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Research Article

Proteolysis of milk proteins lactosylated in model systems

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Five different milk proteins (α-casein, β-casein, κ-casein, β-lactoglobulin, and lactoferrin) and a peptide substrate were applied as substrates for the investigation of how lactosylation affected proteolysis by different proteases. After a lactosylation period of 4 days in aqueous solution, at 65°C and pH 6.8 in a protein: lactose ratio of 1000 the proteins were enzymatically hydrolyzed by the three milk relevant proteases plasmin, cathepsin D, and chymosin. Lactosylation of all substrates affected hydrolysis by plasmin negatively, with the largest effect on the globular proteins. This could be explained by modification of lysine residues, being the preferred cleavage site for plasmin, but also the residue generally preferred for lactosylation. Lactosylation of the caseins and of β-lactoglobulin did not affect subsequent cleavage by cathepsin D and chymosin significantly, but for β-lactoglobulin, both the secondary as well as the tertiary structure were affected by lactosylation. In contrast, decreased hydrolysis by cathepsin D and chymosin was observed for lactoferrin after lactosylation. Decreased hydrolysis may be caused by a more compact tertiary structure induced by lactosylation of lactoferrin, as indicated by fluorescence spectroscopy measurements.

Keywords: Cathepsin D / Chymosin / Lactosylation / Plasmin

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1 Introduction

Thermal treatment is the most common process in dairy industry, and heat-induced changes such as lactosylation of milk proteins and the further Maillard reactions can influence further processing and storage of milk and milk-derived products. Due to the high content of lactose (~50 g/L) and protein (30–40 g/L), milk and milk-derived products constitute excellent media for the occurrence of Maillard reactions. Maillard reaction primarily occurs between the carbonyl group of reducing sugar (in milk; lactose, glucose, and galactose), and the ε-amino group of protein-bound lysine and amino-terminals. Lactosylation, the initial step in Maillard reaction, is known to occur during heat treatment in aqueous systems, and to an even higher extent in dry systems, like milk protein powder [1, 2]. Also in UHT milk, significant lactosylation of whey proteins, including β-lactoglobulin, has been shown to occur [3, 4]. One of the intermediates in the Maillard reactions, the Amadori product, has been detected as furosine after acid hydrolysis in many cheese varieties [5, 6] thus demonstrating heat-induced protein modifications of cheese proteins to occur through the modification of lysines. Depending on the cheese variety, glycation and the further Maillard reaction may hence potentially influence the ripening process by changing the enzymatic hydrolysis of modified milk proteins by proteases present in, or added to milk.

Glycation of the globular whey protein, β-lactoglobulin, and the caseins has been studied earlier. Morgan *et al.* [7] reported lactosylation to be rather nonspecific and to involve most of the α- and ε-amino groups in the structure of β-lactoglobulin, but a specific lactose-binding site in β-lactoglobulin has been identified after heat treatment of whey [3]. After 8 h incubation of β-lactoglobulin and sugars in solid state, lysine residues were found to be the main target of glycation with lactose and galactose, even though the amino terminal Leu residue and Arg 124 were modified as well [8]. The lactosylation of β-lactoglobulin was shown to be independent of its matrix when incubated for a period of 120 h at 60°C whereas lactosylation of α-lactalbumin was enhanced in whole milk compared to the aqueous model system [9]. Lactosylation of β-lactoglobulin was found to increase linearly with heating time [7]. Even mild heat treatment at 40°C for 30 min was sufficient for

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glycation of β-lactoglobulin [10]. Lactosylation of β-lactoglobulin was independent of temperature between 37 and 60°C, whereas the degree of lactosylation of β-casein increased steadily with temperature within the same temperature range [11]. In the caseins, lactose was found to bind specifically to Lys-34 in α_{S1}-casein, and to Lys-107 in β-casein under moderate (72 – 85°C for 15 – 30 s) heat treatment conditions of milk. The number of binding sites was increased to seven for α_{S1}-casein, and five for β-casein after intensive heat treatment (142 – 145°C for 2 – 5 min) [12]. The extent of glycation is dependent on the substrate protein that binds the reducing sugar, and under anaerobic conditions at pH 6.5 and 60°C, 25% of β-lactoglobulin, and 35% of β-casein became modified with lactose [11].

Especially in cheeses, but also in UHT and powder products, proteolysis greatly affects the quality and further storage stability. In milk powders, proteolysis may affect the functionality of the powder product, and in UHT milk proteolysis is undesired, as it may lead to gelation problems [13], while in cheese proteolysis is mandatory for the ripening process [14]. As lactosylation involves covalent modifications of the backbone structure of the lactose-receiving substrate proteins, it is very likely that such modifications have impacts on the accessibility for hydrolysis by proteolytic enzymes.

Chymosin (EC 3.4.23.4), a rennet protease, is responsible for the enzymatic coagulation of milk [14] through the well-known cleavage of the κ-casein into the para-casein and the glycomacropeptide parts [15, 16]. Chymosin furthermore rapidly hydrolyzes α_{S1}-casein at the Phe 23 – Phe 24 bond, hence taking part in the early stages of cheese ripening and in texture development of chymosin-containing cheeses [17].

Proteolysis in good-quality raw milk is mainly caused by the indigenous alkaline serine protease, plasmin (EC 3.4.21.7), but also lysosomal proteases, such as the carboxyl protease, cathepsin D (EC 3.4.23.5) have been found in bovine milk. It has been demonstrated that plasmin contributes to cheese ripening by initiating the proteolytic degradation of the caseins [18]. Furthermore, plasmin and somatic cell proteases may contribute to age gelation of UHT milk [19], but the roles of indigenous bovine proteases for these mechanisms are not clear [20, 21]. The specificity of plasmin is quite strict, with preferred cleavage after basic residues, with cleavage after lysine preferred to arginine [22]. Plasmin activity has been detected in commercial caseins and caseinates [23] as well as in whey protein products [24]. Due to the specificity of plasmin, acting preferentially on lysyl-peptide bonds, it is likely that lactosylation, also involving modifications primarily on lysine residues, affects especially proteolysis mediated by plasmin.

The lysosomal aspartic protease, cathepsin D, hydrolyzes the caseins in a way quite similar to chymosin [25, 26]. Cathepsin D has a general preference for cleavage between two hydrophobic residues in protein substrates [27], but residues located more distant from the susceptible bond are important for the cleavage rate [28]. The enzyme may potentially influence proteolysis of proteins in milk and whey powders, while in cheese, the significance of cathepsin D in the ripening process is often masked by the activity of chymosin. The enzyme may, however, be significant for texture development in, *e.g.*, Swiss type cheeses due to heat inactivation of chymosin or in acid cheeses due to elevated activity of the enzyme at acid pH [29].

Even though most milk undergo some kind of heat treatment resulting in various extents of glycations and Maillard reactions, and proteolysis by indigenous or added proteases being a core element of many dairy products, little is known about the impacts of Maillard reactions on proteolysis. The aim of the present study was to investigate the effect of lactosylation of different types of protein and peptide substrates on their susceptibility for subsequent hydrolysis by the proteolytic enzymes plasmin, cathepsin D, and chymosin, and relate the observed effects of glycation on proteolysis to measurable changes in the secondary and tertiary structures of the substrate proteins. The substrates included examples of globular whey proteins (β-lactoglobulin and lactoferrin), in addition to caseins, which have a more random-coiled structure, and a lysine-containing model peptide substrate.

2 Materials and methods

2.1 Materials

The substrate proteins (α-casein, β-casein, κ-casein, β-lactoglobulin, and lactoferrin), cathepsin D, plasminogen, and TFA were from Sigma – Aldrich Chemie GmBh (Steinheim, Germany). ACN (grade S) was from Rathburn Chemicals Walkerburn (Scotland). The lysine-containing peptide (H-3392) with the sequence VHFFKNIVTARTP [(Ala^{96})-Mylin Basic Protein (87 – 99) (human, bovine, rat)] was purchased from Bachem Holding AG (Bubendorf, Switzerland). Trombolysin was from Immuno Danmark, (Copenhagen, Denmark). Fluorescamine (4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′dione) was purchased from Acros Organics (Geel, Belgium). Trichloroacetic acid and water-free acetone were from Merck (Germany). Chymosin was a gift from Christian Hansen (Hørsholm, Denmark). The 96-well polystyrene plates were obtained from Corning (New York, USA).

2.2 Lactosylation of substrates

The substrate peptide H-3392 and the substrate proteins were incubated with or without inclusion of lactose in concentrations corresponding to that in milk, *i.e.* ~50 g/L. β-Casein, α-casein, κ-casein, and β-lactoglobulin were dissolved in 10 mM phosphate buffer, pH 6.8, to 1.46×10^{-4} M,

corresponding to a molar excess of lactose of 1000 : 1, and incubated at 65°C. Controls without lactose were prepared and incubated in parallel. Lactoferrin was incubated similarly, but at 55°C, due to pilot experiments showing precipitation of lactoferrin when incubated at 65°C. The peptide substrate H-3392 was dissolved in the same buffer to 7.3×10^{-4} M, and was incubated with a molar excess of lactose of 1 : 200 at 65°C. The model peptide was included for comparison of effects of glycation on proteolysis between peptide and protein substrates. This commercially available peptide was selected due to the presence of a lysine (K) residue in the sequence (VHFFKNIVTARTP) being potentially susceptible for lactosylation and due to it being substrate for the three applied proteases. Samples were taken for analysis of glycation over a period of 7 days, and the degree of glycation was quantified by application of the fluorescamine assay, whereby free primary amino groups are determined, yielding decreased response as glycation proceeded. After 4 days of incubation, controls and lactosylated protein and peptide substrates were assayed for their susceptibility for proteolysis by plasmin, cathepsin D, and chymosin. The samples were further investigated for changes in secondary and tertiary structure as a result of lactosylation by circular dichroism (CD) and fluorescence spectroscopy, respectively.

2.3 Proteolysis

Assessment of proteolysis by plasmin was carried out at pH 6.8, while the experiments with the aspartic proteases cathepsin D and chymosin were carried out at pH 5.0 due to a lower pH optimum for these proteases. The controls and the lactosylated samples were each divided into three aliquots, of which two were adjusted to pH 5.0 before addition of cathepsin D or chymosin at enzyme/substrate (E/S) molar ratio of 1 : 600. Plasminogen was activated to plasmin by addition of 0.25 U thrombolysin/μg plasminogen and incubated at 37°C for 30 min. The generated plasmin was added to the pH 6.8 aliquots at an E/S ratio of 1 : 1200. The samples were incubated at 30°C for a period of 48 h. The fluorescamine assay was applied also as a method for the quantification of proteolysis due to the increase in the level of free amino terminals as proteolysis proceeds.

2.4 Determination of primary amino groups determined by fluorescamine assay

The fluorescamine assay [30, 31]; was applied both for the quantification of primary amino groups (N-terminals and lysine side chains) in the peptide and protein substrates after lactosylation, and furthermore for the subsequent measurement of hydrolysis of these substrates after the addition of protease. For the indirect measurement of free primary amino groups present in substrates after lactosylation, the following method was applied: 37.5 μL of each of the samples from the lactosylation reaction (Section 2.2) was diluted in triplicates with 1130 μL of 0.1 M borate buffer, pH 8.0. After the addition of 375 μL of 0.2 mg/mL fluorescamine in water free acetone, 250 μL of each of the triplicates was transferred to wells of a 96-well microtiter plate, incubated for 18 min at room temperature before measurement by fluorescence spectroscopy. Samples were taken at $t = 0$, 15, 44, 68, 96, and 168 h. When the assay was applied as a measurement of enzymatic hydrolysis, the same procedure was used except that the hydrolyzed samples (Section 2.3) were precipitated on ice with 24% TCA (1 : 1 v/v) to stop the enzymatic reaction and to separate liberated peptides from undigested substrate and larger fragments. After precipication the samples were centrifuged at 13 000 rpm for 20 min, and 37.5 μL of the supernatant was diluted with 1130 μL borate buffer before addition of 375 μL 0.2 mg/mL fluorescamine in water-free acetone. This solution (250 μL) was transferred to microtiter wells in triplicates, and fluorescence measured after 18 min of incubation. Samples were taken at $t = 0$, 1, 2, 4, 6, 24, and 48 h for measuring enzymatic hydrolysis. Fluorescence spectroscopy measurements were performed using an LS 50B spectrofluorometer from Perkin-Elmer (Beaconsfield, England). Excitation was performed at 390 nm with a Xenon discharge lamp, and a photomultiplier measured emission at 480 nm with bandwidth of 5 nm. Quantification was carried out according to a leucine standard. Using the fluorescamine assay, initial rates in the kinetic study of enzymatic hydrolysis of lactosylated and nonlactosylated substrates were calculated by linear regression of the first three to four points. Significance was tested applying one tail *t*-test.

2.5 Changes in secondary structure of proteins measured by circular dichroism

Changes in secondary structure upon lactosylation were measured by CD. Far-UV CD-spectra were obtained from 190 to 250 nm in a 0.1 cm cell length and samples were diluted to a protein concentration of 10 μM. Step size was 0.2 nm and the spectra were obtained with a scan speed of 50 nm/min. The time constant factor was set to 4 in all measurements. Spectra were accumulated three times each at 25°C. All measurements were performed on a J-810 spectropolarimeter from Jasco (Tokyo, Japan). Illumination of the samples was performed with a Xenon short arc lamp from Osram (Germany) under a flow of 5.0 mL/min of 99.999% nitrogen. Temperature was controlled with a PTC423S peltier element from Jasco (Tokyo, Japan) coupled with a water bath.

2.6 Changes in tertiary structure of proteins measured by fluorescence spectroscopy

Changes in tertiary structure upon lactosylation were measured by fluorescence spectroscopy. Tryptophan excitation

was performed at 298 nm, and emission spectra were obtained from 310 to 450 nm with a resolution of 0.5 nm. The samples that had been incubated at 65 or 55°C with or without lactose, as described above, were compared with samples kept at 5°C, where the proteins are believed to be in their native structure. The spectra obtained for the samples after thermal incubation were normalized through the maximal emission peak obtained for the native proteins, and difference spectra were subsequently obtained by subtracting the spectra obtained for the lactosylated samples from the spectra obtained for the nonlactosylated controls. Emission spectra were measured in triplicates with a bandwidth of 5 nm.

2.7 Mass spectrometry of lactosylated peptide and proteins

Mass spectra were obtained on a MALDI TOF Tandem Mass Spectrometer, Ultraflex TOF-TOF from Bruker (28359 Bremen, Germany). All spectra were obtained in positive reflector mode using a reflector voltage of 1400 V and an accelerating voltage of 20 kV. The samples analyzed included the peptide (VHFFKNIVTARTP) and substrate proteins incubated with or without lactose for 96 h at the conditions described in Section 2.2. The protein substrates were subsequently hydrolyzed with cathepsin D at 30°C for 24 h at enzyme/substrate (E/S) molar ratio of 1 : 600 prior to MS analysis. Custom-made chromatographic columns were used for concentration and desalting of the substrate peptide and the hydrolyzed solutions prior to mass spectrometric analysis [32]. A column consisting of 100 – 300 nL of Poros 50 R2 was equilibrated with 20 µL 0.1% TFA before 5 µL of sample was accumulated at the column. The column was washed with 50 µL 0.1% TFA and finally eluted directly onto the MALDI target with a solution of saturated α-cyano-4-hydroxy-cinnamic acid in 70% ACN, 0.1% TFA and dried before measurement. The hydrolyzed protein substrates were analyzed in MS mode, while the substrate peptide was analyzed in MS/MS mode. FlexAnalysis version 2.4 (Launch Bruker Daltonics, Germany) was applied for analysis of the mass spectra. The ion accelerating voltage in MS/MS mode was 15 kV.

3 Results

Five different milk proteins (β-casein, α-casein, κ-casein, β-lactoglobulin, and lactoferrin) and the peptide substrate H-3392 were applied for the investigation of effects of lactosylation on proteolysis mediated by the three proteases: plasmin, cathepsin D, and chymosin. The α-casein preparation consisted of a mixture of α_{S1}- and α_{S2}-casein. H-3392 was, in initial experiments, found to be a suitable substrate for all three proteases. By MALDI TOF MS it was shown that the nonlactosylated peptide, H-3392, was cleaved by plasmin with preference for cleavage after the lysine residue (K), but cleavage after the arginine residue (R) was also observed (data not shown). Chymosin and cathepsin D exhibited identical specificity towards this peptide substrate, with preference for cleavage between the two phenylalanines (the F – F bond), but cleavage between asparagine (N) and isoleucine (I) was also seen (results not seen).

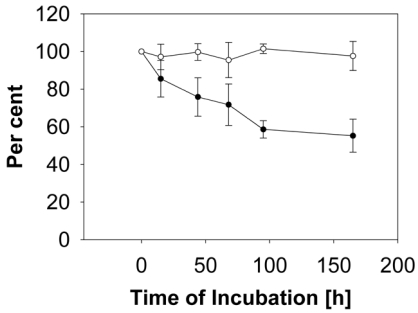

Figure 1. Changes in level of free primary amino groups in α-casein during lactosylation at pH 6.8. Lactosylation was induced by incubation of α-casein and lactose in a molar ratio of 1000:1 at 65°C (●) and control samples were incubated in parallel without lactose (○). The experiments were carried out in triplicates.

The substrates α-casein, β-casein, κ-casein, β-lactoglobulin, lactoferrin, and H-3392 were incubated with lactose for a period of 7 days to obtain lactosylation. Control samples without lactose were incubated at the same conditions. Samples were analyzed for level of accessible primary amino groups (*i.e.* amino terminals and unmodified lysine side chains) by the fluorescamine assay. An example of the development in the level of accessible amino groups during the incubation period is shown for α-casein (Fig. 1). The number of free primary amino groups decreased from $t = 0$ to 4 days (corresponding to 96 h), while after 4 days no further changes were observed in the level of free amino terminals. No significant changes were observed in the control samples incubated in parallel at equivalent conditions, but without lactose. Initial level and the level of free amino groups after 4 days of incubation of each of the substrates with lactose are shown in Table 1. The degree of lactosylation after 4 days of incubation was calculated for each substrate as the decrease in the level of measurable free primary amino groups from 0 to 4 days in relation to the initial level at day 0. The peptide substrate was the one with highest degree of lactosylation after 4 days, while lactoferrin was the substrate with the lowest lactosylation degree. This may reflect that the temperature used for the lactosylation reaction of lactoferrin was lower than for the other substrates. Even though the degree of lactosylation varied, the level of amino groups was found to be significantly lower ($P < 0.05$) after 4 days compared with the initial level.

The lactosylation reaction was verified using MALDI TOF MS analysis of lactosylated substrate proteins and of

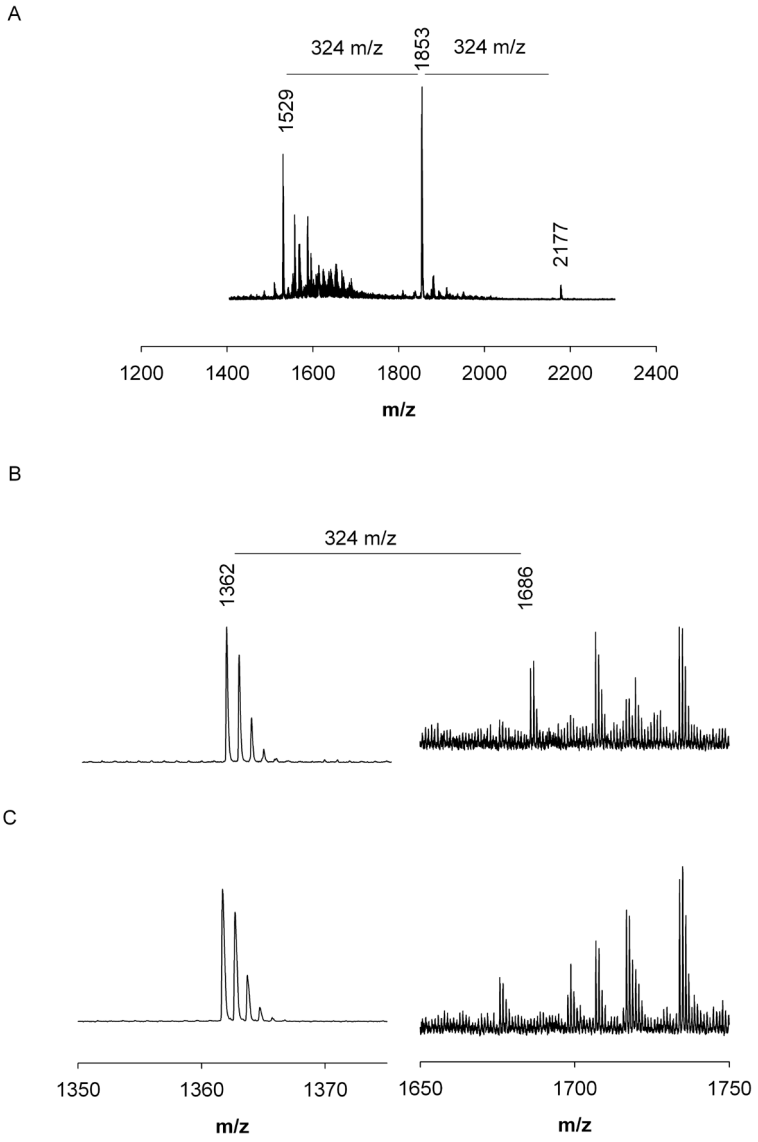

Figure 2. MS spectra of lactosylated H-3392 with a M_r of 1529 g/mol (A) and lactosylated lactoferrin (B) and nonlactosylated lactoferrin (C) after hydrolysis with cathepsin D. Lactosylation induces a mass change of 324 *m/z*, which corresponds to the addition of one lactose molecule, which is not seen in the nonlactosylated sample. In the model peptide H-3392, addition of two lactose molecules is indicated, whereas in the peptide obtained after cathepsin D hydrolysis of lactosylated lactoferrin only includes the binding of one lactose molecule. For simplification only the relevant areas of the spectra are shown.

lactosylated H-3392. The MS analysis of the peptide (unmodified ~1529 *m/z*) indicated binding of one (+324 corresponding to 1853 *m/z*) and two lactose molecules (2177 *m/z*) after 96 h of incubation (Fig. 2 A), but also that some unmodified peptide was still present, which means that the lactosylation was not complete. Additional ions were seen in the spectrum of the lactosylated peptide indicating other side reactions than just binding of one or two lactose molecules. Further MS/MS analysis of the lactosylated H-3392 showed that the peptide was modified both at the lysine residue as well as at the amino-terminal valine residue (data not shown). Lactosylation was also seen in the structure of the substrate proteins. As an example, one of the lactosylated peptides (1686 *m/z*) from lactoferrin obtained after hydrolysis by cathepsin D is shown (Fig. 2B). The unmodified peptide mass (1362 *m/z*) is indicated as well. The mass addition to the nonlactosylated peptide (1362 *m/z*) corresponding to the mass of one lactulosyl molecule (+324 *m/z*) was not present in the nonlactosylated lactoferrin (Fig. 2C). Furthermore, it is seen that not all peptide molecules were modified by lactose, as some unmodified peptide (1361 *m/z*) was still evident in the spectrum after the lactosylation procedure.

After 4 days of incubation, lactosylated substrates and nonlactosylated controls were incubated with plasmin at pH 6.8, or with cathepsin D or chymosin at pH 5.0. Lactosylation of β-casein, α-casein, κ-casein, H-3392, β-lactoglobulin, and lactoferrin highly affected hydrolysis by plasmin (Figs. 3A – F). The generation of peptides, due to hydrolysis by plasmin, was measured as increase in amino terminals by the fluorescamine assay, and was found to be lower for the lactosylated substrates than for the controls. The observed initial rates for hydrolysis were rather low, and were probably due to the fact that only peptides soluble

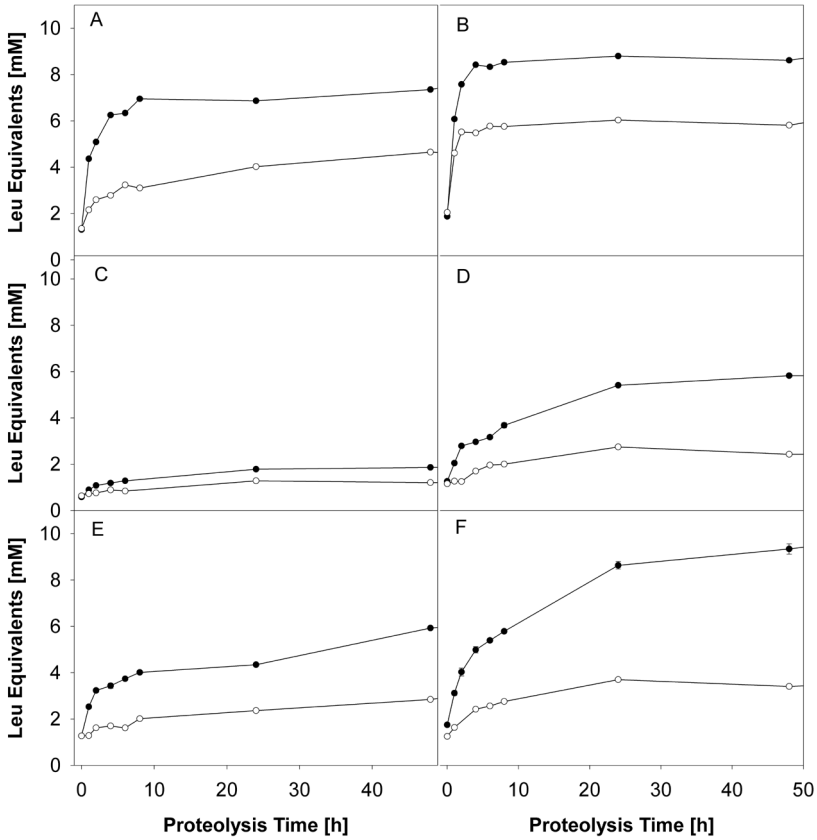

Figure 3. Hydrolysis by plasmin measured as levels of free primary amino groups by the fluoresceamine assay after lactosylation of the substrates. The hydrolysis was measured for lactosylated samples (○) and for control samples (●) and given as the mean of triplicates. (A) β-casein, (B) H-3392, (C) α-casein, (D) β-lactoglobulin, (E) κ-casein, and (F) lactoferrin. Plasmin was incubated at 1:1200 E/S ratio with the substrates at pH 6.8 and 30°C.

Table 1. Level of free primary amino groups in protein samples incubated with molar excess of lactose of 1000:1 at 65°C (lactoferrin 55°C) and at pH 6.8. Changes in free primary amino groups as a result of lactosylation of lysine residues were measured at 0 and 4 days (Leu equivalents). The degree of lactosylation after 4 days, based on the decrease in the level of free amino groups from 0 to 4 days, is given in percentage. The measurements were carried out in triplicates and are given as leucine equivalents [mM]. One tail *t*-test was performed to test for significance in the level of free primary amino groups at $t = 0$ and 4 days of incubation

	0 days		4 days		
	Mean	SD	Mean (%)	SD	*P*-value
α-Casein	2.3	0.38	1.3 (43)	0.19	0.009
β-Casein	2.0	0.14	1.4 (30)	0.19	0.006
κ-Casein	3.4	0.48	1.9 (44)	0.06	0.002
β-Lactoglobulin	2.4	0.31	1.5 (37)	0.34	0.015
Lactoferrin	2.9	0.14	2.4 (17)	0.19	0.008
H-3392	1.2	0.14	0.34 (72)	0.10	0.0004

in 12% TCA were determined in the assay employed, and therefore favors the determination of relatively small peptides.

In contrast with plasmin, lactosylation did not affect the cleavage of β-casein, α-casein, κ-casein or β-lactoglobulin by cathepsin D, whereas the cleavage of the H-3392 peptide substrate and lactoferrin was affected by lactosylation to some extent (Figs. 4A – F). The release of peptides due to enzymatic hydrolysis by cathepsin D was found to be lower for lactosylated H-3392 and for lactosylated lactoferrin compared with the nonlactosylated controls. The hydrolysis by chymosin showed similar hydrolysis pattern as cathepsin D towards these substrates (results not shown).

The hydrolysis of lactosylated substrates by the proteases plasmin, cathepsin D, and chymosin can be characterized both by calculating intial rates as well as by the number of free primary amino groups determined at the end point of the hydrolysis experiment (after 48 h of incubation). Initial rates and end point hydrolysis (48 h) are illustrated as the percent of the same parameters (rates and end point) found after hydrolysis of nonlactosylated substrates (Fig. 5). Values below 100% illustrate lower enzymatic hydrolysis of the lactosylated substates than that of their nonlactosylated controls. The relative initial rate for hydrolysis by plasmin was highly affected by lactosylation for all substrates (Fig. 5A). The highest negative effect of lactosylation for hydrolysis by plasmin was seen for the two globular milk proteins, lactosylated β-lactoglobulin and lactosylated lactoferrin, where the relative initial rates were reduced to 6 and 10%, respectively, of the nonlactosylated control samples. Plasmin hydrolysis of α- and κ-casein was also highly affected by lactosylation resulting in initial rates reduction

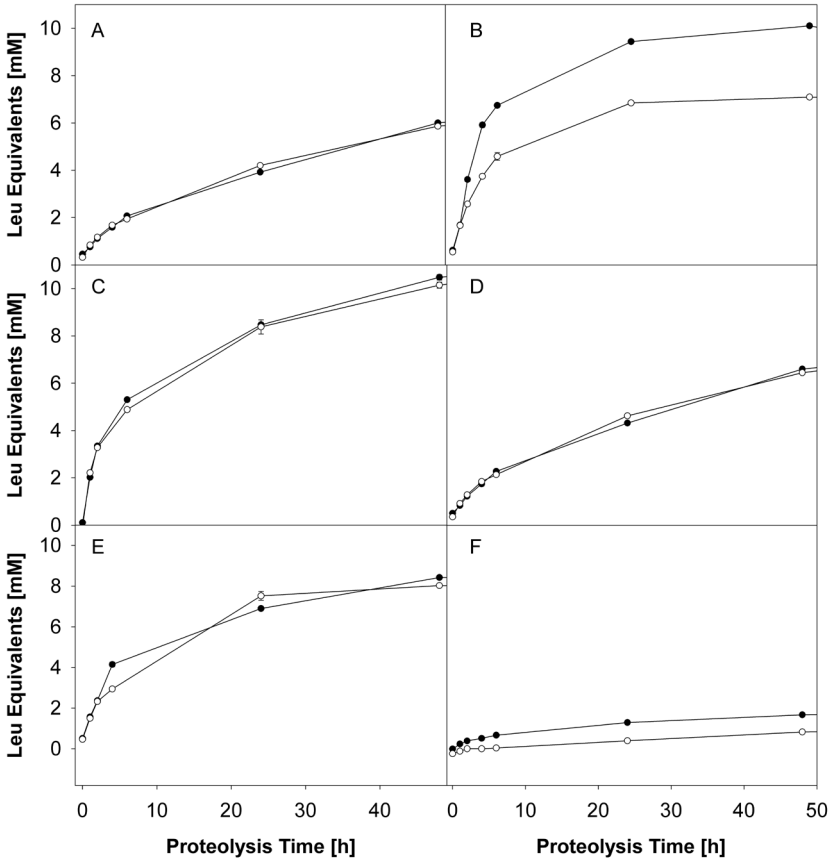

Figure 4. Hydrolysis by cathepsin D measured as levels of free primary amino groups by the fluoresceamine assay after lactosylation of the substrates. The hydrolysis was measured on lactosylated samples (○) and control samples (●) and given as the mean of triplicates. (A) β-casein, (B) H-3392, (C) α-casein, (D) β-lactoglobulin, (E) κ-casein, and (F) lactoferrin. Cathepsin D was incubated at 1 : 600 E/S ratio with the substrates at pH 5.0 and 30°C.

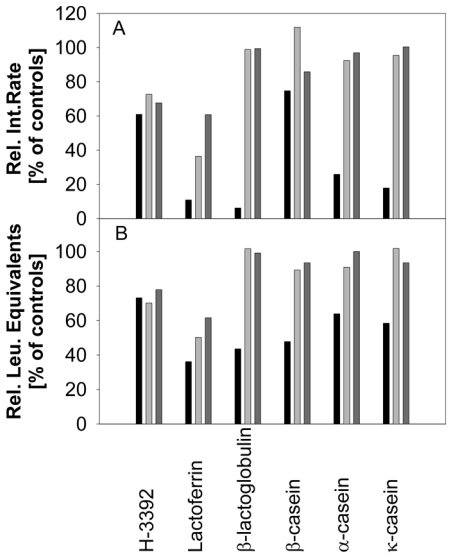

Figure 5. Proteolytic activity measured for the three enzymes plasmin (black), chymosin (light grey) and cathepsin D (dark gray) by the fluorescamine assay. (A) Initial rates for proteolysis determined for lactosylated substrates in relation to initial rates for the cleavage of nonlactosylated controls. (B) End point level for hydrolysis of the lactosylated substrates determined after 48 h of incubation. Initial rates (A) and end point hydrolysis levels (B) are given as percent of the same parameters determined for the nonlactosylated substrates: H-3392, lactoferrin, β-lactoglobulin, β-casein, α-casein, and κ-casein.

to 26 and 18%, respectively. For hydrolysis by plasmin, H-3392 and β-casein were the substrates being less affected by lactosylation. Initial rates for the hydrolysis by cathepsin D and chymosin were mostly affected by lactosylation of the gobular protein lactoferrin, while initial hydrolysis of the caseins and β-lactoglobulin by cathepsin D and chymosin was less affected by lactosylation. A decrease in the initial rate for hydrolysis with chymosin and cathepsin D was also seen for lactosylated H-3392 peptide.

After 48 h of incubation, end point analysis of fluorescamine levels confirmed that hydrolysis by plasmin was highly affected by lactosylation (Fig. 5B). Lactosylation of all the substrate proteins resulted in a decreased hydrolysis by plasmin. No changes in response to lactosylation were seen after 48 h for the hydrolysis of β-lactoglobulin and for the three caseins by chymosin and cathepsin D, whereas lactosylation negatively affected end-point hydrolysis of H-3392 and lactoferrin.

CD was applied for investigation of changes in secondary structure upon lactosylation of the proteins. No changes were seen for the random coil proteins, α, β, and κ-casein in the CD-spectra after lactosylation (data not shown). Loss of β-sheet, which is characterized by a deep signal between 210 – 220 nm in the structure of β-lactoglobulin (Fig. 6A), was seen after incubation at 65°C (control) for 4 days. The CD-spectra for β-lactoglobulin incubated with lactose for 4 days at 65°C showed further loss of β-sheets, and the CD-

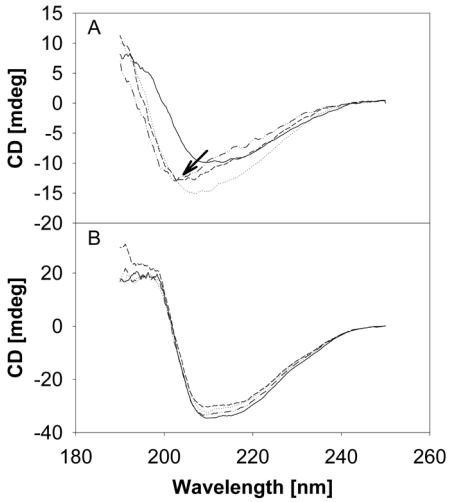

Figure 6. Secondary structure of native, lactosylated, and heat-treated controls was measured by circular dichroism. CD-spectra of native (–), lactosylated for 4 (- - -), and 7 days (– · –) and 4 days heated control (· · ·). (A) β-lactoglobulin and (B) lactoferrin. Loss of β-sheet in β-lactoglobulin is indicated by an arrow. The CD-spectra was obtained as three accumulations with a scan speed of 50 nm/min and the step size was 0.2 nm.

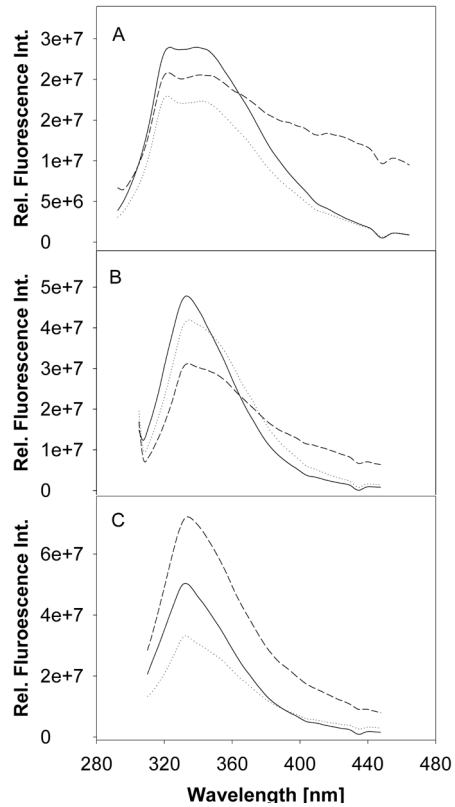

Figure 7. Emission spectra of (A) β-casein, (B) β-lactoglobulin, and (C) lactoferrin. Emission spectra were obtained for native proteins (–), heat-treated controls (· · ·) and samples lactosylated for 4 days (- - -). All spectra were obtained by excitation at 298 nm.

spectra indicated mostly random coil structure, which is characterized by a negative signal at 200 nm. No further changes were seen after 7 days of incubation with lactose. In contrast, no changes were seen in the highly α-helical-structured lactoferrin after incubation at 55°C for 4 or 7 days with or without the addition of lactose (Fig. 6B). The α-helical structure is characterized by a deep signal at 205 and 220 nm.

Changes in tertiary structure induced by lactosylation were further investigated by fluorescence spectroscopy using tryptophan excitation at 298 nm. Emission spectra were obtained for samples of the various substrate proteins incubated at 65°C, or in the case of lactoferrin at 55°C, for 4 days with or without the presence of lactose. Furthermore, a non heat-treated sample of each substrate protein was studied. The emission maximum for κ-casein was located at 335 nm, and for α-casein the emission maximum was at 350 nm (data not shown). β-Casein revealed a less well-defined maximum in the emission spectrum with a broad plateau between 335 and 352 nm (Fig. 7A), whereas the emission maximum for non heat-treated β-lactoglobulin and lactoferrin was observed at 332 nm (Figs. 7B and C). The emission spectra of β-lactoglobulin, κ-casein, α-casein, and β-casein were red-shifted towards wavelengths >350 nm for the lactosylated samples compared to the non-treated samples (Figs. 7A and B for β-casein and β-lactoglobulin as examples), whereas lactosylation of lactoferrin actually revealed a sharper peak at 332 nm with a more narrow half-high peak-width compared to the nontreated sample (Fig. 7C). The heat-treated lactoferrin controls still had emission maximum at 332 nm, but the peak was less intense and well-defined in this region. Difference spectra of the lactosylated samples and their heat-treated controls for β-lactoglobulin, κ-casein, α-casein, and β-casein all showed maxima around 410 nm, while the difference spectra for lactoferrin showed an emission minimum at the same position. These results are summarized in Fig. 8.

4 Discussion

The aim of this study was to investigate the influence of heat-induced lactosylation on the hydrolysis of the substrate proteins lactoferrin, β-lactoglobulin, β-casein, α-casein, and κ-casein, as well as in the lysine containing peptide H-3392 by three different proteases plasmin, cathepsin D and chymosin. Mass spectrometric analysis by MALDI TOF confirmed that lactosylation had occurred after 4 days of incubation of substrate proteins with lactose. This modification was further detected as a decrease in the level of free amino groups due to occupation of lysine residues by lactose by using the fluorescent probe fluorescamine. Degrees of lactosylation of the proteins after 4 days of incubation with lactose varied from 44% for κ-casein to 17% for lactoferrin. However, beyond 4 days of incubation no further

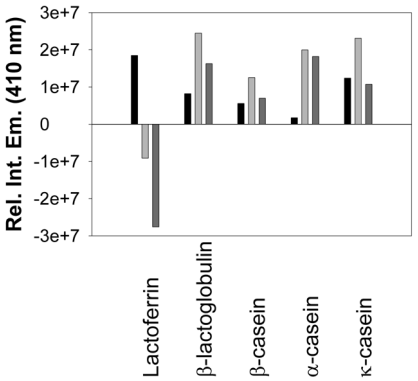

Figure 8. Emission intensities measured at 410 nm after trp-excitation at 298 nm. Heat-treated control, 4 days incubation (black). Samples incubated for 4 days within the presence of lactose in molar excess of 1000 : 1 (light gray). Difference in emission intensities measured at 410 nm between the samples incubated at 65°C (lactoferrin 55°C) with and without the presence of lactose (dark gray). All spectra were normalized through the emission maxima for the nonheat-treated sample before the subtractions were performed. All evaluated spectra were means of triplicate experiments.

changes were obtained in the level of free primary amino groups, even though the lactosylation was not complete as also shown by the MS-analysis. It is possible, however, that further reactions involving these free amino groups occur, as it has been described that intermediates derived from decomposition of Amadori compounds could be susceptible to further reactions with free amino groups [33] via Strecker degradations [34]. Even though further reactions involving the lactosylated adducts formed are likely to occur, these were not detected by the fluorescamine assay used.

The study showed that for all the tested substrates hydrolysis by plasmin was influenced by lactosylation. On the contrary, lactosylation did not affect the hydrolysis by chymosin and cathepsin D of the three casein substrates and of β-lactoglobulin. Instead, decrease in the level of end point hydrolysis as well as decreased initial rates were observed for hydrolysis of lactoferrin and H-3392 by chymosin and cathepsin D.

The decrease in susceptibility for hydrolysis of protein and peptide substrates after lactosylation by plasmin can be explained by the fact that the lactosylation is known to be located primarily at the ε-amino group of lysine residues in the polypeptide backbone [8], which is also the preferred cleavage site for the bovine plasmin [35]. As plasmin is able to cleave after arginine as well, though with less preference, cleavage of the arginyl bonds may therefore contribute to the observed hydrolysis. The fact that plasmin hydrolysis was still observed in lactosylated samples was probably due to the incomplete lactosylation of the lysine residues, but could also be due to hydrolysis of unmodified arginyl bonds. However, lactosylation of arginine residues has been shown to occur to some extent [8], which complicates the interpretation of the data. The end point hydrolysis level after 48 h incubation of lactosylated substrates with enzyme compared to end point hydrolysis level for the non-lactosylated samples showed the influence of lactosylation on the susceptibility for proteolysis by plasmin to be in the following order of magnitude: hydrolysis of H-3392 was least affected by lactosylation followed by α-casein < κ-casein < β-casein < β-lactoglobulin < lactoferrin, *i. e.* that lactosylation of lactoferrin gave the largest decrease in plasmin hydrolysis of the substrates studied. These findings indicate that the effect of lactosylation on plasmin hydrolysis may be dependent on the structure of the substrate proteins, leaving the globular proteins to be the ones that are most highly affected by lactosylation. Comparison of the initial rates for hydrolysis by plasmin revealed a similar relation, as the initial rates for the globular proteins were mostly affected by lactosylation. The tertiary structure has impact on the accessibility of the proteases as cleaving sites may be more or less exposed. Changes in tertiary structure were hence followed by fluorescence spectroscopy. Applying tryptophan-excitation indicated that lactoferrin was the only substrate protein where lactosylation did not induce unfolding of the protein structure. The emission spectra obtained for lactoferrin rather indicated less solvent exposed tryptophans, which may imply a more compact tertiary structure of lactoferrin after lactosylation and thereby some of the tryptophan residues were shielded for the solvent by bound lactose molecules. Lactoferrin has been shown to be able to bind advanced glycation end products (AGEs) [36], and this binding may inhibit the antibacterial effect of lactoferrin [37]. Hence, this noncovalent binding to other AGE-modified lactoferrin molecules may actually stabilize the tertiary structure of lactoferrin without altering the secondary structure of the protein. Binding of sugars to the D-galactose/D-glucose-binding protein from *Escherichia coli* was found to stabilize the conformation of this particular protein against pressure [38], and lactoferrin may be stabilized in a similar way after binding of either lactosylated lactoferrin or lactose. Even though the hydrolysis of both β-lactoglobulin and lactoferrin was highly affected by lactosylation, fluorescence spectroscopy indicated that lactosylation induced unfolding of β-lactoglobulin, while the Trp-excitation results indicated a more compact tertiary structure of lactoferrin after lactosylation. This indicates that both occupation of lysine residues by lactose and the changes in tertiary structure induced by lactosylation are decisive for the plasmin hydrolysis of these substrate proteins.

While plasmin hydrolysis was affected by lactosylation for all substrates, only hydrolysis of the globular protein lactoferrin and of the peptide substrate, H-3392, seemed to be substantially affected by lactosylation when applying chymosin and cathepsin D as proteolytic enzymes. The proposed tighter tertiary structure of lactoferrin, that seemed to

be induced upon lactosylation, may explain the decreased hydrolysis seen by these proteases after lactosylation. Even though lactosylation of lactoferrin affected hydrolysis by these two aspartic proteases, the effect was smaller than observed for hydrolysis by plasmin. Lactoferrin has previously been found to be resistant to hydrolysis by chymosin [39], but in this study, cleavage of lactoferrin was seen for both aspartic proteases. Changes were seen in secondary and tertiary structure after lactosylation of β-lactoglobulin, which however, did not seem to affect the proteolysis by cathepsin D and chymosin. Increased hydrolysis after lactosylation has, however, been observed in another study of β-lactoglobulin cleaved by pepsin [1]. Enhancement in hydrolysis after lactosylation has previously been reported in literature for other proteins. Hence treatment with glucose in low concentrations (up to 20 mM glucose) actually enhanced the proteolysis of BSA by an ATP-dependent proteolytic system from reticulocytes [40]. Increased glucose concentration, however, showed continuous decrease in proteolysis of BSA.

A decrease in hydrolysis by cathepsin D and chymosin was seen for H-3392 after lactosylation. As a lysine residue (K), being the preferred lactosylation site, is located next to the by chymosin and cathepsin D preferred cleaving site (F – F) in the employed peptide substrate H-3392, steric hindrance may explain this observation. The hydrolysis by cathepsin D and chymosin of the caseins was only marginally affected by lactosylation probably due to the less well-defined secondary structure of the caseins. The accessibility by chymosin and cathepsin D for the cleavage sites in the casein structures seemed to be rather unaffected by lactosylation even though fluorescence spectroscopy measurements indicated more solvent-exposed tryptophan residues after lactosylation in all of the three casein structures.

In summary, the results of this study indicate that lactosylation has negative effect on the enzymatic hydrolysis by plasmin. Lactosylation did not seem to have significant impact on the hydrolysis of caseins by the two aspartic acid proteases, chymosin and cathepsin D. Lactosylation may therefore be of minor relevance in relation to proteolysis by chymosin and cathepsin D during cheese manufacture and ripening. The contribution of plasmin in cheese ripening has been widely accepted [41], but is dependent on cheese variety [35]. On this basis, it seems possible that plasmin-mediated proteolysis can be negatively affected in milk products and in cheese varieties where lactosylation occurs.

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