Journal of Peptide Science
J. Peptide Sci. **5**: 221–233 (1999)

Epitope Analysis of the Multiphosphorylated Peptide α_{s1}-Casein(59–79)

JOHN W. PERICH, CATHERINE L. BLACK, N. LAILA HUQ and ERIC C. REYNOLDS*

Biochemistry and Molecular Biology Unit, School of Dental Science, The University of Melbourne, Victoria, Australia

Received 27 May 1998
Accepted 14 September 1998

Abstract: The multiphosphorylated tryptic peptide α_{s1}-casein(59–79) has been shown to be antigenic with anti-casein antibodies. In an approach to determine the amino acyl residues critical for antibody binding we undertook an epitope analysis of the peptide using overlapping synthetic peptides. With α_{s1}-casein(59–79) as the adsorbed antigen in a competitive ELISA only two of five overlapping synthetic peptides at 1 mM significantly inhibited binding of the anti-casein antibodies. Peptides Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu and Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Glu inhibited antibody binding by $20.0 \pm 3.6\%$ and $60.3 \pm 7.9\%$, respectively. The epitope of Glu^{63}-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu^{70} was further localised to the phosphoseryl cluster as the peptide Ser(*P*)-Ser(*P*)-Ser(*P*) significantly inhibited binding of the anti-casein antibodies to α_{s1}-casein(59–79) by $29.5 \pm 7.4\%$. Substitution of Ser(*P*)^{75} with Ser^{75} in the second inhibitory peptide Ile-Val-Pro-Asn-Ser(*P*)^{75}-Val-Glu-Glu also abolished inhibition of antibody binding to α_{s1}-casein (59–79) demonstrating that Ser(*P*)^{75} is also a critical residue for recognition by the antibodies. These data show that the phosphorylated residues in the cluster sequence -Ser(*P*)^{66}-Ser(*P*)-Ser(*P*)^{68} and in the sequence -Pro^{73}-Asn-Ser(*P*)-Val-Glu^{77}- are critical for antibody binding to α_{s1}-casein(59–79) and further demonstrate that a highly phosphorylated segment of a protein can be antigenic. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: multiphosphorylated peptide; ELISA; epitopes; phosphoseryl residues

INTRODUCTION

Antigenicity of an amino acyl sequence has been associated with the hydrophilicity [1], mobility [2,3], surface exposure [4] and propensity for β-turn formation [5] of that sequence. Phosphorylation of a protein can also be associated with hydrophilicity, segment mobility, surface exposure and propensity for β-turn formation [6–8]. Small *et al.* [6] examined 14 different proteins that were highly phosphorylated and found that 80% of the phosphorylation sites existed within regions predicted to be β-turns. Furthermore, phosphorylated serines not predicted to be within turns were often found to be adjacent to predicted turns (± 2 residues) [6]. These results suggest that phosphorylated segments of proteins may be antigenic. However, there are few studies on the antigenicity of phosphorylated proteins. One phosphorylated protein that has been epitope mapped is the bovine milk phosphoprotein α_{s1}-casein [9,10]. This protein contains a highly phosphorylated segment that can be isolated as a tryptic fragment, α_{s1}-casein(59–79) (Gln^{59}-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^{79}).

Abbreviations: BSA, bovine serum albumin; Σ, Ser(*P*); HPR, horse radish peroxidase; OD, optical density; OPD, o-phenylenediamine; NGS, normal goat serum; TBS, Tris buffered saline; TBST, TBS containing 0.05% Tween 20 (w/v); TMB, 3,3′,5,5′-tetramethylbenzidine.

* Correspondence to: Biochemistry and Molecular Biology Unit, School of Dental Science, The University of Melbourne, 711 Elizabeth Street, Melbourne 3000, Victoria, Australia. E-mail: e.reynolds@dent.unimelb.edu.au

Contract/grant sponsor: Australian National Health and Medical Research Council; Contract/grant number: 950568

CCC 1075–2617/99/050221-13$17.50

Using the parameters of Wilmot and Thornton [11,12], the sequences of -Val-Pro-Asn-Ser-, -Pro-Asn-Ser-Val- and -Ile-Ser-Ser-Ser-Glu-Glu- in α_{s1}-casein(59–79) are all predicted to support a β-turn [13]. Furthermore these predictions are supported by NMR observations consistent with the formation of a loop at -Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu- and sequential β-turns at -Val-Pro-Asn-Ser(*P*)- and -Pro-Asn-Ser(*P*)-Val- in α_{s1}-casein(59–79) [13]. These predictions and observations therefore suggest that regions containing phosphoseryl residues in α_{s1}-casein(59–79) may be antigenic. Despite these predictions, it has been reported that these phosphorylated regions of α_{s1}-casein are not recognised by anti-α_{s1}-casein antibodies [9,10]. For example, screening of α_{s1}-casein fragments with anti-α_{s1}-casein antibodies led Ametani *et al.* [9,10] to conclude that the multiple phosphoseryl-containing tryptic fragment α_{s1}-casein(59–79) was not antigenic. However, these authors used a solid phase assay and it is possible that the hydrophilic α_{s1}-casein(59–79) did not adhere to the microtitre plate used in their assay or adhered to the surface in a way that did not allow antibody binding. We have recently demonstrated that the peptide α_{s1}-casein(59–79) was specifically recognised by rabbit anti-α_{s1}-casein antibodies when the peptide was adsorbed onto Nunc-Immuno Maxisorp modules demonstrating that this multiphosphorylated peptide is antigenic [14]. In this current paper using synthetic phosphopeptides in a competitive ELISA with anti-casein antibodies we demonstrate that the phosphorylated residues in the cluster sequence Ser(*P*)-Ser(*P*)-Ser(*P*) and in the sequence Pro-Asn-Ser(*P*)-Val-Glu are critical for antibody binding.

MATERIALS AND METHODS

Preparation of α_{s1}-Casein and Casein Phosphopeptides

The milk protein α_{s1}-casein was prepared using selective precipitation [15]. The purity of the α_{s1}-casein was confirmed by isoelectric focussing [16]. The casein phosphopeptides α_{s1}-casein(59–79) and β-casein(1–25) were selectively precipitated from a tryptic digest of casein using Ca^{2+} and ethanol and further purified by anion exchange FPLC and RP-HPLC [17,18].

Preparation of Synthetic Peptides

All solvents were of Analar grade and THF was distilled from the potassium ketyl of benzophenone immediately prior to use. *N*-Methylmorpholine isobutyl chloroformate, acetic acid and trifluoroacetic acid were obtained from Aldrich Chem. Co. and used without further purification. Acetic acid was sequentially dried over 4 Å sieves and distilled immediately prior to use. Platinum oxide (80%) was obtained from Fluka. ^{13}C-NMR spectra were obtained on a JEOL FX-90Q Fourier transform instrument operating at 22.5 MHz and were referenced to the central resonance of the deuterium triplet set to 77.0 ppm for $CDCl_3$ solutions, to the central resonance of the deuterium heptet set to 39.5 ppm for $(CD_3)_2SO$ solutions and to internal dioxane set to 66.5 ppm for D_2O solutions. The ^{13}C-NMR spectra of Ac-Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-NHMe was recorded as a D_2O solution on a JEOL GX-400 Fourier transform instrument operating at 100 MHz and referenced to internal dioxane set to 66.5 ppm. ^{31}P-NMR spectra were obtained on a JEOL FX-100 Fourier transform instrument operating at 40.26 MHz and were referenced to external 85% H_3PO_4. FAB mass spectra were obtained by analysis of the peptide as an aqueous acetic acid/glycerol mull on a JEOL AX-505H mass spectrometer equipped with a FAB source and argon as ionization gas. HPLC analysis was performed on a Applied Biosystems instrument with a linear CH_3CN gradient provided by a 140A solvent delivery system linked to a 1000S Diode Array detector. Analytical HPLC was performed on a Brownlee CR Aquapore RP300 column (22.0 cm × 4.6 mm diameter) and a flow rate of 1.0 mL/min. Semi-preparative HPLC was performed on a Brownlee C_{18} Aquapore column (25.0 cm × 10.0 mm diameter) at a flow rate of 3.0 mL/min. Amino acid analysis of Ac-Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-NHMe was performed by vapour hydrolysis of the peptide in 5.7 M HCl (24 h at 110°C) followed by analysis of the PTC-derivatized hydrolysate on a Waters HPLC instrument.

Synthesis of Non-Phosphorylated Peptides

Fmoc/solid phase peptide synthesis was performed on an ABI 431A instrument (DCC/HOBt) using HMP-Resin (0.94 mmol/g) (0.266 g, 0.25 mmol) as the resin support.

Gln-Met-Glu-Ala-Glu·TFA. The assembled peptide resin was deprotected using 97:3 TFA/methylethylsulphide for 90 min and the crude product (obtained 0.115 g, expected 0.177 g) was applied to a semi-preparative C_{18}-HPLC column using a linear gradient elution of 8–40% CH_3CN over 10 min to

give pure Gln-Met-Glu-Ala-Glu·TFA (0.083 g, 46.9% based on resin) as a fluffy white solid. δ (^{13}C) (D_2O) 14.1, 16.4, 25.7, 26.2, 26.4, 29.1, 29.7, 29.9, 30.3, 49.5, 51.8, 52.2, 52.6, 53.0, 169.1, 172.4, 172.7, 174.5 (×2), 176.8 (×2). FAB-MS (+ve mode) m/z 607 (expected m/z 607).

Glu-Glu-Ile-Val-Pro-Asn·TFA. The assembled peptide resin was deprotected using 95:5 TFA/water (obtained 0.011 g, expected 0.203 g) and the crude product was applied to a semi-preparative C_{18}-HPLC column using a linear gradient elution of 0–40% CH_3CN over 10 min to give pure Glu-Glu-Ile-Val-Pro-Asn·TFA (6.1 mg, 3.0% based on resin) as a fluffy white solid. FAB-MS (+ve mode) m/z 700 (expected m/z 700).

Ile-Val-Pro-Asn-Ser-Val-Glu-Glu·TFA. The assembled peptide resin was deprotected using 95:5 TFA/water (obtained 0.194 g, expected 0.250 g) and a portion of the crude product (100 mg) was applied to a semipreparative C_{18}-HPLC column using a linear gradient elution of 0–80% CH_3CN over 10 min to give pure Ile-Val-Pro-Asn-Ser-Val-Glu-Glu·TFA (60.0 mg, 46.6% based on resin) as a fluffy white solid. δ (^{13}C) (D_2O) 10.3, 14.1, 17.5, 17.7, 18.2, 18.3, 23.9, 24.6, 25.6, 26.0, 29.4, 29.7, 29.9, 35.9, 36.4, 48.4, 50.65, 51.8, 52.7, 55.6, 57.1, 57.3, 59.5, 60.5, 61.0, 169.2, 171.4, 171.6, 172.4, 172.9, 173.0, 173.7, 174.25, 174.4, 176.8 (×2). FAB-MS (+ve mode) m/z 887 (expected m/z 887). The later-eluting peak was identified as Ile-Val-Pro-Ala(CN)-Val-Glu-Glu·TFA (28.0 mg). δ (^{13}C) (D_2O) (partial) 117.7 {Ala(CN) Cγ}. FAB-MS (+ve mode) m/z 868 (expected m/z 868).

Ile-Val-Pro-Ala-Ser-Val-Glu-Glu·TFA. The assembled peptide resin was deprotected using 95:5 TFA/water (obtained 0.173 g, expected 0.239 g) and a portion of the crude product (20 mg) was applied to a semipreparative C_{18}-HPLC column using a linear gradient elution of 0–80% CH_3CN over 10 min to give pure Ile-Val-Pro-Ala-Ser-Val-Glu-Glu·TFA (19.0 mg, 68.8% based on resin) as a fluffy white solid. δ (^{13}C) (D_2O) 10.4, 14.1, 16.4, 17.5, 17.6, 18.2, 18.3, 24.0, 24.7, 25.6, 26.1, 29.4, 29.8, 30.0, 36.4, 48.4, 49.9, 51.8, 52.75, 55.4, 57.1, 57.4, 59.4, 60.4, 60.9, 65.9, 169.1, 171.3, 171.7, 173.0, 173.7, 174.4, 175.0, 176.7 (×2). FAB-MS (+ve mode) m/z 843 (expected m/z 843).

Synthesis of Ser(*P*)-Containing Peptides

Boc/solution phase peptide synthesis was performed by the REMA method [19] (isobutoxy carbonyl mixed anhydride couplings) and the use of 40% TFA/DCM (30 min) for cleavage of the Boc group from the Boc-peptides. The solvent was evaporated under reduced pressure and the peptide trifluoroacetate precipitated by the addition of diethyl ether.

Boc/Solution Phase Synthesis: General Procedure. A solution of NMM (3.24 equivalents) in THF (1 mL) and IBCF (3.00 equivalents) in THF (1 mL) were successively added to a solution of the Boc-amino acid (3.24 equivalents) in THF (3 mL) at −20°C. After an activation period of 3 min, a solution of the peptide·TFA (1.0 equivalent) and NMM (1.0 equivalent) in DCM or DMF (5 mL) was added to the reaction mixture and the resulting solution stirred for 2 h at −20°C. A solution of 5% $NaHCO_3$ (5 mL) was then added and the solution stirred for a further 30 min at 0°C. Ethyl acetate or $CHCl_3$ (60 mL) was then added and the organic phase washed with 5% $NaHCO_3$ (2 × 30 mL), 1 M HCl (2 × 30 mL), dried (Na_2SO_4) and then filtered. The solvent was then evaporated under reduced pressure to a small volume and diethyl ether (10 mL) was added to precipitate the peptide. The solvent was then evaporated under reduced pressure, the white solid triturated with diethyl ether (2 × 30 mL) and dried under high vacuum.

Boc-Ile-Ser(PO$_3$Ph$_2$)-Ser(PO$_3$Ph$_2$)-Ser(PO$_3$Ph$_2$)-Glu(OBzl)-Glu(OBzl)-NHMe. Boc-Ile-OH (0.441 g, 1.91 mmol)/THF (3 mL); NMM (0.193 g, 1.91 mmol)/THF (1 mL); IBCF (0.242 g, 1.77 mmol)/THF (1 mL); pentapeptide·TFA [24] (0.59 mmol) and NMM (0.060 g, 0.59 mmol)/DCM (5 mL). Ethyl acetate. White solid (0.933 g, 96.5%). δ (^{13}C) {$(CD_3)_2SO$} 11.0, Ile Cδ; 15.4 and 24.4, Ile Cγ, 25.6, NHMe; 27.3, Glu^{5,6} Cβ; 28.1, Boc Me; 30.1, Glu^{5,6} Cγ, 52.1 and 52.9, Glu^{5,6} Cα; 52.1–53.2, Ser^{2,3,4} Cα; 59.1, Ile Cα; 65.5, Glu^{5,6} Bzl CH_2; 67.8 (br d), Ser^{2,3,4} Cβ; 78.2, Boc Cq; 119.9 (d, $J_{P,C}$ 4.9 Hz), Ph C2; 125.5, Ph C4; 127.9 and 128.4, Bzl C2,3,4; 130.0, Ph C3; 136.1, Glu^{5,6} Bzl C 1; 150.0 (d, $J_{P,C}$ 6.6 Hz), Ph C1; 155.4, urethane CO; 167.5, 167.8 and 168.2, Ser^{2,3,4} CO; 170.5, 171.1 and 171.8, Ile CO and Glu^{5,6} CO; 172.1 and 172.2, Glu^{5,6} δ-CO. δ(^{31}P) {$CDCl_3$} −11.1, −11.8 and −11.9.

Boc-Ser(PO$_3$Ph$_2$)-Ile-Ser(PO$_3$Ph$_2$)-Ser(PO$_3$Ph$_2$)-Ser(PO$_3$Ph$_2$)-Glu(OBzl)-Glu(OBzl)-NHMe. Boc-Ser(PO_3Ph_2)-OH (0.708 g, 1.62 mmol)/THF (3 mL); NMM (0.164 g, 1.62 mmol)/THF (1 mL); IBCF (0.205 g, 1.50 mmol)/THF (1 mL); hexapeptide·TFA (0.50 mmol) and NMM (0.51 g, 0.50 mmol)/DCM (5 mL). Ethyl acetate. White solid (0.947 g, 96.7%). δ (^{13}C)

{$(CD_3)_2SO$} 11.0, Ile Cδ; 15.2 and 24.0, Ile Cγ; 25.6, NHMe; 27.2, Glu^{6,7} Cβ; 28.0, Boc Me; 30.1, Glu^{6,7} Cγ, 52.0 and 52.7, Glu^{6,7} Cα; 52.0–53.1, Ser^{1,3,4,5} Cα; 56.8, Ile Cα; 65.5, Glu^{6,7} Bzl CH_2; 67.9 (br), Ser^{1,3,4,5} Cβ; 78.8, Boc Cq; 120.0 (d, $J_{P,C}$ 4.4 Hz), Ph C2; 125.5, Ph C4; 127.9 and 128.4, Bzl C2,3,4; 130.0, Ph C3; 136.1, Glu^{6,7} Bzl C1; 149.9 (d, $J_{P,C}$ 6.6 Hz), Ph C1; 155.3, urethane CO; 167.5, 167.8 and 168.0 ($\times$ 2), Ser^{1,3,4,5} CO; 170.5, 171.05 ($\times$ 2), Ile CO and Glu^{6,7} CO; 172.1 and 172.2, Glu^{6,7} δ-CO. δ (^{31}P) ($CDCl_3$) -11.4, -11.9 and -12.4.

Boc - Glu(OBzl) - Ser(PO_3Ph_2) - Ile - Ser(PO_3Ph_2) - Ser-(PO_3Ph_2)-Ser(PO_3Ph_2)-Glu(OBzl)-Glu(OBzl)-NHMe. Boc-Glu(OBzl)-OH (0.458 g, 1.36 mmol)/THF (3 mL); NMM (0.137 g, 1.36 mmol)/THF (1 mL); IBCF (0.172 g, 1.26 mmol)/THF (1 mL); heptapeptide·TFA (0.42 mmol) and NMM (0.42 g, 0.42 mmol)/DCM (5 mL). Ethyl acetate. White solid (0.871 g, 95.2%). δ (^{13}C) {$(CD_3)_2SO$} 11.0, Ile Cδ; 15.2 and 24.0, Ile Cγ, 25.5, NHMe; 27.2, Glu^{1,7,8} Cβ; 28.1, Boc Me; 30.1, Glu^{1,7,8} Cγ; 52.0–53.5, Glu^{1,7,8} Cα and Ser^{2,4,5,6} Cα; 56.9, Ile Cα; 65.9, Glu^{1,7,8} Bzl CH_2; 67.7 (br), Ser^{2,4,5,6} Cβ; 78.3, Boc Cq; 120.0 (d, $J_{P,C}$ 4.4 Hz), Ph C2; 125.5, Ph C4; 127.9 and 128.4, Bzl C2,3,4; 129.9, Ph C3; 136.1, Glu^{7,8} Bzl C1; 149.9 (d, $J_{P,C}$ 7.7 Hz), Ph C1; 155.3, urethane CO; 167.5, 167.6, 167.7 and 168.1, Ser^{2,4,5,6} CO; 170.4, 171.0, 171.05, 172.0, Ile CO and Glu^{1,7,8} CO; 172.2, Glu^{1,7,8} δ-CO. δ (^{31}P) {$(CD_3)_2SO$} -12.05.

Ac-Glu(OBzl)-Ser(PO_3Ph_2)-Ile-Ser(PO_3Ph_2)-Ser(PO_3-Ph_2)-Ser(PO_3Ph_2)-Glu(OBzl)-Glu(OBzl)-NHMe. AcOH (0.112 g, 1.86 mmol)/THF (3 mL); NMM (0.188 g, 1.86 mmol)/THF (1 mL); IBCF (0.236 g, 1.73 mmol)/THF (1 mL); octapeptide·TFA (0.346 mmol) and NMM (0.35 g, 0.346 mmol)/DMF (5 mL). Chloroform. White solid (0.730 g, 99.6%). δ (^{13}C) {$(CD_3)_2SO$} 11.0, Ile Cδ; 15.2 and 24.0, Ile Cγ; 22.4, acetyl C; 25.6, NHMe; 27.2, Glu^{1,7,8} Cβ; 30.1, Glu^{1,7,8} Cγ; 37.1, Ile Cβ; 52.0–53.0, Glu^{1,7,8} Cα and Ser^{2,4,5,6} Cα; 57.0, Ile Cα; 65.5, Glu^{1,7,8} Bzl CH_2; 67.7 (br), Ser^{2,4,5,6} Cβ; 120.0 (d, $J_{P,C}$ 4.4 Hz), Ph C2; 125.5, Ph C4; 127.9 and 128.4, Bzl C2,3,4; 130.0, Ph C3; 136.1,Glu^{1,7,8} Bzl C1; 149.9 (d, $J_{P,C}$ 6.6 Hz), Ph C1; 167.5, 167.6, 167.7 and 168.1, Ser^{2,4,5,6} CO; 169.6, acetyl CO; 170.4, 171.0, 171.1 and 171.7, Ile CO and Glu^{1,7,8} CO; 172.1 and 172.2 ($\times$ 2), Glu^{1,7,8} δ-CO δ (^{31}P) ($CDCl_3$) -11.0, -12.9, -13.0 and -13.2. δ (^{31}P) {$(CD_3)_2SO$} -12.1.

Ac - Glu - Ser(P) - Ile - Ser(P) - Ser(P) - Ser(P) - Glu - Glu-NHMe. A rapidly stirred solution of octapeptide (0.530 g, 0.25 mmol) in 50% TFA/AcOH (4 mL) containing 80% PtO_2 (0.624 g, 2.20 mmol) was hydrogenated at atmospheric pressure until hydrogen uptake ceased (3 h). The platinum was removed by gravity filtration, washed with TFA (2×2 mL) and the solvent evaporated under reduced pressure. The residue was tritrated with diethyl ether (3×20 mL), dissolved in water (3 mL), passed through a 0.2 pore membrane filter and lyophilized to give the octapeptide (0.307 g, 99.0%) as a fluffy white solid. δ (^{13}C) (D_2O) (400 MHz) 10.16, Ile Cδ; 14.73, Ile Cγ; 21.67, acetyl C; 24.35, Ile Cγ; 25.65, 25.82, 25.97 and 26.20, NHMe and Glu^{1,7,8} Cβ; 29.94, Glu^{1,7,8} Cγ; 36.15, Ile Cβ; 53.05, 53.22 and 53.34, Glu^{1,7,8} Cα; 53.94–54.93 (br), Ser^{2,4,5,6} Cα; 58.32, Ile Cα; 64.00 (br), Ser^{2,4,5,6} Cβ; 170.60, 170.99, 171.22 and 171.35, Ser^{2,4,5,6} CO; 172.98, 173.29, 173.35, 173.54 and 174.34, acetyl CO; Ile CO and Glu^{1,7,8} CO; 176.81, 176.85 and 176.88, Glu^{1,7,8} δ-CO. δ (^{31}P) (D_2O) $+0.3$. FAB-MS ($+$ve mode) m/z 1241 (expected m/z 1241). Amino acid analysis: Glu 2.95 (3), Ser 3.79 (4), Ile 1.00 (1).

Synthesis of Glu-Ala-Glu-Ser(P)-Ile-NHMe

General Coupling Procedure. A solution of NMM (1.4 equivalents) in THF (1 mL) and IBCF (1.3 equivalents) in THF (1 mL) were successively added to a solution of the Boc-amino acid (1.4 equivalents) in THF (3 mL) at -20°C. After an activation period of 3 min, a solution of the peptide·TFA (1.0 equivalent) and NMM (1.0 equivalent) in THF or DMF was added to the reaction mixture and the resulting solution stirred for 2 h at -20°C. A solution of 5% $NaHCO_3$ (5 mL) was then added and the solution stirred for a further 30 min at 0°C. Diethyl ether, ethyl acetate or DCM (60 mL) was then added and the organic phase washed with 5% $NaHCO_3$ (2×30 mL), 1 M HCl (2×30 mL), dried (Na_2SO_4) and then filtered. The solvent was then evaporated under reduced pressure and the product dried under high vacuum.

Boc-Ser(PO_3Ph_2)-Ile-NMHe. Boc-Ser(PO_3Ph_2)-OH (0.306 g, 0.70 mmol)/THF (1 mL); NMM (0.071 g, 0.70 mmol)/THF (1 mL); IBCF (0.089 g, 0.65 mmol)/THF (1 mL); Ile-NHMe·TFA (0.50 mmol) and NMM (0.051 g, 0.50 mmol)/THF (3 mL). Diethyl ether. Oil (0.262 g, 93.1%). δ (^{13}C) ($CDCl_3$) 11.3, Ile Cδ; 15.4 and 24.2, Ile Cγ; 26.0, NHMe; 28.1, Boc Me; 36.7, Ile Cβ; 55.06 (d, $J_{P,C}$ 6.60 Hz), Ser Cα; 58.1, Ile Cα; 67.98 (d, $J_{P,C}$ 6.03 Hz), Ser Cβ; 78.4, Boc Cq; 120.2 (d, $J_{P,C}$ 5.49 Hz), Ph C2; 125.6, Ph C4; 129.8, Ph C3; 150.27 (d, $J_{P,C}$ 7.69 Hz), Ph C1; 155.5, Boc CO; 168.6 and 171.1, Ser and Ile CO. δ (^{31}P) ($CDCl_3$) -11.6.

Boc-Glu(OBzl)-Ser(PO_3Ph_2)-Ile-NMHe. Boc-Glu-(OBzl)-OH (0.226 g, 0.67 mmol)/THF (1 mL); NMM (0.068 g, 0.67 mmol)/THF (1 mL); IBCF (0.085 g, 0.62 mmol)/THF (1 mL); dipeptide·TFA (0.48 mmol) and NMM (0.048 g, 0.48 mmol)/THF (3 mL). Ethyl acetate. White solid (0.292 g, 77.7%). δ (^{13}C) ($CDCl_3$) 11.4, Ile Cδ; 15.3 and 24.5, Be Cγ; 26.0, NHMe; 27.5, Glu Cβ; 28.1, Boc Me; 30.3, Glu Cγ; 36.6, Ile Cβ; 54.0 (d, $J_{P,C}$ 5.49 Hz), Ser Cα; 54.6, Glu Cα; 58.6, Ile Cα; 66.5, Glu Bzl CH_2; 67.7 (d, $J_{P,C}$ 5.49 Hz), Ser Cβ; 80.4, Boc CMe_3; 119.9 (d, $J_{P,C}$ 4.39 Hz), Ph C2; 125.6, Ph C4; 128.1 and 128.4, Bzl C2,3,4; 129.8, Ph C3; 135.6, Glu Bzl C1; 150.1 (d, $J_{P,C}$ 7.7 Hz), Ph C1; 155.8, Boc CO; 168.0, 171.2, 172.6 and 172.8, Ser, Ile, Glu CO and Glu γ-CO. δ (^{31}P) ($CDCl_3$) -11.4.

Boc-Ala-Glu(OBzl)-Ser(PO_3Ph_2)-Ile-NMHe. Boc-Ala-OH (0.204 g, 1.080 mmol)/THF (1 mL); NMM (0.109 g, 1.080 mmol)/THF (1 mL); IBCF (0.137 g, 1.00 mmol)/THF (1 mL); tripeptide·TFA (0.32 mmol) and NMM (0.032 g, 0.32 mmol)/THF (3 mL). Dichloromethane. White solid (0.270 g, 98.9%). δ (^{13}C) {$(CD_3)_2SO$} 11.0, Ile Cδ; 15.3 and 24.2, Ile Cγ; 17.8, Ala Cβ; 25.3, NHMe; 27.6, Glu Cβ; 28.1, Boc Me; 29.7, Glu Cγ; 36.7, Ile Cβ; 49.7, Ala Cα; 51.5, Glu Cα; 52.5 (d, $J_{P,C}$ 7.69 Hz), Ser Cα; 57. l, Ile Cα; 65.4, Glu Bzl CH_2; 67.5 (d, $J_{P,C}$ 3.30 Hz), Ser Cβ; 78.1, Boc CMe_3; 119.9 (d, $J_{P,C}$ 4.39 Hz), Ph C2; 125.5, Ph C4; 127.8 and 127.9, Bzl C2,3,4; 128.3, Ph C3; 136.0, Glu Bzl C1; 149.9 (d, $J_{P,C}$ 6.59 Hz), Ph C1; 155.3, Boc CO; 167.4, 170.8, 171.2, 172.2 and 172.8, Ala, Ile, Ser, Glu CO and Glu γ-CO. δ (^{31}P) {$(CD_3)_2SO$} -12.0.

Boc - Glu(OBzl) - Ala - Glu(OBzl) - Ser(PO_3Ph_2) - Ile-NMHe. Boc-Glu(OBzl)-OH (0.088 g, 0.364 mmol)/THF (1 mL); NMM (0.037 g, 0.364 mmol)/THF (1 mL); IBCF (0.046 g, 0.338 mmol) THF (1 mL); tetrapeptide·TFA (0.260 mmol) and NMM (0.026 g, 0.260 mmol)/DMF (3 mL). Aqueous precipitation (5% $NaHCO_3$). White solid (0.256 g, 91.8%). δ (^{13}C) {$(CD_3)_2SO$} 11.0, Ile Cδ; 15.3 and 24.2, Ile Cγ; 18.1, Ala Cβ; 25.4, NHMe; 27.2 and 27.4, Glu^{1,3} Cβ; 28.1, Boc Me; 30.1, Glu^{1,3} Cγ; 36.7, Ile Cβ; 48.1, Ala Cα; 51.8 and 52.7, Glu^{1,3} Cα; 52.5 (d, $J_{P,C}$ 8.79 Hz), Ser Cα; 57.1, Ile Cα; 65.4, Glu Bzl CH_2; 67.7 (d, $J_{P,C}$ 6.39 Hz), Ser Cβ; 78.1, Boc CMe_3; 119.9, d, $J_{P,C}$ 4.39 Hz, Ph C2; 125.6, Ph C4; 127.8 and 128.4, Bzl C2,3,4; 130.0, Ph C3; 136.1, Glu Bzl C1; 149.9 (d, $J_{P,C}$ 6.59 Hz), Ph C1; 155.3, Boc CO; 167.5, 170.8, 171.2 ($\times 2$), 172.1 and 172.2 ($\times 2$), Ala, Ile, Ser, Glu^{1,3} CO and Glu γ-CO. δ (^{31}P) {$(CD_3)_2SO$} -12.1.

Glu-Ala-Glu-Ser(P)-Ile-NHMe·TFA. A rapidly stirred solution of pentapeptide (0.107 g, 0.1 mmol) in TFA/AcOH (1:1) (4 mL) containing 80% platinum oxide (0.063 g, 0.22 mmol) was hydrogenated at atmospheric pressure until hydrogen uptake ceased (60 min). The platinum was removed by gravity filtration, washed with TFA (2×2 mL) and the solvent evaporated under reduced pressure. The residue was triturated with diethyl ether (2×10 mL) and the white solid was lyophilized from water to give pure Glu-Ala-Glu-Ser(*P*)-Ile-NHMe·TFA as a fluffy white powder (0.068 g, 90.6%). δ (^{13}C) (D_2O) (90 MHz) 10.1, Ile Cδ; 14.7, Ile Cγ; 16.3, Ala Cβ, 24.4, Ile Cγ, 25.7, $NHCH_3$; 25.8 and 26.1, Glu^{1,3} Cβ; 28.9 and 29.8, Glu Cγ; 35.9, Ile Cβ; 49.8, Ala Cα; 52.0 and 52.9, Glu^{1,3} Cα; 53.94 (d, $J_{P,C}$ 7.70 Hz), Ser Cα; 63.46 (d, $J_{P,C}$ 4.40 Hz), Ser Cβ; 168.9, 170.8, 173.1, 173.6 and 174.3 (amides); 176.0 and 176.7, Glu^{1,3} γ-CO. δ (^{31}P) {D_2O} $+0.03$. FAB-MS ($+$ve mode) m/z 641 (expected m/z 641).

Synthesis of Ile-Val-Pro-Asn-Ser(P)-Val-Glu-Glu-NHMe

Boc-Val-Glu(OBzl)-Glu(OBzl)-NHMe. Boc-Val-OH (0.650 g, 3.00 mmol)/THF (3 mL); NMM (0.303 g, 3.00 mmol)/THF (1 mL); IBCF (0.380 g, 2.78 mmol)/THF (1 mL); dipeptide·TFA [24] (2.00 mmol) and NMM (0.202 g, 2.00 mmol)/THF (3 mL). Ethyl acetate. White solid (1.234 g, 92.4%). δ (^{13}C) ($CDCl_3$) 18.0 and 19.0, Val Cγ; 26.1, NHMe C; 27.6 and 27.8, Glu^{2,3} Cβ; 28.3, Boc Me; 30.5, Glu^{2,3} Cγ; 31.3, Val Cβ; 52.4 and 52.8, Glu^{2,3} Cα; 60.0, Val Cα; 66.2 and 66.5, Glu^{2,3} Bzl Cb; 79.8, Boc Cq; 128.1, 128.2, 128.5, Ar C2,3,4; 135.7 and 135.9, Ar C1; 156.2, Boc CO; 171.2, 171.3, 172.2, 172.4 and 173.2, Val, Glu^{2,3} CO and γ-CO.

Boc - Ser(PO_3Ph_2) - Val - Glu(OBzl) - Glu(OBzl) - NHMe. Boc-Ser(PO_3Ph_2)-OH (0.612 g, 1.40 mmol)/THF (3 mL); NMM (0.141 g, 1.40 mmol)/THF (1 mL); IBCF (0.178 g, 1.30 mmol)/THF (1 mL); tripeptide·TFA (1.00 mmol) and NMM (0.101 g, 1.00 mmol)/THF (3 mL). Ethyl acetate. White solid (0.924 g, 93.6%). δ (^{13}C) ($CDCl_3$) 17.9 and 19.0, Val Cγ; 25.6, NHMe C; 26.9 and 27.4, Glu^{3,4} Cβ; 28.0, Boc Me; 30.0, Glu^{3,4} Cγ; 31.1, Val Cβ; 51.9, Glu^{3,4} Cα; 54.5 (d, $J_{P,C}$ 8.79 Hz), Ser Cα; 57.4. Val Cα; 65.5, Glu^{3,4} Bzl Cb; 67.8 (d, $J_{P,C}$ 5.49 Hz), Ser Cβ; 78.8, Boc Cq; 120.0 (d, $J_{P,C}$ 4.39 Hz), Ph C2; 125.5, Ph C4; 127.9 and 128.4, Ar C2,3,4; 129.9, Ph C3; 136.1, Ar C1; 150.0 (d, $J_{P,C}$ 7.13 Hz), Ph C1; 155.2, Boc CO; 168.1, 170.7, 170.8 and 171.1, Ser, Val and Glu^{3,4} CO; 172.1; Glu^{3,4} γ-CO. δ (^{31}P) ($CDCl_3$) -11.6.

Boc - Asn - Ser(PO_3Ph_2) - Val - Glu(OBzl) - Glu(OBzl)-NHMe. Boc-Asn-OH (0.673 g, 2.90 mmol)/DMF (5 mL); NMM (0.293 g, 2.90 mmol)/THF (1 mL); IBCF (0.369 g, 2.70 mmol)/THF (1 mL); tetrapeptide·TFA (0.90 mmol) and NMM (0.091 g, 0.90 mmol)/THF (3 mL). Aqueous precipitation. White solid (two products) (0.675 g, 68%). δ (^{31}P) ($CDCl_3$) -10.8 and -11.8.

Boc-Pro-Asn-Ser(PO_3Ph_2)-Val-Glu(OBzl)-Glu(OBzl)-NHMe. Boc-Pro-OH (0.417 g, 1.94 mmol)/THF (3 mL); NMM (0.196 g, 1.94 mmol)/THF (1 mL); IBCF (0.246 g, 1.80 mmol)/THF (1 mL); pentapeptide·TFA (0.60 mmol) and NMM (0.061 g, 0.06 mmol)/DMF (5 mL). Ethyl acetate. White solid (0.530 g).

Boc - Val - Pro - Asn - Ser(PO_3Ph_2) - Val - Glu(OBzl)-Glu(OBzl)-NHMe. Boc-Val-OH (0.305 g, 1.405 mmol)/THF (3 mL); NMM (0.141 g, 1.405 mmol)/THF (1 mL); IBCF (0.178 g, 1.305 mmol)/THF (1 mL); hexapeptide·TFA (0.435 mmol) and NMM (0.044 g, 0.435 mmol)/DMF (5 mL). Ethyl acetate. White solid (0.556 g).

Boc - Ile - Val - Pro - Asn - Ser(PO_3Ph_2) - Val - Glu(OBzl)-Glu(OBzl)-NHMe. Boc-Ile-OH (0.285 g, 1.234 mmol)/THF (3 mL); NMM (0.125 g, 1.234 mmol)/THF (1 mL); IBCF (0.156 g, 1.146 mmol)/THF (1 mL); heptapeptide·TFA (0.382 mmol) and NMM (0.039 g, 0.382 mmol)/DMF (5 mL). Aqueous precipitation. White solid (0.469 g).

Ile-Val-Pro-Asn-Ser(P)-Val-Glu-Glu-NHMe·TFA. A rapidly stirred solution of impure octapeptide (0.141 g) in TFA/AcOH (1:1) (4 mL) containing 80% platinum oxide (0.10 g, 0.35 mmol) was hydrogenated at atmospheric pressure until hydrogen uptake ceased (60 min). The platinum was removed by gravity filtration, washed with TFA (4 mL) and the solvent evaporated under reduced pressure. The residue was triturated with diethyl ether (3×20 mL) and the crude peptide (99.0 mg) was purified by semi-preparative C_{18}-HPLC using a linear gradient elution of 0–24% CH_3CN over 30 min. Lyophilization of the solution gave pure Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Glu-NHMe·TFA (27.4 mg) as a fluffy white powder. δ (^{13}C) (D_2O) (400 MHz) 10.3, 14.1, 17.5, 18.1, 18.3, 23.9, 24.7, 25.7, 25.8, 26.0, 29.76, 29.78, 29.84, 36.1, 36.3, 48.4, 50.8, 53.1, 53.2, 54.8 (d, $J_{P,C}$ 7.4 Hz), 57.1, 57.4, 60.1, 60.4, 63.8 (d, $J_{P,C}$ 4.4 Hz), 169.3, 171.3, 171.3, 172.7, 173.2, 173.4, 173.5, 173.8, 174.1, 176.8. δ (^{31}P) (D_2O) 0.27. FAB-MS (+ve) m/z 980 (expected m/z 980).

Synthesis of Ile-Val-Pro-Ala-Ser(P)-Val-Glu-Glu-NHMe

Boc - Ala - Ser(PO_3Ph_2) - Val - Glu(OBzl) - Glu(OBzl)-NHMe. Boc-Ala-OH (0.114 g, 0.602 mmol)/THF (3 mL); NMM (0.061 g, 0.602 mmol)/THF (1 mL); IBCF (0.076 g, 0.559 mmol)/THF (1 mL); tetrapeptide·TFA (0.430 mmol) and NMM (0.043 g, 0.430 mmol)/THF (2 mL). Ethyl acetate. White solid (0.447 g, 98%). δ (^{13}C) ($CDCl_3$) 17.8 and 19.0, Val Cγ; 18.0, Ala Cβ; 25.5, NHMe C; 26.8 and 27.3, Glu^{3,4} Cβ; 28.1, Boc Me; 30.0, Glu^{4,5} Cγ; 30.9, Val Cβ; 49.9, Ala Cα; 51.9, Glu^{4,5} Cα; 52.5 (d, $J_{P,C}$ 6.60 Hz), Ser Cα; 57.4, Val Cα; 65.4, Glu^{4,5} Bzl Cb; 67.8 (d, $J_{P,C}$ 4.9 Hz), Ser Cβ; 78.1, Boc Cq; 119.9 (d, $J_{P,C}$ 4.39 Hz), Ph C2; 125.5, Ph C4; 127.8 and 128.3, Ar C2,3,4; 129.9, Ph C3; 136.1, Ar C1; 149.9 (d, $J_{P,C}$ 6.59 Hz), Ph C1; 155.0, Boc CO; 167.7, 170.5, 170.7 and 171.1, Ser, Val and Glu^{4,5} CO; 172.1 ($\times 2$), Glu^{4,5} γ-CO; 173.1, Ala CO. δ (^{31}P) {$(CD_3)_2SO$} -12.0.

Boc-Pro-Ala-Ser(PO_3Ph_2)-Val-Glu(OBzl)-Glu(OBzl)-NHMe. Boc-Pro-OH (0.195 g, 0.909 mmol)/THF (3 mL); NMM (0.092 g, 0.909 mmol)/THF (1 mL); IBCF (0.115 g, 0.844 mmol)/THF (1 mL); pentapeptide·TFA (0.422 mmol) and NMM (0.043 g, 0.422 mmol)/THF (3 mL). Precipitated on addition of water. White solid (0.438 g, 90%). δ (^{13}C) ($CDCl_3$) 17.8 and 19.0, Val Cγ; 18.1, Ala Cβ; 23.1, Pro Cγ; 25.5, NHMe C; 26.9 and 27.3, Glu^{5,6} Cβ; 28.0, Boc Me; 30.0, Glu^{5,6} Cγ, 30.9, Val Cβ; 46.5, Pro Cδ; 49.9, Ala Cα; 51.9, Glu^{5,6} Cα; 52.3 (d, $J_{P,C}$ 4.40 Hz), Ser Cα; 57.4, Val Cα; 59.1, Pro Cδ; 65.4, Glu^{5,6} Bzl Cb; 67.7 (d, $J_{P,C}$ 5.49 Hz), Ser Cβ; 78.1, Boc Cq; 119.9 (d, $J_{P,C}$ 4.39 Hz), Ph C2; 125.5, Ph C4; 127.8 and 128.3, Ar C2,3,4; 130.0, Ph C3; 136.1, Ar C1; 149.9 (d, $J_{P,C}$ 7.69 Hz), Ph C1; 153.3, Boc CO; 167.6, 170.5, 170.7 and 171.1, Ser, Val and Glu^{5,6} CO; 172.0, Pro CO; 172.1 ($\times 2$), Glu^{5,6} γ-CO; 172.6, Ala CO. δ (^{31}P) {$(CD_3)_2SO$} -12.0.

Boc - Val - Pro - Ala - Ser(PO_3Ph_2) - Val - Glu(OBzl) - Glu(OBzl)-NHMe. Boc-Val-OH (0.364 g, 1.67 mmol)/THF (3 mL); NMM (0.169 g, 1.67 mmol)/THF (1 mL); IBCF (0.216 g, 1.55 mmol)/THF (1 mL); hexapeptide·TFA (0.31 mmol) and NMM (0.031 g, 0.31 mmol)/DMF (2 mL). Aqueous precipitation. White solid (two products) (0.352 g). δ (^{31}P) {$(CD_3)_2SO$} -11.08 and -12.14.

Boc - Ile - Val - Pro - Ala - Ser(PO_3Ph_2) - Val - Glu(OBzl)-Glu(OBzl)-NHMe. Boc-Ile-OH (0.187 g, 0.808 mmol)/THF (3 mL); NMM (0.082 g, 0.808 mmol)/THF (1 mL); IBCF (0.102 g, 0.75 mmol)/THF (1 mL); crude heptapeptide·TFA (0.15 mmol) and NMM (0.015 g,

0.15 mmol)/DMF (2 mL). Aqueous precipitation. White solid (two products) (0.185g). δ (^{31}P) {$(CD_3)_2SO$} -12.02.

Ile-Val-Pro-Ala-Ser(P)-Val-Glu-Glu-NHMe·TFA. A rapidly stirred solution of crude octapeptide (0.090 g) in TFA/AcOH (1:1) (4 mL) containing 80% platinum oxide (0.041 g, 0.145 mmol) was hydrogenated at atmospheric pressure until hydrogen uptake ceased (60 min). The platinum was removed by gravity filtration, washed with TFA (4 mL) and the solvent evaporated under reduced pressure. The residue was triturated with diethyl ether (3×20 mL) and the crude product (50.1 mg) was purified by semi-preparative HPLC using a 0–80% CH_3CN linear gradient over 20 min. Lyophilization of the solution gave pure Ile-Val-Pro-Ala-Ser(*P*)-Val-Glu-Glu-NHMe·TFA as a fluffy white powder (20.3 mg). δ (^{13}C) (D_2O) (90 MHz) 10.3, 14.1, 16.2, 17.5, 18.2, 18.3, 23.9, 24.8, 25.7, 25.8, 26.0, 29.76, 29.84, 36.4, 48.4, 50.3, 53.1, 54.5 (d, $J_{P,C}$ 7.69 Hz), 57.1, 57.4, 59.9, 60.3, 63.9 (br d), 169.2, 171.2 ($\times 2$), 173.1, 173.4 ($\times 2$), 173.9, 175.4, 174.7 ($\times 2$). δ (^{31}P) (D_2O) (100 MHz) $+0.09$. FAB-MS ($+$ve) m/z 936 (expected m/z 936).

Antisera

Affinity purified rabbit anti-casein antibodies were purchased from Calbiochem-Behring (La Jolla, CA). Affinity purified goat anti-rabbit IgG(H + L)-HRP conjugate was purchased from Bio-Rad Laboratories (Sydney, Australia).

Competitive ELISA

A competitive ELISA was performed according to the modified method of Otani *et al*. [20]. Nunc-Immuno maxiSorp modules were coated with 100 μL of 2 μg/mL α_{s1}-casein or 100 μg/mL α_{s1}-casein(59–79) (as indicated) in 50 mM Tris–HCl pH 7.4, 200 mM NaCl (TBS) buffer for a minimum of 16 h at 4°C. The modules were then blocked by adding 100 μL of TBS containing 0.05% w/v Tween 20 (TBST) and 2% v/v NGS to the antigen solution [21] for a minimum of 16 h at 4°C. Anti-casein antibody appropriately diluted with TBST containing 1% v/v NGS and 2% w/v polyvinylpyrolidone (antibody diluting buffer) was gently mixed with the same volume of the competing peptides in microcentrifuge tubes and incubated at 37°C for 2 h and then overnight at 4°C. The modules were washed twice with TBST and placed on ice. Aliquots (100 μL) of each mixture of antibody and competing peptide (in triplicate) were added to each well and incubated for 2 h at room temperature. The modules were washed three times with TBST and 100 μL of an appropriately diluted HRP conjugated second antibody was added per well. After incubation for 60 min at 37°C, the modules were washed five times with TBST and 200 μL of substrate solution containing 10 mg/mL 3,3′,5,5′-tetramethylbenzidine (TMB) dissolved in dimethyl sulphoxide, diluted 1/100 with 0.1 M sodium acetate/citric acid buffer pH 6.0 and 0.004% v/v H_2O_2 was added per well [22]. Alternatively the substrate solution contained 0.4 mg/mL o-phenylenediamine (OPD) in 0.1 M citrate/phosphate buffer pH 5.0 and 0.015% v/v H_2O_2. The enzyme reaction was stopped after 30 min incubation at room temperature (in the dark) by the addition of 50 μL of 2 M H_2SO_4 per well. The reaction product formed per well was measured spectrophotometrically (450 nm (TMB) or 490 nm (OPD)) with a Bio-Rad 450 Microplate Reader.

The results of the competitive ELISA were expressed as % inhibition of binding of the antibody to the adsorbed antigen on the wells by competing peptide antigens in solution.

$$\%\ \text{Inhibition} = \frac{\text{Maximum OD} - \text{Test OD}}{\text{Maximum OD} - \text{Minimum OD}} \times 100$$

Maximum OD was absorbance obtained with no competing antigen. Minimum OD was obtained by the addition of the second antibody only to wells coated with antigen. Other controls included the addition of the second antibody to blocked wells containing no adsorbed antigen. All data were analysed using a Wilcoxon rank sum test [23].

RESULTS

Preparation of Synthetic Peptides

The four non-phosphorylated peptides, Gln-Met-Glu-Ala-Glu, Glu-Glu-Ile-Val-Pro-Asn, Ile-Val-Pro-Asn-Ser-Val-Glu-Glu and Ile-Val-Pro-Ala-Ser-Val-Glu-Glu, were readily prepared by automated Fmoc/solid phase peptide synthesis using HMP-resin as the polymer support and DCC/HOBt activation. Analytical C_8-HPLC of the four isolated products showed the target peptide to be the major component (Figure 1, Panels A–D) with both the Asn-containing peptides being contaminated with the dehydrated Ala(CN)-containing derivatives. The four peptides were readily purified to homogeneity by semipreparative C_{18}-RP-HPLC and their structure confirmed by FAB-MS and ^{13}C-NMR spectroscopy.

The synthesis of the four Ser(*P*)-containing peptides, Glu-Ala-Glu-Ser(*P*)-Ile-NHMe, Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Glu-NHMe, Ile-Val-Pro-Ala-Ser(*P*)-Val-Glu-Glu-NHMe and Ac-Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-NHMe, was accomplished by the use of Boc-Ser(PO_3Ph_2)-OH in the Boc/solution phase peptide synthesis [24,25] (mixed anhydride) followed by platinum-mediated hydrogenolysis of the protected Ser(PO_3Ph_2)-containing peptides in TFA/AcOH solution (detailed procedures for the synthesis of Ac-Ser(*P*)-Ser(*P*)-NHMe [26], Ac-Ser(*P*)-Ser(*P*)-Ser(*P*)-NHMe [26] and Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-NHMe [25], have been described previously). In the case of Glu-Ala-Glu-Ser(*P*)-Ile-NHMe, peptide assembly was straightforward with product yields of 93.1, 77.7, 98.9 and 91.8% being obtained from successive Boc-Ser(PO_3Ph_2)-OH, Boc-Glu(OBzl)-OH, Boc-Ala-OH and Boc-Glu(OBzl)-OH couplings to Leu-NHMe. Similarly, peptide assembly for Ac-Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-NHMe proceeded efficiently with the use of 3 equivalents of acylating reagent and was performed as previously described for Ac-Glu-Ser(*P*)-Leu-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-NHMe [24] except that Boc-Ile-OH was substituted for Boc-Leu-OH during peptide assembly (product yields of 96.5, 95.7, 95.2 and 99.6%). RP-HPLC analysis of both isolated phosphopeptides showed greater than 95% purity (Figure 2, Panel A) and demonstrated the efficiency of the solution-based approach for the clean synthesis of Ser(*P*)-containing peptides. Both Glu-Ala-Glu-Ser(*P*)-Ile-NHMe and Ac-Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-NHMe were purified to homogeneity by semi-preparative C_{18}-HPLC and their respective FAB mass spectrum displayed the

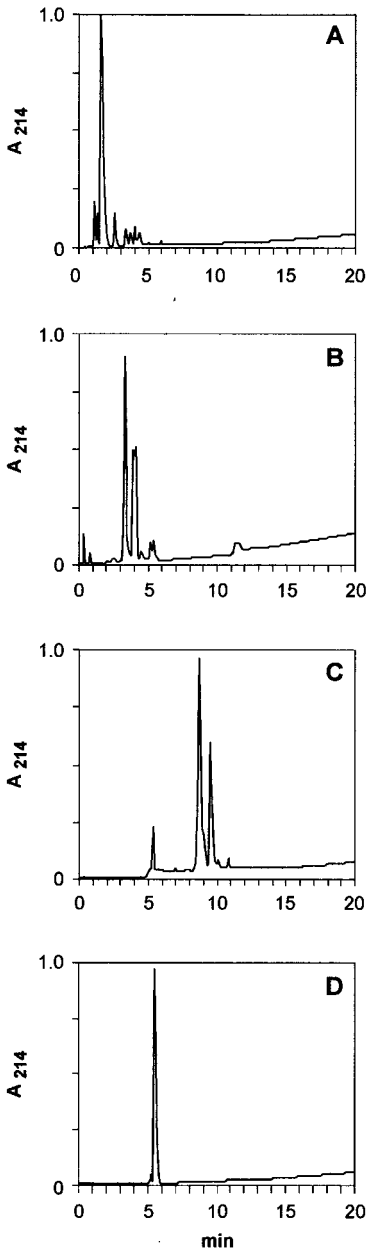

Figure 1 Analytical RP-HPLC traces (0–80% CH_3CN over 20 min) obtained for Gln-Met-Glu-Ala-Glu (Panel A), Glu-Glu-Ile-Val-Pro-Asn (Panel B), Ile-Val-Pro-Asn-Ser-Val-Glu-Glu (Panel C) and Ile-Val-Pro-Ala-Ser-Val-Glu-Glu (Panel D).

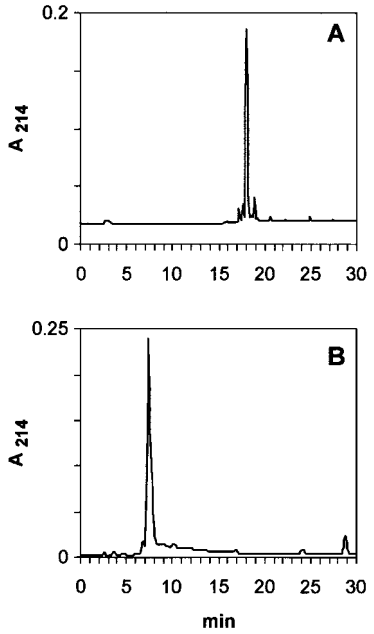

Figure 2 Analytical RP-HPLC traces (0–32% CH_3CN over 32 min) obtained for Glu-Ala-Glu-Ser(*P*)-Ile-NHMe (Panel A) and Ac-Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-NHMe (Panel B).

expected $[M + H]^+$ ion at m/z 641 and 1241, respectively. The presence of the Ser(*P*)-residue in each peptide was confirmed from their ^{13}C-NMR spectra which showed the characteristic phosphorus-coupled doublet resonance for the Cα-carbon of the Ser(*P*)-residue.

In contrast to the straightforward synthesis of the above Ser(*P*)-peptides, the Boc/solution-phase syntheses of Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Glu-NHMe and Ile-Val-Pro-Ala-Ser(*P*)-Val-Glu-Glu-NHMe were problematic due to by-product formation during both the Boc-Asn-OH and Boc-Val-OH couplings. As chromatographic purification of the protected penta- and hexa-peptides was precluded by their low solubility, peptide assembly was continued using the impure protected Ser(PO_3Ph_2)-peptides with the intention of separating the by-products from the target Ser(*P*)-peptide by semi-preparative HPLC. After platinum-mediated hydrogenolytic deprotection of the Ser(PO_3Ph_2)-containing peptides in TFA/AcOH, the RP-HPLC traces obtained for crude Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Glu-NHMe and Ile-Val-Pro-Ala-Ser(*P*)-Val-Glu-Glu-NHMe showed the Ser(*P*)-peptide as the main product along with significant later-eluting by-products (Figure 3, Panels A and B). Both Ser(*P*)-peptides were readily purified by semi-preparative C_{18}-HPLC and gave the expected $[M + H]^+$ ions at m/z 980 and 936, respectively. In addition to HPLC analysis, the purity of each Ser(*P*)-containing peptide was confirmed by capillary electrophoresis prior to epitope analysis studies [18,27]

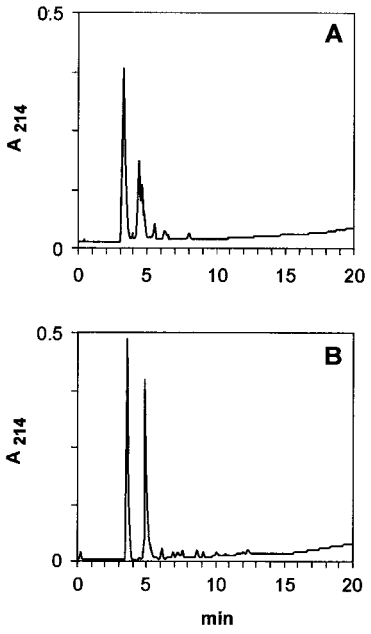

Figure 3 Analytical RP-HPLC traces (0–80% CH_3CN over 20 min) obtained for Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Glu-NHMe (Panel A) and Ile-Val-Pro-Ala-Ser(*P*)-Val-Glu-Glu-NHMe (Panel B).

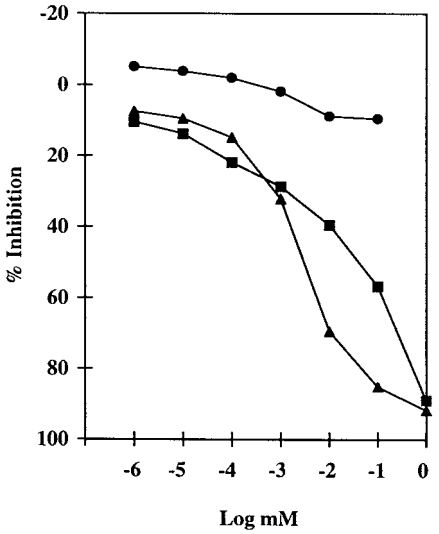

Figure 4 Inhibition of anti-casein antibody binding to the adsorbed antigen α_{s1}-casein(59–79) by α_{s1}-casein(59–79) and β-casein(1–25). The wells were coated with 100 μg/mL α_{s1}-casein(59–79). The anti-casein antibody (1/90000) was preincubated with an equal volume of competing antigen at the specified concentration. The bound antibody was determined using HRP-conjugated second antibody(1/2000) and 3,3′5,5′-TMB. ●, BSA; ■, β-casein(1–25); ▲, α_{s1}-casein(59–79). Each point represents a mean of triplicate values with the coefficient of variation less than 10%. β-casein(1–25): Arg^1-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser(*P*)-Leu-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-Ser-Ile-Thr-Arg^{25}.

Epitope Analysis

In a competitive ELISA with α_{s1}-casein(59–79) as the adsorbed antigen the multiphosphorylated peptide β-casein(1–25) was shown to compete with α_{s1}-casein(59–79) for anti-casein antibody binding (Figure 4). Examination of the amino acyl sequences of α_{s1}-casein(59–79) and β-casein(1–25) revealed that the only region of sequence similarity comprises the residues including the phosphoseryl cluster sequence, Glu-Ser(*P*)-Ile/Leu-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu with Leu in place of Ile in the β-casein peptide.

Utilizing conditions established for the competitive ELISA with α_{s1}-casein(59–79) as the adsorbed antigen (Figure 4) we undertook a more detailed epitope analysis of the peptide α_{s1}-casein(59–79) using overlapping synthetic peptides as shown in

Table 1. The peptide [Glu^{78}]α_{s1}-casein(71–78) was synthesised and used instead of α_{s1}-casein(71–78), where residue 78 is a Gln, due to the difficulties encountered in synthesis of α_{s1}-casein(71–78) and the consideration that the substitution of Gln^{78} for Glu^{78} at the C-terminus was unlikely to affect the antigenicity of the peptide. Only two of the five overlapping synthetic peptides at 1.0 mM significantly inhibited binding of the anti-casein antibodies (Table 1). Peptide α_{s1}-casein(63–70) containing the phosphoseryl cluster sequence inhibited binding by $20.0 \pm 3.6\%$ and peptide [Glu^{78}]α_{s1}-casein(71–78) by $60.3 \pm 7.9\%$. The other synthetic peptides α_{s1}-casein(59–63), α_{s1}-casein(61–65), and α_{s1}-casein(69–74) including the non-phosphorylated counterpart of α_{s1}-casein(63–70) (-Glu-Ser-Ile-Ser-Ser-Ser-Glu-Glu-) at 1.0 mM (data not shown) did not compete for antibody binding as there was no significant difference between the inhibition produced by these peptides and the slight inhibition obtained with 0.1 mM BSA ($9.6 \pm 1.3\%$).

The phosphoamino acid, phosphoserine, did not compete in the ELISA at 1.0 mM and even at concentrations up to 54 mM, similarly no competitive inhibition was found with the dipeptide Ser(*P*)-Ser(*P*) at concentrations up to 5 mM. The tripeptide Ser(*P*)-Ser(*P*)-Ser(*P*), however, did significantly compete giving $29.5 \pm 7.4\%$ inhibition at 1.0 mM and the pentapeptide Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu also significantly inhibited binding by $27.7 \pm 9.0\%$ at the same concentration (Table 2). The single substitution of Ser(*P*)^{75} with Ser^{75} at position 75 in the peptide [Glu^{78}]α_{s1}-casein(71–78) resulted in complete loss of competitive inhibition (Table 3). Further, the substitution of Asn^{74} with Ala^{74} produced a reduction in inhibition to $21.0 \pm 1.9\%$ from $60.3 \pm 7.9\%$ (Table 3).

Table 1 Antigenicity of Overlapping Synthetic Peptides of α_{s1}-Casein(59–79)

Peptide[a]	Sequence[b]	% Inhibition
α_{s1}-Casein (59–63)	QMEAE	10.0 ± 1.7[c]
α_{s1}-Casein (61–65)	EAEΣI	10.9 ± 0.2[c]
α_{s1}-Casein (63–70)	EΣIΣΣΣEE	20.0 ± 3.6[d]
α_{s1}-Casein (69–74)	EEIVPN	10.8 ± 1.0[c]
[Glu^{78}]α_{s1}-casein (71–78)	IVPNΣVEE	60.3 ± 7.9[e]

[a] All peptides used at 1.0 mM ($n = 4$–7).
[b] One letter code where Σ denotes an *O*-phosphoseryl residue.
[c] Not significantly different to the inhibition obtained with 0.1 mM BSA ($9.3 \pm 1.6\%$).
[d,e] Significantly different ($p < 0.01$) to other values not similarly marked and also to the inhibition obtained with 0.1 mM BSA ($9.3 \pm 1.6\%$).

Table 2 Antigenicity of the Synthetic Peptide α_{s1}-Casein(63–70) and Corresponding Smaller Peptides

Peptide[a]	Sequence[b]	% Inhibition
Phosphoserine	Σ	10.0 ± 3.2[c]
Dipeptide	ΣΣ	10.9 ± 2.0[c]
α_{s1}-Casein(66–68)	ΣΣΣ	29.5 ± 7.4[d]
α_{s1}-Casein(66–70)	ΣΣΣEE	27.4 ± 9.0[d]
α_{s1}-Casein(63–70)	EΣIΣΣΣEE	20.0 ± 3.6[d]

[a] All peptides and phosphoserine used at 1.0 mM ($n = 4$–7).
[b] One letter code where Σ denotes an *O*-phosphoseryl residue.
[c] Not significantly different to the inhibition obtained with 0.1 mM BSA ($9.3 \pm 1.6\%$).
[d] Significantly different ($p < 0.05$) to other values not similarly marked and also to the inhibition obtained with 0.1 mM BSA ($9.3 \pm 1.6\%$).

DISCUSSION

The results of this study demonstrate that the multiphosphorylated peptide α_{s1}-casein(59–79) is rec-

Table 3 Antigenicity of the Synthetic Peptide [Glu^{78}]α_{s1}-Casein(71–77) and Analogues

Peptide[a]	Sequence[b]	% Inhibition
[Glu^{78}]α_{s1}-casein(71–78)	IVPNΣVEE	60.3 ± 7.9[c]
[Ala^{74}, Glu^{78}]α_{s1}-casein(71–78)	IVPAΣVEE	21.0 ± 1.9[d]
[Ser^{75}, Glu^{78}]α_{s1}-casein(71–78)	IVPNSVEE	12.0 ± 3.1[e]
[Ala^{74}, Ser^{75}, Glu^{78}]α_{s1}-casein(71–78)	IVPASVEE	10.7 ± 3.4[e]

[a] All peptides used at 1.0 mM ($n = 4$–7).
[b] One letter code where Σ denotes an *O*-phosphoseryl residue.
[c,d] Significantly different ($p < 0.05$) to values not similarly marked and also to the inhibition obtained with 0.1 mM BSA ($9.3 \pm 1.6\%$).
[e] Not significantly different to the inhibition obtained with 0.1 mM BSA ($9.3 \pm 1.6\%$).

ognized by anti-casein antibodies in a competitive ELISA. The multiple phosphoseryl-containing peptide β-casein(1–25) competed with α_{s1}-casein(59–79) for the anti-casein antibodies. The only common sequence in these peptides is the phosphoseryl cluster sequence -Glu-Ser(*P*)-Ile/Leu-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu- suggesting that this sequence is antigenic. Overlapping synthetic peptides of α_{s1}-casein(59–79) confirmed the antigenicity of this multiple phosphoseryl sequence and also the C-terminal region -Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-. The epitope on the peptide containing the multiple phosphoseryl residues was further localized to the three contiguous phosphoseryl residues 66, 67 and 68, as the tripeptide Ser(*P*)-Ser(*P*)-Ser(*P*), but not the dipeptide Ser(*P*)-Ser(*P*) nor the phosphoamino acid Ser(*P*) inhibited antibody binding to α_{s1}-casein(59–79) at an equivalent level to that obtained with the longer peptide α_{s1}-casein(63–70). This, to our knowledge, is the first demonstration of an epitope containing multiple phosphoseryl residues.

The other antigenically active synthetic peptide [Glu^{78}]α_{s1}-casein(71–78) also contained a critical phosphoseryl residue. The critical nature of the phosphorylated serine in [Glu^{78}]α_{s1}-casein(71–78) was demonstrated by the lack of antibody recognition upon substituting Ser(*P*)^{75} with Ser^{75}. The amino acyl sequence of α_{s1}-casein(71–77) Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu, is also repeated in α_{s1}-casein at residues 111–117 in the sequence Ile-Val-Pro-Asn-Ser(*P*)-Ala-Glu except for a Val to Ala substitution at position 116. Ametani *et al.* [9,10] have screened antigenically active fragments of α_{s1}-casein using conventional ELISA and mouse anti-α_{s1}-casein antibodies and demonstrated that the tryptic peptide α_{s1}-casein(106–119) Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-Ser(*P*)-Ala-Glu-Glu-Arg is antigenic. These results are therefore consistent with our findings and would suggest that the epitope on α_{s1}-casein(106–119) identified by Ametani *et al.* [9,10] involves the repeated sequence and that Ser(*P*)^{115} would be critical for antibody binding. Ametani *et al.* [9,10] did not identify α_{s1}-casein(59–79) as an antigenic fragment in their studies. This peptide is not recognised by anti-casein antibodies when used as an adsorbed antigen on conventional microtitre plates at normal coating concentrations, which has been attributed to poor antigen adsorption [14].

Enomoto *et al.* [28] and Shon *et al.* [29] have investigated the location of T-cell and B-cell determinants of whole α_{s1}-casein using synthetic peptides and reported the unphosphorylated peptide α_{s1}-casein(61–80) to be a T-cell determinant; however, antibodies raised to whole α_{s1}-casein did not recognise the same peptide. As the unphosphorylated counterparts of these multiphosphorylated sequences were screened in these studies, antibodies specific for phosphoseryl residues would not have been detected.

Antigenic peptides that cross-react with antibodies raised against a whole protein are frequently observed to be structured in solution [30–33]. We have recently reported NMR evidence that the tryptic phosphopeptide α_{s1}-casein(59–79) exhibits folding in solution. In particular, there is NMR evidence of a loop in the region comprising -Glu^{61}-Ala-Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)^{68}-and β-turns in the region -Ile^{71}-Val-Pro-Asn-Ser(*P*)-Val^{26}- [13]. In the case of β-casein(1–25), there is also NMR evidence of a turn in the region -Ser(*P*)^{17}-Ser(*P*)-Ser(*P*)-Glu-Glu^{21}- (Huq, Cross and Reynolds, unpublished). Hence, the data of the current study together with the previous NMR results demonstrate the presence of an epitope in the peptide α_{s1}-casein(59–79) involving phosphorylated seryl residues in loop or turn structures.

Smith *et al.* [34] have identified an epitope for a monoclonal antibody on the phosphorylated form of phenylalanine hydroxylase and showed that the phosphoseryl residue in the sequence Leu-Ser(*P*)-Asp-Phe-Gly was critical for antibody recognition, as the dephosphorylated form did not bind antibody. The monoclonal antibody did not bind the amino acid, phosphoserine and only bound weakly to phosphoseryl residues in other proteins (e.g. casein) suggesting that other structural features beside the phosphoseryl residue were important for high affinity binding. Levine *et al.* [35] were the first to produce antibodies specific for *O*-phosphorylated seryl residues by immunising with the phosphoamino acid as a hapten coupled to bovine serum albumin. The authors claimed the antibodies detected phosphoseryl residues in commercial preparations of α-, β- and κ-caseins. Although there are prior studies showing that a phosphoseryl residue can be critical for antibody recognition the results of this current paper represent the first demonstration of a cluster of phosphoseryl residues being involved in an antigenic determinant.

Acknowledgements

This study was supported by the Australian National Health and Medical Research Council Re-

search Grant 950568. JWP acknowledges an ARC Research Fellowship.

REFERENCES

1. T.P. Hopp and K.R. Woods (1981). Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA 787*, 3824–3828.
2. E. Westhof, D. Altschuh, D. Moras, A.C. Bloomer, A. Mondragon, A. Klug and M.H.V. Van Regenmortel (1984). Correlation between segmental mobility and the location of antigenic determinants in proteins. *Nature 311*, 123–127.
3. D.G. Reid, A. Salisbury, T. Brown and D.H. Williams (1985). Conformations of two duplex forms of d(TCGA) in slow-exchange equilibrium characterized by NMR. *Biochemistry 24*, 4325–4332.
4. J. Novotny, M. Handschumacher and E. Haber (1986). Location of antigenic epitopes on antibody molecules. *J. Mol. Biol. 189*, 715–721.
5. J.J. Nestor Jr., J.G. Moffat and H.W. Chan (1985). Synthetic peptide sequences useful in biological and pharmaceutical applications and methods of manufacture. US Patent No. 4493795.
6. D. Small, P.Y. Chou and G.D. Fasman (1977). Occurrence of phosphorylated residues in predicted β-turns. Implications for β-turns participation in control mechanisms. *Biochem. Biophys. Res. Commun. 79*, 341–346.
7. C. Holt and M.J.J.M. Van Kemenade in: *Calcified Tissue*, D.W.L. Hukins Ed., p. 175–213, CRC Press, Florida, 1989.
8. B. Prescott, V. Renugopalakrishnan, M.J. Glimcher, A. Bhushan and J.G.J. Thomas (1986). A raman spectroscopic study of hen egg yolk phosvitin: structures in solution and in the solid state. *Biochemistry 25*, 2792–2798.
9. A. Ametani, S. Kaminogawa, M. Shimizu and K.Yamauchi (1987). Rapid screening of antigenically reactive fragments of α_{s1}-casein using HPLC and ELISA. *J. Biochem. 102*, 421–425.
10. A. Ametani, S.M. Kim, S. Karninogawa and K. Yamauchi (1988). Antibody response of three different strains of mice to α_{s1}-casein analysed by using proteolytic and synthetic peptides. *Biochem. Biophys. Res. Commun. 154*, 876–882.
11. C.M. Wilmot and J.M. Thornton (1988). Analysis and prediction of the different types of β-turn in proteins. *J. Mol. Biol. 203*, 221–232.
12. C.M. Wilmot and J.M. Thornton (1990). β-Turns and their distortions: a proposed new nomenclature. *Protein Eng. 3*, 479–493.
13. N.L. Huq, K.J. Cross and E.C. Reynolds (1995). A ^{1}H NMR study of the casein phosphopeptide α_{s1}-casein(59–79). *Biochim. Biophys. Acta 1247*, 201–208.
14. C.L. Black and E.C. Reynolds (1998). An ELISA for a multiphosphorylated peptide, α_{s1}-casein(59–79). *J. Immunol. Methods 214*, 63–71.
15. C.A. Zittle and J.H. Custer (1963). Purification and some of the properties of α_{s1}-casein and κ-casein. *J. Dairy Sci. 46*, 1183– 1189.
16. E.C. Reynolds (1987). The prevention of sub-surface demineralization of bovine enamel and change in plaque composition by casein in an intra-oral model. *J. Dent. Res. 66*, 1120–1127.
17. E.C. Reynolds (1991). Anticariogenic phosphopeptides. US Patent No. 5015628.
18. E.C. Reynolds, P. Riley and N.J. Adamson (1994). A selective precipitation purification procedure for multiple phosphoseryl-containing peptides and methods for their identification. *Anal. Biochem. 217*, 277–284.
19. J. Meienhofer in: *The Peptides: Analysis, Synthesis, Biology*, E. Gross and J. Meienhofer Eds, Vol 1, p. 263–314, Academic Press, New York, 1979.
20. H. Otani, F. Takahashi and F. Tokita (1986). Antigenic reactivities of fragments obtained by cyanogen bromide cleavage of bovine α_{s1}-casein toward antiserum to intact α_{s1}-casein. *Agric. Biol. Chem. 50*, 607–613.
21. M.J. Ianconescu (1985). Simultaneous screening of two different antibodies in ELISA by combining two solid phases: microtiter plate and Falcon assay screening test system lid. *J. Immunol. Methods 76*, 307–316.
22. E.S. Bos, A.A. van der Doelen, N. van Rooy and A.H. Schuurs (1981). 3,3′,5,5′-Tetramethylbenzidine as an Ames test negative chromogen for horse-radish peroxidase in enzyme-immunoassay. *J. Immunoassay 2*, 187–204.
23. M.J. Norusis, *SPSS for Windows: Base System User's Guide*, Release 6.0, SPSS Inc., IL, USA, 1993.
24. J.W. Perich, R.B. Johns and E.C. Reynolds (1992). Synthesis of casein-related peptides and phosphopeptides. XVI. The efficient synthesis of the casein-related *O*-phosphoseryl-containing peptide Ac-Glu-Ser(*P*)-Leu-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-NHMe. *Aust. J. Chem. 45*, 385–394.
25. J.W. Perich, D.P. Kelly and E.C. Reynolds (1992). Efficient solution-phase synthesis of multiple *O*-phosphoseryl-containing peptides related to casein and stratherin. *Int. J. Pept. Protein Res. 40*, 81–88.
26. J.W. Perich and R.B. Johns (1990). The efficient global 'phosphite-triester' phosphorylation of multiple serine-containing peptides using dibenzyl *N*,*N*-diethylphosphoramidite. *Aust. J. Chem. 43*, 1633–1642.
27. N.J. Adamson, P.F. Riley and E.C. Reynolds (1993). The analysis of multiple phosphoseryl-containing casein peptides using capillary zone electrophoresis. *J. Chromatogr. 646*, 391–396.
28. A. Enomoto, D.-H. Shon, Y. Aoki, K. Yamauchi and S. Kaminogawa (1990). Antibodies raised against peptide fragments of bovine α_{s1}-casein cross-react with the intact protein only when the peptides contain both B and T cell determinants. *Mol. Immunol. 27*, 581–586.

29. D.-H. Shon, A. Enomoto, K. Yamauchi and S. Karninogawa (1991). Antibodies raised against peptide fragments of bovine α_{s1}-casein cross-react with the native protein, but recognize sites distinct from the determinants on the protein. *Eur. J. Immunol. 21*, 1475–1480.
30. H.J. Dyson, K.J. Cross, R.A. Houghten, L.A. Wilson, P.E. Wright and R.A. Lerner (1985). The immunodominant site of a synthetic immunogen has a conformational preference in water for a type-II reverse turn. *Nature 318*, 480–483.
31. H.J. Dyson, K.J. Cross, J. Ostresh, R.A. Houghten, I.A. Wilson, P.E. Wright and R.A. Lerner. *Synthetic Peptides as Antigens*, 119 CIBA Foundation, Wiley, London, 1986.
32. H.J. Dyson, M. Rance, R.A. Houghten, P.E. Wright and R.A. Lerner (1988). Folding of immunogenic peptide fragments of proteins in water solution. II. The nascent helix. *J. Mol. Biol. 201*, 201–217.
33. H.J. Dyson, M. Rance, R.A. Houghten, R.A. Lerner and P.E. Wright (1988). Folding of immunogenic peptide fragments of proteins in water solution. I. Sequence requirements for the formation of a reverse turn. *J. Mol. Biol. 201*, 161–200.
34. S.C. Smith, W.J. McAdarn, B.E. Kemp, F.J. Morgan and R.G.H. Cotton (1987). A monoclonal antibody to the phosphorylated form of phenylalanine hydroxylase. Definition of the phosphopeptide epitope. *Biochem. J. 244*, 625–631.
35. L.L. Levine, H.B. Gjika and H. Van Vunakis (1989). Antibodies and radioimmunoassays for phosphoserine, phosphothreonine and phosphotyrosine. Serologic specificities and levels of the phosphoamino acids in cytoplasmic fractions of rat tissues. *J. Immunol. Methods 124*, 239–249.

