

***In vitro* digestion of novel milk protein ingredients for use in infant formulas: Research on biological functions**

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Rather than using lengthy clinical studies, *in vitro* digestion of milk proteins by human neonatal gastric juice at pH values representing those in early neonatal life was investigated. Human milk proteins resisting digestion were immunoglobulins, secretory IgA, α_{s1} -casein, secretory component, osteopontin and MUC-1. Bovine milk proteins resisting digestion at pH 4.0 were immunoglobulins, BSA, caseins, lactadherin, osteopontin, EPV20 and proteose peptone component 3 (PP3). Some proteins were rapidly digested e.g. CGMP. *In vitro* digestion provides rapid information

regarding the susceptibility of proteins to gastric proteases and is a convenient alternative to clinical studies.

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Introduction

Cohort studies have provided evidence that breast feeding has greater health advantages than formula feeding does. One of these studies indicated a reduction in the frequency of infectious episodes during breast feeding (Dewey, Heinig, & Nommsen-Rivers, 1995). A later study, indicated that the mean arterial blood pressure at 13–16 years of age was lower in children assigned banked breastmilk (alone or in addition to mother's milk) than in children assigned preterm formula (Singal, Cole, & Lucas, 2001).

It is known that a protein compositional dissimilarity exists between infant formula and that of human milk (Fig. 1) (Hambreus, Forsum, & Lönnerdal, 1977) and could be responsible for some of the differences in health reported in the cohort studies mentioned earlier. Firstly, bovine milk contains far greater (approximately 4-fold) amounts of protein than human milk. Approximately 80% of this is present as casein, but in contrast, caseins represent only approximately 20% of the total protein in term human milk. Human milk contains far greater amounts of lactoferrin (LF) than in bovine milk. Secondly, bovine milk contains the protein β -Lactoglobulin (β -Lg) which is absent in human milk. These differences could be of great importance. For instance, a nutritional imbalance which is administered to human infants at critical periods during early development leads to consequences which are observed much later in life in the absence of the stimulus that initiated them and this phenomenon is known as metabolic programming (Lucas, 1991).

As a consequence of this knowledge, efforts have been made to improve the protein composition of infant formulas such that they begin to approach that found in human milk. This is possible by purifying individual bovine milk proteins and using these to supplement infant formulas. The antimicrobial protein, LF was one of the first proteins to be purified at industrial scale and subsequently added to infant formulas. LF has been proven to have an antimicrobial effect *in vitro*, by chelating iron required for bacterial growth and thereby provides bacteriostatic and bactericidal activity (Oram

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Nomenclature

LF	lactoferrin
β -Lg	β -lactoglobulin
α -La	α -lactalbumin
CGMP	caseinoglycomacropeptide
OPN	osteopontin
PP3	protease peptone component 3
WPC	whey protein concentrate
α_{S1} -CN	α_{S1} -casein
sIgA	secretory IgA
β -CN	β -casein
κ -CN	κ -casein.

& Reiter, 1979). Following the introduction of LF as an ingredient for infant formulas, other novel milk protein ingredients have been recently marketed. Examples of these are alpha-lactalbumin (α -La) (Chatterton, Nielsen, Holst, Berthelsen, & Albertsen, 1999), kappa caseinoglycomacropeptide (CGMP), a glycosylated and phosphorylated peptide (Holst & Chatterton, 2002) and osteopontin (OPN) a phosphorylated & glycosylated protein (Sørensen, Ostensen, Chatterton, Holst, & Albertsen, 2001). Current interest is focused on the use of milk fat globule membrane proteins as ingredients for infant formulas, as they contain biologically interesting proteins such as MUC-1 and lactadherin (Peterson *et al.*, 1998; Pallesen *et al.*, 2001).

Despite knowledge of the biological effects of some of these intact proteins, little is known regarding whether these proteins resist the process of digestion following ingestion. Digestion could effect the half life of a biologically active milk protein, changing the biological activity, or conversely release biologically active fragments which could have new biological properties when compared with the intact protein. For instance, gastric pepsin cleavage of bovine and human LF has been reported to release the peptide lactoferricin (Bellamy, Takase, Yamauchi, Wakabayashi, Kawase, & Tomita, 1992). This peptide has broad-spectrum antimicrobial properties and leads to rapid loss of colony-forming ability (Bellamy *et al.*, 1992; Tomita, Takase, Wakabayashi, & Bellamy, 1994). Although large amounts of LF remained undigested, this peptide has also been observed to be released during digestion in human adults (Kuwata, Yip, Yip, Tomita, & Hutchens, 1998). These observations are interesting because the bactericidal effects of lactoferricin have been described to be 10–100 times greater than that of the intact protein (Bellamy *et al.*, 1992).

The process of digestion can be assessed by the *in vitro* digestion model. The usefulness of this model depends upon a consideration of particular physiological factors.

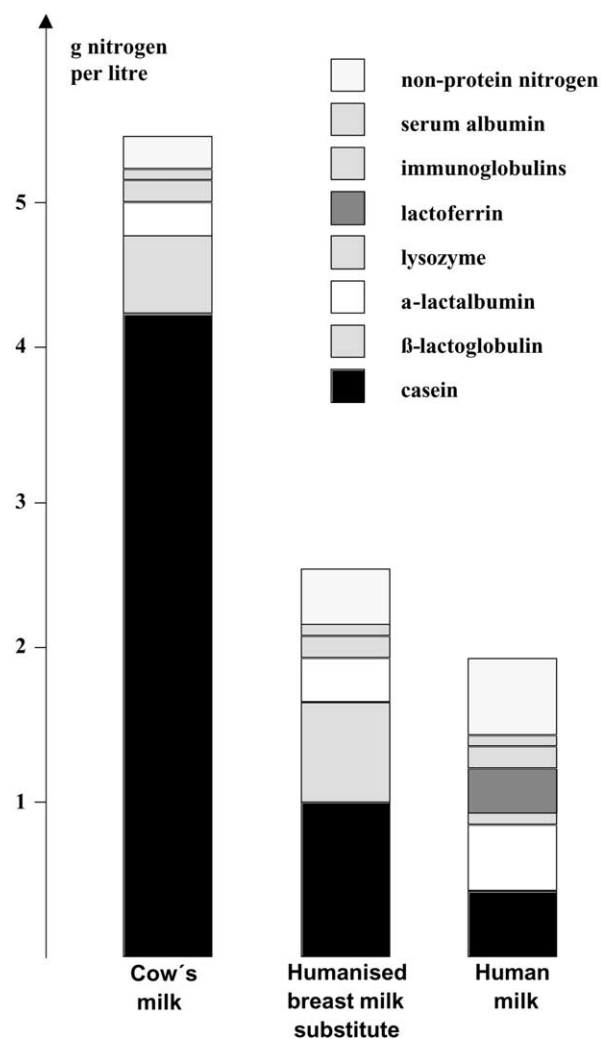


Fig. 1. Protein composition of cow's milk, humanized infant formula (adapted formula) and human milk. Nitrogen derived from the various proteins and non-protein nitrogen are represented as grammes of nitrogen per litre (From Hambreus *et al.*, 1977)

Firstly, rates of gastric emptying reported for newborn infants can be utilized to determine the length of incubation of milk protein with gastric proteases. Mean half emptying rates of 48 min for breast fed infants and between 65 and 78 min for formula fed infants have been reported (Van Den Driessche, Peeters, Marien, Ghoos, Devlieger, & Veereman-Wauters, 1999; Cavell, 1982). These data compare with studies we have performed which indicated that in 2 week old infants, approximately 90% of human milk was emptied from the stomach within 60 min (Chatterton, unpublished data).

Secondly, the digestion of milk proteins used in infant formulas is complicated by the changes in gastric pH which occur in human infants. Gastric pH is known to be high in early neonatal life and the pH of gastric

contents has been measured to be pH 6.0 in term newborn infants but decreased abruptly during the first 24 h of life (Ebers, Smith, & Gibbs, 1956; Ahn & Kim, 1963; Widstrom, Christensson, Ransjo-Arvidson, Mathiesen, Winberg, & Uvnas-Moberg, 1988). However, these age-related decreases in gastric pH, have not accounted for the cyclic changes in intraluminal pH which occur during feeding of human milk or infant formula. Mean gastric pH has been reported to rise shortly after feeding in 25 term infants fed human milk, reaching pH values of 6.4 within 30 min after a bolus of human milk, gastric pH subsequently decreasing with time (Fig. 2) (Mason, 1962). Similar results have been observed in pre-term infants (Mitchell, McClure, & Tubman, 2001). These observations indicate that the pH in the stomach of newborn infants is far from the pH optimum of the gastric enzymes pepsin and gastricsin. As a result, this will have consequences regarding the digestion of milk protein. Unfortunately, the digestion of milk protein under these conditions is still poorly understood. Some studies have investigated the digestion of milk proteins by using duodenal juice (Jakobsson, Lindberg, & Benediktsson, 1982), or in short-bowel patients (Mahe, Messing, Thuillier, & Tome, 1991). However, these studies have only provided information regarding major proteins such as caseins, α -La and β -Lg.

The aims of this study are therefore to investigate the effects of *in vitro* digestion on novel milk protein ingredients for use in infant formulas and to determine the stability of these milk proteins. To determine this, human milk, whey protein concentrate and infant formula were digested with either porcine pepsin or human

neonatal gastric juice at a range of pH values simulating those commonly found in the stomach of human newborn infants. Some of these proteins have previously been subjected to extensive studies in our laboratory. These proteins were MUC-1 (Pallesen *et al.*, 2001), OPN (Sørensen, Højrup, & Petersen, 1995), Lactadherin (Hvarregaard, Andersen, Berglund, Rasmussen, & Petersen, 1996), PP3 (Lactophorin) (Sørensen & Petersen, 1993) and EPV20, a secretory glycoprotein (Larsen, Ravn, Boisen, Berglund, & Petersen, 1997).

Materials and methods

Materials

Gel electrophoresis molecular mass standards were from Bio-Rad laboratories. All tested protein samples were from Arla Foods, Videbaek, Denmark, with the exception of the infant formula, SMA from Wyeth Laboratories (Philadelphia, USA). Whey protein concentrate (WPC) is commercially known as Lacprodan-80. Pooled human milk was obtained from Aarhus University Hospital, Denmark. ProBlott™ membranes and reagents used for sequence analysis were obtained from Applied Biosystems (Foster City, CA, USA). Polyclonal primary antibodies against bovine and human OPN, Lactophorin (PP3), bovine Lactadherin, MUC-1 and EPV20 were raised in rabbits by DAKO A/S (Glostrup, Denmark). The immunoglobulin fraction was recovered from antiserum by affinity chromatography on protein-A-Sepharose according to the instruction of the manufacturer. Mouse anti-human MUC-1 Ab-2 (GP1.4) was from NeoMarkers (Fremont, CA, USA). Alkaline phosphatase conjugated swine immunoglobulins to rabbit immunoglobulins were from DAKO A/S (Glostrup, Denmark). Tween^R-20, 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt, nitro blue tetrazolium were supplied by Sigma Chemicals Co. (St Louis, MO, USA).

In vitro proteolysis studies with human gastric juice

A neonate presenting with anal atresia was operated at UC Davis Medical Center, California, USA. Two days following surgery, a sample of gastric juice was collected and centrifuged at 15,000g for 10 min. The supernatant was collected and immediately frozen at -20°C . Gastric aspirates were obtained from two infants aged 8 days and 28 days at 1 and 3 h following ingestion of a bolus of human milk. For *in vitro* proteolysis studies, 10 μl of gastric juice was added to 900 μg of either mature human milk or WPC. The pH was adjusted to pH 6.5, 5.0, 4.0, 3.5, 3.0 and 2.0 by addition of 80 mM HCl, and the samples incubated for 1 h at 37°C , simulating the holding time of human milk in the stomach. Following incubation, samples were immediately heated to 100°C for 3 min with non-reducing SDS sample buffer and analysed by SDS PAGE.

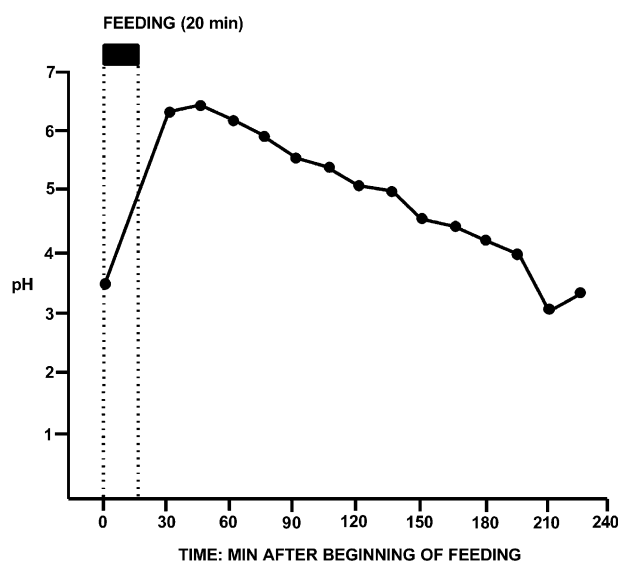


Fig. 2. Gastric pH during feeding Mean values of pH of the stomach contents of 25 newborn infants during the interval between feeds (Modified from Mason, 1962).

Identification of proteins

Proteins were analysed by SDS PAGE using 16% Tris/Tricine gels and stained with Coomassie blue R-250. Electrophoresis was performed using ProBlott™ membranes.

Peptides from glycomacropeptide digests were separated by reverse-phase HPLC on a Vydac C₄ column (25×0.45 cm). The separation was performed on a Pharmacia LKB system (Pharmacia, Uppsala, Sweden), consisting of a 2248 LC gradient pump connected to a 2252 LC controller and a 2510 Uvicord SD detector equipped with a 226 nm filter and a flow cell with a 2.5 mm path length. The peptides were separated in 0.1% trifluoroacetic acid (buffer A) and eluted with a gradient of 90% acetonitrile in 0.1% TFA (buffer B), developed over 50 min (0–5 min: 0% B; 5–40 min: 0–50% B; 40–50 min: 50–95% B) at a flow rate of 0.85 ml/min.

N-terminal sequence analysis was carried out using an Applied Biosystems model 477A sequencer with on-line phenylthiohydantoin analysis on an Applied Biosystems 120A HPLC. Electrophoresed proteins were sequenced using standard programmes.

For Western Immunoblotting, the membranes were blocked with 50 mM Tris, pH 7.4, 0.5 M NaCl, 2% Tween 20. Washing and incubation buffer was 50 mM Tris, pH 7.4, 0.5 M NaCl, 0.1% Tween 20 (TBS-T). For MUC-1 identification, mouse anti-human MUC-1 Ab-2 (GP1.4) was used as the primary antibody (7 µl/ml in TBS-T). For identification of OPN and PP3, rabbit anti bovine OPN or PP3 and rabbit anti human OPN IgG was used (5 µg/ml in TBS-T). Following incubation with primary antibody, the membranes were washed and incubated with secondary antibody for 1 h. For MUC-1, alkaline phosphatase-conjugated rabbit immunoglobulins to mouse immunoglobulins were diluted 3000-fold in TBS-T. For OPN and PP3 alkaline phosphatase conjugated swine immunoglobulins to rabbit immunoglobulins were diluted 3000-fold in TBS-T. Colour was developed by the addition of 50 ml 0.1 M Tris, 0.1 M NaCl, 50 mM MgCl₂, pH 9.0, containing 100 µl 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt stock solution (100 mg in 1.33 ml 70% dimethylformamide). The reaction was stopped by rinsing the membrane with water, followed by air drying.

Results

Several human milk proteins resist *in vitro* digestion with gastric juice in a pH dependant manner even at pH 2.0

To investigate the effects of gastric proteases on the digestion of human milk proteins, we incubated human milk with human neonatal gastric juice at pH values ranging from pH 6.5–2.0. Significantly lower hydrolysis occurred in the pH range 6.5–4.0 than at lower values of pH where a pronounced hydrolysis of some proteins occurred (Fig. 3A). We observed that specific proteins were

particularly resistant to the effects of gastric proteases, even at pH 2.0. These proteins were identified as immunoglobulins, secretory IgA (sIgA), α_{S1} -casein (α_{S1} -CN) heteromers, and secretory component. Other proteins were partially resistant, evidenced by LF, β -casein (β -CN) and α -La. Using N-terminal amino acid sequencing we identified a lower molecular weight fragment of β -CN arising from proteolysis by endogenous milk proteases and commencing at amino acid 24 in the amino acid sequence of β -CN. This moiety was observed at all pH values during incubation with gastric juice, demonstrating its resistance to further digestion by gastric juice.

Western immunoblotting using either antibodies specific to phosphorylated human osteopontin or human MUC-1 provided evidence that significant amounts of human MUC-1 (presenting as two main protein bands of molecular weight ~400 kDa) and OPN resist digestion even at pH 2.0 (Fig. 3B & C, respectively). Human OPN antibody recognised major protein bands at ~78 kDa (intact glycosylated and phosphorylated protein), but also at 34 and 23 kDa which correspond to peptide fragments formed by endogenous proteases contained in milk. A slight loss of the 34 and 23 kDa bands occurred at pH 4.0, indicating their lability when compared to the intact OPN. Taken collectively, these data indicate that *in vitro* digestion of milk proteins could provide useful information regarding the stability of biologically interesting milk proteins.

Several human milk proteins resist *in vivo* digestion in the stomach of human new born infants

For further elucidation of the usefulness of *in vitro* digestion of milk proteins, we examined the digestion of human milk *in vivo* by sampling gastric aspirates from human infants aged 8 days and 28 days. Samples were collected at timed intervals of 1 h and 3 h following administration of a bolus of human milk (Fig. 3D). In agreement with *in vitro* digestion studies, analyses indicated that large amounts of intact protein survived digestion for at least 1 h following ingestion of human milk. For instance, significant quantities of α -La, LF and secretory component were detected after 1 h of digestion, irrespective of age of the infant. However, some exceptions were observed for marginally resistant proteins for instance β -CN which was detected after 1 h in the 8 day-old infant, but little was observed in the 28 day-old infant despite both samples having comparable values of pH. In contrast, marked hydrolysis of milk protein occurred in 3 h samples irrespective of age.

Many bovine milk proteins resist *in vitro* digestion with gastric juice for at least one hour at pH values as low as pH 4.0

In comparison to human milk, significantly fewer WPC proteins were resistant to digestion by gastric juice at pH levels below 4.0 (results not shown). In particular,

β -Lg and small amounts of α -La were the only proteins identified remaining, following 1 h of incubation with gastric juice at pH 2.0. In comparison to human milk, LF was observed to be susceptible to hydrolysis below pH 5.0, but lower levels of this protein are present in WPC compared to human milk. Importantly, several proteins resisted digestion at pH values of 4.0 and above (Fig. 4A). For instance, bovine immunoglobulins, serum albumin and caseins were still detectable at this pH. Analyses using Western immunoblotting confirmed that at these pH values, bovine lactadherin, OPN, EPV20 and PP3 were also stable to digestive enzymes (Fig. 4B, C, D & E, respectively). Lactadherin was

identified as two main protein bands of molecular weight ~ 47 and ~ 50 kDa corresponding to that found previously and thereby indicating no detectable proteolysis by gastric proteases (Hvarregaard *et al.*, 1996). Bovine OPN antibody recognised bands of molecular weight ~ 180 ; ~ 60 ; ~ 45 ; ~ 40 ; ~ 20 kDa.

Antibody raised against purified EPV20 recognised two forms of EPV20 of ~ 20 and ~ 16 kDa which, although stained weakly, indicated that EPV20 was not cleaved at pH 4.0 and above and therefore likely to pass into the intestine in intact form.

PP3 also presented two main bands in WPC. One of these corresponded to the 28 kDa intact protein,

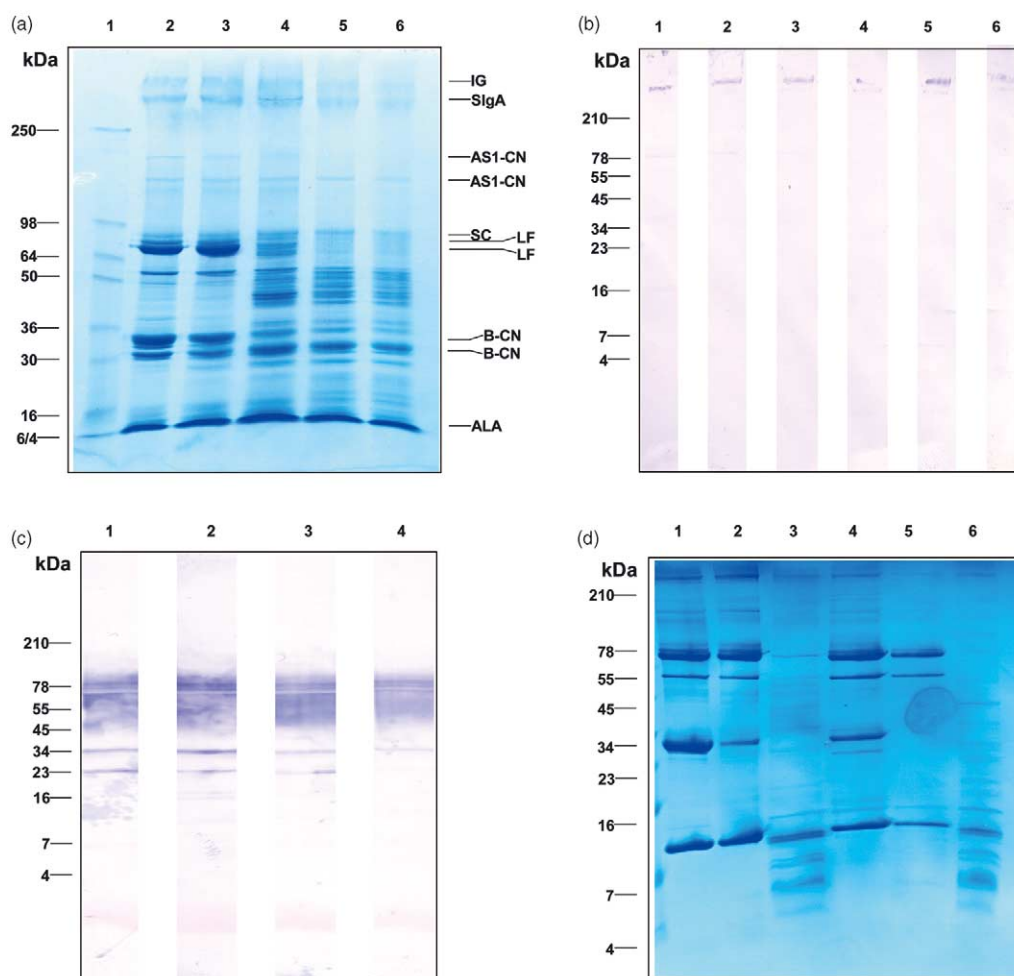


Fig. 3. Digestion of human milk. (A) SDS PAGE of human milk treated with human neonatal gastric juice for 1 h at 37°C. Lane 1: Molecular weight marker; Lane 2: pH 6.5; Lane 3: pH 4.0; Lane 4: pH 3.5; Lane 5: pH 3.0; Lane 6: pH 2.0. Electrotransferred proteins were excised from PVDF membranes and identified N-terminal amino acid sequencing. IG: Immunoglobulins; SIgA: Secretory IgA; AS1-CN: Alpha S1-Casein; SC: Secretory Component; LF: Lactoferrin; B-CN: Beta Casein; ALA: Alpha-lactalbumin. (B) Western immunoblots of human milk treated with human neonatal gastric juice for 1 h at 37°C, using primary antibody against human MUC-1. Lane 1: Untreated human milk; Lane 2: pH 6.5; Lane 3: pH 4.0; Lane 4: pH 3.5; Lane 5: pH 3.0; Lane 6: pH 2.0. (C) Western immunoblots of human milk treated with human neonatal gastric juice for 1 h at 37°C, using primary antibody against human OPN. Lane 1: pH 6.5; Lane 2: pH 4.0; Lane 3: pH 3.5; Lane 4: pH 3.0. (D) Gastric aspirate samples from two human newborn infants. Lane 1: Human milk before administration to an 8 day-old infant; Lane 2: Gastric aspirate from an 8 day-old infant, sampled 1 h post ingestion; Lane 3: Gastric aspirate from an 8 day-old infant, sampled, 3h post ingestion; Lane 4: Human milk before administration to a 28 day-old infant; Lane 5: Gastric aspirate from a 28 day-old infant, sampled 1 h post ingestion; Lane 6: Gastric aspirate from a 28 day-old infant, sampled 3 h post ingestion.

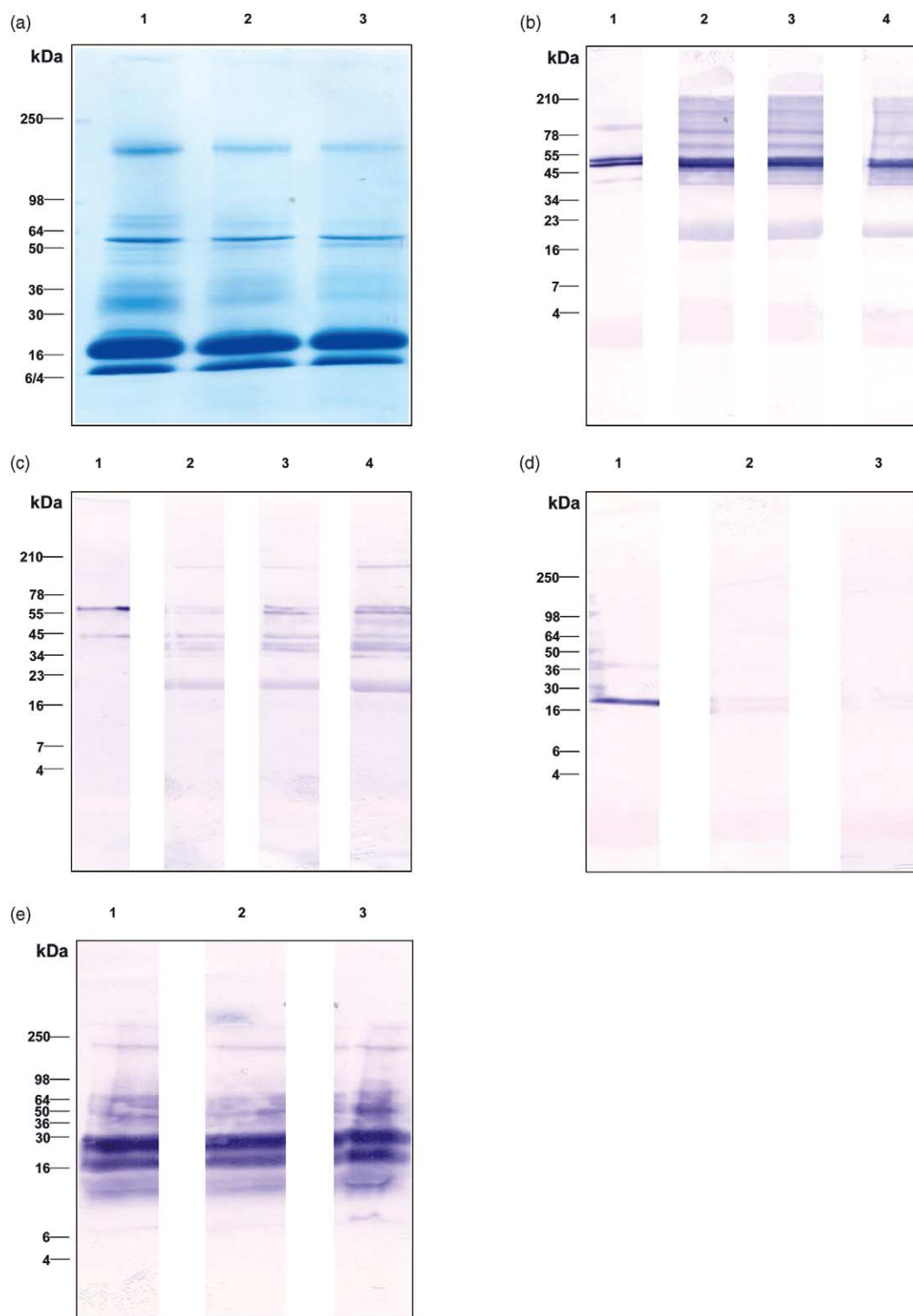


Fig. 4. Digestion of bovine milk proteins. (A) SDS PAGE of bovine milk proteins contained in WPC, treated with human neonatal gastric juice for 1 h at 37°C. Lane 1: Untreated WPC; Lane 2: pH 6.5; Lane 3: pH 4.0. (B) Western immunoblot of WPC treated with human neonatal gastric juice for 1 h at 37°C using primary antibody against bovine Lactadherin. Lane 1: Purified bovine lactadherin standard; Lane 2: Untreated WPC; Lane 3: pH 6.5; Lane 4: pH 4.0. (C) Western immunoblots of WPC treated with human neonatal gastric juice for 1 h at 37°C using primary antibody against bovine Osteopontin. Lane 1: Purified bovine osteopontin standard; Lane 2: Untreated WPC; Lane 3: pH 6.5; Lane 4: pH 4.0. (D) Western immunoblots of WPC treated with human neonatal gastric juice for 1 h at 37°C using primary antibody against bovine EPV20. Lane 1: Purified bovine EPV20; Lane 2: Untreated WPC; Lane 3: pH 4.0. (E) Western immunoblots of WPC treated with human neonatal gastric juice for 1 h at 37°C using primary antibody against PP3. Lane 1: WPC, untreated; Lane 2: pH 6.5; Lane 3: pH 4.0.

whereas the lower fragment was ~ 17 kDa corresponding to a plasmin cleavage (Sørensen, 1993).

We examined the stability of a major milk peptide, CGMP, to gastric proteases by using comparable concentrations of porcine pepsin as those reported to be present in the stomach of newborn infants. CGMP was rapidly cleaved by gastric proteases at pH 2.0 (Fig. 5). No differences were observed when proteolysis was performed at pH 4.5 (results not shown).

The positive relationship between high pH and resistance of milk proteins to proteolysis lead us to speculate whether proteins contained in infant formula would be stable to gastric proteases in a similar manner to those

in WPC. To elucidate this possibility, we examined the stability of milk proteins contained in a commercially available infant formula (SMA). Caseins in this infant formula resisted digestion for 1 h at pH 5.0, but were cleaved below this pH (results not shown)

Human and bovine milk α -La appears to be digested in a similar manner

Proteolysis studies described above indicated that α -La is digested in a similar manner, irrespective of whether it is contained in human milk, WPC, or infant formula. Although significant amounts of α -La appear to resist the action of gastric proteases, close inspection

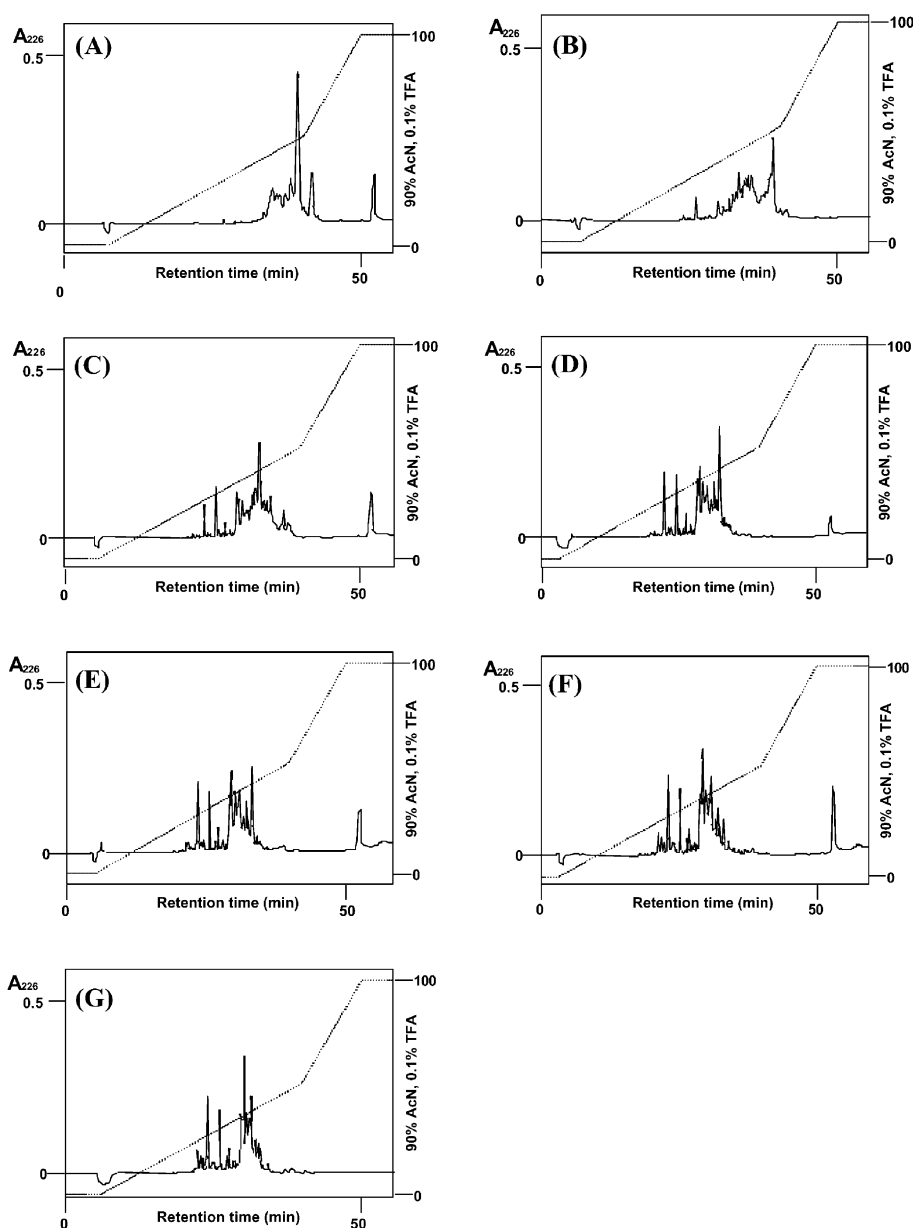


Fig. 5. Digestion of glycomacropeptide. RP-HPLC chromatograms of glycomacropeptide digested by porcine pepsin for 1 h at 37°C at pH 2.0; (A) 0 min; (B) 1 min; (C) 5 min; (D) 10 min; (E) 15 min; (F) 30 min; (G) 1 h.

of the Tris-Tricine PAGE gels indicated the formation of a higher molecular weight band observed between that of α -La and β -Lg.

Discussion

This study has investigated the digestion of proteins contained in human milk, whey protein concentrate and infant formula by gastric juice in the pH range 6.5–4.0. Furthermore, this study has compared the *in vitro* digestion of these proteins to the *in vivo* digestion of human milk proteins in human infants.

During *in vitro* digestion studies, pH values were chosen to represent the variation of pH existing in the stomach of newborn infants during normal feeding. The average duration between feeds has been reported to be between 2 and 3 h during the first month of life (de Carvalho, Robertson, Merkatz, & Klaus, 1982; Benson, 2001). Under these conditions, gastric pH is not expected to fall below pH 4.0 in healthy term infants (Mason, 1962; Mitchell *et al.*, 2001). This present study has indicated that several proteins present in human milk, WPC and infant formula are resistant to the effects of proteolytic enzymes present in human neonatal gastric juice in the pH range 6.5–4.0. These findings are interesting in terms of the potential bioactivity of these proteins and suggest that intact proteins could have a biologically active role in the intestine of the newborn infant, or could be cleaved further in the intestine, possibly releasing biologically active peptides. Previous studies have reported that some human milk proteins such as sIgA are stable during digestion (Donovan, Atkinson, Whyte, & Lönnerdal, 1989). This study indicates that similar results were obtained using *in vitro* digestion of milk proteins with human neonatal gastric juice. Not only was sIgA observed to be stable to the effects of digestion, but several other proteins resisted the effects of digestion. One of these proteins was in the form of heteromers of α_{S1} -CN, the presence of which after incubation with gastric juice for 1 h at pH 2.0 indicated a significant biological role for this human milk protein. It is likely that these heteromers of α_{S1} -CN could be further cleaved in the small intestine by pancreatic enzymes such as trypsin, one of the major proteolytic enzymes. This could release peptides with angiotensin inhibitory activity. In support of this, trypsin has been shown to release peptides with an angiotensin-1-converting enzyme inhibitory activity from recombinant human α_{S1} -CN (Kim, Yoon, Yu, Lönnerdal, & Chung, 1999).

Another milk protein, MUC-1, has been reported to be present in the faeces of breast-fed infants, however it has not been determined whether faecal MUC-1 arises from endogenous sources or from human milk itself (Patton, 1994). Our present findings indicated that MUC-1 contained in human milk remained stable to the action of gastric proteases *in vitro*. These findings

are interesting in terms of the potential biological activity of MUC-1, which has been reported to inhibit binding of S-fimbriated *Escherichia coli* to buccal mucosa (Peterson *et al.*, 1998).

Intact human milk OPN was stable to proteolysis at pH 4.0 and above. These data indicate that it is likely to pass in intact form into the intestine. In contrast to these results, we also observed that OPN fragments of molecular weight ~ 34 and ~ 23 kDa were slightly degraded by gastric proteases. These results are worth attention, as the effects of these peptides and indeed human milk OPN remain unclear and require further investigation.

In the present study, we observed that human milk LF was resistant to the effects of digestion at pH 4 and above, in agreement with recent studies which have indicated that native human milk LF is stable during digestion with pepsin at pH 3.8 for 30 min (Suzuki *et al.*, 2003). Significant digestion occurred though at pH 3.5 and at lower pH values complete degradation occurred when analyzed by Tris-Tricine PAGE (results not shown). These findings correlate with those published previously which have shown that although the biologically active peptide lactoferricin is released during digestion, the digestion of LF itself has been shown to be incomplete (Kuwata *et al.*, 1998).

Human milk β -CN resisted digestion at pH 4.0 and above. Additionally, so too did a β -CN fragment commencing at amino acid residue 24 in the β -CN sequence. The detection of this peptide implies that the first 23 amino acids of β -CN are released in human milk. This region of β -CN is highly phosphorylated and has been suggested to play a role in the solubilization and uptake of calcium, zinc and iron (Tsuchita, Sekiguchi, Kuwata, Igarashi, & Ezawa, 1993; Peres *et al.*, 1998; Bouhallab *et al.*, 2002).

When the digestion of human milk was studied *in vivo*, in human infants, a great degree of proteolysis of human milk proteins was observed at 3 h following ingestion of human milk when compared to at 1 h. Approximately 90% of milk protein was emptied from the stomach at 1 h following ingestion. Therefore, the great degree of hydrolysis occurring 3 h following ingestion of human milk can be explained by the small amounts of residual milk protein in the stomach being subjected to greater amounts of gastric proteases than in the 1 h samples. Overall, the *in vivo* digestion results indicated a close correlation between *in vivo* and *in vitro* investigations. Furthermore, they reinforce observations gained from the *in vitro* studies that large amounts of proteins remain undigested at 1 h following ingestion of human milk.

Not only were many human milk proteins resistant to the effects of digestion—so too were many bovine milk proteins. Previous studies have reported that in human subjects, bovine LF is cleaved to produce the

biologically active fragment lactoferricin, *in vivo*. However, a large proportion of LF was not completely digested (Kuwata *et al.*, 1998). In the present study we observed that bovine LF was not hydrolyzed at pH 6.5 and at pH 5.0, pH values occurring after ingestion of human milk in human newborn. However, bovine LF was hydrolysed below pH 5.0, indicating a greater susceptibility to proteolysis than other bovine milk proteins. These observations indicate that LF could have a dual role in infants, partly as a result of the intact protein, and partly as a result of peptides produced early during the gastric phase of digestion.

Bovine OPN resisted digestion at pH 4.0 in a similar manner to human milk OPN. Furthermore, in contrast to human milk OPN, smaller OPN cleavage fragments resisted digestion. We observed a 60 kDa band which corresponded to previous reports for intact, glycosylated and phosphorylated OPN (Sørensen & Petersen, 1993). Like human milk, cleavage fragments of ~46; ~30 (N-terminal fragment) and ~20 kDa (C-terminal fragment) have been reported by others (Bayless, Davis, & Meininger, 1997; Salih, Zhou, & Glimcher, 1996). Furthermore, OPN has been reported to be present as dimers and dimers have been reported to bind to monomers (Goldsmith, Labrosse, McIntosh, Maenpaa, Kaartine, & McKee, 2002). Thus, the observation of several OPN moieties indicates the great heterogeneity of OPN.

Polyclonal antibody was prepared against purified EPV20 as described earlier (Larsen *et al.*, 1997). The antisera recognized both a ~20 kDa and a ~16 kDa protein. It is known that EPV20 is glycosylated at one Asn residue, however variation in this oligosaccharide chain have been reported and could be responsible for the formation of the ~16 kDa band (Larsen *et al.*, 1997). Furthermore, EPV20 is synonymous with a protein known as Epididymal Secretory Protein E1 (HE-1). This protein is a major secretory protein of human epididymis and specifically binds cholesterol. The protein has been reported to be glycosylated and to exist as both a 19 kDa and 16 kDa protein, both having the same N-terminal sequence. De-glycosylation of both of these purified proteins with N-glycosidase F resulted in a fragment having the same mobility of 14.5 kDa on SDS-PAGE (Okamura *et al.*, 1999). This 14.5 kDa fragment corresponds to the molecular weight of EPV20 calculated from the amino acid sequence alone. Therefore, it is likely that the 16 kDa band observed in our studies is a partially deglycosylated variant of EPV20. The resistance of both forms of EPV20 is very interesting and could suggest that EPV20 plays a role in cholesterol transport in newborn infants.

The two known variants of lactadherin formed major bands of ~47 and ~50 kDa as previously reported (Hvarregaard *et al.*, 1996). The presence of these moieties at pH 4.0 indicates that these are undigested. In

addition to these, we observed a series of extra bands. The C-terminal portion of lactadherin (amino acids 206–364) is known to form fibrils when in isolation from the intact protein and this phenomenon could be responsible for the higher molecular weight moieties observed by western immunoblotting of bovine lactadherin (Häggqvist *et al.*, 1999). Lactadherin has been suggested to provide resistance to rotavirus infection (Yolken, Peterson, Vonderfecht, Fouts, Midthun, & Newburg, 1992; Newburg *et al.*, 1998). These data also indicate that large amounts of lactadherin are likely to survive the process of digestion and could protect against rotavirus infection.

At least two forms of PP3 were observed in this study. Up to 7 forms of PP3 have been reported (Kanno, 1989). PP3 remained undigested down to pH 4.0. Although little is known regarding the effects of this protein, it shows homology with mouse and rat GlyCAM-1, a protein which has been shown to act as a ligand for lymphocytes. Furthermore, it has been found to be expressed in mammary gland, but not in peripheral lymph nodes, Peyer's patches, lung, spleen, heart and muscle (Johnsen, Sørensen, Petersen, & Berglund, 1995). These observations could indicate a possible specific function of PP3 in milk. This is hypothesis is reinforced by the observation that PP3 binds to cell membranes, the C-terminal portion of PP3 contains an amphipathic helix which is responsible for this membrane binding (Bak *et al.*, 2000; Campagna, Cosette, Molle, & Gaillard, 2001).

Both human and bovine α -La appeared to resist digestion. However, close inspection of the Tris–Tricine PAGE gels indicated the presence of a higher molecular weight band formed in both proteins during digestion. This new band is the result of nicking of α -La which releases the immune stimulating tripeptide glycyl-leucyl-phenylalanine (GLF) both *in vitro* and *in vivo* (Chatterton, unpublished observations).

Bovine caseins were cleaved by gastric juice at pH values under 4.0. This would result in release of the C-terminal portion of κ -CN known as CGMP, a highly glycosylated, phosphorylated peptide. CGMP is rapidly cleaved by one of the enzymes contained in gastric juice, pepsin. This C-terminal portion of κ -CN is highly glycosylated and has been proposed to have particular biological functions. For instance, CGMP has been shown to be released during digestion from human milk (Chobert, Mercier, Baby, & Haze, 1976) and is a strong growth promoting factor for *Bifidobacterium bifidum* (Bezkorovainy, Grohlich, & Nichols, 1979; Azuma, Yamauchi, & Mitsuoka, 1984). *Bifidobacterium bifidum* is an acid-generating anaerobe present at high concentrations in the intestine and stools of breast fed infants that reduces the growth of intestinal pathogenic microorganisms. Pepsin digestion of CGMP at pH 2.0 and at pH 4.5 rapidly cleaved CGMP and it is therefore

likely that the biological effects of CGMP are mediated via peptides not the intact protein.

In comparison to human milk, and to WPC, where many biologically relevant proteins were stable at pH 4.0, proteins present in infant formula were stable to digestion at pH 5.0 and above thereby indicating that thermal processing of these proteins during formulation resulted in a slightly increased susceptibility to proteolysis possibly linked to thermal processing of these proteins during formulation (results not shown).

Future trends and conclusion

In conclusion, the present study has provided information regarding the *in vitro* digestion of novel milk protein ingredients for use in infant formulas. The study has suggested the possible contribution of intact milk proteins as potential bioactive substances in the intestine of the newborn infant and further indicated some proteins which act as potential biologically active substances at a peptide level following digestion by gastric proteases. Because of the limitation in conducting clinical investigations designed to investigate the stability of milk proteins, *in vitro* digestion is an alternative tool for this purpose. The advantages that this method offers are that an insight into the mechanism by which a biologically active protein might act can be obtained by determining if a protein acts via its intact moiety or by its peptides.

Further studies are important to address the effects of heat treatment during formulation of these novel milk protein ingredients. Therefore, careful evaluations will be needed to gain a greater knowledge of the potential usefulness of milk protein ingredients incorporated in infant formulas.

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