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Analysis of phosphopeptides by capillary electrophoresis and matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry

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Abstract

Capillary electrophoresis was used for the qualitative as well as quantitative analysis of phosphopeptides. Three pairs of phosphorylated peptides and non-phosphorylated analogues were analyzed by capillary electrophoresis. Each sequence of phosphopeptides contained one phosphoserine, phosphothreonine or phosphotyrosine residue. The capillary electrophoresis analyses were performed in a fused-silica capillary 50 μm in internal diameter and 100 cm in length. For detection of analyte peptides, absorbance at 214 and 229 nm wavelength in the UV region was measured. To obtain the best resolution, the concentration of analyte and the pH of the electrolyte buffer were optimized. Matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry was used to confirm the molecular mass of the phosphorylated peptides and non-phosphorylated analogues. For quantitative analysis of the phosphorylated peptides using capillary electrophoresis, the linearity of the response of the absorbance at 214 nm was examined. The response of the absorbance for the phosphorylated peptides was linear; thus this validates the method for quantifying phosphopeptides using capillary electrophoresis. The results of our study show that capillary electrophoresis can be widely applied to analyze and characterize many biologically active phosphopeptides and phosphoproteins.

Keywords: Phosphopeptides; Peptides; MALDI–TOF mass spectrometry

1. Introduction

Phosphorylation is regarded as the most important post-translational modification in the regulation of cellular processes [1–3]. Specific serine, threonine and tyrosine residues in substrate proteins become phosphorylated by the action of various protein kinases in vivo.

Synthesis of phosphopeptides related to the sequences of certain phosphoproteins is necessary for studies of both the structure and the activity of the phosphoproteins. As a general approach to phosphopeptide synthesis, the method of solid-phase peptide synthesis has been developed [4–7]. Phosphorylated amino acid precursors can be incorporated into the course of solid-phase peptide synthesis. This approach is quite straightforward and the coupling proceeds with high efficiency.

However, during the course of synthesis, a partial loss of phosphoryl-protecting group may occur. Especially, dephosphorylation during the final deprotection step circumvents the preparation of global phosphorylation of the peptides. Steric effects are another important hindrance where multiphosphorylation in a peptide is required. Therefore,

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development of a powerful technique for detection of hydrolysis after synthesis of phosphopeptides is essential.

High-performance capillary electrophoresis (CE) is a newly developed and powerful technique with great potential for the high resolution separation and determination of various molecules ranging in size from small low-molecular-mass species to biological macromolecules [8–19]. Like liquid chromatography, separation by CE is not limited to a single mode. Analytes can be determined using charge/mass ratio, hydrophobicity, size, affinity, absorption, etc. [20–24]. Thus, one can create a CE separation based on almost any type of physical differentiation of compounds.

In the past, mass spectrometry has been used mostly for the analysis of small species, such as steroids. More recently, however, the problems associated with vaporizing and ionizing proteins and peptides have been solved. Matrix-assisted laser-desorption ionization coupled with time-of-flight mass spectrometry (MALDI–TOF-MS) is an excellent analytical tool for the mass determination of macromolecular biomolecules [25]. Along with electrospray ionization mass spectrometry (ESI–MS), MALDI–TOF-MS is the foundation of the new field of biological MS, a tool that can sequence blocked proteins, define N- and C-terminal sequence heterogeneity and identify sites of phosphorylation, deamidation and glycosylation [26–28].

In this study, we have used CE to analyze three pairs of phosphorylated peptides and their non-phosphorylated analogues. Analytical parameters such as concentration, type and mobile phase pH were optimized to obtain the best results. Feasibility of quantitative analysis for the phosphorylated peptides using CE was performed. MALDI–TOF-MS was used in a separate analysis for further confirmation of the molecular mass of the phosphorylated and non-phosphorylated peptides. In addition, off-line MALDI–TOF-MS analysis for one pair of the peptides was performed after the CE separation.

2. Experimental

2.1. Synthesis of phosphopeptides

Pairs of phosphorylated and non-phosphorylated peptides were synthesized using the 9-fluorenylmethoxycarbonyl (FMOC)-solid-phase peptide synthetic method. The YEEI and YpEEI peptides for this study were synthesized at this facility. The remaining two pairs of peptides were synthesized in the Protein and Carbohydrate Facility at University of Michigan (Ann Arbor, MI, USA). Following synthesis, the peptides were purified by HPLC [29] and characterized either by amino acid analysis or by MS.

2.2. Capillary electrophoresis

The CE instrument used for the most of this study was a Quanta 4000 CE system (Millipore, Waters Chromatography Division, Milford, MA, USA). The detector for the Quanta 4000 system was a fixed type wavelength UV detector equipped with a zinc lamp for 214 nm, a cadmium lamp for 229 nm and a mercury lamp for 185 nm wavelength.

The applied potential to the CE column was at a constant voltage of 20–30 kV. Fused-silica capillary tubes of 100 cm×50 μm I.D. were used. All experiments were carried out at ambient temperature (ca. 25–28 °C). Sample injections were done through hydrodynamic mode by raising the sample reservoir for 10 s. The electropherograms were recorded on a Waters 746 data module.

The capillary tube preparation was carried out by flushing the tube with 0.1 *M* potassium hydroxide solution for 10 min, washing with water for 10 min and then purging with the electrolyte buffer for 10 min. In addition, the capillary tube was purged for 2 min automatically with the working buffer before each injection.

2.3. Sample preparation for capillary electrophoresis analysis

Deionized water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Water of 18 MΩ was used for the preparation of all the solutions and electrolyte buffers. All the sample solutions were passed through a 0.45-μm membrane filter unit (Millex-HV13, Sartorius, Germany) and carefully degassed before use.

Two different kinds of buffer, 20 m*M* 3-(cyclohexylamino)propane-3-sulphonic acid (CAPS) buffer (pH 11.0) and 50 m*M* citric acid buffer (pH 2.5)

were used for the analysis of the peptides. As an internal standard, FMOC-O-dimethylphospho-L-Tyr or FMOC-L-Arg-OH was injected with the analyte peptides. We prepared FMOC-O-dimethylphospho-L-Tyr solution in 20 m*M* CAPS buffer and FMOC-L-Arg-OH solution in the mixture of citric acid buffer–dimethylformamide (DMF) (4:1). Each peptide was dissolved in appropriate buffer solutions as listed in Table 1.

2.4. MALDI–TOF mass spectrometry

MALDI–TOF-MS was performed on a HP G2025A (Hewlett–Packard, Palo Alto, CA, USA) linear type time-of-flight mass spectrometer operating in the positive-ion mode of detection. The ion accelerating potential was +28 kV and the length of flight tube was 1 m. Operating pressure for studies on the HP G2025A MALDI–TOF-MS were lower than 4×10^{-6} Torr (1 Torr=133.322 Pa). A nitrogen laser was set to deliver 337 nm wavelength pulses (3 ns duration) onto the sample. The laser was operated at a rate of 5 Hz. Data was analyzed with the HP G2025A MALDI–TOF-MS system software version A.02.00. The optimum energy power for desorption was found to be in the range of 0.5–1.0 μJ. The spectra presented in this paper were generated from the sum of 32–53 laser shots.

The MALDI–TOF matrix solution was prepared by making a saturated solution of 4-hydroxy-α-cyanocinnamic acid (4HCCA) with water–acetonitrile (2:1). The solution was thoroughly vortexed and centrifuged to obtain a clear working matrix solution of a mixture. Sample preparations for the analysis using MALDI–TOF-MS were performed as in the following: typically 10 μl of the matrix stock solution was placed in an eppendorf tube and 1 μl of each sample peptide solution (0.1 mg/ml) was added. The solution was briefly mixed using vortex stirring. One μl of the matrix–peptide mixture was applied onto the one of the ten-position gold-plated sample probes. Then the sample probe was allowed to sit at ambient temperature to dry and crystallize the sample mixtures. Cold distilled water was placed over the crystals for a few seconds to remove any water soluble contaminants and then subsequently removed with the HP G2024A sample preparation accessory. Calibration was accomplished using known compounds as internal mass standards.

2.5. CE separation for off-line MALDI–TOF-MS analysis

The CE instrument used for the study of off-line MALDI–TOF-MS analysis was a P/ACE CE system equipped with a diode array detector and fraction collection system (Beckman, Fullerton, CA, USA.).

The applied potential to the CE column was at a constant voltage of 28 kV. Fused-silica capillary tube of 67 cm×50 μm I.D. was used. Experiments were carried out at a constant temperature of 25 °C. Sample injections were done through hydrodynamic mode with pressure of 0.5 p.s.i. for 10 s (1 p.s.i.= 6894.76 Pa). Buffer solutions used for sample preparation and CE separation for the study of off-line MALDI–TOF-MS analysis was same as used above for the CE analysis.

3. Results and discussion

3.1. Separation of phosphopeptides

The initial phase of this investigation was centered around evaluation and development of a method for checking the purity of phosphotyrosine containing phosphopeptides after organic synthesis since the phosphate group is labile. High-performance CE was investigated for its potential as a powerful method for characterization of synthetic phosphopeptides. Then, the developed method was generalized for the separation of other kinds of phosphopeptides. To explore further applications of the method, a quantitative analysis of phosphopeptides was also performed.

Since phosphorylation of proteins is an important step in many cellular regulatory processes such as in signal transduction process, the need for the synthesis of phosphopeptides related to the sequences of certain signalling proteins, for the studies of both the activity and the structure, has increased. In our laboratory, we have also synthesized several pairs of peptides which contain phosphopeptides and non-phosphorylated analogues mainly related to receptor-type protein tyrosine kinases for the purpose of other biochemical studies of macromolecular interactions. Three pairs of peptides were chosen for this study, each of which contained one phosphotyrosine, phosphoserine, or phosphothreonine residue in their se-

Table 1
Non-phosphorylated and phosphorylated peptides that were analyzed by capillary electrophoresis

	Structure	Internal standard	Buffer solution	Wavelength (nm)
1	YEEI YpEEI	FMOC-O-dimethylphospho-L-tyrosine	CAPS buffer (pH 11)	214
2	KRTIRR KRTpIRR	FMOC-L-arginine	Citric acid buffer (pH 2.5)	229
3	KRPSQRHGSKY-amide KRPSpQRHGSKY-amide	FMOC-L-arginine	Citric acid buffer (pH 2.5)	229

quences. Table 1 shows the sequences of the three pairs of the peptides for the study.

Determination of the phosphopeptides and non-phosphorylated analogues by CE was carried out using extreme pH conditions of the electrolyte buffer as shown in Table 1. The reason for this extreme buffer pH is mainly due to the well known problem of adsorption of peptides to the capillary wall. General approaches to control this problem, particularly for peptides, have included the use of extreme pH conditions of the electrolyte buffer. If the electrolyte buffer is kept at acidic pH, the fused silica inside the capillary remains neutral. Proteins and peptides under these conditions will be positively charged and will not electrostatically interact with the wall, although hydrophobic interactions may still occur. Keeping the electrolyte buffer at basic pH but at least two pH units above the p*I* of the analyte will, although the fused silica becomes negatively ionized silanol groups under these conditions, cause the analyte protein or peptide molecules to also be negatively ionized as well. Therefore, the problem of the adsorption of protein or peptide molecules does not occur in either case of the extreme pH conditions of the electrolyte buffer that we used for this study.

Fig. 1 shows the separation of a pair of tyrosine containing peptides. The separation was carried out in 20 m*M* CAPS buffer (pH 11.0) and the UV detection was performed using the zinc lamp at 214 nm wavelength. Non-phosphorylated, tyrosine containing peptide (YEEI) was eluted at 11.98 min and phosphorylated tyrosine containing analogue (YpEEI) was separated at 12.98 min. For the reference peak, 0.2 μ*M* of *N*-FMOC-*O*-dimethylphospho-L-tyrosine (Sigma, St. Louis, MO, USA) in CAPS buffer was used and the peak was eluted at 9.03 min.

Figs. 2 and 3 depict the separations of pairs of serine or threonine containing peptides, respectively.

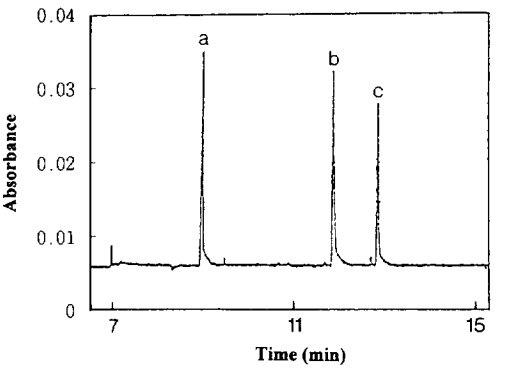

Fig. 1. Electropherogram of tyrosine containing peptides. Conditions: 20 m*M* CAPS buffer (pH 11.0); 28 kV; 10 s injection, Hydrostatic. 100 cm×50 μm I.D. capillary. UV detection at 214 nm. Peaks: a=N-FMOC-O-dimethylphospho-L-tyrosine; b=YEEI; c=YpEEI.

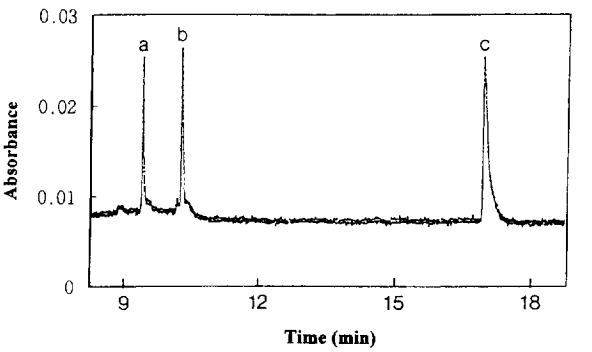

Fig. 2. Electropherogram of serine containing peptides. Conditions: 50 m*M* citric acid buffer (pH 2.5); 30 kV; 10 s injection, Hydrostatic; 100 cm×50 μm I.D. capillary. UV detection at 229 nm. Peaks: a=KRPSQRHGSKY; b=KRPSpQRHGSKY; c=N-FMOC-Arg-OH.

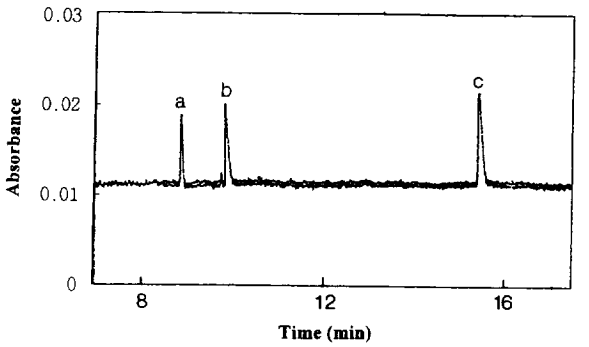

Fig. 3. Electropherogram of threonine containing peptides. Conditions were same as in Fig. 2. Peaks: a=KRTIRR; b=KRTpIRR; c=N-FMOC-Arg-OH.

The separations were carried out in 50 m*M* citric acid buffer (pH 2.5) and the UV detection was performed using a cadmium lamp at 229 nm wavelength.

The serine containing peptide (KRPSQRHGSKY) appeared 9.54 min after the injection and phosphorylated serine analogue (KRPSpQRHGSKY) appeared after 10.44 min (Fig. 2). For the separation of threonine containing peptides, a peak of non-phosphorylated peptide (KRTIRR) appeared at 8.93 min and that of phosphorylated analogue (KRTpIRR) at 9.91 min (Fig. 3). FMOC-L-Arg-OH (Sigma) was used as a reference in both cases.

One of the useful characteristics of the method of capillary electrophoretic analysis is that the elution order of each of the components in the electropherogram is predictable. The internal standard of N-FMOC-O-dimethylphospho-L-tyrosine contains one carboxyl group in its structure. On the other hand, the peptide, YEEI, contains two carboxyl group from the two glutamic acid residues and one hydroxyl group from the tyrosine residue. Therefore, the intensity of negative ionization of the peptide, YEEI, is much higher than that of the internal standard (N-FMOC-O-dimethylphospho-L-tyrosine) in CAPS buffer system at pH 11.0. Furthermore, the phosphopeptide, YpEEI, contains one phosphate group in the position of the hydroxyl group of tyrosine residue which also contributes to the intensity of negative ionization.

The difference in the intensity of negative ionization as well as the CE direction of the electric field of the CE system, from positive toward negative, allows us to predict the order of elution of each component as follows: (1) the internal standard, (2) YEEI, (3) YpEEI. This prediction is verified experimentally by results shown in the electropherogram of Fig. 1.

The electropherograms of the serine and threonine containing peptides in Figs. 2 and 3 were also well matched. The internal standard of *N*-FMOC-L-Arg-OH contains one amino group in its structure. On the other hand, the serine containing peptide, KRPSQRHGSKY and threonine containing peptide, KRTIRR, possess four amino groups which contribute to the intensity of positive ionization in their structures. Thus, the more positive ionization of the peptides relative to the internal standard can promote the migration of the peptides and allow them to elute faster than the internal standard. This characteristic of predictability in analysis using capillary electrophoresis enhances the validity of the analysis method.

3.2. Quantitative analysis using CE

For quantitative analysis of peptides using CE, the pair of serine containing peptides was arbitrarily selected. Various dilutions of each peptide ranging from 10–250 n*M* concentrations were used and the peak area of each dilution versus the concentration of the peptides was plotted.

As an example, Fig. 4 shows the data for the peptide KRPSpQRHGSKY. The linearity of UV

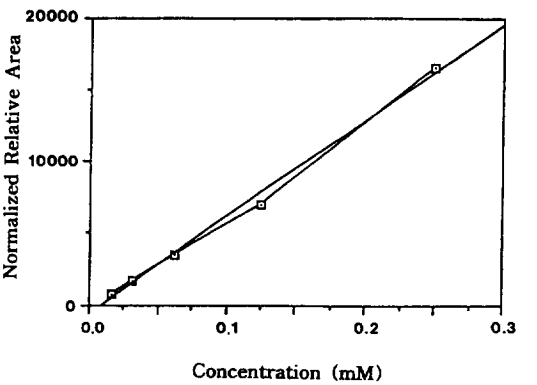

Fig. 4. Quantitative analysis of serine containing phosphopeptide (KRPSpQRHGSKY). Normalized relative peak area of the peptide versus amount of peptide was plotted. Electrophoretic conditions were same as in Fig. 2. $y=3.4\cdot10^4x-650$, $R=0.99$.

absorbancy at 229 nm wavelength versus the amount of peptide was highly acceptable. The correlation of variants, R value, was 0.99 for the phosphopeptide in the range 10–250 nM concentrations and the R value for the non-phosphorylated analogue (data not shown) was also very good at 1.0. As other investigators have reported, the observation of good linearity in this study suggests the validity of the method for quantitative analysis not only for peptides but also phosphopeptides [30].

The effect of lamp wavelength on sensitivity was also considered. The responses for phosphorylated and non-phosphorylated peptides at both 229 nm and 185 nm were compared. When a 185 nm filter with a mercury lamp was used, sensitivity was improved by a factor of 8 for both phosphorylated and non-phosphorylated peptides. The use of sub-200 nm lamp wavelengths in HPLC and CE detection has been limited due to the low intensity of light sources as well as the low transmission of most solvents in this range. However, in CE with a fixed-type wavelength detection system, UV detection below 200 nm is more reasonable owing to the short path length of the detection cell and the consistency of the separation medium [31].

3.3. Mass confirmation using MALDI–TOF-MS

For the analysis of proteins or peptides by MALDI–TOF-MS, the most commonly used matrix solution is 4-hydroxy-α-cyanocinnamic acid (4HCCA) with trifluoroacetic acid (TFA) and acetonitrile. In this study, however, the matrix solution was prepared by making a saturated solution of 4HCCA with mixture of water–acetonitrile (2:1) instead [32]. This is possible because the molecular mass of all the peptides of interest is lower than 2000.

In order to find the best way to form crystals of our small phosphorylated and non-phosphorylated peptides, we compared the MALDI mass spectra from two different methods of crystallization: (1) the dried-drop method where the sample remains at an ambient temperature for about 10 min and (2) the rapid crystallization method using vacuum. Other investigators have suggested the use of the rapid crystallization method for the mass analysis of lower-molecular-mass analytes [33]. In this system, however, the spectrum from the rapid crystallization method using the matrix solution of 4HCCA–water–acetonitrile was comprised mostly of salt-adduct peaks. Therefore, the dried-drop method of crystallization was used for the MALDI–TOF-MS analysis throughout the study.

Fig. 5 shows the MALDI mass spectra of the peptides. Panel I shows the spectrum of the pair of tyrosine containing peptides. Peak A represents YEEI and peak B represents YpEEI. Panel II and panel III depict the spectrum of the pair of threonine and serine containing peptides, respectively. Peak C and E represent KRTIRR and KRPSQRHGSKY, respectively and peak D and F represent their phosphorylated analogues, KRTpIRR and KRPSpQRHGSKY, respectively, which show the expected molecular mass increase of 80 for the phosphate group.

As shown in Table 2, the observed mass of all peptides with or without phosphate group are almost perfectly matched with the calculated mass. This study of MALDI-MS analysis was performed in an attempt to confirm the molecular mass of the phosphorylated and non-phosphorylated peptide samples.

3.4. Off-line MALDI–TOF-MS analysis after CE separation

For off-line MALDI–TOF-MS analysis after CE separation and fractionation, the pair of threonine containing peptides was arbitrarily selected. Excessive concentrations of KRTIRR and KRTpIRR were prepared to inject into fused-silica capillary column mounted in the Beckman P/ACE system for collecting fractions of the peptides. The peptide fractions were collected between 4 and 6.5 min after injection. Since the length of the capillary column used for this analysis was shorter (67 cm) than the tube length (100 cm) for the CE analysis above using the Waters Quanta 4000 system, peaks of the peptides appeared faster (data not shown) than previous analysis. Due to the excess amount of the sample peptides for CE injection, the shapes of the peaks were not as good as earlier analysis of this study (data not shown). The peptide fractions were collected several times repeatedly into 10 μl of buffer in collection vials of the P/ACE system. The collected sample (2 μl) was

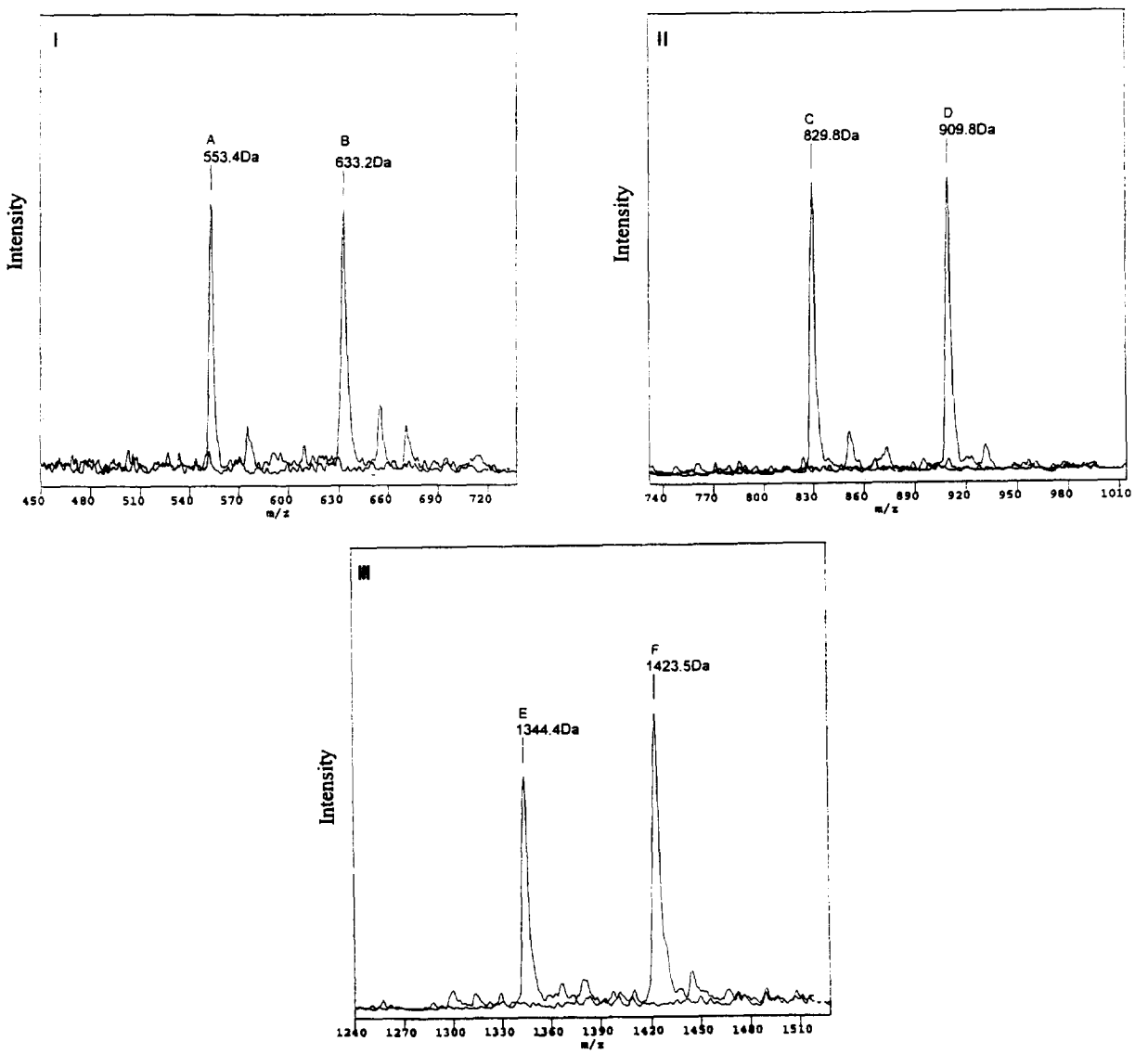

Fig. 5. MALDI–TOF mass spectra of the peptides. The matrix solution of 4-hydroxy-α-cyanocinnamic acid (4HCCA) with water–acetonitrile (2:1) was used. The ion accelerating potential was +28 kV and the length of flight tube was 1 m. Panels: I=tyrosine containing peptides; II=threonine containing peptides; III=serine containing peptides. Peaks: A=YEEI; B=YpEEI; C=KRTIRR; D=KRTpIRR; E=KRPSQRHGSKY; F=KRPSpQRHGSKY. Da=dalton.

added to 5 μl of the matrix solution. Crystallization and MALDI–TOF-MS analysis of the peptide fractions were done as previously.

Fig. 6 shows the MALDI mass spectrum of the off-line MALDI–TOF-MS analysis. Two peaks of M_r 830.2 and 910.1 represent KRTIRR and KRTpIRR, respectively. We assume that a peak of M_r 212.2 may represent the citric acid of the CE electrolyte buffer, because the mass spectrum of the separate analysis of MALDI–TOF did not show any peak of M_r 212.2. Peaks of M_r 190.2 as well as 379.2 come from the matrix solution.

Table 2
Comparison of exact molecular mass with observed mass using MALDI–TOF-MS

Peptides	Exact mass	Cal. $M+H^+$	Obs. $M+H^+$	Error (%)
YEEI	552.58	553.58	553.40	0.032
YpEEI	632.58	633.58	633.20	0.060
KRTIRR	829.01	830.01	829.80	0.025
KRTpIRR	909.01	910.01	909.80	0.023
KRPSQRHGSKY-amide	1343.51	1344.51	1344.40	0.008
KRPSpQRHGSKY-amide	1423.51	1424.51	1423.50	0.071

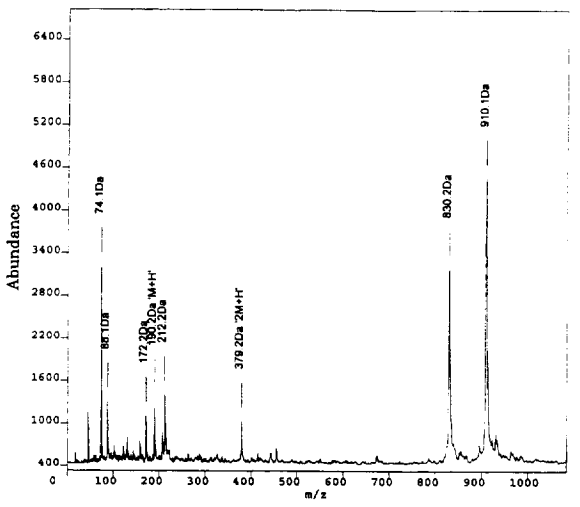

Fig. 6. Mass spectrum of off-line MALDI–TOF-MS analysis of the threonine containing peptides. The matrix solution and conditions of the MALDI–TOF analysis were same as Fig. 5.

Acknowledgments

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