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Food Chemistry 96 (2006) 234–241
Food Chemistry
www.elsevier.com/locate/foodchem

β-CN-5P and β-CN-4P components of bovine milk proteose–peptone: Large scale preparation and influence on the growth of cariogenic microorganisms

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Received 15 August 2003; received in revised form 9 February 2005; accepted 9 February 2005

Abstract

Indigenous proteolytic activity in milk, mostly due to plasmin, gives rise to many casein-derived peptides that subsequently are found in the proteose–peptone fraction of milk where they comprise 10% or more of the total whey protein. Prominent amongst proteose–peptone components are β-CN-5P (β-casein residues, 1–105/107) and β-CN-4P (β-casein residues 1–28). Many peptides have potentially valuable functional or biological properties that differ from those of the parent proteins, and this paper describes simple, rapid and cost-effective preparation of these two milk peptide components in a high degree of purity, and in gramme quantities, for evaluation of such properties. The purification process was more efficient if β-casein was used as starting material. In this work, we prepared 46 g of β-casein from sodium caseinate in a simple rapid DEAE-cellulose ion-exchange chromatography stage. This was followed by in vitro hydrolysis with plasmin and precipitation and gel filtration steps to yield 4.8 g of highly purified β-CN-5P and 1.2 g of β-CN-4P. Utilising either unfractionated sodium caseinate, or milk itself, as starting material was satisfactory but gave less purified material containing other peptide impurities. Peptides similar to these proteose–peptone components have been implicated in the protective effects of milk and dairy products against dental caries in teeth. The mechanism(s) by which this protection occurs is unclear, but some antibiotics are peptides. However, we have found that, even at peptide concentrations as high as 0.5 mg/ml, neither β-CN-5P nor β-CN-4P had any effect on the in vitro growth of cariogenic *Streptococcus mutans* bacteria, ruling out a simple antibiotic mechanism.

Keywords: Proteose–peptones; β-CN-5P; β-CN-4P; Large scale preparation; *S. mutans* growth

1. Introduction

The proteose–peptone fraction of bovine milk is partially made up of a number of heat-stable minor proteins and glycoproteins but also, largely, of casein-derived peptides (Andrews & Alichanidis, 1983; Paquet, 1989). These are generated in vivo by the action of indigenous proteinases (mainly plasmin) on all of the four main casein proteins (Andrews, 1983; de Rham & Andrews, 1982; Eigel et al., 1984; Kaminogawa, Mizobuchi, & Yamauchi, 1972).

Simple one-dimensional gel electrophoresis reveals at least 40 readily-detectable peptide bands in the proteose–peptone fraction (Andrews & Alichanidis, 1983). The three main ones, however, were originally termed components 3-, 5- and 8-fast (Larson & Rolleri, 1955; Kolar & Brunner, 1970), with component 3 now known to be a heat-resistant phosphorylated glycoprotein (Girardet & Linden, 1996; Sorensen & Petersen, 1993).

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doi:10.1016/j.foodchem.2005.02.039

Component 5 (now termed β-CN-5P) contains two main constiuents, β-casein fragments 1–105 and 1–107, and component 8-fast (now termed β-CN-4P) is β-casein fragment 1–28 (Andrews, 1978b).

In fresh good quality milk, the proteose–peptone fraction is typically 1–2 mg/ml or about 10–20% of the whey protein (Paquet, 1989; Swaisgood, 1992) while the concentrations of β-CN-5P and β-CN-4P are approximately 0.2–0.4 mg/ml and 0.1–0.15 mg/ml, respectively (i.e., up to about 5% and 2% of the total whey protein), so these two components are very significant milk constituents. Values are often much higher in stored milk where greater hydrolysis may have occurred.

In recent years, there have been a number of reports of food protein-derived peptides exhibiting interesting biological activities, among which the protective effect of milk casein phosphopeptides (CCP) against dental caries in teeth is now well established (Reynolds, 1987, 1997; Reynolds et al., 1995). These CCP contain the partial amino acid sequence SerP–SerP–SerP–Glu–Glu which occurs in α_{S1}-, α_{S2}-(twice) and β-caseins and has been implicated in the protective process. In β-casein, this sequence occurs at amino acid residues 17–21 (Ribadeau-Dumas, Brignon, Grosclaude, & Mercier, 1972) so it is present in both β-CN-5P and β-CN-4P, which can both therefore be regarded as CPP and natural milk components. The mechanism whereby CPP exert their protective effect is not clear and a number of alternative pathways are possible.

In this paper, we describe a simple and rapid procedure for the laboratory-scale purification of several grammes of β-casein as starting material, followed by optimisation of in vitro hydrolysis with plasmin and subsequent fractionation, to generate the required β-CN-5P and β-CN-4P in quantities sufficient for an in vitro examination of their effects on the growth of a cariogenic strain of *Streptococcus mutans*.

2. Materials and methods

2.1. Materials

Polyethylene glycols of differing average molecular masses (PEG1, PEG2, PEG4, PEG8 or PEG10), sodium caseinate, dextran T500, DEAE-cellulose (DE-52) and plasmin (E.C.3.4.21.7) were supplied by Sigma Chemicals (Poole, Dorset, UK). A standard proteose–peptone fraction (TPP, total proteose–peptone) was prepared from fresh skimmed bovine milk heated at 90± 2 °C for 20 min, as described by Andrews and Alichanidis (1983). Polyacrylamide gel electrophoresis (PAGE) was performed with $T = 12.5\%$, $C = 5\%$ gels with Coomassie Blue G-250 staining (Andrews & Alichanidis, 1983).

2.2. β-Casein purification

DEAE-cellulose (sufficient to give a bed volume of approximately 1 l) was suspended in 5 l of 0.1 M Tris–HCl pH7.0 buffer and the pH re-adjusted while stirring to 7.0 if necessary. The slurry was then poured into a large (150 mm diameter) Buchner funnel and packed under gentle vacuum into a bed of approximately 80–100 mm in height. This was then washed with 500 ml of 0.05 M Tris–HCl, pH 7.0, buffer or until the pH of the filtrate was also 7.0 ± 0.5. The DE-52 bed was then washed with 500 ml of this same buffer containing 90 g urea (i.e., 3 M urea), and the sodium caseinate sample applied (40 g sodium caseinate + 180 g urea in a total volume of 500 ml 0.05 M Tris–HCl pH 7.0 buffer). The sample was permitted to flow onto the bed under gravity to minimise uneven loading. Non-adhering proteins (and also κ- and γ-caseins) were then removed with 2 l of 0.05 M Tris–HCl, pH 7.0, buffer containing 3 M urea and 0.03 M NaCl. Stepwise elution of β-casein was achieved with 2 l of this same buffer containing 0.12 M NaCl. Remaining bound proteins were then washed off the bed with 2 l of buffer containing 0.3 M NaCl, and followed by 2 l of salt-free buffer to prepare the bed for the next run.

The β-casein-containing fraction was diluted to 5 l with H_2O to reduce the molarity of urea present and the pH adjusted to 4.5 with 1 M HCl. Most of the casein precipitated out (1% w/v of trichloroacetic acid can be added to complete precipitation if required). After standing at 4 °C overnight, most of the supernatant liquid was decanted off and precipitated caseins collected from the remaining mixture by centrifugation at 3000*g* for 15 min. The β-casein was washed once by suspension and re-centrifugation, redissolved at pH 7 and stored at −20 °C. The yield (typically 62% of theoretical from sodium caseinate) was not particularly good but sodium caseinate is relatively inexpensive and the method was simple, fast, used minimal equipment and gave β-casein of good electrophoretic purity.

2.3. Preparation of β-CN-5P and β-CN-4P from β-casein

On the basis of preliminary time course experiments (monitored by PAGE analysis), to determine the best conditions for casein hydrolysis by plasmin, it was found that an E:S = 1:30,000 with 120 min incubation time gave approximately 50% hydrolysis of the β-casein. This generated substantial amounts of β-CN-5P with little further breakdown to β-CN-4P and the β-casein fragment 29–105. Accordingly, 748 ml of β-casein solution, containing 46 g β-casein, was warmed to 37 °C and 15 ml of plasmin solution (0.1 mg/ml) added with stirring. After 120 min at 37 °C the pH was adjusted to 4.2–4.5 with 1 M HCl and the mixture heated to 90 ± 2 °C for 5 min. The pH adjustment virtually

stopped the hydrolysis reaction and the heating step then inactivated the plasmin to avoid regain of activity in subsequent steps. The mixture was then cooled, the pH re-adjusted if necessary to 4.2–4.5, and centrifuged at 3000*g* for 15 min to separate precipitated casein (residual β-casein plus γ-casein products). The supernatant was carefully collected and the casein pellet re-suspended in 300 ml H_2O, stirred for 30 min and re-centrifuged. The supernatant washings were combined with the first supernatant and TCA added to 5% (w/v) to precipitate a crude β-CN-5P fraction which was collected by filtration through Whatman No. 1 filter paper. The precipitate on the filter paper was washed three times with 50 ml portions of acetone (to remove most of the TCA) and air-dried at room temperature to yield approximately 9.2 g of crude β-CN-5P.

The washed casein centrifuge pellet, obtained as above, still contained a large proportion of the β-casein which could either be recycled or used for large scale preparation of β-CN-4P by a generally similar procedure. Since this is an end-product of the plasmin hydrolysis of β-casein, there is no fear of excessive hydrolysis and either a 24 h incubation, under conditions as above, or a 10-fold higher proportion of plasmin to β-casein were used. The only other difference was that the TCA concentration used for precipitation after hydrolysis was increased to 10% (w/v).

2.4. Purification by gel filtration

The crude β-CN-5P and β-CN-4P, prepared as above, were further purified by gel filtration on a column (550 × 22 mm) of Sephadex G-75 made up in and equilibrated with volatile 0.1 M NH_4HCO_3 (pH 8.0–8.5) buffer. Samples of 0.5–0.7 g of crude β-CN-5P or β-CN-4P were dissolved in 5–7 ml of buffer (with addition of a few μl of 1 M NaOH to neutralise residual traces of TCA and aid dissolution) and applied to the column. Flow rate was adjusted to 0.5 ml/min and 5 ml fractions were collected. Column monitoring was at 230 nm. Alternate fractions were also analysed by PAGE to facilitate pooling of appropriate fractions. For subsequent column runs, UV monitoring alone was adequate. Pooled fractions were lyophilised to give salt-free product.

2.5. Purification by aqueous two-phase systems

For the small scale analysis of the β-CN-5P content in β-casein hydrolysates or in the milk TPP fraction, 1 ml aqueous two-phase systems (ATPS) volumes were used. For this, stock solutions of PEG and dextran in H_2O, at 2.5 times the desired final concentration, were prepared and 0.4 ml of each added to Eppendorf microcentrifuge tubes, followed by 0.1 ml of 0.1 M Tris–HCl buffer of various pH values and 0.1 ml of sample in H_2O at 10.0 mg ml^{-1}. After mixing briefly in the vortex mixer, samples were centrifuged at 10,000*g* for 15 min to ensure complete separation of the phases. Portions of each phase were then analysed with a Superose 12 HR 10/30 gel filtration FPLC column (Pharmacia Biotechnology, Milton Keynes, UK) attached to a Dionex DX500 HPLC with AD20 UV monitor (Dionex UK Ltd., Camberley, UK), using 0.1 M Tris–HCl, pH 7.0, buffer containing 0.125 M NaCl as eluent at a flow rate of 0.5 ml/min. Absorption was monitored at 215 nm. The column was calibrated with β-CN-5P and β-CN-4P prepared by conventional methods (Andrews, 1978a, 1978b). Partition coefficients (*K*) were determined by analysing both the PEG and dextran phases and calculating the peak areas, *K* being the concentration in the upper (PEG) phase divided by that in the lower aqueous phase. β-CN-5P was a large peptide and separated well from other small peptide components under these gel filtration conditions so, although somewhat laborious, this approach allowed *K* for β-CN-5P to be assessed accurately without interference from other peptides that might have occurred if a dye-binding type of assay (widely used in conjunction with ATPS) had been used for quantification.

2.6. S. mutans growth experiments

The strongly cariogenic strain of *S. mutans* NCTC 10449 (National Type Culture Collection, Colindale, UK) was used in all experiments.

The lyophilised culture was inoculated into sterile brain heart infusion Broth (BHI, 37 g/l, Oxoid, UK) and incubated at 30 °C for 24 h. The culture was grown at 37 °C and maintained at 4 °C on brain heart infusion agar plates and slopes (BHI 37 g/l and bacteriological agar No. 1, 10 g/l, Oxoid, UK) using standard methods.

A stock culture from a BHI agar slope was inoculated into 100 ml of sterile BHI broth and incubated at 37 °C overnight to provide working cultures for each experiment. The working cultures were serially diluted to 1×10^{-3} in sterile maximum recovery diluent (MRD, 9.5 g/l, Oxoid) to give an inoculum containing approximately 1×10^5 CFU/ml.

Six 100 ml portions of BHI broth were prepared. To four of these portions, were added β-CN-5P (5 or 50 mg) or β-CN-4P (5 or 50 mg) while two portions were retained as peptide-free controls. All broths were autoclaved at 121 °C for 15 min before being cooled to 37 °C, inoculated with 1 ml of the diluted overnight working culture and incubated at 37 °C for 8 h.

The broths were sampled aseptically at hourly intervals (1 ml volume) and the number of viable *S. mutans* colony-forming units/ml estimated on BHI agar plates following incubation at 37 °C for 24 h.

3. Results and discussion

3.1. β-Casein purification

The major casein components are well separated by ion-exchange chromatography on DEAE-cellulose, so, in this study, we chose a simple and rapid stepwise elution, consisting of just three steps. Two column volumes of buffer for each step were a minimum, but adequate, for each step. We also found that, as long as casein micelles and aggregates had been disrupted by adding high levels of urea (6–8 M) to the sample prior to chromatography, ion-exchange columns could then be run satisfactorily in urea at 2.8–3.0 M, a level lower than that employed in most previous reports (Andrews & Alichanidis, 1983; Andrews, Taylor, & Owen, 1985; Humphrey & Newsome, 1984; Lorient, Closs, & Courthaudon, 1989). Further reduction to 2.5 M gave less satisfactory results. Reducing urea levels, not only saved on reagent costs, but also facilitated recovery of caseins from the eluates by a simple cost-effective dilution and precipitation, as high urea levels inhibit precipitation. Five or six column runs could be performed in a single day, giving about 50 g of high purity β-casein.

3.2. β-CN-5P and β-CN-4P production and purification

PAGE analysis of β-casein hydrolysates, obtained in preliminary experiments with plasmin at E:S = 1:30,000 or 1:6000, for various times, is shown in Fig. 1. Since β-CN-5P is not an end-product of β-casein hydrolysis it was important to control the reaction carefully. Visually, if 50–60% of the β-casein was hydrolysed, this gave a good yield of β-CN-5P with minimal further hydrolysis. Fig. 1 also shows that the desired degree of breakdown was achieved in 120 min at E:S = 1:30,000 or in 20 min at E:S = 1:6000. For larger preparative scale experiments, we chose the lower enzyme level for: (i) ease of control, as less enzyme meant slower rate of change; (ii) to minimise enzyme costs.

Analytical gel filtration on the FPLC Superose 12 column (Fig. 2(a)) confirmed that both high and low molecular weight components, usually seen in the TPP fraction from milk, were missing from the β-casein hydrolysate, confirming the benefits of using β-casein as starting material. The peak seen at about 16 min (Fig. 2) represented the column void volume (totally excluded material) and the included volume was at approximately 40 min, so later eluting peaks represented UV absorbing salts or retarded amino acids and peptides. All peptides of interest in this work were eluted in the range of 20–35 min. β-CN-5P, β-CN-4P and β-CN-1P were clearly identified by PAGE analysis. The peptide fragment β-CN-1P is β-casein residues 29–105 and so represents, essentially, β-CN-5P less β-CN-4P and is one of the first products in the further hydrolysis

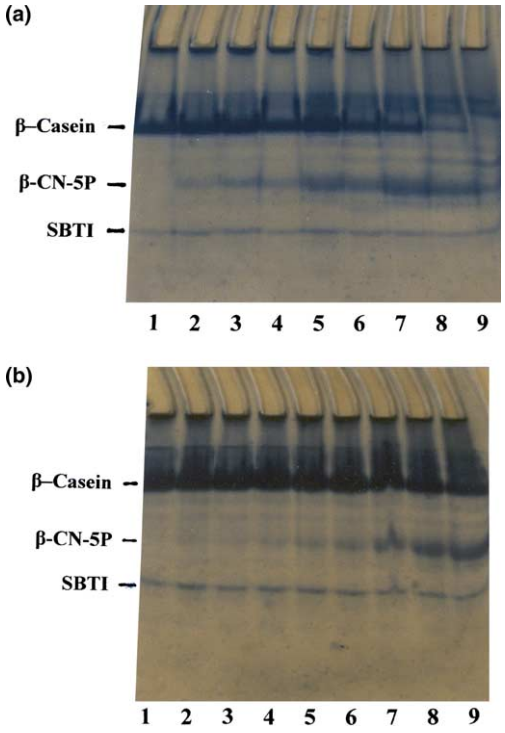

Fig. 1. Time course showing the progressive hydrolysis of β-casein by plasmin (E:S = 1:30000 and E:S = 1:6000) at pH 7.0 and 37 °C: (a) plasmin at the higher level (E:S = 1:6000); (b) at the lower level (E:S = 1:3000). Left to right: Lane 1, pure β-casein control (0 min incubation time); Lanes 2–9, incubation with plasmin for 2, 5, 10, 20, 30, 60, 120 and 240 min. (*Note*: β-casein was heavily overloaded on the gels to facilitate the detection of any impurities in the β-casein and to improve the visibility of plasmin peptide zones.)

of β-CN-5P (Andrews & Alichanidis, 1983; Le Roux, Girardet, Humbert, Laurent, & Linden, 1995).

In spite of the relatively poor resolution capabilities of the preparative scale G-75 Sephadex column, which resulted in a single broad elution peak (data not shown) because β-CN-5P was larger than other peptides present in the hydrolysate, it was possible, with guidance from PAGE patterns, to pool fractions that were of high purity and gave a single sharp peak on FPLC analysis (Fig. 2(b)) as well as a single PAGE band (Fig. 3(D)). Fig. 3 also shows (A) the profile of milk TPP, (B) β-CN-5P purified from TPP (Andrews, 1978a) and (E) the crude β-CN-5P fraction from the β-casein hydrolysate after adjustment to pH4.4 and before G-75 purification

From the respective molecular weights, 46 g of β-casein could theoretically give as much as 23 g of β-CN-5P. The combined yield we obtained from several gel filtration runs was 4.8 g, equivalent to only about 21% overall yield, although it gave only a single electrophoretic band with no visible contaminants, so was of excellent purity.

In a typical β-casein recycling experiment aimed at β-CN-4P production, we obtained 1.24 g of high purity β-CN-4P following gel filtration, which represented approximately 18% of the theoretical maximum yield.

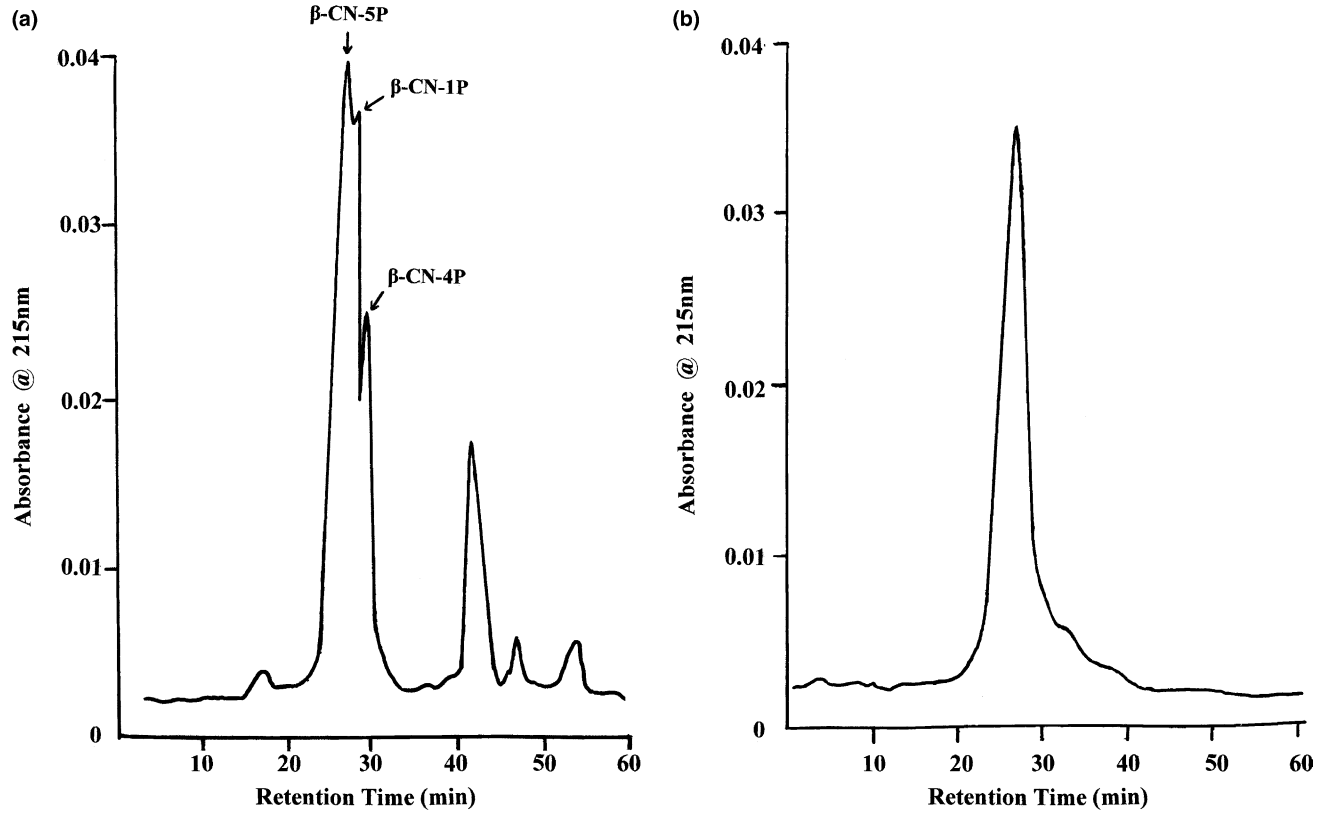

Fig. 2. Analytical-scale gel filtration on an FPLC Superose 12 HR column run in 0.1 M Na phosphate buffer, pH 7.0, containing 0.1 M NaCl with a flow rate of 1.0 ml/min of: (a) crude β-CN-5P material from a plasmin hydrolysate of β-casein (see text); (b) β-CN-5P further purified on a preparative column of G-75 Sephadex (see text).

However, it should be noted that only the recycled portion of the β-casein was used here in the interests of economy and the plasmin hydrolysis step was not optimised for β-CN-4P production. Much higher yields could have been expected had this been the primary objective.

3.3. ATPS purification of β-CN-5P

Hydropathic index plots (Kyte & Doolittle, 1982) for the four caseins have been published in a number of dairy texts (e.g. Swaisgood, 1992).

These showed that, in general, the distribution of hydrophilic and hydrophobic regions along the amino acid sequences was relatively uniform. The main exception was that, although β-casein was the most hydrophobic of the casein molecules, it nevertheless included the most pronounced hydrophilic region seen in any of them. This comprised residues 14–55 and was followed by an extended region (56–101) that was markedly hydrophobic. β-CN-5P (residues 1–105/107) corresponds closely to this region and so represented a large (M_r = 12,258) and very polar peptide sequence, with strongly hydrophilic N-terminal and hydrophobic C-terminal regions. Likewise, β-CN-4P, although much smaller (M_r = 3469), represents the N-terminal residues 1–28 of β-casein and would also be polar, with a hydrophobic N-terminal end, followed by the hydrophilic C-terminus. β-CN-1P corresponds to β-casein residues 29–105/107 (Andrews & Alichanidis, 1983; Girardet & Linden, 1996) so is less polar and intermediate in size (M_r = 8790).

Because of these differences in size and polarity between them and most other proteose–peptone components, it was considered likely that ATPS might be useful for their purification. Molecular properties, such as size, charge and hydrophobicity, are crucial for determining partitioning behaviour and other factors influencing partitioning tend to be of a much less importance (Albertsson, 1986; Walter, Brooks, & Fisher, 1985). In PEG-salt ATPS, the high ionic strength reduces the influence of further added salt ions and, hence, the role of protein molecular charge, although pH remains an important factor. However, in PEG-Dextran ATPS, all of these factors are fully operative and these appeared therefore to have the greatest potential for proteose–peptone fractionation. Generally, high molecular weight PEG has the best potential for protein separations (Albertsson, Cajarville, Brooks, & Tjerneld, 1987; Diamond & Hsu, 1989) and our results confirmed

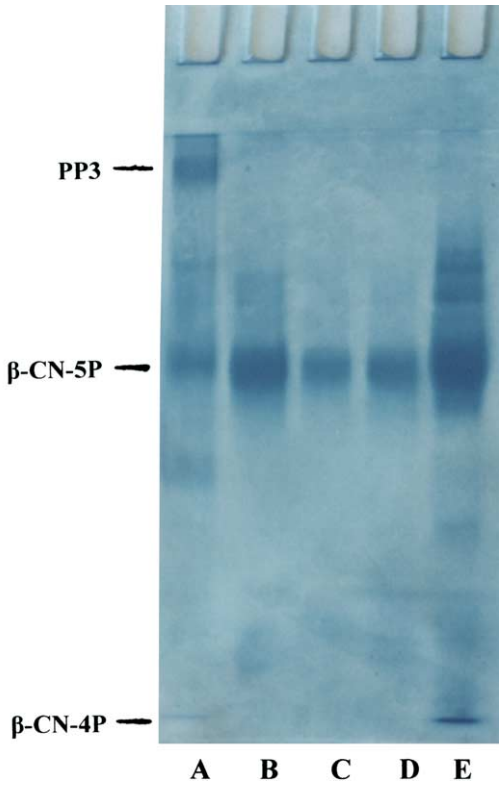

Fig. 3. PAGE analysis on a $T = 12.5\%$, $C = 5\%$ gel of: (A) TPP fraction from heated bovine milk; (B) β-CN-5P purified from milk TPP fraction; (C) β-CN-5P material from recycled β-casein (see text); (D) β-CN-5P purified from plasmin hydrolysate of β-casein; (E) crude β-casein hydrolysate material.

that PEG8 and PEG10 were indeed better in this study than PEG1, PEG2 or PEG4, which gave very little selective partitioning (data not shown).

Using appropriate concentrations of PEG8 and dextran T500, in 0.01 M Tris–HCl buffer, pH 7.0, to give two phases of equal volume (Table 1), so-called equivalent systems (Niven, Smith, Scurlock, & Andrews, 1990), the PEG concentration was varied over the range 4.0–6.0% (w/w) and the influence this had on β-CN-5P partitioning behaviour is shown in Fig. 4(a), where it can be seen that, as PEG concentration increased, so did the proportion of β-CN-5P found in the upper PEG phase. The addition of neutral salts, such as NaCl, is widely used to alter partitioning behaviour in ATPS (Johansson, Karlström, Tjerneld, & Haynes, 1998) and the effect

Table 1
Concentrations of PEG8 and dextran T500 required to form "equivalent systems" with two phases of equal volume

PEG8 (% w/w)	Dextran T500 (% w/w)
4.0	5.3
4.2	7.0
4.5	8.0
5.0	9.2
5.5	10.3
6.0	11.1

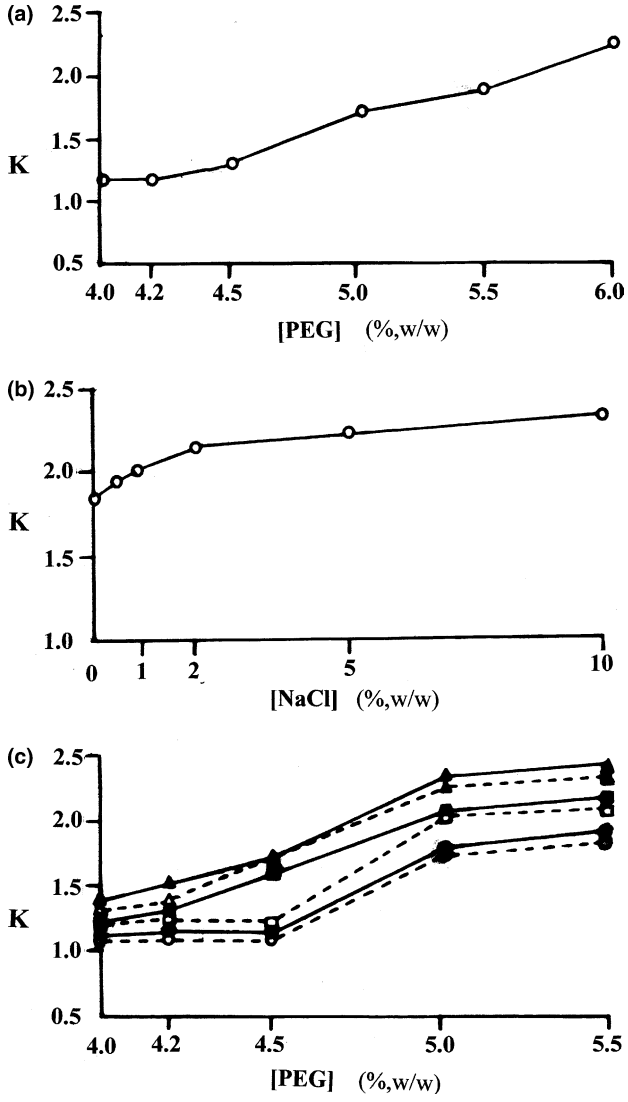

Fig. 4. The effects on the partition coefficient (K) of β-CN-5P in 10 mM Tris–HCl buffer pH 7.0 and "equivalent" PEG 8 – dextran ATPS systems (see text for details) of: (a) changing PEG concentration; (b) adding solid NaCl up to a concentration of 10% (w/w) to a system containing 5.5% (w/w) of PEG; (c) the combined effects of changing both PEG and NaCl concentrations: ○–○, no NaCl; ●–●, 0.5% NaCl; □–□, 1% NaCl; ■–■, 2% NaCl; △–△, 5% NaCl; ▲–▲, 10% NaCl.

of adding up to 10% NaCl (w/w) to a 5.5% (w/w) PEG system on the partitioning of β-CN-5P is shown in Fig. 4(b). K increased from 1.85 in the salt-free system to 2.20 in the presence of 10% NaCl. The increment was not large but still represented a useful gain, most of which could in fact be achieved by addition of NaCl at a level of 2% (w/w) (Fig. 4(b)). The effects of NaCl addition and increasing PEG concentration were additive (Fig. 4(c)) and, under the optimal conditions, when $K = 2.4$, a yield of 72.5% of β-CN-5P of high purity and giving a single peak on Superose 12 FPLC analysis was obtained in the PEG phase.

The best separation conditions for the components of milk TPP were similar to the above, i.e., pH 7.0, using

PEG 8 (or PEG 10) with a 5–10% addition of NaCl. Milk-derived β-CN-5P was then recovered from the PEG phase in a good state of purity, but there were some peptide contaminants and the purification was not as successful as that from β-casein hydrolysates. Good conditions for the purification of β-CN-4P were not found.

3.4. Effects on growth of cariogenic bacteria

There are a number of possible mechanisms whereby CPP could provide protection for teeth from attack by acid generated by cariogenic bacteria, such as *S. mutans*. These include:

1. By binding to calcium and then becoming localised in dental plaque the CPP could provide a source of acid-buffering counterions within the plaque.
2. Similarly, by localising calcium close to the tooth surface, mass action effects might inhibit dissociation of further calcium ions from the tooth surface itself.
3. The CPP might form a more passive, protective coat over the tooth surface.
4. Some antibiotics are peptide in character (e.g., nisin, bacitracin, the lantibiotics) so CPP might have antibiotic activity toward the growth of cariogenic bacteria.
5. CPP might inhibit the adhesion of microbial cells to the tooth surface and reduce biofilm formation.

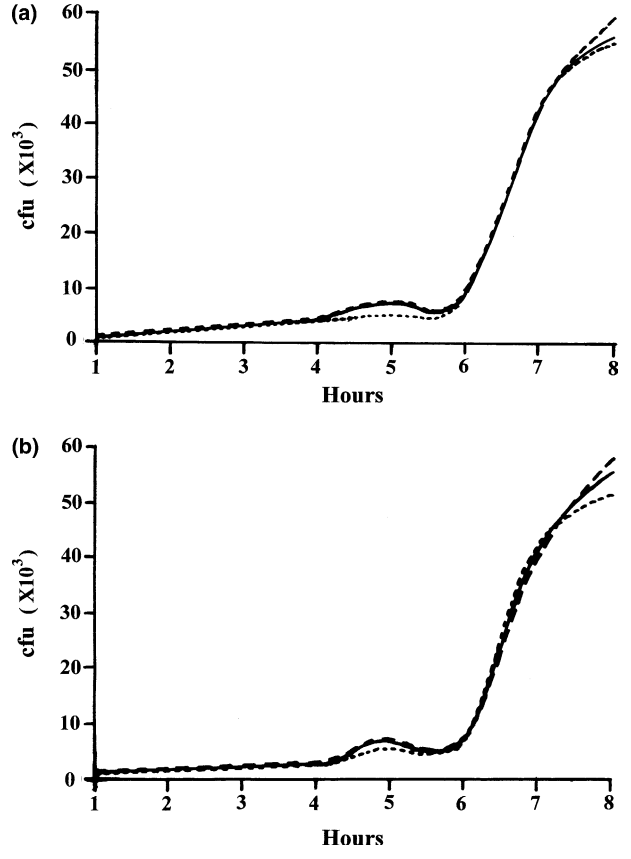

Fig. 5. The growth of cariogenic *S. mutens* bacteria at 3 °C in brain heart infusion broth in the presence of added proteose–peptone peptide components: (a) β-CN-5P added at 0.05 mg/ml (– – –), or 0.5 mg/ml (. . .), control with no added peptide (—); (b) β-CN-4P at 0.05 mg/ml (– – –), or 0.5 mg/ml (. . .), or control with no added peptide (—).

As part of our studies on protective mechanisms, we added β-CN-5P and β-CN-4P at concentrations of 0.05 and 0.5 mg/ml to cultures of *S. mutans* and monitored growth over eight days. Even at the higher concentration (very much higher than would ever occur in any in vivo situation) neither β-CN-5P (Fig. 5(a)) nor β-CN-4P (Fig. 5(b)) had any effect and, in all cases, the growth curves closely followed those of control cultures with no added proteose–peptone constituents. Thus, it was considered that the anti-cariogenic activity of CPP, such as these two β-casein fragments, is very unlikely to be due to a simple antibiotic mechanism.

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