

Detection of caseinophosphopeptides in the distal ileostomy fluid of human subjects

H. Meisel^{1*}, H. Bernard², S. Fairweather-Tait³, R. J. FitzGerald⁴, R. Hartmann¹,
C. N. Lane⁵, D. McDonagh⁵, B. Teucher³ and J. M. Wal²

¹Federal Dairy Research Centre, Institute for Dairy Chemistry and Technology, Kiel, Germany

²Laboratoire d'Immuno-Allergie Alimentaire, INRA-CEA, SPI, Gif sur Yvette, France

³Institute of Food Research, Norwich Research Park, Norfolk, UK

⁴University of Limerick, Life Sciences Department, Limerick, Republic of Ireland

⁵Teagasc, Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Republic of Ireland

(Received 23 April 2002 – Revised 16 October 2002 – Accepted 31 October 2002)

Caseinophosphopeptides (CPP) were detected for the first time in ileostomy fluid, collected at 2 h intervals for 10 h post milk and CPP ingestion, from human volunteers with an ileostomy. The level of CPP present in ileostomy fluid obtained from milk-fed volunteers was markedly higher than that from volunteers fed with selected CPP preparations. The findings are based on HPLC analysis in combination with peptide-bound P determination, thin-layer electrophoresis and amino acid analysis, together with ELISA studies using polyclonal antibodies raised against a set of CPP to detect immunoreactive CPP in ileostomy fluid. These procedures allowed the detection of nm concentrations of CPP. CPP, which can be released during intestinal digestion, may function as bioactive constituents and carriers for different minerals, especially Ca, and may be used as ingredients in functional foods or pharmaceutical preparations.

Caseinophosphopeptides: Milk: Human ileostomy samples: Bioactive peptides

Food-derived bioactive peptides represent a source of health-enhancing components that may be incorporated in functional foods and in pharmaceutical preparations. These peptides are potential modulators of various regulatory processes in the body. Milk proteins are precursors of many different biologically active peptides, e.g. peptides having opioid, hypotensive (angiotensin-converting enzyme inhibitory), immunomodulating or antimicrobial activities (for reviews, see Meisel, 1997, 2001; FitzGerald & Meisel, 2000; Meisel & FitzGerald, 2000). The intrinsic bioactivities of the peptides encrypted in major milk proteins are latent until they are released and activated by enzymatic hydrolysis, e.g. during gastrointestinal digestion and/or food processing. Several studies have been performed in the last two decades on caseinophosphopeptides (CPP), which may function as carriers for different minerals, especially Ca (for review, see FitzGerald, 1998). It has been proposed that CPP, which form soluble complexes with $\text{Ca}_3(\text{PO}_4)_2$ *in vitro*, may lead to enhanced Ca absorption by limiting the precipitation of Ca at alkaline conditions pertaining in the distal ileum. However,

until now it had not been established whether CPP can be obtained in the human ileum following digestion of a milk meal (Meisel *et al.* 2001). For their function as mineral carriers CPP must, at least partially, resist enzymatic digestion during intestinal passage. In the present study (see also Meisel *et al.* 2001), intestinal fluid samples obtained from human subjects during feeding trials with milk and selected CPP preparations were analysed for the presence of CPP.

Material and methods

Milk and caseinophosphopeptide preparations

The same brand of a fresh pasteurized, homogenized full-fat cows' milk was obtained from a local food retailer for the duration of the present study. CPP were generated as previously described (McDonagh & FitzGerald, 1998) from bovine sodium caseinate, which was hydrolysed with four different commercially available food-grade proteinase preparations, i.e. PTN 3-0S and Alcalase (both obtained

from Novo Nordisk, Novo Alle, Bagsvaerd, Denmark), Bioprotease P(conc) (Quest International, Carrigaline, Cork, Republic of Ireland) and Proteinase DS (Rhône-Poulenc, Stockport, Cheshire, UK), resulting in CPP preparations designated CPP1, CPP2, CPP3 and CPP4 respectively. CPP were aggregated by inclusion of Ca (Adamson & Reynolds, 1995) and the resultant aggregates were collected by ultrafiltration through 10 kDa cut-off membranes (Brule, 1982; McDonagh & FitzGerald, 1998). The CPP preparations were decalcified (McDonagh & FitzGerald, 1998) prior to spray-drying. The N:P for these different preparations were: 3.11:1.00, 3.15:1.00, 4.0:1.00, and 3.59:1.00 respectively. All four preparations had >90% peptide materials with a molecular mass <1 kDa.

Ileostomy study

One male and three female volunteers with an ileostomy participated in the present study. The mean age of the volunteers was 51.5 (SD 7.9) years. All four volunteers were without any acute digestive problems and were taking routine antacid medication to help digestion; no medication was taken during the experimental days. The study protocol was approved by the Norfolk (UK) District Ethics Committee and informed consent was obtained from each volunteer prior to participation. Volunteers were chosen on the basis that they had no prior history of adverse reactions to the consumption of milk proteins. Cytochemical assessment of CPP *in vitro* (Caco-2 cells) revealed no cytotoxic potential, and no adverse influence on epithelial properties and differentiation (Hartmann & Meisel, 2002).

In the present study, which was carried out within 2 months, each volunteer consumed either 250 g full-fat cows' milk or one of four different CPP preparations (1.52 g in 20 ml water), together with a standard milk-protein-free breakfast, on two separate occasions following an overnight fast. Ileostomy fluid was collected at baseline (*t* 0) after a 10 h overnight fast and following the test meal in 2 h intervals for 10 h (*t* 1–5). The two tests were separated by at least 2 weeks. To avoid consumption of milk products, volunteers were provided with a diet free of milk protein for 2 d prior to both test days. During the 10 h experimental period, volunteers were confined to the premises of the Human Nutrition Unit at the Institute of Food Research (Norwich, Norfolk, UK). The same milk-protein-free lunch was provided for all volunteers throughout the study. Ileostomy fluid was immediately centrifuged at 5000 *g* for 10 min at 4°C after collection and the pellet and supernatant fractions were stored at –18°C prior to freeze-drying. According to the volunteer and the meal ingested, the ileostomy samples were designated as 01-1:CPP2, 01-2:Milk, 02-1:CPP4, 02-2:Milk, 03-1:Milk, 03-2:CPP1, 04-1:Milk, 04-2:CPP3.

Extraction of ileostomy samples

Addition of perchloric acid prevented the degradation of CPP by proteolytic and/or peptideolytic action in reconstituted ileostomy samples, presumably by inhibition of enzymes of intracellular or bacterial origin, which may be liberated after freezing and thawing (D McDonagh,

H Meisel, C Lane and RJ FitzGerald, unpublished observations). Lyophilized ileostomy sample (100 mg) was suspended in 2 ml perchloric acid (172 mM, pH 1.2 in the final extract). The suspension was vortexed vigorously for 1 min and then treated in an ultrasonic bath for 3 min. After centrifugation at 14 000 *g* for 3 min, the perchloric acid-soluble portion of ileostomy samples was used for further analyses. Ileostomy samples, which had been collected at various time intervals (*t* 1–5), were pooled for analysis of CPP in order to maximize the likelihood of detecting CPP.

Adsorptive microconcentration

Microcon strong cation exchange (SCX) units (Amicon, Inc., Beverly, MA, USA) containing a SCX membrane were used for adsorptive microconcentration of phosphopeptides. The manufacturer's protocol was followed with minor modifications. To wet the membrane, 0.5 ml methanol and then 0.5 ml water were centrifuged at 7000 *g* for 15 s through the SCX unit. Sample (4 × 0.5 ml) was loaded into the SCX unit. The filtrate (SCX-A) obtained after centrifugation at 1200 *g* for 10–30 min was collected for further analyses. Thereafter, the membrane was washed with 10 mM-HCl to remove residual contaminants. For elution of adsorbed peptides, 50 µl desorption reagent (0.5 ml methanol, 0.1 ml NH₃ (250 ml/l), 0.4 ml water) was pipetted into the unit and was then centrifuged at 14 000 *g* for 1 min; this step was repeated once and the respective filtrates (microconcentrate SCX-B) were combined for further analyses.

Analytical and semi-preparative reverse-phase HPLC

The reverse-phase HPLC-system consisted of two 422 Master pumps (Bio-Tek Kontron, Neufahrn, Germany), two mixers M800 (Bio-Tek Kontron), injector 7125 (Rheodyne, Rohnert Park, CA, USA), column oven (constructed by the authors), diode array detector 440 (Bio-Tek Kontron), Foxy fraction collector (ISCO, Lincoln, NE, USA) and data system KromaSystem 2000 (Bio-Tek Kontron). A LiChroCART Supersphere 100 C₁₈ endcapped 124 × 4 mm column (16855; Merck, Darmstadt, Germany) was used for analytical and semi-preparative separations; column temperature was 30°C. Solvent A was trifluoroacetic acid (1 ml/l water) and solvent B was trifluoroacetic acid (1 ml/l aqueous acetonitrile (500 ml/l)). Both solvents were purged with He. Freeze-dried samples were taken up in eluent A. Sample solutions (100 µl extracted material from 100 mg freeze-dried powder reconstituted in 2 ml perchloric acid) were injected and eluted at a flow rate of 1 ml/min. The gradient used was 50 ml B/l for 5 min and 50–900 ml B/l in 85 min. For rechromatography, a shallower gradient (50 ml B/l for 5 min and 50 to 450 ml B/l in 80 min) was used. Peaks were detected by absorbance at 215, 235 and 280 nm. Peptide fractions were collected and solvents were removed by freeze-drying (Speed Vac concentrator; Savant, Holbrook, NY, USA).

Determination of phosphorus content

P content was measured after wet ashing of freeze-dried samples, which converts organic P to inorganic phosphate. Inorganic phosphate was quantified as the phosphomolybdate complex by an ultramicro method (Meisel & Frister, 1988).

Amino acid analysis

Freeze-dried samples were hydrolysed with 6 M-HCl-phenol (10 ml/l) in evacuated tubes at 110°C for 24 h. Amino acid analysis was performed with an Alpha Plus analyser (type 4151; Amersham Pharmacia Biotech, Freiburg, Germany) using an Ultrapac 8 ion-exchange resin (80-202845; Amersham Pharmacia Biotech) and sodium citrate buffer system (Amersham Pharmacia Biotech). SerP content per mg lyophilized ileostomy sample was obtained by extrapolation of the SerP concentration in acidic hydrolysates (6, 12, 24 h) to zero time. Calibration was achieved by running a mixture of standard amino acids (AA-S18; Sigma, St Louis, MO, USA) and SerP (Sigma) respectively.

Thin-layer electrophoresis

Thin-layer electrophoresis was performed using a thin-layer electrophoresis double chamber connected to a mains supply unit 1200/200 (DESAGA, Heidelberg, Germany). Samples (5–10 µl) of acidic hydrolysates (*t* 3) of ileostomy samples were dotted on microcrystalline cellulose sheets (Polygram CEL 400, 0.20 × 0.20 m, 0.1 mm thickness; Macherey-Nagel, Düren, Germany); an additional set of the same samples were spiked with a SerP solution (5 nmol/0.5 µl water). Separations were carried out in pyridine-acetic acid-water (5:50:945, by vol.), pH 3.5, at 400 V/15–20 mA for 1 h. Components of the partial hydrolysates were detected by spraying with a solution of ninhydrin (0.1 mg/50 ml methanol) and heating for 5 min at 110°C.

Preparation and purification of a synthetic phosphopeptide

A consensus phosphopeptide with a sequence of VESLΣΣΣEESIL (Σ depicts SerP residues) was synthesized using a Milligen 9050 apparatus (Biosearch Inc., Novato, CA, USA) and standard solid-phase synthesis by the 9-fluorenylmethoxycarbonyl continuous-flow method. Synthetic peptide was purified by successive anion-exchange chromatography using a fast protein liquid chromatography system with a mono Q HR 10/10 column (Amersham Pharmacia Biotech), and reverse-phase HPLC using a Waters system (Milford, MA, USA) and a 300 A C₁₈ Vydac column (250 × 10 mm; Shandon, France).

Immunogen preparation

Tryptic CPP isolated by Ca²⁺-ethanol precipitation from whole casein were coupled to keyhole limpet (megatura crenulata) haemocyanin via its amino groups, using glutaraldehyde. Briefly, 5 mg CPP and 20 µl glutaraldehyde were successively added to 10 mg keyhole limpet haemocyanin

dissolved in 10 ml 0.1 M-phosphate buffer, pH 7.4. After 18 h stirring at 4°C, the mixture was divided into 1 ml portions and stored at –20°C.

Immunization

Anti-whole CPP antisera was obtained by immunizing adult male white Blanc-de Bouscat rabbits as follows: 1 ml immunogen solution (250 ml/l water) was emulsified with 1 ml Freund's complete adjuvant and injected subcutaneously into two rabbits (no. 1494 and no. 1495). Booster injections were given 6 weeks later and once per month thereafter. Rabbits were bled 10 d after each immunization. Sera were stored at 4°C after addition of sodium azide (0.1 g/l).

Enzymatic tracer preparation

Enzymatic tracer was prepared by covalent linkage of consensus synthetic phosphopeptide to the tetrameric form of acetylcholinesterase. This method involved the reaction of thiol groups introduced into phosphopeptide using *N*-succinimidyl-*S*-acetyl-thioacetate with maleimido groups previously incorporated into acetylcholinesterase with *N*-succinimidyl-4-(maleido-methyl)-cyclohexane-1-carboxylate as previously described (Caruelle *et al.* 1988).

Competitive enzyme immunoassay

All the dilutions were performed in enzyme immunoassay (EIA) buffer corresponding to 0.1 M-potassium phosphate pH 7.4, containing 0.15 mol NaCl/l, 1 g bovine serum albumin/l and 0.1 g sodium azide/l. Competitive EIA were performed using ninety-six-well microtitre plates coated with mouse monoclonal anti-rabbit immunoglobulin G (10 µg/ml 50 mM-phosphate buffer, pH 7.4 for 18 h at 4°C before saturating with EIA buffer), in order to ensure separation of bound and free moieties of the enzymatic tracer during the immunological reaction. The total volume of the immunological reaction was 150 µl, each component (enzymatic tracer, rabbit polyclonal antisera and standard or sample) being added in a volume of 50 µl. The enzymatic tracers were used at a concentration of 2 Ellman units/ml (Ellman *et al.* 1961), while the working dilutions for the corresponding rabbit antisera were previously determined by serial dilution experiments. After 4 h incubation at 20°C, the plates were washed (with 0.01 M-phosphate buffer pH 7.4 containing 0.5 ml Tween 20/l) before the enzyme activity of the bound immunological complex was quantitated by addition of 200 µl Ellman's reagent (7.5 × 10⁻⁴ M acetylthiocholine iodide, substrate; 5 × 10⁻⁴ M 5,5'-dithiobis(2-nitrobenzoic acid), reagent for thiol colorimetric measurement) per well. After 1 or 2 h of gentle shaking in the dark at room temperature, the absorbance of each well at 414 nm was measured using an automatic plate reader. Results were expressed in terms of B/B0 (%) as a function of the dose (logarithmic scale), where B and B0 represent the bound activity in the presence or absence of competitor respectively. All measurements for standards or samples were made in duplicate, and in quadruplicate for B0 values. A linear log-logit transformation was used to fit the standard

curve. The assay sensitivity was characterized by the dose of standard inducing a 50% reduction of the binding observed in absence of competitor (B/B0 50%). The minimum detectable concentration corresponds to an 80% reduction of the binding (B/B0 80%).

Results

(Phospho)peptide pattern of human ileostomy fluid

The peptide profiles of ileostomy extract samples pooled over the time points t 1–5 were quite similar between one subject and another (four volunteers with an ileostomy), and were largely independent of the test meal ingested, i.e. milk or CPP (Fig. 1). From the HPLC profiles

of the different ileostomy fluid samples, no distinctive peptide could be assigned to a component or digestion product of the ingested milk and CPP preparations. The chromatograms measured in the fasting state (t 0) also revealed complex but different patterns (results not shown) as compared with those that were obtained 2 to 10 h post-dose (t 1–5).

Using HPLC analysis in combination with the determination of organically bound P, CPP were found in the perchloric acid-soluble peptide fraction of ileostomy samples obtained from volunteers who ingested milk (Fig. 2, Table 1). However, no CPP were detected in ileostomy samples of volunteers fed specific CPP preparations or in pre-meal samples collected before ingestion of a test meal (t 0). This observation also pertained even if adsorptive (SCX) microconcentration was applied as an additional

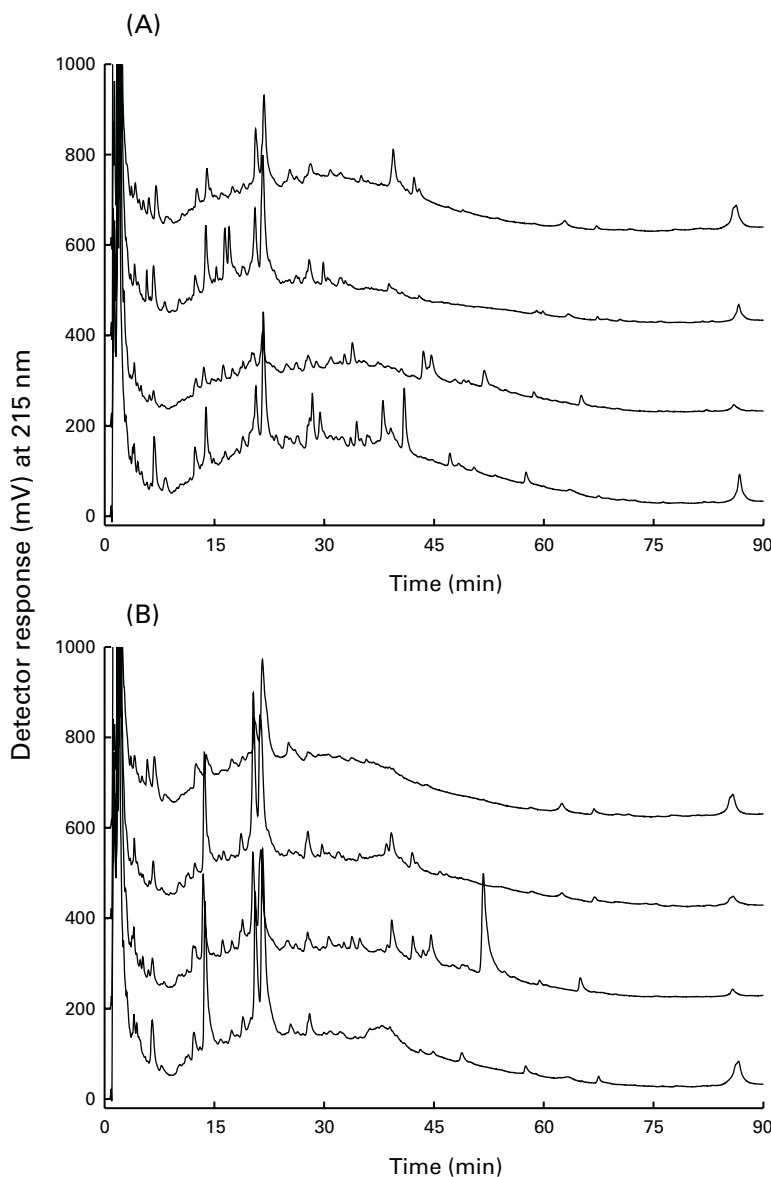


Fig. 1. HPLC peptide patterns of ileostomy fluids collected for 10 h post-dose at 2 h intervals (pooled over the time points t 1–5) from four human volunteers after ingestion of a milk meal (A) and caseinphosphopeptides (CPP) meal (B). Test sample reverse-phase HPLC profiles were overlaid for direct comparison purposes. The magnitude of the detector response for all samples was similar. For details of subjects and test meals, see p. 352. (A), patterns represent (vertically, from top) 01-2:Milk, 02-2:Milk, 03-1:Milk, 04-1:Milk; (B), 01-1:CPP1, 02-1:CPP4, 03-2:CPP1, 04-2:CPP3 (for details of sample classification, see p. 352).

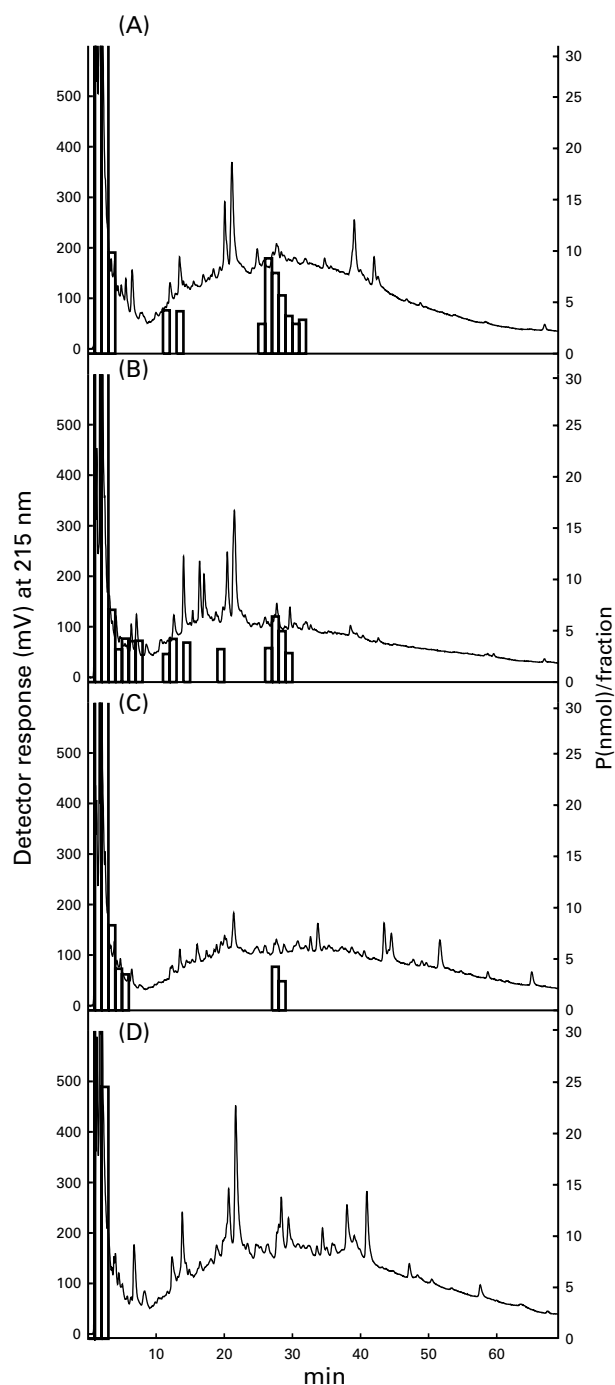


Fig. 2. HPLC peptide pattern and P content of ileostomy fluid (perchloric acid-soluble fraction) collected for 10 h post-dose at 2 h intervals (pooled over the time points t 1–5) from four human volunteers after ingestion of a milk meal. Elution of inorganic P in fractions 1–5, organic (peptide-bound) in fractions >5. For details of subjects and test meals, see p. 352. (A), 01-2: Milk; (B), 02-2: Milk; (C), 03-1: Milk; (D), 04-1: Milk (for details of sample classification, see p. 352).

step to enrich for CPP. In earlier experiments, adsorptive microconcentration (SCX) enabled the enrichment of CPP from ileostomy extract spiked with pure phosphopeptides. Non-phosphorylated peptides were also enriched to some extent, but the concentration of CPP (measured by peak area) was more than 7-fold greater in the corresponding

microconcentrate. Therefore, the use of adsorptive microconcentration for the enrichment of CPP from intestinal chyme may compensate for the 3- to 5-fold dilution of the meal within the intestine (Borgström, 1957).

The results from HPLC analysis showing the presence of CPP in ileostomy fluid were confirmed by thin-layer electrophoresis as a specific qualitative method to detect SerP in hydrolysates of ileostomy samples as well as by quantitative amino acid analysis to measure the SerP content (Table 1).

The composition of the CPP in intestinal fluid changed with time post-dose following ingestion of milk (Fig. 3), and the amount of CPP decreased after prolonged digestion (≥ 8 h). An increase in total peak area and number of peaks after more than 6 h (e.g. t 4 in Fig. 3) may be due to digestion of lunch, which was taken 3 h after ingestion of the meals (milk or CPP preparation).

Immunoreactive caseinophosphopeptides in human ileostomy fluid

The EIA system has been developed using specific rabbit polyclonal antibodies raised against a mixture of CPP prepared by tryptic hydrolysis of whole casein and an acetylcholinesterase-labelled synthetic phosphopeptide. This test system appears to be sensitive, allowing a range of detection between 1 and 10 nmol/ml depending on the polyclonal antibodies used. The binding of acetylcholinesterase-labelled synthetic phosphopeptide to rabbit antibodies was easily inhibited by CPP preparations, e.g. CPP1, 2, 3 and 4, which presented similar inhibition values. Moreover, no differences were observed in the inhibition curves between CPP preparations with or without perchloric acid extraction. The EIA system was then applied to detection of CPP in ileostomy samples (Fig. 4). The validity of the competitive EIA system was supported by the very good agreement in the results obtained with anti-CPP antibodies obtained from two animals (rabbits no. 1494 and no. 1495). Pre-meal (t 0) ileostomy extract samples displayed some low inhibitory effects at a 1:5 dilution, which disappeared at a 1:25 dilution. However, as shown in Fig. 4, extracts from ileostomy samples pooled over the time points from human subjects fed either milk or CPP preparations markedly inhibited the binding even at a dilution greater than 1:625. In agreement with results from HPLC and thin-layer electrophoresis analyses (Table 1), greater inhibition (at least a 100-fold difference) was observed for ileostomy samples obtained from human subjects fed milk as opposed to CPP.

Discussion

The reverse-phase HPLC peptide patterns (samples pooled over the time points t 1–5) were not characteristic of the test meal ingested, and complex elution profiles were also obtained in the fasting state (t 0). Therefore, it would appear that the reverse-phase HPLC profiles also contained many endogenous components secreted into the intestinal fluid.

CPP were detected in ileostomy fluid obtained from human volunteers using two separate approaches, i.e. using HPLC analysis (in combination with peptide-bound

Table 1. Identification of phosphopeptides in pooled ileostomy fluid collected for 10 h at 2 h intervals (*t* 1–5) after ingestion of a test meal*

Ileostomy sample†	Peptide-bound P (HPLC)‡		Peptide-bound SerP		Immunoreactive CPP (EIA; nmol/mg)¶
	nmol/fraction	Fraction no.	TLE (ninhydrin-positive spot)§	AAA (nmol/mg)	
Subject 1					
01-01:CPP2	nd	nd	nd	nd	0.05
01-02:Milk	8	12–14	+	7.3	7.10
	36	26–32			
Subject 2					
02-01:CPP4	nd	nd	nd	nd	0.09
02-02:Milk	15	5–8	+	4.8	4.40
	11	12–15			
	3	21			
	18	27–30			
Subject 3					
03-01:Milk	7	28–29	+	3.1	2.60
03-02:CPP1	nd	nd	nd	nd	0.02
Subject 4					
04-01:Milk	nd	nd	+	2.8	2.60
04-02:CPP3	nd	nd	nd	nd	0.02
Pre-meal**	nd	nd	nd	nd	<0.002

TLE, thin-layer electrophoresis; SerP, phosphoserine; AAA, amino acid analysis; EIA, enzyme immunoassay; CPP, caseinophosphopeptide; nd, not detected.

* For details of subjects and test meals, see p. 352.

† The soluble fraction obtained after extraction with perchloric acid (172 mM) was analysed. For details, see p. 352.

‡ HPLC analysis and determination of total organically bound P in HPLC fractions.

§ TLE of partial acid hydrolysates (6 h) and ninhydrin-positive spot of SerP.

|| AAA of acidic hydrolysates (6, 12, 24 h). SerP content per mg lyophilized ileostomy sample was obtained by extrapolation to zero time; non-hydrolysable material having the same elution position as SerP (equivalent to ≤ 0.5 nmol/mg) was present in all samples; SerP was not detectable in the insoluble part of extracted ileostomy samples.

¶ Competitive EIA using rabbit (no. 1495) polyclonal antiserum and acetylcholinesterase-labelled synthetic phosphopeptide as tracer. CPP concentration equivalent to the amount of synthetic VESLSSSEESIL (Σ = SerP) per mg lyophilized ileostomy sample.

** The pre-meal sample represents a pool of all samples collected in the fasting state (*t* 0).

P and SerP determination) of ileostomy extract, as well as the application of a unique EIA system. Both the immunochemical and chromatographic-peptide chemical approach to detect CPP enabled the semi-quantification of CPP in human ileostomy fluid of milk-fed volunteers (Table 1). However, the quantity of CPP in ileostomy samples from volunteers fed with CPP preparations was too small to be detected by HPLC or SerP analyses. The EIA system combined a broad specificity suitable for the detection of numerous different phosphopeptides and a high sensitivity that allows detection of trace amounts in complex media such as in biological fluids or food matrices.

The reason for looking at CPP generated with four different proteolytic preparations was that there were differences in the Ca^{2+} binding and solubilizing abilities of CPP *in vitro* depending on the enzyme preparation used in hydrolysate generation (McDonagh & FitzGerald, 1998). However, this has not proved to be relevant in the current *in vivo* study. There is obviously an effect of the food matrix that protects CPP from degradation in the gut, provided that they are ingested as milk constituents and not as isolated CPP ingredients. CPP are not found naturally in good quality milk, but are encrypted in the primary structure of the caseins and are released by proteolytic action. Therefore, the form in which phosphopeptides exist in milk *v.* that in which they exist in the CPP meal is quite different, and this may account for potential differences in proteolytic susceptibility during gastrointestinal transit. It is worth noting that CPP released during milk digestion appeared to be stable for up to 8 h in ileostomy contents (Fig. 3). Milk seems to be a good 'wrapping' or 'carrier'

for CPP, i.e. the corresponding protein-bound sequences are partly protected against proteolytic attack.

Based on the fact that the ingested amount of milk (250 g) is equivalent to 6.5 g casein, which accounts for 55 mg (1.775 mmol) organically bound P (P_o ; Douglas *et al.* 1982), a maximal recovery of 15% peptide-bound P for the digestion products of milk can be estimated from the content of peptide-bound SerP (Table 1) multiplied by the total weight of freeze-dried ileostomy samples. Casein theoretically yields 126 mg CPP/g, which is released predominantly during tryptic *in vitro* proteolysis (Meisel, 1998). This would suggest that about 120 mg potentially available CPP could be liberated into the ileum after a meal of 250 g milk. This estimation is supported by an alternative calculation, which assumes that the CPP in ileostomy fluid have predominantly two SerP groups and that their average relative molecular mass was 1000.

The Ca-binding constants for different CPP preparations are reported to be in the order of 10^2 – 10^3 l/mol (Berrocal *et al.* 1989; Sato *et al.* 1991; Meisel & Olieman, 1998). Interestingly, the amount of Ca solubilized per mol CPP is far in excess of the estimated amount of SerP that binds an almost equivalent number of Ca^{2+} . Accordingly, Berrocal *et al.* (1989) estimated that the effective CPP concentration required to inhibit the formation of insoluble $\text{Ca}_3(\text{PO}_4)_2$ *in vitro* was 10 mg/l. Furthermore, it has been shown that relatively low concentrations of CPP (10–50 mg/l) can stimulate cultivated immunocompetent human cells (Hata *et al.* 1998, 1999). Therefore, it is postulated that the quantity of CPP found in ileostomy fluid in the present study could be of physiological importance.

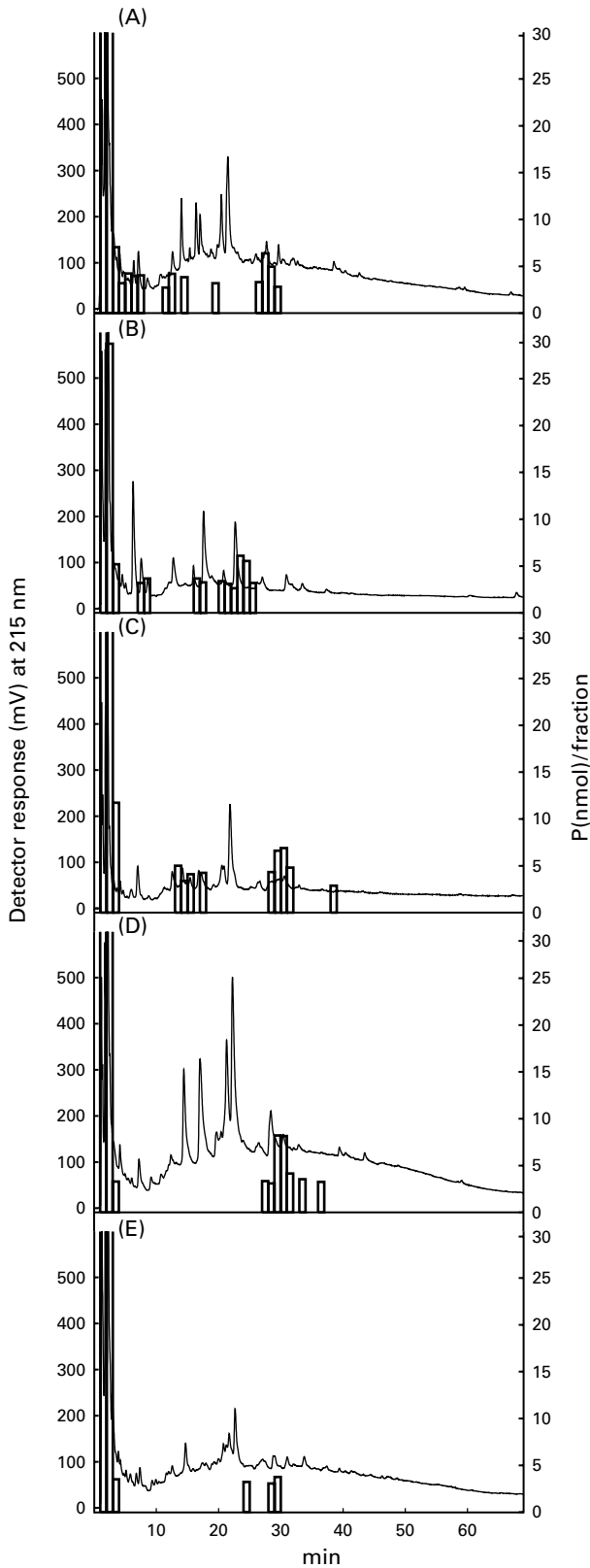


Fig. 3. HPLC peptide patterns and P content of ileostomy fluids (perchloric acid-soluble fraction) measured at different time points post-dose (2 h intervals t 1–5) after ingestion of a milk meal (subject 2 milk). (A), t 1–5 (pooled samples 2–10 h); (B), t 1 (2 h); (C), t 3 (6 h); (D), t 4 (8 h); (E), t 5 (10 h). For details of subjects and test meals, see p. 352.

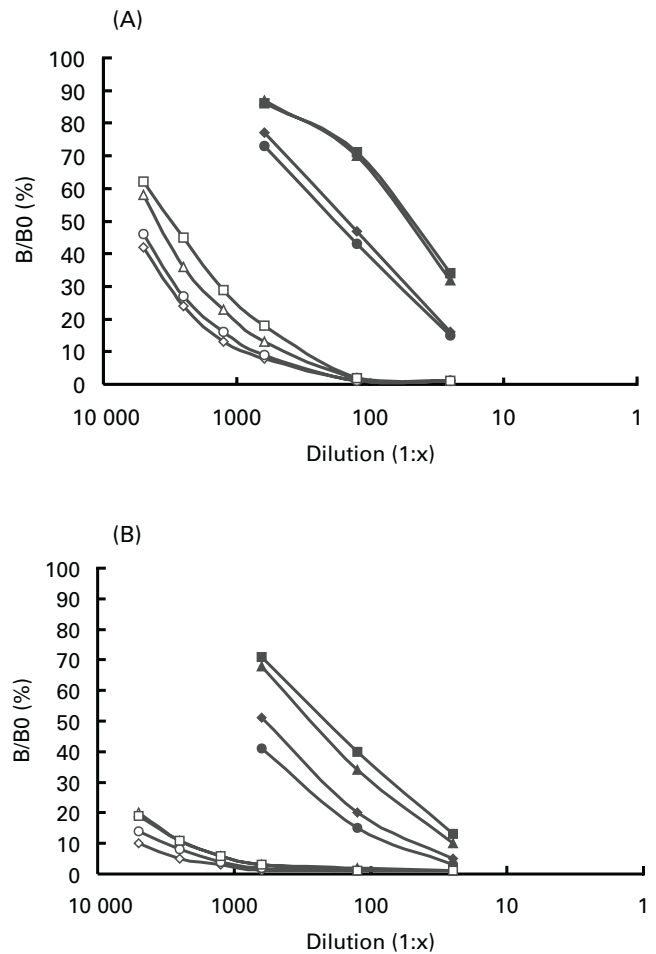


Fig. 4. Inhibition of the binding of specific rabbit anti-caseinophosphopeptides (CPP) antibodies (A, rabbit no. 1494; B, rabbit no. 1495) to synthetic phosphopeptide VESL $\Sigma\Sigma\Sigma$ EESIL-acetylcholinesterase, where Σ is phosphoserine) by different ileostomy samples from four human volunteers after ingestion of a milk meal (\diamond 01-2:Milk; \circ 02-2:Milk; \square 03-1:Milk; \triangle 04-1:Milk) and CPP meal (\blacklozenge 01-1:CPP2; \bullet 02-1:CPP4; \blacksquare 03-2:CPP1; \blacktriangle 04-2:CPP3). The y-axis represents the bound enzyme activity in presence (B) or absence (B0) of competitor. For details of subjects and procedures, see p. 352.

The *in vivo* formation of CPP derived from bovine casein was demonstrated in the small intestinal fluid of minipigs after ingestion of a diet containing casein (Meisel & Frister, 1988). Chabance *et al.* (1998) recently detected CPP in the stomach and duodenum of adult human subjects after ingestion of milk or yogurt. Our present studies demonstrate, for the first time, that phosphopeptides can survive the prolonged intestinal passage to the distal small intestine (ileum) *in vivo*, which is a prerequisite for their function as bioactive substances. Vitamin D-independent paracellular transport of Ca is known to play a major role in maximal passive Ca absorption from the distal small intestine (Bronner, 1987). However, the physiological significance of CPP as potentially bioactive substances in the ileum has not yet been clarified. Preliminary results indicate that consumption of CPP by human subjects does not significantly increase the fractional absorption of dietary Ca (B Teucher, G Majsak-Newman, JR Dainty,

D McDonagh and S Fairweather-Tait, unpublished observations). Nevertheless, it has recently been demonstrated that CPP mediate Ca uptake in rat intestinal everted sacs (Erba *et al.* 2001) and into epithelial (HT29) cells (Ferraretto *et al.* 2001). Furthermore, the production of immunoglobulin G was stimulated by CPP in immunocompetent human cells (Hartmann *et al.* 2000). The latter finding is in support of the suggestion (Hata *et al.* 1998; Otani *et al.* 2000) that CPP may act as immunostimulators, which are beneficial to the enhancement of mucosal immunity. Therefore, irrespective of their possible function as Ca carriers, CPP are of particular interest as dietary components that may modulate specific bodily functions and thus have functional food ingredient potential for promoting better health and reducing the risk of developing specific diseases.

Acknowledgements

The studies have been carried out with financial support from the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD programme, CT98-3077, 'Caseinophosphopeptides: Nutraceutical/Functional Food Ingredients for Food and Pharmaceutical Applications'; the present publication does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area.

References

- Adamson NJ & Reynolds EC (1995) Characterisation of tryptic casein phosphopeptides prepared under industrially relevant conditions. *Biotechnology and Bioengineering* **45**, 196–204.
- Berrocal R, Chanton S, Juillerat MA, Pavillard B, Scherz J-C & Jost R (1989) Tryptic phosphopeptides from whole casein: II. Physicochemical properties related to the solubilisation of calcium. *Journal of Dairy Research* **56**, 335–341.
- Borgström B, Dahlqvist A, Lundh G & Sjövall J (1957) Studies of intestinal digestion and absorption in the human. *Journal of Clinical Investigation* **36**, 1521–1536.
- Bronner F (1987) Intestinal calcium absorption: Mechanisms and applications. *Journal of Nutrition* **117**, 1347–1352.
- Brule G, Roger L, Fauquant J & Piot M (1982) Phosphopeptides from casein-based material. *U. S. Patent*, No. 4,358,465.
- Caruelle D, Grassi J, Courty J, Groux-Muscatelli B, Pradelles P, Barritault D & Caruelle JP (1988) Development and testing of radio and enzyme immunoassays for acidic fibroblast growth factor. *Analytical Biochemistry* **173**, 328–339.
- Chabance B, Marteau P, Rambaud JC, Migliore-Samour D, Boynard M, Perrotin P, Guillet R, Jollès P & Fiat AM (1998) Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie* **80**, 155–165.
- Douglas FW, Tobias J, Groves ML, Farrell HM & Edmondson LF (1982) Quantitative determination of total protein, casein, and whey protein of processed dairy products. *Journal of Dairy Science* **65**, 33–45.
- Ellman GL, Courtney KD, Andres V & Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology* **7**, 88–95.
- Erba D, Ciappellano S & Testolin G (2001) Effect of casein phosphopeptides on inhibition of calcium intestinal absorption due to phosphate. *Nutrition Research* **21**, 649–656.
- Ferraretto A, Signorile A, Gravaghi C, Fiorilli A & Tettamanti G (2001) Casein phosphopeptides influence calcium uptake by cultured human intestinal HT-29 tumor cells. *Journal of Nutrition* **131**, 1655–1661.
- FitzGerald RJ (1998) Potential uses of caseinophosphopeptides. *International Dairy Journal* **8**, 451–457.
- FitzGerald RJ & Meisel H (2000) Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *British Journal of Nutrition* **84**, Suppl. 1, 33–37.
- Hartmann R, Günther S, Martin D, Meisel H, Pentzien A-K, Schlimme E & Scholz N (2000) Cytochemical model systems for the detection and characterization of potentially bioactive milk components. *Kieler Milchwirtschaftliche Forschungsberichte* **52**, 61–85.
- Hartmann R & Meisel H (2002) Cytochemical assessment of phosphopeptides derived from casein as potential ingredients for functional food. *Nahrung/Food* **46**, 427–431.
- Hata I, Higashiyama S & Otani H (1998) Identification of a phosphopeptide in bovine α_{S1} -casein digests as a factor influencing proliferation and immunoglobulin production in lymphocyte cultures. *Journal of Dairy Research* **65**, 569–578.
- Hata I, Ueda J & Otani H (1999) Immunostimulatory action of a commercially available casein phosphopeptide preparation, CPP-III, in cell cultures. *Milchwissenschaft* **54**, 3–7.
- McDonagh D & FitzGerald RJ (1998) Production of caseinophosphopeptides (CPP) from sodium caseinate using a range of commercial protease preparations. *International Dairy Journal* **8**, 39–45.
- Meisel H (1997) Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers* **43**, 119–128.
- Meisel H (1998) Overview on milk protein-derived peptides. *International Dairy Journal* **8**, 363–373.
- Meisel H (2001) Bioactive peptides from milk proteins: a perspective for consumers and producers. *Australian Journal of Dairy Technology* **56**, 83–92.
- Meisel H, Bernard H, Fairweather-Tait S, FitzGerald RJ, Hartmann R, Lane CN, McDonagh D, Teucher B & Wal J-M (2001) Letter to the Editor: Nutraceutical and functional food ingredients for food and pharmaceutical applications. *British Journal of Nutrition* **85**, 635.
- Meisel H & FitzGerald RJ (2000) Opioid peptides encrypted in intact milk protein sequences. *British Journal of Nutrition* **84**, Suppl. 1, 27–31.
- Meisel H & Frister H (1988) Chemical characterization of a caseinophosphopeptide isolated from *in vivo* digests of a casein diet. *Biological Chemistry Hoppe-Seyler* **369**, 1275–1279.
- Meisel H & Olieman C (1998) Estimation of calcium binding constants of casein phosphopeptides by capillary zone electrophoresis. *Acta Chimica Acta* **372**, 291–297.
- Otani H, Kihara Y & Park M (2000) The immunoenhancing property of a dietary casein phosphopeptide preparation in mice. *Food and Agricultural Immunology* **12**, 165–173.
- Sato R, Shindo M, Gunshin H, Noguchi T & Naito H (1991) Characterisation of phosphopeptide derived from bovine β -casein: An inhibitor of intra-intestinal precipitation of calcium phosphate. *Biochimica et Biophysica Acta* **1077**, 413–415.