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EFFECT OF CLOTTING IN STOMACHS OF INFANTS ON PROTEIN DIGESTIBILITY OF MILK

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Abstract

Differences in clotting between human and cow's milk in the stomachs of infants are discussed. Gastric pH, after ingesting milk, of an infant up to 6 months of age stays at a pH range of 4-5, near the isoelectric point of casein, and never reaches the value of 2, which is found in adults. Pepsin C (or gastricsin) can hydrolyze proteins at this pH range. Gastric emptying time is shorter with human milk than with cow's milk which appears to be correlated to the smaller size of human milk clots. Elimination of a readily coagulable fraction of casein from cow's milk by restricted rennet action produced β-casein rich milk with similar coagulating properties to that of human milk. Although pepsin digestibility at pH 2 was greater for bovine whole casein than bovine β-casein-rich fraction or human casein, this difference was minimized or even reversed at pH 4. This was ascribed to the difference in clotting behavior of α_{s1}-casein and β-casein, namely a harder clot of the former. Therefore, the difference in clotting and proteolytic properties between human milk and cow's milk in an infant's stomach can be explained from the difference in chemical properties of their major caseins, i.e., β-caseins and α_{s1}-caseins in human milk and cow's milk, respectively.

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Introduction

Digestion of protein in the stomach by pepsin is generally considered as a preliminary step to the digestion in the small intestine by more powerful proteases, i.e., trypsin, chymotrypsin and several peptidases. Since pepsin hydrolyzes the sites in peptide linkages which are different from the sites of hydrolysis by proteases in duodenal juices, the role of digestion in the stomach cannot be ignored, though it may be supplemental in the complete digestion of proteins in the digestive tract. In the case of infants, it is generally agreed that up to 3 months of age peptic activity is low and that minimal protein digestion occurs in the stomach (Berfenstam et al., 1955). Buchs (1973), however, suggested that the main physiological role of pepsin was to split off a few amino acids or peptides which stimulated the release of gastrointestinal hormones after they had reached the duodenal lumen.

Because of the important roles played by stomach digestion at the early, but essential, stage of complete digestion of proteins, it may be useful to know the meaning of clotting in the stomach in the digestion of milk (Ruegg and Blanc, 1982). The main concerns are the significance of clotting of cow's milk in comparison to that of human milk within the stomachs of human infants.

Gastric Functions in Infants

Gastric pH

Hydrochloric acid production is observed in the stomachs of infants soon after birth. Although newborn infants have a neutral to slightly alkaline gastric pH, within 24 hours after birth, gastric acid secretion reaches a peak comparable to that in a 3-year-old child. However, 2 days after birth, gastric acid secretion decreases rapidly, and a low level is maintained for at least 3 weeks (Harris and Fraser, 1968). The mean pH in the stomachs of 1-2 day old infants has been reported to be 3.0 - 3.1 but after the intake of milk the pH quickly rises due to a strong buffering capacity of milk. Hydrogen ion concentration in the stomach of full-term infants is estimated to be less than 30% of that in the adult stomachs (Lebenthal et al., 1983). Because of this difference, while pH in the stomachs of adults decreases to below 2 within 2 hours after ingestion of milk, in the case of infants up to 5 months of age, pH frequently stays between 4-5, even 2-3 hours after the intake of milk (Nakai, 1962).

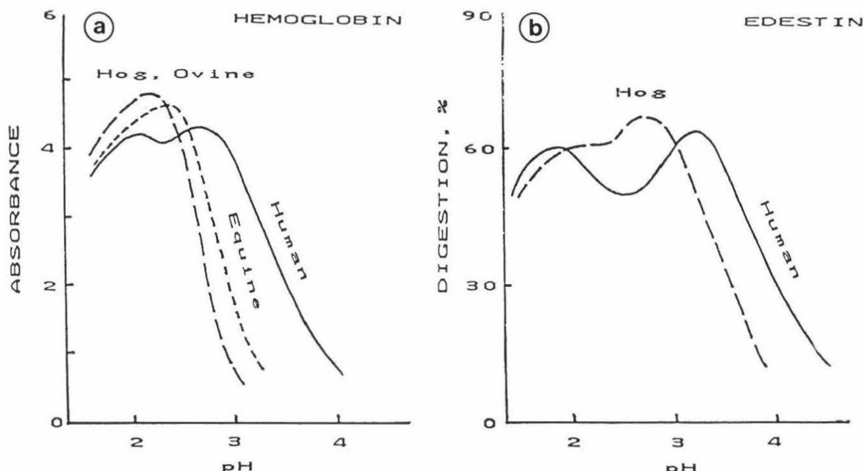

Fig. 1. pH-Activity profiles of pepsins in stomach juices from different mammals.

(a) Hemoglobin profile: a mixture of diluted stomach juice and 2% hemoglobin after pH adjustment was incubated at 35°C for 30 min and A_{280} of the 12% trichloracetic acid filtrate was measured. A_{280} value for the original juices before dilution was plotted.

(b) Edestin profile: a mixture of diluted stomach juice and 0.5% edestin after pH adjustment was incubated at 35°C for 30 min. The turbidity after addition of sulfosalicylic acid was measured as A_{660} and digestion rate (%) was computed from a standard curve (linear for A_{660} vs edestin).

It is interesting to note that immunoglobulins (Ig), which are useful for preventing pathogenic infection in an infant's intestine, can pass through the stomach without destruction as Ig are not stable at pH below 4, but are stable above pH 4 (Kaneko et al., 1985).

Pepsin secretion

Agunod et al. (1969) and Deren (1971), reported that during the first day of life, the peptic activity was one-fifteenth that in the adult. During the next 4-month period, the pepsin output increased sevenfold. Over this period, the pepsin secretion changed in accordance with the hydrogen ion secretion. In infants who were 2 years old, pepsin output per kg body weight became roughly comparable to that observed in adults.

When the pH-activity profile was measured, human stomach juice had 2 peaks compared to a single peak for stomach juices from most other mammals (Fig. 1). This double-peak property is dependent on the substrate. For example, infantile stomach juices had pH optima at 1.5 and 3.2 for edestin and 1.9 and 2.8 for hemoglobin, while only one peak of pH 2.2 was observed with casein (Nakai, 1962). However, this double peak property was not absolutely distinct, as purified hog pepsin showed a double-peak property at pH 1.8 and 2.6 using edestin with a smaller pH difference between the 2 peaks than human pepsin, while a single peak was observed for hemoglobin and casein.

It was postulated that there could be another protease present in the stomach with a higher optimum pH than pepsin, which would explain digestibility in the infantile stomach even at pH higher than the optimum pH < 2 of pepsin. This other enzyme has been isolated and identified as gastricsin by Tang et al. (1959). Since gastricsin is produced from the same zymogen as pepsin depending on gastric pH (Tang, 1970), and also it is as strongly proteolytic as pepsin (pepsin A, EC 3.4.23.1), it was categorized as pepsin C (EC 3.4.23.3) by the Nomenclature Committee (1984) for the International Union of Biochemistry.

Despite having an optimum pH (pH 2.8) similar to that of chymosin (pH 3.8 on hemoglobin), gastricsin is proteolytic, and not as milk clotting as chymosin (EC 3.4.23.4). There was no evidence for the presence of chymosin in the stomach of infants, even in those fed with cow's milk-based formula (Komura et al., 1957; Malpress, 1967).

Gastric emptying

Effects of age on the gastric emptying time are evident, although comparison of published data from different studies must be made with caution in order to match data using identical liquid meals in volumes appropriate to the size of the stomachs. Gastric emptying time is usually measured from the percentage of a meal remaining in the stomach (residual volume, V%) plotted against time after consumption of the meal. The stomach contents are withdrawn at certain time intervals after intake of the test meals containing an indicator, e.g., phenol red. From the

concentration of the indicator in the samples withdrawn, V is calculated. The half time is frequently used as the time required for V to fall to 50%.

Hunt and Spurrell (1951), Blumenthal et al. (1979), and Pildes et al. (1980) reported an average half-emptying time of 21.8 min and 44.6 min for adults and infants, respectively, when carbohydrate solutions were ingested. For cow's milk which clots in the stomach, the half time was extended to 45 min (Heading et al., 1976) and 87 min (Signer and Fridrich, 1975), respectively.

It is generally accepted that gastric emptying is delayed in premature infants compared to full-term infants. Gupta and Brans (1978) showed that during the first 12 hours of life, preterm infants emptied a smaller portion of dextrose solution than full-term infants.

In infants it appears that the type of meal affects emptying time. Most infants receiving breast milk had a rapid early phase with logarithmic decline followed by a linear phase of emptying (Cavell, 1979). In contrast, most of the infants fed a cow's milk formula had either a delayed early emptying phase followed by a linear emptying pattern or a linear emptying pattern from the beginning. In general, the overall gastric emptying time was slower with cow's milk than with human milk. An example with premature infants showed the half emptying time of 25.1 min vs. 51.9 min for human milk and cow's milk, respectively (Cavell, 1979).

Milk Clotting and Proteolytic Activity

Pepsin vs. chymosin

Although almost all proteinases clot cow's milk, the enzymes which have optimum pH in the acidic side (pH < 5) have a similar molecular structure and contain aspartic acid residues essential for proteolysis. Therefore, the enzymes in this category are called "aspartyl proteases".

These enzymes have similar sequences: a high level of similarity (57%) was observed between pepsin and chymosin, while 25% similarity was observed between chymosin and a bacterial rennet (Mucor miehei proteinase). However, penicillopepsin (the acid proteinase produced from the mold Penicillium janthinellum) had similarity values of only 25% and 27% with chymosin and pepsin, respectively (Yada, 1984). The milk clotting activity of proteinases, in units, has been defined as the amount of proteinase which clots 10 ml of reconstituted skim milk in 100 sec at 30°; the specific activity is then expressed as milk clotting activity per mg proteinase. Proteolytic activity may be determined as the ability to hydrolyze sodium caseinate and expressed as amount of tyrosine released per mg proteinase (Yada and Nakai, 1986). Milk clotting abilities expressed as the ratio of milk clotting to proteolytic activity were 95.8 and 47.0 for chymosin and pepsin, respectively, compared to 60-70 for microbial rennets and less than 0.8 for the proteinase from Asp. saitoi and for penicillopepsin. It appears, therefore, that the protein sequence itself may be relatively unimportant for milk clotting activity.

The three-dimensional structures of aspartyl proteases are bean shaped with a long substrate binding cleft between two domains (Drenth, 1981). The major catalytic functions of chymosin are derived from the carboxyl groups of Asp 32 and 215.

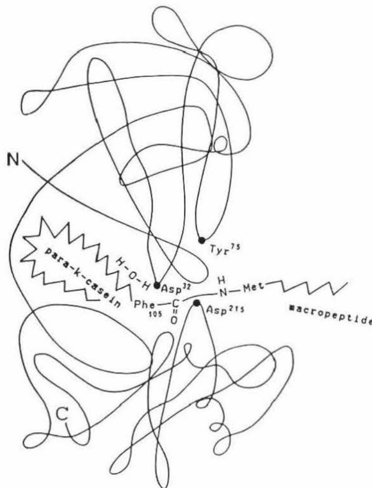

Fig. 2. Hypothetical illustration of the action of chymosin on κ-casein.

The ionized carboxyl group of Asp 32 activates a water molecule. The proton donor is Tyr 75 which is rather mobile; upon binding with the substrate, its phenolic OH-group moves near the NH-group of the susceptible peptide bond of the substrate (Fig. 2).

Discriminant analysis of electrical and hydrophobic parameters, zeta potential, surface hydrophobicity, and circular dichroism data showed the importance of β-sheet, β-turn, and random structure for milk clotting activity of proteinases (Aishima et al., 1987). For the coagulation of casein micelles, the κ-casein component, particularly its hydrophobic moiety, i.e., para-κ-casein portion, should be located near the two active Asp residues and Tyr residue in the vicinity of the cleft area of the enzymes (Fig. 2). Hydrophobic property inside the cleft may assist in the access to and proper orientation of κ-casein. More detailed information on the three dimensional structures of the substrate and enzymes is required for explaining the milk clotting activity, especially the difference in the mechanisms between pepsin and chymosin which are similar in sequence.

Clotting of Cow's Milk and Human Milk

Clotting of milk in the abomasum by chymosin is important for the digestion and absorption of protein in calves fed with milk. It has been well established that chymosin splits the peptide bond between Phe 105 and Met 106 of the κ-casein molecule yielding para-κ-casein and a macropeptide. This destroys the protective functions of κ-casein, resulting in precipitation of α_{s1}- and β-caseins in the presence of Ca^{++}. Stomach clots delay gastric emptying and thus

improve protein and fat digestion (Huber, 1969). When calves were fed with whole milk, clot prevention treatments resulted in a decrease in weight gains, feed efficiency and digestibility of dry matter, and marked increase in post-feeding levels of plasma amino acids and urea nitrogen (Jenkins and Emmons, 1982). Therefore, clotting of milk in the stomach is a physiological prerequisite for the completion of the protein digestion mechanism in calves.

In the case of human infants, the situation is reversed. The gastric emptying time is faster with human milk than with cow's milk (Cavell, 1979). Slight or no clotting of human milk is observed in the infantile stomach.

In vitro experiments using adult rats to study the effect of clotting on gastric emptying and digestion of bovine caseins have been reported (Miranda and Pelissier, 1981). The diet containing bovine skim milk clotted in the rat stomach, and resulted in a significantly higher amount of sediment remaining in the stomach 30 minutes after ingestion, indicating a reduction in the rate of gastric emptying compared to an unclotted diet based on 3% whole bovine casein solution in water. Electrophoresis of the remaining stomach contents indicated little proteolytic degradation in the clotted diet, whereas breakdown products from α_{s1}-, β- and κ-casein could be identified from the unclotted diet.

Ultrastructural studies of the milk curd in the gastric lumen of suckling rats (Berendsen, 1982) indicated an appearance similar to that reported for bovine milk curd and cottage cheese (Kalab, 1981). There was no ultrastructural evidence of intragastric proteolysis in suckling rats up to 15 days of age.

To reproduce milk clotting in the human stomach, the American Dairy Science Association (1941) proposed a standard method for measuring curd tension using a pepsin-HCl solution simulating gastric juice. "Soft curd milk" was defined as milk with a curd tension value below 20 g compared to values of 50-60 g for regular cow's milk. Curd tensions of both human milk and evaporated cow's milk were 0 g, meaning very fine coagulum formation or no clotting at all.

However, the pH of the clot according to this method was about 6.2 which was excessively high, thus corresponding only to a very early stage of stomach digestion even for infants. When an in vitro digestion test was carried out using a pepsin-HCl solution to simulate the physiological conditions, it was found that "soft-curd milk" no longer produced soft curd, and even evaporated cow's milk formed discernible curds while human milk showed either no clot or almost undetectable very fine curds (Fig. 3). None of the methods suggested for making soft-curd milk, i.e., dilution, heating, calcium reduction, and homogenization, were effective in simulating the clot of human milk under the physiological conditions in the infantile stomach (Nakai, 1963a). To withstand the stomach pH of a young infant, prevention of acid clot of casein, in which calcium is not involved in the coagulation mechanism, is essential.

Humanization of Cow's Milk

Cow's milk is reported to contain approximately 3.5 g protein/100 ml, whereas human milk usually averages 1.2 g/100 ml (George and Lebenthal, 1981). In addition, there are significant differences in the

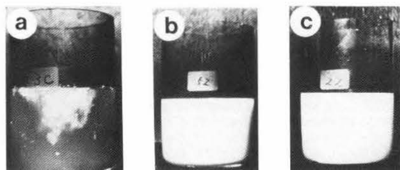

Fig. 3. Milk clotting after in vitro digestion test in **(a)** cow's milk, **(b)** modified milk, and **(c)** human milk. To 100 ml of milk sample at 37°C, 0.2% 3000X pepsin in 0.1 N HCl was added slowly at a flow rate of 15ml/h while the enzyme-milk mixture was gently stirred.

protein composition of the two milks, human milk having a much lower ratio of casein to whey proteins. Furthermore, it is generally agreed that the major casein in human milk is a β-casein-like fraction. Traditionally, casein has been classified into α-, β- and γ-caseins based on their electrophoretic mobilities. Nagasawa et al. (1967) found that β-casein was the major casein in human milk, which did not contain calcium-sensitive α_s-casein. Toyoda and Yamauchi (1972) concluded from the sedimentation rate, optical rotatory dispersion and circular dichroism data that the major fraction of human casein was similar to bovine β-casein, based on the temperature-dependent polymerization and molecular structure. Human β-casein, like bovine β-casein, produced a γ-casein-like degradation product as a result of plasmin hydrolysis (Azuma et al., 1985). In contrast, cow's milk contains about 45% α_{s1}-casein (Packard, 1982; Schmidt, 1982), a fraction which is either absent or present in minute quantities in human milk.

Upon acid precipitation, human milk produces a much finer protein floc than cow's milk (Fig. 4). The fine clot of human milk in the stomach apparently shortens the gastric emptying time as compared to the coarser cow's milk clot. Although the casein micelles of human milk are much smaller and presumably more digestible than those of cow's milk, they remain unchanged for 3 hours after nursing (Hadorn, 1981). In vitro studies demonstrated that the initial rate and extent of hydrolysis by pepsin were much greater at pH 2 for bovine milk than for human milk (Li-Chan and Nakai, manuscript submitted). At pH 4, the initial rate of hydrolysis was higher for human milk, but the extent of hydrolysis after 60 minutes was greater for bovine milk.

Preliminary experiments indicated that upon acidification to pH 4 in the presence of 11 mM $CaCl_2$, a fine soft floc was formed in the case of bovine β-casein, in contrast to a sticky hard clot for bovine α_{s1}-casein. In the absence of calcium ions, clear solutions were formed at pH 2 with both caseins, while at pH 4, a hard clot was observed for α_{s1}-casein, whereas β-casein solution was turbid with no visible evidence of clotting. Figure 5 shows the time course of hydrolysis of these casein fractions by pepsin, measured as absorbance at 280 nm (A_{280}) of the 2.5% trichloracetic acid-soluble fraction during proteolysis. At pH 2, the increase in A_{280} was more rapid and extensive for α_{s1}-casein than β-casein (Fig. 5a); on the other hand, at pH 4, most rapid and

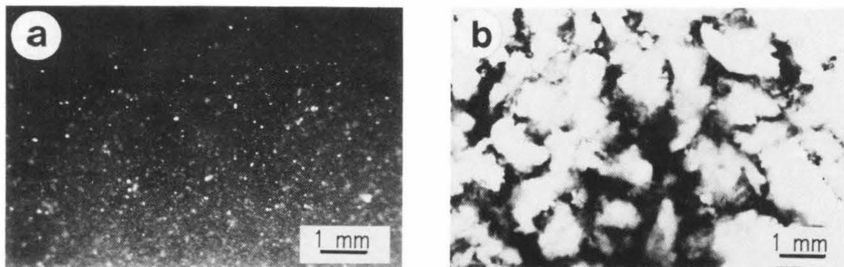

Fig. 4. Micrographs of **(a)** 1.25% human milk, and **(b)** 1.25% bovine milk samples acid clotted at pH 4.

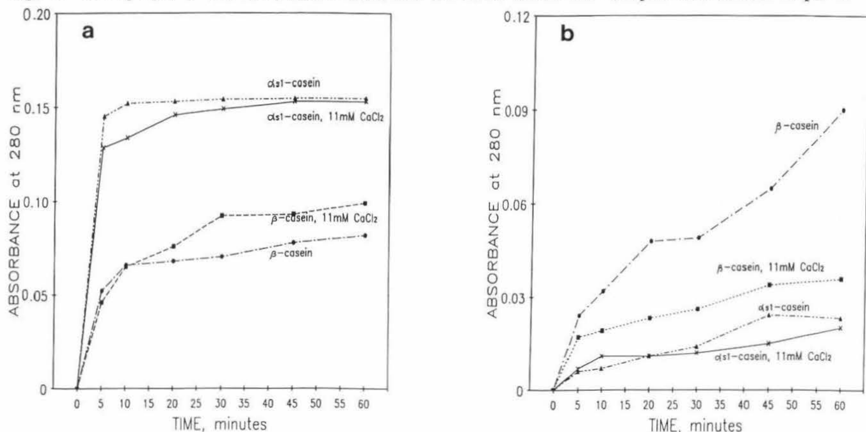

Fig. 5. Pepsin hydrolysis curves at (a) pH 2 and (b) pH 4 of bovine α_{s1}-casein and β-casein with and without 11 mM $CaCl_2$. (Hydrolysis conditions: 0.49% casein, 0.005% pepsin, 37°C).

extensive hydrolysis was observed with β-casein, especially in the absence of $CaCl_2$ (Fig. 5b). Tam and Whitaker (1972) also reported that the initial rates and extents of hydrolysis of caseins by both chymosin and pepsin generally decreased with increasing pH from 3.0 to 6.0; the initial rates of hydrolysis decreased in the order of α-, κ- and β-casein, at pH 3.0, 5.5 and 6.0. However, for β-casein, the extent of hydrolysis by 4 different enzymes (chymosin, pepsin, M. pusillus protease and E. parasitica protease) was greater at pH 3.5 than at 3.0, and hydrolysis at pH 3.5 was more extensive for β-casein than the other caseins. This is probably related to the formation of harder clots with α_{s1}-casein than with β-casein as the clotting pH approaches the isoelectric points of these proteins.

By careful control of the reaction conditions for chymosin activity to give only "partial" clotting, it was possible to prepare a modified milk with coagulability similar to that of human milk (Fig. 3). A limited amount of rennet was used which would result in coagulation of part of the protein after heating (Nakai, 1963b).

Recently, we have re-investigated the soluble casein fraction, recovered after mild rennet modification at neutral pH for partial coagulation of bovine casein, especially with respect to studying its clotting behavior and hydrolysis by pepsin at acidic pH (Li-Chan and Nakai, manuscript submitted). The process for rennet[a] modification of a 2% bovine casein

[a] Rennet is a common term referring to the commercial crude preparation which contains chymosin. In the work described here, the source of enzyme was "rennin" (Product R7751 from Sigma Chemical Co., St. Louis, MO) and the terms "rennet" and "rennin" have been used interchangeably.

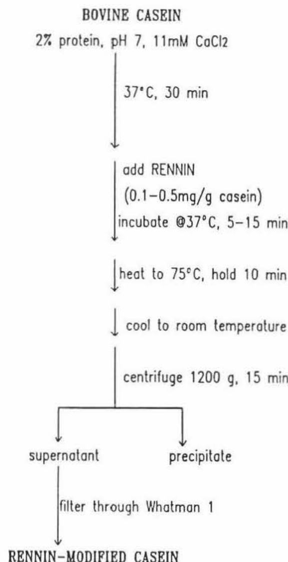

Fig. 6. Flow-chart for modifying bovine casein with rennet (source of enzyme: "rennin" from Sigma Chemical Co).

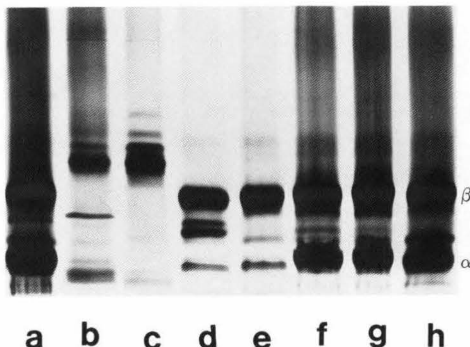

Fig. 7. Electrophoresis pattern of caseins and milks detected by silver staining. Samples: **a**: bovine milk, **b**: human milk, **c**: human casein, **d-g**: rennet-modified bovine casein (0.15, 0.10, 0.05 and 0.05mg rennin/g casein, respectively), **h**: bovine casein.

solution is shown in Figure 6. Analysis of electrophoretic patterns demonstrated that the ratio of β- to α_{s1}-casein was increased from 0.7 in the unmodified bovine casein, to higher than 3.0 in the soluble fraction recovered after rennet treatment (Fig. 7). Protein recovery in this fraction, rich in soluble β-casein, was 20-25%, yielding a 0.5% protein solution. This falls within the average range for casein concentration normally found in human milk. Thus, the preferential coagulation of α_{s1}-casein by this process can result in a β-casein-rich cow's milk that more closely resembles human milk with respect to casein concentration and composition.

Upon acidification of these casein solutions, it was observed that hardness of the clot formed at pH 2 or 4 decreased in the order of bovine, rennin-modified and human casein (Fig. 8). Large hard clots were formed by the acidification of bovine casein, especially at pH 4, whereas smaller clots were observed with rennin-modified casein. Turbid suspensions containing very fine protein flocs were observed upon acidification of human casein solutions.

Scanning electron microscopy of the fine flocs of human casein in solution acidified to pH 4 showed very tightly clustered protein particles (Fig. 9a). In contrast, the larger bovine casein clots consisted of a fairly dense lattice-like network of protein particles (Fig. 9b), while rennin-modified bovine casein formed a looser network (Fig. 9c). Rennin modified bovine milk also formed a loose structure but the globular particles were larger than those observed for casein (Fig. 9d).

At pH 2, the extent of hydrolysis by pepsin after 60 min incubation followed the order bovine > rennin-modified > human casein (Fig. 10a). On the other hand, at pH 4, the extent of pepsin proteolysis was greatest for rennin-modified casein (Fig. 10b). Both experiments were carried out at a protein concentration of 0.5%. The trends for pepsin hydrolysis of bovine control and rennin-modified caseins thus resemble those for α_{s1}- and β-caseins, respectively (Figs. 5a, 5b). Both bovine whole casein and α_{s1}-casein showed much greater susceptibility to pepsin hydrolysis at pH 2 than pH 4, which may be related to the harder clot formed as well as the lower activity of pepsin at the higher pH. On the other hand, β-casein and rennin-modified casein (which is predominantly β-casein) were still hydrolyzed at a moderate rate, even at pH 4, which may be related to the open loose structure of these caseins. Similar trends were observed when the protein concentration of casein solutions as substrate for pepsin hydrolysis was increased to 2% or when milk samples (1.25% protein) were used as substrates. However, when the protein substrate concentration was only 0.1%, none of the casein samples yielded large clots at either pH 2 or 4, and the differences in their rates and extent of hydrolysis were also minimal. These results suggest that both the clot formation as well as the composition of the casein fraction (e.g., α_{s1}- vs. β-casein) affect proteolysis, especially at pH 4 which is similar to the gastric conditions of infants.

A possible reason why bovine α_{s1}-casein was more digestible than β-casein at pH 2 may be due to the accessibility of peptide linkages susceptible to pepsin hydrolysis. Since A_{280} was used to monitor release of 2.5% TCA soluble peptides, the higher aromatic amino acid content of α_{s1}-casein than β-casein may also partly explain the differences in A_{280} as a function of hydrolysis time. However, reversion of

Fig. 8. Micrographs of **(a)** 0.5% human casein, **(b)** 2% bovine casein, and **(c)** 2% rennin-modified casein, clotted at pH 4.

Fig. 9. Scanning electron micrographs of (a) human casein, (b) bovine casein, (c) rennin-modified casein and (d) rennin-modified milk clotted at pH 4. Black bar = 2 μm; 20kV; arrows in (a) point to dense clusters of casein particles. Samples acidified to pH 4 were fixed with 2.5% glutaraldehyde. After rinsing with 0.1M sodium cacodylate buffer (pH 7.4), the particles were treated with 1% osmium in cacodylate buffer, rinsed, then further fixed with 2% uranyl acetate, prior to dehydration (30-100% alcohol) and critical point drying. Dried samples, mounted on slabs with conductive silver cement, were coated under argon with 30nm thickness of gold and observed in a Cambridge 250T scanning electron microscope.

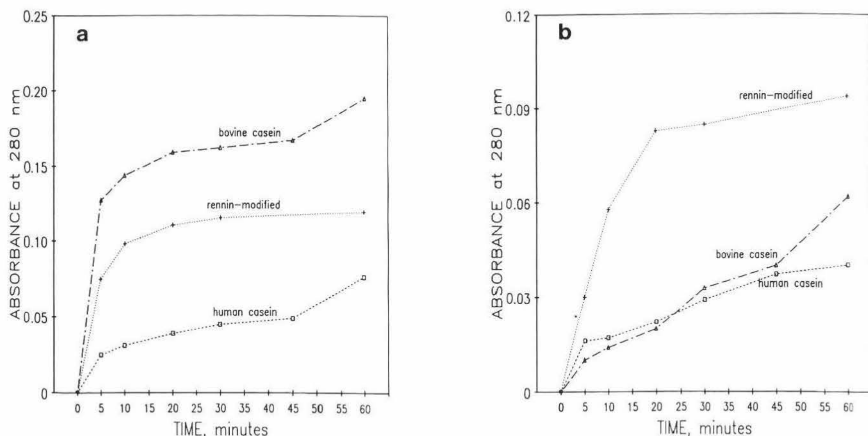

Fig. 10. Pepsin hydrolysis curves at **(a)** pH 2, and **(b)** pH 4, of human casein, bovine casein and rennin-modified casein. (Hydrolysis conditions: 0.49% casein, 0.005% pepsin, 37° C).

this situation at pH 4 may have been due to the clotting difference at pH close to the isoelectric points of the caseins. Because the α_{s1}-casein clots were firmer, pepsin could not penetrate through and the degree of hydrolysis was considerably decreased.

The reasons for the difference in clotting at the isoelectric points between the two caseins are unknown. In general, protein solubility is explained based on the interrelation between charge and hydrophobicity (Hayakawa and Nakai, 1985). If this concept is accepted, the solubility of proteins at the isoelectric point should be controlled solely by hydrophobicity, as the net charge is minimal at the isoelectric point. However, the content of hydrophobic amino acid residues is higher in β-casein than α_{s1}-casein (Eigel et al. 1984). This means that β-casein should form harder clots than α_{s1}-casein, which is opposite to what is observed. A possible explanation is that most of the hydrophobic side chains in β-casein are not fully exposed due to some steric hindrance. The degree of phosphorylation is in the order of α_{s1}-casein, β-casein and human β-casein (8, 5 and 0-5 moles of phosphate per molecule, respectively). Although the formation of strong salt bridges at the isoelectric point in the presence of calcium is unlikely, it is possible that some salt bridge formation due to a strong dissociating ability of the phosphate radicals at pH 4.6 contributes to the formation of firm clots.

Although human and bovine β-caseins have been reported to be homologous with respect to the amino acid sequences (Greenberg et al., 1984), the temperature of polymerization of human β-casein is higher than that of bovine β-casein, i.e., 20°C vs. 8.5°C (Toyoda and Yamuchi, 1972) and antigenic reactivities are different between the two β-caseins (Otani et al., 1984). Furthermore, human κ-casein differs from its bovine counterpart in having a much higher carbohydrate content, i.e, 40% vs. 5%, and shows a greater stabilization of α_{s1}-casein than bovine κ-casein in the presence of calcium ions (Yamauchi et al., 1981). Unlike bovine κ-casein, human κ-casein is present in a monomeric form at pH 7 (Azuma et al., 1984). These differences in β- and κ-caseins may be a reason for differences in casein micelle formation and acid clotting between human and cow's milks. The tight protein clot structure observed by SEM for acidified human casein compared to the looser structure of the bovine casein clot (Fig. 9) may explain the lower extent of proteolysis by pepsin of human milk than cow's milk.

Preferential coagulation of bovine α_{s1}-casein fraction by rennet treatment yields a β-casein rich milk which forms softer clots and is more susceptible to pepsin digestion at pH 4 than bovine milk. β-casein enrichment in cow's milk has also been reported by Rose (1968) by ultra-centrifuging the milk at 4°C to sediment α_{s1}-casein rich micelles. Thus simulation of human milk clots in the infant stomach is now feasible to a certain extent. However, the significance of the more detailed differences between human and bovine species in their α-and β-caseins as well as other protein and non-protein constituents requires further studies, before the true meaning of this simulation for human feeding can be assessed.

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Discussion with Reviewers

Reviewer IV: In Figs. 5 and 10, have the authors considered (or corrected for) effects of differing aromatic amino acids in α_{s1}- and β-caseins? This might affect the relative positions of their curves.

Authors: It is true that the differing aromatic amino acid contents of α_{s1}- and β-casein would affect their absorbance at 280 nm (Tyr+Trp+Phe contents are approximately 14 and 24 residues per monomer for β-casein and α_{s1}-casein, respectively (Thompson, 1971), while A_{280} values at 1% are 4.6 and 10.1, respectively, (Sober, 1972)). However, it is not possible to directly correct the absorbance values shown in Figs. 5 and 10 for these differences since there may not be any direct correlation between the total content of aromatic amino acid residues in a protein and the extent of their release by pepsin in the form of 2.5% TCA-soluble peptides. The differing aromatic acid contents may explain in part the observation that A_{280} values of the soluble fraction after pepsin hydrolysis at pH 2 for 20-60 min have been almost double for α_{s1}-casein compared to β-casein (Fig. 5a). However, the reversal of this trend at pH 4 (Fig. 5b), which shows higher A_{280} for the soluble fractions from β-casein than α_{s1}-casein, strongly suggests that the extent of hydrolysis was greater for β-casein at pH 4. To confirm this, a different method to quantitate hydrolysis in the TCA-soluble fraction would be required, e.g., Kjeldahl nitrogen or biuret reaction. In this regard, the results of Tam and Whitaker (1972, text reference) using 2,4,6-trinitrobenzenesulfonic acid to follow peptide bond hydrolysis indicated higher extents of hydrolysis of α-casein at pH 3.0 and 6.0, but higher extents of hydrolysis of β-casein at pH 3.5.

Reviewer IV: What is the yield of rennin-modified casein (in the process shown in Fig. 6)? What do the authors propose to do with the residual α_{s1}-κ complex?

Authors: The yield of rennin-modified casein is approximately 20-25% of the starting casein. The residual casein complex could be useful as a food ingredient, or perhaps as a process cheese ingredient.

P.B. Berendsen: Although the magnifications in Figures 9a, b, c, and d are the same, the casein micelles in Figure 9d appear to be larger. Has this simply resulted from choice of areas or did the treatment of the sample shown in Figure 9d enlarge the casein micelles?

Authors: Firstly, the particles in these samples should not be referred to as micelles. The caseinate samples (Figs. 9a and b) were prepared by acidifying milk to pH 4.6 at 40°C. The rennet modification process to prepare samples in Figs. 9c and d would also destroy the micellar structure. The differences in particle sizes were definitely not due to a choice of area; the micrographs depict typical sizes of particles in each of the samples after clotting at pH 4. The difference between samples in Figs. 9a, b and c, versus that in Fig. 9d, is that the latter represents rennin-modified milk at pH 4 whereas the other three were casein samples at pH 4. The basis for these differences is not clear to us.

P.B. Berendsen: Have you done, or are you aware of any studies which compare the caseins of colostral milk or milk at the initiation of suckling with those later during milk secretion? If so in what way do they differ?

Authors: Ruegg and Blanc (1982, text reference) have published a review on the structure and properties of particulate constituents of human milk, including changes at different stages of lactation. These changes include the absence of the characteristic band of β-casein in electrophoretic patterns of colostral or early milk (1 to 4 days post partum), and a decrease in citrate concentration and increase in average diameter of casein particles with advancing lactation.

J.J. Strandholm: What, if anything, is known regarding the effects of longer gastric emptying time of cow's milk compared to human milk on the nutrition or health of infants?

Authors: We are not aware of any published studies that deal with this question.

Additional refernces

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