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# THE USE OF SYNTHETIC PHOSPHOPEPTIDES FOR EPITOPE MAPPING OF THE $\alpha_{s1}$-CASEIN PHOSPHOPEPTIDE SEGMENT 59-79

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**Abstract** : Through the use of synthetic Ser- and Ser(*P*)-containing peptides, both sequences -Ser(*P*)-Ser(*P*)-Ser(*P*)- and Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Glu of the tryptic phosphopeptide segment 59-79 of $\alpha_{s1}$-casein were shown to be recognized by anti-$\alpha_{s1}$-casein polyclonal antibodies in a competitive ELISA. Since replacement of Ser(*P*) with Ser in the later peptide resulted in complete loss of antibody recognition, this indicates that the phosphorylated seryl residue is a critical residue for antibody recognition.

The 21-mer phosphopeptide obtained from the tryptic digest of $\alpha_{s1}$-casein[1] (sequence 59-79) (shown below) is a particularly unusual peptide since it contains five Ser(*P*)-residues of which three Ser(*P*)-residues occur in a clustered arrangement. While epitope mapping has been used to identify the active regions of many protein-antibody systems,[2] this has not been possible with phosphopeptides due to past difficulties in the preparation of complex Ser(*P*)-containing peptides. In this paper, we report the use of synthetic Ser- and Ser(*P*)-containing peptides to identify the antigenic determinants of the $\alpha_{s1}$-casein 59-79 segment and establish that the phosphorylated seryl residue of the *C*-terminal sequence is critical for antibody recognition.

Gln-Met-Glu-Ala-Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Gln-Lys
59 63 68 73 78

Since the chemical synthesis of Ser(*P*)-containing peptides is laborious and time-consuming, the phosphopeptide was sectioned into five segments which covered the 59-63, 61-65, 63-70, 69-74 and 71-78 sequences. The heavily phosphorylated sequence 63-70 was kept intact since this region is conserved in both bovine and human β-casein and is considered to be an important structural feature of the caseins.[3] Also, sequence 69-74 was selected to cover the non-phosphorylated region between the Ser(*P*)-residues at positions 68 and 75, and sequence 61-65 was selected to cover the *N*-terminal Ser(*P*)-residue. The synthesis of the three Ser(*P*)-containing peptide segments was accomplished using a methodology[4,5] which employed Boc-Ser($PO_3Ph_2$)-OH in the Boc mode of solution phase peptide synthesis followed by hydrogenolytic deprotection (platinum) of the protected Ser($PO_3Ph_2$)-peptides in 50% TFA/AcOH. For sequence 71-78 though, Gln-78 was changed to Glu so as to avoid possible difficulties in the synthesis of peptides which contain a *C*-terminal Gln residue. The synthesis of the two peptides corresponding to sequences 59-63 and 69-74 was accomplished by Fmoc/solid phase peptide synthesis using DCC/HOBt activation.[6]

The analysis of peptides 1-6 by competitive ELISA[7] using $\alpha_{s1}$-(59-79) as the adsorbed antigen showed that peptides 3 and 5 were both recognized by the anti-$\alpha_{s1}$-casein antibodies, having % inhibitions of 20.0 ± 3.6 and 60.3 ± 7.9 respectively. The lack of competition of the two peptide sequences flanking the Ser(*P*)-Ser(*P*)-Ser(*P*)- cluster sequence suggests that this heavily phosphorylated cluster is an important feature of the determinant recognized by the polyclonal antibodies. In addition to this sequence, the *C*-terminal Ser(*P*)-containing sequence (peptide 5) was found to strongly inhibit in the competitive ELISA. In order to test the importance of the Ser(*P*)-residue of peptide 5 in antibody recognition, the non-phosphorylated peptide was prepared by Boc/solution phase peptide synthesis (peptide 6). Subsequent analysis of this peptide in the competitive ELISA showed that the change of Ser(*P*) to Ser resulted in a complete loss of competition. As this result indicates that the phosphate group of the seryl residue is an important factor for antibody recognition, NMR spectroscopy studies are currently underway to establish the structural conformation of peptides 5 and 6.

| | Peptides [8] | % Inhibition [9] |
|---|---|---|
| 1. | Gln-Met-Glu-Ala-Glu | 10.0 ± 1.7 |
| 2. | Glu-Ala-Glu-Ser(*P*)-Ile-NHMe | 10.9 ± 0.2 |
| 3. | Ac-Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-NHMe | 20.0 ± 3.6* |
| 4. | Glu-Glu-Ile-Val-Pro-Asn | 10.8 ± 1.0 |
| 5. | Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Glu-NHMe | 60.3 ± 7.9* |
| 6. | Ile-Val-Pro-Asn-Ser-Val-Glu-Glu-NHMe | 12.0 ± 3.1 |
| 7. | BSA (control) | 9.3 ± 1.6 |

*significantly different by ANOVA ($p < 0.001$) to other values not similarly marked.

In conclusion, both sequences -Ser(*P*)-Ser(*P*)-Ser(*P*)- and Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Glu of $\alpha_{s1}$-casein (59-79) were shown to be recognized by anti-$\alpha_{s1}$-casein antibodies with the Ser(*P*)-residue in the later peptide being critical.

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**References and Notes**

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6. Performed on a ABI 431 instrument using HMP-Resin (ABI) and 20% piperidine/DMF for Fmoc cleavage.
7. Performed using Nunc-Immuno Modules (MaxiSorp) and the procedure of Otani, H.; Takahashi, F.; Takita, F. *Agric. Biol. Chem.* **1986**, *50*, 607. The anti-$\alpha_{s1}$-casein antibody was purchased from Calbiochem-Behring. For colour development, 3,3',5,5'-tetramethylbenzidine was used and the reaction product was measured spectrophotometrically at 450 nm using a Bio-Rad 450 Microplate reader.
8. All peptides were found to be homogeneous by $C_8$ HPLC and were readily characterised by $^{13}C$ NMR spectroscopy, FAB mass spectrometry and amino acid analysis.
9. All peptides were analysed at a concentration of 1.0 mM.