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Dephosphorylation of sodium caseinate, enzymatically hydrolyzed casein and casein phosphopeptides by intestinal alkaline phosphatase: implications for iron availability

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Abstract

Clusters of phosphoserine residues in casein bind iron with high affinity. Casein inhibits iron absorption in humans but partial hydrolysis of casein prior to ingestion diminishes this inhibition. The objective of this study was to test two hypotheses: 1. Partial hydrolysis of the peptide bonds in casein exposes phosphoserine residues to attack by intestinal alkaline phosphatase (IAP). 2. Hydrolysis of the phospho-ester linkage in phosphoserine residues in casein by IAP releases bound iron or inhibits iron chelation, thereby allowing its absorption. Test of hypothesis 1: Suspensions of sodium caseinate (SC), enzymatically hydrolyzed casein (EHC), and casein phosphopeptides (CPP) were subjected to an in vitro pepsin/pancreatin digestion and subsequently incubated in the presence of calf IAP. The rate of release of inorganic phosphate was measured with the following results (expressed as μmol phosphate released/unit of IAP/min): 0.081, 0.104, 0.139 for SC, EHC, and CPP, respectively. These results are consistent with hypothesis 1. Test of hypothesis 2: ^{59}Fe-citrate or ^{59}Fe-citrate + CPP in minimum essential media were spiked with a Na_2WO_4 solution or water (Na_2WO_4 is a known inhibitor of IAP) and placed on Caco-2 cell monolayers. Uptake of ^{59}Fe by the cells was used as an index of iron bioavailability. Na_2WO_4 did not affect ^{59}Fe uptake from samples containing only iron but did slightly inhibit (by 10%) uptake from samples containing iron + CPP. These results are consistent with hypothesis 2 and provide a possible explanation for the observation that partial hydrolysis of casein improves iron bioavailability. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Iron; Bioavailability; Alkaline phosphatase; Casein; Casein phosphopeptides

1. Introduction

Casein binds iron and other cations with affinities that vary with the concentration and properties of the cations [1]. When soluble iron salts are added to milk, most of the iron quickly binds to caseins [2]. These interactions between minerals and caseins provide a possible explanation for the effects of dairy products on mineral bioavailabilities. For example, casein inhibits iron absorption in humans. This inhibitory effect is diminished when casein is partially hydrolyzed prior to ingestion [3]. Hydrolyzed casein has higher solubility than intact casein under physiological conditions in the gastrointestinal tract and this may be the reason for the enhancing effect of casein hydrolysis on iron bioavailability [3].

Dephosphorylation of phosphoserine residues in casein decreases iron binding by casein [4,5]. Therefore, the high affinity of α_{s1}, α_{s2}, and β-casein for ferric iron is likely due to the presence of clusters of phosphoserine residues in the caseins. The iron binding affinity of these clusters surpasses that of both nitrilotriacetate and citrate [6]. Furthermore, clusters of phosphoserine residues in proteins form stable complexes with ferric iron even at pH 2.5 [1].

Iron solubility in an aqueous system decreases as pH increases above 3 unless it is bound to soluble ligands. Presumably, iron must be soluble in the digesta before it can be absorbed [7]. Iron absorption is believed to take place mainly in the duodenum, where the pH varies with the distance from the pylorus, the nature of food consumed, and the pH and emptying rate of stomach contents. However, a microclimate between the intestinal lumen and the brush

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border surface of the intestine provides a relatively neutral pH at the intestinal cell surface. This microclimate is created and maintained by pancreatic secretions, HCO_3^- secretion by enterocytes, mucin, and mucosal cell surface-attached glycoproteins. Presumably, the microclimate protects cells against damage from digestive enzymes and low pH and provides a suitable pH for membrane-anchored enzymes.

Intestinal alkaline phosphatase is a tissue specific phosphatase. It anchors itself to the apical microvilli of brush border membranes through a glycosyl-phosphatidylinositol linkage [8]. Although most phosphorus is absorbed in the form of free phosphate [9] and the presumed function of intestinal alkaline phosphatase is to improve phosphate absorption [10,11], it has broad substrate specificity for hydrolyzing phosphoester bonds in many compounds. Calf intestinal alkaline phosphatase is capable of hydrolyzing clustered phosphoserine residues but its activity toward whole casein is low. It hydrolyzed only 10% of the clustered phosphoserine residues present in a 2% whole casein suspension when incubated at 37°C for 60 min (25–50 mg/g casein). When isolated α_{s1}- and β- caseins were treated individually with the same amount of enzyme, over 50% of phosphoserine residues were hydrolyzed in 30 min [12]. This difference in hydrolysis rate may be due to low accessibility of phosphoserine residues in whole casein.

The objective of this study was to test two hypotheses: 1. Partial hydrolysis of the peptide bonds in casein exposes phosphoserine residues to attack by intestinal alkaline phosphatase (IAP). 2. Hydrolysis of the phospho-ester linkage in phosphoserine residues in casein by IAP releases bound iron, thereby allowing its absorption.

2. Materials and methods

Chemicals were obtained from Sigma Chemicals (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ) or Mallinckrodt Baker (Paris, KN) and were grade ACS or better. All water was double de-ionized. Calf intestinal alkaline phosphatase (CIAP, Cat. #18009−019) and cell culture solutions were obtained from Life Technologies (GIBCO BRL, Rockville, MD). Spray-dried sodium caseinate (SC) and casein hydrolytic products [casein phosphopeptides (CPP) and enzymatically hydrolyzed casein (EHC)] were obtained from DMV International Nutritionals (Fraser, NY). CPP is a casein hydrolysate fraction purified to the extent that 90% of all peptides in the preparation contain clustered phosphoserine residues. EHC is a 27% DH casein hydrolysate. DH (degree of hydrolysis) is the ratio of NH_2-terminal groups to the total number of NH_2-terminals that would be present if all peptide bonds were hydrolyzed (i.e., total number of amino acid residues). Caco-2 cells obtained at passage 17 from American Type Culture Collection (Rockville, MD) were used between passage 32–35. Cell culture conditions and the seeding protocol of experimental plates were as described by Glahn et al. [13]. Caco-2 cells were grown in Dulbecco's modified eagle medium with 1% antibiotic/antimycotic and 10% fetal calf serum in a 95% air, 5% CO_2, and 37°C incubator. Experimental plates were collagen treated and seeded at a density of 50,000 cells/cm^2 (6-well cell culture dishes, Costar, Cambridge, MA). Medium was changed every 2–3 days.

2.1. Phosphate determination

Total and free phosphate concentrations were determined on all samples. Total phosphate concentration was defined as the concentration of inorganic phosphate after the removal of organic matter. Free phosphate concentration was defined as the concentration of inorganic phosphate that was readily measurable without the removal of organic matter. To determine *total phosphate,* samples were ashed to eliminate organic matter prior to analysis with a colorimetric method described below. *Free phosphate* was measured with the same colorimetric method but samples were not ashed prior to assay.

For ashing, samples were transferred to glass tubes and heated at 250°C for 30 min in a muffle furnace. Without removing the glass tubes, the temperature of the furnace was raised to 450°C. Samples were left at this temperature for 24 hr. After equilibration to room temperature, the resulting ash was dissolved in 2 ml of concentrated nitric acid and slowly dried in a 130°C heating block. The glass tubes extended about 4 inches above the heating block, allowing for some refluxing of nitric acid which served to wash down the sides of the tubes. When all of the nitric acid had evaporated, the ash was dissolved in concentrated HCl and stored for subsequent phosphate measurements.

Phosphate concentrations were determined using the Fiske Subbarow colorimetric method as modified by Chen et al. [14]. This method measures inorganic (free) phosphate but not phosphate esterified with organic molecules. Samples were analyzed as follows. A 2.0 ml aliquot of sample, previously diluted to within the range of measurement, was first transferred into a glass tube. Then, 2.0 ml of a freshly prepared reagent containing 0.6 mol/L sulfuric acid, 0.5% ammonium molybdate and 2.0% ascorbic acid was added. The samples were mixed, incubated in a 50°C water bath for 20 min and cooled in a water bath at room temperature for 20 min. Absorbance at 820 nm was measured against a reagent blank. A standard curve was prepared for each experiment using desiccated KH_2PO_4. Total and free phosphate concentrations of samples are presented in Table 1.

2.2. Dephosphorylation of caseinate and casein hydrolytic products with or without added iron

Preparations of 9% sodium caseinate (SC), 9% enzymatically hydrolyzed casein (EHC) and 2% casein phosphopeptides (CPP) were prepared by dispersing in water. These concentrations of protein or peptides in each preparation, after dilution, provided approximately 0.44−0.53 μmol/ml

Table 1
Total and free phosphate concentrations in sodium caseinate, enzymatically hydrolyzed casein and casein phosphopeptides

Sample[†]	Total phosphate	Free phosphate	Phosphate from phosphoserine residues[‡]
	mmol P/g of sample[§]		
SC	0.222 ± 0.021	0.027 ± 0.0009	0.195
EHC	0.255 ± 0.024	0.034 ± 0.0012	0.221
CPP	1.093 ± 0.129	0.046 ± 0.0061	1.047

[†] SC: Sodium caseinate; EHC: Enzymatically hydrolyzed casein; CPP: Casein phosphopeptides

[‡] Phosphate from phosphoserine residues is the difference between total and free phosphate.

[§] Mean ± standard deviation. n = 3.

phosphate from phosphoserine clusters. The preparations were heated to no more than 80°C to aid in dispersion. Then, 6.25 ml aliquots of each sample mixture were transferred into 25-ml beakers. To these beakers, 12.5 ml of 100 mmol/L Tris-HCl at pH 9 and 1.25 ml ferric chloride solution in 1% HCl (+Fe) (Atomic absorption standard, Sigma Chemicals; 1000 μg/ml Fe) or 1% HCl (−Fe) were added. The pH 9 buffer prevented the acidic iron solution from coagulating the caseinate and maintained the pH at slightly above 7.6. Samples were then adjusted to pH 7.6 with 1 mol/L HCl and quantitatively transferred into 25-ml volumetric flasks. The mixtures were brought to final volumes with de-ionized H_2O and pHs were checked. Prior to dephosphorylation, aliquots of each buffered sample were diluted 10-fold with water.

Aliquots (0.8 ml) of the diluted, buffered samples were pre-incubated with 0.1 ml of dephosphorylation buffer at 37°C in a water bath for 10 min. The dephosphorylation buffer contained 1 mmol/L Mg and 0.1 mmol/L Zn as described in the dephosphorylation protocol from the manufacturer (GIBCO). Next, 0.1 ml calf intestinal alkaline phosphatase (CIAP), containing 1.3 units of the enzyme, was added to start the dephosphorylation. One unit of CIAP is defined as the amount of enzyme that will hydrolyze 1 mmol ρ-nitrophenyl phosphate (PNPP) in 1 min at 37°C. After 30 min of incubation, 1 ml of 15% trichloroacetic acid solution (TCA) was added to arrest enzyme activity. Control samples received TCA prior to the addition of alkaline phosphatase. Inorganic phosphate concentration of samples was measured as described earlier and the difference between samples with and without enzymatic dephosphorylation represented the amount of phosphate released by CIAP.

2.3. Dephosphorylation of digested caseinate and casein hydrolytic products

Preparations of caseinate and casein hydrolytic products were prepared by dispersing 2 g of SC, 1.8 g of EHC or 0.4 g of CPP in 10 ml of water in 50-ml capped conical tubes with heating to no more than 80°C in a water bath. Ferric chloride solution was added to yield a final iron concentration of 269 μmol/L (15 mg/L) of each digested sample. The in vitro digestion method described by Glahn et al. [15] was performed with slight modifications. Sample pHs were adjusted to 2 with 1 mol/L HCl and 1 ml of 2% pepsin solution in 0.1 mol/L HCl was added. They were then incubated on a rocking platform at 70 oscillations per minute in a 37°C incubator for 1 hr. After incubation with pepsin, the pH of samples was brought above 3.5 with solid $NaHCO_3$ and 1 mol/L NaOH was added to raise the pH to 6 [16]. At this point, samples were combined with 2.5 ml of a 2% solution of pancreatin in 0.1 mol/L $NaHCO_3$ and the pH was raised to 7 with 1 mol/L NaOH. Water was added to bring the volume of samples to 30 ml. Samples were incubated at 37°C on rocking platform for an additional 2 hr. The digested samples were placed in a boiling water bath for 30 min in order to inactivate the digestive enzymes. Water was added to bring the final volume to 40 ml. All digested samples were stored at 4°C overnight until alkaline phosphatase treatment.

The digested samples were adjusted to pHs of 6.8, 7.0, 7.2 or 7.4. To do so, 13 ml aliquots of digested sample were mixed in a 50/50 ratio with a buffer containing 100 mmol/L each of HEPES, MES and PIPES. Then, 5 ml buffered/digested sample was adjusted to the desired pH with 1 mol/L HCl and diluted to 10 ml with buffer solution of appropriate pH (100 mmol/L HEPES, MES and PIPES at pHs of 6.8, 7.0, 7.2 or 7.4).

Aliquots (0.9 ml) of each buffered digested sample were subjected to dephosphorylation for 0, 5, 10, 15 or 20 min. Each digested sample was pre-incubated in a 37°C water bath for 10 min prior to the start of enzymatic dephosphorylation. Enzymatic dephosphorylation began by addition of 1 U of CIAP (0.1 ml of enzyme in dephosphorylation buffer). After incubating at 37°C for the allotted time, TCA solution was added to stop further dephosphorylation. Phosphate released from casein products after incubation with CIAP was measured as described above.

2.4. Iron uptake by Caco-2 cell monolayers with or without inhibition of the Caco-2 cell-associated alkaline phosphatase activity

Radioactive iron solutions were prepared by mixing $^{59}FeCl_3$ (NEN Products, Boston, MA) with stock $FeCl_3$ solution. This stock $FeCl_3$ solution was prepared by diluting an iron atomic absorption standard solution (1000 μg/ml) with 1% HCl to 1.79 mmol Fe/L. Next, aliquots of 40 mmol/L citric acid solution were added to these radioactive iron solutions to yield a molar ratio of iron to citric acid of 1:20. These iron citrate chelate solutions were then diluted to 10 and 200 μmol/L, iron and citric acid, respectively, using minimum essential medium (MEM) with or without dissolved CPP. The ratio of iron to CPP was 12 mg to 1.5 g.

Inhibition of intestinal alkaline phosphatase activity of

Caco-2 cells was achieved by adding 1 mmol/L Na_2WO_4 to MEM. Four iron uptake mediums were formulated. They were MEM with Fe-citrate; MEM with Fe-citrate and Na_2WO_4; MEM with Fe-citrate and CPP; and MEM with Fe-citrate, CPP and Na_2WO_4.

The iron uptake study was conducted at 21 days post seeding of Caco-2 cells. Caco-2 cell monolayers grown in 6-well plates were first rinsed twice with MEM (no added iron). Then, a 2 ml aliquot of each of the above prepared MEM solutions, containing 2.38KBq of ^{59}Fe, was transferred to the respective wells. The plates were placed on a rocking platform set at 20 oscillations per minute located inside a 37°C; 5% CO_2 cell culture incubator and incubated for 2 hr. After incubation, the MEM solutions were removed by aspiration and monolayers were rinsed once with 140 mmol/L NaCl, 5 mmol/L KCl solution and incubated twice for 10 and 5 min with 1mmol/L bathophenathroline disulfonic acid, 5 mmol/L sodium hydrosulfite solution to remove non-specific, surface-bound iron [17]. Cell viability was monitored by capacity to exclude trypan blue dye. Cell monolayers were harvested in 1 ml of 0.5 mol/L NaOH and radioactivity of monolayers was measured. The effectiveness of Na_2WO_4 as an alkaline phosphatase inhibitor was evaluated using the remaining 2 wells of the 6-well plate. The hydrolysis of PNPP (10 mmol/L), with or without the presence of 1 mmol/L Na_2WO_4 at 37°C, by Caco-2 cell surface alkaline phosphatase was monitored for 30 min.

All experiments were repeated at least 3 times. Duplicates were run for all replications. Means were compared using Tukey's multiple comparison test when ANOVA was significant. Means were concluded to be significantly different when alpha was less than 0.05.

3. Results

Fig. 1 shows the extent of dephosphorylation of samples of SC, EHC and CPP with or without added iron. Dephosphorylation of SC was not affected by the presence of 71.6 μmol/L (4 μg/ml) iron. However, iron inhibited dephosphorylation of CPP and EHC significantly (about 7%). The percentages dephosphorylation of CPP +Fe and CPP −Fe were 57% and 64%, respectively. For EHC, the percentages dephosphorylation were 60% and 67% for +Fe and −Fe.

When in vitro digested SC, EHC and CPP with added iron were incubated with CIAP from 0 to 20 min, phosphate release followed a linear pattern with time. Fig. 2 shows linear regression equations of phosphate release over time (r^2 = 0.94, 0.96, and 0.98 for SC, EHC, and CPP respectively). This suggests that dephosphorylation of samples progressed at V_{max} for the entire 20 min of incubation under our experimental conditions. CPP had the highest rate of dephosphorylation followed by EHC while dephosphorylation of SC progressed at the slowest rate (Fig. 2). The amount of phosphate released form each sample treatment is shown in Fig. 3. More phosphate was released at pH 7.4

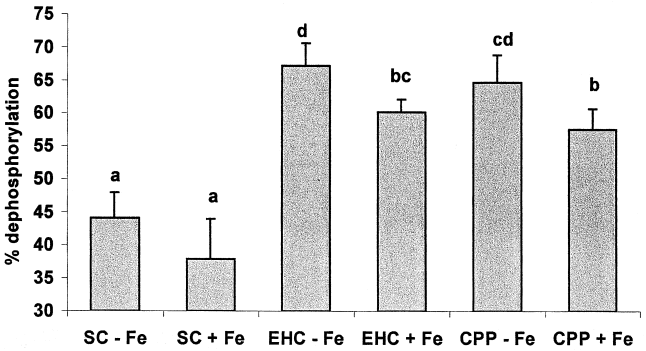

Fig. 1. Extent of Dephosphorylation of Caseinate and its Hydrolytic Products with or without Added Iron. Dephosphorylation of 0.225% sodium caseinate (SC), 0.225% enzymatically hydrolyzed casein (EHC) and 0.05% casein phosphopeptides (CPP) using 1.3 U calf intestinal alkaline phosphatase with (+Fe) or without (−Fe) the presence of 71.6 μmol/L (4 ppm) iron. Sample solutions were incubated at 37°C for 30 min. Bar values (mean ± SEM; n = 3) having no letter in common are significantly different from each other.

than at pH 6.8 from CPP and EHC at several time points but dephosphorylation of SC did not increase with increasing pH.

Specific activities of CIAP on SC, EHC and CPP are listed in Table 2. Specific activity is defined as μmol phosphate released from peptides per unit of CIAP per min at 37°C. The substrate as well as pH affected specific activity of CIAP. Among different substrates, CPP was the most suitable form of substrate tested for CIAP followed by EHC with SC being the least suitable. In general, CIAP activity increased with increasing pH.

Table 3 shows the results of iron uptake by Caco-2 cells with or without intestinal alkaline phosphatase inhibitor (Na_2WO_4). CPP inhibited iron uptake by 38% in the absence of Na_2WO_4 and 44% in the presence of Na_2WO_4 when compared to Fe-citrate. Na_2WO_4 inhibited iron uptake from CPP by 10%. However, it had no effect on iron uptake in the absence of CPP. Therefore, Na_2WO_4 did not affect normal iron uptake by the cells. It affected iron uptake only in the presence of CPP. The presence of Na_2WO_4 inhibited the hydrolysis of PNPP by 67.88 ± 5.90% (mean ± std dev).

4. Discussion

Iron deficiency affects approximately two billion people in the world [18]. Infants, children and women of reproductive age are at higher risk for iron deficiency. In the US, 9% of toddlers 1–2 years of age, 11% teenage girls and 9% of women of reproductive age are iron deficient [19]. These same population groups are also at risk for low calcium intakes. This creates a dilemma since milk and other dairy products are excellent sources of calcium but may inhibit iron absorption from other foods taken at the same time. Thus, it is important to increase our understanding of the

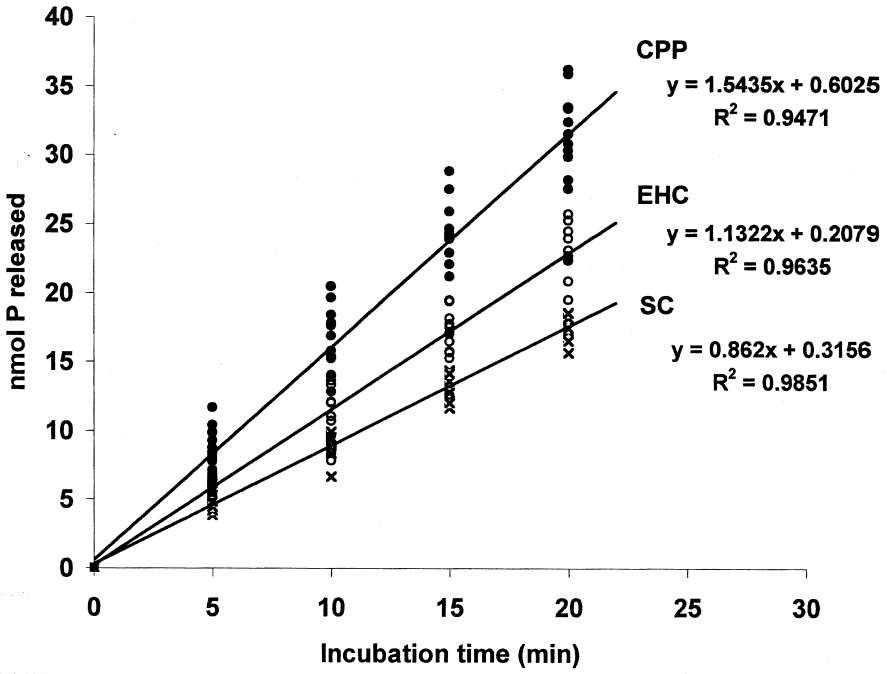

Fig. 2. Regression Analysis of Phosphate Released From In-vitro Digested Caseinate and Casein Hydrolytic Products on incubation time. Solutions of in vitro digested sodium caseinate (SC), hydrolyzed casein (EHC) and casein phosphopeptides (CPP) in the presence of iron were incubated with calf intestinal alkaline phosphatase for 0, 5, 10, 15 and 20 min. Concentration of ferric iron was at 53.8 μmol/L (3 μg/ml). One mL of solution containing 2.2 μmol phosphate in the form of phosphoseryl residues and 1 U of calf intestinal alkaline phosphatase was incubated at 37°C. At each time point, the amount of phosphate released from each sample, regardless of buffer pH, were combined together in calculating the linear regression equation. (×) = SC, (○) = EHC and (●) = CPP. n = 3.

mechanisms by which milk components affect iron absorption.

The rate of dephosphorylation by intestinal alkaline phosphatase decreased when ferric iron was added to casein hydrolytic products. However, ferric iron has not been reported to inhibit intestinal alkaline phosphatase [20]. We hypothesize that this decrease in dephosphorylation rate is substrate specific. Since ferric iron is likely to be associated with phosphoserine clusters in casein [2,4,5], it possibly hinders hydrolysis by intestinal alkaline phosphatase. Nevertheless, due to a high variance, dephosphorylation of SC +Fe samples was not significantly different from SC −Fe samples.

The lower accessibility of phosphoserine residues of SC to CIAP became apparent when dephosphorylation of in vitro digested SC and casein hydrolytic products was carried out at various pHs. Dephosphorylation by CIAP of EHC and CPP was faster than dephosphorylation of SC (Fig. 2). In addition, dephosphorylation of SC was insensitive to increasing pH unlike the dephosphorylation of CPP and EHC. The two hydrolytic products of casein we chose contain shorter peptides than SC and we anticipated their phosphoserine clusters to be more exposed than those of SC. This higher accessibility of hydrolytic products of casein may explain the observation of Hurrell et al. [3] that iron is more bioavailable in the presence of hydrolyzed casein than intact casein.

Hurrell et al. [3] postulated that increased iron dialyzability of casein hydrolytic products could play a role in their improved iron bioavailabilities over casein and that pepsin-pancreatin digestion of casein may result only in relatively long peptides when compared to commercial casein hydrolytic products. Although they speculated on the involvement of casein phosphoserine clusters on iron availability, they did not pursue the question. In the present study, we examined a role for intestinal alkaline phosphatase in the iron uptake process in the presence of CPP. We sought to define this role through the use of an intestinal alkaline phosphatase inhibitor, Na_2WO_4.

Although intestinal alkaline phosphatase activity was reported to be inhibited by cysteine, phenylalanine, EDTA, arsenate, molybdate, vanadate, tungstate, iodine, and certain phosphate compounds [20–22], choosing an appropriate intestinal alkaline phosphatase inhibitor for this study was difficult. Initially, vanadate and tetramisole (an inhibitor which was reported to inhibit liver and non tissue-specific alkaline phosphatase) were tested but failed to satisfactorily inhibit the alkaline phosphatase activity of Caco-2 cells. Arsenate was also tested but it affected Caco-2 cell viability (results not shown). Phenylalanine only inhibits at pHs that are too alkaline for our experiment [23,24]. The use of EDTA or cysteine could affect iron uptake since they can directly affect the availability of the added iron. Finally, Na_2WO_4 was selected for our study. In order to monitor its appropriateness, controls (Fe-citrate with or without added Na_2WO_4) were incorporated into our experiment to assure

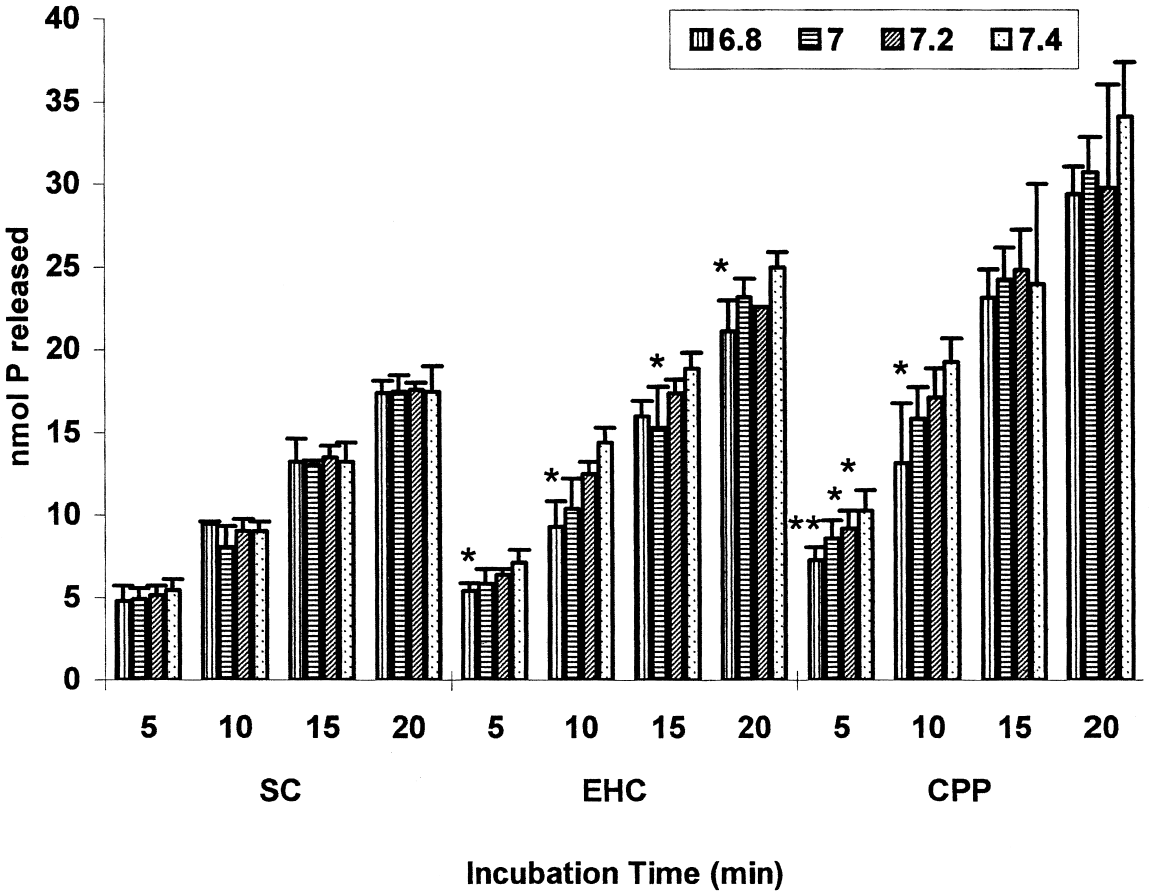

Fig. 3. Amount of Phosphate Released From In-vitro Digested Sodium Caseinate and Casein Hydrolytic Products After Incubation at Various pHs and Times. Solutions of in vitro digested sodium caseinate (SC), enzymatically hydrolyzed casein (EHC) and casein phosphopeptides (CPP) in the presence of ferric iron were incubated with calf intestinal alkaline phosphatase at 37°C from 0 to 20 min. Error bars are standard error of means. Means within each protein/peptides groupings and time point were compared with each other. * Mean is significantly lower than mean of pH 7.4. ** Mean is significantly lower than all others. n = 3.

that Na_2WO_4 itself did not affect iron uptake. Our results clearly show that $NaWO_4$ did not alter iron uptake by Caco-2 cells in the absence of CPP (Table 3).

Table 2
Specific activities of calf intestinal alkaline phosphatase (CIAP) toward sodium caseinate, enzymatically hydrolyzed casein and casein phosphopeptides[§]

pH	6.8	7	7.2	7.4	Mean[†]
SC	0.081	0.078	0.082	0.083	0.081[a]
EHC	0.092	0.097	0.107	0.119	0.104[b]
CPP	0.123	0.137	0.142	0.155	0.139[c]
Mean[‡]	0.0985[a]	0.104[b]	0.110[bc]	0.119[c]	

[§] Specific activity is defined as μmol phosphate released from peptides per unit* of CIAP per min at 37°C. Assay condition: solution of peptides containing approximately 2.3 μmol of phosphoserine residue per 0.9 mL were dephosphorylated for 0, 5, 10, 15 and 20 mins using 0.1 mL buffered enzyme (1 unit) at 37°C in 53.8 μmol/L (3 μg/mL) iron. *Unit definition: 1 unit of CIAP hydrolyzes 1 mmol of PNPP in 1 min at 37°C. [†] SEM = 0.00199. [‡] SEM = 0.00229. Means, within a column or row, with no letter in common are significantly different (Tukey's multiple comparison test, α = 0.05). n = 3.

We expected that iron uptake in the presence of CPP would be lower relative to uptake from Fe-citrate alone. Casein phosphoserine clusters have higher affinity for iron than citrate [6], therefore, we expect that Fe-citrate will transfer its iron to casein phosphoserine residues and this binding of iron will inhibit uptake. Inhibiting IAP with Na_2WO_4 decreased iron uptake from CPP samples by an additional 10% supporting our hypothesis that hydrolysis of the phospho-ester linkage in phosphoserine residues in casein by IAP releases bound iron, thereby allowing its absorption. Since intestinal alkaline phosphatase is anchored to the mucosal cell membrane, it likely hydrolyzes clustered phosphoserine residues at very close proximity to where iron is absorbed.

Casein phosphopeptides bind other cations besides iron [1] raising the possibility that binding sites might have been saturated with other cations prior to the addition of iron. Therefore, we analyzed the CPP preparation for several other minerals prior to the study. The analysis revealed that molar ratios of P to Ca, Zn, Cu, and Mg were at least 500:1. Furthermore, after we added iron to the CPP, there was a

Table 3
Effects of CPP and Na_2WO_4 on iron uptake by Caco-2 cells

		Iron uptake (%)		% decrease in the presence of Na_2WO_4
		No Na_2WO_4[‡]	1 mmol/L Na_2WO_4	
MEM[†]	Fe-citrate	1.58 ± 0.46^a	1.57 ± 0.41^a	0.7
	Fe-citrate + CPP	0.970 ± 0.43^b	0.873 ± 0.38^c	10.0
% inhibition by CPP[§]		38.6	44.4	

[†] Minimum essential medium. [‡] Intestinal alkaline phosphatase inhibitor. [§] The difference in iron absorption from Fe-citrate and from Fe-citrate + CPP was expressed as a percentage of iron absorption from Fe-citrate. Means (± standard deviation) with different superscripts were significantly different. n = 5.

70-fold molar excess of Fe over Ca, the predominant cation besides iron in the CPP preparation (data not shown). Thus, the high concentration of iron relative to other cations, the large excess of phosphate groups, and the high affinity of CPP for iron, insure that CPP did bind the added iron.

In summary, adding iron reduced dephosphorylation by CIAP, possibly through binding to casein phosphoserine clusters. Furthermore, dephosphorylation by CIAP of casein phosphopeptides and enzymatically hydrolyzed casein was faster than dephosphorylation of sodium caseinate. This may be related to the accessibility of these clusters. Finally, inhibition of Caco-2 cell IAP lowered iron uptake suggesting possible IAP involvement in iron uptake from casein phosphoserine clusters.

Although casein is considered a highly digestible protein and a large percentage of casein's amino acids is absorbed by humans, successive in vitro digestion of casein with pepsin and trypsin yields peptides that contain phosphoserine clusters [25]. Since phosphoserine clusters bind iron and alkaline phosphatase can hydrolyze phosphoserine clusters at physiological pH, it is possible that iron bound to casein becomes more available after phosphate is cleaved from the phosphopeptide by intestinal alkaline phosphatase. This observation is similar to the well-documented effect of phytase on iron bioavailability. Phytase is another phosphoesterase that has been shown to enhance iron absorption in humans and in animal models [26,27]. Phytate is a myo-inositol hexaphosphate that is found in most plant foods. Phytate binds iron and inhibits its absorption. When phytase was added to diet containing phytate, iron bioavailability improved.

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