Original article

Maillard glycation of β-lactoglobulin with several sugars: comparative study of the properties of the obtained polymers and of the substituted sites

François CHEVALIER[a], Jean-Marc CHOBERT[a]*,
Daniel MOLLÉ[b], Thomas HAERTLÉ[a]

[a] Laboratoire d'Étude des Interactions des Molécules Alimentaires,
INRA, BP 71627, 44316 Nantes Cedex 3, France
[b] Laboratoire de Recherches de Technologie Laitière,
INRA, 65 rue de Saint Brieuc, 35042 Rennes Cedex, France

Abstract — Maillard reactions represent a major cause of structural and chemical modifications of proteins during industrial food processing and storage. Nutritional and functional properties of food proteins are highly dependent on this reaction. A better knowledge of this modification and of its structural consequences presents a great interest for the food industry. In this study, β-lactoglobulin was heated in solution at 60 °C in the presence of arabinose, galactose, glucose, lactose, rhamnose and ribose. Protein polymerization and glycation site specificity were investigated according to the nature of sugar used for modification of β-lactoglobulin. Among the six common sugars used, arabinose and ribose induced the highest degree of modification. Glucose, galactose and rhamnose were less reactive and lactose generated the lowest degree of modification. Proteins substituted with ribose or arabinose formed polymers stabilized by sugar induced covalent bonds. When other sugars were used, part of the aggregated proteins were stabilized only by hydrophobic interaction and disulfide bonds. The site specificity for the attachment of each sugar could not be clearly demonstrated. According to mass spectrometry analysis, leucine 1 (N-teminal amino acid), lysine 14 and lysine 47 were modified in the presence of galactose, glucose or lactose. Lysines 69, 75 and 135 were modified only in the case of protein glycated with glucose. Lysine 100 was modified only when protein was glycated with lactose. No glycation site could be detected for proteins glycated with ribose or arabinose due to the higher degree of modification and polymerization which inhibited the tryptic hydrolysis used before mass spectrometry analysis.

β-lactoglobulin / Maillard reaction / glycation / polymer / trypsinolysis

---

* Correspondence and reprints
Tel.: (33) 2 40 67 50 85; fax: (33) 2 40 67 50 84; e-mail: chobert@nantes.inra.fr

Résumé — Glycation de la β-lactoglobuline par réaction de Maillard à l'aide de différents sucres : étude comparative des propriétés des polymères obtenus et des sites de substitution. Les réactions de Maillard sont une cause majeure de modifications structurale et chimique des protéines au cours des traitements thermiques alimentaires et du stockage. Les propriétés nutritionnelles et fonctionnelles des protéines alimentaires dépendant beaucoup de cette réaction, une meilleure connaissance de cette modification et de ses conséquences structurales présente un grand intérêt pour les industriels. Dans cette étude, la β-lactoglobuline a été chauffée en solution à 60 °C en présence d'arabinose, de galactose, de glucose, de lactose, de rhamnose et de ribose. Le type de polymérisation obtenu ainsi que les sites de glycation ont été déterminés en fonction de la nature du sucre utilisé. L'arabinose et le ribose conduisaient au plus haut degré de modification. Le glucose, le galactose et le rhamnose étaient moins réactifs alors que le lactose était à l'origine du taux de modification le plus bas. Les protéines substituées par le ribose ou l'arabinose formaient des polymères stabilisés par des liaisons covalentes induites par les sucres. En utilisant d'autres sucres, une partie des protéines agrégées était stabilisée uniquement par des interactions hydrophobes et des ponts disulfure. Aucun site spécifique d'attachement d'un sucre particulier n'a pu être clairement démontré. L'analyse en spectrométrie de masse des hydrolysats trypsiques des protéines modifiées a montré que la leucine 1 (résidu N-terminal), les lysines 14 et 47 étaient modifiées après glycation par le galactose, le glucose ou le lactose. Les résidus lysyle 69, 75 et 135 n'étaient modifiés qu'après glycation par le glucose. La lysine 100 n'était modifiée que lorsque la protéine était modifiée en présence de lactose. Le taux élevé de glycation et la formation de polymères obtenus par l'usage de ribose ou d'arabinose, inhibant toute action de la trypsine sur les résidus lysyle de la protéine, n'a pas permis de détecter des sites particuliers de glycation par ces sucres.

β-lactoglobuline / réaction de Maillard / glycation / polymère / trypsinolyse

1. INTRODUCTION

The Maillard reaction or non-enzymatic browning is largely encountered during industrial processing and particularly during heat treatment of foods rich in reducing sugars [4, 12, 33].

The reaction, which was first described by the French biochemist Louis Maillard at the beginning of the 20th century [25] is now extensively studied because of the high quantity and complexity of the products formed during its three different reaction stages [16, 22]. The early stage consists of a condensation of a reducing sugar with an amino group and leads, via the formation of a Schiff's base and the Amadori rearrangement, to the so-called Amadori product. The second stage involves the formation of Advanced Maillard reaction Products (AMP), including numerous essential fission sugar-amino compounds. The third stage results in the final Maillard reaction products containing condensation and polymerization products of proteins, inducing brown pigments called melanoidins.

Protein browning has different effects on food quality depending on the extent of the reaction [3, 20]. In domestic cooking, the reaction is frequently exploited to enhance color, aroma and flavor. The Maillard reaction is accompanied by a reduction of the nutritive value of a protein due to the addition of sugars on the lysyl residues which are then no longer available for enzymatic (tryptic) digestion [2], and to the formation of toxic compounds in the latter stage of the reaction [7, 33].

In the human body, long-lived proteins were identified to be modified by the Maillard reaction, inducing their polymerization [5, 14, 34]. This phenomenon is particularly visible among diabetics who have a higher concentration of circulating glucose [10, 43]. Besides oxidation, glycation

is a well-known factor of age-related diseases such as cataracts [23] and Alzheimer's disease [19, 40].

The mechanism of protein polymerization via the Maillard reaction is not yet well understood [1]. Some studies have shown that reducing sugars can create a covalent bond between two lysyl residues to produce a lysyl-lysine imidazolium cross-linked Advanced Glycation End product or AGE [18, 35, 36].

Due to its good nutritional and functional properties, bovine β-lactoglobulin, the major whey protein, is used frequently by the food industry as an ingredient [41]. In milk, this protein is known to react with lactose, producing the protein-bound Amadori product lactulosyllysine [24, 44]. Mass spectrometry analysis has indicated some preferential sites of lactosylation during the modification of β-lactoglobulin in a powder state [15, 28].

The aim of this work was to modify β-lactoglobulin with several common sugars in an aqueous state at neutral pH. An electrophoretic study of the different polymers separated by gel filtration was performed and binding site specificity of the different sugars was analyzed by mass spectrometry.

2. MATERIALS AND METHODS

2.1. Protein purification

β-Lactoglobulin (BLG), variant A was purified from fresh milk as described by Mailliart and Ribadeau Dumas [26]. The purity of BLG (99%) was assessed by RP-HPLC.

2.2. Reagents

D-arabinose, D-galactose, D-glucose, D-lactose, D-rhamnose, D-ribose monohydrates and TPCK-treated trypsin were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other reagents were of analytical grade.

2.3. Glycation experiments

BLG (0.217 mmol·L^{-1}) and the different sugars (0.217 mol·L^{-1}) were dissolved in 0.1 mol·L^{-1} phosphate buffer, pH 6.5. After filtration on 0.22 μm acetate cellulose filters (Millipore), mixtures of protein and sugar were put in well-capped flasks and heated in a water bath at 60 °C for 72 h. This mild heat treatment was chosen to limit self-aggregation of BLG. All experiments were performed under strictly anaerobic and sterile conditions, all media were purged and saturated with N$_2$. After heating, the different fractions were dialyzed against distilled water, freeze-dried, and stored at –20 °C. BLG heated without sugar (heated control) was named "heated BLG", and BLG heated in the presence of sugar was named "glycated BLG".

2.4. Determination of available amino groups

The available amino groups were determined by the modified ortho-phthaldialdehyde (OPA) method [17]. The OPA reagent was prepared daily by mixing 40 mg of OPA (dissolved in 1 mL of methanol), 50 mL of 0.1 mol·L^{-1} sodium borate buffer, pH 9.3, 100 mg of N-dimethyl-2-mercaptoethyl-ammonium chloride (DMMAC), and 1.25 mL of 20% (w/w) SDS in water. 50 μL of protein solution (2 g·L^{-1} in 50 mmol·L^{-1} sodium phosphate buffer, pH 7.8) was added to 1 mL of OPA reagent. The absorbance was read at 340 nm after a minimal delay of 5 min. A calibration curve was obtained by using 0.25–2 mmol·L^{-1} L-leucine as a standard.

2.5. Gel filtration chromatography

Native, heated and glycated proteins were dissolved at 5 mg·mL^{-1} in 0.1 mol·L^{-1} Tris-HCl buffer, pH 8 containing 0.5 mol·L^{-1}

NaCl (elution buffer). Samples were filtered through a 0.22 μm membrane filter (Millipore) to eliminate insoluble aggregates and 1 mL of each sample was applied to a High Performance Liquid Chromatography system (BIOcad SPRINT System, Perkin-Elmer, USA) monitored with BIOcad software (version 2.06). Separations were performed on a Superdex 75 prep grade column (separation domain 3–70 kg·mol^{-1}) (Pharmacia, Sweden) equilibrated with elution buffer at a flow rate of 0.5 mL·min^{-1}. Fractions of 2 mL were collected, and UV absorption was measured at 214 and 278 nm. The molecular masses of all samples were calculated according to the elution time of commercial protein standards (Gel Filtration Calibration Kit, Pharmacia, Sweden).

2.6. Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis experiments (SDS-PAGE in reducing or non-reducing conditions) were performed using a Mini Protean II gel electrophoresis apparatus (Bio-Rad, USA) as described by Laemmli [21]. After completion, polyacrylamide gels were silver stained according to Nesterenko et al. [32].

2.7. Tryptic hydrolysis of BLG

BLG (1 mg·mL^{-1}) was dissolved in 0.1 mol·L^{-1} Tris-HCl, pH 8. TPCK-treated trypsin, previously solubilized in 10 mmol·L^{-1} HCl (1 mg·mL^{-1}) was added to the reaction mixture at an E/S ratio of 0.5% (w/w). The mixture was incubated at 37 ºC for 24 h. The reaction was stopped by the addition of 0.5 mol·L^{-1} HCl.

2.8. Reversed phase high performance liquid chromatography (RP-HPLC)

The HPLC equipment consisted of an auto sampling injector (Model 231, Gilson, France), two solvent delivery systems (Model 510, Waters) and a photodiode Array Detector (Model 996, Waters). The HPLC system was driven with the Millennium software program (Waters). Tryptic peptides of BLG were separated by RP-HPLC on a Symmetry 300A C$_{18}$ column (3.9 mm × 150 mm, Waters, USA) equilibrated in 90% solvent A (0.115% TFA in H$_2$O) and 10% solvent B (80% acetonitrile, 19.9% H$_2$O, 0.1% TFA). The column was eluted with linear gradients: 75% solvent A and 25% solvent B for the first 3 min, 45% solvent A and 55% solvent B for the next 30 min and 100% solvent B for the last 3 min. The temperature of the column and solvent was maintained at 30 ºC. The flow rate was 0.6 mL·min^{-1}. Eluted peaks were detected by UV-absorbance (214 nm) and analyzed by ESI-MS.

2.9. Electrospray ionization – mass spectrometry (ESI-MS)

Tryptic peptides of BLG were identified by on line RP-HPLC/ESI-MS on a Perkin-Elmer Sciex (Thornhill, Ontario, Canada), Model API III$^+$ mass spectrometer. Tryptic peptides were separated by RP-HPLC on a symmetry C$_{18}$ (2.1 mm × 150 mm, Waters, Milford, MA, USA) at a flow rate of 0.25 mL·min^{-1} (40 ºC) with the split to the MS ionization source (30 μL·min^{-1}). Ion detection was performed in positive mode, and molecular masses were determined from the m/z charge using Biomultiview 1.2 (Software package Sciex).

3. RESULTS AND DISCUSSION

3.1. Glycation experiments

During the three days of mild heat treatment at 60 ºC, an increasingly intense yellow color appeared in the flasks containing BLG and sugar, and particularly in those containing arabinose and ribose, indicating

the formation of Advanced Glycation End products (AGE) such as melanoidins. The control, made up of BLG only, exhibited an increasingly intense white opaque color as a function of time, which could be explained by a progressive aggregation of BLG monomers.

3.2. Determination of the modification degree

Results of amino groups determination with the OPA method are presented in Figure 1. Only 80% of the 16 amino groups of native BLG (15 lysyl and 1 α-NH$_2$) could be detected. Since no amino groups were modified on native BLG, all results were reported relative to 100% of the amino groups of native BLG. In the heated control, one amino group appeared to be modified. This artifact could be explained by a structural modification of BLG (polymerization for example) inducing a masking of a single amino group. On average, 8.8, 6.7, 6.6, 5.5, 6.5 and 11.0 amino groups were modified in the cases of proteins heated in the presence of arabinose, galactose, glucose, lactose, rhamnose and ribose, respectively. Thus, the sugars can be classified from the slowest to the fastest reactant for glycation of BLG as follows:

lactose < rhamnose ≈ glucose ≈ galactose < arabinose < ribose.
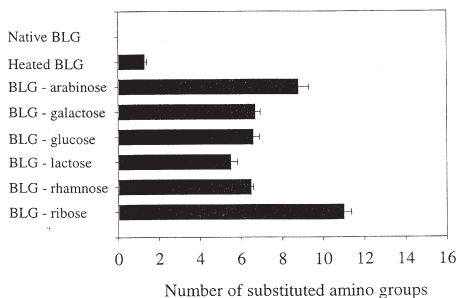
Figure 1. Degree of modification of BLG as determined by the OPA method.

As expected and according to previous works, the degree of modification was in direct relation to sugar size [31, 37]. The shorter the carbonic chain length of the sugar, the more the open chain form exists and the more the sugar reacts with amino groups of proteins.
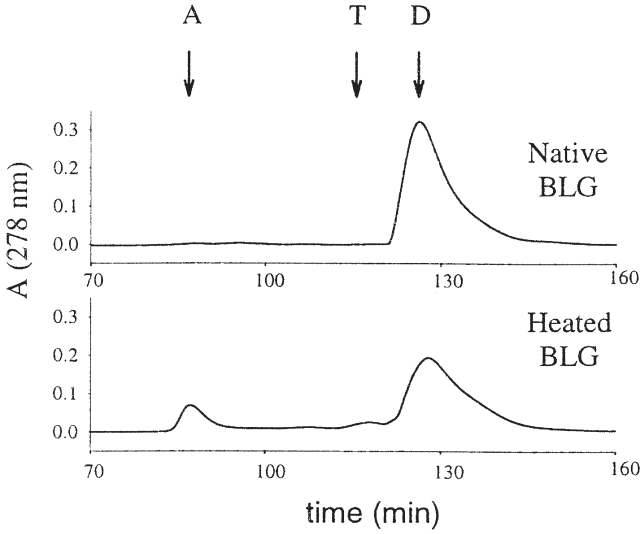
3.3. Size exclusion chromatography (SEC) experiments

SEC was performed with native, heated and glycated BLG at pH 8 on a Superdex 75 column, which allows for a separation of molecules between 3 and 70 kg·mol^{-1} in size. Molecular masses were calculated with standard proteins: ribonuclease A (13.7 kg·mol^{-1}); chymotrypsinogen A (25 kg·mol^{-1}); ovalbumin (43 kg·mol^{-1}); bovine serum albumin (67 kg·mol^{-1}); Blue dextran (2000 kg·mol^{-1}).

Figure 2 shows the absorbance at 278 nm of native and heated BLG after filtration on a 0.22 μm filter (only soluble material was analyzed in the SEC experiments). As expected, native BLG eluted as dimers (D ∼ 37 300 g·mol^{-1}). Ruminant BLGs are dimeric over the pH range 5 to 8 [27, 42].

Heating BLG for 72 h at 60 °C induced the formation of a trimeric form (T ∼ 51 200 g·mol^{-1}) and of aggregated forms in addition to the dimeric form (D ∼ 35 300 g·mol^{-1}), which is in accordance with previous studies on heat-induced aggregation of BLG [38].

Figure 3 shows the SEC elution profiles of glycated proteins. Some of the obtained aggregates had molecular masses greater than the upper limit of separation of the column as recommended by the manufacturer. A linear extrapolation of the calibration curve was used in the range of 70–79 kg·mol^{-1} in order to determine the polymeric nature of some of the glycated molecules.

Figure 2. Elution profiles from the size exclusion chromatography of native and heated BLG.
D: dimer; T: trimer; A: aggregate.
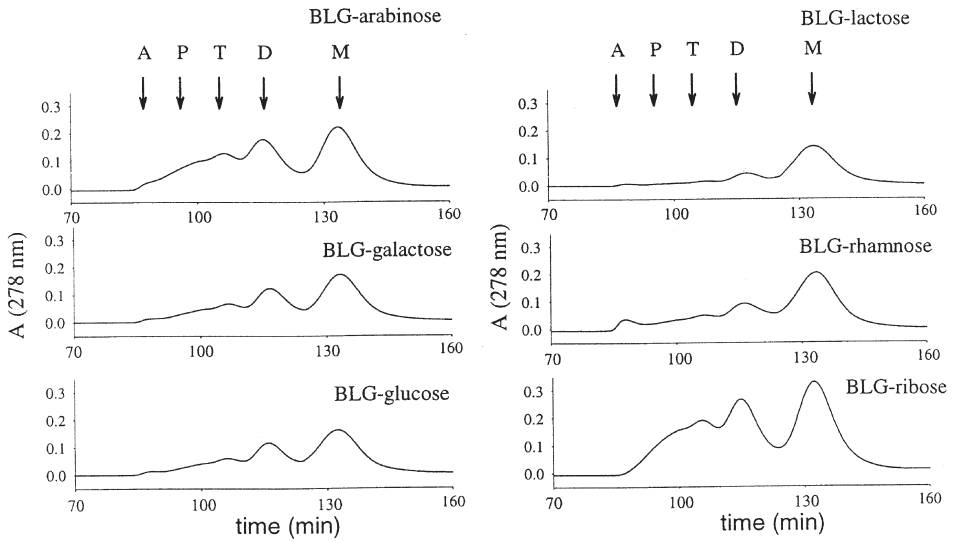
Figure 3. Elution profiles from the size exclusion chromatography of glycated BLG.
M: monomer; D: dimer; T: trimer; P: polymer; A: aggregate.

Monomeric (M), dimeric (D), trimeric (T), polymeric (P), and aggregate (fractions not retained on the column) (A) forms were obtained in the case of glycated proteins. It seemed that the dimers observed in the absence of sugar (for native and heated proteins) dissociated in the presence of sugar. It could be supposed that glycation at the surface of the BLG molecule increased electrostatic repulsions between monomers,

explaining the presence of monomeric forms of glycated protein in contrast with what was observed in the case of native and heated BLG.

An apparent increase of the molecular masses of the monomeric, dimeric and trimeric fractions was observed with the modified proteins. For example, the dimeric fraction of glycated BLG eluted at about 115 min (Fig. 3) seemed to have the same molecular mass as compared with the trimeric fraction of heated BLG eluted at about the same time (Fig. 2).

The separation of proteins by size exclusion chromatography is not only governed by their molecular masses, but the shape of the molecule including its degree of hydration and its physicochemical characteristics (net charge, hydrophobicity) may also play a role in their elution behavior. Recent studies [11] have shown that glycation of BLG with glucose in the same conditions induced a modification of the pI (essentially an acidification of the whole protein). This could explain the observed increase in molecular masses of the glycated samples. Since molecular masses of the glycated proteins determined by SEC

did not give useful results, the polymeric nature of these samples was defined on the basis of the PAGE experiments (see next section).

Relative amounts of the different fractions eluted were calculated from SEC profiles (Tab. I). Very different proportions were determined for the different proteins. The monomeric form was only observed in the case of glycated proteins. The relative quantity of the monomeric form was proportional to the degree of modification. The more the protein was modified, the less it contained a monomeric form. The opposite was observed for the trimeric and polymeric forms. Proteins modified with arabinose and ribose (the most reactive sugars) contained the highest amount of trimeric and polymeric forms. Proteins modified with lactose (the less reactive sugar) showed only 6.7% of trimeric forms and no polymeric form.

3.4. SDS-PAGE experiments

Aliquots of SEC fractions corresponding to the different forms of BLG (Figs. 2

Table I. Relative amount of different fractions separated by SEC (%).

Samples	A	P	T	D	M
Native BLG	0	1.6	0	98.4	0
Heated BLG	23.4	2.7	7.3	66.6	0
BLG-arabinose	4.1	15.2	19.7	27.3	33.7
BLG-galactose	2.9	10.5	15.8	29.5	41.3
BLG-glucose	3.3	10.4	15.3	29.7	41.3
BLG-lactose	3.4	0	6.7	20.5	69.4
BLG-rhamnose	9.2	8.8	12.2	22.0	47.8
BLG-ribose	0	15.8	20.6	28.6	35.0

A = aggregate; P = polymer; T = trimer; D = dimer and M = monomer.
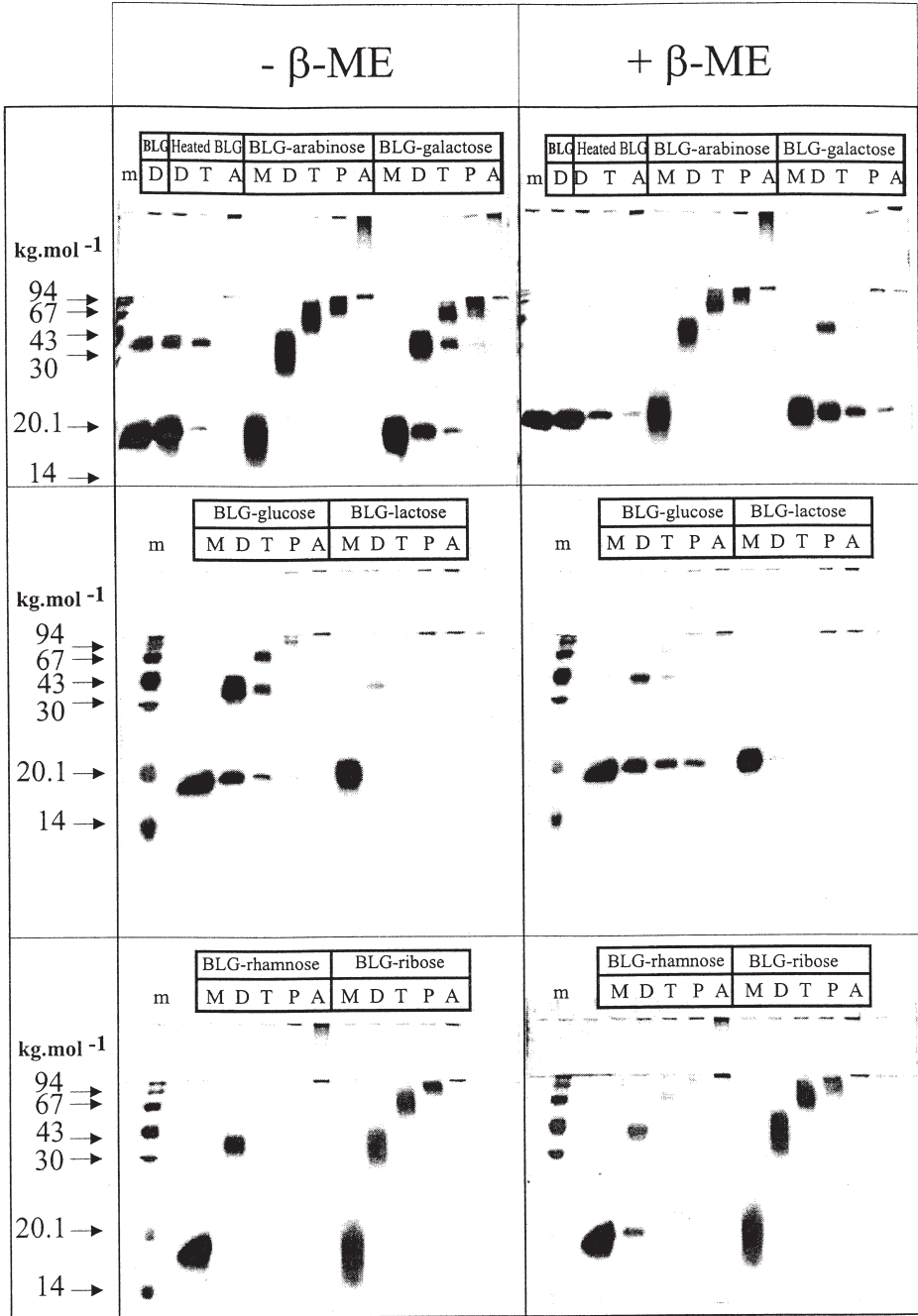
Figure 4. SDS-PAGE of native, heated and glycated BLG under reducing (right) or non-reducing (left) conditions.
M: monomer; D: dimer; T: trimer; P: polymer; A: aggregate.
m: molecular mass marker.

and 3) were collected and analyzed by SDS-PAGE under reducing and non-reducing conditions (Fig. 4). Different types of bonds can form polymers of BLG: non-covalent bonds could be the result of hydrophobic interactions; covalent bonds could be formed by a disulfide or by a sugar-induced bond as previously described [11]. Since SEC does not suppress hydrophobic interactions or covalent bonds, the nature of the polymers formed during the glycation of BLG was analyzed by SDS-PAGE.

Under non-reducing conditions, due to SDS, only hydrophobic interactions were impeded, whereas under reducing conditions besides the disappearance of hydrophobic interactions, disulfide bonds are broken. This allows establishment of the different kinds of bonds stabilizing polymeric forms of BLG.

The dimeric forms of BLG obtained from SEC were almost totally transformed into monomers during SDS-PAGE. Under non-reducing conditions, a faint band remained, corresponding to the dimeric form; this band completely disappeared in the presence of β-mercaptoethanol (β-ME). Consequently, dimeric BLG results from both hydrophobic interactions and disulfide bonds. For a pH value up to 8, a rearrangement of the three-dimensional structure of BLG occurs, leading to disulfide exchange induced by the presence of free cysteine 121 [13].

The dimeric, trimeric and aggregate fractions of heated BLG were fully reduced under reducing conditions, verifying the presence of the previously studied heat-induced disulfide bond [27].

Among the proteins modified with sugar, three groups could be observed according to the type of bonds contributing to their polymerization.

In the first group, formed by the proteins glycated with ribose or arabinose, the resulting polymers were stable in the presence of SDS or under reducing conditions. Consequently, sugar induced covalent bonds most likely stabilize dimers, trimers, polymers and aggregates formed in the presence of ribose and arabinose. Several authors have previously described such bonds. Pongor et al. [36] studied the "FFI" product that appeared to be a lysyl-lysyl cross-link. "Pentosidine" may be another potential cross-link product [39].

In the present study, the presence of hydrophobic interactions and disulfide bonds could not be totally eliminated. For example, some of the dimers could be formed first by hydrophobic interactions, then by disulfide bonds after heating, and then, after the reaction of the sugars with the amino groups, by sugar induced covalent bonds. Such molecules are very complex; their structural studies could yield some information on the nature of the polymeric interfaces.

In the second group, composed of proteins glycated with galactose, glucose and rhamnose, a portion of the polymers was broken by SDS (Fig. 4, left column) and a portion of the polymers stable in SDS was broken by β-ME (Fig. 4, right column). Consequently, one part of the polymers formed during the reaction with these three sugars was stabilized only by hydrophobic interactions, one part by disulfide bonds, and another part by sugar-induced covalent bonds.

As observed in SEC profiles (Fig. 3), only a small amount of dimers was obtained in the case of BLG modified in the presence of lactose. This dimeric fraction did not disappear in the presence of SDS, but was reduced by β-ME. Consequently, disulfide bonds stabilized dimeric forms of lactosylated BLG, and no sugar-induced bonds were observed, which is in accordance with the results of Bouhallab et al. [6].

3.5. Identification of glycation sites

Glycation sites were identified analyzing tryptic peptides of modified BLG by
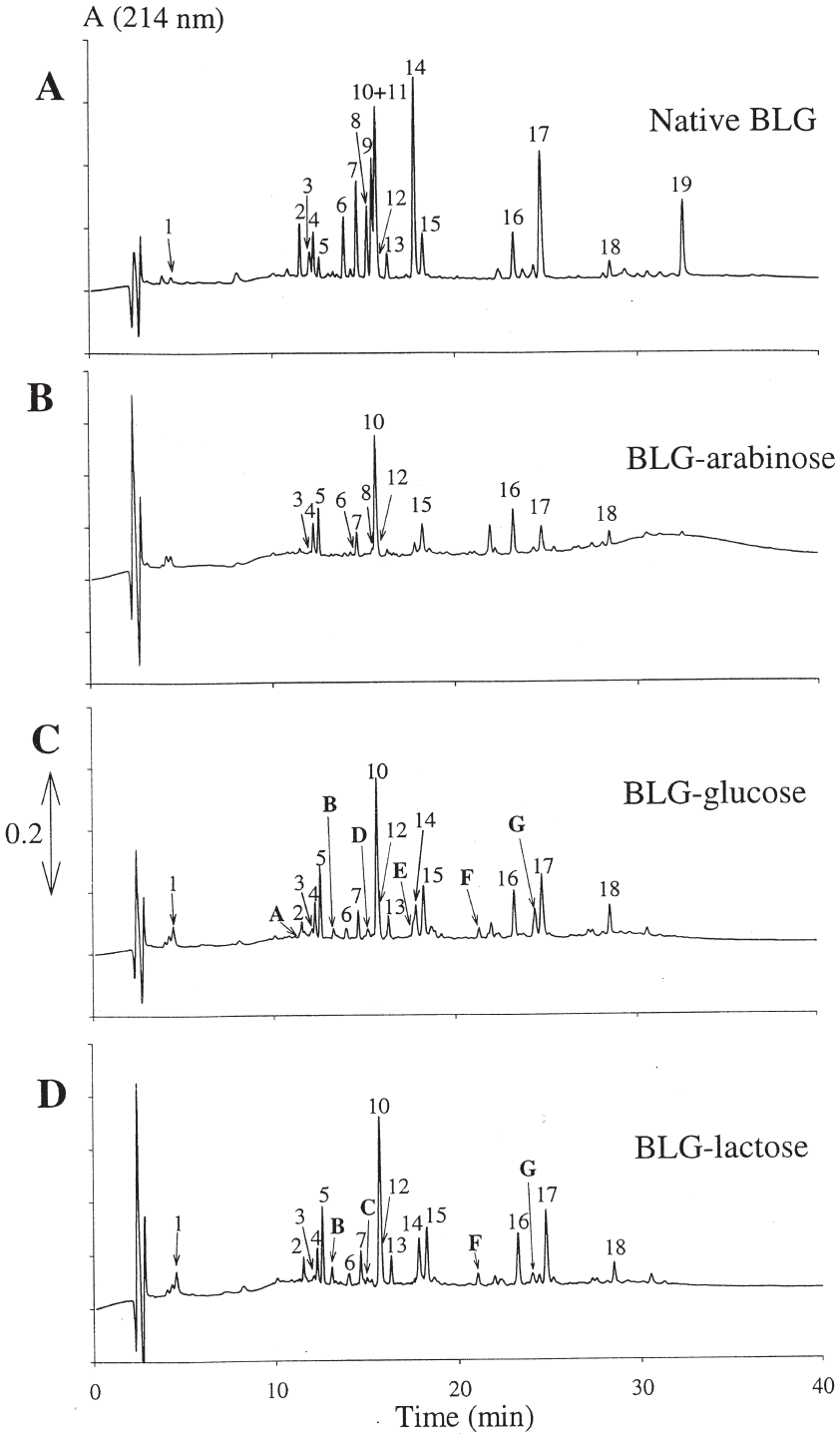
Figure 5. RP-HPLC elution profiles of the tryptic hydrolysates of native BLG and BLG glycated with arabinose, glucose or lactose. Peptides indicated with numbers or letters are described in Table II.

RP-HPLC coupled with ESI-MS. Tryptic peptides of native BLG were identified according to their molecular masses (MM) as compared with theoretical MM of tryptic peptides of BLG. Modified peptides corresponded to the sum of the MM of the sugar and the MM of the unmodified peptide. Tryptic peptide profile identification is shown in Figure 5 for the hydrolysates of native BLG (A) and BLG modified with arabinose (B), glucose (C) and lactose (D).

The present study was based on analyzing both disappearing and newly formed peptides in order to determine a possible correlation between the nature of the sugar used during glycation and the glycation site (Tab. II). All the results are only based on tryptic hydrolysates since all the resulting peptides always contained at least one lysyl or arginyl C-terminal residue, which is necessary for detection by mass spectrometry.

Hydrolysis conditions allowed peptides containing cysteyl residues to make a disulfide bond. Consequently, peptide [149–162] which contains Cys 160 was linked with three different peptides: peptide [61–70] which contains Cys 66; peptide [61–69] which also contains Cys 66; and peptide [102-124] which contains Cys 106. This disulfide reorganization was clearly observed and was not an impediment for peptide identification.

Glycation inhibits tryptic hydrolysis since glycation sites are located on lysyl and arginyl residues that are tryptic cleavage sites [2, 11]. Consequently, when such a residue is glycated by the Maillard reaction, the peptide bond in which it is involved cannot be hydrolyzed by the protease any more. It would have been more practical to use another proteinase which is not a priori inhibited by glycation (for example pepsin). However, in this case, all the peptides devoid of lysyl or arginyl residues should not have been detected and interpretation of both disappearing and newly formed peptides should be very difficult. In addition, due to its least specificity, pepsin

gives numerous peptides which are more difficult to separate and to identify.

BLG modified with arabinose and ribose were the most glycated derivatives. Many peptides obtained in the hydrolysate of native BLG were absent in the hydrolysates of BLG glycated with arabinose (Fig. 5 B) and ribose (profile not shown but comparable with that of arabinose). This indicated that numerous cleavage sites for the trypsin were substituted. However, no additional peptides could be identified as the low availability of protonation of modified side chains of arginyl and lysyl residues precluded ionization by ESI-MS.

Polymerization of BLG modified with ribose and arabinose could be another factor playing a role in decreasing accessibility for the protease.

Tryptic hydrolysates of BLG modified with galactose, glucose, lactose or rhamnose show 3, 6, 4 and 1 new peptides, respectively, as compared with the tryptic peptide profiles of native BLG. These peptides are shown on tryptic profile of glucosylated (Fig. 5 C) and lactosylated (Fig. 5 D) BLG.

The hydrolysate of glucosylated BLG presented the largest number of detectable modified peptides. However, as shown in Figure 1, a similar degree of modification occurred in the case of glucosylated and galactosylated BLG. In addition, D-glucose and D-galactose are epimers at C-4, since they differ only in their configuration at a single asymmetric center on carbon 4. However, this single difference cannot explain the three specific sites of glycation observed in the case of glucosylated BLG as compared with galactosylated BLG. Some of the additional peptides observed (for example peptides A and D in Fig. 5 C) were at the limit of the detection, which could be the basis of this difference. Therefore, slight difference in the protein concentration used for hydrolysis could influence peptide detection.

Table II. Identification of tryptic peptides of native and glycated BLG.

Peak	Retention time (min)	Mass (g·mol^{-1})	Peptide	Native BLG	BLG-arabinose	BLG-galactose	BLG-glucose	BLG-lactose	BLG-rhamnose	BLG-ribose	Potentially modified amino acids
							Non-appearing peptides				
1	4.482	408.3	[136-138]		X					X	Lys 135 or Lys 138
2	11.535	572.4	[71-75]		X					X	Lys 70 or Lys 75
3	12.268	915.6	[84-91]							X	Lys 83 or Lys 91
4	12.602	672.6	[9-14]							X	Lys 8 or Lys 14
5	12.7	1244.84	[125-135]								/
6	13.968	932.84	[1-8]							X	**Leu 1** or Lys 8
7	14.668	836.84	[142-148]							X	Lys 141 or Arg 148
8	15.235	1636.04	[125-138]			X	X	X	X		Arg 124 or **Lys 135** or Lys 138
9	15.502	1193.09	[92-101]		X	X	X	X	X		Lys 91 or **Lys 100**
10	15.702	695.7	[15-20]								/
11	15.702	902.84	[76-83]		X	X	X	X			**Lys 75** or Lys 77 or Lys 83
12	15.8	673.5	[78-83]							X	Lys 77 or Lys 83
13	16.335	1064.84	[92-100]		X					X	Lys 91 or Lys 100
14	17.835	2907.73	[61-70]-[149-162]		X					X	Lys 60 or **Lys 69** or Lys 70 or Arg 148
15	18.302	2779.63	[61-69]-[149-162]							X	Lys 60 or Lys 69 or Arg 148
16	23.235	2030.54	[21-40]							X	Arg 40
17	24.735	2313.98	[41-60]							X	Arg 40 or **Lys 47** or Lys 60
18	28.2	4331.08	[102-124]-[149-162]							X	Lys 101 or Arg 124 or Arg 148
19	32.502	2708.38	[15-40]			X	X	X		X	**Lys 14** or Arg 40

Peak	Retention time (min)	Mass (g·mol^{-1})	Peptide	Native BLG	BLG-arabinose	BLG-galactose	BLG-glucose	BLG-lactose	BLG-rhamnose	BLG-ribose	Modified amino acids
							New peptides				
A	11.4	802.50 + M	[71-77]*				+				**Lys 75**
B	13.307	932.84 + M	[1-8]*			+	+	+			**Leu 1**
C	14.7	1193.51 + M	[92-101]*					+			**Lys 100**
D	15.207	1636.04 + M	[125-138]*				+				**Lys 135**
E	17.8	2907.32 + M	[61-70]-[149-162]*				+				**Lys 69**
F	21.307	1352.40 + M	[9-20]*			+	+	+	+		**Lys 14**
G	24.407	2313.98 + M	[41-60]*			+	+	+			**Lys 47**

X : non appearing peptides ; + : presence of new peptides ; * : modified peptides.

M = molecular mass of the corresponding sugar (galactose and glucose M = 162.1 g·mol^{-1} ; ribose and arabinose M = 132.1 g·mol^{-1} ; rhamnose M = 146.1 g·mol^{-1} ; lactose M = 324 g·mol^{-1}). Peaks 1 to 19 and A to G were reported in Figure 5.

Nevertheless, some differences of glycation site specificity exist in the proteins modified with glucose, lactose or rhamnose. Peptides [1–8], [9–20] and [41–60] of the tryptic hydrolysate of BLG glycated with glucose and lactose were detected by adding the MM of a sugar to the MM of the tryptic peptides, indicating that leucine 1, lysine 14 and lysine 47 were modified, respectively. Lysines 75, 135 and 69 were modified only in glucosylated BLG, and lysine 100 only in lactosylated BLG. Glycation of BLG with rhamnose induced only one additional tryptic peptide corresponding to the modification of lysine 14. This site was well recognized, as it was the only residue to be modified in the presence of galactose, glucose, lactose and rhamnose. Two sites (leucine 1 and lysine 47) were recognized by galactose, glucose and lactose. On the other hand, the disappearance of some tryptic peptides can also yield further information on glycation sites.

In the case of proteins modified with galactose, glucose, lactose or rhamnose, only four tryptic peptides were not obtained. For all these peptides, the glycation of a residue at the extremity of or within the peptide chain could explain their disappearance. Glycation sites found in new peptides detected and the putative modified amino acids responsible for the loss of a peptide are shown in bold in Table II.

Peptide [15–40] was not observed in the case of BLG modified with galactose, glucose or lactose. The potential amino acids composing it, which can be modified, are lysine 14 and arginine 40. Only lysine 14 was found inside the modified peptide [9–20] when glycation was performed in the presence of galactose, glucose, lactose or rhamnose. Consequently, the loss of peptide [15–40] could be attributed to the glycation of lysine 14.

Peptide [92–101] disappeared in tryptic profile of BLG modified with galactose, glucose, lactose or rhamnose. Its potentially modified amino acids are lysine 91, lysine 100 and lysine 101. Lysine 100 was only found to be modified in the peptide [92–101] from the tryptic hydrolysate of BLG modified with lactose. Although peptide [92–101] was not observed in the case of glycation of BLG with galactose, glucose or rhamnose, no additional peptide containing the modified lysine 100 could be detected in tryptic hydrolysate of BLG modified with these sugars. This could be explained as follows: either peptide [92–101] containing modified lysine 100 was undetected due to its low concentration, or lysine 92 or lysine 101 were modified too, inhibiting trypsin attack and inducing the absence of this peptide. Although in the case of proteins modified with lactose, the absence of peptide [92–101] could be due to the glycation of lysine 100, in the case of proteins modified with galactose, glucose or rhamnose, its absence could not be attributed to the glycation of a precise site. There is certainly a high probability for the same site of glycation to be responsible for the absence of peptide [92–101] independent of the sugar used, but such a hypothesis could not be demonstrated via this technique.

The fact that peptides [125–138] and [76–83] were not observed in the case of proteins modified with galactose, glucose or lactose raised the same questions. For the protein modified with glucose, it could be explained by the substitution of lysine 135 and lysine 75. However, in the case of the other sugars used, the sites of glycation responsible for the absence of these peptides could not be determined.

4. CONCLUSION

The study of tryptic hydrolysates profiles of BLG and modified BLG was

very useful for the identification of additional peptides and for the localization of sites of glycation. However, regarding the low number of peptides obtained after tryptic hydrolysis of BLG glycated with arabinose and ribose, the use of tryptic hydrolysis followed by mass spectrometry does not seem to be adequate for the identification of modified peptides of highly glycated proteins. Moreover, it is still hazardous to compare the site specificity of modification since some glycation sites could not be observed due to the low concentrations of the resulting peptides. Additionally, all of the obtained results are an average of the particular glycation of each BLG molecule.

Previous studies on lactosylation of BLG have suggested preferential sites of modification. Fogliano et al. [15] identified "Lysyl 100" to be a preferential site of lactosylation during industrial milk treatment. "Lysyl 47" was observed by Léonil et al. [24] to be a specific lactosylation site of BLG during the initial stage of the Maillard reaction. Multiple lactosylation sites were also found [9] and led to heterogeneity of the modified sites [28–30].

The nature of the sugars and their different reactivities are at least partially responsible for site-specific glycation. However, structural accessibility of lysyl residues is the main factor responsible for preferential modification sites [8, 45]. Structural accessibility may evolve quite significantly during ongoing substitution of nucleophilic groups on the BLG surface.

ACKNOWLEDGEMENTS

REFERENCES

[1] Adrian J., Billaud C., Potus C., Les manifestations de la réaction de Maillard en nutrition et pathologie, Med. Nutr. 36 (2000) 69–89.

[2] Alonzo M.L., Zapico J., Change in sugars and lysine in baby foods during storage, J. Food Biochem. 18 (1995) 393–403.

[3] Ames J.M., Control of the Maillard reaction in food systems, Trends Food Sci. Technol. 1 (1990) 150–154.

[4] Ames J.M., Application of the Maillard reaction in the food industry, Food Chem. 62 (1998) 431–439.

[5] Azevedo M.S., Maillard compounds as a cause of aging, Acta Med. Port. 3 (1990) 126–128.

[6] Bouhallab S., Morgan F., Henry G., Mollé D., Léonil J., Formation of stable covalent dimer explains the high solubility at pH 4.6 of lactose-beta-lactoglobulin conjugates heated near neutral pH, J. Agric. Food Chem. 47 (1999) 1489–1494.

[7] Brands C.M., Alink G.M., van Boekel M.A., Jongen W.M., Mutagenicity of heated sugar-casein systems: effect of the Maillard reaction, J. Agric. Food Chem. 48 (2000) 2271–2275.

[8] Brown E.M., Pfeffer P.E., Kumosinski T.F., Greenberg R., Accessibility and mobility of lysine residues in beta-lactoglobulin, Biochemistry 27 (1988) 5601–5610.

[9] Burr R., Moore C.H., Hill J.P., Evidence of multiple glycosylation of bovine β-lactoglobulin by electrospray ionization mass spectrometry, Milchwissenschaft 51 (1996) 488–492.

[10] Chellan P., Nagaraj R.H., Protein crosslinking by the Maillard reaction: dicarbonyl-derived imidazolium crosslinks in aging and diabetes, Arch. Biochem. Biophys. 1 (1999) 98–104.

[11] Chevalier F., Chobert J.M., Dalgalarrondo M., Haertlé T., Characterization of the Maillard reaction products of β-lactoglobulin glucosylated in mild conditions, J. Food Biochem. 25 (2001) 33–55.

[12] Chuyen N.V., Maillard reaction and food processing. Application aspects, Adv. Exp. Med. Biol. 434 (1998) 213–235.

[13] Dunnil P., Green D.W., Sulphydryl groups and the N-R conformational change in β-lactoglobulin, J. Mol. Biol. 15 (1965) 147–151.

[14] Dyer D.G., Blackledge J.A., Katz B.M., Hull C.J., Adkisson H.D., Thorpe S.R., Lyons T.J., Baynes J.W., The Maillard reaction in vivo, Z. Ernahrungswiss. 30 (1991) 29–45.

[15] Fogliano V., Monti S.M., Visconti A., Ritieni A., Identification of a β-lactoglobulin lactosylation site, Biochim. Biophys. Acta 1388 (1998) 295–304.

[16] Friedman M., Food browning and its prevention: an overview, J. Agric. Food Chem. 44 (1996) 631–653.

[17] Frister H., Meisel H., Schlimme E., OPA method modified by use of N,N-dimethyl-2 mercaptoethyl-ammoniumchloride as thiol component, Fresenius Z. Anal. Chem. 330 (1988) 631–633.

[18] Frye E.B., Degenhardt T.P., Thorpe S.R., Baynes J.W., Role of the Maillard reaction in aging of tissue proteins. Advanced glycation end product-dependent increase in imidazolium cross-links in human lens proteins, J. Biol. Chem. 273 (1998) 18714–18719.

[19] Horie K., Miyata T., Yasuda T., Takeda A., Yasuda Y., Maeda K., Sobue G., Kurokawa K., Immunohistochemical localization of advanced glycation end products, pentosidine, and carboxymethyllysine in lipofuscin pigments of Alzheimer's disease and aged neurons, Biochem. Biophys. Res. Commun. 236 (1997) 327–332.

[20] Hurrel R. F., Carpenter K. J., Nutritional significance of cross-link formation during food processing, in: Friedman, M. (Ed.), Protein crosslinking: Nutritional and Medical consequences, Plenum, New York, USA, 1977, pp. 225–238.

[21] Laemmli U.K., Cleavage of structural protein during the assemblage of the head of bacteriophage T4, Nature 225 (1970) 650–657.

[22] Ledl F., Schleicher E., New aspects of the Maillard reaction in foods and in the human body, Angew. Chem. Int. 29 (1990) 565–594.

[23] Lee K.W., Mossine V., Ortwerth B.J., The relative ability of glucose and ascorbate to glycate and crosslink lens proteins in vitro, Exp. Eye Res. 67 (1998) 95–104.

[24] Léonil J., Mollé D., Fauquant J., Maubois J.L., Pearce R.J., Bouhallab S., Characterization by ionization mass spectrometry of lactosyl β-lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site, J. Dairy Sci. 80 (1997) 2270–2281.

[25] Maillard L.C., Action des amines sur les sucres : formation des mélanoïdines par voie méthodique, C. R. Acad. Sci. 2 (1912) 154–166.

[26] Mailliart P., Ribadeau Dumas B., Preparation of β-lactoglobulin and β-lactoglobulin-free proteins from whey retentate by NaCl salting out at low pH, J. Food Sci. 53 (1988) 743–752.

[27] McKenzie H. A., β-lactoglobulin, in: McKenzie H.A. (Ed.), Milk Proteins-II, Academic Press, New York, USA, 1971, pp. 257–330.

[28] Mollé D., Morgan F., Bouhallab S., Léonil J., Selective detection of lactolated peptides in hydrolysates by liquid chromatography/electrospray tandem mass spectrometry, Anal. Biochem. 259 (1998) 152–161.

[29] Morgan F., Léonil J., Mollé D., Bouhallab S., Nonenzymatic lactosylation of bovine β-lactoglobulin under mild heat treatment leads to structural heterogeneity of the glycoforms, Biochem. Biophys. Res. Commun. 236 (1997) 413–417.

[30] Morgan F., Bouhallab S., Mollé D., Henry G., Maubois J. L., Léonil J., Lactolation of β-lactoglobulin monitored by electrospray ionisation mass spectrometry, Int. Dairy J. 8 (1998) 95–98.

[31] Nacka F., Chobert J.M., Burova T., Léonil J., Haertlé T., Induction of new physicochemical and functional properties by the glycosylation of whey proteins, J. Protein Chem. 17 (1998) 495–503.

[32] Nesterenko M.V., Tilley M., Upton S.J., A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels, J. Biochem. Biophys. Meth. 28 (1994) 239–342.

[33] O'Brien J., Morrissey P.A., Nutritional and toxicological aspects of the Maillard browning reaction in foods, Crit. Rev. Food Sci. Nutr. 28 (1989) 211-248.

[34] Paul R.G., Bailey A.J., Glycation of collagen: the basis of its central role in the late complications of ageing and diabetes, Int. J. Biochem. Cell Biol. 28 (1996) 1297–1310.

[35] Pellegrino L., van Boekel M.A.J.S., Gruppen H., Resmini P., Pagani M.A., Heat-induced aggregation and covalent linkages in beta-casein model systems, Int. Dairy J. 9 (1999) 255–260.

[36] Pongor S., Ulrich P.C., Bencsath F.A., Cerami A., Aging of proteins: isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose, Proc. Nat. Acad. Sci. USA 81 (1984) 2684–2688.

[37] Prabhakaram M., Ortwerth B.J., Determination of glycation crosslinking by the sugar-dependent incorporation of [^{14}C]lysine into protein, Anal. Biochem. 216 (1994) 305–312.

[38] Schokker E.P., Singh H., Pinder D.N., Norris D.N., Creamer L.K., Characterization of intermediates formed during heat-induced aggregation of β-lactoglobulin AB at neutral pH, Int. Dairy J. 9 (1999) 791–800.

[39] Sell D.R., Monnier V.M., Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process, J. Biol. Chem. 264 (1989) 21597–21602.

[40] Smith M.A., Taneda S., Richey P.L., Miyata S., Yan S.D., Stern D., Sayre L.M., Monnier V.M., Perry G., Advanced Maillard reaction end products are associated with Alzheimer disease pathology, Proc. Nat. Acad. Sci. USA 91 (1994) 5710–5714.

[41] Smithers G.W., Ballard F.J., Copeland A.D., de Silva K.J., Symposium: Advances in dairy foods proceeding and engineering. New opportunities from the isolation and utilization of whey proteins, J. Dairy Sci. 79 (1996) 1454–1459.

[42] Swaisgood H.E., Chemistry of milk protein, in: Fox, P.F. (Ed.), Development in dairy chemistry-1, Applied Science Publishers, London, England, 1982, pp. 1–59.

[43] Thorpe S.R., Baynes J.W., Role of the Maillard reaction in diabetes mellitus and diseases of aging, Drugs Aging 9 (1996) 69–77.

[44] van Boekel M.A.J.S., Effect of heating on Maillard reaction in milk, Food Chem. 62 (1998) 403–414.

[45] Wu S.Y., Perez M.D., Puyol P., Sawyer L., β-lactoglobulin binds palmitate within its central cavity, J. Biol. Chem. 274 (1999) 170–174.