure, only two lysine moieties are clearly free from possible disturbance of charged groups. The flattening of the trigalacturonic acid curve at a lower average DSP compared to that of galacturonic acid can be explained by the incorporation of three negative charges per trigalacturonic acid molecule, in contrast to one charge per

galacturonic acid molecule. The reaction rate decrease with increasing DP and the curve flattening have also been observed before to some extent, when the Maillard reaction between ovalbumin and glucuronic acid, galacturonic acid, or pectic hydrolysates was monitored using free $-NH_2$ analysis.[6,18]

3.1.3. Dispersity of the Reaction Products. In Table 2, a generic trend can be observed regarding the product dispersity index. It increases when the reaction proceeds and slowly decreases when the maximum number of substitutions has been reached (12 + 1, based on Lys residues and the terminal amino group). This index, being the standard deviation of the DSP, indicates how close the different DSP values within one sample are clustered around the average DSP. The product dispersity observed indicates that not each individual protein molecule reacts equally. The maximum dispersity is not dependent on the type of saccharide, except for trigalacturonic acid, as a result of its low average DSP. A higher amount of reducing ends present did not increase the product dispersity, but slightly increased the reaction rate, as was tested for glucose. A reaction rate increase of ∼17% was observed when the molar ratio Lys:glucose was increased from 1:2 to 1:5, and a reaction rate increase of ∼14% from 1:5 to 1:10. A further change to 1:16 did not lead to an extra increase of the reaction rate (based on 50 mg of α-lactalbumin per sample, after 2 h of incubation).

By means of the detailed product composition data provided by ESI-TOF-MS, including the proportion of each glycated (DSP) species in the product mixture, a relative reaction rate of each individual DSP species within one reaction mixture can be calculated per time interval (rate DSP 0 → 1, rate DSP 1 → 2, etc.). The relative reaction rates provide more insight into the factors determining the course of the Maillard reaction. Calculations were made for the incubation between α-lactalbumin and glucose. After a start-up phase, the relative reaction rates of protein molecules tended to decrease when the number of saccharide units attached to it decreased (rate DSP 1 → 2 > rate DSP 6 → 7 > rate DSP 10 → 11), but rates for the same reaction also decreased when the incubation time increased. This led to the conclusion that reducing end availability may have become a limiting factor during the reaction, which was performed in the solid state. This limiting factor would make it impossible to draw mechanistic conclusions. Generation of data with a wider range of saccharides and a higher excess of reducing ends is needed to provide more details.

3.1.4. Advantages of MS Techniques Generating Multiply Charged Ions. Limited protein glycation has, under dilute conditions, little or no effect on the protein ionization potential.[19] In our research, the α-lactalbumin sample incubated for 8 h with glucose, resulting in an average DSP of 9.7, was examined for a decrease in ionization potential because it is highly substituted and still shows clear peaks. The results after deconvolution of the highly charged section of the spectrum and the lowly charged part of the spectrum were compared. In the lowly charged part, the abundance of the highly substituted protein molecules was relatively higher, resulting in an average DSP for this section of 10.0. The highly charged part contained a relatively low amount of substituted protein molecules, resulting in an average DSP of 9.4 for this section. This illustrates the slight decrease in ionization potential when DSP increases. Hence, for calculations of the average DSP of the samples, as shown in Table 2, the complete spectrum was taken into account, meaning that a correct view on the sample composition was obtained.

As indicated by these observations, the generation of molecular ions with multiple charges by ESI-TOF-MS represents an advantage compared to MS techniques generating mainly monovalent molecular ions, such as MALDI-TOF-MS. A possible decrease in ionization potential due to structure modifications would in the case of monovalent ions lead to an incorrect view on the sample composition, because part of the molecules would not be ionized at all. In the case of the generation of multiply charged molecular ions, molecules that are less sensitive to ionization might indeed carry fewer charges as a result of structure modifications, but will still be taken into account in a correct way.

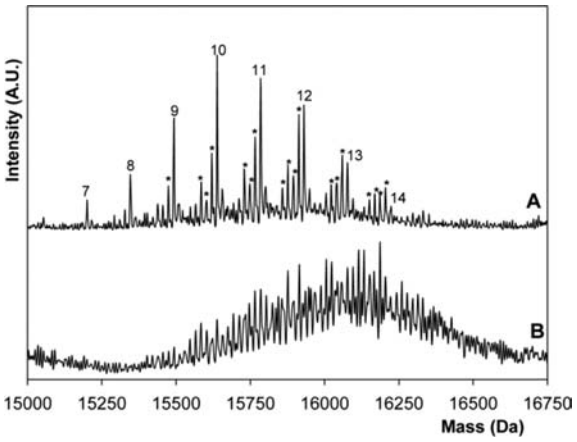

Figure 4. Deconvoluted UPLC−ESI-TOF mass spectra of α-lactalbumin after incubation with rhamnose for (A) 8 h and (B) 24 h. Dehydrated products in spectrum A are indicated by an asterisk; the number of rhamnose moieties attached is also provided. Intensities are scaled to the highest peak.

3.1.5. Analysis of Intermediate Maillard reaction Products. For a number of samples, the DSP could not be completely determined using UPLC−ESI-TOF-MS due to degradation reactions, resulting in a product mixture which was too disperse for accurate analysis. This was the case for α-lactalbumin incubated with arabinose (>2 h), rhamnose (>8 h), galacturonic acid (>4 h), and trigalacturonic acid (>8 h). Apparently, instability is saccharide structure-dependent and is not automatically related to the initial Maillard reactivity. This is illustrated by the relatively high stability of the products substituted with glucose and glucose oligomers. Mass spectra of dehydrated Amadori products are provided in Figure 4. Spectrum A represents the α-lactalbumin sample incubated for 8 h in the presence of rhamnose. Apart from the main peaks representing the protein carrying 6−14 anhydrorhamnose moieties, a number of single or multiple additional 18 Da mass losses compared to these main peaks can be observed. The number of additional 18 Da mass losses increases with increasing DSP. Degradation via 2,3-enolization is favored by a high pH, but this pathway does not include dehydration of saccharides linked to the protein molecule. Therefore, the observed dehydration of the rhamnose moieties linked to α-lactalbumin is probably a result of degradation via the 1,2-enolization pathway, although this would only be expected at low pH values.[20,21] A maximum of one water loss per rhamnose moiety can occur before hydrolysis and amino group release occurs.[21] The number of dehydrations observed per protein molecule logically increases when the number of rhamnose moieties attached increases. The average DSP of this sample can still be calculated, with or without taking the dehydration products into account (average DSPs of 11.3 and 10.5, respectively). Hydrolysis and release of amino groups, as mentioned before, makes the amino groups available

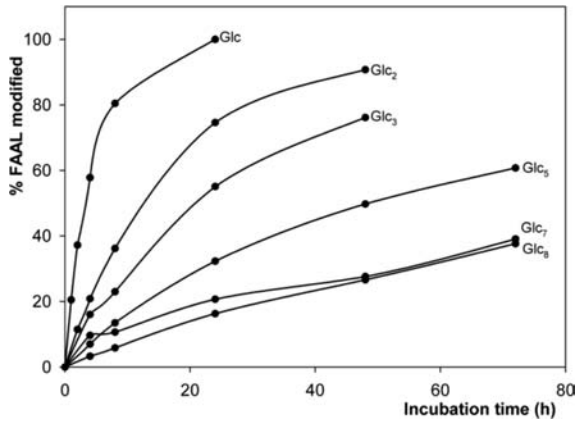

Figure 5. Course of the Maillard reaction of a series of glucose and maltooligosaccharides with FAAL, expressed as the percentage of FAAL in the sample having reacted with a saccharide.

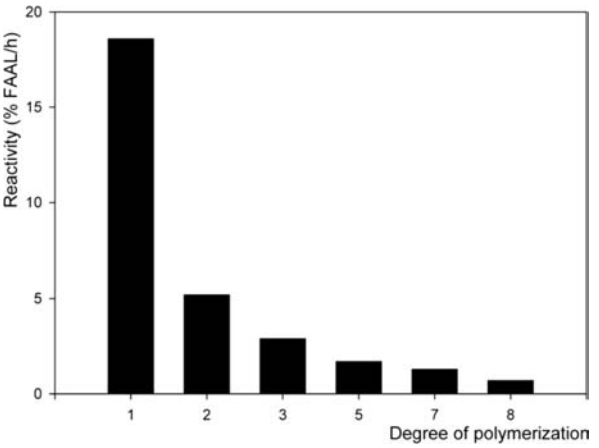

Figure 6. Maillard reaction rate of glucose and maltooligosaccharides with different degrees of polymerization with model peptide FAAL. Data are deduced from the linear part of the curves in Figure 5, and the reaction rate is expressed as % FAAL reacting per hour.

for attachment of new saccharide moieties or other reactive compounds. This explains the high product dispersity even after a relatively long incubation time. The dehydration effect has been observed before at a lower resolution using ESI-ion trap-MS after the reaction of ovalbumin with glucose, and the dehydrated products were referred to as deoxyosone derivatives.[22]

Analysis of the α-lactalbumin/rhamnose mixture after 24 h of incubation yields the mass spectrum as shown in Figure 4B. Due to degradation reactions, the sample composition has become too disperse for an adequate interpretation. In general, the extent of degradation was found to be dependent on both the incubation time and the stability of the saccharide involved. The highly reactive products formed as a result of dehydration, deamination, and fission of Amadori products may have reacted with free amino groups via their carbonyl groups,[23] yielding an even more complex set of products.

The detailed view on the early stage of product dehydration obtained by UPLC−ESI-TOF-MS, as shown in Figure 4A, could lead to a better understanding of these reactions. In addition, detailed monitoring enables a better control of the Maillard reaction in, e.g., the production of food ingredients, making this pathway less "notoriously difficult to control"[23] and thus more exploitable than generally assumed.

3.2. Maillard Reaction of Saccharides with a Model Peptide. As mentioned before, the reaction rate observed can be expected to be a function of more factors than the relative open-ring proportion, such as lysine accessibility and substrate mobility. To exclude the accessibility and mobility factors, additional experiments were performed with a lysine-containing model peptide instead of α-lactalbumin. For this, the dipeptide FAAL was selected because of its blocked terminal amino group, good accessibility of Lys due to the compact Ala moiety, and the possibility to quantify the peptide using RP-UPLC−PDA analysis (304 nm). The reactivity of a series of glucose and pure maltooligosaccharides with increasing DP toward the peptide was investigated. The series of maltooligosaccharides was chosen to study solely the effect of DP increase on the Maillard reaction rate, without the influence of other factors such as saccharide type. The course of the reaction between these saccharides and FAAL can be found in Figure 5.

As observed earlier in the incubation using α-lactalbumin, there is a clear correlation between the DP of the glucose oligosaccharides and the Maillard reaction rate with the FAAL peptide. The higher the DP, the lower the reaction rate. The trends of the curves indicate that, when incubation time suffices, 100% FAAL modification will be reached for all saccharides. Comparison of the initial reaction rates in the linear parts of the curves is facilitated by Figure 6. The effect of a DP increase on the Maillard reaction rate tends to decrease when DP increases, so the largest difference in reaction rate is found between glucose and maltose, the smallest difference between maltoheptaose and maltooctaose. This difference is a result of two factors: saccharide reactivity and substrate mobility. Saccharide reactivity is mainly a result of the relative proportion present in the open-chain form.[1] The solid-state conditions used in this research make it difficult to draw conclusions on substrate mobility, but it most probably decreases when the size of the oligomer increases. On the basis of these data, it cannot be concluded what the main factor for the decrease in reaction rate is. The fast reaction rate decrease with increasing saccharide DP and the expected high mobility of FAAL, however, suggest that the saccharide reactivity is the major factor responsible for the effects observed. More data about the relative open-chain proportion are needed to confirm this.

3.3. Comparison of Saccharide Reactivity toward FAAL and α-Lactalbumin. To be able to draw conclusions concerning the influence of the protein characteristics on the rate of its modification by the Maillard reaction, a comparison was made between the results obtained using the α-lactalbumin protein and the FAAL peptide. To do this, the theoretical substitution of all 12 lysine residues present in α-lactalbumin was set at 100%, and the average number of lysines substituted with glucose, maltotriose, and maltoheptaose at several time points (Figure 3) was expressed as a percentage of this. Overlay graphs were then made in combination with the data presented in Figure 5 to facilitate comparison between the saccharide reactivities toward α-lactalbumin and FAAL. These graphs are shown in Figure 7.

According to the data in Figure 7A, the course of the Maillard reaction using glucose as the saccharide is similar for FAAL and α-lactalbumin. This means that the α-lactalbumin structure does not have an influence on the lysine accessibility for glucose. After a 24 h incubation between glucose and α-lactalbumin, more than the theoretical number of lysine moieties has reacted, which means that also a small number of other groups has taken part in the reaction. The terminal α-amino group of the peptidic chain

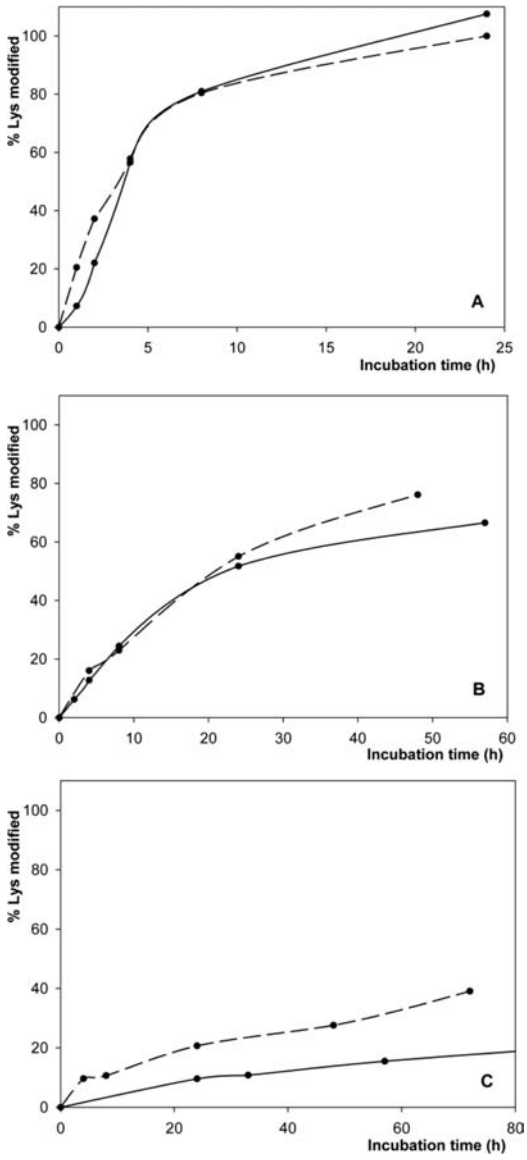

Figure 7. Comparison of the Maillard reactivity of (A) glucose, (B) maltotriose, and (C) maltoheptaose toward α-lactalbumin (solid line) and FAAL (dashed line). Reactivity is expressed as the percentage of lysine moieties in the substrate being modified.

could be one of these, and the guanidyl group of arginine and the thiol group of cysteine may also participate.[23,24] This is also clear from the DSP range as shown in Table 2, which exceeds the number of lysines present[12] in a number of cases.

When the reactivities of maltotriose and maltoheptaose in the Maillard reaction with FAAL and α-lactalbumin are compared (Figure 7 B,C), clear differences compared to glucose are observed. For maltotriose, the curves for FAAL and α-lactalbumin in the first 24 h of incubation follow a similar trend, but the reaction rate when using α-lactalbumin is lower afterward. This would mean that about 50% of the lysine residues present in α-lactalbumin are easily available for maltotriose and that, as a result of the protein structure, the reaction rate from this point onward is influenced by the reduced lysine accessibility. This was not observed for glucose, as a result of its smaller molecular size. For maltoheptaose, the reaction rate using α-lactalbumin is lower than the rate when using FAAL already from the start of the reaction. The protein structure is, apparently, of major influence on the course of the reaction. This can be explained by the molecular size of maltoheptaose, which reduces its mobility and apparently hinders its diffusion to or reaction with the lysine moieties.

It has been discussed before that, apart from lysine, also other groups present in the protein structure may also participate in the Maillard reaction. When this effect is taken into account, the curves for α-lactalbumin in Figure 7 display a slight downshift. This would even strengthen the conclusions about the influence of lysine availability and substrate mobility on the Maillard reaction rate.

In conclusion, it can be stated that a high-throughput UPLC–ESI-TOF-MS-assisted technique can be applied to reveal the dispersity of the Maillard reaction product mixture as well as the average DSP. Furthermore, it visualizes dehydration steps in the more advanced Maillard reaction stage. The product dispersity was mostly independent of the type of saccharide used. The Maillard reaction rate varies between saccharides types and is for charged saccharides influenced by electrostatic repulsion. The decreasing reaction rate with increasing oligomer size is a result of both decreased saccharide reactivity and reduced lysine accessibility in the protein.

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REFERENCES

(1) van Boekel, M. A. J. S. Kinetic aspects of the Maillard reaction: A critical review. *Nahrung* **2001**, *45*, 150–159.

(2) Shepherd, R.; Robertson, A.; Ofman, D. Dairy glycoconjugate emulsifiers: Casein-maltodextrins. *Food Hydrocolloids* **2000**, *14*, 281–286.

(3) Hayward, L. D.; Angyal, S. J. A symmetry rule for the circular dichroism of reducing sugars, and the proportion of carbonyl forms in aqueous solutions thereof. *Carbohydr. Res.* **1977**, *53*, 13–20.

(4) Laroque, D.; Inisan, C.; Berger, C.; Vouland, É.; Dufossé, L.; Guérard, F. Kinetic study on the Maillard reaction. Consideration of sugar reactivity. *Food Chem.* **2008**, *111*, 1032–1042.

(5) Chevalier, F.; Chobert, J.-M.; Mollé, D.; Haertlé, T. Maillard glycation of beta-lactoglobulin with several sugars: Comparative study of the properties of the obtained polymers and of the substituted sites. *Lait* **2001**, *81*, 651–666.

(6) Aoki, T.; Hiidome, Y.; Sugimoto, Y.; Ibrahim, H. R.; Kato, Y. Modification of ovalbumin with oligogalacturonic acids through the Maillard reaction. *Food Res. Int.* **2001**, *34*, 127–132.

(7) Broersen, K.; Voragen, A. G. J.; Hamer, R. J.; de Jongh, H. H. J. Glycoforms of β-lactoglobulin with improved thermostability and preserved structural packing. *Biotechnol. Bioeng.* **2004**, *86*, 78–87.

(8) Morris, G. A.; Sims, I. M.; Robertson, A. J.; Furneaux, R. H. Investigation into the physical and chemical properties of sodium

caseinate-maltodextrin glyco-conjugates. *Food Hydrocolloids* **2004**, *18*, 1007–1014.

(9) Oliver, C. M. Insight into the glycation of milk proteins: An ESI- and MALDI-MS perspective (review). *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 410–431.

(10) Heijnis, W. H.; Wierenga, P. A.; van Berkel, W. J. H.; Gruppen, H. Directing the oligomer size distribution of peroxidase-mediated cross-linked bovine α-lactalbumin. *J. Agric. Food Chem.* **2010**, *58*, 5692–5697.

(11) Wierenga, P. A.; van Norél, L.; Basheva, E. S. Reconsidering the importance of interfacial properties in foam stability. *Colloids Surf., A* **2009**, *344*, 72–78.

(12) Chevalier, F.; Chobert, J. M.; Dalgalarrondo, M.; Choiset, Y.; Haertlé, T. Maillard glycation of *β*-lactoglobulin induces conformation changes. *Nahrung* **2002**, *46*, 58–63.

(13) Fenaille, F.; Campos-Giménez, E.; Guy, P. A.; Schmitt, C.; Morgan, F. Monitoring of beta-lactoglobulin dry-state glycation using various analytical techniques. *Anal. Biochem.* **2003**, *320*, 144–148.

(14) Fenaille, F.; Morgan, F.; Parisod, V.; Tabet, J.-C.; Guy, P. A. Solid-state glycation of *β*-lactoglobulin monitored by electrospray ionisation mass spectrometry and gel electrophoresis techniques. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1483–1492.

(15) French, S. J.; Harper, W. J.; Kleinholz, N. M.; Jones, R. B.; Green-Church, K. B. Maillard reaction induced lactose attachment to bovine *β*-lactoglobulin: electrospray ionization and matrix-assisted laser desorption/ionization examination. *J. Agric. Food Chem.* **2002**, *50*, 820–823.

(16) Chevalier, F.; Chobert, J.-M.; Popineau, Y.; Nicolas, M. G.; Haertlé, T. Improvement of functional properties of beta-lactoglobulin glycated through the Maillard reaction is related to the nature of the sugar. *Int. Dairy J.* **2001**, *11*, 145–152.

(17) http://www.pdb.org. Access code 1f6r (accessed June 7, 2011).

(18) Aoki, T.; Hiidome, Y.; Kitahata, K.; Sugimoto, Y.; Ibrahim, H. R.; Kato, Y. Improvement of heat stability and emulsifying activity of ovalbumin by conjugation with glucuronic acid through the Maillard reaction. *Food Res. Int.* **1999**, *32*, 129–133.

(19) Yeboah, F. K.; Yaylayan, V. A. Analysis of glycated proteins by mass spectrometric techniques: Qualitative and quantitative aspects. *Nahrung* **2001**, *45*, 164–171.

(20) Hodge, J. E. Dehydrated foods, chemistry of browning reactions in model systems. *J. Agric. Food Chem.* **1953**, *1*, 928–943.

(21) Nursten, H. *The Maillard Reaction. Chemistry, Biochemistry and Implications*; The Royal Society of Chemistry: Cambridge, U.K., 2005.

(22) Yeboah, F. K.; Alli, I.; Yaylayan, V. A.; Konishi, Y.; Stefanowicz, P. Monitoring glycation of lysozyme by electrospray ionization mass spectrometry. *J. Agric. Food Chem.* **2000**, *48*, 2766–2774.

(23) Martins, S. I. F. S.; Jongen, W. M. F.; van Boekel, M. A. J. S. A review of Maillard reaction in food and implications to kinetic modelling. *Trends Food Sci. Technol.* **2001**, *11*, 364–373.

(24) Sikorski, Z. E.; Pokorny, J.; Damodaran, S. Physical and chemical interactions of components in food systems. In *Fennema's Food Chemistry*, 4th ed.; Damodaran, S., Parkin, K. L., Fennema, O. R., Eds.; Taylor & Francis Group, LLC: Boca Raton, FL, 2008; pp 849−883.