Characterization and Kinetics of Evacuation of Peptides Resulting from Casein Hydrolysis in the Stomach of the Calf

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A quantitative method for HPLC analysis of the peptides was developed in order to quantify and to compare the peptides leaving the abomasum of preruminant calves fed three different diets: skim milk, aqueous casein solution, casein solution in a mineral medium simulating ultrafiltered milk permeate. It was found that during the first phase of the digestion (0–90 min) the main peptides released from the two casein diets were fragment 106–169 of κ-casein (casein macropeptide, CMP), fragments 1–23 and 165–199 of α_{s1}-casein, and fragment 193–209 of β-casein. During the same period, slightly more CMP was released after ingestion of skim milk, while the amounts of the other fragments were respectively 10, 2, and 2 times lower than those obtained with casein diets. Thus, CMP was almost the only peptide to be released during the first hour. After that, casein was degraded into small peptides with the three diets, but the amounts released with skim milk were slower than with casein solutions. It was concluded that coagulation contributed to slow down casein degradation and also to retain some peptides. It was supposed that the stomach's role is not only to regulate the release of milk protein but also to select peptides that enter the gut. Taking into consideration the kinetics of liberation of some particular casein peptides, their supposed physiological activities such as regulation in gastric and pancreatic secretions, morphine-like activity, and role in calcium transport were discussed.

The in vivo digestion of milk proteins is initiated in the stomach by pepsins and, in some species including ruminants, chymosin. In vitro hydrolysis of caseins by gastric proteinases has been thoroughly investigated (Visser, 1981; Kaminogawa, 1981; Pelissier, 1984). These proteinases quickly hydrolyze the Phe^{105}–Met^{106} bond of κ-casein (Delfour et al., 1965), causing the caseins to coagulate when calcium is present. These enzymes also rapidly cleave the Phe^{23}–Phe^{24} bond of α_{s1}-casein (Hill et al., 1974) and some bonds of β-casein (Creamer et al., 1971). However, nothing is known on the fate of these proteins in vivo. The present work has been undertaken in order to get information on the precise role of the stomach in the proteolysis of the calf's natural food proteins, caseins. Indeed it could be inferred that peptides early released in the duodenum might have physiological functions such as the regulation of gastric and pancreatic secretions (Chernikov et al., 1974; Stan and Chernikov, 1982) or an opioid activity (Brantl et al., 1979; Loukas et al., 1983; Chiba and Yoshikawa, 1985) or a role in calcium transport (Naito and Suzuki, 1974).

Reversed-phase HPLC (RP HPLC) has been used to separate milk proteins and their fragments (Diosady and Bergen, 1980; Hobbs et al., 1981; Pearce, 1983; Carles and Ribadeau Dumas, 1984, 1985) and to determine the kinetic parameters relative to the action of chymosin on the most sensitive bonds of α_{s1}-, β-, and κ-caseins (Carles and Ribadeau Dumas, 1984, 1985). In our work RP HPLC analysis of gastric effluents has given quantitative information about the overall process of gastric digestion of caseins, which includes hydrolysis by gastric proteinases, casein retention in the form of coagulum (in the presence of calcium) or of precipitate (by acidification), and gastric emptying.

MATERIAL AND METHODS

Diets. Three types of test meals were prepared from the same milk of a single cow homozygous at the four caseins loci: I, 3% (w/v) whole casein in water; II, 3% (w/v) whole casein in a mineral medium simulating ultrafiltered milk permeate (Jenness and Koops, 1962); III, skim milk. All three were at the pH of fresh milk.

Table I. Peak Height Variations for Peptides To Be Assayed in Relation to TFA Concentrations Used for Casein Precipitation (Trials on First Peptidic Fraction of Effluent Sampled between 40 and 50 min after Ingestion of Diet I)

		peak height, mm			
TFA, %	pH	1–23 α_{s1}	CMP_0	165–199 α_{s1}	193–209 β
0.10	4,86	93	25	30	130
0.15	4,22	100	24	20	95
0.20	3,06	97	25	12	62
0.25	2,24	100	21	10	57
0.30	1,99	105	20	8	46

Collection of Gastric Effluents. The experimental details of sample collection have been described previously (Pelissier et al., 1983; Yvon et al., 1984 a). Two preruminant calves were fitted with double proximal duodenum cannula (Ash, 1962). They were given 5 kg of a test meal. The whole effluent from the stomach was then collected over 7 h, fractions being taken at 10-min intervals during the first hour, 15-min intervals during the second hour, and 30-min intervals up to the end of the 7-h period.

Each sample was immediately precipitated with trichloroacetic acid (TCA) to a final concentration of 12% and centrifuged at 2000*g* for 20 min. The pellet was resuspended in 200 mL of water. An aliquot of this suspension was brought to pH 7.0 and then added to 0.1% trifluoroacetic acid (TFA) and centrifuged as above. The TFA-soluble fraction thus obtained corresponded to the "first peptidic fraction". The fraction soluble in 12% TCA was adjusted to pH 2 with 1 N NaOH and corresponded to the "second peptidic fraction". Each fraction was analyzed by RP HPLC.

RP HPLC. Separation was carried out on Waters μ-Bondapak columns (Milford, MA). The equipment consisted of a pump, an S.P. 8700 system controller, and an SP 8750 distributor fitted with an injection valve (Spectra Physics, San Jose, CA). Peptides were detected at 220 nm on a variable-wavelength spectrophotometer (Philipps, LC871). The column was equilibrated in solvent A (0.11% TFA), and the peptides were eluted by a linear gradient

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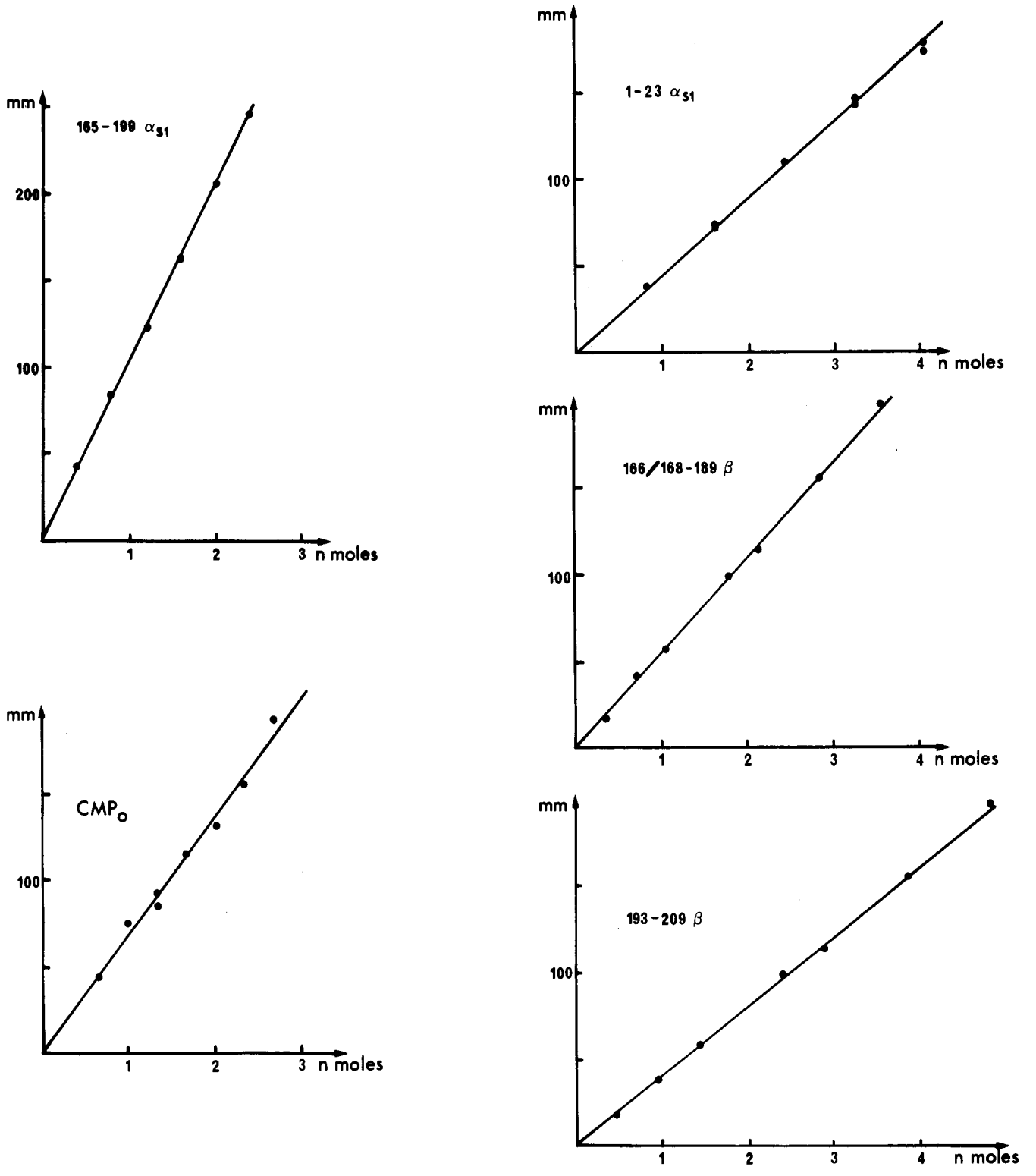

Figure 1. Relationship between peak height at 220 nm and injected amount of control peptides in a 0.1% TFA solution. See text for conditions of HPLC.

of 0–60% solvent B (0.11% TFA, 60% CH_3CN). The column and the solvents were kept at 40 °C in a water bath. Elution rate was 2 mL/min.

Peptide Identification. The HPLC fractions were collected after several injections and pooled. The solvent was removed by a Speed Vac evaporator (Savant, France), and before analysis, each fraction was rechromatographed by the same elution system. Then, the peptides were identified by their amino acid composition. These were determined after acid hydrolysis (110 °C, 24 h, 5.7 N HCl, under vacuum) using the method of Spackman et al. (1958) with a Biotronik LC5000 analyzer (Munich, RFA). The C-terminal amino acids were identified by amino acid analysis after carboxypeptidase A (Sigma, St. Louis, MO) action and the N-terminal amino acids by recurring Edman degradation using Tarr's technique (1982).

Quantitative Assays. The peptides of interest were prepared from in vitro casein hydrolysates. Figure 1 shows that, in RP HPLC, the relation between peak height at 220 nm and injected quantity (0–4 nmol, determined by amino

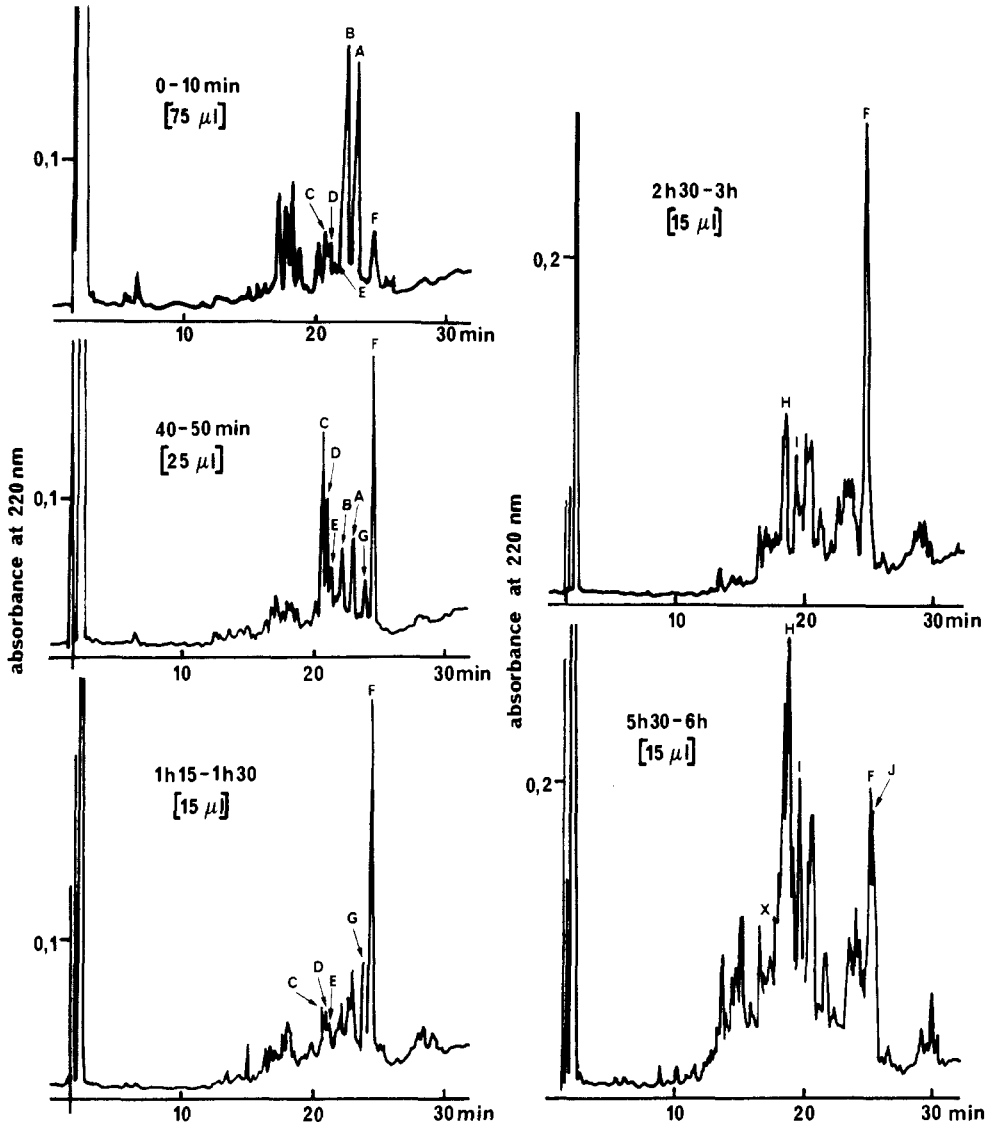

Figure 2. Chromatograms of the first peptidic fraction collected between 0 and 10 min, 40 and 50 min, 1 h 15 min and 1 h 30 min, 2 h 30 min and 3 h, 5 h 30 min and 6 h after ingestion of meal I (casein solution). For fractions A–J see Table II.

acid analysis) was linear for the peptides rapidly released in vitro from caseins: casein macropeptide 0 (CMPo), i.e. sequence 106–169 from nonglycosylated κ-casein; 1–23 and 165–199 from α_{s1}-casein; 193–209 and 166–189 from β-casein.

The pH of the TFA-soluble fraction varied with the sample, although the same amount of TFA was added. Furthermore, it was difficult to precisely adjust it because of the small volumes used. As shown in Table I, peak height corresponding to the peptides to be assayed varied widely when the pH of precipitation varied by some tenths. In order to evaluate the amount of each peptide in the samples, taking into account the precipitation yield of each peptide, three precipitations with 0.1% TFA were carried out for each assay. Two of these were performed directly on the sample. The third was carried out on the sample to which known amounts of each control peptide had been added to determine their yields of precipitation.

RESULTS

1. Characterization of the First Peptidic Fraction. The chromatograms of the first peptidic fraction obtained with meals I (Figure 2) and II were very similar while meal III gave different profiles (Figure 3). In any case there was a clear overall increase with time in peak number and height. However, peptide evacuation was considerably slower with meal III due to milk coagulation in the stomach.

The 10 main peptides (fractions A–J) were collected and identified. The results are reported in Table II. Their identification demonstrates in vivo hydrolysis of some peptide bonds of α_{s1}-, β-, and κ-casein. All these bonds had been previously shown to be split in vitro by the action of pepsin or chymosin (Pelissier et al., 1974; Visser and Slangen, 1977). The same major peptides were found whatever the diet was.

Fractions C–E (Figures 2 and 3) all corresponded to α_{s1}-casein sequence 1–23. The difference in their chromatographic behavior can probably be explained by the binding to this peptide of endogenous substances of nonprotein origin during transit through the stomach. These products could be bound on fragment 8–22 of this peptide: indeed fragment 8–22 obtained by tryptic hydrolysis of fractions C–E displayed the same behavior. Fractions A and B corresponded to CMP, which is known to occur under different forms differing by degree of glycosylation.

2. Characterization of the Second Peptidic Fraction. Figures 4 and 5 show chromatograms obtained with

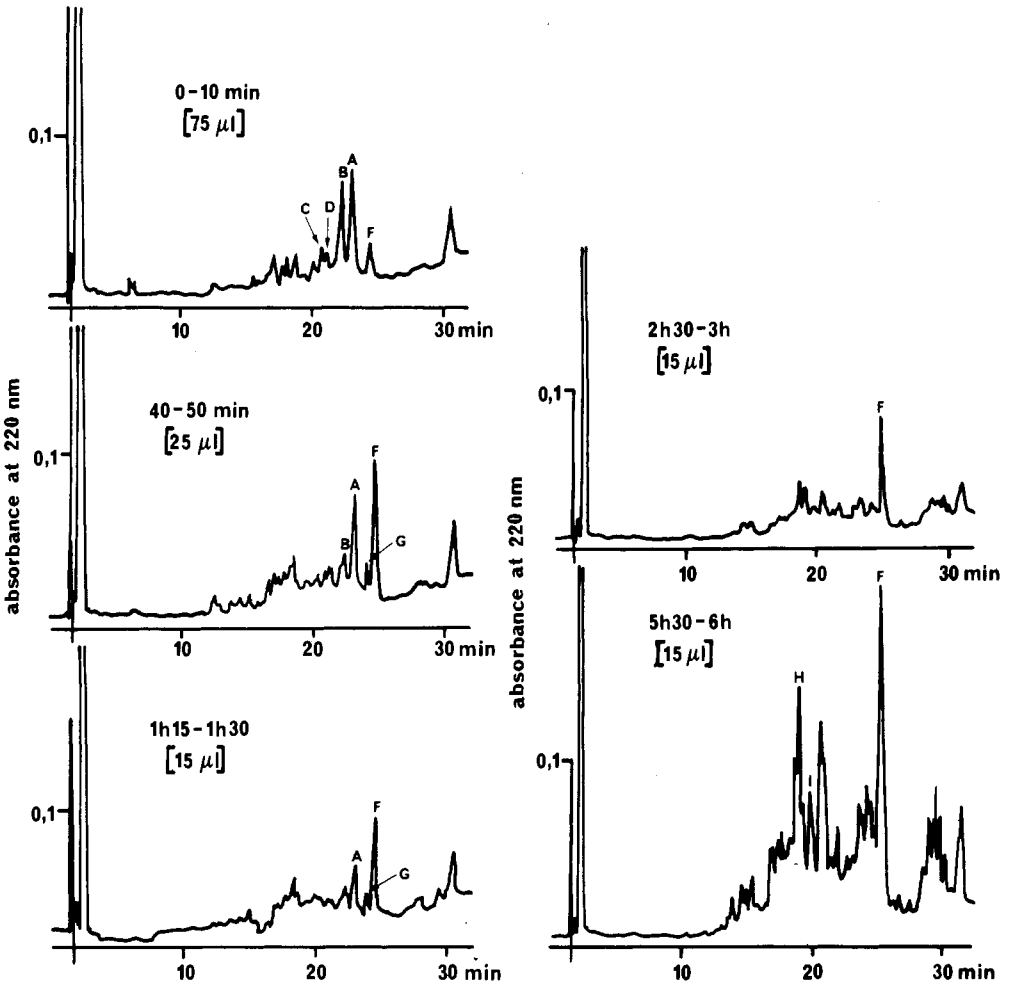

Figure 3. Chromatograms of the first peptidic fraction collected between 0 and 10 min, 40 and 50 min, 1 h 15 min and 1 h 30 min, 2 h 30 min and 3 h, and 5 h 30 min and 6 h after ingestion of meal III (skim milk). For fractions A–J see Table II.

meals I and III. As for the first peptidic fraction, there was a clear increase in peak number and height with time. Meals I and II, which gave similar profiles, appear to be proteolyzed faster than meal III. Six peptides were characterized in these samples as demonstrated in Table III. They all are originating from α_{s1}-casein, and the bonds split in vivo have been previously shown to be cleaved in vitro (Pelissier et al., 1974).

3. Emptying Kinetics of Identified Peptides. (*a*) *Semiquantitative Analysis.* Figure 6 is a schematic representation of the kinetics of the gastric release of the different isolated peptides. In this representation, differences in color intensity correspond to height differences of the peaks and, consequently, to different evacuated quantities. However, it is not possible to compare a peptide with another one because of their different extinction coefficients.

Some peptides appeared rapidly in the effluent and then disappeared, i.e. fragments 1–23 and 165–199 of α_{s1}-casein and CMP. Others peptides progressively appeared with time. Peptide quantities were smaller with "milk" diet than with "casein" diets. This shows an important decrease of the proteolysis when coagulation occurs in the stomach. Important quantitative differences are also observed with the peptides that appeared rapidly.

(*b*) *Quantitative Analysis of the First Peptides Released.* The peptides resulting from the hydrolysis of the most sensitive bonds by abomasal proteinases were assayed quantitatively by RP HPLC. Figure 7 gives the concentrations (mg/L) of the various peptides studied, non-glycosylated casein macropeptide (CMP_0) of κ-casein, and fragments 1–23 and 165–199 of α_{s1}-casein and 193–209 of β-casein in the effluents collected over the first 7 h of digestion.

The appearance of CMP_0 in the effluent within the first 10 min confirmed that the Phe^{105}–Met^{106} bond of κ-casein was preferentially hydrolyzed by gastric enzymes; this has been shown to occur in vitro many times (Visser, 1981; Kaminogawa, 1981; Pelissier, 1984). The quantity emptied for diets I and II represented only about 10% of the κ-casein ingested compared to 15% with diet III since emptying lasted longer with this diet. The disappearance of this peptide could be due to proteolysis because pepsin-sensitive bonds exist in CMP (Mercier et al., 1972), particularly when the pH of the reactions medium is acidic (Hill and Hocking, 1978).

α_{s1}-Casein bond 23–24 was quickly hydrolyzed. With diets I and II, peptide 1–23 appeared in effluent after 10 min. Its disappearance from the effluent after 1 h to 1 h 30 min coincided with that of the caseins. With the same diets the total amount released corresponded to about 12% of the ingested α_{s1}-casein compared to 1% with the milk diet. When a whole-casein hydrolysate was precipitated at pH 4.6 in vitro, 50% of peptide 1–23 was found in the insoluble fraction, thus indicating coprecipitation with the caseins. The peptide was released by lowering to pH 2. Moreover, the detection of the H-Leu-Arg-Phe-OH (sequence 21–23) peptide shows that fragment 1–23 was cleaved further by the action of abomasal proteinases. These observations explain the small amount detected and

Table II. Identification of Peptides from the First Peptidic Fraction (Result (mol/mol) Obtained after 24-h Hydrolysis; Theoretical Values of Identified Fragment in Parentheses)

	A	B	C	D	E	F	G	H	I	J
AA compn										
Asx	5.3 (5)	5 (5)	2 (2)	2 (2)	2.1 (2)		3.8 (4)		1 (1)	1.9 (2)
Thr	11.4 (11)	9.7 (11)					3.5 (4)		1.7 (2)	1.1 (1)
Ser	5.7 (6)	5.7 (6)					3.9 (4)		1 (1)	
Glx	10.8 (10)	9.6 (10)	3.8 (4)	3.8 (4)	3.8 (4)	2.1 (2)	4.5 (3)	1 (1)	1.1 (1)	1.2 (1)
Pro	8.5 (8)	6.7 (8)	3.2 (3)	3.3 (3)	3.5 (3)	4 (4)	7.5 (5)	1.1 (1)	2 (2)	2.3 (2)
Gly	1.1 (1)	1.6 (1)	1 (1)	1 (1)	1 (1)	2 (2)	2 (2)		1 (1)	
Ala	5 (6)	3.7 (6)					1.4 (1)	0.9 (1)	1 (1)	
Cys										
Val	5.4 (6)	4.3 (6)	1 (1)	1 (1)	1 (1)	2.5 (3)	1.9 (1)		1 (1)	0.9 (1)
Met	1 (1)	0.6 (1)					1.4 (1)			
Ile	5.7 (7)	4.4 (7)	0.9 (1)	1 (1)	1 (1)	1 (2)	2 (2)			
Leu	1.4 (1)	1.5 (1)	3.8 (4)	3.9 (4)	3.6 (4)	1.4 (1)	2.9 (2)	1.1 (1)	1 (1)	4.5 (4)
Tyr						0.8 (1)	2.7 (3)	1.7 (2)	2.6 (3)	
Phe			1 (1)	1 (1)	1.1 (1)	1 (1)	1.3 (1)	1 (1)	1 (1)	
His			2 (2)	1.9 (2)	1.8 (2)					1 (1)
Lys	3.1 (3)	2.7 (3)	2 (2)	2 (2)	2 (2)		1.4 (1)			
Arg			1.9 (2)	1.9 (2)	1.7 (2)	0.9 (1)				
C-terminal seq	Thr-Ala-Val	ND	Phe	Phe	Phe	Ile-Ile-Val	Leu-Trp	ND	Phe	ND
N-terminal seq	Met-Ala-Ile	Met-Ala	ND	ND	ND	Tyr-Gln	Tyr-Tyr-Val	Ala-Tyr-Phe	Tyr-Tyr-Val	Thr-Asp-Val-Glu
peptides	CMP	CMP	1–23	1–23	1–23	193–209	165–199	143–149	165–179	128–139
	κ-casein	κ-casein	α_{s1}-casein	α_{s1}-casein	α_{s1}-casein	β-casein	α_{s1}-casein	α_{s1}-casein	α_{s1}-casein	β-casein

Table III. Identification of Peptides from the Second Peptidic Fraction (See Table II)

	K	L	N	O	P	Q	R	S
AA compn								
Asx	++					1.1 (1)		
Thr	++							
Ser	++					1 (1)		
Glx	+++			1.1 (1)		+	+	1.2 (1)
Pro						1.3 (1)	1.5 (1)	1.3 (1)
Gly	+		1 (1)			0.8 (1)	+	
Ala	+					1.4 (2)		0.9 (1)
Cys							+	
Val	+						1 (1)	
Met								
Ile								
Leu			1 (1)	1 (1)	1 (1)		1.3 (1)	0.9 (1)
Tyr		++	0.9 (1)	0.8 (1)		1 (1)	1.6 (2)	1.6 (2)
Phe					1 (1)			0.9 (1)
His	++ (free)						+	
Lys	++						+	
Arg	+				0.8 (1)			
C-terminal seq			Tyr	Leu	ND	Gly-Ala-Trp	Leu	Leu
N-terminal seq			Leu-Gly-Tyr	Tyr-Gln-Leu	Leu-Arg-Phe	Asp-Ala-Tyr	Tyr-Tyr-Val	Ala-Tyr-Phe
peptides		free Tyr	92–94 α_{s1}-casein	154–156 α_{s1}-casein	21–23 α_{s1}-casein	157–164 α_{s1}-casein	165–169 α_{s1}-casein	143–149 α_{s1}-casein

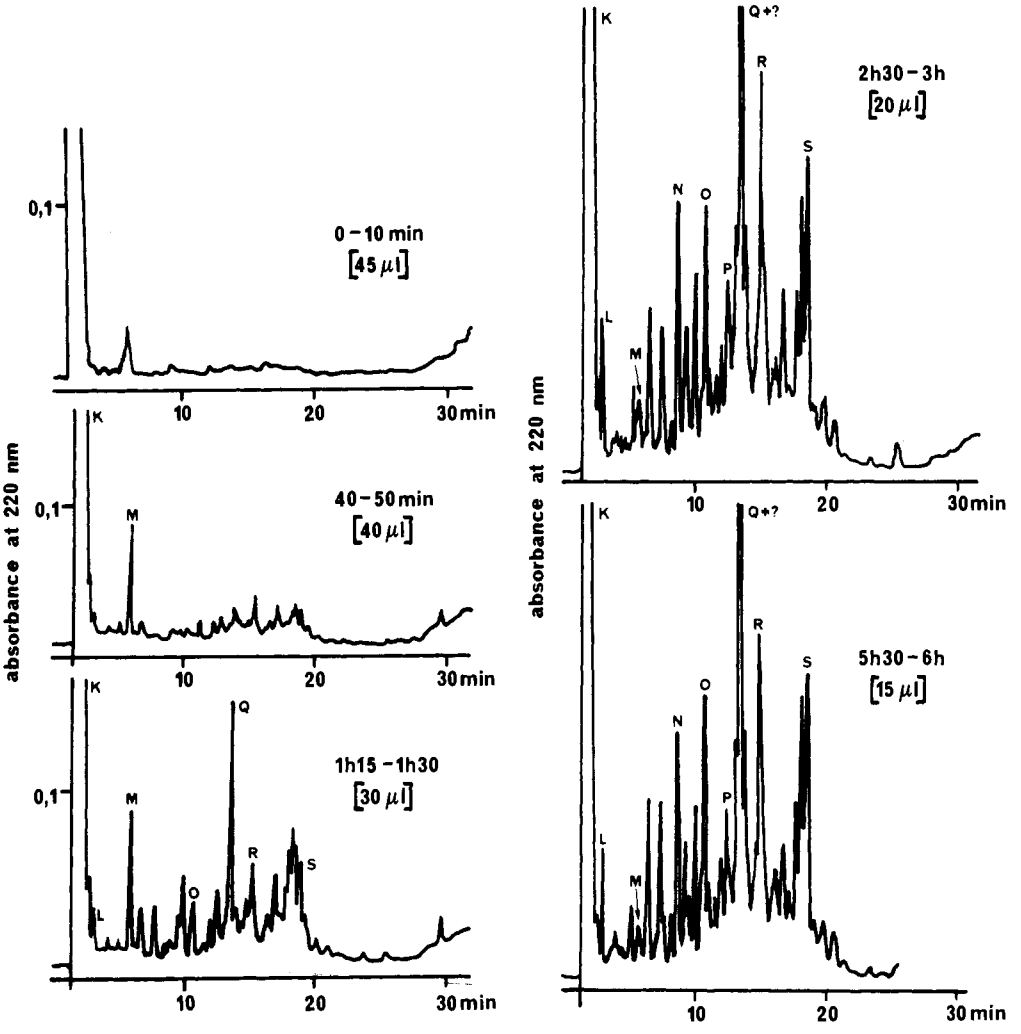

Figure 4. Same as Figure 2. Second peptidic fraction. For fractions K–S see Table III.

the disappearance of this peptide from the effluent. With diet III, coagulation increased the retention of peptide 1–23 in the stomach. We have confirmed these findings by in vitro assays.

Fragment 165–199 was the second α_{s1}-casein peptide to be observed in the effluent. The total quantity evacuated was very small (about 2% of the casein ingested with diets I and II). This peptide did not coprecipitate in vitro with caseins at pH 4.6 (more than 80% of the peptide occurred in the supernatant) but was trapped during coagulation; only 10% of the amount released diffused into the supernatant. The disappearance of this peptide is explained by its further hydrolysis. This was confirmed by detection of fragments 165–179 and 165–169 in the effluent.

The hydrolysis of β-casein bond 192–193 was slower than that of α_{s1}-casein bond 23–24. On the other hand, the total quantity of peptide 193–209 evacuated in 7 h was higher, corresponding to 50% of the casein with diets I and II compared to 17% with diet III. The in vitro assays showed that this peptide coprecipitated little with casein at pH 4.6 (80% occurred in the soluble fraction) but that most of it was trapped in the coagulum that formed. As this peptide is resistant to gastric proteinases, it could be evacuated later.

DISCUSSION

During the first phase of the digestion of the two casein diets, peptides resulting from casein proteolysis left the abomasum without any important differences between these diets. The main peptides were CMP (200–300 mg of CMP_0 + glycosylated CMP), fragment 1–23 of α_{s1}-casein (500–600 mg), fragment 165–199 of α_{s1}-casein (100–150 mg) and peptide 193–209 of β-casein (300–400 mg).

During the same period after ingestion of the skim milk diet, slightly more CMP_0 was emptied (450 mg). On the contrary, the amounts of fragments 1–23 and 165–199 of α_{s1}-casein and 193–209 of β-casein were 10, 2, and 2 times lower, respectively, than those obtained with casein diets. These results show that coagulation contributed to slower casein degradation and also to the retention of some peptides. Then, CMP is almost the only one to be released during the first hour. This observation is especially interesting because this peptide appears to have physiological functions. First, Chernikov et al. (1974) and Stan and Chernikov (1982) reported that the CMP or a fragment from CMP could inhibit gastrin and then the acid secretion in the stomach. On the other hand, this peptide could be involved in the liberation of gastrointestinal hormones (CCK-PZ, secretine, somatostatine, VIP). Consequently, secretion of pancreatic proteinases would be maintained for a certain time at a low level. Then it is possible that the whey proteins that leave the stomach quickly (Yvon et al., 1984b) are not hydrolyzed by pancreatic proteinases and could play a role in the establishment of the intestinal flora and the immunity of the young (Reiter, 1978), particularly during the colostral period. This hypothesis

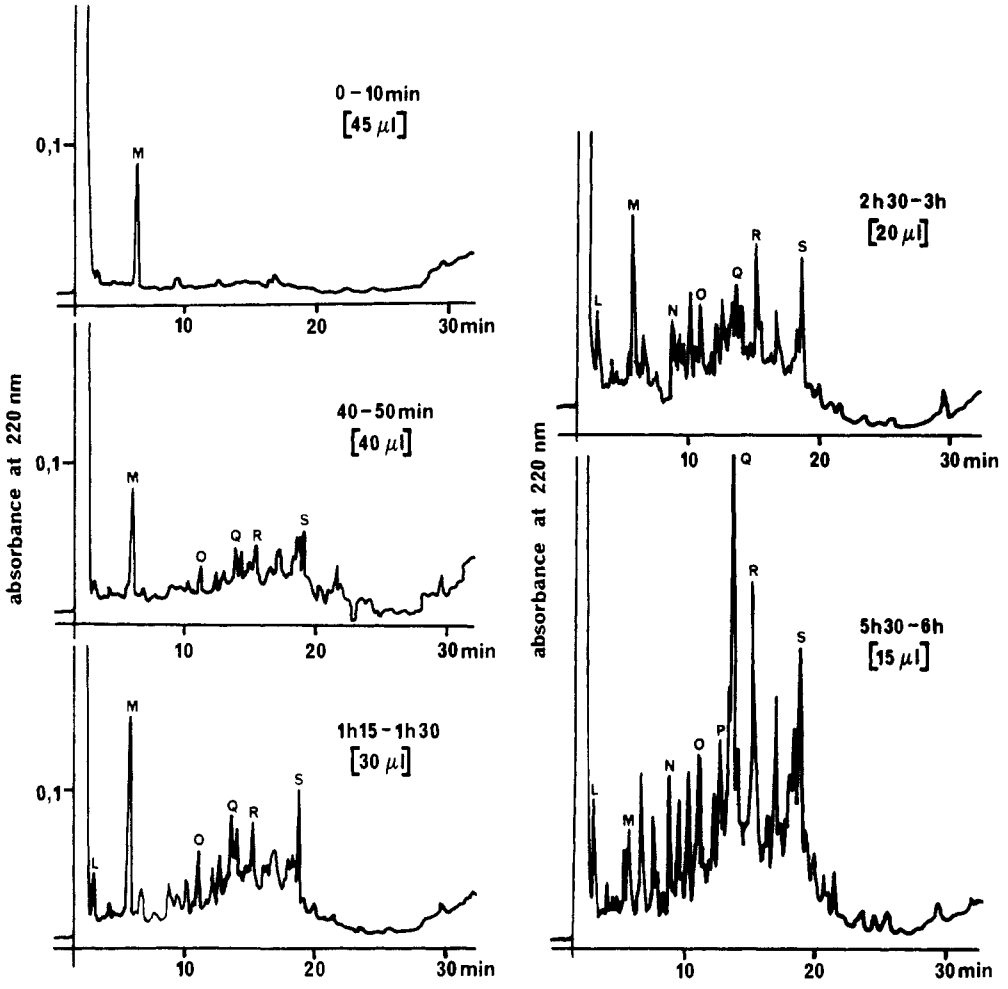

Figure 5. Same as Figure 3. Second peptidic fraction. For fractions K–S see Table III.

agrees with that suggested by Foltmann et al. (1981) on the importance of chymosin (high milk clotting and low general proteolytic activities) for postnatal uptake of immunoglobulins. At least Stan et al. (1983) showed that a CMP hydrolysate has an opium-like activity.

Lahav (1967) found that a casein mild hydrolysate had antibacterial properties and suggested that fragment 1–23 of α_{s1}-casein was responsable for this property. Milk coagulation causes retention of this peptide in the stomach and its partial degradation (release of the tripeptide 21–23). Furthermore, this peptide is modified in the region 8–22 while passing through the stomach. The nature of the modification remains to be established as well as the possibly higher resistance of the peptide to the proteinases of the digestive tract. Only if higher resistance to hydrolysis is found, the peptide may play a role in the development of the intestinal flora.

With both types of diet, the casein was then degraded into small peptides whose number and quantity increased progressively with time. The degradation products observed over a postprandial 7-h period were identical and originated mainly from α_{s1}-casein, but smaller amounts were emptied with the milk diet. Three hours after ingestion of casein meals, casein proteolysis increased, as also observed with the milk diet but a little later (about 5 h after the meal). The drop in abomasal pH (slower with the milk diet) probably caused abomasal proteolysis to increase, particularly that due to pepsin. These peptides released after 90 min of digestion are composed of 3–17 residues (Figure 8). Hydrophobic residues (Trp, Ile, Phe, Leu, Pro, Val) account for more than 56% of the total residues of these peptides, while they account only for 35% of the residues of whole casein. In particular proline and leucine contents are 2 times higher. It is interesting to note that the proportion of tyrosine is about 4 times higher than in whole casein. This residue is often situated at the N-terminal part of peptides while the C-terminal amino acid is almost always a hydrophobic residue. C-terminal amino acids released by the action of pancreatic carboxypeptidases (A and B) may possibly reinduce secretion of pancreatic proteinases. As a matter of fact, hydrophobic amino acids, especially tryptophan and phenylalanine, infused into the proximal gut stimulate the secretion of pancreatic proteins. This has been demonstrated for dog (Meyer et al., 1976) and for man (Go et al., 1970). Considering their sequence, these peptides should be resistant to pancreatic proteinases. Therefore, they should reach the intestinal barrier almost unbroken with probably just a few C-terminal residues split off. Peptides rich in proline and aromatic residues would thus be excellent substrates for proteinases of the brush border (Kenny and Maroux, 1982; Vonk and Western, 1984).

These observations show that the stomach's role is not only to regulate the release of milk protein but also to select peptides that enter the gut.

Naito and Suzuki (1974) postulated that phosphopeptides could play a role in calcium transport. However, we have not identified the phosphorylated fragments from α_{s1}-, α_{s2}-, and β-casein in the gastric effluent during postprandial 7 h. During our investigation only 30–40%

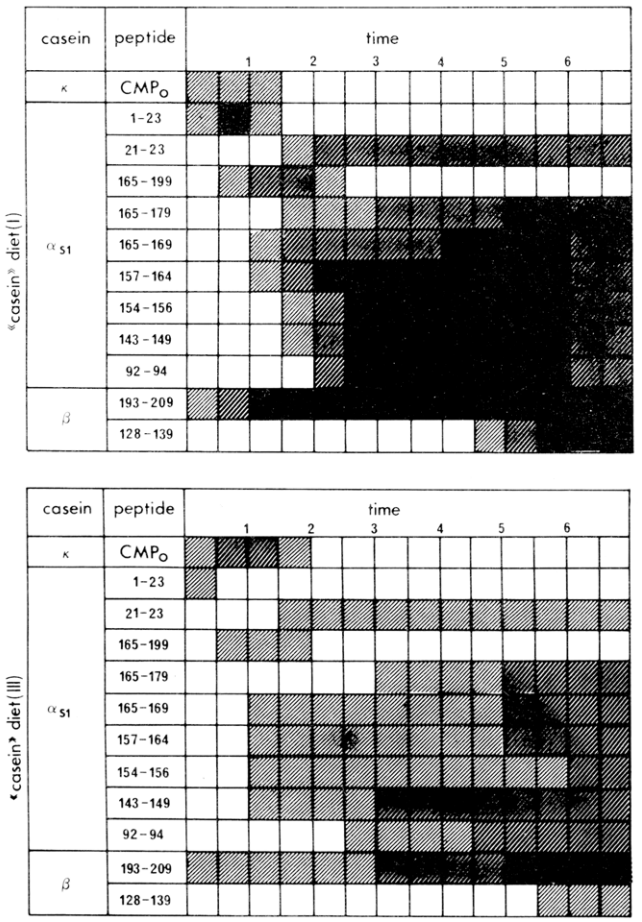

Figure 6. Schematic representation of the kinetics of gastric emptying of peptides from κ-, α_{s1}-, and β-caseins.

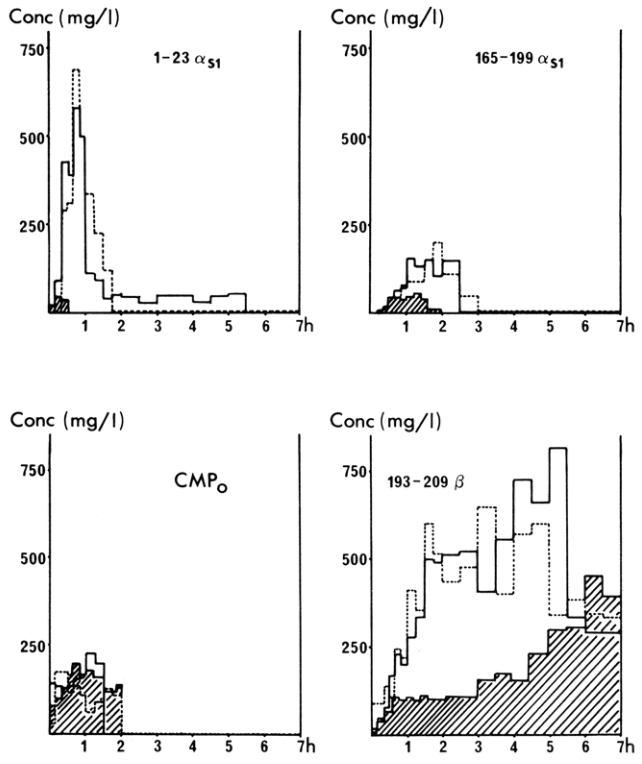

Figure 7. Changes in peptide concentration: CMP_0 of κ-casein; 1–23 and 165–199 of α_{s1}-casein; 193–209 of β-casein in the effluents obtained after the ingestion. Diets: I (—); II (- - -); III (▨).

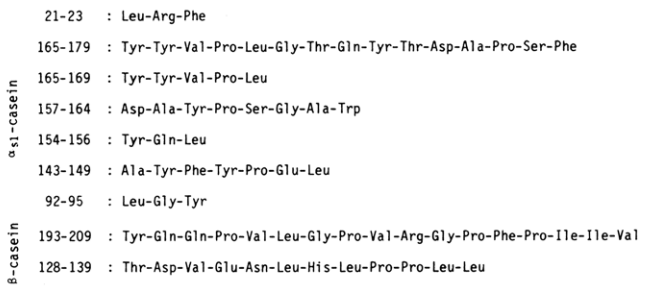

Figure 8. Amino acid sequence of peptides released from the stomach between 1 h 30 min and 7 h postprandial.

of the ingested protein products was released (Yvon et al., 1984a,b). The same observation have been described by other authors (Ternouth and Roy, 1973; Meisel and Hengemeister, 1984). These fragments could be released later or following the next meal (meal given at intervals of 7–8 h) and participate in calcium absorption at that time.

Finally, three casein fragments are known for their opioid activity: 60–65 from β-casein (Brantl et al., 1979), 33–38 from κ-casein (Chiba and Yoshikawa, 1985), 90–95 from α_{s1}-casein (Loukas et al., 1983). The latter is hydrolyzed by gastric proteinases and can therefore have no in vivo action (we isolated fragment 92–94). On the other hand, neither β-casomorphin nor larger fragments that might be precursors are released after 7-h digestion. In further experiments, particular attention has to be payed to these fragments. Indeed, the length and the N-terminal sequence of the precursor reaching the brush border are essential for the survival of these peptides to the action of the dipeptidyl amino peptidase IV and to reach their putative receptors (Caporale et al., 1985).

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Rapid HPLC Assay for the β-Exotoxin of *Bacillus thuringiensis*

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A high-performance liquid chromatography (HPLC) assay has been developed for β-exotoxin in fermentation beers and formulation samples. The assay is rapid and specific, and results correlate well with the fly bioassay. Detector response is linear over a concentration range of 0.0–0.56 mg/mL β-exotoxin. The assay is capable of detecting both the phosphorylated and dephosphorylated forms of β-exotoxin.

A number of strains of *Bacillus thuringiensis* produce a low molecular weight, heat-stable insecticidal toxin known as β-exotoxin (Sebesta et al., 1981). β-Exotoxin (thuringiensen, $C_{22}H_{32}N_5O_{19}P_1 \cdot 3H_2O$) is a nucleotide composed of adenosine, glucose, and allaric acid (Figure 1). It is believed that the phosphate group on allaric acid is essential for activity and that the toxicity of β-exotoxin results from its inhibition of RNA biosynthesis by competing with ATP for binding sites (Farkas et al., 1976).

Fly bioassay (Ignoffo and Gard, 1970) up to now has been the most commonly used method of detecting the presence of β-exotoxin in fermentation beers. The bioassay monitors the ability of β-exotoxin to prevent pupae of housefly larvae from developing into normal complete adults. However, the fly bioassay suffers from the fact that it is quite variable and nonspecific, and it takes 9 days to complete a single assay.

In the last few years, two high-performance liquid chromatography (HPLC) assay methods for β-exotoxin have appeared in the literature (Oehler et al., 1982; Danilova and Kruglyak, 1983). Unfortunately the methods either lack specificity for β-exotoxin in complex fermentation mixtures (Johnson and Peterson, 1983) or require gradient elution techniques with long analysis times (Danilova and Kruglyak, 1983). Recently, we developed a rapid, specific, quantitiative HPLC assay for β-exotoxin. β-Exotoxin samples can be assayed in 20–30 min vs. an external β-exotoxin standard.

EXPERIMENTAL SECTION

Reagents and HPLC Standard. Analytical reagent-grade potassium dihydrogen phosphate (KH_2PO_4) and 85% phosphoric acid obtained from Mallinckrodt (Paris, KY) were used to prepare the mobile phase. Deionized water was obtained from a Milli Q purification system (Millipore Corp., Bedford, MA). Abbott Laboratories β-exotoxin reference standard (lot 635-19), having a purity of 80.9%, was used to assay β-exotoxin samples.

Liquid Chromatography. HPLC assays were performed isocratically on a Waters μ-Bondapak C_{18} column (30 cm × 3.9 mm i.d.) maintained at a constant temperature between 25 and 35 °C. A 50 mM KH_2PO_4 (pH 3.0) mobile phase and a flow rate of 2.0 mL/min were used. The mobile phase was filtered through a 0.4-μm polycarbonate membrane (Nuclepore Corp., Pleasanton, CA). Solid samples and standards were dissolved in deionized water. Solid samples that did not readily dissolve in deionized water were acidified to pH 3 with phosphoric acid. Liquid samples and dissolved solid samples were diluted to a concentration of approximately 0.1 mg/mL β-exotoxin and filtered through 0.45-μm disposable nylon 66 syringe filters (Alltech Associates Inc., Deerfield, IL). Samples were then injected into the HPLC. Instrumentation included an M6000A pump (Waters Associates, Milford, MA), a 501 autosampler (Beckman Instruments, Berkeley, CA) equipped with a 20-μL sample loop, a SF 770 variable-wavelength UV detector set at 260 nm (Kratos Analytical Instruments, Ramsey, NJ), and a Chromatopac C-R3A integrator (Shimadzu Corp., Kyoto, Japan).

Fly Factor Bioassay. A CSMA fly larva Medium No. 5060 (Ralston Purina Co., St. Louis, MO) was used for bioassay of β-exotoxin samples against 3-day-old larve of *Musca domestica*. Ten larvae in quadruplicate at each test level were transferred into a 4-dram shell vial containing 5 mL of diluted sample and 2 g of CSMA fly larvea media. Each vial was covered with a double thickness of paper toweling and secured with a rubber band. Vials were incubated at 27 °C for 9 days. The number of larve, pupae, normal adults, and deformed adults was noted. Deformed

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