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The analysis of multiple phosphoseryl-containing casein peptides using capillary zone electrophoresis

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ABSTRACT

Multiple phosphoseryl-containing sequences of peptides and proteins stabilize amorphous calcium phosphate at neutral and alkaline pH and have been implicated in the nucleation/regulation of biomineralization. In an approach to analyze these peptides using capillary zone electrophoresis (CZE) we have attempted to relate the absolute electrophoretic mobility of various casein phosphopeptides to their physicochemical properties. Multiple phosphoseryl-containing peptides were selectively precipitated from enzymic digests of sodium caseinate and further purified using RP-HPLC and anion-exchange fast protein liquid chromatography. Purified fractions were then analyzed by CZE. Absolute electrophoretic mobilities of 13 peptides were determined by measurement of migration times relative to that of a neutral marker, mesityl oxide. A linear relationship ($r^2 = 0.993$) was obtained between absolute electrophoretic mobility and $q/M_r^{2/3}$ where q is the net negative charge of the peptide calculated using relevant pK_a values and M_r is the molecular mass. $M_r^{2/3}$ is a measure of the surface area of a sphere that has a volume proportional to the M_r of the peptide and relates to the frictional drag exerted on the peptide during electrophoretic migration. As absolute electrophoretic mobility is influenced by charge and size CZE can be used to monitor peptide phosphorylation, dephosphorylation, deamidation and truncation. This technique therefore would be suitable for quantitative analysis of peptide substrates in kinase and phosphatase studies. In conclusion CZE is a rapid and efficient technique for the resolution of multiple phosphoseryl-containing peptides from enzymic digests of casein.

INTRODUCTION

Multiple phosphoseryl-containing sequences of peptides and proteins stabilize amorphous calcium phosphate at neutral and alkaline pH and have been implicated in the nucleation/regulation of biomineralization [1–3]. Casein tryptic peptides containing multiple phosphoseryl residues have potential as toothpaste, mouthwash and food additives for the prevention of dental caries [4]. The structure of these sequences and the relationship between structure and function, particularly in calcium phosphate stabilization, anticariogenicity and biomineralization is as yet unclear. To obtain insight into the structure and function of these peptides it is first essential to develop analytical techniques for their purification and characterization.

Capillary zone electrophoresis (CZE) has been used extensively in the separation and characterization of proteins and peptides. The advantages of CZE are high resolution, rapid automated analyses and minimal sample requirements [5–11]. CZE has also shown potential as a useful tool for the determination of structure when used to exploit the orientation of molecules in free solution under the influence of an electric field [12]. Relatively few workers however have attempted to relate the absolute electrophoretic mobilities (μ) of peptides and proteins to their physicochemical properties. Offord [13] has shown a linear relationship between

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paper electrophoretic mobilities of a series of charged peptides and their molecular mass to the power of $-2/3$. Grossman *et al.* [5] proposed a semi-empirical model which related the electrophoretic mobilities of a range of positively charged peptides to their size, charge and hydrophobicity. The effects of size and charge were determined independently and then combined to show a linear relationship between μ and $\ln(q+1)/n^{0.43}$ where n is the number of amino acids in the polypeptide chain and q is the net negative charge of the peptide. Rickard *et al.* [11] using CZE have recently shown a linear correlation of absolute electrophoretic mobility for various peptides with $q/M_r^{2/3}$ over a range of pH values. The correlation was the best of three relationships investigated proving more suitable than mobility against $q/M_r^{1/3}$ or $q/M_r^{1/2}$.

Absolute electrophoretic mobility (μ) is proportional to the net charge and inversely proportional to the frictional properties of a molecule, the latter being determined by its size and shape [11,12]. Assuming that the structure of the peptides in the capillary can be approximated by a sphere of constant density, then the radius of the sphere would be proportional to the molecular mass raised to the 1/3 power ($M_r^{1/3}$). Hence, if frictional drag is governed by Stokes law then absolute electrophoretic mobility would be related to the radius of the species *i.e.* proportional to $M_r^{1/3}$ [11]. However, if frictional drag is related to the surface area (or cross-sectional area) of the molecule absolute electrophoretic mobility would be proportional to $M_r^{2/3}$ [13].

From studies of synthetic polymers it has been shown that the average radius of gyration is proportional to the square root of the number of polymer units multiplied by the length of a single unit [14]. Rickard *et al.* [11] concluded from this that if the frictional drag were proportional to the average radius of gyration it would be proportional to the square root of the number of residues (*ca.* $M_r^{1/2}$).

In an approach to characterize multiple phosphoseryl-containing peptides using capillary zone electrophoresis we have related the absolute electrophoretic mobility of various casein phosphopeptides to their physicochemical properties.

MATERIALS AND METHODS

Sodium caseinate was obtained from Murray Goulburn (Melbourne, Australia). Trypsin (E.C. 3.4.21.4) was purchased from Sigma (St. Louis, MO, USA) and pancreatin from Southern Cross Laboratories (Dural, NSW, Australia). Mesityl oxide was obtained from BDH (Melbourne, Australia), disodium tetraborate from AJAX (Sydney, Australia) and trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA). All other chemicals were of the highest purity analytical grade available.

Preparation of multiple phosphoseryl-containing casein peptides

Multiple phosphoseryl-containing peptides were selectively precipitated from enzymic digests of casein using calcium and ethanol as described previously [4,15]. Sodium caseinate [96% (w/w) protein, 20 mg/ml] was dissolved in 20 m*M* Tris–HCl pH 8.0 to which trypsin or pancreatin was added at 0.4 mg/ml. Hydrolysis was carried out at 50°C for 2 h and stopped by the addition of 1 *M* HCl to pH 4.6. Insoluble material was removed by centrifugation (12000 *g*, 15 min) and the multiple phosphoseryl-containing peptides selectively precipitated from the hydrolysate by the addition of $CaCl_2$ to 1.0% (w/v) and ethanol to 50% (v/v). The selectively precipitated casein phosphopeptides were further purified using reversed-phase HPLC (Brownlee AQUAPORE RP-300, 220 × 4.6 mm, C_8 column, ABI, Australia) and anion-exchange fast protein liquid chromatography (FPLC) (Mono Q HR 5/5, Pharmacia-LKB, Australia) and characterized using amino acid composition and sequence analyses [4].

The multiple phosphoseryl-containing casein peptides obtained and used in this study are listed in Table I together with their physicochemical properties.

Analysis of multiple phosphoseryl-containing casein peptides by capillary zone electrophoresis

Peptides were dissolved in Milli Q water or 0.1% TFA (1 mg/ml) and analyzed by capillary zone electrophoresis (CZE) using an Applied

TABLE I

PHYSICOCHEMICAL PROPERTIES AND ABSOLUTE ELECTROPHORETIC MOBILITIES OF VARIOUS MULTIPLE PHOSPHOSERYL-CONTAINING PEPTIDES FROM CASEIN TRYPTIC AND PANCREATIC DIGESTS

Peptide[a]	M_r [b]	Net negative charge[c] pH 9.2	μ[d] $(cm^2/Vs) \cdot 10^4$
β(1–25)	3125	13.91	3.223 ± 0.069
β(2–25)	2969	14.91	3.487 ± 0.111
α_{s1}(59–79)	2721	15.04	3.629 ± 0.017
[Glp^{59}]α_{s1}(59–79)	2721	15.07	3.660 ± 0.016
[Glu^{59}]α_{s1}(59–79), [Glu^{78}]α_{s1}(59–79)	2721	16.04	3.785 ± 0.015
α_{s2}(46–70)	3009	16.06	3.629 ± 0.017
α_{s2}(3–21)	2505	12.00	3.223 ± 0.069
α_{s2}(2–21)	2619	12.06	3.193 ± 0.009
β(7–24)	2200	12.99	3.596 ± 0.013
α_{s1}(61–76)	2076	14.91	4.082 ± 0.022
α_{s1}(64–75)	1648	12.99	4.236 ± 0.026
α_{s1}(61–75)	1977	14.91	4.267 ± 0.009

[a] Primary structure of peptides β(1–25), RELEELNVPGEIVESLSSSEESITR; α_{S1}(59–79), QMEAESISSSEEIVPNSVEQK; α_{S2}(46–70), NANEEEYSIGSSSEESAEVATEEVK; α_{S2}(2–21), NTMEHVSSSEESIISQETYK where S = Ser(P).

[b] Calculated using free acid residue masses.

[c] Calculated using Ser(P) pK_{a1} 1.5, pK_{a2} 6.48 [16]; Lys p$K_{a\varepsilon}$ 10.30; Arg pK_a(guanidino) 12.50 [17]; Glu p$K_{a\gamma}$ 4.85; Asp p$K_{a\beta}$ 4.85 [18]; N-terminus and C-terminus pK_a values from Rickard *et al.* [11].

[d] cm = Capillary length (72 cm), V = volts applied (30 kV), s = seconds.

Biosystems 270A instrument. Separation conditions consisted of 30 kV applied voltage at 30°C with 20 m*M* sodium tetraborate pH 9.2 as the running buffer. Samples were introduced at the anode into a capillary of length 72 cm by creating an intracapillary vacuum (17 kPa) for a specified time (0.5–1.0 s). Peptides were detected by UV absorbance at 200 nm using a variable wavelength detector situated 50 cm along the capillary. The instrument was hardwired to an IBM AT compatible PC and data were collected by a Galactic LAB CALC. data processing and acquisition software package. Absolute electrophoretic mobility of the purified peptides was calculated from measurement of migration time relative to that of a neutral marker, mesityl oxide. Absolute electrophoretic mobility, μ is defined as migration velocity per unit electrical field strength. Measurements made by CZE result in the determination of apparent electrophoretic mobility, μ_{app} which is dependent upon the electrophoretic migration of the analyte (μ) as well as the electroendosmotic flow of the buffer. μ_{app} is defined by the equations

$$\mu_{app} = \mu + \mu_{eo} \quad (1)$$

$$\mu_{app} = \nu_{net}/E = L_d L_t / t_m V \quad (2)$$

where μ_{eo} is the electroendosmotic component of the apparent mobility, ν_{net} is the net (measured) migration velocity, E is the electrical field strength, L_d is the length of the capillary from the injection end to the detector, L_t is the total length of the capillary, V is the applied voltage over that length, and t_m is the measured electrophoretic migration time in seconds. Similarly μ_{eo} is defined by the relationship

$$\mu_{eo} = \nu_{eo}/E = L_d L_t / t_{eo} V \quad (3)$$

where ν_{eo} is the electroendosmotic velocity and t_{eo} is the migration time for a neutral molecule whose motion through the capillary is due solely to electroendosmosis. Measurement of the migration time for a neutral molecule hence allows

the calculation of μ_{eo} from eqn. 3. Absolute electrophoretic mobility of each multiple phosphoseryl-containing casein peptide was calculated from its measured electrophoretic migration time using eqns. 1, 2 and 3. Net charge of the peptides at pH 9.2 was calculated using Ser(P) pK_{a1} 1.5, pK_{a2} 6.4 [16]; Lys $pK_{a\epsilon}$ 10.30; Arg pK_a (guanidino) 12.50 [17]; Glu $pK_{a\gamma}$ 4.85; Asp $pK_{a\beta}$ 4.85 [18]; N-terminus and C-terminus pK_a values from Rickard *et al.* [11].

RESULTS

Representative electropherograms obtained from CZE of selectively precipitated multiple phosphoseryl-containing peptides from a tryptic (A) and pancreatic (B) digest of casein are shown in Fig. 1. Thirteen peptides were purified from these selective precipitates using anion-exchange FPLC and reversed-phase HPLC and the electrophoretic migration times measured relative to that of a neutral marker, mesityl oxide using CZE. The peptides obtained were β(1–25) and truncated forms, α_{s1}(59–79) and its deamidated and truncated forms and minor amounts of

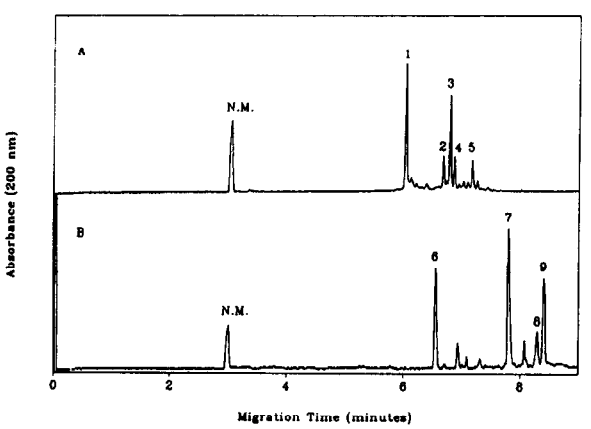

Fig. 1. Electropherogram from CZE of a mixture of multiple phosphoseryl-containing peptides purified from (A) casein tryptic and (B) pancreatic digests. Peak 1 = β(1–25) and α_{s2}(3–21); peak 2 = β(2–25); peak 3 = α_{s1}(59–79) and α_{s2}(46–70); peak 4 = [Glp^{59}]α_{s1}(59–79); peak 5 = [Glu^{59}]α_{s1}(59–79) and [Glu^{78}]α_{s1}(59–79); peak 6 = β(7–24); peak 7 = α_{s1}(61–76); peak 8 = α_{s1}(64–75) and peak 9 = α_{s1}(61–75). Peak "N.M." is the neutral marker peak, mesityl oxide. Conditions: 30 kV applied voltage, 30°C, 20 m*M* sodium tetraborate pH 9.2, capillary length = 72 cm, detection λ = 200 nm, detector 50 cm along capillary.

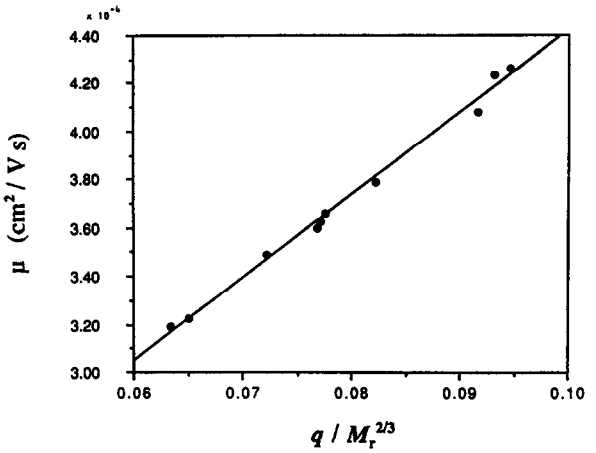

Fig. 2. The relationship between absolute electrophoretic mobility (μ) and $q/M_r^{2/3}$ for 13 multiple phosphoseryl-containing peptides purified from casein tryptic and pancreatic digests.

the multiple phosphoseryl-containing peptides from α_{s2}-casein, α_{s2}(46–70) and α_{s2}(2–21). The truncation presumably resulted through exopeptidase activity in the crude pancreatin. Absolute electrophoretic mobilities (μ) were calculated using the migration times obtained for the purified peptides and are presented in Table I along with the net negative charge and molecular mass of each peptide. Absolute electrophoretic mobility was related to $q/M_r^{2/3}$ (Fig. 2), $q/M_r^{1/2}$ (Fig. 3), $\ln(q+1)/n^{0.43}$ (Fig. 4) and $q/M_r^{1/3}$

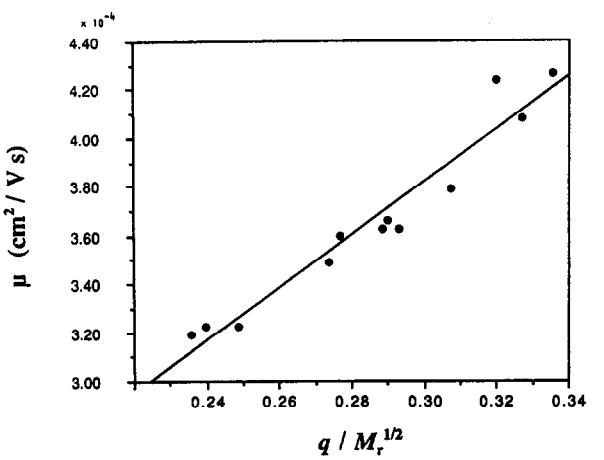

Fig. 3. The relationship between absolute electrophoretic mobility (μ) and $q/M_r^{1/2}$ for 13 multiple phosphoseryl-containing peptides purified from casein tryptic and pancreatic digests.

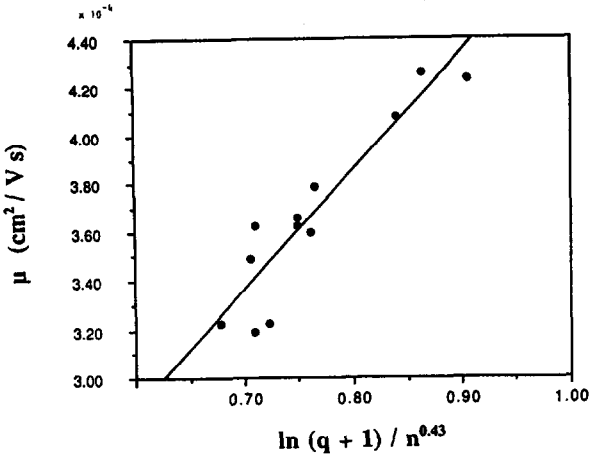

Fig. 4. The relationship between absolute electrophoretic mobility (μ) and $\ln(q+1)/n^{0.43}$ for 13 multiple phosphoseryl-containing peptides purified from casein tryptic and pancreatic digests.

(Fig. 5). All relationships were linear, however absolute electrophoretic mobility was most closely correlated ($r^2 = 0.993$) with $q/M_r^{2/3}$, followed by $q/M_r^{1/2}$ ($r^2 = 0.941$), $\ln(q+1)/n^{0.43}$ ($r^2 = 0.861$) and then $q/M_r^{1/3}$ ($r^2 = 0.755$).

DISCUSSION

In this study we have shown capillary zone electrophoresis (CZE) to be a rapid method for the resolution and characterization of multiple phosphoseryl-containing casein peptides. Analysis of a selective precipitate of phosphorylated peptides from a tryptic casein hydrolysate revealed baseline resolution of 5 major peaks (Fig. 1A) in less than 8 min. The peptides of this selective precipitate were purified using a combination of reversed-phase HPLC and anion-exchange FPLC into 8 different phosphopeptides however this proved a much more complex and time consuming procedure [4].

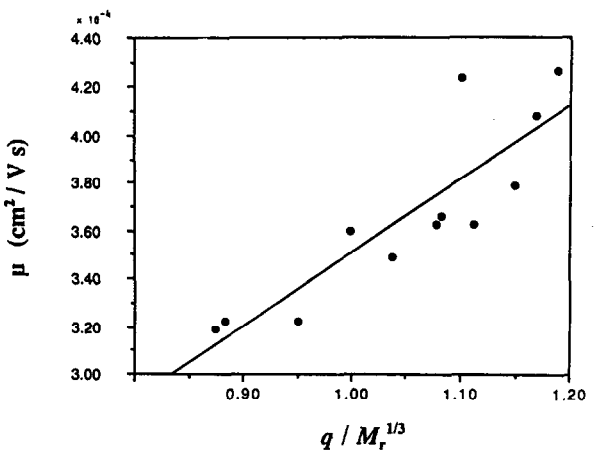

Fig. 5. The relationship between absolute electrophoretic mobility (μ) and $q/M_r^{1/3}$ for 13 multiple phosphoseryl-containing peptides purified from casein tryptic and pancreatic digests.

Absolute electrophoretic mobility (μ) of the multiple phosphoseryl-containing peptides was most closely correlated with $q/M_r^{2/3}$. Rickard *et al.* [11] also found that the absolute electrophoretic mobility of a set of non-phosphorylated peptides with isoelectric points of 3.35–13.10 correlated closely with $q/M_r^{2/3}$. The results presented here therefore confirm and extend this relationship to include multiple phosphoseryl-containing peptides with isoelectric points of 2.0 and less. It is interesting to note however that we obtained a closer association between, $q/M_r^{1/2}$ and μ with the casein phosphopeptides than did Rickard *et al.* [11] with non-phosphorylated peptides. Spectroscopy studies on multiple phosphoseryl-containing proteins, for example phosvitin [19] and phosphophoryn [20] indicate that multiple phosphorylation appears to destabilize secondary and tertiary structure promoting flexibility. Although little work has been done on the conformation of the multiple phosphoseryl-containing casein peptides some information is available on the structures of the caseins. Optical rotatiory dispersion, circular dichroism and [^{31}P]NMR measurements all indicate that α_{s1}-casein and β-casein have a rather open structure in solution with many amino acid side chains exposed to solvent and relatively flexible [21]. [^{31}P]NMR relaxation measurements indicate that Ser(P) residues are relatively mobile in β-casein [19]. Further, Chaplin *et al.* [22] have reported the secondary structure of the casein phosphopeptide β(1–28) to be composed of β-structure and random coil. It is possible that the better correlation obtained between μ and $q/M_r^{1/2}$ with multiple phosphoseryl-containing casein peptides relates to their relative flexibility during capillary migration compared with the

structures of the peptides studied by Rickard *et al.* [11].

The results of this study show that the absolute electrophoretic mobility of multiple phosphoseryl-containing peptides is influenced by charge and size such that CZE is an efficient technique for monitoring peptide phosphorylation, dephosphorylation, deamidation and truncation. The ability of CZE to rapidly resolve peptides which exhibit varying degrees of phosphorylation would make it ideal for the rapid and quantitative analysis of peptide substrates in kinase and phosphatase studies. In conclusion CZE is a rapid and efficient technique for the resolution of multiple phosphoseryl-containing peptides from a tryptic digest of casein.

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