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# Influence of hydrophobicity on positive- and negative-ion yields of peptides in electrospray ionization mass spectrometry

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**RATIONALE:** The influence of hydrophobicity originating from an amino acid phenylalanine (Phe) residue on the ion yields of peptides has been empirically evaluated using positive- and negative-ion electrospray ionization (ESI) mass spectrometry. The enhancement effect of hydrophobicity was compared with that of the presence of basic and acidic residues of peptides.
**METHODS:** In order to empirically understand the ion yields in soft ionization methods, we have divided the total ionization process into ionization efficiency of analyte molecules and the rate of desorption or vaporization of molecules. The ion yields of protonated and deprotonated molecules of peptides were evaluated.
**RESULTS:** The presence of a Phe residue resulted in an increase in the ion yields of both the analyte ions $[M + nH]^{n+}$ and $[M–nH]^{n–}$. The relationship between the ion yields and hydrophobicities of peptides was evaluated using the partition coefficient measured by thin-layer chromatography (PACTLC). A peptide containing a Phe residue at its C-terminus gave a higher ion yield than when it was at the N-terminus.
**CONCLUSIONS:** The ion yields of peptides increased with increasing hydrophobicity both in positive- and negative-ion ESI. The enhancement effect of hydrophobicity on the ion yields was higher than that of basicity and acidity of the peptides in ESI. Copyright © 2014 John Wiley & Sons, Ltd.

Mass spectrometry (MS) is a useful method for analyzing various kinds of organic analytes. The soft ionization methods such as matrix-assisted laser desorption/ionization (MALDI)[1–3] and electrospray ionization (ESI)[4,5] have been developed into more sensitive analytical tools allowing the analysis of peptides, proteins and other biological polymers without decomposition. Methods for proteomics analysis have been established using ESI-MS interfaced with liquid chromatography.[6] It has been reported that high sequence coverage and database search scores are obtained using both positive- and negative-ion data in proteomic analysis.[7] If all the peptide ion peaks from a protein digest can be detected in a mass spectrum, accurate identification of the protein can be achieved using the database. However, it is difficult to detect the all tryptic digests because of the different ion yields for each peptide fragment. These ion yields are strongly dependent upon the physicochemical properties which originate from the individual nature of the side chains of the constituent amino acids.

In order to understand empirically the ion yields in soft ionization methods, Nishikaze and Takayama[8] and Asakawa *et al.*[9] have divided the total ionization processes $Ji$ into ionization efficiency of analyte molecules $I$ [ions/molecules] and the rate of desorption or vaporization of molecules $Jv$ [molecules/cm$^2$ s] as follows:

$$Ji = I\,Jv \quad (1)$$

The efficiency $I$ can be related to thermochemical quantities such as proton affinity, gas-phase basicity, ionization energy or electron affinity of analytes. The quantity $Jv$ represents the ability of the analyte molecules to desorb or vaporize from the liquid or solid phase to the gas phase. Amino acids, which constitute peptides and proteins, have a wide variety of physicochemical properties such as acidity, basicity, hydrophobicity and hydrophilicity originating from the characteristics of the amino acid side chain. It has been reported that the presence of aromatic amino acid residues enhances the ion yields of the peptides in MALDI and/or fast atom bombardment (FAB).[8,9] In MALDI, an enhancement effect of basic and aromatic amino acids such as arginine (Arg), lysine (Lys) and phenylalanine (Phe) has been reported.[9] In ESI, a number of studies have been reported about the ionization mechanism and the ion yields of various samples.[10–15] Tang and Kebarle have developed the equations for Iribarne's ion evaporation theory in which the increase of the surface charge density as a result of evaporating solvent in a droplet leads to a Coulombic repulsion.[11–14] They suggested that the roll-off observed in positive-ion ESI calibration curves in the high concentration region is due to a limit in the surface charge density. Enke has reported the effect of nonpolar or hydrophobic regions of analyte tripeptides on the ion yield in positive-ion ESI.[15]

Here we report the effects of the hydrophobic amino acid Phe residue and the influence of its position in peptides on the positive- and negative-ion yields in ESI. The enhancement effect

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of hydrophobicity on the ion yields was compared with that of the presence of basic and acidic residues of peptides. The model peptides threonine (T)-cluster and adrenocorticotropic hormone (ACTH)-based series were used for the evaluation of the ion yields. The relationship between the ion yields and hydrophobicities of peptides was evaluated using the partition coefficient measured by thin-layer chromatography (PACTLC).

## EXPERIMENTAL

### Materials

All peptides were purchased from the Peptide Institute (Minoh, Osaka, Japan). The sequences are summarized in Table 1. Acetic acid and acetonitrile (HPLC grade) were purchased from Wako Pure Chemicals (Osaka, Japan). All the reagents were used without any further purification. Water used in all the experiments was purified using a MilliQ water purification system from Millipore (Billerica, MA, USA).

### Sample preparation and ESI-MS

Each peptide was dissolved in 50% acetonitrile at a concentration of 1 pmol/μL with 0.1% acetic acid. The solutions were injected at a flow rate of 1 μL/min into the ESI interface of an HCT ultra ETDII ion trap mass spectrometer (Bruker Daltonics Inc., Bremen, Germany). The instrument parameters of ESI-MS were as follows: $N_2$ drying gas of 3 L/min and 250 °C, $N_2$ nebulizing gas of 6 psi, and capillary voltages of –3.3 kV for positive-ion mode and 3.1 kV for negative-ion mode. The positive- and negative-ion yields of peptides were calculated as follows:

$$Ji^{+} = \sum[M + n\mathrm{H}]^{n+}(n = 1\text{–}4)$$
$$Ji^{-} = \sum[M - n\mathrm{H}]^{n-}(n = 1\text{–}2)$$

where Σ is the sum of the areas under the curves of all charge states of the peptides. The ion yields of peptides were evaluated by taking the average of five measurements.

## RESULTS AND DISCUSSION

### Ion yields of threonine (T)-cluster peptides

In the high-concentration range of peptide solutions, an effect of competition for excess charge is observed.[10–13,15] In LC/MS experiments, mobile phases prepared with $H_2O$ and acetonitrile with 0.1% acetic acid are usually used. Therefore, low-concentration peptide solutions (1 pmol/μL) dissolved in 50% acetonitrile with 0.1% acetic acid were used in this experiment. The ion yields of protonated and deprotonated molecules, $[M+H]^+$ and $[M–H]^-$, of T-cluster peptides were estimated, as shown in Fig. 1. In positive-ion mode, the analyte peptides T6R, RT5F and FT5R gave relatively high ion yields, while T7, RT6 and KT6 gave low ion yields. RT5F gave the highest ion yield in both positive- and negative-ion mode.

The conversion of a Thr residue into an aromatic hydrophobic amino acid, namely Phe (RT6 into RT5F and T6R into FT5R), resulted in increases in the ion yields in both positive- and negative-ion mode. The enhancement effect of the Phe residue on the peak abundances of $[M+nH]^{n+}$ and $[M–H]^-$ was clearly observed by comparing the Phe-containing peptides RT5F and FT5R with the Phe-free peptides RT6 and T6R, respectively. It was previously reported that the hydrophobic property relating to the liquid surface activity, in the ESI process[8] is an important factor in the migration of peptide ions from the interior of the droplet to the surface. Therefore, it is conceivable that hydrophobic peptides containing Phe residue(s) are potentially advantageous to the vaporization process from the charged droplet to the gas phase in ESI, resulting in increased total ion yields in both positive- and negative-ion ESI. The comparison of ion yields for the Phe-containing peptides RT5F and FT5R demonstrates that RT5F gives a higher ion yield than FT5R in both ion modes. The results obtained above indicate that the presence of the Phe residue contributes to the increase in the ion yields, and suggest that the peptide containing a Phe residue at its C-terminus gives a higher ion yield than when it is at the N-terminus. The positive influence of the Phe residue on the ion yields of $[M+nH]^{n+}$ and $[M–H]^-$ may be attributed to the rate of vaporization $Jv$ from the droplet surface.

**Table 1.** Sequence and monoisotopic mass (Mm) of T-cluster and ACTH-based peptides

| Peptide | Sequence | Mm | R | K | E | D | F | P |
|---|---|---|---|---|---|---|---|---|
| T7 | TTTTTTT | 725.35 | 0 | 0 | 0 | 0 | 0 | 0 |
| RT6 | RTTTTTT | 780.41 | 1 | 0 | 0 | 0 | 0 | 0 |
| T6R | TTTTTTR | 780.41 | 1 | 0 | 0 | 0 | 0 | 0 |
| RT5F | RTTTTTF | 826.43 | 1 | 0 | 0 | 0 | 1 | 0 |
| FT5R | FTTTTTR | 826.43 | 1 | 0 | 0 | 0 | 1 | 0 |
| KT6 | KTTTTTT | 752.4 | 0 | 1 | 0 | 0 | 0 | 0 |
| ACTH18-35 (A1) | RPVKVYPNGAEDESAEAF | 1977.95 | 1 | 1 | 3 | 1 | 1 | 2 |
| [pTry$^{23}$]-ACTH18-35 (A1p1) | RPVKVpYPNGAEDESAEAF | 2057.93 | 1 | 1 | 3 | 1 | 1 | 2 |
| [pTry$^{23}$, pS$^{31}$]-ACTH18-35 (A1p2) | RPVKVpYPNGAEDEpSAEAF | 2137.91 | 1 | 1 | 3 | 1 | 1 | 2 |
| [Arg$^{36}$]-ACTH18-36 (A2) | RPVKVYPNGAEDESAEAFR | 2134.05 | 2 | 1 | 3 | 1 | 1 | 2 |
| [Arg$^{36}$]-ACTH19-36 (A3) | PVKVYPNGAEDESAEAFR | 1977.95 | 1 | 1 | 3 | 1 | 1 | 2 |
| ACTH19-36 (A4) | PVKVYPNGAEDESAEAFP | 1918.9 | 0 | 1 | 3 | 1 | 1 | 3 |
| ACTH22-39 (A5) | VYPNGAEDESAEAFPLEF | 1983.88 | 0 | 0 | 4 | 1 | 2 | 2 |
| [Arg$^{22}$]-ACTH22-39 (A6) | RYPNGAEDESAEAFPLEF | 2040.91 | 1 | 0 | 4 | 1 | 2 | 2 |



  

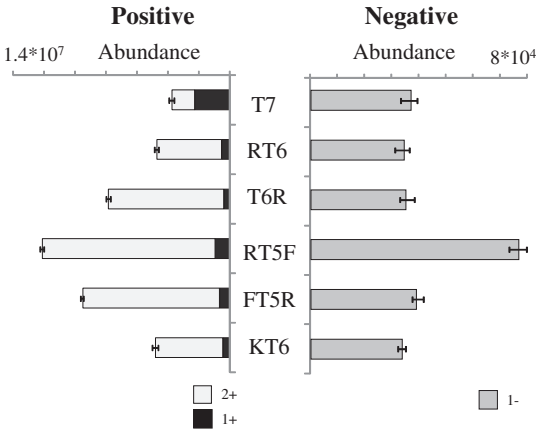


**Figure 1.** Positive- and negative-ion yields in ESI of analyte ions of 1 pmol/μL T-cluster peptides.

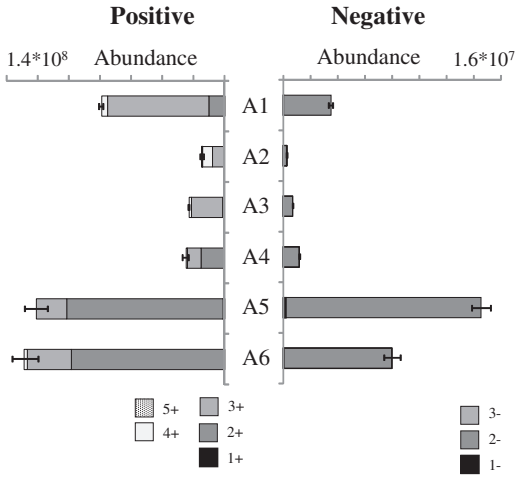


**Figure 2.** Positive- and negative-ion yields in ESI of analyte ions of 1 pmol/μL ACTH-based peptides.

The enhancement effect of the Arg residue on the peak abundance of $[M+nH]^{n+}$ was observed by comparing Arg-containing peptides RT6 and T6R with the Arg-free peptide T7. A Lys-containing T-cluster peptide KT6 also gave a higher ion yield than T7. The ion yield of T6R was higher than that of RT6. This result indicates that the presence of the Arg residue contributes to the increase in the positive-ion yield of $[M+nH]^{n+}$, and suggests that the peptides containing an Arg residue at their C-terminus result in a higher ion yield than those having the residue at the N-terminus. The ion yield of KT6 was higher than that of T7 and was similar to that of RT6, even though the basicity of Arg is higher than that of Lys. In the negative-ion ESI mode, the presence of the Arg and Lys residues did not affect the ion yields of T-cluster peptides. The positive influence of the Arg and Lys residues on the positive-ion yields of $[M+nH]^{n+}$ can be attributed to the ionization efficiency *I*. The results imply that the influence of the rate of desorption on the ion yields is higher than the effect of ionization efficiency in ESI.

### Ion yields of ACTH-based peptides

The ion yields of protonated and deprotonated molecules of ACTH-based peptides were obtained from the peak abundance, as shown in Fig. 2. In both positive- and negative-ion ESI, the peptides A1, A5 and A6 resulted in relatively high ion yields, while the peptides A2, A3 and A4 gave low ion yields.

The enhancement effect of a Phe residue at the C-terminus on the peak abundance of both protonated and deprotonated peptides was observed upon comparison of A1, A5 and A6 with A2, A3 and A4. Two Phe-containing peptides A5 and A6 gave extremely high ion yields in both positive- and negative-ion mode. The observed positive trends for A1, A5 and A6 on the ion yields were in accordance with that observed with the Phe-containing T-cluster peptide RT5F. The analyte A1 containing an Arg residue at its N-terminus and a Phe residue at its C-terminus gave higher ion yields than that of A3 containing an Arg residue at its C-terminus.

An enhancement effect of an Arg residue on the peak abundance of protonated molecules was observed upon comparison of the Arg-containing peptides A1 and A6 with the Arg-free peptides A4 and A5. The low ion yield of A2 which contains two Arg residues at both the N- and C-termini may be explained by the low hydrophobic properties of Arg residues. In negative-ion ESI, the higher ion yield of A5 compared to that of A6 may be due to the high hydrophobicity of A5. In comparing A5 with A6, the factor of hydrophobicity is the most important influence on the negative-ion yields because the ionization efficiencies *I* of A5 and A6 which contain five acidic amino acid residues are almost the same.

### Influence of hydrophobicity of peptides on ESI ion yields

Both positive- and negative-ion yields of peptides obtained above were dependent on their hydrophobic and hydrophilic natures, as well as on the presence of basic and acidic residues. Nishikaze *et al.* applied the Bull and Breeze (B&B) hydrophobic index[16] to evaluate the ion yields of peptides in FAB, MALDI and ESI-MS.[7] The hydrophobicities of peptides were calculated from the summation of the values of the B&B index of constituent amino acids. The B&B index, estimated from the surface tension of each amino acid in 0.1 M NaCl aqueous solution, has been widely used to represent the hydrophobicity of amino acids. It was found that the hydrophobic values of peptides obtained from the B&B index are affected by the hydrophobic amino acid residues without a large degree of influence from hydrophilic residues. Therefore, the ion yields of the peptides used here could not be rationalized strictly from the B&B index. In this study, the positive- and negative-ion yields of ACTH-based peptides were correlated with the hydrophobicity calculated from the partition coefficient measured by thin-layer chromatography (PACTLC) using various solvents,[17] as shown in Fig. 3. The PACTLC values of peptides were calculated from the summation of the values of functional groups of each peptide shown by Pliška *et al.*[17] The hydrophobicities calculated for the peptides are summarized in Table 2. The positive-ion yields of the peptides increased with increasing hydrophobicity as measured by PACTLC. Even though it may be expected that the most hydrophobic peptide A5 would give a highest ion yield, the analyte A6 gave a higher ion yield than A5 in positive-ion mode. The higher ion yield of A6 can be attributed to both the effects

  

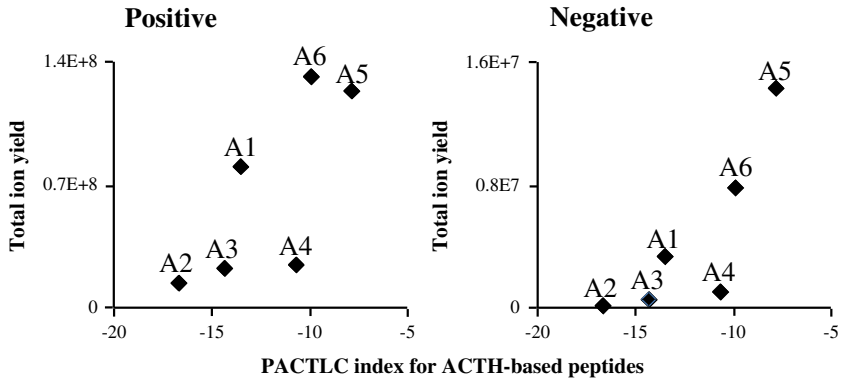


**Figure 3.** Correlations between ion yield and PACTLC hydrophobicity index in positive- and negative-ion ESI.

**Table 2.** Calculated values of B&B, PACTLC and HYPA indices of analyte peptides

| Peptide | B&B | PACTLC[a] | HYPA[b] |
|---|---|---|---|
| A1 | 2.45 | -13.64 | -15.0 |
| A2 | 3.14 | -16.84 | -19.5 |
| A3 | 2.45 | -14.47 | -15.0 |
| A4 | 1.59 | -10.79 | -12.1 |
| A5 | 1.93 | -7.92 | -7.70 |
| A6 | 3.37 | -10.01 | -16.4 |
| T7 | 2.03 | -6.52 | -4.9 |
| RT6 | 2.43 | -7.75 | -8.7 |
| T6R | 2.43 | -7.75 | -8.7 |
| RT5F | 0.62 | -6.45 | -5.2 |
| FT5R | 0.62 | -6.45 | -5.2 |
| KT6 | 2.20 | -7.99 | -8.1 |

[a]PATLC is the hydrophobicity calculated from partition coefficient measured by TLC.
[b]HYPA is the number representing the hydrophobic property by the hydrophathy index.

of the N-terminal Arg residue and the C-terminal Phe residue which gives high hydrophobicity. Since the PACTLC correlation shown in Fig. 3 does not include the enhancement effect of basic amino acid residues, the correlation in positive-ion mode did not give the linear relationship. Similarly, with the exception of A4, the negative-ion yields of the peptides also increased with increasing hydrophobicity. The analyte A5 gave the highest negative-ion yield due to the presence of the highest hydrophobicity and acidic amino acid residues. In both positive- and negative-ion ESI, the low ion yields of A2 can be attributed to its low hydrophobicity. Analytes A3 and A4 containing two or three proline (Pro) residues at the N- and C-termini gave low ion yields. Although the Pro residue is recognized as a hydrophobic amino acid, the hydropathy (HYPA) index reported by Kyte *et al.* shows Pro to have a high hydrophilicity, as shown in Table 2.[18] Thus, Pro is recognized as a residue more hydrophilic than serine (Ser) and Thr residues as measured by the HYPA index. The low ion yields of A3 and A4 containing Pro residues may be due to the hydrophilic nature. This means that the high hydrophilicity of the Pro residue caused low desolvation efficiency in the charged droplets in ESI. The results obtained here indicate that ESI ion yields of peptides are rather influenced by the vaporization factor *Jv* in Eqn. (1) than the ionization efficiency factor *I*.

### Influence of phosphorylation on the ion yields of ACTH-based peptides

To evaluate the ion yields of phosphorylated peptides in ESI-MS, ACTH18-35, a non-modified peptide A1 and mono- and di-phosphorylated peptides, A1p1 and A1p2, were used. In the positive-ion mode, the doubly, triply and quadruply charged analytes were observed as $H^+$ and $Na^+$ and $K^+$ adduct ions. In the negative-ion mode, the doubly charged deprotonated peptides $[M–2H]^{2–}$ were mainly observed. Figure 4 shows the positive- and negative-ion yields of the ACTH peptides. A decrease in the yields of the phosphorylated peptides A1p1 and A1p2 compared with non-phosphorylated peptide A1 was observed in both positive- and negative-ion ESI. The results indicate that the phosphorylation contributes to the suppression of both positive- and negative-ion yields. Even though the negative-ion yields of phosphorylated peptides were expected to be high due to the acidic nature of the phosphate group, A1p1 and A1p2 gave lower ion yields than A1. This suggests that the highly hydrophilic properties of phosphorylated peptides contributed to the low desorption efficiency from the charged droplets in ESI by means of the rate of desorption of analyte

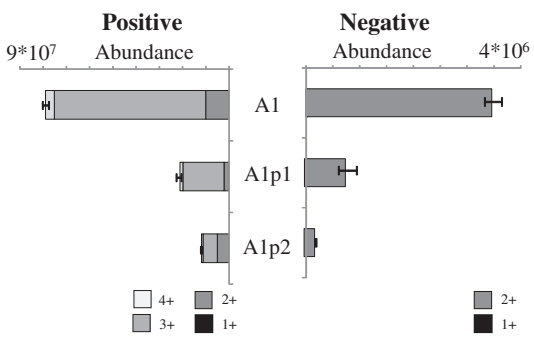


**Figure 4.** Positive- and negative-ion yields in ESI of analyte ions of non-, mono- and di-phosphorylated ACTH-based peptides.



  

molecules, $Jv$ in Eqn. (1). These results indicate that the effect of the desorption rate on the ion yields is high compared to the effect of ionization efficiency in ESI.

## CONCLUSIONS

The influence of hydrophobicity on the ion yields of model peptides was studied in both positive- and negative-ion ESI-MS. In ESI, the ion yields were governed by both ionization (protonation/deprotonation) efficiency and desorption (or vaporization) rate of peptides. The ionization efficiency and desorption rate of peptides are strongly dependent upon their physicochemical properties which originate from the individual nature of the side chains of the constituent amino acids. The presence of an aromatic hydrophobic amino acid Phe in the peptides was advantageous for the desorption of peptide ions in the charged droplets generated by ESI, and resulted in the enhancement of the ion yields of both analyte ions $[M+nH]^{n+}$ and $[M–nH]^{n–}$. The peptide containing a Phe residue at its C-terminus gave a higher ion yield than when the same residue is present at its N-terminus. The positive influence of the Phe residue on the ion yields was attributable to the desorption rate. The positive-ion enhancement effect of basic amino acid residues such as Arg and Lys on the peak abundance of $[M+nH]^{n+}$ was also observed upon comparison of Arg-containing peptides with Arg-free peptides for increasing the ionization efficiency. The ion yield of KT6 was higher than that of T7 and was similar to that of RT6, even though the basicity of Arg is higher than that of Lys. These results seemed to be affected by the difference in vaporization efficiency of Arg and Lys. The highest ion yield of $[M+nH]^{n+}$ was observed with the peptide containing both hydrophobic and basic amino acid residues. However, the peptide containing two Arg residues gave lower a ion yield due to the high hydrophilic property of Arg. Additionally, the high hydrophilic property of the phosphate group contributes to suppression of the ion yields of both $[M+nH]^{n+}$ and $[M–nH]^{n–}$. The results indicate that the enhancement in the ion yields of peptides in ESI would be governed more by the effect of hydrophobicity than that of ionization efficiency.

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