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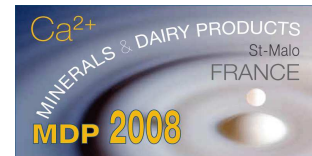
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## Phosphorylation variants of equine $\beta$ -casein: purification of caseinophosphopeptides and determination of the phosphorylation sites

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Equine caseins have variable degrees of phosphorylation. Indeed, the different isoforms of  $\alpha_{s1}$ -casein contain phosphate groups varying from two (2P) to six (6P) or eight (8P) according to exon skipping. The  $\beta$ -casein has 3P to 7P variants; variants with 5P and 6P are the most abundant in Haflinger mare's milk. It would be interesting to study the influence of the phosphorylation level and sequences of equine  $\beta$ -casein on chelation capacity of its caseinophosphopeptides (CPPs) for minerals of nutritional interest. For this purpose, the different phosphorylation variants of  $\beta$ -casein were purified and the phosphorylated sites were determined after purification of the corresponding CPPs. Equine  $\beta$ -casein was isolated from whole sodium caseinate by hydrophobic interaction chromatography. An ion-exchange chromatography (IEC) of the purified  $\beta$ -casein allowed separating the different variants of phosphorylation. Each fraction obtained by IEC was hydrolyzed by trypsin linked to agarose. To achieve a selective enrichment of CPPs, each hydrolysate obtained was separated onto a titanium dioxide microcolumn. The non-phosphorylated peptides were not retained onto the microcolumn and were eluted directly whereas the CPPs interacted with the titanium dioxide column. The retained fractions were first analysed by LC/ESI-MS to confirm the selectivity of titanium dioxide column for CPPs. For each phosphorylation variant, the isolated CPPs corresponded to fragments 1-34 and 4-34 of  $\beta$ -casein. In the purpose to identify the phosphorylated sites, the CPPs of each variant were submitted to hydrolysis by endoproteinase GluC which generated peptides with a molecular weight between 500 and 2000 Da compatible with a good MS/MS fragmentation. The MS/MS results allowed locating the phosphorylation sites of each phosphorylation variant. Indeed,  $\beta$ -casein 4P was phosphorylated on Ser-9, Ser-23, Ser-24 and Ser-25.  $\beta$ -Casein 5P was phosphorylated on Ser-18 in addition to the previous sites. The sixth site of phosphorylation of  $\beta$ -casein 6P was provided by Thr-12. The supplementary phosphorylation site of  $\beta$ -casein 7P was Ser-10. So, the common sites of phosphorylation of the different variants were Ser-9, Ser-23, Ser-24 and Ser-25; the additional sites were successively Ser-18, Thr-12 and Ser-10. Only one phosphorylation sequence was found for the different variants, suggesting that phosphorylation was not at random but occurred in a sequential way.