

It is well known that reversible phosphorylation of proteins plays an important role in biological sample, such as signal transduction. In many analyses of phosphorylated proteins, the main objective is to extract phosphorylated peptides from enzymatic digest of proteins. However, it is very hard to extract phosphorylated peptides since a trace amount of phosphorylated peptides are only available.

In this technical note, an efficient method for the selective extraction of phosphorylated peptides using "Titansphere Phos-TiO" was developed. "Titansphere Phos-TiO" contains a titania ( $\text{TiO}_2$ ) packing fixed to the tip column. Titania has been reported to be the most efficient and effective adsorbent to selectively extract or enrich phosphorylated peptides.

Phosphorylated angiotensin II was selectively extracted from mixture of angiotensin II and phosphorylated angiotensin II by only 40 minutes.

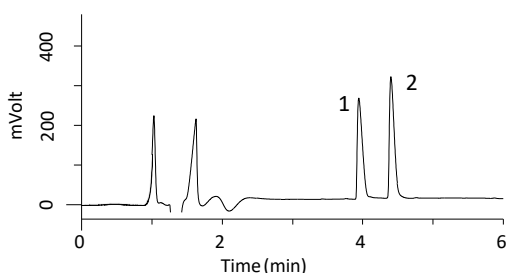
(C. Aoyama)

## HPLC Conditions

|                   |  |
|-------------------|--|
| <b>System</b>     | : LC800 System   |
| <b>Column</b>     | : InertSustain C18 HP<br>(3 $\mu\text{m}$ , 150 $\times$ 2.1 mm I.D.)  |
| <b>Eluent</b>     | : A) 0.1% HCOOH in $\text{H}_2\text{O}$<br>B) 0.1% HCOOH in $\text{CH}_3\text{CN}$ A/B =<br>85/15 – 5 min – 70/30, v/v |
| <b>Flow Rate</b>  | : 0.3 mL/min   |
| <b>Col. Temp.</b> | : 40 $^\circ\text{C}$  |
| <b>Detection</b>  | : UV 210 nm  |
| <b>Inj. Vol.</b>  | : 10 $\mu\text{L}$   |

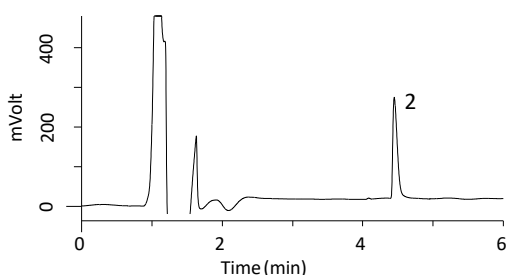
## Standard Solutions

1. Angiotensin II 50 mg/L
2. [Tyr( $\text{PO}_3\text{H}_2$ )<sup>4</sup>]-Angiotensin II 50 mg/L



## After Pretreatment with Phos-TiO Kit

Recovery Rate ( $n = 3$ ):  $81.6 \pm 1.3\%$



\* Buffer A was made by adding 4 times of Acetonitrile of Solution A (v/v) contained in Titansphere Phos-TiO Kit.

\* Buffer B was made by adding 3 times of Buffer A of Solution B (v/v) contained in Titansphere Phos-TiO Kit.

## Sample preparation method with Titansphere Phos-TiO Kit

- Set the centrifuging rate at 3,000  $\times$  g.
- Use digestive peptide after finishing the reduction and alkylation procedure.
- Make sure that the solution is completely eluted from the Spin Tip after centrifuge operation.
- Regardless the Spin Tip volume (10  $\mu\text{L}$  or 200  $\mu\text{L}$ ), use the same amount of Buffer except the Buffer B for adsorption (e. g.): Use 20  $\mu\text{L}$  of conditioning for both types.

### Spin Tip

- Connect a centrifugal adaptor to a Waste Fluid Tube, and insert the Spin Tip into tube (Fig. 1).

### Conditioning

- Add 20  $\mu\text{L}$  of Buffer A, then centrifuge (3,000  $\times$  g, 2 min, RT).  
※ RT = Room Temperature

### Equilibration

- Add 20  $\mu\text{L}$  of Buffer B then centrifuge (3,000  $\times$  g, 2 min, RT).  
- Remove the 40  $\mu\text{L}$  of effluent from the Waste Fluid Tube.

### Adsorption

- Add 15  $\mu\text{L}$  ~ 50  $\mu\text{L}$  of a sample and 100  $\mu\text{L}$  of Buffer B into the Spin Tip.  
※ In the case of 10  $\mu\text{L}$  tip, add 15  $\mu\text{L}$  of the sample and 50  $\mu\text{L}$  of Buffer B into the Spin Tip.
- To mix the sample with Buffer B, repeat pipetting three times within the Spin Tip (Fig. 2).
- Centrifuge (1,000  $\times$  g, 10 min, RT)  
※ High centrifugal speed may lead to determination in recovery rate.
- Put the sample in the tube back into the Spin Tip again and centrifuge (1,000  $\times$  g, 10 min, RT).
- Remove the sample and buffer B collected in the tube.

### Rinsing

- Add 20  $\mu\text{L}$  of Buffer B and centrifuge (3,000  $\times$  g, 2 min, RT).  
- Add 20  $\mu\text{L}$  of Buffer A and centrifuge (3,000  $\times$  g, 2 min, RT).  
※ Add of buffer A repeat twice

### Elution

- Put the Spin Tip into the recovery tube.
- Add 50  $\mu\text{L}$  of 5% ammonium hydroxide solution and centrifuge (1,000  $\times$  g, 5 min, RT)
- Add 50  $\mu\text{L}$  of 5% pyrrolidine solution and centrifuge (1,000  $\times$  g, 5 min, RT)

### Phosphopeptide

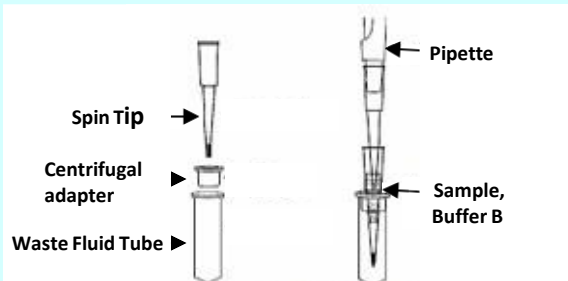
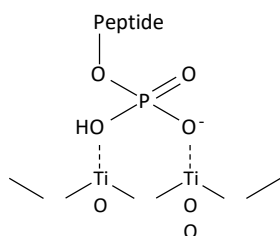


Fig. 1 Setting of Spin Tip

Fig. 2 Mixing of Sample and Buffer B



Structures are created using Chemistry 4-D Draw which is provided by ChemInnovation Software.



It is assumed that titania ( $\text{TiO}_2$ ) has functionality to act on phosphate group as shown on the left figure. This interaction tends to be strong under acidic condition, and to be weak under alkaline condition. In this application note, ammonia aqueous solution was used to elute phosphorylated peptides from Phos-TiO column. Also, high concentrated phosphate solution can be used to elute phosphorylated peptides from Phos-TiO column.

\* Titansphere Phos-TiO products were developed with corporation by Prof. Ishihama in Kyoto University.

### Titansphere Phos-TiO Kit

|                     |                        |          |
|---------------------|------------------------|----------|
| Cat. No. 5010-21309 | 1 mg/10 $\mu\text{L}$  | 24 times |
| Cat. No. 5010-21310 | 1 mg/10 $\mu\text{L}$  | 96 times |
| Cat. No. 5010-21311 | 3 mg/200 $\mu\text{L}$ | 24 times |
| Cat. No. 5010-21312 | 3 mg/200 $\mu\text{L}$ | 96 times |

- Titania packing material which has strong affinity is fixed to the tip column.
- The tip column can be connected to the 1.5 mL micro tube using a dedicated centrifugal adaptor.
- All steps are done by centrifugation which eliminates human error.
- Requires only 40 minutes of sample preparation time.

### Titansphere Phos-TiO (Syringe barrel type)

|                     |             |           |
|---------------------|-------------|-----------|
| Cat. No. 5010-21290 | 50 mg/3 mL  | 25 pcs/pk |
| Cat. No. 5010-21291 | 100 mg/3 mL | 25 pcs/pk |

For large sample volume, the above syringe barrel type is available for higher efficiency.

InertSustain C18 HP (3  $\mu\text{m}$ , 2.1 x 150 mm) Cat. No. 5020-14415

InertSustain C18 show superior inertness to typically any analytes and is compatible with wide pH analysis.

MonoSpin C18 100 pk Cat. No. 5010-21701      50 pk Cat. No. 5010-21700 MonoSpin C18

MonoSpin is a spin column for sample preparation by centrifugation. It is ideal for desalting of peptides, etc.

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