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Effect of pH and protein composition on proteolysis of goat milk proteins by pepsin and pancreatin

Kasper Hettinga [a,*], Linette Pellis [b,1], Wolf Rombouts [b,2], Xiaogu Du [c], Gabriela Grigorean [d], Bo Lönnerdal [c]

[a] Dairy Science and Technology, Food Quality & Design Group, Wageningen University, 6708WG Wageningen, the Netherlands
[b] Ausnutria B.V., 8025BM Zwolle, the Netherlands
[c] University of California, Department of Nutrition, Davis, CA 95616, USA
[d] Proteomics Core, University of California, Davis, CA 95616, USA

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ABSTRACT

The roles of protein composition, pH and enzymes in goat milk protein hydrolysis is still unclear and the proteolysis of low abundant goat milk proteins has received limited attention. The aim of this study was to study the impact of protein composition and proteolytic conditions on goat milk protein hydrolysis in a simplified digestion model. Both whole milk and infant formula were hydrolyzed at pH 2 and 4, using pepsin as well as pepsin combined with pancreatin. Intact proteins were separated from digests using spin filters, followed by bottom-up proteomics of the separated proteins. Results show that under all conditions, caseins are hydrolyzed quickly. Goat casein hydrolysis in infant formula was slightly faster than in goat whole milk, possibly due to less casein coagulation during pepsin hydrolysis at both pH 2 and 4. Several low abundant immunoactive goat milk proteins, especially immunoglobulins, GLYCAM-1 and osteopontin, resisted proteolysis more than high abundant proteins, independent of the pH and enzyme used for hydrolysis. Fast hydrolysis of casein and slow hydrolysis of immunoactive proteins may indicate a good balance between protein utilization and protection of the infant by goat milk proteins.

1. Introduction

Goat milk is receiving increased attention for its nutritional properties (Clark & Mora García, 2017). These are thought to be related to the easy digestibility of goat milk, due to differences in its lipid droplet size as well as protein composition. It has been shown that digestion kinetics of goat milk proteins are different, both under adult (Almaas et al., 2006; Li et al., 2022) and infant conditions (Maathuis, Havenaar, He, & Bellmann, 2017; Ye, Cui, Carpenter, Prosser, & Singh, 2019), compared to cow milk proteins. Due to these digestion kinetics in infants, goat milk is increasingly used for the production of infant formula. Three parameters that may impact protein digestion of goat milk and goat infant formula are casein micelle composition, the casein to whey protein (CW) ratio, and digestive conditions.

The goat milk casein micelle is known to contain a larger amount of β-casein and a smaller amount of α_{s1}-casein compared to cow milk (Ye et al., 2019). This is believed to be the major reason for the formation of a softer clot during gastric digestion of goat milk products. In turn, this softer clot may enhance gastric casein hydrolysis and increase the speed of gastric emptying, enhancing the overall rate of casein digestion and absorption (Hodgkinson, Wallace, Boggs, Broadhurst, & Prosser, 2018; Ye et al., 2019). A study comparing dynamic *in vitro* digestion of cow and goat milk infant formula as well as human milk showed that indeed gastric digestion of goat milk infant formula was faster than cow milk infant formula, although this did not affect the final degree of intestinal proteolysis, indicating that differences are mainly in digestion kinetics and not in overall digestibility (Maathuis et al., 2017).

The CW ratio has also been shown to play an important role in gastric clot formation and protein hydrolysis kinetics (Phosanam, Chandrapala, Huppertz, Adhikari, & Zisu, 2021; Ye et al., 2019). Infant formula usually has a lower CW ratio, as well as a lower protein content, than the milk it is made from; these changes are made so that infant formula

* Corresponding author at: Kasper Hettinga, Bornse Weilanden 9, 6708WG Wageningen, the Netherlands.
E-mail address: kasper.hettinga@wur.nl (K. Hettinga).
[1] Present address: FrieslandCampina, 3818 LE Amersfoort, the Netherlands.
[2] Present address: Danone Nutricia Research, 3584CT Utrecht, the Netherlands.

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better resembles human milk (Clemens, Hernell, & Michaelsen, 2011). Under infant digestion conditions, it has been shown that changing the CW ratio of goat milk-based infant formula impacts its digestion kinetics (Ye et al., 2019). Whereas native whey proteins from both goat and cow milk generally resist pepsin hydrolysis (Hodgkinson et al., 2018), caseins are faster broken down by gastric digestion. This higher digestion rate for goat milk caseins seems to be related to the structure of the gastric casein clot formed (Roy et al., 2020, 2021).

When studying the digestion of whey proteins, this is usually focused on the most abundant whey proteins, α-lactalbumin and β-lactoglobulin. However, milk contains a wide range of whey proteins, amongst those involved in host defense are of particular interest (K. Hettinga et al., 2011). Proteolysis of these functional whey proteins by digestive enzymes has, however, received limited attention. Only two studies on human milk have been performed, which both show that these functional proteins are more resistant to digestion compared to the major milk proteins (Elwakiel, Boeren, Wang, Schols, & Hettinga, 2020; Zhang et al., 2014). However, whether this is also the case for milk of other mammals needs further study.

Gastric proteolysis depends not only on the protein composition of the product, but also on the specific gastric digestion conditions. Whereas in the adult stomach, the pH is rather low (around 2–3), the pH in the infant stomach is much higher (4–6) with pH decreasing with increasing age (Ménard et al., 2014). The higher pH in the infant's stomach is expected to lead to a much reduced proteolytic activity by the main gastric protease pepsin, as has been shown in several studies on goat milk protein digestion (Hodgkinson et al., 2018; Ye et al., 2019).

Although the impact of protein composition, especially CW ratio, as well as proteolytic conditions (such as pH) on milk protein hydrolysis has been studied previously, especially in cow milk but not for goat milk, the relative impact of multiple changes in these parameters in parallel has not been studied. Our aims were therefore to study in a simplified model of digestion, consisting of only pH modification and enzyme addition, the relative impact of both protein composition, i.e. goat milk vs. goat milk-based infant formula, and proteolytic conditions, i.e. pH 2 vs pH 4; pepsin & pancreatin, on the proteolysis kinetics. Further, proteolysis of some specific low abundant immunoactive whey proteins from goat milk was studied to determine whether this differs under the different digestion conditions.

2. Materials and methods

2.1. Samples

Pasteurized whole goat milk (80 °C for 15 sec) and goat milk-based infant formula (IF), with a CW ratio of 40:60, were provided by Ausnutria B.V (Zwolle, The Netherlands). The liquid samples were shipped on dry ice to UC Davis (Davis, CA, USA). Whole goat milk samples were thawed, whereas IF samples were freshly made according to the manufacturers' instruction (13.5 g of powder was mixed with 90 mL of water).

2.2. Pepsin and pancreatin hydrolysis of milk proteins

A fixed protein concentration of 1 mg/mL was used in a simplified *in vitro* digestion assay, as also used for example by Wada and Lönnerdal (2015a), Wada and Lönnerdal (2015b), mimicking adult and infant gastrointestinal digestion conditions. Samples were acidified to pH 2.0 and 4.0, respectively, with 1 mM HCl. Porcine pepsin (Sigma-Aldrich, St. Louis, MO, USA. Catalog number P6887) with protein purity of 70–100 % and ≥ 3200 units/mg protein was added as 2 % pepsin in 1 mM HCl in phosphate buffered saline (PBS), at a ratio of 22uL per 25 mL of sample, to achieve a protein:pepsin ratio of 12.5:1. Pepsin activity was 56 U/ml of sample. Pepsin and pancreatin to protein ratio was the same for pH 2.0 and pH 4.0 hydrolysis conditions. After pepsin addition, samples were incubated at 37 °C for 15 and 30 min. For samples incubated for 15 and 30 min with pepsin, pH was adjusted back to 7.0 using $NaHCO_3$, after which to part of the samples pancreatin (Sigma, St. Louis, MO, USA; catalog number: P7545) was used at 8xUSP specifications (1×USP: 25 USP protease units/mg; 25 USP amylase units/mg; 2 USP lipase units/mg) as a 0.4 % solution in 1 mM $NaHCO_3$ was added to the samples at a ratio of 4 uL pancreatin per 25 mL of milk, to achieve a protein:pancreatin ratio of 62.5:1. After pancreatin addition, samples were incubated for 30 min and enzymes were then inactivated by placing the samples in a water bath at 85 °C for 1 min.

2.3. Proteomics analysis

Sample preparation; The Bicinchoninic Acid Assay (BCA; ThermoFisher Scientific, Waltham, MA, USA) was used to determine the protein content for all samples. Based on this, 100 μg total protein from each sample was used to perform protein digestion via suspension-trap devices (S-Trap) (ProtiFi, Huntington, NY, USA). S-Trap is a powerful Filter-Aided sample preparation (FASP) method that consists of trapping acid aggregated proteins in a quartz filter prior to enzymatic proteolysis, and allows for reduction, alkylation, and tryptic proteolysis in a single system. Disulfide bonds were reduced with dithiothreitol and alkylated with iodoacetamide in 50 mM TEAB buffer. Samples were subjected to three washes with a wash buffer composed of 90 % methanol and 100 mM triethyl ammonium bicarbonate (TEAB). Enzymatic digestion was performed by first adding trypsin in a 1:25 enzyme:protein ratio (w/w), incubating at 37 °C for 4 h, followed by a boost addition of trypsin using the same ratio followed by overnight digestion at 37 °C. Peptides were eluted from the S-Trap by sequential elution buffers of 100 mM TEAB, 0.5 % FA, and 50 % acetonitrile (ACN) in 0.1 % formic acid (FA). Eluted tryptic peptides were dried in a vacuum centrifuge and re-constituted in 0.1 % trifluoroacetic acid (TFA). These samples were then subjected to LC-MS/MS analysis.

LC-MS/MS analysis; LC separation was done on a Proxeon Easy-nLC II HPLC (ThermoFisher Scientific, Waltham, MA, USA) with a Proxeon nanospray source. One μg of each sample was loaded onto a 100 um × 25 mm Magic C18, 100 Å 5 um reverse phase trap, where they were desalted online by washing them for 5 min with 0.1 % FA. After this, they were eluted onto the analytical column for separation. The analytical column was a 75 um × 150 mm Magic C18 200 Å 3 um reverse phase column. Peptides were eluted into the MS/MS using a 2-buffer gradient: buffer A being 0.1 % FA in water, buffer B being 0.1 % FA in 100 % ACN, at a flow rate of 300 nL/min. A 95-min gradient was run with 5 % to 40 % buffer B over 80 min, 35 % to 80 % buffer B over 5 min, 80 % buffer B for 3 min, 80 % to 5 % buffer B over 2 min, and finally held at 5 % buffer B for 5 min. MS/MS spectra were collected on an Orbitrap QExactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) in a data-dependent mode with one MS precursor scan, resolution 70,000, followed by 15 MS/MS scans, resolution 17,500.

Data Analysis; LC-MS/MS data were analyzed using The Andromeda search engine of the MaxQuant software v2.0.3.0 was used to analyze the raw LC-MS/MS data (Cox et al., 2011). Data analysis was carried out as described before (K. A. Hettinga et al., 2015) using a database downloaded from Uniprot containing all Capra Hircus protein (downloaded from UniProtKB on 26-June-2021). Carbamidomethylation on cysteine was set as fixed modification, whereas variable modifications included M oxidation, N&Q deamidation and S, T, Y phosphorylation. FDR of 1 % was set at both the peptide and protein level. We accepted identifications with at least two peptides, of which at least one was unique. The IBAQ algorithm of MaxQuant was used to obtain quantitative data on the protein level (Schwanhäusser et al., 2011).

2.4. Data analysis

Protein IBAQ values were averaged over triplicates and mean and standard deviations were calculated. This was normalized to undigested whole milk or infant formula, which was set as 100 %. These resulting

data were visualized using R for Windows 4.1.0 (R Core Team, 2022), using the ggplot2 package (Wickham, 2016).

3. Results

3.1. Pepsin and pancreatin hydrolysis of high abundant whole milk proteins

After low temperature heat treatment whole milk was hydrolyzed with pepsin, at pH 2 and pH 4, followed by pancreatin hydrolysis. Fig. 1 shows the pepsin and pancreatin-induced hydrolysis of the most abundant milk proteins.

Fig. 1 shows that proteolysis of caseins, especially α_{s1}-casein and κ-casein, proceeds faster than the proteolysis of the whey proteins at both pH values. For the samples with combined pepsin and pancreatin hydrolysis, the caseins were almost completely absent, whereas still a considerable amount of intact whey proteins was present. Proteolysis of the caseins was rather similar between the pepsin-induced hydrolysis at pH 2 and pH 4. At pH 2, both the major whey proteins remained to some extent during proteolysis by pancreatin, whereas at pH 4 more β-lactoglobulin remained, and α-lactalbumin was degraded to a larger extent.

3.2. Pepsin and pancreatin hydrolysis of high abundant infant formula proteins

Goat milk infant formula was also hydrolyzed, using the same conditions as for the whole milk. In comparison to whole milk, infant formula has undergone more extensive heat treatment and has a different CW ratio. Fig. 2 shows the disappearance of intact high abundant goat milk proteins after pepsin and pancreatin hydrolysis at pH 2 (panel A) and pH 4 (panel B).

Compared to whole milk, casein proteolysis seems to be even faster, with almost complete disappearance of α_{s1}-casein and β-casein already during proteolysis by pepsin, whereas α_{s2}-casein and κ-casein appear to be somewhat resistant to pepsin-induced proteolysis. Whey proteins in infant formula are hydrolyzed at an equally slow rate as in whole milk, with relatively more β-lactoglobulin remaining at pH 4 compared to pH 2.

3.3. Pepsin and pancreatin hydrolysis of low abundant whole milk proteins

Analyses of the high abundant goat milk proteins consistently showed relatively fast proteolysis of caseins and slow proteolysis of the most abundant whey proteins. However, goat milk also contains a wide range of low-abundant whey proteins, many of which are related to immune protection. Fig. 3 shows the disappearance in whole milk of some specific immunoactive low-abundant goat milk proteins after pepsin and pancreatin hydrolysis at pH 2 and pH 4.

Based on the results in Fig. 3, it becomes clear that there is a major difference among immunoactive proteins in their rate of degradation upon proteolysis. Especially immunoglobulins, GLYCAM-1 and osteopontin seem to be degraded to a relatively small extent. Results at pH 2 (Fig. 3A) are very similar to the results at pH 4 (Fig. 3B). It is remarkable that immunoglobulins show an initial decrease upon proteolysis by pepsin, with higher levels found after proteolysis by pancreatin at both pH values.

3.4. Pepsin and pancreatin hydrolysis of low abundant infant formula proteins

Besides retention of low-abundant immunoactive protein in whole milk, retention of these proteins in infant formula upon hydrolysis was determined. Fig. 4 shows the disappearance of these specific low abundant goat milk proteins after pepsin and pancreatin hydrolysis at pH 2 and pH 4.

Similar to whole milk, the immunoglobulins, GLYCAM1, and osteopontin to a lesser extent, in infant formula were resistant to hydrolysis. The results show relatively lower retention of these proteins in the infant formula matrix compared to the whole milk matrix, with no clear differences between pH 2 and pH 4.

4. Discussion

The objective of this study was to investigate, in a simplified model of protein digestion, the relative impact of both protein composition and proteolytic conditions on the disappearance of intact high- and low-abundant goat milk proteins.

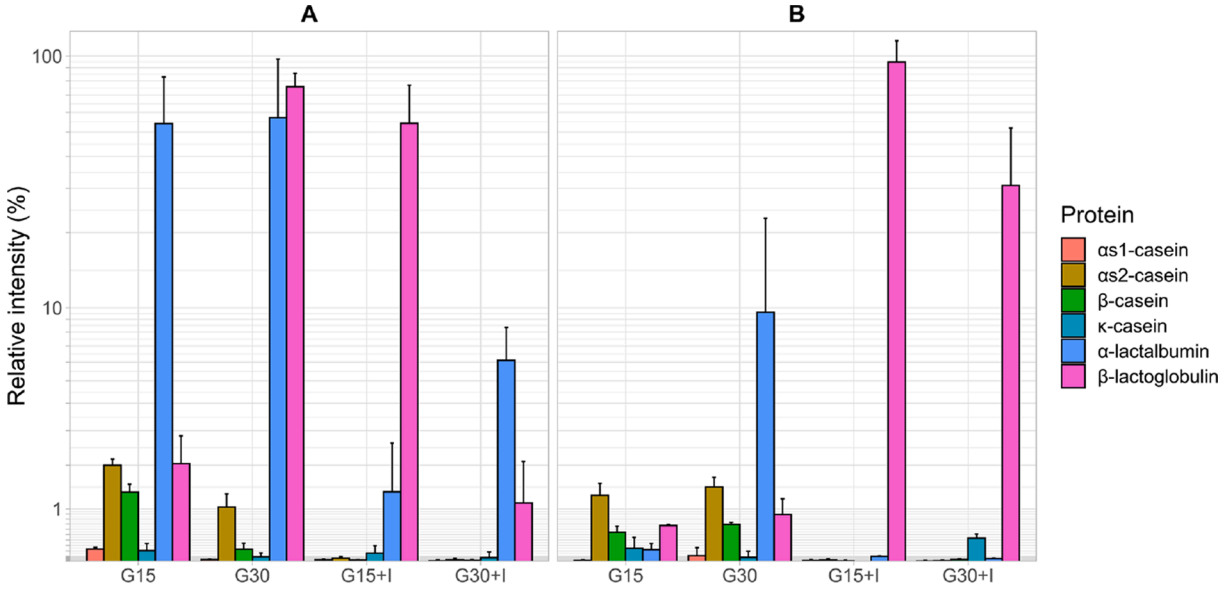

Fig. 1. Relative abundance (as percentage relative to non-digested whole milk) of the six main proteins after proteolysis of whole milk with pepsin at pH 2 (panel A) and pH 4 (panel B) for 15 or 30 min (G15 & G30) as well as with pancreatin at pH 7 for 30 min following either 15 or 30 min of pepsin hydrolysis (G15 + I & G30 + I).

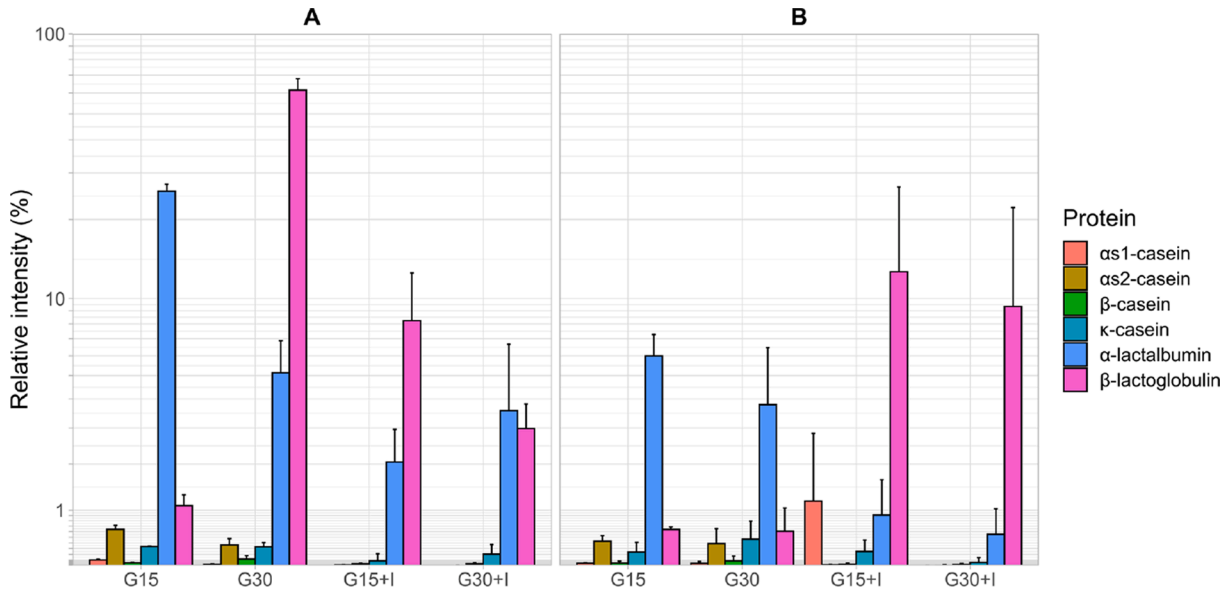

Fig. 2. Relative abundance (as percentage relative to non-digested infant formula) of the six main proteins after proteolysis of infant formula with pepsin at pH 2 (panel A) and pH 4 (panel B) for 15 or 30 min (G15 & G30) as well as with pancreatin at pH 7 for 30 min following either 15 or 30 min of pepsin hydrolysis (G15 + I & G30 + I).

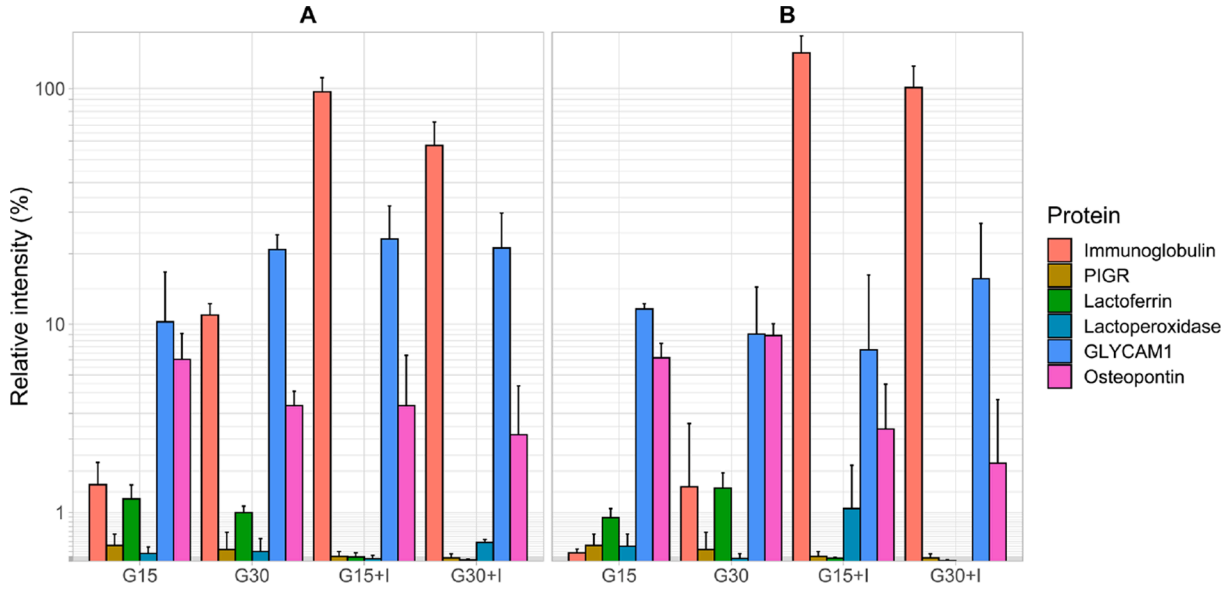

Fig. 3. Relative abundance (as percentage relative to non-digested whole milk) of six low abundant immunoactive goat milk proteins after proteolysis with pepsin at pH 2 (panel A) and pH 4 (panel B) for 15 or 30 min (G15 & G30) as well as with pancreatin at pH 7 for 30 min following either 15 or 30 min of pepsin hydrolysis (G15 + I & G30 + I).

When looking at the 6 most abundant proteins (4 caseins, α-lactalbumin and β-lactoglobulin) in either milk or infant formula, hydrolyzed by pepsin at either pH 2 and 4 and then by pancreatin, it is clear that intact caseins disappear faster than intact whey proteins in both products and under both conditions (Figs. 1 & 2). This matches results from previous studies on bovine, goat and human milk, where this phenomenon has been ascribed to the easier proteolysis of caseins by pepsin due to casein's almost complete absence of both secondary and tertiary structure, while most whey proteins have a tightly folded tertiary structure making them more resistant to gastric digestion (Hodgkinson et al., 2018; Zenker, Van Lieshout, Van Gool, Bragt, & Hettinga, 2020), independent of the use of adult or infant digestion parameters. Whey proteins are generally more extensively digested by pancreatin, due to the combination of the higher pH during proteolysis and the specific proteases involved in this enzyme mixture, allowing extensive hydrolysis of these proteins (Sousa, Portmann, Dubois, Recio, & Egger, 2020). Of the different whey proteins, β-lactoglobulin had the highest resistance to proteolysis by both pepsin and pancreatin (Figs. 1 & 2). Previous *in vitro* as well as *in vivo* studies have also shown β-lactoglobulin to be the high-abundant milk protein most resistant against gastrointestinal digestion, at the low pH of adult gastric digestion conditions, with intact β-lactoglobulin being detected until the end of digestion (Egger et al., 2019). This resistance of β-lactoglobulin, a protein absent in human milk, has also been shown to be physiologically relevant, as its limited digestibility does not fully destroy its allergenic potential (Bossios et al., 2011). Overall, the faster proteolytic disappearance of casein than whey protein in our system is similar to the difference in digestion rate of these proteins, independent of different digestion models and parameters.

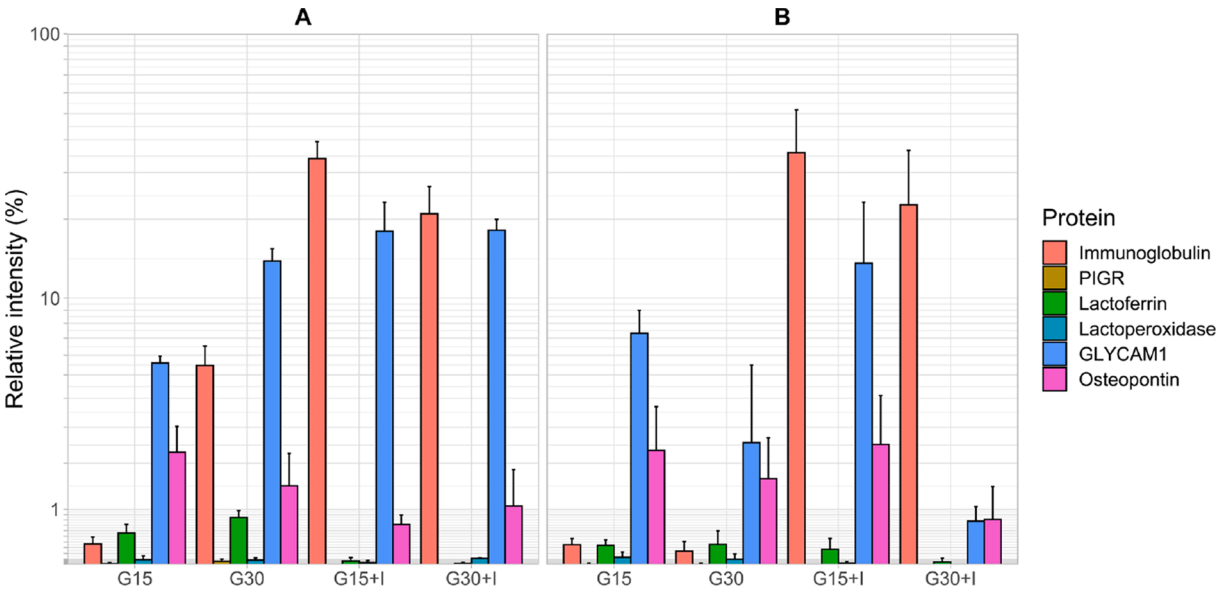

Fig. 4. Relative abundance (as percentage relative to non-digested whole milk) of six low abundant immunoactive goat milk proteins after proteolysis of infant formula with pepsin at pH 2 (panel A) and pH 4 (panel B) for 15 or 30 min (G15 & G30) as well as with pancreatin at pH 7 for 30 min following either 15 or 30 min of pepsin hydrolysis (G15 + I & G30 + I).

When comparing the two different pH values used during proteolysis (Fig. 1A vs B), the major difference is the increased breakdown of α-lactalbumin at pH 4. This may be due to the molten globule structure of α-lactalbumin, which differs depending on the pH (Rösner & Redfield, 2009). In the molten globule structure, most of the secondary structure of the protein is retained, but it loses part of its tertiary structure (Lönnerdal & Lien, 2003). The absence of a tertiary structure makes the protein more prone to proteolytic degradation (Elwakiel et al., 2020; Lönnerdal & Lien, 2003). Differences in the molten globule structure between pH 2 and 4 may thus underlie the enhanced proteolysis of this protein if the pepsin-induced proteolysis takes place at pH 4. α-lactalbumin is important for iron and zinc uptake in the gastrointestinal tract (Lönnerdal, 2016).

When comparing proteolysis of whole milk (Fig. 1) with that of infant formula (Fig. 2), it can be seen that especially proteolysis of caseins by pepsin seems to be faster in the infant formula. This may be due to the fact that there is less casein coagulation during pepsin digestion, due to the decreased CW ratio in infant formula as compared to whole milk, combined with the lower absolute amount of casein present (Ye et al., 2019). It is important to realize that the data collected in this study is on disappearance of intact protein. It has previously been shown that casein are broken down quickly in the stomach, but are subsequently broken down and absorbed slower than the whey proteins (Abrahamse, Thomassen, Renes, Wierenga, & Hettinga, 2022).

Besides the six high-abundant milk proteins, the proteolysis of a number of immunoactive low-abundant milk protein was studied. From the results in Figs. 3 & 4, it is evident that these proteins are generally more resistant towards proteolytic degradation than the major milk proteins. Especially immunoglobulins, GLYCAM-1, and osteopontin were degraded to a relatively limited extent. Lactoferrin also showed partial retention during pepsin-induced proteolysis, but was broken down completely during pancreatic proteolysis. This does, however, not necessarily mean a loss of function, as many of the peptides formed upon lactoferrin hydrolysis were shown to have a wide range of functions (Wang, Timilsena, Blanch, & Adhikari, 2017).

The relative resistance of immunoglobulins and lactoferrin in goat milk towards proteolysis in general, but especially proteolysis by pepsin, has been shown previously (Hodgkinson et al., 2018; Ma, Hou, Xie, Zhang, & Zhou, 2021) both when using adult or infant *in vitro* digestion parameters, with one of these studies also showing that these proteins from goat milk were more resistant to proteolysis than those from bovine milk (Ma et al., 2021). Remarkably, the immunoglobulins show an initial sharp decrease during pepsin hydrolysis in both types of samples and at both pH values. One could speculate that the large immunoglobulins are initially trapped in casein coagulates. This agrees with previously published data on the gastric clot formed during goat and bovine milk digestion, where the SDS-PAGE data show bands at the position of the immunoglobulin heavy chain (Ren, Boiani, He, Wichers, & Hettinga, 2023; Ye, Cui, Dalgleish, & Singh, 2016), although no formal identification of this band was performed in either study. Upon further digestion, the casein coagulates disintegrate, which may then also release the trapped immunoglobulins, explaining the increased presence during longer hydrolysis as shown in Figs. 3 & 4. The relative stability of lactoferrin has not only been shown for goat milk, but also for both bovine (Wang et al., 2017) and human milk (Elwakiel et al., 2020), using adult and infant *in vitro* digestion parameters, respectively. For bovine lactoferrin, its proteolytic degradation has been shown to increase upon heat treatment, which was especially increasing its gastric digestion rate (Xiong, Boeren, Vervoort, & Hettinga, 2021). This may explain why in the current study, the gastric degradation of lactoferrin in the relatively mildly heat-treated whole milk was lower than for the more intensely heat-treated infant formula. The main explanation for this resistance to proteolysis is the combination of a tightly folded structure and post-translational modifications, especially glycosylation (Abrahamse et al., 2022; Elwakiel et al., 2020; Van Veen, Geerts, Van Berkel, & Nuijens, 2004). Overall, the proteolysis of immunoactive goat milk proteins in our model systems did not strongly depend on pH during proteolysis, which agrees with previous studies reporting similar findings under both adult and infant *in vitro* digestion conditions.

With regard to the other low abundant whey proteins studied, limited prior literature is available. One study on bovine milk ingredients did show that GLYCAM-1 as well as osteopontin were relatively resistant to digestive enzymes (Chatterton et al., 2004). Bovine milk osteopontin has been shown to partly resist both *in vitro* and *in vivo* digestion in animal models (Jiang, Tran, & Lönnerdal, 2021; Jiang & Lönnerdal, 2020).

Overall, the results for the immunoactive proteins show that these are resistant to hydrolysis, especially by pepsin. Many functional milk proteins need to be in their native, intact form to be able to exert their biological function (Lönnerdal, 2003). The resistance to hydrolysis may

be due to a combination of compact folding and post-translational modifications, making it more difficult for enzymes to reach their cleavage sites (Egger et al., 2019; Van Veen et al., 2004), which agrees with previous data on protein degradation during *in vitro* digestion of human milk and bovine milk-based infant formula under infant digestion conditions (Abrahamse et al., 2022; Elwakiel et al., 2020). Our data on goat milk and goat milk-based infant formula show similar results, although no quantitative comparisons can be made due to differences in hydrolysis conditions.

5. Conclusions

The results in this study, using the enzymes pepsin and pancreatin, show that caseins are the goat milk proteins that are hydrolyzed the fastest, with only small differences between different pH values used for proteolysis by pepsin. A slightly faster casein degradation was seen for the goat milk based infant formula, which may be related to the lower rate of casein coagulation that would be expected in infant formula, as well as the lower casein concentration. Several low abundant immunoactive goat milk proteins, especially immunoglobulins, GLYCAM-1 and osteopontin, resist proteolysis to a larger extent that most high abundant proteins. No major differences were found between either whole goat milk and infant formula or the two pH values tested. These proteins may have beneficial effects for the healthy development of the newborn infant consuming such proteins.

CRediT authorship contribution statement

Kasper Hettinga: Methodology, Formal analysis, Visualization, Software, Writing – original draft. **Linette Pellis:** Conceptualization, Resources, Project administration, Supervision, Writing – review & editing. **Wolf Rombouts:** Conceptualization, Resources, Supervision, Writing – review & editing. **Xiaogu Du:** Methodology, Investigation, Data curation, Writing – review & editing. **Gabriela Grigorean:** Methodology, Investigation, Data curation, Writing – review & editing. **Bo Lönnerdal:** Conceptualization, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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