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Identification of phosphopeptides by mass spectrometry

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Phosphopeptides can be identified by ion spray mass spectrometry. The method was tested with phosphokemptide and with a proteolytic digest of one subunit (δ-subunit) of the nicotinic acetylcholine receptor. In the latter one peptide containing tyrosine phosphate, one with two serine phosphates, and two different peptides each containing one serine phosphate were unambiguously identified. Thus it is proven that ion spray mass spectrometry can be applied for the localization of phosphorylation sites in a known primary structure.

Protein phosphorylation; Mass spectrometry; Acetylcholine receptor

1. INTRODUCTION

Phosphorylation by protein kinases is one of the major posttranslational modifications of proteins. A host of regulatory phenomena are correlated with this phenomenon. Phosphate groups are transferred in most cases from ATP to $-OH$ groups of serine threonine, and tyrosine residues. Esterification of these amino acid side chains causes conformational changes in the protein and as a consequence altered activity or stability.

The site of phosphorylation in a protein is of special interest because of its key role in regulating a protein's properties. Localization of phosphoamino groups in a primary structure is tedious and in not all cases possible. The phosphate-ester bond in serine and threonine phosphate is labile and does not withstand protein chemical methods like acid hydrolysis (for amino acid analysis) or Edman sequencing. It has been proposed to convert the phosphate esters of serine by an elimination/addition reaction into a stabile product [1] which can be detected by standard sequencing methods. These modifications do not apply to phosphothreonine and phosphotyrosine. With the introduction of mass spectrometry into protein chemistry it has become possible to analyze molecules of high molecular weights by non-chemical means. Here we describe the application of an ion spray/MS mass spectrometer [2,3] to localize phosphorylation sites in the known primary structures of polypeptides, in phosphokemptide as a model peptide, and in one of the subunits of the nicotinic acetylcholine receptor isolated from *Torpedo californica* electric tissue.

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2. MATERIALS AND METHODS

The experiments were performed with a Sciex API III mass spectrometer (MDS Health Group Ltd, Thornhill, Canada) working on-line with an HPLC system. Ionization of the molecules to be investigated was accomplished by the ion spray method. Kemptide was from Sigma (Deisenhofen, FRG). Phosphokemptide was prepared by phophorylation with ATP catalyzed by protein kinase A (PKA). Preparation of the nicotinic acetylcholine receptor (AchR) and isolation of its δ-subunit were performed as described elsewhere [4,5]. All other materials and chemicals were of the highest purity commercially available.

Protein digestion was accomplished by incubation with endoproteinase Lys-C. To 3 nmol desalted purified receptor subunit in 25 mM Tris-HCl buffer, pH 8.5, 1 mM EDTA and 5% acetonitrile (final volume 2 ml) 10 μg Lys-C dissolved in 100 μl of the same buffer were added. After 16 h incubation at 37°C another 5 μg were added and incubation was continued for a further 8 h. This digest was applied without further treatment to an HPLC system using an ABI Aquapore C18 column attached on-line to the mass spectrometer.

3. RESULTS

To investigate whether mass spectrometry is able to detect phosphate groups in a polypeptide we used kemptide as a model. Kemptide is a heptapeptide of relative molecular mass 771.9 which is a substrate for the cAMP-dependent protein kinase (PKA). The mass spectrum of kemptide (Fig. 1) shows two signals corresponding to the mono $(M + H)^+$ and double $(M + 2H)^{2+}$ protonated peptide . The mass is correctly given as 771.5 (one mass unit for the proton has to be subtracted). Fig. 2 shows the corresponding mass spectrum of the phosphorylated peptide. The signal of the mono charged ion indicates a molecular mass of 852 which is within one mass unit, the mass of kemptide plus the mass of one phosphate group (M_r of H_2PO_3: 80.98).

Subsequently we attempted to identify phosphopeptides in the Lys-C digest of the AChR δ-subunit. Fig. 3

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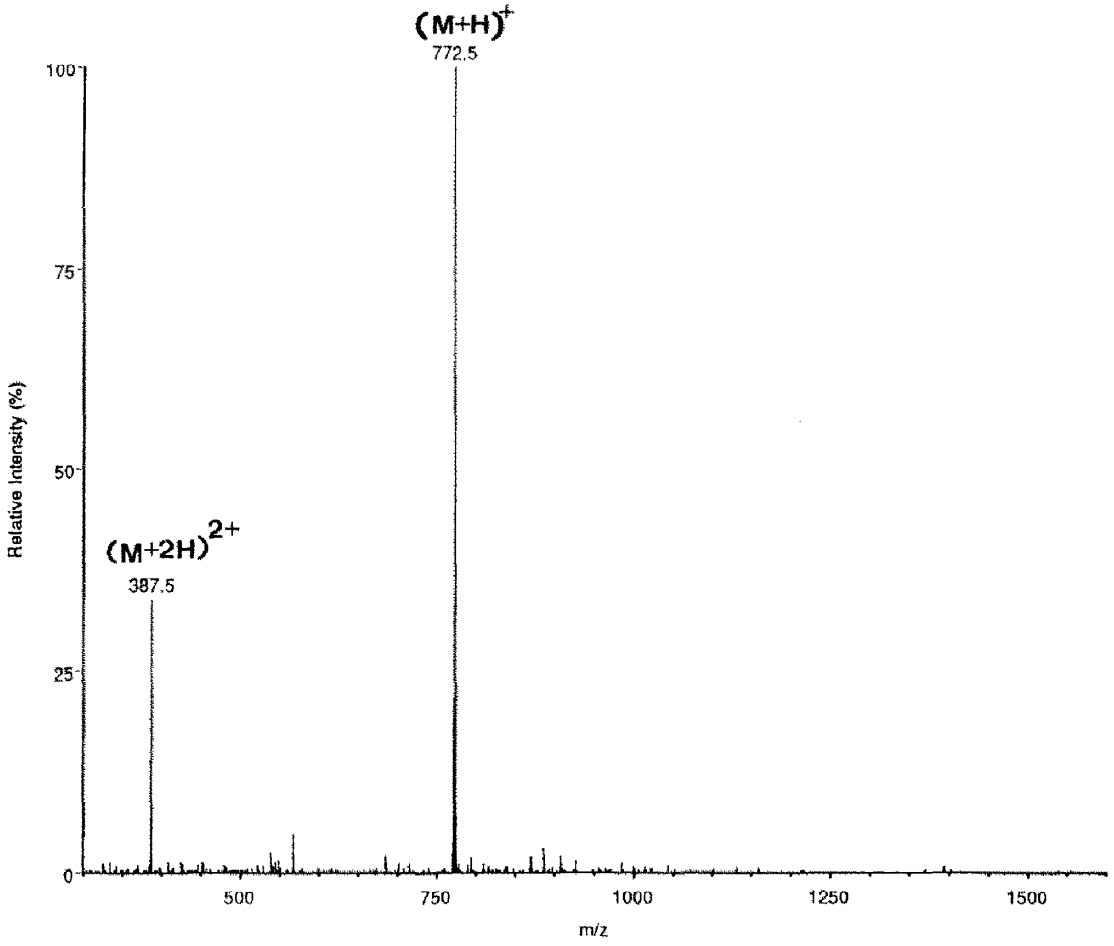


Fig. 1. Mass spectrum of kemptide.

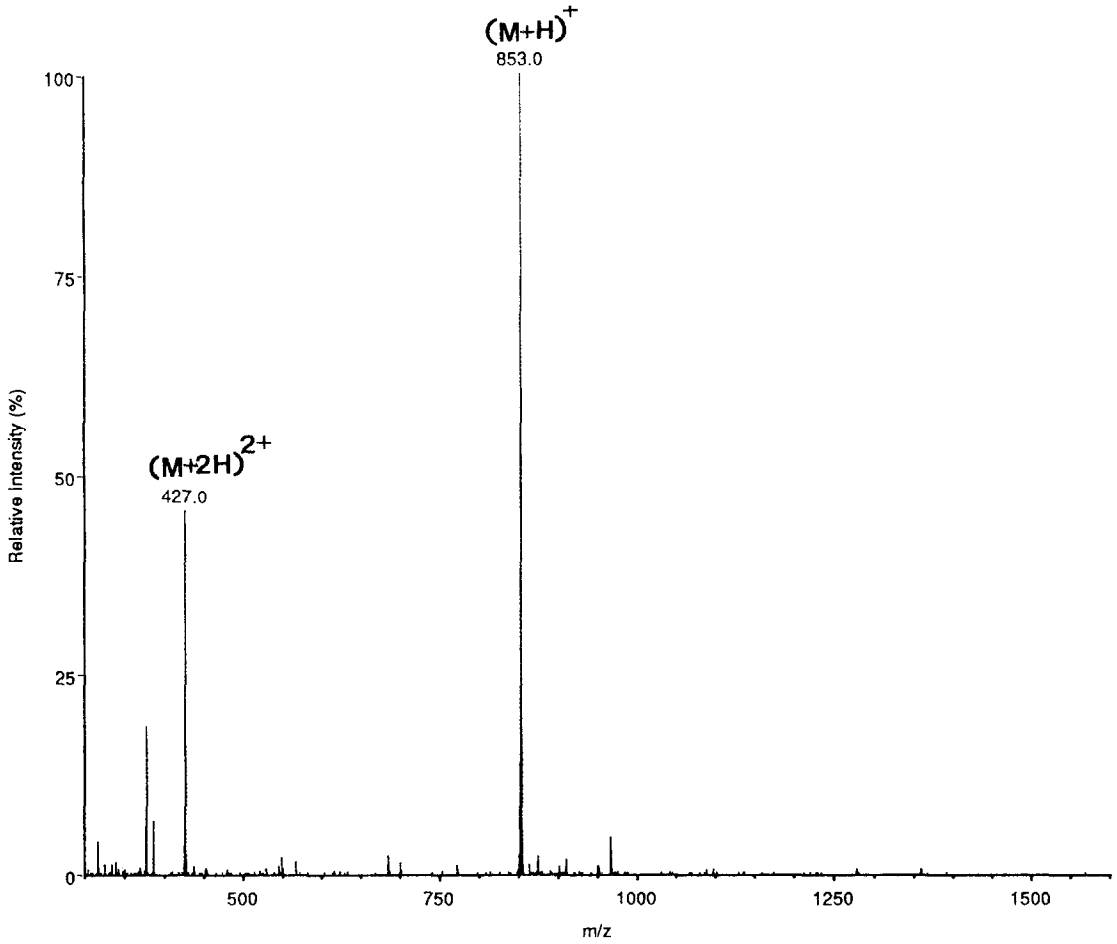
shows the 'total ion current' of an HPLC run of the digest which was subjected on-line to the mass spectrometer. As a matter of fact this scan shows the peak profile of the eluant; the mass spectrometer is used instead of the usual UV monitoring. Each of the main peaks was subjected to further mass analysis.

The mass spectrum of peak 1 in Fig. 3 is shown in Fig. 4. The signal indicates an m/z value of 757.0 for the double-protonated and 1513.5 for the mono-protonated ion, corresponding to a mass of 1512 Da. The only peptide matching this mass is the Lys-C peptide of the δ-subunit having the sequence LRRSSSVGYISK (position 357–368) containing two phophate groups (mass 1513 Da) [6]. In addition the monophosphorylated peptide can be identified (m/z value 717.0; $(M + 2H)^{2+}$ which seems to be present as a minor component.

Peak 2 in Fig. 3 gives the mass spectrum shown in Fig. 5. The m/z value of 603.5 ($(M + 2H)^{2+}$) and 1208.5 ($(M + H)^{+}$) corresponds to a mass of 1205 and 1207.5 Da, respectively. Again only one Lys-C peptide matches this mass, the monophosphorylated peptide SRSELMFEK (position δ377–385; mass 1206), predicted to contain a PKC substrate site [7]. Additionally the MS spectrum shows m/z values of 1092.5 and 547, corresponding to the mono- and double-charged ions, respectively. Peptide AQEYFNIK, position δ369–δ376, phosphorylated at its tyrosine residue is the only peptide obtainable by digestion with endoproteinase Lys-C having this mass (1092 Da). This proves that the predicted tyrosine kinase phosphorylation site [7] of the AChR δ-subunit is phosphorylated in our receptor preparations.

Fig. 2. Mass spectrum of phosphokemptide.

Fig. 3. Total ion current of all masses. Lys-C digest of acetylcholine receptor (δ-subunit); amount injected 300 pmol. On-line from HPLC ABI Aquipore C18, 1 mm × 10 cm. Arrows indicate peptides later identified as phosphopeptides (see Figs. 4 and 5).


(M+H)+
853.0
(M+2H)2+
427.0
Relative Intensity (%)
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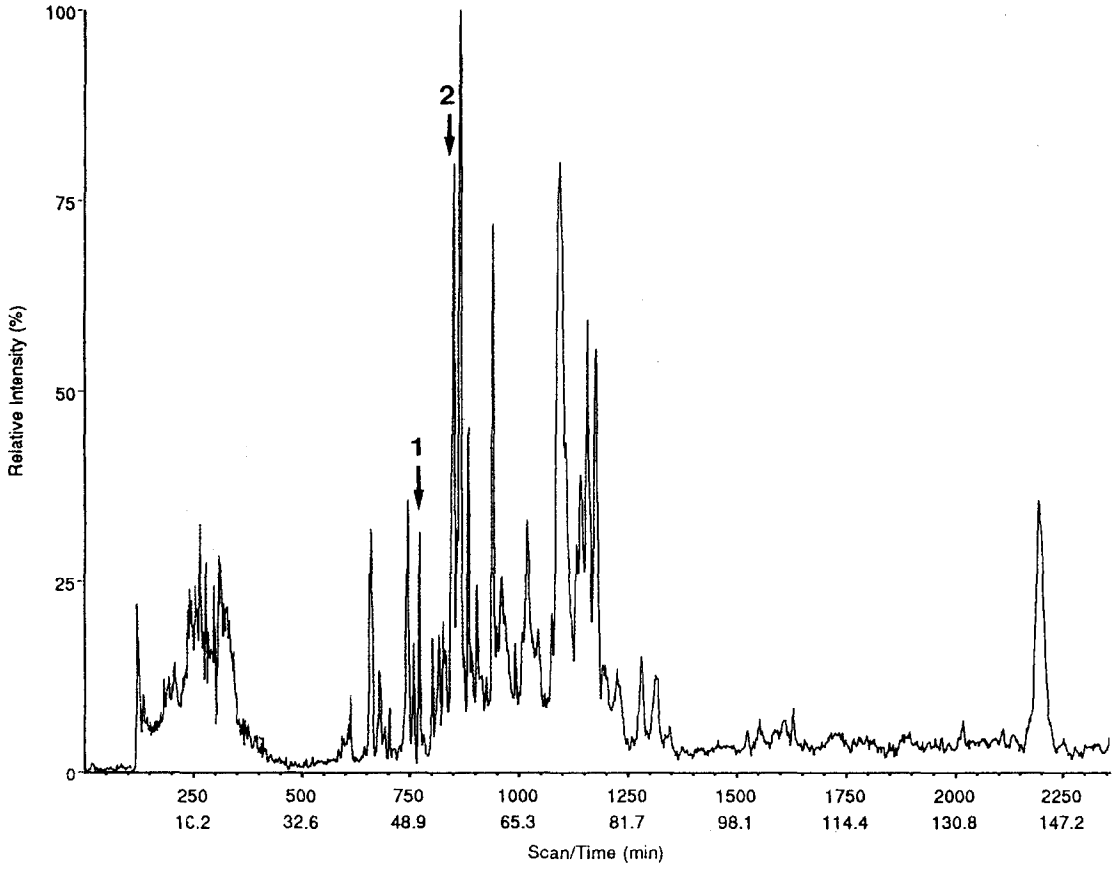
75
50
25
0
500
750
1000
1250
1500
m/z


1
2
Relative Intensity (%)
100
75
50
25
0
250
500
750
1000
1250
1500
1750
2000
2250
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130.8
147.2
Scan/Time (min)

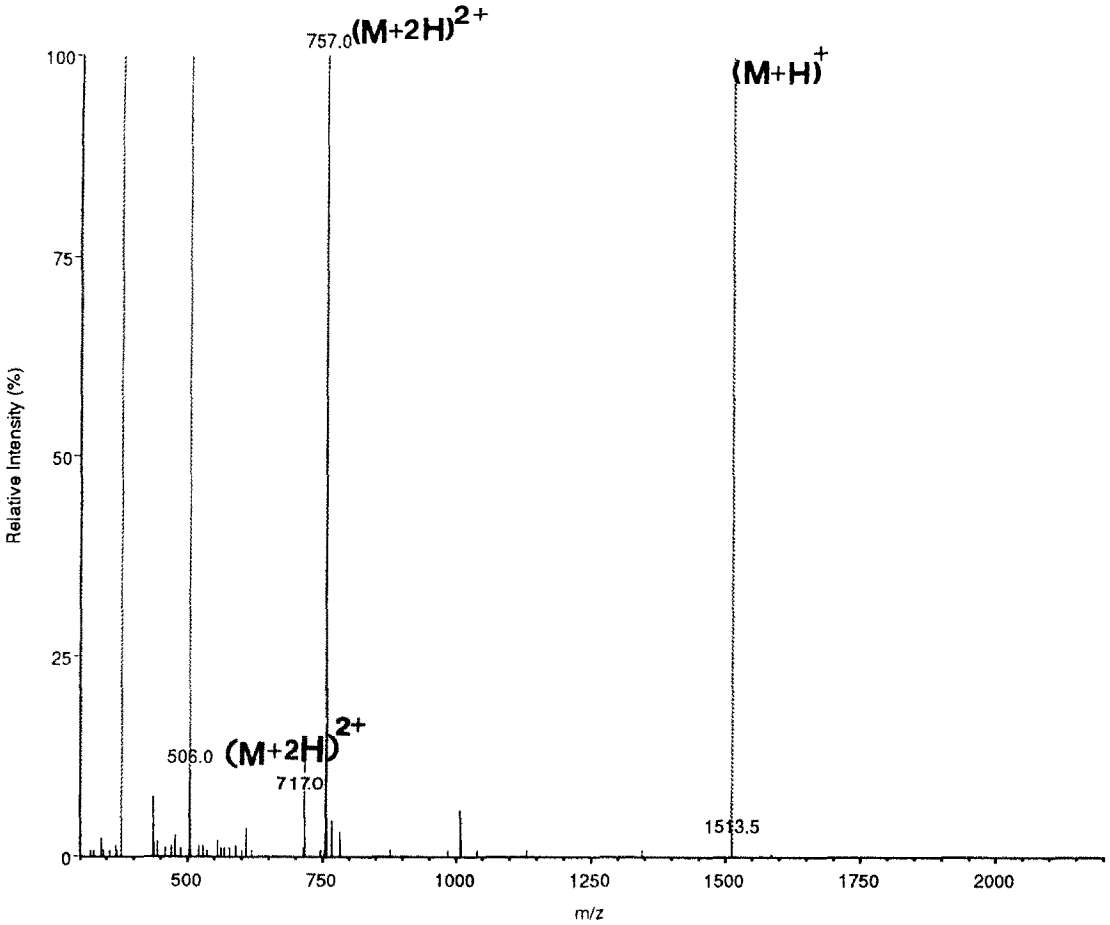

Fig. 4. Mass spectrum of peak 1 of Fig. 3. The spectrum shows mono- and double-charged molecular ions of peptide δ357–δK368. The molecular mass corresponds to the mass of the peptide plus two phosphate groups. The minor component $(M+2H)^{2+}$ 717.0 represents the same but monophosphorylated peptide. The other peaks were not identified.

4. DISCUSSION

Mass spectrometry can unambiguously identify phosphorylated peptides in a proteolytic digest of a protein. Kemptide and its phosphorylated counterpart can be easily discriminated. This shows the accuracy of the method and it proves that ion spray ionisation does not cleave phosphate esters and that it does not produce to a significant extent fragmentation artifacts to be expected by harsher ionisation methods.

In the δ-subunit of the nicotinic acetylcholine receptor a diphosphorylated peptide and monophosphorylated peptides are detectable. With the help of the known sequence [8] of this protein both can be unambiguously assigned to a position in the primary structure. The diphosphorylated peptide identified in the Lys-C digest of the actylcholine receptor δ-subunit was recently discovered by us by classical protein chemistry [6,9]. The experiment described here confirms this result which proved that the receptor subunit contains stable phosphate groups and that in position δS362 there is a phosphate located which was not predicted on the basis of the known consensus substrate sequence of the protein kinase PKC. Despite this fact we were able to phosphorylate in a synthetic peptide the position corresponding to δS362 with PKC [6]. Phosphorylation of the receptor in this position by external PKA could, in the past, not be shown [10].

The δ-subunit peptide with the molecular mass of 1206 Da contains a typical consensus phosphorylation site for PKC. Therefore it is confirmed here by mass spectrometry that this site is indeed phosphorylated in the receptor. And finally, the predicted phosphorylation site for a tyrosine kinase was also unambiguously detected in the mass spectrum. In both cases the non-phosphorylated peptides were identified. This serves as a control for the complete cleavage and for the stability of the peptides. Coincidental occurrence of an identical mass peak originating from an unrelated protein fragment can be considered extremely unlikely.

Recently in a similar approach no phosphorylation sites were detected by mass spectrometry [11]. But this was possibly due to a loss of the phosphorylated peptides on the HPLC column preceding the mass spectrometer. Phosphopeptides have a tendency to stick to metal filters and fittings of HPLC columns. Extending

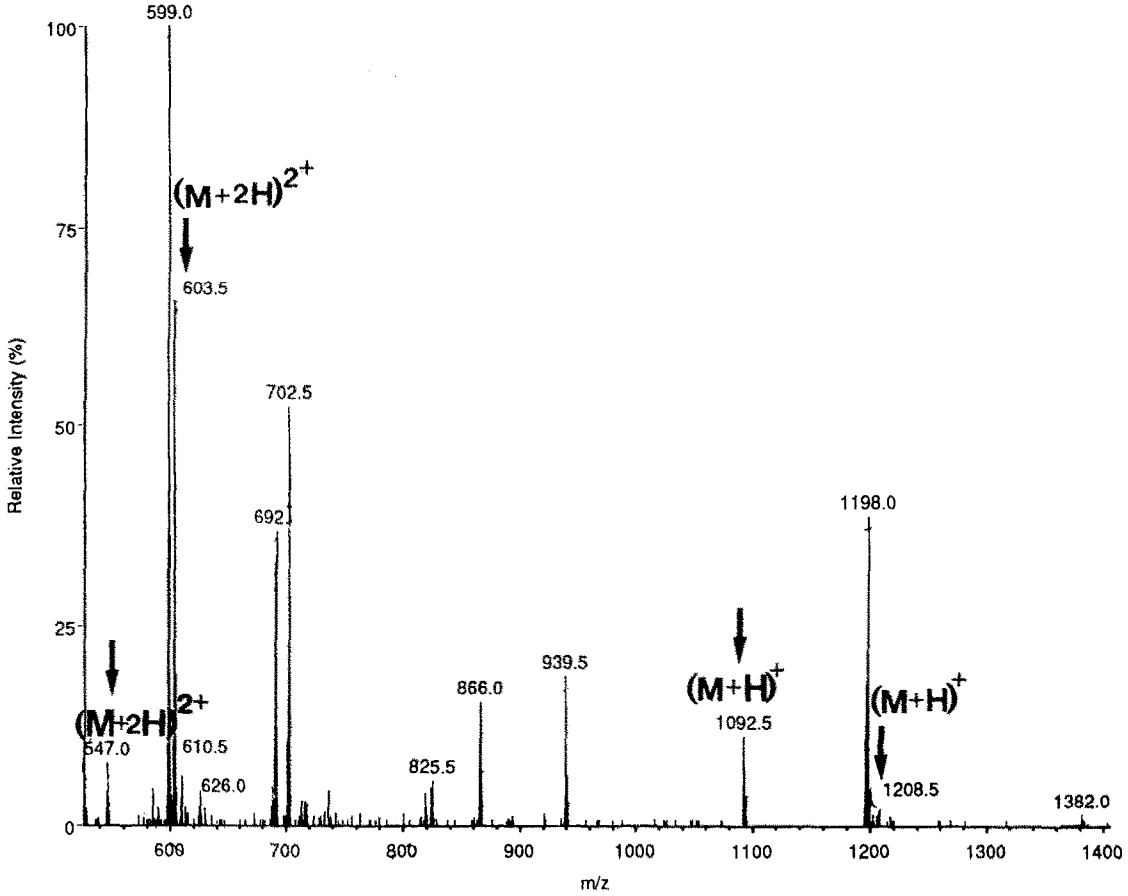

Fig. 5. Mass spectrum of peak 2 of Fig. 3. Two phosphorylated peptides were identified: *m/z* 1092.5 represents the mono-charged molecular ion of the phosphorylated peptide δA369–δK376. *m/z* 547 represents the corresponding double-charged molecular ion. These peaks indicate that the δ-subunit was phosphorylated at tyrosine 372. *m/z* 1208.5 represents the mono-charged molecular ion of δS377–δK385 plus one phosphate group, *m/z* 603.5 represents the corresponding double-charged ion. The peptide identified comprises the site phosphorylated by PKC.

this procedure to MS MS tandem mass spectrometry it should be possible to identify even the substituted amino acid within the sequence.

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