In vitro evaluation of caseinophosphopeptides from different genetic variants on bone mineralization

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ABSTRACT - Casein phosphopeptides (CPPs) have been shown to enhance calcium solubility and to increase the calcification by *in vitro* analyses. The aim of our study was to investigate the effects of four selected casein peptides, which differ in the number of phosphorylated serines, on osteoblast mineralization *in vitro*. The chosen peptides, related to different casein genetic variants, were obtained by chemical synthesis and tested on murine osteoblast cell line (MC3T3-E1). Our results suggest that the distinct peptides in protein hydrolysates may differentially affect calcium deposition in the extracellular matrix and that the genetic variation within the considered peptides is involved in their differential effect.

Key words: Caseinophosphopeptide, Milk protein, Biological effect, Bone.

Introduction - Milk contains different protein components, many of which showing biological activities in addition to their nutritional value, and produced in their active form, i.e. lysozyme, lactoferrin, growth factors, hormones (Lorenzini *et al.*, 2007). Among the biologically active molecules, the caseinophosphopeptides (CPPs) are strongly phosphorylated peptides known to exert an effect on calcium metabolism but also on other minerals (Bouhallab and Bouglé, 2004). The CPPs have a strong capacity to fix nutritionally interesting divalent cations such as calcium, iron or zinc, thus making them stable and soluble in different physico-chemical conditions in particular of pH (Brulé and Fauquant, 1982; Meisel and Schlimme, 1993). The CPPs obtained by enzymatic *in vitro* hydrolysis of caseins have been shown to enhance calcium solubility and to increase the calcification of embryonic rat femora and tibiae *in vitro* (Gerber and Jost, 1986).

From the genetic point of view, the biological activity of peptides released from milk protein digestion may be affected by amino acid exchanges or deletions resulting from gene mutations. The aim of our study was to investigate the effects of four selected peptides, which differ in the number of phosphorylated serine, on osteoblast *in vitro* mineralization. The peptides considered were related to different casein genetic variants.

Material and methods - We used a murine osteoblast cell line (MC3T3-E1) that has the potency to differentiate during culture in the presence of ascorbic acid and glycerol-2-phosphate forming a mineralized extracellular matrix. The two copies of peptides were obtained by chemical synthesis (Table 1). Peptides were synthesised by CRIBI (Padova, Italy) (peptide-1 and -2), and PE & Elephants (Germany) (Peptide-3 and -4). The main difference between peptide-1 and -2 is the occurrence of one phosphorylated serine at position 35 of the mature β-casein in the latter peptide. Moreover, Glu (E) at position 37 is substituted with Lys (K) in variant C. Both peptide-3 and -4 carry two phosphorylated serines separated by one amino acid which differs in the two sequences (Ile *vs.* Thr). From a genetic

point of view, it is to highlight that β-casein C variant is not common in cattle, and is mainly found in local breeds, whereas α_{s2}-casein C was found in *Bos grunniens* (Formaggioni *et al.*, 1999). Peptide-2 and -4 also occur in the known genetic variants of β-casein and α_{s2}-casein in *Capra hircus* and *Ovis aries* (Chessa *et al.*, 2009). Calcium content of the cell monolayer lysates was determined as previously described by van Driel *et al.* (2006) after a 3-week long treatment.

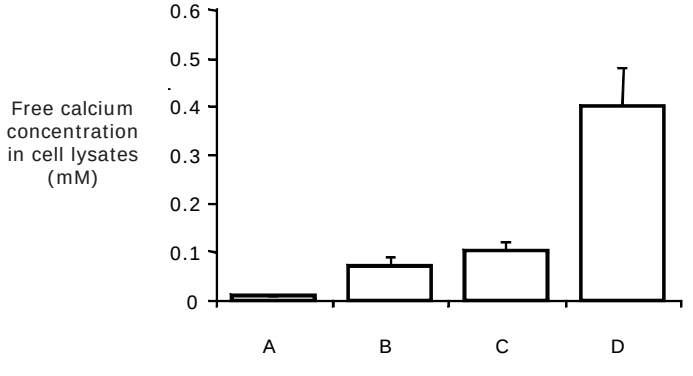
Table 1. Peptides obtained by chemical synthesis and genetic variation involved. Phosphorylated serines are sub lined. Amino acid exchanges between the two peptide couples are bolded.

	Sequence	Bovine casein variant
Peptide-1	KIEKFQSE**K**QQQT	β-casein C
Peptide-2	KIEKFQS**E**E**E**QQQT	β-casein A[1, 2, 3, 4], B, F, G, H[1, 2], I
Peptide-3	EQLS**I**SEENS	α_{s2}-casein C
Peptide-4	EQLS**T**SEENS	α_{s2}-casein A, B, D

Results and conclusions - Calcium concentration of the culture medium (alfa-MEM) was inadequate to support detectable mineralization of cell monolayer, based on the sensitivity of our calcium assay. When calcium lactate (0.5 mM) was added to the culture medium, increased calcium deposition was observed (Figure 1). Hence, in order to study the effects of the four selected peptides on *in vitro* mineralization, cells were grown in the presence of calcium lactate supplement.

Peptide-1 did not affect significantly final calcium content as compared with control wells. Peptide-2, which contains one phosphorylated serine, increased moderately calcium content (128±8% *vs.* control, N=4, P<0.05) at the higher dose tested (40 μM). Lower doses (1-10 μM) were ineffective. Peptide-3 and -4, containing 2 phosphorylated serines, reduced final calcium content to 42±10% and 34±8% *vs.* control, respectively (N=5, P<0.01 and P<0.001) at 40 μM concentration (Figure 2). Lower doses (0.4-4 μM) did not affect significantly mineralization.

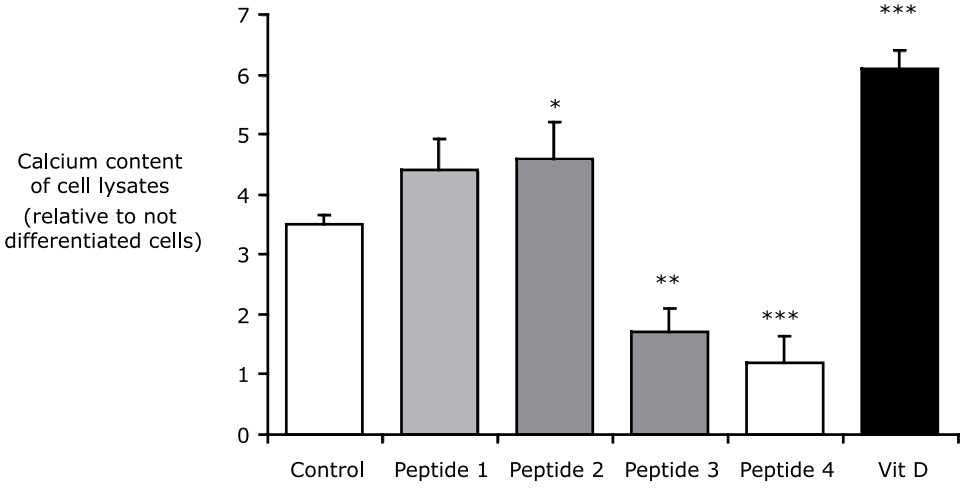
Figure 1. Effects of calcium lactate addition to culture medium on cell mineralization. A, not differentiated cells. B, cells growing in differentiating medium (alpha-MEM supplemented with 100 μg/ml ascorbic acid, 5 mM glycerol-2-phosphate). C, not differentiated cells growing in medium supplemented with 0.5 mM calcium lactate. D, cells growing in differentiating medium supplemented with calcium lactate.



The only peptide showing a significant positive effect on bone mineralization (peptide-2) carried one phosphorylated serine, and occurs in most bovine β-casein variants as well as in sheep and goat β-casein. As for α_{s2}-casein, the negative effect on bone mineralization was less evident for the *Bos grunniens* C variant.

Our preliminary results suggest that the distinct casein peptides in protein hydrolysates

Figure 2. Mineralization trial results. Control=differentiated cells growing in medium supplemented with 100 µg/ml ascorbic acid, 5 mM glycerol-2-phosphate, 0.5 mM calcium lactate. Peptide concentrations=40 µM. Vit D=1alpha, 25-dihydroxyvitamin D3 (positive control). Results are the mean±SD of 4 to 5 replicate wells. *, P<0.05. **, P<0.01. ***, P<0.001 (one-way ANOVA, Dunnett' t test).



may differentially affect calcium deposition in the extracellular matrix. The higher content of phosphorylated serines in the peptide might compete with calcium deposition, probably subtracting Ca^{++} from the culture medium. Moreover, the genetic variation within the considered peptides may influence their differential effect on osteoblast *in vitro* mineralization.

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