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Cross-Linking Behavior and Foaming Properties of Bovine α-Lactalbumin after Glycation with Various Saccharides

Ruud ter Haar, Yvonne Westphal,† Peter A. Wierenga, Henk A. Schols, and Harry Gruppen*

Laboratory of Food Chemistry, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands

ABSTRACT: α-Lactalbumin was glycated via the Maillard reaction in the dry state using various mono- and oligosaccharides. The reaction resulted not only in coupling of the saccharides to α-lactalbumin but also in cross-linked proteins. The glycation rate and the extent of cross-link formation were highly dependent on the saccharide used. Glycation by arabinose and xylose led to a very fast protein cross-link formation, whereas glucose showed a relatively low protein cross-linking ability. The stability of foams, created using the various glycated protein samples, depended on the type of saccharide used, the extent of glycation, and possibly the amount of cross-linked protein. Compared to nonmodified α-lactalbumin, glycation with rhamnose and fucose improved foam stability, whereas application of glucose, galacturonic acid, and their oligosaccharides did not exert a clear effect. Mass spectrometric analysis revealed that dehydration of the Amadori products is an indicator of the formation of protein cross-links.

KEYWORDS: Maillard reaction, protein glycation, conjugation, cross-link, foam, saccharides

INTRODUCTION

The Maillard reaction is a reaction between an amino group, for example, an amino acid or a protein, and a carbonyl group, mostly a reducing saccharide.[1] Via several reaction steps, Amadori products are formed, which are vulnerable to further degradation reactions. No extraneous chemicals are needed to induce the Maillard reaction.[2] The reaction occurs spontaneously during food processing and is also suitable for directed protein glycation. The Maillard reaction is known to result in cross-linked proteins after degradation of the Amadori compounds. On the basis of the first coherent mechanism proposed,[3] Amadori compound degradation mainly occurs via 1,2-, or 2,3-enolization, depending on the pH. Via various hydrolysis, fission, and dehydration steps, highly reactive species, such as ketoaldehydes, dicarbonyls, and reductones, are formed.[4] These eventually react with one or more amino groups to form melanoidins. The latter have been defined as "brown nitrogenous polymers and co-polymers".[5] The mechanism of protein cross-linking via the Maillard reaction in vivo has been the subject of a large number of literature studies (see ref 6 and references cited therein). Recent research has revealed that Amadori compounds may degrade to dideoxyosones, leading to the major in vivo lysine−arginine cross-links glucosepane and pentosidine. These pathways involve several dehydration steps, but do not include deamination.[7,8] It is, however, not clear to which extent these findings are applicable to food systems. The complexity of food products and the wide range of conditions applied in food processing make the elucidation of the chemical pathways and the resulting cross-links a challenge.

From a protein functionality point of view, a number of studies on the modification of food protein properties by using the Maillard reaction have been published, as reviewed by Oliver et al.[9] The emulsification properties and the solubility at low pH of caseinate improved after conjugation with maltodextrins.[10] Another study showed that the change in the functional properties of β-lactoglobulin is related to the nature of the saccharide used for modification.[11] Conjugation of caseins with dextrans improved the properties of the emulsions created using this protein under acidic conditions.[12] Cross-linking of food proteins was also found to have a profound effect on their functional properties.[6,13]

In recently published research, a method for the quick and accurate determination of the exact number of saccharide units attached per protein molecule, involving UPLC-ESI-TOF MS, was developed.[14] By this method, also the dispersity of Maillard reaction products could be visualized. The current paper provides an initial study of the relationship between the extent of glycation of the protein, protein cross-linking, and glycated protein foam stability after the Maillard reaction between α-lactalbumin and various saccharides. Although additional research will be needed to elucidate precise mechanistic details, the results illustrate the importance of saccharide selection and control of the glycation process for successful protein modification.

MATERIALS AND METHODS

Materials. Bovine α-lactalbumin was obtained as a commercial powder (BioPURE, Davisco Foods International Inc., Le Sueur, MN), containing 95% (w/w) protein of which 90% (w/w) was α-lactalbumin, according to the manufacturer's specifications. Arabinose (Ara, A6085), glucose (Glc, G7528), rhamnose monohydrate (Rha, R3875), maltotriose (M8378), maltoheptaose (M7753), and trigalacturonic acid (T7407) were purchased from Sigma-Aldrich (St. Louis, MO). Galacturonic acid (GalA) was from Fluka Biochemica (Buchs, Switzerland), xylose (Xyl) from Merck (Darmstadt, Germany), fucose (Fuc) from Alfa Aesar (Ward Hill, MA), and D-methionine from Acros Organics

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(Geel, Belgium). The water used was purified by using a Milli-Q Gradient A10 system (Millipore Corp., Billerica, MA).

Methods. *Synthesis and Purification of Glycated α-Lactalbumin.* To glycate α-lactalbumin, 50 mg of α-lactalbumin (~4.2×10^{-5} mol of lysine residues) was mixed with 8.5×10^{-5} mol of saccharide in 10 mL of water (molar ratio lysine/reducing end groups 1:2). The pH of this solution was set to 8.0 using 0.1% ammonia. Samples were subsequently freeze-dried. Incubation was performed for different time intervals at 60 °C in a desiccator. The relative humidity (RH) was kept at 65% by equilibrating the atmosphere in the desiccator with a saturated $NaNO_2$ solution. To remove nonreacted saccharides, samples were subsequently dissolved in 5 mL of water and dialyzed for 24 h against demineralized water, using dialysis tubes with a molecular mass membrane of 12−14 kDa, followed by freeze-drying. Per time interval, two samples were incubated to obtain sufficient amounts of material. These samples were pooled prior to dialysis.

Determination of the Average Degree of Substitution of α-Lactalbumin. Determination of the average degree of substitution per protein molecule (DSP) and the product dispersity index of the glycated proteins was performed by applying ultraperformance liquid chromatography coupled to electrospray ionization time of flight mass spectrometry (UPLC-ESI-TOF-MS) for sample analysis, as described previously.[14] When part of the product was insoluble, analysis was performed on the soluble part. To indicate the dispersity of the reaction mixture, the standard deviation of the average DSP was calculated. This value will be referred to as the dispersity index.

Molecular Mass Distribution. (Glycated) protein samples were analyzed by high-performance size exclusion chromatography using a Superdex 75 10/300 GL column (GE Healthcare, Uppsala, Sweden). Procedure and equipment have been described previously.[15] In our case, 10 mM sodium phosphate buffer, pH 7.0 containing 150 mM NaCl and 6 M urea was used as eluent. Urea was added to exclude noncovalent protein aggregation. When part of the product was insoluble, analysis was performed on the soluble part. Eluting compounds were monitored using UV absorbance at 280 nm. The proportion of cross-linked protein was determined by comparing the area of the peak of the monomeric protein with the area of the peaks representing polymeric protein (complexes of >1 protein molecules). UV responses for mono- and polymeric material were assumed to be similar. Bovine serum albumin (67000 Da), β-lactoglobulin (36300 Da, dimer in solution), carbonic anhydrase (29400 Da), and ribonuclease A (13700 Da) were analyzed used for column calibration.

Protein Content Determination. After dialysis and freeze-drying of the glycated α-lactalbumin samples, the nitrogen content of samples used for functionality tests was determined using a Thermo Flash EA 1112 Element Analyzer (Thermo Fisher Scientific Inc., Waltham, MA), according to the manufacturer's instructions. A calibration curve was made using D-methionine. For each measurement, about 2−4 mg of sample was used. Analyses were performed in duplicate. For conversion of nitrogen content to protein content, a factor of 6.26 was used, as calculated for α-lactalbumin on the basis of its amino acid sequence.[16]

Determination of Sample Solubility. To test the solubility of the samples, about 10 mg of sample was added to a reaction tube. Subsequently, 1 mL of 10 mM sodium phosphate buffer (pH 7.0) was added, and the tubes were left shaking for 1 h at room temperature. Samples were then centrifuged (17500*g*), and the supernatant was decanted. Then, 1 mL of water was added to each of the samples, followed by mixing, centrifugation (17500*g*), and decanting of the supernatant. The latter was done to remove salts from the tubes. Samples were then dried at 90 °C using a stream of air. The amount of nonsolubilized material was determined gravimetrically and was expressed as a percentage of the original amount of sample. Analysis was done in duplicate.

Protein Foam Stability Testing. Foam stability was investigated according to a procedure applied previously,[17] using a foam beaker with a radius of 3.1 cm. Soluble samples were dissolved in 10 mM sodium phosphate buffer, pH 7.0, to 0.4 mg protein/mL (based on the analysis as described under Protein Content Determination). Thirty milliliters of this solution was added to the foam beaker. Nitrogen gas was sparged through the solution at a flow rate of about 0.85 L/min, until the resulting foam reached a height of 8 cm. The foam height was subsequently recorded as a function of time. The time point at which the foam height had decreased to 5 cm was defined as the foam half-life time. This number was used for foam stability comparisons. Samples were analyzed at least in duplicate, using a fresh protein solution for each measurement.

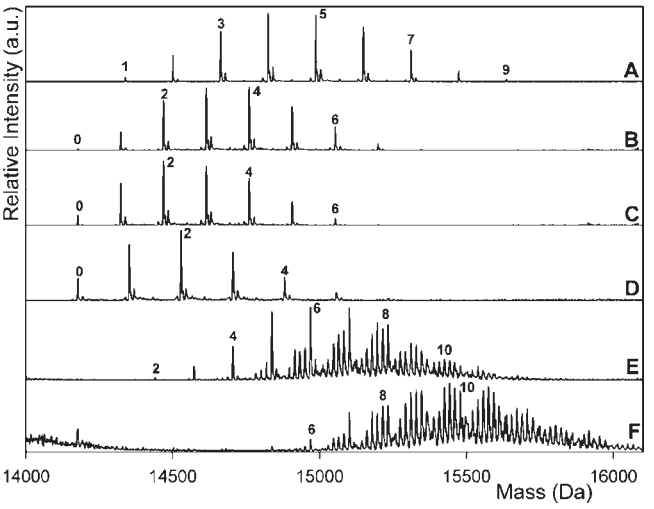

Figure 1. Deconvoluted UPLC-ESI-TOF mass spectra of α-lactalbumin after Maillard-mediated glycation (60 °C, 65% RH) with (A) glucose (2 h), (B) rhamnose (1.5 h), (C) fucose (1.5 h), (D) galacturonic acid (1 h), (E) arabinose (1.5 h), and (F) xylose (1.5 h). Degrees of substitution per protein molecule are indicated.

RESULTS AND DISCUSSION

Modification Extent after α-Lactalbumin Glycation. Via the Maillard reaction, α-lactalbumin was glycated using a selection of neutral and charged mono- and oligosaccharides. Samples with a different extents of glycation, defined as average degree of substitution per protein molecule (DSP), were created to study the relationship between average DSP, protein cross-linking, and protein foam stability. Experiments were based on the results from previous research,[14] in which the average DSP was determined as a function of incubation time using mass spectrometry. When all lysine residues are modified, a DSP of 12 would be reached. In the present research, it was attempted to reach a DSP of around 3−4 for the different mono- and oligosaccharides applied by adjusting the incubation time. Samples subjected to a longer incubation were included to create highly modified and, possibly, cross-linked proteins. After incubation, the average DSP of the samples was determined using UPLC-ESI-TOF-MS. In Figure 1, mass spectra obtained after deconvolution of m/z spectra of α-lactalbumin modified using various monosaccharides are given. In Table 1, incubation times, the calculated average DSP, the product dispersity index (standard deviation),[14] the protein content, and the extent of cross-linking (vide infra) of the various modified α-lactalbumin samples are provided.

The DSPs mentioned above were not always achieved as a result of the high initial reaction rates of the monosaccharides

Table 1. Average Degree of Substitution per Protein Molecule (DSP), Product Dispersity Index (Standard Deviation), Percent Cross-Linked Protein, Percent Insoluble Sample, and Protein Content after Dialysis of Proteins Glycated via the Maillard Reaction (60 °C, 65% RH)

type of saccharide	incubation time (h)	av DSP[a]	dispersity index	% cross-linked protein	% insoluble	protein content (%)
none (α-lactalbumin only)	2			0	0	92.6
	24			0	0	96.1
glucose	2	4.6	1.7	0	0	87.7
	24	12.7	1.3	34	0	84.7
rhamnose	1.5	3.6	1.5	0	0	86.5
	24	~13	NA[b]	62	10	84.6[c]
fucose	1.5	2.7	1.4	0	0	89.4
	24	~ 13	NA	64	NA	83.3[c]
arabinose	1.5	7.4	1.6	~40	NA	87.9[c]
	24	NA	NA	>90	NA	75.7[c]
xylose	1.5	~10.5	NA	~60	NA	87.0
	24	NA	NA	>90	NA	86.7
galacturonic acid	1	2.1	1.6	0	0	92.6
	24[d]	NA	NA	68		
trigalacturonic acid	15	1.4	1.0	0	0	67.4
	55[d]	NA	NA	57		
maltotriose	8	2.4	1.3	0	0	86.6
	57[d]	8.0	1.7	18		
maltoheptaose	96	2.1	1.3	0	NA[e]	75.3
	192[d]	3.7	1.7	0		

[a] Degree of substitution per protein molecule. For partly insoluble products, analysis was performed on the soluble part. [b] NA: could not be analyzed due to sample complexity, inhomogeneity or (nearly) complete insolubility. [c] Only indicative due to product inhomogeneity. [d] These samples were included only to study the extent of cross-linking after long incubation times. [e] Amount of material not sufficient for proper analysis, by eye: 0% insoluble.

and the corresponding challenge to control the synthesis of proteins with a low average DSP. The average DSPs after the short incubations are, however, in the same order of magnitude for glucose, rhamnose, and fucose. The Amadori products of these saccharides can be clearly observed in Figure 1. For example, for glucose (Figure 1A), a series of 162 Da mass increase steps can be observed compared to the original non-modified α-lactalbumin signal (14177 Da). The 162 Da mass increase can be explained by the addition of glucose (mass 180 Da) to an amino group of α-lactalbumin, which is followed by loss of a water molecule (18 Da) under formation of a Schiff base. This compound will then rearrange to the Amadori product.[1] The same applies for the other saccharides, for which a similar series of (saccharide M_w − 18 Da) mass increases is observed.

In the monosaccharide-containing samples subjected to a 1.5−2 h incubation, the DSP is in some cases higher than expected on the basis of previous data.[14] The order of reactivity of the different saccharides, nevertheless, is the same. The lower reaction rates observed in previous research could be a result of the frequent sample-taking involved in those experiments and the consequent destabilization of the relative humidity of the reaction mixture.[14]

For the most reactive saccharides, arabinose and xylose, the DSP is hard to control. The dehydration of the Amadori products proceeds quickly (Figure 1E,F), as discussed before for arabinose.[14] The dehydration steps are visible as a series of 18 Da mass decrease steps compared to the peaks representing the Amadori products. Due to the high extent of dehydration and possible other degradation products present in the xylose-containing sample after 1.5 h of incubation, the average DSP was estimated from the mass spectrum and a dispersity index could not be calculated. The same applies for samples incubated with rhamnose and fucose for 24 h (data not shown). DSP analysis of the arabinose- and xylose-containing samples after 24 h of incubation could not be performed due to sample insolubility and complexity.

Glycation with oligosaccharides was also successful (Table 1). As expected, high levels of modification were not reached due to the relatively low reactivity, electrostatic repulsion for the charged oligomers, and steric factors, as reported previously.[14]

The protein content, in general, inversely correlates with the average DSP and the size of the saccharides attached. Some of the values deviate from the expected levels, probably caused by the hampered removal of nonreacted saccharides, especially trigalacturonic acid.

Molecular Size Distribution after Protein Glycation. The development of brown color in a number of samples during synthesis indicated the progress of the Maillard reaction into more advanced stages. Furthermore, insolubility of some of the products was observed. For these reasons, the development of protein cross-linking or complex formation was suspected. To examine differences between saccharides in cross-link-inducing tendency, samples were analyzed using size exclusion chromatography under denaturing conditions. α-Lactalbumin incubated in the absence of any saccharide was also included in the analysis. In Figure 2, a selection of the resulting elution profiles is given. Table 1 provides information on the extent of cross-link formation for all samples analyzed, based on comparison of the areas of the monomeric and polymeric (>1 protein molecule) protein peaks.

In the α-lactalbumin blank, incubated in the absence of saccharide, the major compound eluting around 11.2 mL is α-lactalbumin. No conjugates are observed in this sample, also not after 2 and 24 h of incubation (Table 1). The elution volume for α-lactalbumin is lower than expected on the basis of the column calibration. The α-lactalbumin elution volume of 11.2 mL would correspond to a molecular mass of ~22.5 kDa. The reason for this deviation from the actual value (14.2 kDa) is unknown, but similar results were obtained in previous research using this column.[18] Different degrees of calibration protein unfolding in urea could be a possible cause. Nevertheless, conclusions can be drawn based on these data, because all samples were analyzed within the same run.

When saccharides are present during incubation, the elution pattern observed is different compared to the pattern of the α-lactalbumin blank. As can be seen in Figure 2A, polymeric protein molecules elute between 7.5 mL (void) and 10−10.5 mL. The monomeric protein molecules that are glycated show a slightly lower elution volume compared to the α-lactalbumin blank, as a result of their mass increase due to conjugation with the saccharides (Figure 2A). This effect seems to be relatively strong for the charged saccharides in Figure 2B. Although the charged groups were aimed to be shielded by 150 mM NaCl present in the eluent, they still seem to have an increased effect on the apparent molecular weight compared to neutral saccharides.

When the behavior of the monosaccharides is compared more precisely, on the basis of the elution profiles in Figure 2 and the data in Table 1, some differences can be observed. Arabinose and xylose show a very fast conjugation with α-lactalbumin, and cross-linked protein is observed already in an early stage of the reaction. After 24 h of incubation, the cross-linking of proteins induced by these monosaccharides led to mostly insoluble products. The other monosaccharides generate cross-linked proteins after longer incubation times. Glucose clearly has the lowest tendency to generate cross-linked proteins. Oligosaccharides generate less cross-linked protein formation compared to their corresponding monosaccharides (Figure 2B). The oligosaccharide with the highest DP used in this study, maltoheptaose, generates no cross-linked protein at all within the incubation times applied (Table 1).

When the appearance of cross-linked protein is compared to the obtained mass spectra, a parallel can be noted. The appearance of cross-linked protein is related to the appearance of dehydrated Amadori products. This is visualized in Figures 1 and 3 by the presence of peaks for dehydrated Amadori products, depending on the saccharide type and incubation time applied.

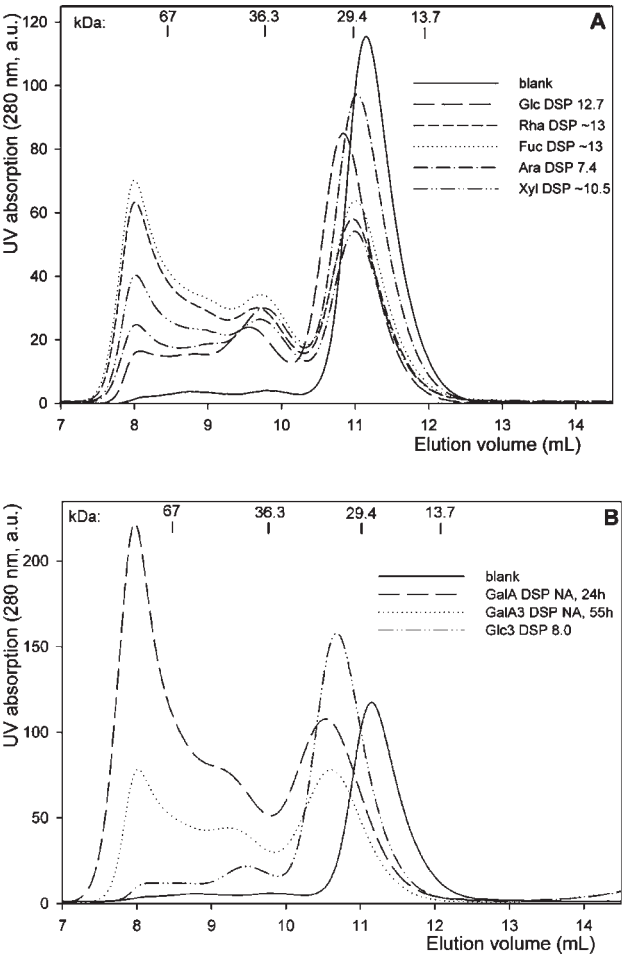

Figure 2. Size exclusion chromatography elution profiles under denaturing conditions (6 M urea, 150 mM NaCl in 10 mM sodium phospate, pH 7.0) of α-lactalbumin samples glycated using neutral monosaccharides (A) and others (B). The average degree of substitution per protein molecule of the samples is indicated. Elution volumes of proteins used for column calibration are included. DSP, average degree of substitution per protein molecule' DSP NA, sample too complex to determine extent of glycation' GalA3, trigalacturonic acid; Glc3, maltotriose.

The only two saccharides inducing cross-linking in an early reaction stage were arabinose and xylose (Table 1). As can be seen in Figure 1E,F, only α-lactalbumin molecules carrying Amadori products of these two saccharides show dehydration in this stage of the reaction. This already indicates that dehydration and protein cross-linking may be connected. Furthermore, Figure 3 shows that the Amadori product of glucose is clearly less susceptible to dehydration than the Amadori product of rhamnose and fucose. The latter two yield a very complex product mixture, whereas α-lactalbumin conjugated with glucose clearly shows the main Amadori product peaks accompanied by a number of dehydrated conjugates. As mentioned, glucose also showed the lowest cross-linked protein inducing tendency. The formation of cross-links is, apparently, strongly related to the instability of the Amadori compounds involved. The relatively low reactivity of glucose in the Maillard reaction due to its stable ring structure has been observed before.[19]

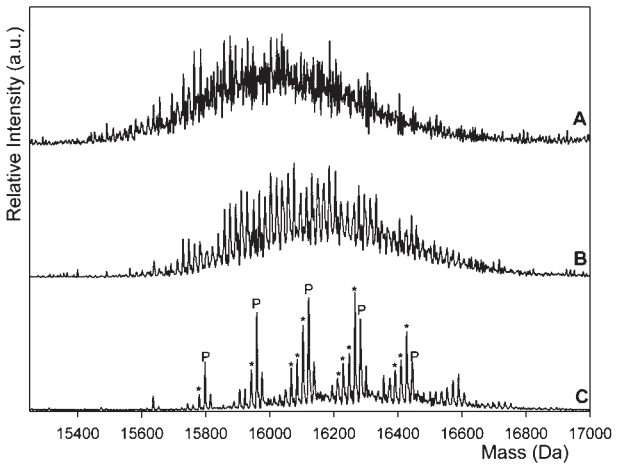

Figure 3. ESI-TOF MS spectra of α-lactalbumin incubated for 24 h (60 °C, 65% RH) with (A) fucose, (B) rhamnose, and (C) glucose. Amadori products (P) and a number of dehydrated Amadori products (∗) are indicated.

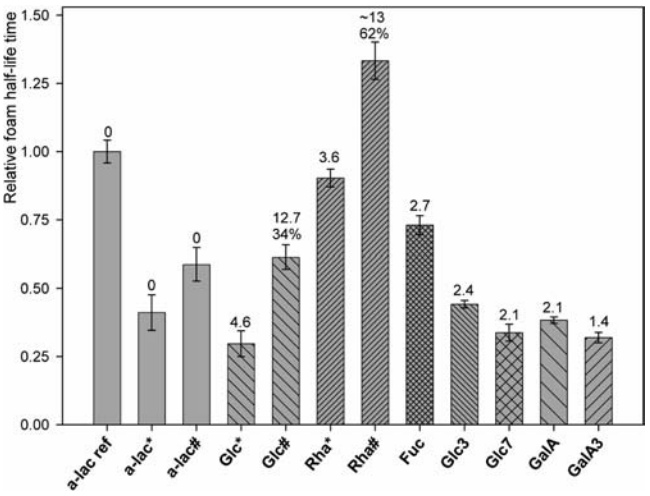

Figure 4. Relative half-life times of foams created with various (modified) α-lactalbumin samples. Data are presented relative to the half-life time of foam created using untreated α-lactalbumin. The average degree of substitution per protein molecule of the glycated α-lactalbumin samples is provided, together with the percentage of cross-linked protein in the sample (if applicable). ∗, 1.5−2 h of incubation; #, 24 h of incubation (60 °C, 65% RH, Table 1); Glc3, maltotriose; Glc7, maltoheptaose; GalA3, trigalacturonic acid.

The fact that cross-linked protein is observed only during or after dehydration steps of the Amadori products can be explained by the fact that reactive compounds have to be formed from the Amadori products to induce this cross-linking. A number of proposed mechanisms, including cross-linking after 1,2-enolization[1,3] and via dideoxysones to glucosepane and pentosidine,[7,8] involve dehydration of the original Amadori compound. A high Maillard reactivity of a saccharide, resulting in a fast average DSP increase, strengthens this effect, because it leads to a fast formation of these Amadori compounds, which can subsequently dehydrate. The statement that the often doubted importance of Amadori compounds in protein cross-linking should be reconsidered[8] is underscored by these observations.

The similar behavior of a number of the saccharides used in this research in terms of glycation rate and cross-link formation seems to be related to their molecular structure. Arabinose and xylose are both aldopentoses, which could, for example, lead to cross-link formation via pentosidine.[8] Rhamnose and fucose are both deoxyhexoses, and this is also results in similar behavior. High Maillard reactivity of a saccharide and fast formation of cross-links are, however, not automatically related to each other. Arabinose and xylose show the highest glycation rate and, indeed, show the highest extent of cross-linking. Fucose, rhamnose, and glucose, however, are also similar to each other in terms of glycation rate, but glucose is clearly less inclined to cross-link formation.

Cross-linked protein formation was also induced by trigalacturonic acid and to a lower extent by maltotriose (Figure 2B; Table 1) after conjugation to α-lactalbumin. This corresponds with the higher cross-link-inducing capacity of galacturonic acid compared to glucose. An increase in the DP of the saccharides results in a decrease in the cross-linking behavior, which is underscored by the behavior of maltoheptaose, inducing no cross-links. This effect of the DP is a result of two factors. On the one hand, α-lactalbumin samples glycated using neutral oligosaccharides have a lower average DSP than the samples conjugated with monomers. A lower DSP automatically results in a lower probability of the formation of reactive species, which decreases the probability of cross-link formation. On the other hand, the cross-linking induced by conjugated oligosaccharides may take place by a different mechanism than the cross-linking induced by conjugated monosaccharides. This is indicated by the MS spectrum of maltotriose with an average DSP of 8.0, for which no dehydration of the conjugated saccharides is visible (data not shown), whereas cross-linking has been observed for this sample (Figure 2B; Table 1). A stabilizing effect of, for example, methyl groups or saccharide moieties coupled to the Amadori product of glucose has been observed before, when the development of the Maillard reaction between ovalbumin and glucose, methylglucose, and lactose was compared.[20] It has also been concluded that mainly 1,4-dideoxyhexosulose is formed from oligo- or polysaccharides during the Maillard reaction, but the assignment of all compounds and mechanisms involved remains difficult.[21] It was, however, not the goal of this initial study to examine the detailed mechanism of formation of cross-linked proteins in these specific systems.

Foam Stability of Glycated and Cross-Linked Proteins. With a number of samples mentioned in Table 1, foams were created. These foams were evaluated for their stability by measuring their half-life time. Samples that were (partly) insoluble due to complex formation were not tested. Whereas changed foam properties may not automatically be reflected in the interfacial properties,[17] they can at least indicate a change in functional properties. Nonmodified α-lactalbumin was used as a reference, which means that the half-life time of its foam was set at 1. Half-life times of other foams are expressed relative to this. Results can be found in Figure 4.

The stability of foams created with α-lactalbumin is greatly reduced after protein incubation for 2 h in the absence of any saccharide. Additional experiments, however, indicate that mostly the preceding freeze-drying and dialysis steps are responsible for this effect, and not the incubation at 60 °C (data not shown). After 24 h of incubation, a slight increase of the foam stability is observed compared to the sample incubated for 2 h. This may be the result of an additional change in the conformation of the protein as a

result of the heating step. When the stability of the foams created with various glycated protein samples is evaluated, it appears that this stability depends on the extent of glycation of the protein as well as the type of saccharide used for protein glycation.

Attachment of glucose to α-lactalbumin does not have a major influence on the stability of its foams. A slight increase in stability is observed when the DSP increases to 12.7, but this corresponds to an incubation time of 24 h, and about 34% of the protein is cross-linked. Compared to the sample incubated for 24 h without any saccharide present, there is no difference in foam stability. This is a remarkable finding, as conjugation with glucose has been reported to improve the foam stability of other proteins such as β-lactoglobulin[11,22] and lysozyme.[17] The cross-linking observed for the 24 h incubated sample containing glucose may be an explanation for its low foam stability, as enzymatically cross-linked α-lactalbumin was found to have antifoam properties.[23] This may mask a positive effect of the glucose units attached. Also, conjugation with charged saccharides and maltooligosaccharides does not induce a very clear effect on the foam stability. The degree of substitution may be too low to have an influence. The low stability of the Amadori products and the presence of charges make it hard to create monomeric proteins with a high degree of substitution using (tri)galacturonic acid.[14] Furthermore, long incubation times are needed to reach a high extent of glycation with maltooligosaccharides. These glycation difficulties may make the application of these saccharides in protein property modification through the Maillard reaction difficult. A different synthesis route for saccharide−protein coupling, as proposed previously,[24] may be more applicable in this case, but was not included in the present study.

A clear positive influence on the foam stability is observed after conjugation of α-lactalbumin with rhamnose and, to some extent, also with fucose. For α-lactalbumin glycated using rhamnose, an increase of the average DSP resulted in higher foam stability. The stability of the foam created with α-lactalbumin with an average DSP of ∼13 exceeds the foam stability of the original α-lactalbumin sample. The latter might even be an underestimation due to the high amount of cross-linked protein in this sample, which was in previous research, involving enzymatic cross-linking, found to reduce the foam stability of α-lactalbumin.[23] These findings are in contrast to results obtained using glycated β-lactoglobulin, with which conjugation with rhamnose did not exert a clear effect on the foam stability.[11] The incorporation of fucose in the α-lactalbumin structure also increased its foam stability already at a low DSP (Figure 4). The similarity in the structures of rhamnose and fucose, and the expected similarity in their glycation mechanisms and sites, is reflected in their effect on the foam stability of α-lactalbumin glycated using these saccharides.

In conclusion, it can be stated that the amount of cross-linked protein observed after glycation of α-lactalbumin by the Maillard reaction is, apart from reaction time and conditions, highly dependent on the type of saccharide applied. The dependency on the saccharide type also accounts for the effect on the foam stability of the glycated protein. Dehydrated Amadori products in the more advanced stages of the Maillard reaction, as monitored by UPLC-ESI-TOF-MS, were a clear indicator for the formation of cross-linked protein.

AUTHOR INFORMATION

Corresponding Author

*Phone: +31 317 483211. Fax: +31 317 484893. E-mail: Harry.Gruppen@wur.nl.

Present Addresses

†Unilever R&D, Vlaardingen, The Netherlands.

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