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In vitro digestion of bovine and caprine milk by human gastric and duodenal enzymes

Hilde Almaas[a,*], Anne-Laure Cases[a], Tove G. Devold[a], Halvor Holm[b], Thor Langsrud[a], Lars Aabakken[c], Tormod Aadnoey[d], Gerd E. Vegarud[a]

[a]*Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P. Box 1432-Ås, Norway*
[b]*Department of Nutrition, University of Oslo, P. Box 1046 Blindern, 0316 Oslo, Norway*
[c]*University of Oslo, Gastrointestinal Endoscopy, Rikshospitalet, 0027 Oslo, Norway*
[d]*Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, P. Box 1432-Ås, Norway*

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Abstract

In vitro digestion was performed by human proteolytic enzymes on bovine and caprine individual milks. Two types of caprine milk were investigated: with high and low contents of α_{S1}-casein (CN). In addition the influence of heating of the milk on digestion was examined. The digestion was performed in two steps using human gastric and duodenal juice. Protein and peptide profiles were studied by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF). Caprine milk proteins were digested faster than bovine milk proteins. This was confirmed by the degradation profile obtained for both cows' and goats' milk, and was most evident for β-lactoglobulin. Comparing the digestion of milk protein from two groups of goats, high and low in α_{S1}-CN content, respectively, did not show significant differences. Heat treatment of milk had a strong and significant effect on the level of digestion. Raw milk was degraded faster than the heat-treated milk, and the effect of heating was different for bovine and caprine milk.
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Keywords: Digestion; Human proteolytic enzymes; Caprine milk; Bovine milk; Genetic polymorphism; Heat treatment

1. Introduction

Milk proteins provide a major dietary source for humans, supplying amino acids for the synthesis of proteins and other nitrogen-containing compounds (Munro, 1969; Millward & Pacy, 1995; Young & Pellet, 1989). In addition, some of these proteins contain bioactive peptides released by hydrolysis that may affect the human health. These effects include mineral binding, growth factors, blood pressure reduction (Tomé & Debabbi, 1998) and protective properties against different micro-organisms and viruses (Meisel & Schlimme, 1996; Pihlanto & Korhonen, 2003). The nutritional efficiency of milk proteins clearly depends on the content of essential amino acids that is delivered during the digestion of the proteins, and the absorption in the gut of amino acids and peptides released (Bos, Gaudichon, & Tomé, 2000).

There has recently been an increased attention on cows' milk allergy, particularly among infants (Paupe, Paty, de Blic, & Scheinmann, 2001; Sampson, 2004). As a result, alternative sources for milk have been asked for. This has lead to an increasing interest in and demand for caprine and equine milk. Milk from the goat differs from that of the cow in the composition of many components, which may influence the digestibility of the milk. The composition and structure of the fat, for instance, is quite different in both types of milk. Goats' milk contains smaller fat globules and higher amounts of short-chain fatty acids. The naturally emulsified fat of goats' milk is, from a human health standpoint, much easier to digest (Haenlein, 1992).

Also, the protein composition and structure of milk of these animals differ, again with possible consequences for the digestibility. Although the general distribution of

*Corresponding author. Tel.: +47 64945866; fax: +47 64965901.
E-mail address: hilde.almaas@umb.no (H. Almaas).

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proteins is quite similar in the milk of the two species, variations in both the whey and casein (CN) fractions occurs. Focus on genetic polymorphism of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) has revealed important differences between the bovine and caprine milk (Hill et al., 1997; Moatsou, Hatzinaki, Samolada, & Anifantakis, 2005; Trujillo, Casals, & Guamis, 2000). A recent study demonstrated major differences between hydrolysis of bovine and ovine β-LG by pepsin, due to small variations in the tertiary structure (El-Zahar et al., 2005). The ovine β-LG was hydrolysed faster. No comparative investigations have been performed on caprine and bovine whey proteins so far.

Studies of the genetic polymorphism of bovine and caprine CNs have shown that the composition of α-, β-, and κ-CN is different in the milk from the two species (Buchberger & Dovc, 2000; Ikonen, Ojala, & Syvaoja, 1997; Trujillo et al., 2000). The polymorphism of α_{S1}-CN has received considerable interest due to its strong influence on the technological properties of the milk. Bovine milk has nine different genetic variants, however, type B is dominant in Europe (Bech & Kristiansen, 1990; Lien et al., 1999; McLean, Graham, Ponzoni, & McKenzie, 1984). For caprine milk, eighteen different alleles are identified. Four different levels of α_{S1}-CN content in the milk have been found, referred to as "strong", "medium", "weak" and "null". The strong variant contains about $3.6\,g\,L^{-1}$ α_{S1}-CN, while the "null" variant completely lacks this protein. These differences are due to deletions, substitutions and insertions in the DNA, defective mRNA processing and loss of mRNA stability (Chianese, Ferranti, Garro, Mauriello, & Addeo, 1997; Martin, Ollivier-Bousquet, & Grosclaude, 1999; Martin, Szymanowska, Zwierzchowski, & Leroux, 2002). In the Norwegian Dairy Goat breed, a remarkable high frequency (70%) is lacking the α_{S1}-CN in the milk (Vegarud et al., 1999).

Besides depending on the type and amount of protein present in the milk, its digestibility may also be affected by heat denaturation. It has been reported that the heat stability of caprine milk is lower than that of bovine milk (Morgan, Jacquet, Micault, Bonnin, & Jaubert, 2000). The degree of denaturation is dependent on several factors as heating temperature, time, pH, ionic strength and the concentration of soluble calcium and phosphate. CNs have an open and flexible structure, giving a rather high stability towards heat treatment. However, it is reported that heating up to 90 °C may induce changes in the size of the CN micelles (Devold, Brovold, Langsrud, & Vegarud, 2000; Singh & Creamer, 1992). When milk is heated complex reactions take place between whey proteins and CNs. Three types of aggregates are formed; CN and whey protein aggregates, CN micelles coated with whey proteins, and internal aggregates between the different whey proteins (Singh, 1995; Singh & Creamer, 1992).

The goal of the present study was to compare the in vitro digestion of the proteins in samples of milk using human gastric and duodenal enzymes, with major focus on three aims: (i) comparison of the degradation of proteins in bovine and caprine milk; (ii) evalution of difference in digestibility of caprine milk with high content of α_{S1}-CN and without this protein, respectively; (iii) the influence of heat treatment of milk on the digestibility of the proteins in bovine and caprine milk.

2. Materials and methods

2.1. Collection and preparation of milk samples

Milk from 16 goats was collected individually from the university farm in the southern part of Norway, in addition to milk from six genetically typed goats in northern Norway. All animals were of the breed Norwegian Dairy Goat, undergoing the same type of traditional feeding. The goats were divided in to two groups: eight goats lacking the α_{S1}-CN in the milk (G0), and eight goats expressing high amounts of α_{S1}-CN (GS). Grouping was done by genetic typing, or by analysis of the α_{S1}-CN content in the milk by isoelectric focusing (IEF). Milk from eight Norwegian red cattle cows from the university farm was also obtained individually.

Analyses were carried out on skimmed milk. Fat was removed by heating for 20 min at 37 °C, followed by centrifugation at $3100 \times g$ (Bench top Beckman GPR Refrigerated Centrifuge, swinging bucket rotor GH 3.7, Beckman Coulter, CA, USA) for 20 min. After storage at −20 °C for 20 min, fat was removed by a spatula.

The effect of heating was studied on mixtures of 20 mL milk from each individual animal. These mixtures were prepared by pooling samples of milk from goats with low content of α_{S1}-CN, from goats with high amount of α_{S1}-CN and cows, respectively. Each of the mixtures was divided into three different fractions, two undergoing different heat treatments, pasteurisation (72 °C, 15 s) and high heating (100 °C, 1 min), while the third fraction was kept untreated as a reference. Each mixture contained milk from eight individual animals, and all measurements were repeated twice.

2.2. Protein content

Determination of total nitrogen (TN) and non-casein nitrogen (NCN) in the milk was performed by Kjeldahl analysis (IDF, 1993) according to standard protocol (Kjeltec 1035 Analyser, Tecator, Höganäs, Sweden). CN nitrogen was calculated as the difference between TN and NCN. The conversion factor of 6.38 was used to obtain the content of crude protein (CP) and CN. The NCN fraction was obtained in skimmed milk, after precipitation of the CNs, according to a modified method of Aschaffenburg and Drewry, (1959), and Pierre, Michel, and Le Graet (1995).

2.3. Human gastric and duodenal enzymes

Human proteolytic enzymes were obtained in the activated state by collecting human gastric (HGJ) and duodenal juice (HDJ) according to Holm, Hanssen, Krogdahl, and Florholmen (1988). All gastric and duodenal enzymes used in this study were obtained from one person. In brief, a three-lumen tube enabled both simultaneous instillation of saline in the duodenum, and aspiration of gastric and duodenal juice from the volunteer. Saline ($100\,mL\,h^{-1}$) was instilled close to the papilla of Vater to stimulate the production of proteolytic enzymes, and duodenal juice was aspirated some 18 cm distally. Aspirates were collected on ice and frozen in aliquots. Before further use the individual samples of HGJ and HDJ were mixed into two batches to avoid differences in enzyme activity between the samples.

Proteolytic activity in the HGJ was assayed according to Sanchez-Chiang, Cisternas, and Ponce (1987). The pepsin activity was measured with bovine haemoglobin at pH 3.0 as a substrate. In HDJ the concerted action of proteases and peptidases named "Total proteolytic activity" was assayed at pH 8.0 with CN as substrate according to Krogdahl and Holm (1979). The reactions were stopped after 20 min of incubation by addition of 10% TCA. After centrifugation, absorbance at 280 nm of the trichloroacetic acid soluble hydrolysis product was used as measure of proteolysis. One unit (U) of enzyme activity is defined as the amount of enzyme that gives an absorbance of 1.0 at 280 nm in 20 min at 37 °C.

2.4. Digestibility assay—pH drop method

A modified digestibility assay, in vitro protein digestion (AOAC Official Method 982.30; Rasco, 1994), was performed in two steps, using HGJ and HDJ. The procedure developed to mimic a "normal digestion" in the human gastro-intestinal tract consisted of two incubation periods, imitating both the human stomach and the duodenum. Each period lasted 30 min at 37 °C. Previous results showed that no new peptides were produced with an extended reaction time (unpublished results). First, 10 mL of skimmed milk acidified to pH 2.5 with 2 M HCl were incubated with 50 μL (0.4 U) HGJ. Then the pH was adjusted to 7.5 with 1 M NaOH, 400 μL (13.0 U) HDJ was added and the mixture was incubated again with continuous stirring. The change in pH in the milk during the degradation with HDJ was measured every minute, and the corresponding pH curves were plotted. Aliquots (0.5 mL) were also taken out for gel electrophoresis at different times during the incubation. To stop the proteolytic reactions, samples were put on ice, frozen and freeze dried.

The assay was performed with individual milk samples from eight animals of each group: two groups of goats, and one group of cows. Each sample was run in duplicate, and results are presented as the average of all 16 measurements within each group.

2.5. Gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to evaluate the protein profile after each step of hydrolysis (PhastSystem^{TM}, Pharmacia Laboratory Separation Division, Amersham Biosciences, Uppsala, Sweden). The assay was performed according to standard protocols (Laemmli, 1970), using 20% acrylamide gels (PhastSystem^{TM} Homogeneous 20 gels, Amersham Biosciences). The molecular mass markers used were the low molecular weight standard kit (LMW Calibration kit, Amersham Biosciences). Staining was performed according to standard procedure (Amersham Biosciences). Gels from SDS-PAGE were scanned, and the amount of protein was quantified by analysis using Image Master 1D quantification software (Amersham Biosciences). The amount of protein was divided with the total protein content in the milk (see Section 2.2), in order to be able to compare results between different milk samples.

Genetic variants of the α_{S1}-CN from individual goats were determined by IEF using ultra thin (0.3 mm) urea containing polyacrylamide gels according to a modified method of Erhardt (1989) (Devold et al., 2000; Vegarud et al., 1989). A mixture of ampholytes was chosen in order to give a maximum resolution of the caprine α_{S1}-CN-complex; Ampholine pH 3.5–5.0, Pharmalyte pH 4.2–4.9 and Pharmalyte pH 5.0–6.0 (Amersham Biosciences) were used in the ratio 3:4:1. Coomassie Brilliant Blue R-250 was used for staining. The different α_{S1}-CN-variants of goats were identified according to lyophilised CN samples from goats known to be strong or lacking the α_{S1}-CN in the milk (kindly provided by Prof. F. Grosclaude, INRA, France).

2.6. Statistics

Student's t tests (two-sample, assuming equal variances) were run to compare the protein and CN contents in the different types of goats' milk (assuming one-tail alternative). Differences were considered significant when p values were less than 0.05, here and in the following analyses. For the digestion studies the drop in pH during the first 5, 10 and 30 min of hydrolysis with HDJ were studied. The drop in pH was modelled as dependent on the groups of goats and cows (in proc GLM of SAS) using the model:

$$\text{Drop in pH} = \text{mean} + \text{milk group} + \text{error}. \qquad (1)$$

Milk group was either from goats lacking the α_{S1}-CN (G0), from goats expressing the α_{S1}-CN (GS) or from cows. The term "error" is the effect of each of the eight individuals in a group, in addition to random error. Significances of pairwise milk-type comparisons, and the contrast of goat versus cow were estimated.

When comparing the effect of heat treatment on mixtures of milk samples from the eight individual animals

in each group, this model was used:

$$\text{Drop in pH} = \text{mean} + \text{milk group} + \text{heating} + (\text{milk type} \times \text{heating}) + \text{error}. \quad (2)$$

Milk group was in this model 2 the group of which the milk samples were mixed. The heating methods used were pasteurisation, high heat treatment/sterilisation and no heat treatment (raw milk). The term "error" is from duplicate samples of the same bulk milk. Significances of pair-wise milk-type or pair-wise heating comparisons were estimated.

3. Results

3.1. Comparison between hydrolysates from individual bovine and caprine milk by gel electrophoresis

The degradation products from cows' and goats' milk, after treatment with human gastric (pH 2.5) and duodenal (pH 7.5) enzymes were studied by SDS-PAGE. Both steps of hydrolysis were carried out during 30 min at 37 °C. The protein profile after the two digestion steps, for individual samples of both bovine and caprine milk is illustrated in Fig. 1. The amount of proteins in each lane was quantified by Image Master 1D quantification software. Fig. 1 shows that the digestion with HGJ caused a major degradation of the native proteins in milk from both cow and goat (lane C1 and G1). The CNs were rapidly hydrolysed in milk from both species. This was also observed for α-LA, serum albumin, immunoglobulins and lactoferrin (LF). However, the degradation of β-LG in cows' and goats' milk differed. Most of the β-LG remained undigested (100%) during the first degradation step with HGJ in bovine milk, while in caprine milk approximately 35% remained intact. After this first degradation step, digestion of cows' milk also seemed to produce more low molecular weight peptides than digestion of goats' milk.

During the second enzymatic step (Fig. 1, lane C2 and G2), further degradation of both CNs and whey proteins were observed. The most obvious difference between cows' and goats' milk was the digestibility of β-LG. Data from Image Master showed that only about 23% of β-LG remained undigested in the caprine milk, while the amount of native β-LG in bovine milk was 83% after treatment with HDJ.

IEF was used to identify the expression of α_{S1}-CN in milk from individual animals. The results of the protein profile of milk from the individual Norwegian goats grouped as G0, GW and GS are shown in Fig. 2A. Seventy percent of Norwegian goats seem to lack the α_{S1}-CN, and most Norwegian animals belong to the group G0. Analysis of the total CN content in the milk (*t* test, see Section 2.6) revealed significant differences ($p = 0.04$) between the two groups of goats, G0 and GS. However, no significant difference in total protein content was found between the groups ($p = 0.12$).

The degradation of milk containing high amount of α_{S1}-CN (GS) is shown in Fig. 2B. The digestion profile of the proteins showed a fast degradation of the α_{S1}-CN with HGJ, while β-CN, κ-CN and β-LG seemed to be degraded

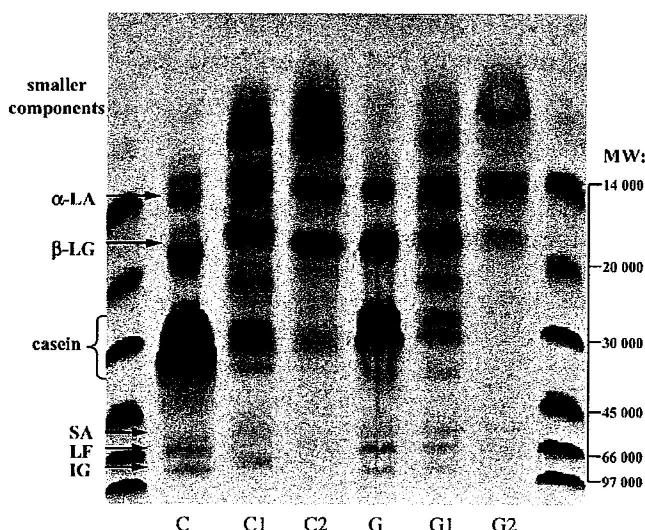

Fig. 1. SDS-PAGE (20%) of skimmed milk from cow and goat (low amount of α_{S1}-casein), digested with human gastric and duodenal enzymes. Arrows indicate the major bands; immunoglobulins (IG), lactoferrin (LF), serum albumin (SA), casein, β-lactoglobulin (β-LG), α-lactalbumin (α-LA) and peptides. The wells contain: (C) native cows' milk, (C1) cows' milk hydrolysed with HGJ (30 min, 37 °C), (C2) cows' milk hydrolysed with HGJ (30 min, 37 °C) and HDJ (30 min, 37 °C), (G) native goats' milk, (G1) goats' milk hydrolysed with HGJ (30 min, 37 °C), (G2) goats' milk hydrolysed with HGJ (30 min, 37 °C) and HDJ (30 min, 37 °C). Standard molecular weight markers are shown on each side of the gel.

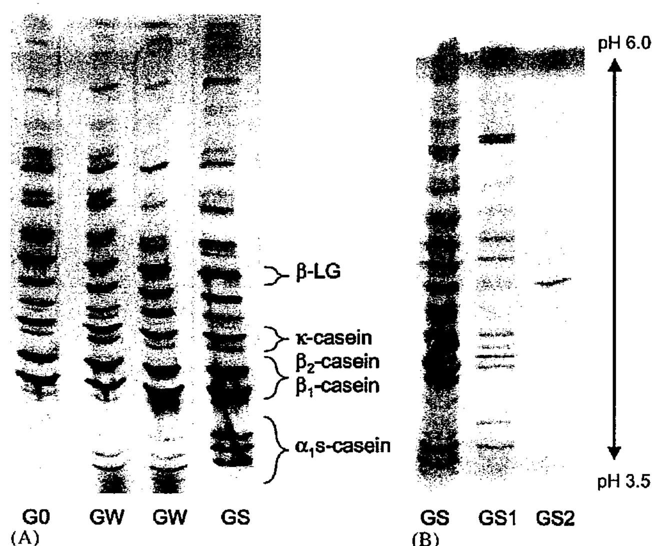

Fig. 2. IEF of caprine milk with various expression of the α_{S1}-casein. Gel A shows three different levels of α_{S1}-casein; no expression (G0), weak expression (GW) and strong expression (GS). Gel B demonstrates the degradation of the strong type (GS) by digestion with human proteolytic enzymes. GS: native form, GS1: digested with HGJ (30 min, 37 °C), and GS2: digested with HGJ (30 min, 37 °C) and HDJ (30 min, 37 °C).

more moderately. Further hydrolysis with HDJ showed that most of the CNs and the whey proteins were digested except β-LG.

3.2. Comparison of digestion between individual caprine and bovine milk by the pH-drop method

The degradation of caprine and bovine milk by human proteolytic enzymes was compared using the pH-drop method. To investigate the influence of α_{S1}-CN content on the degradation of caprine milk, the milk the G0 and GS groups of goats were studied. The results for bovine milk and the two groups of goats are presented in Fig. 3.

A faster drop in pH was observed for milk from the two groups of goats compared with that for milk from cows. After 30 min the drop in pH of the bovine milk was significant different ($p = 0.049$) from that of caprine milk. These results showed that human gastric and duodenal enzymes degraded the caprine milk faster than the bovine milk. The milk from goat group G0 showed a faster pH drop within the first 5 min compared with the milk from group GS. After 10 and 30 min the slope of the curves were identical. No significant statistical differences were observed between the milk from the two goat groups, G0 and GS (see model 1, Section 2.6).

3.3. Effect of heat treatment on the digestion of bovine and caprine milk

Two types of heat treatment were performed, low (72 °C, 15 s) and high (100 °C, 1 min), on mixtures of milk samples

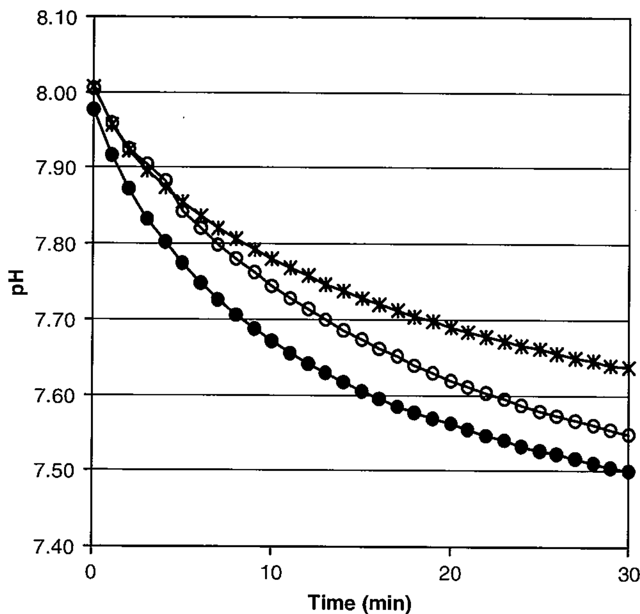

Fig. 3. Decrease in pH during enzymatic treatment with human duodenal juice (HDJ) on goats' and cows' milk at 37 °C. Three groups of milks were compared; G0 goats' milk lacking the α_{S1}-casein (●), GS goats' milk with a high expression of the α_{S1}-casein (○), and cows' milk (*). Each curve shows the mean of milk from eight individual animals.

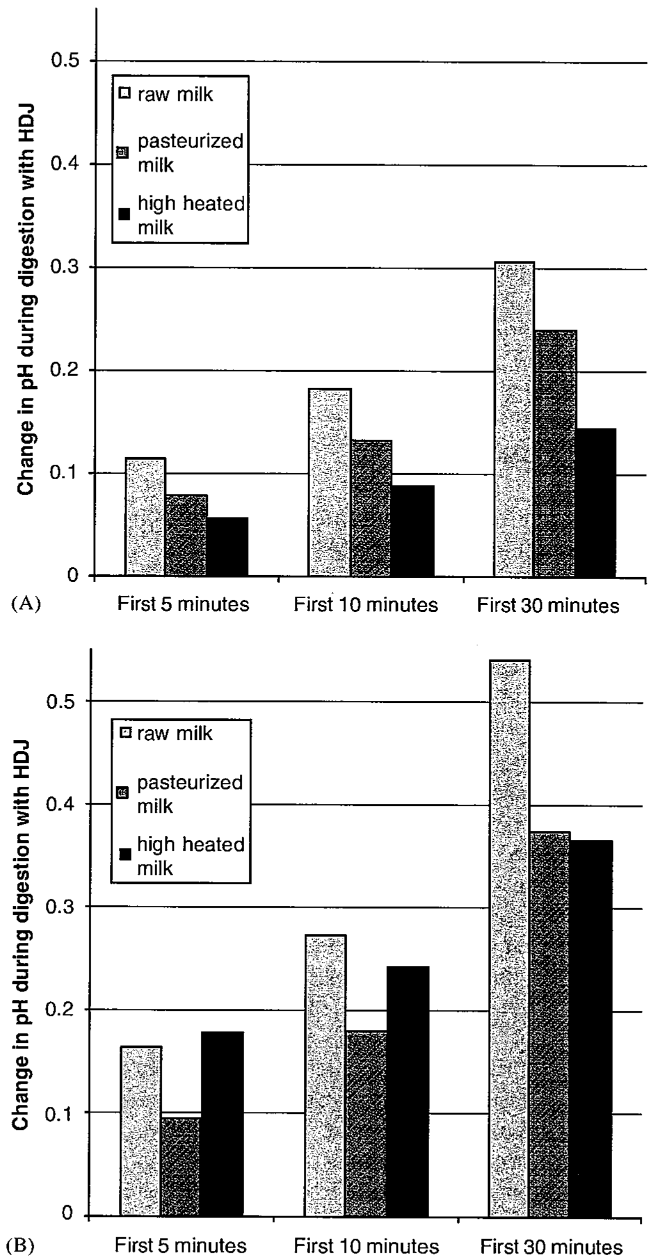

Fig. 4. Decrease in pH during treatment with human duodenal juice (HDJ) at 37 °C after 5, 10 and 30 min for cows' and goats' milk. Raw, pasteurised (15 s, 72 °C) and sterilised/high heated (100 °C, 1 min) milk samples were tested: (A) bulk cows' milk, mixed from eight individuals and (B) bulk goats' milk, mixed from eight individuals.

from group G0 and GS goats and from cows. The effect of degradation by HGJ and HDJ on raw, pasteurised and high heated milk was studied (Fig. 4). The data showed a significant difference between cows' and goats' milk ($p < 0.0001$). However, no difference between the two groups of goats' milk was observed (applying model 2, Section 2.6).

Results from the pH drop experiments showed that raw milk from cows and goats was digested by the enzymes faster than the heated milk, as shown by a "larger" drop in

pH (Figs. 4A and B). Statistical analysis of the data obtained after 30 min hydrolysis showed significant differences between raw and heated milk from both cows and goats ($p<0.0001$). A significant effect of interaction between the two variables, group of animal and heat treatment, was also found ($p = 0.0036$). This implies that the effects of heat treatment on the subsequent protein degradation are different for the various groups of animals. The data obtained for bovine milk also indicated a higher degradation of the pasteurised milk than the high heated milk. The type of heat treatment did not seem to have any important impact on the degradation of protein in caprine milk.

4. Discussion

Milk proteins represent an important dietary source for humans, provided that they are digested suitably. The present work has, to mimic the in vivo situation, focused on the in vitro digestion of bovine and caprine milk by enzymes from the human stomach and duodenum. Previous work (unpublished results) has shown that commercial pepsin, trypsin and chymotrypsin from pig give rise to different peptide profiles after hydrolysis of milk. Using gastric and duodenal enzymes will give better knowledge about the degradation of milk in humans, and can reveal important issues with regard to the proteins in nutrition. The results obtained may be relevant for development of easy digestable products for consumer groups with special needs, such as infants, athletes and the elderly. However, it should be kept in mind that this is only an in vitro model system, and that clinical studies will be needed in order to make any clear conclusions.

A comparison of the protein patterns from SDS-PAGE (Fig. 1), illustrates the major protein profile after degradation of bovine and caprine milk by human proteolytic enzymes. Most noticeable is the difference observed with β-LG. After treatment with both HGJ and HDJ, image analysis of the gel showed that only small amounts (approximately 23%) of the caprine β-LG still remained undigested, while the amount of intact bovine β-LG was about 83%. Similar results from hydrolysis of ovine and bovine β-LG by commercial pepsin were obtained by El-Zahar et al. (2005). Ovine β-LG was degraded faster due to its tertiary structure, and also surface hydrophobicity, being slightly different from those of bovine β-LG (El-Zahar et al., 2005). In order to confirm this for caprine β-LG, further investigations will be needed. The studies on the digestion of milk from both bovine and caprine individuals with the pH drop method confirmed the differences. Caprine milk was digested significantly faster than bovine milk. The reason for this may probably be due to the more resistant β-LG in bovine milk. However, other variations in protein composition between bovine and caprine milk could also contribute to this effect. Other factors like variations in the tertiary structure due to amino acid differences and genetic polymorphism may also influence the result. An additional possibility is that the enzymatic activity of the human juice may differ slightly in the milk from the two species, due to variations in the amount of minerals, carbohydrate content, buffer capacity, etc.

IEF (Fig. 2) of individual milk samples showed different levels of α_{S1}-CN in the milk. It was demonstrated that α_{S1}-CN was partly degraded by HGJ, and then totally hydrolysed with HDJ. This indicates that milk with a high level of α_{S1}-CN might take a longer time to be degraded than milk lacking the protein. However, the results demonstrated no significant difference in pH-drop between the two groups of goats. The study showed an apparent variation between the individual goats in each group. This variation was probably due to other differences in between the chosen individuals, such as genetic factors, feeding, stage of lactation and other seasonal effects (Devold et al., 2000).

Results on digestion of both cows' and goats' milk showed that heated milk in general was more resistant to hydrolysis. This is probably due to structural changes in the proteins caused by denaturation and aggregation of the whey proteins during heat treatment. New linkages between the κ-CN and the β-LG could be formed (Singh, 1995; Singh & Creamer, 1992), and the potential for these protein aggregates to be attacked by human proteolytic enzymes is different from that for native protein in raw milk.

The significant interaction between the two variables, group of animals (cows, G0 and GS) and heat treatment, also showed that heating influenced the bovine milk in a different way than the caprine milk. Degradation of high-heated and pasteurised cows' milk differed, while various heat treatments on goats' milk did not result in differences. This may be due to the variations in the protein composition of bovine and caprine milk as discussed previously. Other differences between milk from the two species might be of importance as well, such as variations in the content of salts and carbohydrates. Further work in this field will be needed to make a clear conclusion.

5. Conclusions

The present study on in vitro digestion by human proteolytic enzymes of caprine and bovine milk proteins has provided new knowledge. Human proteolytic enzymes degraded milk proteins from goat more rapidly than those from cow. Most noticeable was the difference observed in digestibility of β-LG. No significant effect was observed between the digestion of goats' milk with a high level of α_{S1}-CN, and milk from typical Norwegian goats lacking this protein. Raw milk was digested significantly faster with human proteolytic enzymes than pasteurised and high-heated milk. This was the case for both bovine and caprine milk.

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