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ScienceDirect
Food Chemistry 107 (2008) 1015–1021
Food Chemistry
www.elsevier.com/locate/foodchem

Caseinophosphopeptides and dental protection: Concentration and pH studies

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Received 1 June 2007; received in revised form 20 July 2007; accepted 6 September 2007

Abstract

Casein-derived phosphopeptides (CPP) can protect teeth from acids produced by bacteria causing dental caries or directly from acidic foods and beverages causing dental erosion. Neither the CPP concentrations required for protection nor the effective pH range has been published and the protective mechanism is still open to debate. Using experimental protocols mimicking practical situations in which CPP and acid were present together in a hydroxyapatite model system, or in which excess CPP was removed before acid exposure and also using either Ca-saturated CPP or Ca-reduced CPP, we found that in all cases protection was 50–60% at pH 4.2. Protection increased exponentially with CPP concentration to about 10.0 mg/ml as measured in mixtures before acid addition, with little further improvement above that. Most of the protection was achieved at a more economical 4.0–6.0 mg/ml. The extent of protection was almost constant over the pH range 2.5–4.5. Our findings suggested a mechanism involving the formation of a protective coat of CPP over the mineral particle surfaces.

Keywords: Caseinophosphopeptides; Dental caries; Dental erosion

1. Introduction

In recent years an increasing number of reports has shown that peptides produced by the digestion of food proteins can have powerful physiological effects which can be either beneficial or harmful. Even in the limited area of milk proteins, all the major individual caseins and whey proteins can produce bioactive peptides (see Fox & Flynn, 1992, for a review).

Our recent interest (Andrews et al., 2006; Grenby, Andrews, Mistry, & Williams, 2001; Warner, Kanekanian, & Andrews, 2001) in casein-derived peptides has concerned their protective action against dental erosion caused by acid attack on tooth mineral. In dental caries the acid is generated during the metabolism of dietary sugars by oral and dental plaque bacteria but this is only one source of oral acid and there can also be direct attack by acidic foods and beverages. While some recent ideas (e.g. Loimaranta et al., 1999) such as vaccination or manipulation of oral bacteria are aimed at combating dental caries, they would not be expected to have any influence on the more general non-microbial and purely chemical processes giving rise to dental erosion. Dental erosion has become a major problem in recent years due to the increased popularity of carbonated beverages and fruit juices with children and young adults. There is now substantial evidence that intact milk proteins (Guggenheim, Schmid, Aeschlimann, Berrocal, & Neeser, 1999; Rugg-Gunn, 2001; Vaccasmith, Vanwuyckhuyse, Tabak, & Bowen, 1994) have anti-cariogenic properties, perhaps by selectively adsorbing onto the tooth surface (Devold et al., 2005; Guggenheim, Giertsen, Schupbach, & Shapiro, 2001) but their possible role in protection against the wider problem of dental erosion is less well established. Several groups, including especially the pioneering work of Reynolds and his colleagues (Adamson & Reynolds, 1995; Adamson, Riley, & Reynolds, 1993; Reynolds, 1987; Reynolds, 1999; Reynolds, Riley, &

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doi:10.1016/j.foodchem.2007.09.013

Adamson, 1994) have indicated that caseinophosphopeptides (CPP), heavily phosphorylated peptides derived from milk casein proteins, are even more active. CPP contain the amino acid sequence -SerP-SerP-SerP-Glu-Glu- (which occurs in α_{s1}-casein, β-casein and twice in α_{s2}-casein) and bind calcium ions strongly to form soluble complexes (Holt, Wahlgren, & Drakenberg, 1996; Ono, Ohotowa, & Takagi, 1994). They are probably involved in calcium transport within various biological tissues, including enhanced absorption across intestinal mucosa (see review by Kitts & Yuan, 1992), thereby increasing the bioavailability of calcium in milk (Tsuchita, Suzuki, & Kuwata, 2001).

Adamson and Reynolds (1995) suggested that CPP could find practical applications for improving dental health by addition to toothpastes, mouthwashes, etc. or by addition to foods and beverages. While the overall picture of tooth protection by CPP is now clear, the mechanism of action is still open to debate, as we discussed previously (Andrews et al., 2006). There have also been no reported studies on the concentrations of CPP required for protection or of the pH range over which protective action is manifest. Another important factor which requires consideration is the mode of exposure of teeth to both CPP and acid. For example, if CPP are added as an ingredient to an acid food or beverage the tooth mineral will be exposed to both CPP and acid at the same time, so the CPP must be able to compete effectively with H^+ ions. However if the CPP is used as a prophylactic in toothpaste or a mouthwash, then unbound CPP is likely to be rinsed away by washes and/or saliva and the exposure to acid may occur substantially later. This paper seeks to address these factors and thereby aid a thorough evaluation of CPP for practical applications in this important area.

2. Materials and methods

2.1. Materials

Sodium caseinate (Product No. C8654), bovine trypsin (E.C.3.4.21.4; Product No. T8642) and crystalline hydroxyapatite (HA) suspension (Type 1; 27% solids; Product No. H0252) were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Dowex 50 (H^+) ion-exchange resin was supplied by BioRad, Poole, UK.

2.2. Preparation of hydroxyapatite

Immediately before use, 20 g of the HA suspension was mixed thoroughly into 750 ml H_2O and centrifuged at 3000*g* for 10 min. The pelleted material was washed again by suspension in 750 ml H_2O and centrifugation to completely remove soluble phosphate present in the initial HA suspension. The pellet was then resuspended in 300 ml of 0.05 M Tris–HCl pH 7.5 buffer for use. Fresh suspensions were prepared daily.

2.3. Calcium and phosphate determination

Calcium contents were determined by atomic absorption spectroscopy at 422.7 nm with lanthanum suppression of interference. Phosphate was measured colorimetrically as the phosphomolybdate complex according to Allen (1940).

2.4. Preparation of trypsin digests

For preparation of the soluble trypsin hydrolysates fraction, 20 g sodium caseinate was dissolved in 200 ml 0.05 M Tris–HCl buffer, the pH adjusted to 7.5 and then mixed with 20 mg trypsin and incubated at 37 °C for 2 h. The reaction was stopped by heating to 90 °C for 5 min and the mixture then cooled and pH adjusted to 4.5 with 1 M HCl. After stirring for 10 min precipitated protein was removed by centrifugation (3000*g*, 10 min) and the supernatant collected and lyophilised to give a powdered fraction referred to here as trypsin hydrolysate, which would contain CPP and some other peptides but no intact casein proteins.

2.5. Preparation of CPP fractions

CPP was prepared from this essentially as described by Adamson and Reynolds (1995). Trypsin hydrolysate material starting with 100 g sodium caseinate was prepared as above and the resulting lyophilised powder dissolved in H_2O and made up to 1 l. $CaCl_2$ (8.0 g) was added followed by 1 l of 95% ethanol to precipitate the CPP fraction. After standing for 1 h the precipitate was collected by centrifugation (3000*g*, 30 min), the supernatant discarded and the CPP pellet washed briefly by resuspension in 80 ml 50% ethanol and recentrifugation for 10 min. The washed CPP pellet was spread on a plastic dish and allowed to air dry at room temperature to yield 15.3 g CPP. Being prepared in this way all Ca binding sites on the peptides would be saturated with Ca so this was referred to as CPP + Ca.

A portion of CPP + Ca (5.0 g) was dissolved in 50 ml H_2O and added to 120 g of washed Dowex 50 ion-exchange resin (H^+ form). After stirring for 40 min the pH was found to have fallen to 1.10 and the supernatant was collected by decantation. The resin was washed with 100 ml H_2O for 2 min with stirring. The pH of the combined initial supernatant and washings was adjusted to 4.0 with 1 M NH_4OH and lyophilised to yield 3.5 g of CPP from which approx. Seventy percent of the Ca had been removed (as determined by atomic absorption spectroscopy) and was therefore referred to as CPP − Ca or calcium-reduced CPP.

2.6. Concentration dependency experiments

2.6.1. Protocol A

Typically about 15 ml of stock solutions of peptide or protein (25.0 mg/ml) in 0.05 M Tris–HCl buffer pH 7.0 were prepared from sodium caseinate, trypsin hydrolysate, CPP + Ca and CPP − CA.

The prewashed HA suspension described above was placed on a magnetic stirrer and while stirring continuously 3.0 ml portions were withdrawn and added to a series of 15 ml centrifuge tubes arranged in groups of three. For this about 3–4 mm was cut off standard disposable pipette tips to give a tip orifice of 1–2 mm diameter and avoid any sieving effect of HA particles during pipetting and thereby to ensure that each tube contained the same weight of HA (approx. 40 mg dry weight). Triplicated portions of stock peptide/protein solution plus 0.05 M Tris–HCl buffer pH 7.0 were then added to the tubes to give a series of dilutions in an added volume of 2.0 ml (for example different groups of three tubes would have added to them 2.0, 1.0, 0.5, 0.25, 0.12 or 0.06 ml of peptide solution plus 0.0, 1.0, 1.5, 1.75, 1.88 or 1.94 ml of buffer, respectively). Thus in a typical experiment with triplicated samples and all four peptide and protein materials there would be 72 centrifuge tubes each containing the same amount of HA but differing amounts of peptide in a final volume of 5.0 ml. Blank and control samples containing HA only (no protective peptide so equivalent to zero % protection) or peptide/protein only (no HA, so no Ca or phosphate solubilisation and hence equivalent to 100% protection) were also prepared. All samples were mixed thoroughly and allowed to stand for 15 min with intermittent stirring to permit maximum peptide/protein binding to the HA. Then 5.0 ml portions of a concentrated acidic buffer (0.25 M sodium acetate pH 4.2) were added to all tubes and blanks as rapidly as possible, the contents briefly stirred and after standing for precisely 10 min all the tubes were centrifuged at 3000*g* for 10 min. Supernatants were immediately decanted off into clean tubes for subsequent Ca and phosphate analyses. Repeat experiments with more concentrated peptide/protein stock solutions extended readings to higher concentration values.

2.6.2. Protocol B

This was performed as for protocol A except that before acidic buffer addition all tubes were centrifuged (3000*g*, 10 min), the supernatants discarded and the HA pellets washed once by resuspension in 5.0 ml of 0.05 M Tris–HCl buffer pH 7.0 and recentrifugation. The washed pellets were then finally resuspended again in 5.0 ml of the Tris–HCl buffer before continuing as above by adding 5.0 ml of the acidic 0.25 M acetate pH 4.2 buffer. These added steps removed excess peptide or protein not bound to the HA particles before acid addition.

2.7. pH dependency of protection

2.7.1. Protocol A

HA suspension was prepared and used as above except that for these experiments 4.0 ml of suspension was added to each centrifuge tube (five groups of 18 tubes). Solutions of the four peptide and protein materials were prepared at 25 mg/ml in 0.05 M Tris–HCl pH 7.0 buffer and 1.0 ml portions added to four of the groups of tubes. The fifth group provided controls with 1.0 ml of buffer added in place of the peptide solution. This control group represented the maximum solubilisation of HA by the acidic buffers at the different pH values studied (i.e. zero protection). All tubes were mixed and allowed to stand for 10 min. Each group of tubes was then divided into six sub-groups of three which were then mixed with 5.0 ml of 0.25 M sodium acetate buffer of pH 2.5, 3.0, 3.5, 4.0, 4.5 or H_2O. The H_2O sub-group represented blanks where there had been no acid attack (i.e. equivalent to 100% protection). The acetate buffers had been preadjusted to the required pH with glacial acetic acid (for pH 4.5, 4.0 and 3.5) or formic acid (for pH 3.0 and 2.5). Once mixed with acidic buffer all samples were stirred immediately, allowed to stand for precisely 10 min and centrifuged (3000*g*, 10 min). Supernatants were then rapidly decanted off into clean tubes for later Ca and phosphate analyses.

2.7.2. Protocol B

As for protocol A, except that as above for the concentration dependency experiments a buffer washing step was included to remove excess peptide/protein not bound to the HA particles before the acidic buffer addition.

3. Results and discussion

3.1. General

Model systems always have their limitations and there may be concerns about how closely the model reflects the real life situation. However, in this study it is probable that the use of a crystalline HA suspension as a substitute for teeth or tooth mineral was an even more rigorous test of the effects of CPP on acid attack since the greater surface area of HA per unit weight would facilitate the dissolution of the mineral in acidic media. Measurements of the amount of calcium and/or phosphate solubilised from HA under the different experimental conditions can be used to evaluate the extent of protection afforded by CPP against the action of acid.

HA suspensions have a range of particle sizes but this is likely to be narrower than in ground tooth material and also more homogeneous in composition and both of these factors will improve accuracy and reproducibility. When working, as here, on a small scale with only about 40 mg of HA per sample tube, any heterogeneity in HA particle size would be especially damaging, as samples with predominantly smaller particles would have a larger surface area than those with larger particles and consequently rates of Ca and phosphate solubilisation would be greater. Because of this it is particularly important to stir the initial HA suspension continuously and vigorously to avoid sedimentation of the larger HA particles when pipetting aliquots into the sample tubes. Even with these precautions and maintaining tight and accurate control of timings and all other stages of the experimental protocols, there was often considerable tube-to-tube variation, so it was

essential to always at least triplicate samples and when practical to pentuplicate them. With routine triplication of the calcium and phosphate assays as well this resulted in a minimum of 18 readings for each sample. Although at first plotted separately it was found that when results were calculated as percentage change from controls the results from calcium analyses closely followed those for phosphate, so it was possible to combine them for greater accuracy and clarity. Thus each data point shown in figures is an average of 18 individual readings. No statistical analysis was required beyond taking simple averages because all readings were relative to appropriate controls and by calculating percentage protection values we sought to highlight differences and trends rather than absolute values, which in model systems would have been of limited interest for practical applications.

3.2. Influence of peptide concentration

The results shown in Fig. 1 for protocol A (CPP and acid present at the same time) reveal a number of interesting features:

- All the casein-derived materials showed at least some protection indicating that they could compete effectively with H^+ ions for HA binding sites and reduce its solubilisation.
- Protection increased with increasing concentration.
- CPP + Ca and CPP − Ca gave generally similar levels of protection.

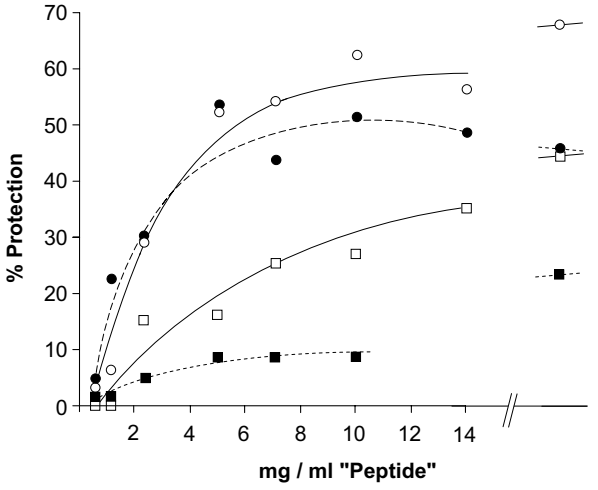

Fig. 1. Inhibition of hydroxyapatite (HA) solubilisation by acidic buffer at pH 4.2 by caseinophosphopeptides (CPP) at different concentrations using protocol A, where CPP and acidic buffer were present together. See text for details. Percentage protection is relative to the extent of HA solubilisation in the absence of any added peptide or protein. Mg/ml peptide is the peptide or protein concentration in the 5 ml of mixture volume immediately prior to acidic buffer addition. Calcium-saturated CPP, ○–○; calcium-reduced CPP, ●--●; unfractionated trypsin hydrolysate, □–□; sodium caseinate, ■--■.

- Maximum degree of protection under our experimental conditions was 60%, achieved with 10.0 mg/ml of CPP + Ca.
- Unfractionated hydrolysate was less effective than either of the CPP materials but gave a useful level of protection, especially at the higher concentrations tested.
- Sodium caseinate itself gave about one-third of the protection given by the hydrolysate and about 10% of that given by CPP. At the higher concentrations used there was a major problem of protein solubility in acid conditions, so the data points at high concentration values were very variable and unreliable.

The economics of using these materials in a practical application would be improved by minimising the cost of preparation stages. Unfortunately these results show that sodium caseinate would be ineffective at acceptable levels of concentration. High concentrations would be unacceptable on solubility as well as textural and organoleptic grounds. Unfractionated hydrolysate would almost certainly be unacceptable organoleptically because many casein-derived peptides have a very bitter taste, in particular the more hydrophobic peptides (Swaisgood, 1992). It is interesting to note however that although the hydrolysate contained exactly the same amino acid composition as the parent casein, both qualitatively and quantitatively, the hydrolysate was the more active. This suggests that due to steric effects small peptides packed more efficiently around the HA particles than possible with the relatively large protein molecules. The ethanolic precipitation step for CPP preparation removes the hydrophobic peptides by precipitating only the more hydrophilic Ca binding-peptides which are unlikely to be bitter, although this remains to be clearly established. It also removes many other inactive peptides and therefore increases protective activity per unit weight, enabling smaller weights of peptide to be added to any product to give the required effect, thereby minimising potentially undesirable side-effects caused by adding too large a proportion of peptide. Alternatively more CPP could be added for greater effects. Further treatment to reduce the amount of peptide-bound calcium appears to be unjustified as there was little or no advantage over CPP + Ca, which was therefore the material of choice.

Perhaps the most interesting feature found with protocol B (Fig. 2), where excess unbound CPP was removed by washing before acid addition, was that a very substantial amount of the protective action remained and indeed was not greatly different to that seen with protocol A. Likewise the efficiency of the four materials in protective action was in the same order as for protocol A, with CPP + Ca, and CPP − Ca giving similar levels of protection, hydrolysate being less effective and sodium caseinate giving the least.

Repeat experiments with peptide/protein levels increased to 28 mg/ml confirmed that there was no advantage to increasing to levels beyond the 10.0 mg/ml used above in either protocol. CPP + Ca and CPP − Ca were again always very similar quantitatively in their protective

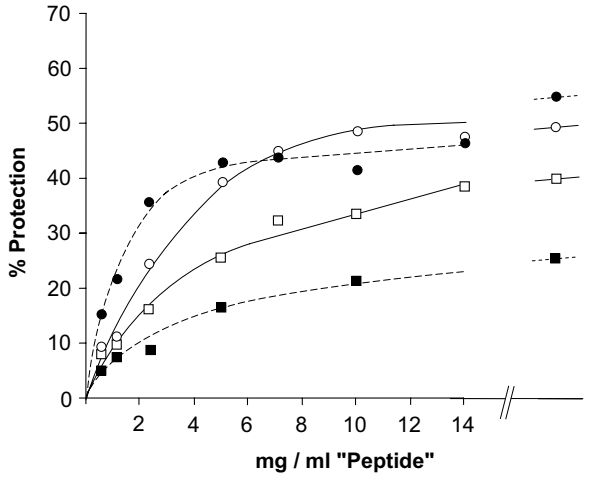

Fig. 2. Inhibition of hydroxyapatite (HA) solubilisation by acidic buffer at pH 4.2 by caseinophosphopeptides (CPP) at different concentrations using protocol B, in which unbound CPP was removed before acid addition. See text for details. Percentage protection is relative to the extent of HA solubilisation in the absence of any added peptide or protein. Mg/ml peptide is the peptide or protein concentration in the 5 ml of mixture volume before the centrifugal washing step to remove excess unbound material and acid addition. Calcium-saturated CPP, ○–○; calcium-reduced CPP, ●--●; unfractionated trypsin hydrolysate, □–□; sodium caseinate, ■--■.

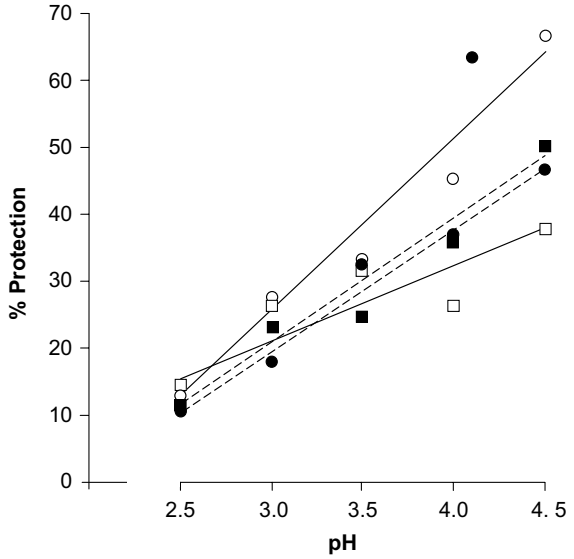

Fig. 3. The influence of caseinophosphopeptides (CPP) etc. at 5 mg/ml (immediately prior to acid addition) on hydroxyapatite (HA) solubilisation by acidic buffers of varying pH. See text for details. Protocol A. Calcium-saturated CPP, ○–○; calcium-reduced CPP, ●--●; unfractionated trypsin hydrolysate, □–□; sodium caseinate, ■--■.

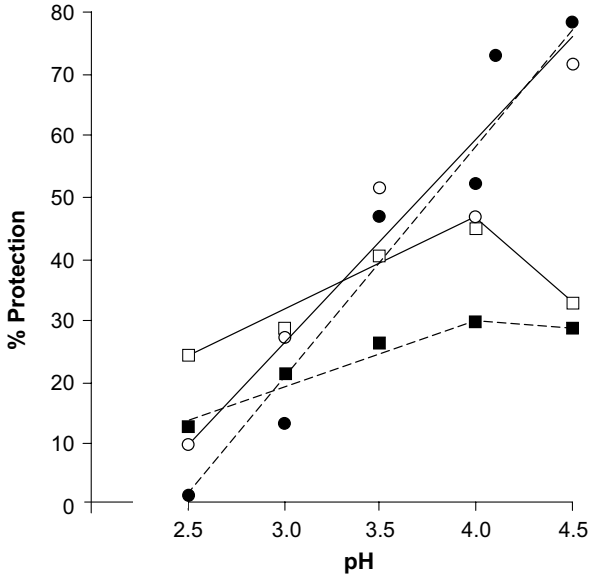

Fig. 4. The influence of caseinophosphopeptides (CPP) etc. at 5 mg/ml (immediately prior to acid addition) on hydroxyapatite (HA) solubilisation by acidic buffers of varying pH. See text for details. Protocol B. Calcium-saturated CPP, ○–○; calcium-reduced CPP, ●--●; unfractionated trypsin hydrolysate, □–□; sodium caseinate, ■--■.

action and the results for protocols A and B were also always similar.

All these results clearly show firstly that calcium bound to CPP is relatively unimportant (little difference between CPP + Ca and Ca-reduced CPP) and secondly that CPP must bind strongly to HA particles and not be easily removed by simple washing steps so that a protective coat remains.

3.3. Influence of pH on protection

At pH 5.0 or above, foods and beverages cause very little dental erosion and few have a pH below 2.5, so the critical range is 2.5–4.5. The percentage protections given by the four materials under investigation over this pH range are shown in Fig. 3 using protocol A and Fig. 4 with protocol B. Perhaps surprisingly the data are best presented as straight line plots with protection values in the pH 4.0–4.5 region being generally similar to those reported in Figs. 1 and 2. As previously, CPP + Ca and CPP − Ca being most effective with unfractionated hydrolysate being less so and sodium caseinate least effective. When plotted in this way as percentage protection versus pH, it would appear that the extent of protection declined as pH fell, but this is somewhat misleading because the overall dissolution of HA increases the more acid the pH. It was found that when expressed in absolute terms the amount of protection afforded was almost constant, at least over the pH range 3.0–4.5 (Fig. 5). The figure shows the amounts of phosphate released from HA averaged for protocols A and B. Calcium analysis data (not shown) was very similar. The extent of protection by the test materials is represented in Fig. 5 by the displacement of the test plots below the top plot, which is the control plot with H_2O in place of peptide solution. Considering the pH 4.0 and 4.5 data points it can be seen that protective action was qualitatively and quantitatively consistent with the data in Figs. 1 and 2. Although it remains to be clearly established it also appears from Fig. 5 that at pH 3.0 and 3.5 hydrolysate and sodium caseinate give protection at similar levels to those afforded by CPP peptides and at pH 2.5 it is better. As pH declined there

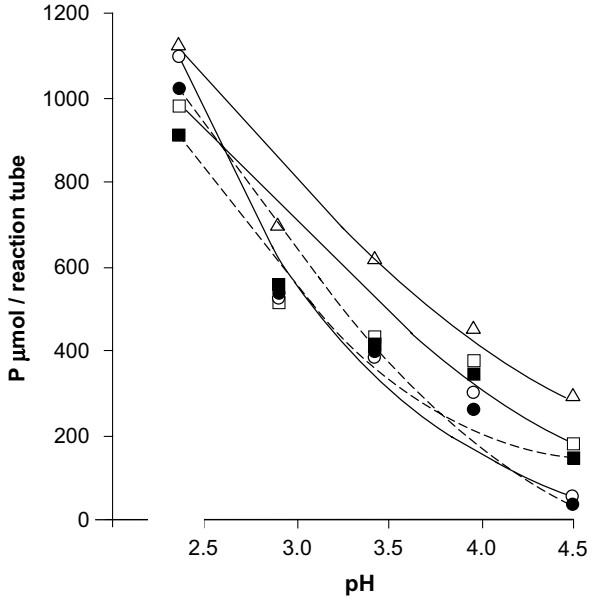

Fig. 5. Reduction in hydroxyapatite (HA) solubilisation by acidic buffers of varying pH afforded by caseinophosphopeptides and the other test materials at a concentration of 5 mg/ml (as measured prior to acid addition), measured in absolute terms as molar release of phosphate. See text for details. Calcium-saturated CPP, ○–○; calcium-reduced CPP, ●--●; unfractionated trypsin hydrolysate, □–□; sodium caseinate, ■--■.

appeared to be a trend of diminishing effectiveness for the CPP peptides. The first ionisation point (pK_{a1}) for the phosphate group is at pH 2.14, so as this is approached binding between the peptide serine phosphate groups and the surface binding sites of HA would be expected to become weaker, and this may prevent the formation of an adequate and stable protective coat over the HA particle surfaces. With larger intact casein protein molecules other interactions with the mineral surface are possible and these may help to stabilise the coat.

3.4. Mechanism of development of protective action

We have referred to this briefly at various places in the above, but discussed possible alternative pathways more explicitly in an earlier paper (Andrews et al., 2006) in which we showed that CPP possessed no antibiotic activity which might influence dental caries bacteria (although such activity would not influence other non-bacterial dental erosion processes). Likewise the protection could not be due to any interference by CPP with bacterial binding to tooth surfaces. Alternative hypotheses in which Ca or Ca clusters bound to CPP could buffer acids in dental plaque, or by localising large amounts of Ca at the tooth surface could reduce the dissolution of Ca ions from the tooth surface via mass action effects, are not consistent with our findings in which no bacteria were involved and in which there was little difference between protocols A and B. Our experiments showing that CPP + Ca was no more effective than Ca-reduced CPP likewise argued against such mechanisms. We therefore concluded that the probable requirement for protection was the formation of a stable protective coat over the HA (or tooth) surfaces via a purely physicochemical adsorption process.

References

Adamson, N. J., & Reynolds, E. C. (1995). Characterisation of multiply phosphorylated peptides selectively precipitated from a pancreatic digest. *Journal of Dairy Science, 78*, 2653–2659.

Adamson, N. J., Riley, P. F., & Reynolds, E. C. (1993). Analysis of multiple phosphoseryl-containing casein peptides using capillary zone electrophoresis. *Journal of Chromatography, 646*, 391–396.

Allen, R. J. (1940). The estimation of phosphorus. *Biochemical Journal, 34*, 858–864.

Andrews, A. T., Williams, R. J. H., Brownsell, V. L., Isgrove, F. H., Jenkins, K., & Kanakanian, A. D. (2006). β-CN-5P and β-CN-4P components of bovine milk proteose-peptone: Large scale preparation and influence on the growth of cariogenic microorganisms. *Food Chemistry, 96*, 234–241.

Devold, T. G., Rykke, M., Isabay, D., Sorensen, E. S., Christiansen, B., Langsrud, T., et al. (2005). *In vitro* studies of adsorption of milk proteins onto tooth enamel. *International Dairy Journal*.

Fox, P. F., & Flynn, A. (1992). Biological properties of milk proteins. In P. F. Fox (Ed.). *Advanced dairy chemistry* (Vol. 1, pp. 255–284). London, NY: Elsevier Applied Science.

Grenby, T. H., Andrews, A. T., Mistry, M., & Williams, R. J. H. (2001). Dental caries-protective agents in milk and milk products: Investigations *in vitro*. *Journal of Dentistry, 29*, 83–92.

Guggenheim, B., Giertsen, E., Schupbach, P., & Shapiro, S. (2001). Validation of an *in vitro* biofilm model of supragingival plaque. *Journal of Dental Research, 80*, 363–370.

Guggenheim, B., Schmid, R., Aeschlimann, J. M., Berrocal, R., & Neeser, J. R. (1999). Powdered milk micellar casein prevents oral colonisation by *Streptococcus sobrinus* and dental caries in rats: A basis for the caries-protective effect of dairy products. *Caries Research, 33*, 446–454.

Holt, C., Wahlgren, N. M., & Drakenberg, T. (1996). Ability of a β-casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters. *Biochemical Journal, 314*, 1035–1039.

Kitts, D. D., & Yuan, Y. V. (1992). Caseinophosphopeptides and calcium bioavailability. *Trends in Food Science and Technology, 3*, 31–35.

Loimaranta, V., Laine, M., Söderling, E., Vasara, E., Rokka, S., Marniba, P., et al. (1999). Effects of bovine immune and non-immune whey proteins on the composition and pH response of human dental plaque. *European Journal of Oral Science, 107*, 244–250.

Ono, T., Ohotowa, T., & Takagi, Y. (1994). Complexes of caseinophosphopeptide and calcium phosphate prepared from casein micelles by tryptic digestion. *Bioscience, Biotechnology and Biochemistry, 58*, 1378–1380.

Reynolds, E. C. (1987). The prevention of subsurface demineralisation of bovine enamel and change in plaque composition by casein in an intra-oral model. *Journal of Dental Research, 66*, 1120–1127.

Reynolds, E. C. (1999). Anticariogenic casein phosphopeptides. *Protein and Peptide Letters, 6*, 295–303.

Reynolds, E. C., Riley, P. F., & Adamson, N. J. (1994). A selective precipitation purification procedure for multiple phosphoseryl-containing peptides and methods for their identification. *Analytical Biochemistry, 217*, 277–284.

Rugg-Gunn, A. J. (2001). Nutrition, diet and oral health. *Journal of the Royal College of Surgeons of Edinburgh, 46*, 320–328.

Swaisgood, H. E. (1992). Chemistry of the caseins. In P. F. Fox (Ed.). *Advanced dairy chemistry* (Vol. 1, pp. 63–110). London, NY: Elsevier Applied Science.

Tsuchita, H., Suzuki, T., & Kuwata, T. (2001). The effect of casein phosphopeptides on calcium absorption from calcium-fortified milk in growing rats. *British Journal of Nutrition, 85*, 5–10.

Vaccasmith, A. M., Vanwuyckhuyse, B. C., Tabak, L. A., & Bowen, W. H. (1994). The effect of milk and casein proteins on the adherence of *Streptococcus mutans* to saliva-coated hydroxyapatite. *Archives of Oral Biology, 39*, 1063–1069.

Warner, E. A., Kanekanian, A. D., & Andrews, A. T. (2001). Bioactivity of milk proteins: 1. Anticariogenisity of whey proteins. *International Journal of Dairy Technology, 54*, 151–153.