In Vitro Simulation of Gastric Digestion of Milk Proteins

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On the basis of in vivo data obtained during digestion of milk proteins in preruminant calf stomach, an in vitro method is proposed to simulate the gastric emptying of digestive products. The reaction vessel, containing 500 mL of skim milk and liquid calf rennet, was placed in a shaken water bath. Peristaltic pumps continuously provided, at a variable flow rate, additional rennet and HCl and allowed the collection of digested products. Evacuated material was collected in trichloroacetic acid at a final concentration of 12%. The pH of the incubation medium was measured during digestion, and the effluents were analyzed for nitrogen content by the Kjeldahl method, liberation of milk proteins by electrophoresis, and some characteristic peptides by HPLC. The model provided in vitro results that showed an excellent correlation with in vivo data.

Milk proteins represent 20–30% of dietary proteins in human nutrition of the industrialized world (Hambraeus, 1982). It has been shown in vivo that milk coagulation in the stomach slows down casein degradation. In the first part of digestion four main peptides are released from caseins. Three of them appear rapidly in the effluent (1–23 and 165–199 of α_{s1}-casein and 106–169 of κ-casein or CMP) and then disappear whereas the last one (193–209 of β-casein) appears progressively with time (Miranda and Pêlissier, 1981, 1983; Kaufmann, 1984; Meisel and Hengemeister, 1984; Yvon et al., 1984; Yvon and Pêlissier, 1987). On the other hand, β-lactoglobulin and α-lactalbumin are resistant to hydrolysis (Yvon et al., 1984). Slow degradation of caseins into smaller peptides takes place afterward, and α-lactalbumin is hydrolyzed when stomach pH is under 4 (Yvon et al., 1984; Miranda et al., 1987; Yvon and Pêlissier, 1987).

Technological treatments of milk modify its gastric digestion (Kaufman, 1984; Meisel and Hengemeister, 1984; Miranda and Pêlissier, 1987). In vivo experiments on animal or man are relatively difficult to realize, in particular for deontological reasons. The study of the effect of technological treatments of milk on its digestion could be facilitated by using in vitro analysis. A number of methods combining enzymatic hydrolysis with nonprotein nitrogen quantification, amino acid analysis, or pH modification have been used to estimate the digestibility and the nutritional value of proteins (Camus and Sautier, 1972; Satterlee et al., 1982; Raghunath and Narasinga Rao, 1984; Mitchell and Grundel, 1986; Oh and Hoff, 1986; Philipps and Baker, 1987; Berger and Possompes, 1987). These assays often consist of a batch peptic predigestion of proteins, generally followed by digestion with pancreatic enzymes; analysis are then performed on end products. To overcome the problems of the accumulation of digestion products and subsequent inhibition of proteolysis, a method based on the enzymatic hydrolysis of proteins with simultaneous dialysis of digested products has been developed to simulate the pancreatic digestion (Gauthier et al., 1982; Savoie and Gauthier, 1986). However, the digestibility measurements established in vitro and in vivo in rats did not correlate well (Vachon et al., 1987). In fact, in these methods, gastric digestion was reduced to a peptic batch predigestion of proteins, and one can therefore suppose that in such in vitro assays the kinetics of digest evacuation from the stomach was one factor not taken into consideration. Yvon and Pêlissier (1987) showed that the stomach role was not only to ensure a peptic hydrolysis of proteins but also to regulate the release of proteins and to select peptides that enter the gut. This progressive sequential evacuation of peptides into the duodenum could have an important role in the regulation of gastric and pancreatic secretions and thereafter in the absorption of amino acids and peptides.

The aim of the present work was to simulate several of the most important phenomena observed in vivo in the stomach during digestion of milk proteins, to obtain an in vitro model for studying gastric digestion of proteins.

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Table I. Tests Performed To Check Parameters Chosen To Simulate in Vivo Digestion

parameter	requested phenomena (in vivo observns)	test
total evac N	−20 to +40% of total ingested N at end of assay	Kjeldahl
acidification of med	pH 2.0 at end pH 3.5 at half-time	pH
addn of gastric proteinas	retention of casein hydrolysis of α-lactalbumin below pH 4	SDS-PAGE
	kinetics of evacuation of four peptides	RP-HPLC
gastric emptying (way, rate)	kinetics of evacuation of four peptides	RP-HPLC
shaking conditions	retention of caseins	SDS-PAGE
motor-drive helix (fermentor)	homogenization of incubn med	pH curve
oscillation (Erlenmeyer flask)	sequential evac of digestion products	RP-HPLC

MATERIALS AND METHODS

Products. Milk was collected from a single cow, homozygous for the four caseins (α_{s1}-B, α_{s2}-A, β-A1, κ-A), skimmed, lyophilized, and stored frozen (−20 °C). It was reconstituted just before use with deionized water. Liquid rennet (520 mg of chymosin, 290 mg of pepsin/L) was obtained from Boll-Hansen (France). All other chemicals were purchased from Prolabo (France) and Merck (FRG).

Gastric Digestion Unit. A thermostated fermentor or a 1-L Erlenmeyer flask, fixed in a shaking water bath (Haake SWB 20, Munich, FRG), regulated at 37 °C was used to simulate the stomach. Acidification of the medium and enzyme supply were ensured through two peristaltic pumps (Pharmacia, Upsala, Sweden) with variable flow rates. Another peristaltic pump allowed the collection at the bottom of the flask (lower part collection) or at the liquid–air interface of the medium (upper part collection) of the whole digestion products during 3-h assay. The pH of the incubation medium was measured during digestion.

Milk Digestion and Sample Collection. Table I indicates the parameters that were chosen to simulate in vitro the main phenomena that have been observed in vivo in the preruminant calf (Pêlissier et al., 1983; Yvon et al., 1984; Yvon and Pêlissier, 1987) and to take into account in vivo acidic and enzymic gastric secretions, which could represent up to 200% of ingested volume

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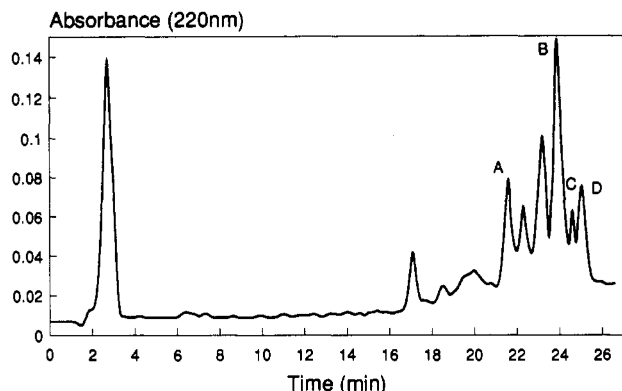

Figure 1. RP-HPLC chromatogram of 12% TCA insoluble and 0.1% TFA soluble fractions, collected between 15 and 20 min after beginning the digestion. For identification of fractions A–D, see Table II.

(Bernier, 1984; Guilloteau, 1986).

A 500-mL portion of skim milk was used for each experiment. Liquid rennet was added to obtain an initial enzyme/substrate (E/S) ratio of 1/2500 (w/w). Ratios from 1/10000 (w/w) to 1/2500 (w/w) were also tested. Acidification was obtained by adding 143 or 286 mM HCl. Different flow rates for adding enzyme or collected digesta and agitation mode (helix in a fermentor or shaken water bath) were tested.

To obtain a constant volume in the stomach model, the following conditions were finally retained: 143 mM HCl at a constant flow rate of 2.0 mL/min; diluted enzymes (2% liquid rennet v/v) at variable flow rates, 5.5 mL/min during the first 15 min, 3.5 mL/min during the following 45 min, and 1.0 mL/min up to the end of experiment (3 h). Emptying rates were 7.5 mL/min for 15 min, 5.5 mL/min up to 1 h, and then 2.5 mL/min up to 3 h. The contents were shaken at 150 oscillations of 5 mm/min not only to homogenize the medium with its HCl and rennet content but also to ensure the formation of the coagulum. The total amounts of effluent were collected in 17 samples over a period of 3 h. During the first 30 min, six samples were collected every 5 min; during the following 30 min, three samples were collected every 10 min; during the remaining 2 h, eight samples were collected every 15 min.

Samples Analysis. Each sample was immediately precipitated with trichloroacetic acid (TCA) at a final concentration of 12% and centrifuged at 1600*g* for 10 min. The supernatant was analyzed for nitrogen. After homogenization in water, the pellet was extracted eight times with an equal volume of diethyl ether to eliminate TCA, brought to pH 7.0 with 1 N NaOH, and lyophilized. This fraction was analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and for nitrogen. An 1.5% water solution of lyophilized pellet was brought to pH 4.6 with trifluoroacetic acid (TFA) and centrifuged at 8500*g* for 15 min. The soluble fraction, corresponding to the "non-casein" products, was analyzed by RP-HPLC. Four peptides were chosen as markers of the gastric emptying digestion from the in vivo data of Yvon and Pélissier (1987): 1–23 and 165–199 of α_{s1}-casein, 106–169 of κ-casein, 193-209 of β-casein. These peptides were collected by RP-HPLC (Figure 1) and identified by their amino acid composition and N- and C-terminal sequences (Table II).

Techniques. Nitrogen level was determined according to the Kjeldahl method with the colorimetric technics of Koops et al. (1975).

SDS-PAGE measurements were made according to the method of Laemmli (1970) adapted to milk proteins by Trieu-Cuot and Gripon (1981).

RP-HPLC analyses were performed on a Spectra Physics LC system. The column (C_{18} μBondapack, Waters) was equilibrated with 0.11% TFA, and the peptides were eluted with a linear gradient to 0.10% TFA, 60% acetonitrile for 42 min. The column was kept at 40 °C. Elution rate was 2 mL/min. Peptides were detected at 220 nm.

Amino acid compositions were determined after acid hydrolysis (110 °C, 24 h, 5.7 N HCl, under vacuum) according to Spackman et al. (1958) with a Biotronik LC 5000 analyzer (Munich, RFA).

Table II. Identification of the Four Peptides Chosen as Markers (Amino Acid Composition and Sequence)[a]

peptide	A	B	C	D
Asx	2.0 (2)	4.8 (5)	2.7 (4)	
Thr		11.0 (12)	3.4 (4)	
Ser		5.4 (6)	3.4 (4)	
Glx	3.8 (4)	9.6 (10)	3.7 (3)	1.9 (2)
Pro	2.3 (3)	8.2 (8)	5.2 (5)	3.6 (4)
Gly	1.0 (1)	1.6 (1)	2.0 (2)	1.8 (2)
Ala		4.8 (5)	1.4 (1)	
Val	1.2 (1)	5.4 (6)	1.7 (1)	2.4 (3)
Met		0.8 (1)	0.8 (1)	
Ile	1.2 (1)	5.2 (6)	1.7 (2)	1.1 (2)
Leu	3.6 (4)	1.0 (1)	1.7 (2)	1.0 (1)
Tyr			2.4 (3)	0.8 (1)
Phe	1.0 (1)		1.0 (1)	0.9 (1)
His	2.0 (2)			
Lys	2.0 (2)	3.0 (3)	1.2 (1)	
Arg	1.8 (2)			0.8 (1)
C-ter	Phe	Thr,Ala,Val	ND	Ile,Val
N-ter	Arg-Pro-Lys	Met-Ala	ND	Tyr-Gln
origin	1–23 α_{s1}-casein	106–169 κ-casein	165–199 α_{s1}-casein	193–209 β-casein

[a] Results (mole/mole) were obtained after 24-h hydrolysis. Figures in parentheses are theoretical values. ND = not determined. For name of peptide, see Figure 1.

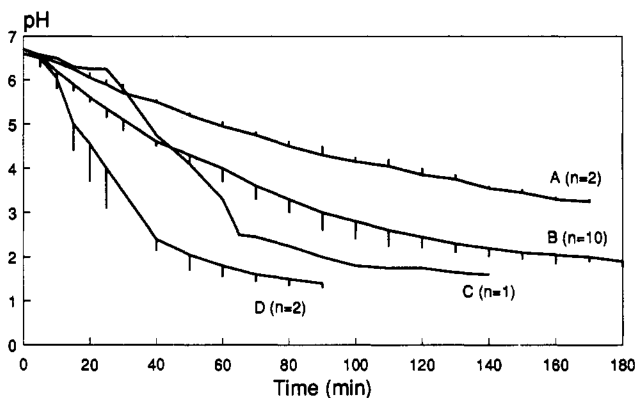

Figure 2. pH decrease in the incubation medium during in vitro gastric digestion of skim milk (*n* = number of the experiment; — = mean; | = standard deviation): (A) 143 mM HCl, 1.5 mL/min, constant shaking; (B) 143 mM HCl, 2.0 mL/min, constant shaking; (C) 143 mM HCl, 2.0 mL/min, irregular stirring; (D) 286 mM HCl, 3.0 mL/min, regular stirring.

C-Terminal sequence was determined after the action of carboxypeptidase A (Sigma, St Louis, MO) and their N-terminal sequence by recurring Edman degradation using Tarr's technique (1982).

RESULTS

Nitrogen Emptying. Change of the different parameters studied did not significantly modify the total nitrogen emptying. The quantity evacuated during 3 h represented around 35% of the added nitrogen. This corresponded with the data obtained in vivo during 6-h assays (Ternouth and Roy, 1973; Pélissier et al., 1983).

Acidification of the Incubation Medium. The progressive acidification of the medium is described in Figure 2. At a high acidification rate (curve D, 286 mM HCl at 3.0 mL/min) the pH drop is too fast, whereas the acidification was incomplete with a low acidification rate (curve A, 143 mM HCl at 1.5 mL/min). Satisfactory acidification was obtained by adding 143 mM HCl at 2.0 mL/min (curve B). The initial pH was between 6.70 and 6.80, and it reached the value 3.5 at about half of digestion time and the value 2.0 at the end of the assay. Conditions and rate of emptying and initial E/S ratio had no effect on the

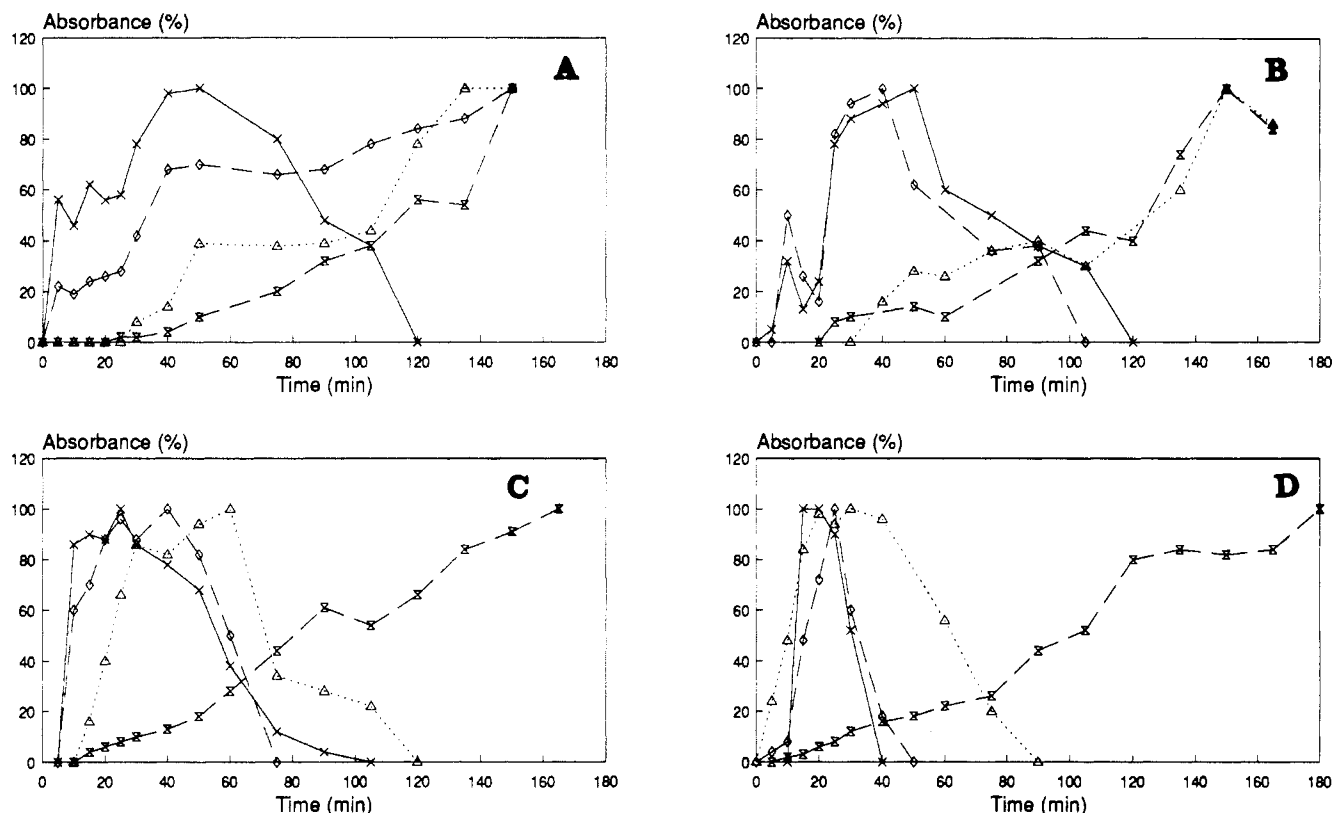

Figure 3. Schematic representation of the kinetics of in vitro gastric emptying of four peptides from caseins: ×, CMP; ⧖, 193–209 from β-casein; ◇, 1–23 from α_{s1}-casein; △, 165–199 from α_{s1}-casein. 100% correspond to the maximal area observed during the assay for each peptides. Key: (A) emptying, bottom part of fermentor, constant rate, E/S ratio 1/5000; (B) emptying, upper part of fermentor, constant rate, E/S ratio 1/5000; (C) emptying, upper part of fermentor, constant rate, E/S ratio 1/2500; (D) emptying, upper part of shaken water bath, variable rate, E/S ratio 1/2500.

acidification curve, except for the stirring conditions (curve C).

Mode of Emptying and Effect of Initial E/S Ratio. Figure 3 is a schematic representation of the evacuation kinetics of the peptides used to characterize the digestion. Gastric emptying from the upper part of the flask instead of the lower part ensured the retention of peptide 1–23 in the coagulum and its progressive disappearance from the effluent (Figure 3A,B). The increase of the initial E/S ratio from 1/5000 to 1/2500 confirmed the further hydrolysis of this peptide by gastric enzymes (Figure 3B,C).

Finally, introducing a variable emptying rate, 3 times faster during the first 15 min, correlates with in vivo results (Yvon and Pélissier, 1987) whatever the model used (fermentor or shaken water bath, Figure 3D): i.e., rapid appearance and disappearance of three peptides (CMP from κ-casein, 1–23 and 165–199 from α_{s1}-casein) and a regular appearance of peptide 193–209 from β-casein.

Shaking Conditions and Coagulation of Caseins. Different modes of shaking and homogenizing of the incubation medium were checked to ensure coagulation in about 15 min (Table III). Coagulation is characterized by the disappearance of caseins in the collected samples (Figure 4).

With irregular or slow shaking, the coagulation was obtained during the first 10 min, but the decrease in pH and homogenization of the medium were not satisfactory (Figure 2C). On the other hand, when the incubation medium was vigorously shaken, caseins did not coagulate and peptides originating from casein hydrolysis appeared in the effluents as soon as caseins disappeared.

An intermediate and constant mode of agitation in a shaken water bath was therefore chosen. Frequence of agitation, 150 oscillations of 5 mm/min, were settled upon

Table III. Influence of the Mode of Shaking on Retention of Caseins in the Coagulum[a]

shaking condition	pH curve (Figure 3)	coaguln time, min	consequences on acidificn and hydrolysis
	Helix Agitation (Fermentor)		
regular	A	40 (−)	hydrolysis and appearance of peptides too long
	B, D	25 (−)	good acidificn and release of peptides
irregular	C	10 (+)	irreg acidificn and release of peptides
	Oscillation Water Bath (Erlenmeyer)		
100 osc/min		10 (+)	irreg acidificn
200 osc/min	D	60 (−)	no coaguln
150 osc/min	B	15 (+)	progressive acidificn and appearance of peptides

[a] Key: (−) coagulation too slow (caseins in the effluent); (+) good coagulation (no caseins after the time indicated).

so that the 500 mL of skim milk could be shaken in a 1-L Erlenmeyer flask. Under such conditions, caseins could be identified by SDS-PAGE only during the first 15-min period (Figure 4). There was little or no degradation of β-lactoglobulin; α-lactalbumin disappeared after about 90 min. It was probably hydrolyzed simultaneously with the decrease of pH. These results simulate well the processes observed in vivo (Yvon et al., 1984; Yvon and Pélissier, 1987).

DISCUSSION

It is technically impossible to reproduce exactly the entire in vivo process of the gastric protein digestion, but it is important to take into account the essential phenomena observed: progressive acidification, hydrolysis by gastric proteinases, gastric emptying, endogenous se-

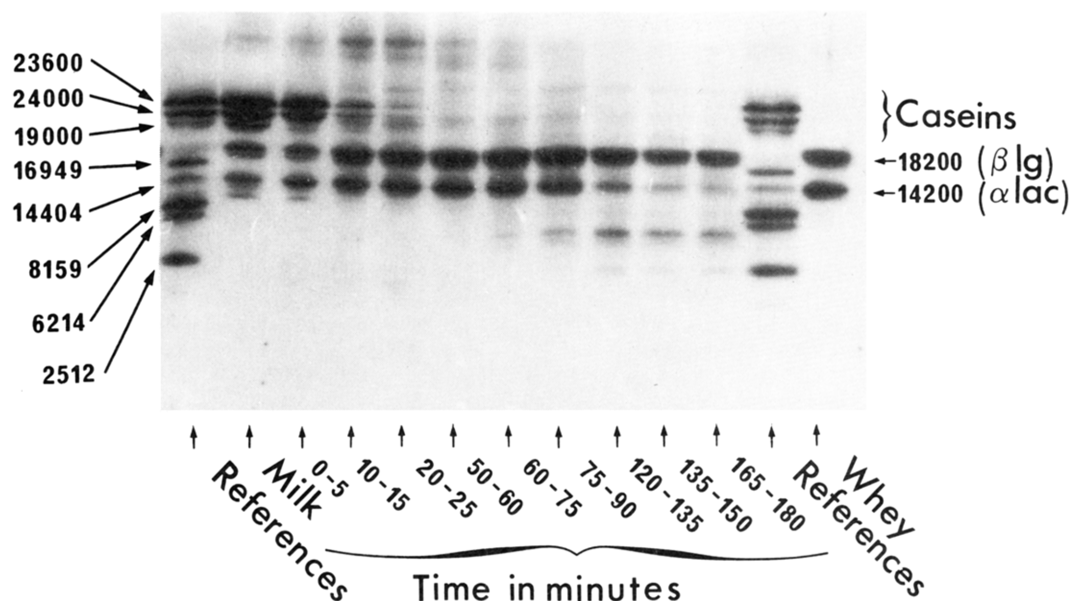

Figure 4. SDS-polyacrylamide gel electrophoresis of samples collected under the final conditions: HCl, 143 mM at 2.0 mL/min; 2% liquid rennet at 5.5 mL/min (15 min), 3.5 mL/min (45 min), 1.0 mL/min (2 h); emptying, 7.5 mL/min (15 min), 5.5 mL/min (45 min), 2.5 mL/min (2 h); 150 oscillations of 5 mm/min. Time in minutes.

cretions, and, in the case of milk proteins, casein retention in the form of coagulum.

The choice of pH conditions is a very important factor affecting the activity of enzymes and thus the composition of the evacuated products. The pH of the stomach does vary during in vivo digestion: It increases rapidly from pH 2 (pH of gastric juice) to the pH of the diet during the period just following the meal and then decreases progressively toward its initial value (Guilloteau et al., 1975; Bernier, 1984). This slow decrease of the pH is necessary to ensure emptying of intact proteins or large peptides that could play a role in the establishment of immunity of the young (Reiter, 1978) or in the regulation of digestive secretions. Such a curve was obtained by using 143 mM HCl, which corresponds to the molarity of acidic secretions in human beings (Bernier, 1984).

Gastric digestion of proteins is rather incomplete: Polypeptides of high molecular weight and only few small peptides are rapidly evacuated and submitted to pancreatic enzyme action. In the calf, enzyme secretions are maximal during the period following the meal (Ash, 1964; Williams et al., 1976). Taking into consideration the quantities of chymosin and pepsin secreted in the preruminant calf (Guilloteau and Toullec, 1983) the E/S ratio was estimated as being around 1/3000 (w/w) during the first hour following the meal. Finally an initial E/S ratio of 1/2500 (w/w) gave in vitro a correct coagulation and hydrolysis of milk proteins.

The gastric emptying process is not monophasic (Decuypere et al., 1986), and there is a critical concentration of digestion products at which pepsin is no longer active (Camus and Sautier, 1972). By the use of simultaneous evacuation of noncoagulated digestive products and acid and enzyme arrival in a three-step emptying, a good simulation of in vivo digestion (Yvon and Pélissier, 1987) was obtained: i.e., N emptying, coagulation of caseins, appearance and sometimes disappearance of peptides and α-lactalbumin, pH evolution.

Finally, by means of biochemical techniques it was possible to follow and reproduce the kinetics of the gastric digestion of milk proteins. The data obtained by SDS-PAGE and RP-HPLC confirmed observations on the incubation medium during the digestion assay. The clotting of milk is complete in less than 15 min, when the two phases (coagulum and whey) are clearly differentiated. The nature of products leaving the stomach changed with time. At first, whole milk is evacuated. Then, just after coagulation, products similar to whey appeared. Finally, because of coagulum hydrolysis, a mixture of whey and casein fragments was obtained.

The in vitro study of protein digestion is without any doubt more economic, faster, and easier than in vivo assays. The excellent reproducibility of the results obtained in vitro permits standardization and automation of this method for measurements of the effects of technological processing on protein digestion. A sequential use of the stomach model with a pancreatic digestion system could be explored.

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