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Short Communication

Identification of O-phosphoamino acids in urine hydrolysate by gas chromatography–mass spectrometry

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ABSTRACT

The occurrence of O-phosphoserine (P-Ser), O-phosphothreonine (P-Thr) and O-phosphotyrosine (P-Tyr) in the hydrolysate of human urine was demonstrated. Urine samples were separated into non-adsorbed and adsorbed fractions by DEAE-cellulose column chromatgraphy. After acid and base hydrolyses of these fractions, the O-phosphoamino acids in the hydrolysates were converted into their N-isobutoxycarbonyl methyl ester derivatives and identified by gas chromatography–mass spectrometry. By gas chromatography with flame photometric detection, the urinary contents of P-Ser, P-Thr and P-Tyr were estimated to be 945 ± 3, 109 ± 3 and 2.9 ± 0.2 ng/ml, respectively.

INTRODUCTION

Post-translational modification of proteins by the formation of phospho monoesters of serine, threonine and tyrosine residues is one of the major regulatory mechanisms for the control of diverse intracellular functions [1–5]. Particularly, it is noteworthy that abnormal protein phosphorylation reactions caused by genetic mutations, the introduction of viral genomes, or chemical treatment are responsible for malignant neoplasia or the uncontrolled growth of cells [6,7]. Furthermore, it is reported that Tau proteins from Alzheimer's disease are abnormally phosphorylated [8–10]. Therefore, if these abnormal phosphorylation levels are reflected in biological fluids, a measurement of the phosphorylated amino acids in these samples could be important in the diagnosis of these diseases. However, these phosphorylated amino acids have not yet been found in biological fluids.

Recently, we developed a selective and sensitive method for determining O-phosphoamino acids by gas chromatography (GC) with flame photometric detection (FPD) [11,12], and analysed the contents of O-phosphoamino acids in the tissue samples [13] and the proteins that were phosphorylated by protein kinases [14]. When the hydrolysates of human urine samples were analysed by this method, peaks having the same retention times as those of O-phosphoserine (P-Ser), O-phosphothreonine (P-Thr) and O-phosphotyrosine (P-Tyr) were observed in the chromatograms. Therefore, we attempted to identify these peak components by GC with mass spectrometry (MS).

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EXPERIMENTAL

Chemicals

O-Phospho-L-serine (P-Ser), O-phospho-D,L-threonine (P-Thr) and O-phospho-L-tyrosine (P-Tyr) were purchased from Sigma (St. Louis, MO, USA). Standard solutions were prepared containing 0.1 mg/ml of each compound in distilled water and then stored at 4°C. Isobutyl chloroformate was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). N-Methyl-N-nitroso-*p*-toluenesulphonamide to generate diazomethane was obtained from Nacalai Tesque (Kyoto, Japan). DEAE-cellulose SS was purchased from Nacalai Tesque. Sephadex G-25 was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Dowex 1-X8 (100–200 mesh) and Dowex 50W-X8 (100–200 mesh) were purchased from Muromachi Kagaku Kogyo (Tokyo, Japan). All other chemicals were of analytical grade.

Sample preparation for GC–MS analysis

Normal human urine samples were collected under toluene and kept frozen if not analysed immediately. The following sample treatments were carried out for the identification of O-phosphoamino acids in 24-h urine (880 ml) (see Fig. 1).

DEAE-cellulose column chromatography. A portion (40 ml) of 24-h urine was applied to a DEAE-cellulose column (40 cm × 2.5 cm I.D.) pre-equilibrated with distilled water. The column was washed with 450 ml of water, and then eluted with 0.1 *M* ammonium hydrogencarbonate (pH 8.3). The effluent (flow-rate 55 ml/h) was collected in 15-ml fractions. The UV absorption of each fraction was determined manually in a Shimadzu UV-140 double-beam spectrophotometer at 280 nm. The O-phosphoamino acids in the fractions were analysed by the previously reported GC–FPD method [11,13] after acid and base hydrolyses of 0.1–0.5 ml of each fraction. Remaining 24-h urine was treated by same procedure. The non-adsorbed fraction (containing P-Ser and P-Thr) and the adsorbed fraction (containing P-Tyr) were separately collected, and called fraction A and fraction B, respectively.

Isolation of P-Ser and P-Thr in fraction A. The combined fraction A (1320 ml) was concentrated to *ca.* 180 ml in a rotary evaporator at 40–45°C and then precipitated by addition of 20 ml of 50% trichloroacetic acid (TCA). After centrifugation at 15 000 *g* for 15 min at 4°C, the pellet was washed three times with 20 ml of diethyl ether and dissolved in 10 ml of 75% methanol. This solution was equally divided into 32 glass tubes (50 mm × 5 mm I.D.), dried in a Model RD-41 centrifugal evaporator (Yamato Kagaku, Tokyo, Japan), and then hydrolysed with 0.2 ml of 6 *M* hydrochloric acid in the vapour phase for 2 h at 110°C with a Pico-Tag workstation (Waters Assoc., Milford, MA, USA). Each hydrolysate was extracted twice with 0.4 ml of distilled water, combined and neutralized with 2 *M* sodium hydroxide. The hydrolysate extract was made up to 40 ml with water, and an aliquot (5 ml) of this solution was applied to a Dowex 1-X8 (AcO^- form) column (5 cm × 0.7 cm I.D.). The column was washed with 15 ml of water and then

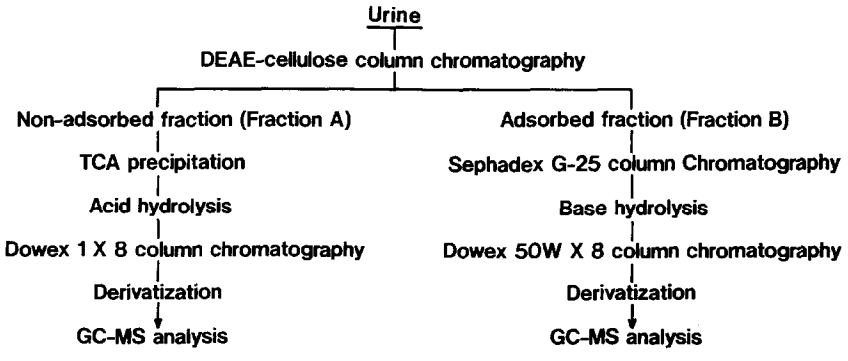

Fig. 1. Sample treatment used for the identification of urinary O-phosphoamino acids.

eluted with 0.1 *M* hydrochloric acid. The initial 14 ml of the eluate was discarded, and the following 4 ml of the eluate (pH 1–2) was collected. The remaining hydrolysate extract was treated by the same procedure. The collected eluates were derivatized by the derivatization procedure described below, and the derivatized samples were combined and used for GC–MS analysis.

Isolation of P-Tyr in fraction B. The combined fraction B (330 ml) was concentrated to *ca.* 10 ml in a rotary evaporator at 40–45°C and then applied to a Sephadex G-25 column (20 cm × 3.5 cm I.D.) pre-equilibrated with distilled water. The column was eluted with water and the effluent (flow-rate 118 ml/h) was collected in 7.5-ml fractions. The initial 150 ml of the eluate was discarded, and the following 15 ml of the eluate was collected. This fraction was evaporated to dryness in a rotary evaporator at 40–45°C, and dissolved in 2 ml of water. After this solution was equally divided into ten polypropylene tubes (9 cm × 0.75 cm I.D.), 0.2 ml of 10 *M* potassium hydroxide was added to each tube, and the mixture was hydrolysed for 1 h at 130°C in a Pico-Tag workstation. Each hydrolysate was neutralized with 6 *M* hydrochloric acid and made up to 5 ml with water. Each solution was applied onto a Dowex 50W-X8 (H^+ form) column (6.2 cm × 0.7 cm I.D.). The column was eluted with water and the initial 10 ml of the eluate was collected. Each collected eluate was derivatized by the derivatization procedure described below, and the derivatized samples were combined and used for GC–MS analysis.

Derivatization procedure

O-Phosphoamino acids were derivatized as previously described [11]. After the sample was adjusted to pH 10 with 2 *M* sodium hydroxide if necessary, 0.1 ml of isobutyl chloroformate was added and the mixture was shaken with a shaker set at 3000 rpm (up and down) for 10 min at room temperature. The reaction mixture was acidified to pH 1–2 with 2 *M* hydrochloric acid and extracted with 3 ml of diethyl ether in order to remove the excess of reagent. The ether extract was discarded, and the aqueous layer was saturated with sodium chloride and extracted twice with 3 ml of diethyl ether containing 10% 2-propanol. The pooled ether extracts were methylated by bubbling diazomethane, generated according to the micro-scale procedure of Schlenk and Gellerman [15], through the solution until a yellow tinge became visible. After standing for 5 min at room temperature, the solvents were removed by evaporation to dryness at 80°C under a stream of dry air. The residue was dissolved in 0.1 ml of ethyl acetate, and 1 μl of this solution was analysed by GC or GC–MS.

Gas chromatography

GC analysis was carried out with a Shimadzu 14A gas chromatograph equipped with a flame ionization detector and flame photometric detector (P-filter). Fused-silica capillary columns containing cross-linked DB-1701 (J & W, Folsom, CA, USA, 15 m × 0.53 mm I.D., 1.0 μm film thickness, for the P-Ser and P-Thr analysis) or DB-5 (J & W, 15 m × 0.53 mm I.D., 1.0 μm film thickness, for the P-Tyr analysis) were used. Column temperatures: 220°C for DB-1701, and programmed at 3°C/min from 230 to 280°C for DB-5. Injection and detector temperatures: 260°C for DB-1701, and 290°C for DB-5. The nitrogen flow-rate was 10 ml/min.

Gas chromatography–mass spectrometry

A Hewlett-Packard 5890A gas chromatograph was operated in conjunction with a VG Analytical 70-SE mass spectrometer and a VG 11-250J mass data system. Fused-silica capillary columns containing cross-linked OV-1 (Quadrex, New Haven, CT, USA, 50 m × 0.25 mm I.D., 0.25 μm film thickness, for the P-Ser and P-Thr analysis) or OV-17 (Quadrex, 25 m × 0.25 mm I.D., 0.25 μm film thickness, for the P-Tyr analysis) were used. Column temperature: isothermal at 190°C for 15 min, then programmed at 6°C/min to 260°C (for OV-1) or at 3°C/min from 220 to 260°C for OV-17; injection temperature, 260°C; ion-source temperature, 250°C; ionizing voltage, 40 eV; helium flow-rate, 8 ml/min.

RESULTS AND DISCUSSION

When the acid and base hydrolysates of human urine samples were directly analysed by GC–FPD, which is highly selective for phosphorus compounds, peaks having the same retention times as those of P-Ser, P-Thr and P-Tyr were observed in the chromatograms. However, these peaks could not be confirmed by non-selective GC with flame ionization detection (FID) because of interferences. Therefore, we attempted to identify these peak components by GC–MS after partial purification of urine samples. The sample preparation for GC–MS is outlined in Fig. 1. In the first place, urine samples were chromatographed on a DEAE-cellulose column. Representative data of the elution profile and the O-phosphoamino acid contents are shown in Fig. 2. The O-phosphoamino acids in each fraction were measured by GC–FPD method [11,13] after release from proteins by acid and base hydrolyses. Most of P-Ser and P-Thr were eluted in the non-adsorbed fraction (fraction A), but P-Tyr was not detected in this fraction. On the other hand, P-Tyr was eluted with 0.1 *M* ammonium hydrogencarbonate in the adsorbed fraction (fraction B) as single peak, and P-Ser was also detected in this fraction. These results indicate that several proteins containing O-phosphoamino acids exist in urine, and that these proteins differ in their properties.

Fraction A was precipitated with TCA after concentration. The resulting pellet was hydrolysed with 6 *M* hydrochloric acid for release of P-Ser and P-Thr from proteins, because the O-phosphate linkages of serine and threonine residues are more stable to acid than to base. The resulting acid hydrolysate was purified by Dowex 1-X8 column chromatography. On the other hand, fraction B was desalted by Sephadex G-25 column chromatography and hydrolysed with 5 *M* potassium hydroxide for release of P-Tyr from proteins, because the O-phosphate linkage of tyrosine residue is more stable to base than to acid. The resulting base hydrolysate was purified by Dowex 50W-X8 column chromatography.

The O-phosphoamino acids isolated from fractions A and B could be conveniently converted into their N-isobutoxycarbonyl methyl ester derivatives by the previous method [11] and identified by GC–MS. As shown in Fig. 3A, C and E, a molecular ion peak (M^+) was not observed for each of the authentic derivatives but the prominent fragment ion peaks, $M^+ - 59$ ($COOCH_3$), $M^+ - 73$ [$(CH_3)_2CHCH_2O$] and *m/z* 109 [$PO(OCH_3)_2$] were characteristic and useful for

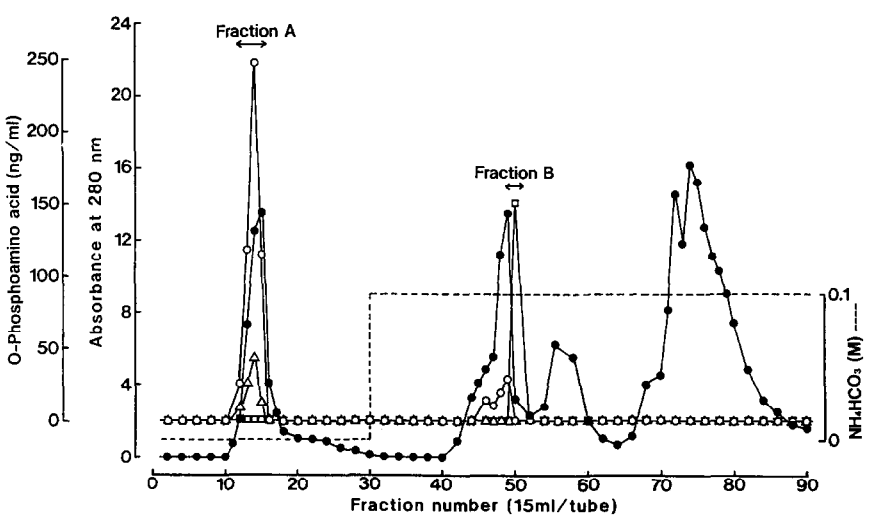

Fig. 2. Column chromatography of a urine sample containing O-phosphoamino acids on DEAE-cellulose. Chromatographic conditions as in Experimental. (●) Absorbance at 280 nm; (○) P-Ser; (△) P-Thr; (□) P-Tyr.

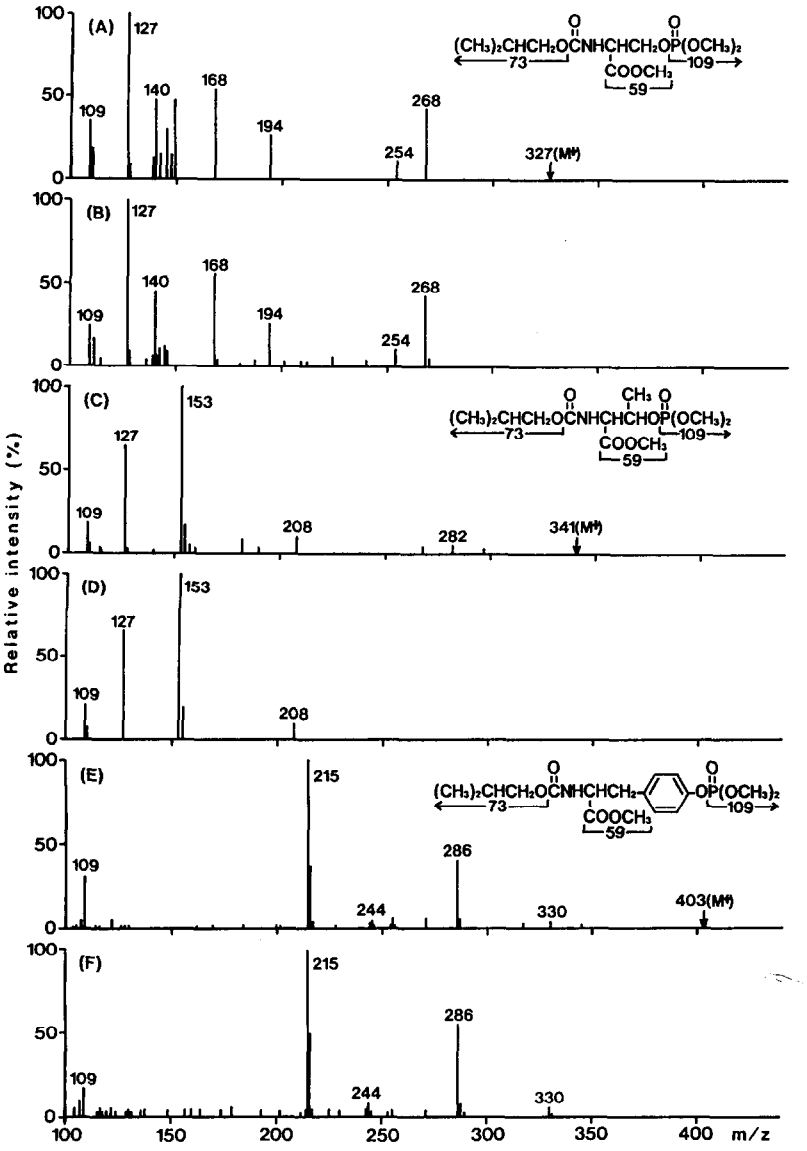

Fig. 3. GC–MS spectra obtained from N-isobutoxycarbonyl methyl ester derivatives of authentic P-Ser, P-Thr and P-Tyr, and from urine hydrolysate. (A) Authentic P-Ser; (B) peak identified as P-Ser in acid hydrolysate of urine; (C) authentic P-Thr; (D) peak identified as P-Thr in acid hydrolysate of urine; (E) authentic P-Tyr; (F) peak identified as P-Tyr in base hydrolysate of urine.

structure elucidation. As shown in Fig. 3B, D and F, the mass spectra obtained from the urine samples agreed with those of the authentic derivatives of O-phosphoamino acids. These results clearly indicate that P-Ser, P-Thr and P-Tyr were present in urine hydrolysates.

The urinary contents of P-Ser, P-Thr and P-Tyr, as measured by GC–FPD after partial acid and base hydrolyses of urine sample according to the previous method [13], were estimated to be 945 ± 3, 109 ± 3 and 2.9 ± 0.2 ng/ml (n = 4), respectively. On the other hand, the total serine, threonine and tyrosine contents, measured by the previous GC–FID method [16] after complete hydrolysis of urine sample with 6 M hydrochloric acid at 150°C for 1 h, were 118 ± 6, 57 ± 3 and 126 ± 31 μg/ml (n = 4), respectively. These results show that P-Ser, P-Thr and P-Tyr in urine

were less than 0.8, 0.2 and 0.002% of total serine, threonine and tyrosine, respectively. In addition, free O-phosphoamino acids in non-hydrolysed urine sample were measured by the previous GC–FPD method [17] after clean-up of the sample by ion-exchange column chromatography. Free P-Ser was found in urine at a concentration of 33 ± 2 ng/ml (n = 4) but free P-Thr and P-Tyr were not detected at all. These results indicate that most of O-phosphoamino acids exist in the bound form in urine. Similarly, these O-phosphoamino acids were also found in other healthy human urine samples by GC–FPD method.

The present results are interesting in connection with protein phosphorylation in the tissues. Although the physiological significance and origin of urinary O-phosphoamino acids are not known, the existence and occasional contents of these O-phosphoamino acids in urine suggest that the urinary O-phosphoamino acid level may reflect the *in vivo* phosphorylation level. An investigation of the behaviour of O-phosphoamino acids *in vivo* is now in progress in our laboratory.

REFERENCES

1 E. G. Krebs, *Biochem. Soc. Trans.*, 13 (1985) 813.
2 P. Cohen, *Proc. R. Soc. London*, B234 (1988) 115.
3 D. R. Sibley, J. L. Benovic, M. Caron and R. Lofkowitz, *Endocrinol. Rev.*, 9 (1988) 38.
4 R. B. Bourret, K. A. Borkovich and M. I. Simon, *Ann. Rev. Biochem.*, 60 (1991) 401.
5 T. Hunter and M. Karin, *Cell*, 70 (1992) 375.
6 S. Giordano, M. F. DiRenzo, D. Cirillo, P. Chiado and P. M. Camoglio, *Int. J. Cancer*, 39 (1987) 482.
7 B. J. Druker, H. J. Mammon and T. M. Roberts, *N. Engl. J. Med.*, 321 (1989) 1383.
8 S. Flament, A. Delacourte and D. M. A. Mann, *Brain Res.*, 516 (1990) 15.
9 I. P. Shapiro, E. Masliah and T. Saitoh, *J. Neurochem.*, 56 (1991) 1154.
10 D. P. Hanger, J. P. Brion, J. M. Gallo, N. J. Cairns, P. J. Luther and B. H. Anderton, *Biochem. J.*, 275 (1991) 99.
11 H. Kataoka, N. Sakiyama and M. Makita, *J. Chromatogr.*, 438 (1988) 67.
12 H. Kataoka, Y. Ueno and M. Makita, *Agric. Biol. Chem.*, 55 (1991) 1587.
13 H. Kataoka, K. Nakai, Y. Ueno and M. Makita, *Biosci. Biotech. Biochem.*, 56 (1992) 1300.
14 H. Kataoka, Y. Ueno and M. Makita, *J. Pharm. Biomed. Anal.*, 10 (1992) 365.
15 H. Schlenk and J. L. Gellerman, *Anal. Chem.*, 32 (1960) 1412.
16 M. Makita, S. Yamamoto and M. Kono, *J. Chromatogr.*, 120 (1976) 129.
17 H. Kataoka, N. Sakiyama and M. Makita, *J. Biochem.*, 109 (1991) 577.