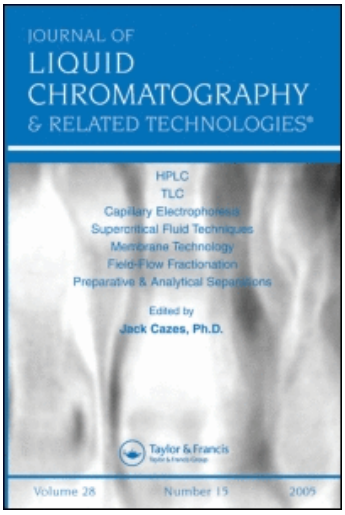

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Successful and Rapid Verification of the Presence of a Phosphate Group in Synthetic Phosphopeptides Using the Conditions of Standard Dabs-Cl Amino Acid Analysis

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SUCCESSFUL AND RAPID VERIFICATION OF THE PRESENCE OF A PHOSPHATE GROUP IN SYNTHETIC PHOSPHOPEPTIDES USING THE CONDITIONS OF STANDARD DABS-CL AMINO ACID ANALYSIS

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ABSTRACT

The increased importance of phosphopeptides, and the currently used post-assembly phosphorylation protocol in synthetic peptide laboratories, requires a rapid and sensitive method to verify the presence of the phosphate group in synthetic phosphopeptides. A reversed-phase high-performance liquid chromatography protocol has been developed to verify the success of the phosphorylation reaction in synthetic phosphopeptides after hydrolysis and derivatization with 4-dimethylamino-azobenzene-4'-sulphonyl chloride. Phosphoamino acid standards and model phosphopeptides were used to study the optimal elution and hydrolysis conditions of phosphoamino acids. A 1.5-hour, gas-phase acidic hydrolysis condition liberated the phosphoamino acids from the phosphopeptides, and still did not destroy them. After hydrolysis, the dabsylated free phosphoamino acids were baseline separated from the other acidic amino acids and were eluted from the reversed-phase column in the following order: phosphoserine, phosphothreonine, and phosphotyrosine. The utility of the approach was demonstrated by the phosphoamino acid analysis of several synthetic phosphopeptides, in which the amino acid environment of the phosphorylated serine or tyrosine was different. This method may not only be applicable for phosphopeptides, but also for verifying the presence of phosphate groups in phosphoproteins.

INTRODUCTION

In the last decade, understanding the biological functions of post-translationally modified proteins has become the focus of interest, and it has elevated the importance of synthetic phospho- and glycopeptides. The negatively charged phosphoryl groups are on the surface of proteins, and are known to play a crucial role in the recognition processes at both the macromolecular and cellular levels (1-5). Our laboratory is particularly interested in the physical, chemical, and biological properties of phosphorylated peptides. One series of peptides we have studied corresponds to various regions of the low molecular-weight, microtubule-associated protein τ. The neurofibrillary tangles (one of the hallmark lesions of Alzheimer's disease) are made of hyperphosphorylated forms of protein τ (6). Using synthetic phosphopeptides, we have demonstrated that conformational and immunological alterations in the protein are due to the incorporation of phosphate groups into amino acids that lack the phosphate in normal τ (7-10).

Recognition of the increasing importance of phosphopeptides demands the development of appropriate analytical methods. Analysis of unmodified peptides is most often accomplished by amino acid analysis, fast atom bombardment mass spectroscopy (FAB-MS), nuclear magnetic resonance (NMR) spectroscopy, and sequencing. All these methods are equally appropriate to verify the presence of phosphate groups on synthetic peptides, but all have drawbacks. FAB-MS and NMR are not common in the peptide synthesis laboratories. Although sequencing is more often available, all of the above listed methods tend to be expensive for the analysis of a series of phosphopeptides. Moreover, phosphopeptides tend to remain in the matrix during FAB-MS spectroscopy, especially when other charged groups are present (11). Loss of the phosphate group is also reported during NMR spectroscopy in an acidic aqueous solution (12). The conventional organic phosphate analysis (13) is a good alternative, but it requires samples in the several hundred μg range. Alternatively, the hydrophilic character of the phosphate group presents a chromatography-based analysis, since the phosphopeptides exhibit a decreased retention time compared to their non-phosphorylated analogs on reversed-phase high-performance liquid chromatography (RP-HPLC) (11,14). This may apply to the phosphorylated amino acids as well.

The standard procedure for amino acid analysis of synthetic peptides follows hydrolysis, post- or precolumn derivatization and HPLC analysis. Verification of the presence of the phosphate group on the phosphopeptides requires the detection of phosphorylated amino acids in the hydrolyzate utilizing the different conditions of amino acid analysis. The stability of phosphoamino acids under acidic conditions

is well characterized (15-16), and various derivatization methods are used for identification of phosphoamino acids, such as O-phthalaldehyde (17-21), 9-fluorenylmethyl chloroformate (FMOC) (22), phenyl isothiocyanate (PITC) (23) and 4-dimethyl-aminoazobenzene-4'-sulphonyl chloride (DABS-Cl) (24-25).

Most of the currently used phosphopeptide synthetic protocols utilize a post-assembly phosphorylation procedure (26). In an ideal case, only the phosphopeptides are present after the phosphorylation reaction, but in our experience the reaction is never complete and two peaks, corresponding to the phosphorylated and non-phosphorylated peptides, are detected after cleavage from the resin. The growing number of synthetic phosphopeptides requires a rapid, unambiguous and sensitive method to verify the success of this phosphorylation reaction. Since it was previously reported that partial acidic hydrolysis of phosphopeptides can liberate phosphoserine from the peptides (15), our aim was to work out a method to analyze phosphopeptides using standard amino acid analysis conditions. Precolumn derivatization offers a sensitivity range at a low-picomole level (27), and, in addition to high sensitivity, the advantages of the DABS-Cl method include the use of visible wavelength detection and the stability of the derivatized amino acids (28-29). Our efforts were boosted by our most recent successful development of DABS-Cl amino acid analysis of the similar acid-sensitive glycopeptides (30).

We report here the advantageous application of DABS-Cl amino acid analysis for the compositional study of synthetic phosphopeptides. Most of the studied peptides correspond to phosphorylated fragments of protein aggregates of Alzheimer's disease.

MATERIALS AND METHODS

Chemicals

Unmodified and phosphorylated peptides were synthesized and purified as previously described (26). The following peptides were investigated (* marked amino acids were phosphorylated):

GS: Z-Gly-Ser[OP(OBzl)$_2$]-OMe;

PKSPV: H-Pro-Lys-Ser*-Pro-Val-NH$_2$;

GDSKG: H-Gly-Asp-Ser*-Lys-Gly-NH$_2$;

GDRSG: H-Gly-Asp-Arg-Ser*-Gly-NH$_2$;

GDSRG: H-Gly-Asp-Ser*-Arg-Gly-NH_2;

T3: H-Gly-Ala-Glu-Ile-Val-Tyr-Lys-Ser*-Pro-Val-Val-Ser-Gly-Asp-NH_2;

T3Ala: H-Gly-Ala-Glu-Ile-Val-Tyr-Lys-Ser*-Pro-Val-Val-Ala-Gly-Asp-NH_2;

Ac-TGV11: Ac-Gly-Asp-Thr-Ser*-Pro-Arg-His-Leu-Ser-Asn-Val-NH_2;

TDG16: H-Asp-Ala-Gly-Leu-Lys-Glu-Ser*-Pro-Leu-Gln-Thr-Pro-Thr-Glu-Asp-Gly-NH_2;

HNFM 1-17: H-Glu-Glu-Lys-Gly-Lys-Ser*-Pro-Val-Pro-Lys-Ser*-Pro-Val-Glu-Glu-Lys-Gly-NH_2;

T1NM-GSR: H-Gly-Asp-Arg-Ser*-Gly-Tyr-Ser*-Ser*-Pro-Gly-Ser*-Pro-Gly-Thr-Pro-Gly-Ser*-Arg-NH_2;

TR2: H-Val-Lys-Ser-Lys-Ile-Gly-Ser*-Thr-Glu-Asn-Leu-Lys-His-Gln-Pro-Gly-Gly-Gly-NH_2;

T3+9: H-Gly-Ala-Glu-Ile-Val-Tyr-Lys-Ser-Pro-Val-Val-Ser-Gly-Asp-Thr-Ser*-Pro-Arg-His-Leu-Ser-Asn-Val-NH_2;

AT8C: H-Tyr-Ser-Ser-Pro-Gly-Ser*-Pro-Gly-Thr-Pro-Gly-Ser-Arg-Ser-Arg-Thr-NH_2;

T3TYR: H-Gly-Ala-Glu-Ile-Val-Tyr*-Lys-Ser-Pro-Val-Val-Ser-Gly-Asp-NH_2;

TGNL: H-Asn-Gln-Leu-Tyr*-Asn-Glu-Leu-NH_2;

TGRL: H-Arg-Glu-Glu-Tyr*-Asp-Val-Leu-NH_2;

Ac-PRH-Camk: Ac-Pro-Arg-His-Leu-Ser*-Asn-Val-Ser-Ser-Thr-Gly-Ser-Ile-Asp-Met-Val-Asp-NH_2, and

APP645-661: H-Leu-Val-Met-Leu-Lys-Lys-Lys-Gln-Tyr-Thr-Ser*-Ile-His-His-Gly-Val-Val-NH_2.

All peptides were characterized by conventional amino acid analysis and organic phosphate analysis (13). Phosphoamino acid standards, O-Phospho-L-Serine (L-2-Amino-3-hydroxypropanoic acid 3-phosphate), O-Phospho-L-Threonine (L-2-Amino-3-hydroxybutanoic acid 3-phosphate), and O-Phospho-L-Tyrosine (L-3-[4-Hydroxyphenyl]alanine 4'-phosphate), were purchased from Sigma Chemical Company (St. Louis, MO, USA). Hydrolysis and dabsylating reagents were purchased from Beckman (San Ramon, CA, USA), HPLC solvents and all the rest of the chemicals were from Aldrich (Milwaukee, WI, USA).

Gas-Phase Hydrolysis

Lyophilized samples (12 in 600 µl vials) and 700 µl of 6 M HCl were placed in a hydrolysis vessel (provided by Beckman; volume 113 cm^3), and then flushed with argon and evacuated at 0.1 millibar (0.00145 p.s.i.; 1 p.s.i. = 6894.76 Pa) for 1 to 2 min. The vessels were placed in a drying oven at 110 °C for 1.5 hours.

Dabsylation

The amino acid mixture (the result of hydrolysis of 2 to 5 nmol peptide or phosphoamino acid standards) was dissolved in 20 µl $NaHCO_3$-NaOH buffer (pH = 8.3), and 40 µl DABS-Cl solution (40 µg in 40 µl acetonitrile) was added (27-29). The vials were closed and placed in a drying oven at 70 °C for 12 to 14 min. After derivatization, samples were diluted with 440 µl of ethanol:water mixture (1:1), and 8% of the diluted sample was injected for HPLC analysis.

High-Performance Liquid Chromatography

The Beckman System Gold HPLC apparatus consisted of a 126 programmable solvent delivery module, a 167 scanning UV-visible detector module operating at 436 nm, an Altex 210A injector and a C_{18} Ultrasphere-DABS column (4.6 x 250 mm). The system was controlled by an IBM system 2 model 55SX personal computer with Beckman System Gold Personal Chromatography software version 6.0. The chromatographic conditions were as follows: Solvent A (final pH 6.50 ± 0.05) contained 100 ml of 0.11 M sodium citrate (pH 6.51), 860 ml HPLC water, and 40 ml N,N-dimethyl formamide (DMF). Solvent B contained 300 ml solvent A, 672 ml acetonitrile, and 28 ml DMF. The modified solvent A contained 115 ml of 0.11 M sodium citrate buffer, 845 ml HPLC water, and 40 ml DMF. The flow rate was 1.4 ml/min. The gradient conditions were as listed in Table 1. The solvent vessels were continuously flushed with argon. All runs were done at room temperature.

RESULTS AND DISCUSSION

Phosphoamino Acid Standards and Hydrolysis Conditions

We studied the elution profile of the dabsylated phosphoamino acids in our system, utilizing the regular amino acid analysis conditions. The unhydrolized and

TABLE 1
Solvent Composition During Reversed-Phase HPLC

	Time (min)	Solvent A%	Solvent B%	Duration (min)
Gradient	Start	71	29	
	0	49	51	24
	24	14	86	10
	40	0	100	1
	47	71	29	0.25
	55			End of run

derivatized phosphoamino acids and amino acid peaks were eluted in the following order: phosphoserine, aspartic, phosphothreonine, glutamic, and phosphotyrosine, as has been reported before (24). When the amino acids and phophoserine standards were co-injected, the peaks were baseline separated. In order to fully separate the dabsylated phosphothreonine and phosphotyrosine from dabsylated aspartic (Asx) and glutamic (Glx), respectively, a modification of the content of solution A was necessary. When the concentration of citric acid in the eluents was increased from 11 mM to 12.6 mM, the dabsylated phosphothreonine and phosphotyrosine peaks were baseline separated. In addition, when different amounts of unhydrolized phosphoamino acid standards were derivatized, the peak areas were directly proportional to the measured amounts.

In order to find an optimal hydrolysis time for studying the phosphopeptides, we examined the effect of hydrolysis on the recovery of the phosphoserine standard. We found that 1.5 hours was optimal, a value slightly less than that previously reported (18) 2- to 4-hour period. It needs to be mentioned that the longer hydrolysis time was proposed based on experiments with 6 N HCl in solution, in contrast to our gas-phase conditions. In our hands, hydrolyzing the phosphoserine standard as much as 3 hours caused a 25 to 50% decrease in the final quantity of phosphoserine.

The chromatogram of the hydrolized phosphoserine standard (Figure 1A) contained the dabsylated serine peak, indicating that the decrease of phosphoserine began after 1.5 hours of hydrolysis. Co-injecting the hydrolyzed amino acid and phosphoserine standard produced peaks that were baseline separated (Figure 1B), and no extra peak was found compared to the blank (Figure 1C), except the peak of phosphoserine at 8.09 min. The hydrolized and dabsylated aspartic was eluted at 8.59 min, and the glutamic was eluted at 9.44 min (Figure 1B).

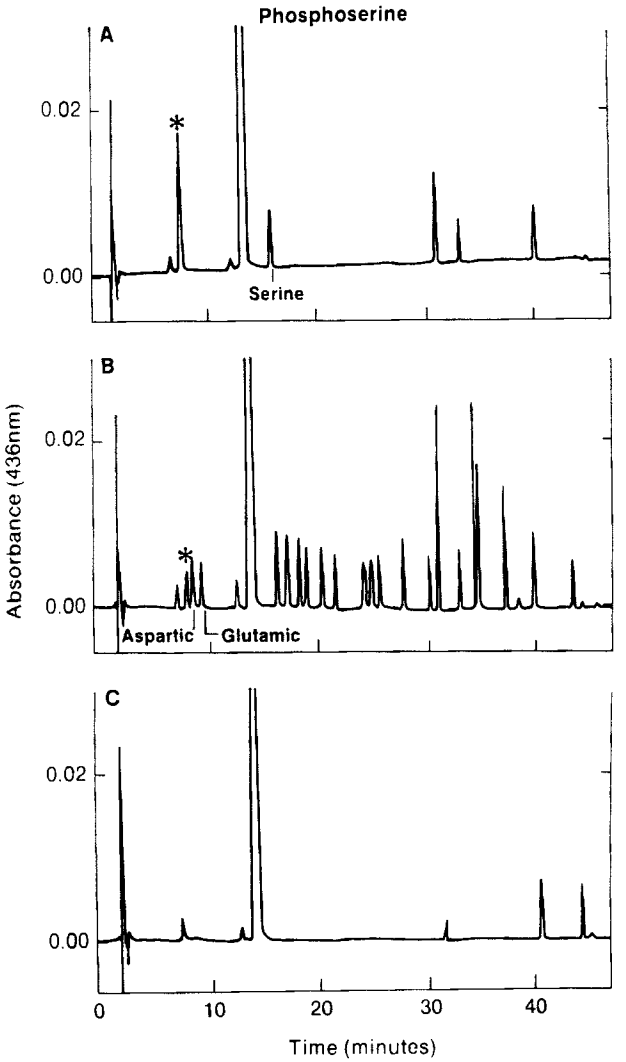

Figure 1. Reversed-phase chromatography of hydrolyzed and dabsylated phosphoserine standard (A), hydrolyzed and dabsylated amino acid standard coinjected with phosphoserine standard (B), and blank (C). The peaks of the phosphoserine were marked with an asterisk. On chromatogram A, 8% of 5 nmol phosphoserine standard was injected; on chromatogram B, 1 nmol of each amino acid and 0.8 nmol of phosphoserine were mixed for coinjection, and 8% of the mixture was injected. On chromatogram B the dabsylated phosphoserine peak was eluted at 8.09 min, the Dabs-aspartic (Asx) was eluted at 8.59 min and the Dabs-glutamic (Glx) peak was observable at 9.44 min.

Model Peptides

Five well-characterized synthetic model phosphopeptides (Table 2) and their non-phosphorylated analogs were examined in preliminary studies. All of these peptides were synthesized in our laboratory. We selected short, phosphoserine-containing peptides because (i) they can be reliably characterized by FAB-MS; (ii) they can be hydrolyzed without major difficulties; (iii) serine-phosphorylation is much more frequent in cytoskeletal proteins than the other two most commonly phosphorylated amino acids; and (iv) phosphoserine is often adjacent to glycine or positively charged amino acids (31).

Using 1.5 hours for hydrolysis of each of the 10 peptides, we found an extra peak only on the chromatograms of phosphopeptides eluting before the Dabs-aspartic. The chromatograms of non-phosphorylated analogs lacked this extra peak. Figure 2 demonstrates this result. The extra peak was eluted at the same position as the dabsylated and hydrolyzed phosphoserine standard. Co-injecting the dabsylated hydrolyzate of phosphopeptides with Dabs-phosphoserine standard, the extra peak overlapped with the peak of the standard. However, because of the partial hydrolysis of peptide bonds, 1.5 hours of hydrolysis time was not enough for quantitative analysis. Nevertheless, it was still good enough to verify the presence of the phosphate group in hydrolyzates of phosphopeptides.

Phosphopeptides

Based on these results, we extended the analysis for immunologically active, medium-sized phosphorylated fragments of protein aggregates of Alzheimer's disease. As Table 3 shows, these peptides contained either phosphoserine or phosphotyrosine. We hydrolyzed and dabsylated several different phosphopeptides. The results were the same as with the model peptides. The extra peak was observed in the dabsylated hydrolyzates of the phosphopeptides (indicating the presence of a phosphate group in the peptides), but was absent from the chromatograms of non-phosphorylated analogues. Figure 3 illustrates this result. The Dabs-phosphoserine peak was eluted before the Dabs-aspartic, while the chromatogram of the non-phosphorylated peptide lacked the Dabs-phosphoserine peak. The Dabs-phosphotyrosine eluted after the Dabs-glutamic in an almost baseline separated manner (Figure 4). The retention times were in agreement with the amino acid and phosphoamino acid standards. The dabsylated aspartic was eluted at 8.55 min, the glutamic was eluted at 9.41 min and the extra peak was found at 9.77 min (Figure 4), while the peak of the dabsylated phosphotyrosine standard was observable at 9.78 min (see insert on Figure 4).

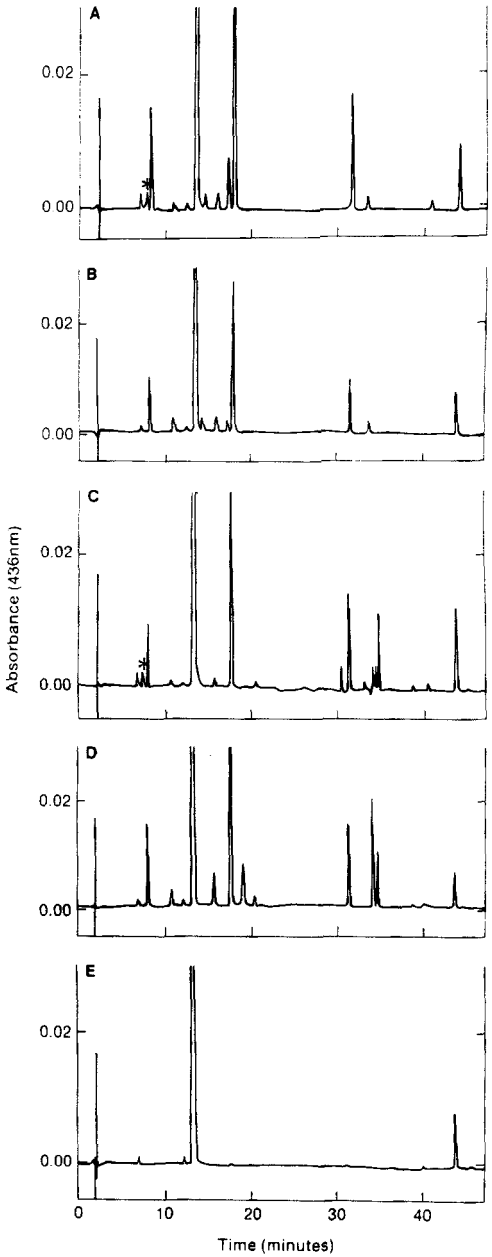

Figure 2. Reversed-phase chromatograms of hydrolyzed and dabsylated model peptides. Panels A and B show the chromatograms of phosphorylated and nonphosphorylated analogs of peptide GDSRG, respectively, panels C and D show the chromatograms of phosphorylated and non-phosphorylated GDSKG peptide, respectively. The phosphoserine peaks are marked with an asterisk. The peak that elutes after the labelled peak corresponds to the dabsylated aspartic. The peak of Dabs-aspartic is observed also on the chromatograms of non-phosphorylated peptides (B, D). Chromatogram E is the blank.

TABLE 2

Detection of Phosphoserine in Synthetic Model Phosphopeptides

Sequences	Peak of phosphoserine	FAB-MS ([M+H$^+$],m/z)
GS(P)*	+	571
PKS(P)PV	+	605, 627[M+Na$^+$]
GDS(P)KG	+	542
GDRS(P)G	+	570
GDS(P)RG	+	570

* This peptide contained three protecting groups (see Materials and Methods).

TABLE 3

Detection of the Presence of a Phosphate Group in Synthetic Phosphopeptides

Sequence	Peak of phosphoserine	Peak of phosphotyrosine
GAEIVYKS(P)PVVSGD	+	
GAEIVYKS(P)PVVAGD	+	
GDTS(P)PRHLSNV	+	
DAGLKES(P)PLQTPTEDG	+	
EEKGKS(P)PVPKS(P)PVEEKG	+	
GDRS(P)GYS(P)S(P)PGS(P)PGTPGS(P)R	+	
VKSKIGS(P)TENLKHQPGGG	+	
GAEIVYKSPVVSGDTS(P)PRHLSNV	+	
YSSPGSPGTPGSRSRT	+	
GAEIVY(P)KSPVVSGD		+
NQLY(P)NEL		+
REEY(P)DVL		+

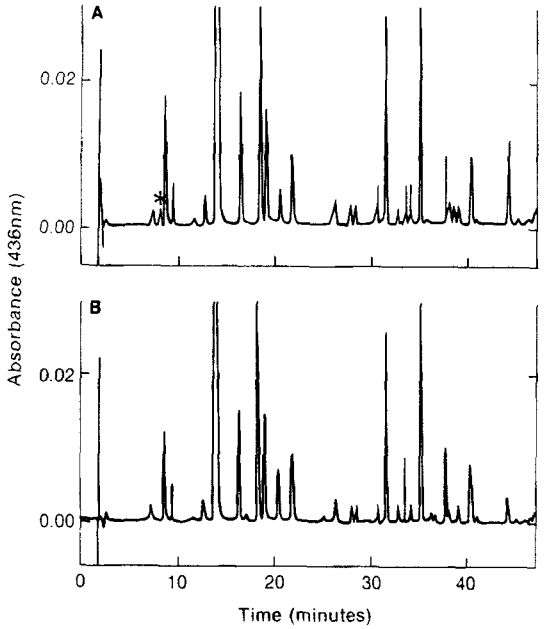

Figure 3. Chromatograms of hydrolyzed and dabsylated peptide T3. Panel A shows the chromatogram of the phosphorylated peptide, panel B its nonphosphorylated analog. The asterisk marks the phosphoserine peak. The peaks, eluted after the marked peak, are the dabsylated aspartic and glutamic, respectively. These two peaks are observed also on the chromatogram of the nonphosphorylated peptide (B). In both cases, the same amounts of peptides (3 nmol) were hydrolyzed and dabsylated, and 8% of them were injected.

The period of hydrolysis is important because short hydrolysis does not liberate the phosphoserine, while longer hydrolysis destroys it very quickly. We found that less than 1 hour of hydrolysis did not liberate the free phosphoamino acids. After 3 hours of hydrolysis of the T3 peptide (see Materials and Methods), we still could determine the presence of phosphoserine, although the amount of it (calculated by peak areas) was two thirds of that obtained by 1.5 hours of hydrolysis. Studying APP and Ac-PRH-Camk phosphopeptides (see Materials and Methods) the presence of phosphoserine could not be verified after 1.5 hour hydrolysis. In these peptides one of the neighboring amino acids of phosphoserine was leucine or isoleucine. The Ile-Ser and the Leu-Ser peptide bonds are likely to be resistant to acidic hydrolysis similar to the earlier reported resistance of Leu-Thr (15), and the bonds probably could not be destroyed during the 1.5 hour hydrolysis. By increasing

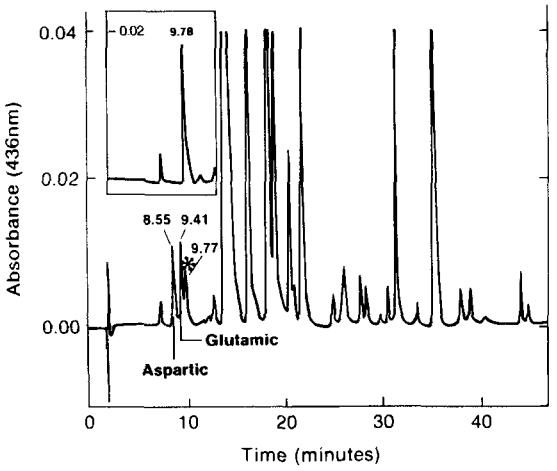

Figure 4. Chromatogram of hydrolyzed and dabsylated T3TYR phosphopeptide. This peptide was phosphorylated on the tyrosine residue. The dabsylated aspartic was eluted at 8.55 min, the dabsylated glutamic was eluted at 9.41 min, and the extra peak (marked with an asterisk) was found at 9.77 min. The dabsylated phosphotyrosine standard (see insert) was eluted at 9.78 min using the modified solvents (see Materials and Methods). In both cases, 5 nmol of standard or peptide were hydrolyzed and dabsylated, and 8% each was injected.

the hydrolysis time to 3 hours, a low intensity phosphoserine peak was observed, but this could not be considered as a reliable detection. Three hours of hydrolysis, which partially destroyed the Ile-Ser and Leu-Ser bonds, has already destroyed the liberated phosphoserine. These results seem to support the idea that each individual peptide sequence has a specific influence on the detectability of phosphoserine. Different sequences may require different hydrolysis times to demonstrate the presence of phosphoserine.

The partial hydrolysis of peptide bonds does not offer optimal circumstances for quantitative analysis, because short hydrolysis does not destroy the peptide bond entirely, and the sensitivity of phosphoserine similarly increases the analytical difficulties. Quantitative analysis of phosphoamino acid standards and phosphopeptides after hydrolysis and derivatization showed a high variability. As Table 4 illustrates, the peak area of the phosphoserine standard was less variable than that of phosphoserine from phosphopeptides. However, upon hydrolyzing numerous standards, we observed more than 40% variability in the standard phosphoserines. As far as phosphoserine from phosphopeptides is concerned, the

TABLE 4

Recovery of Phosphoserine in Different Phosphopeptides and in Phosphoamino Acid Standard

Name	Peak area of 80 pmole phosphoserine
Phosphoserine standard	0.32
DAGLKES(P)PLQTPTEDG	0.18
GDRS(P)G	0.07
GDS(P)RG	0.12
Phosphoserine standard	0.33
VKSKIGS(P)TENLKHQPGGG	0.03
GDTS(P)PRHLSNV	0.05

area of phosphoserine peaks showed very different results. Since the detected amount of phosphoserine in phosphopeptides seems to be sequence-dependent, we did not use the results for quantitative analysis. Correction based on the amount of phosphoamino acid standard is also useless because a longer time is required for the cleavage of peptide bonds, while the phosphoamino acid is free during the short hydrolysis. As Table 4 shows, after hydrolyzing and dabsylating the same amounts of phosphoserine and phosphopeptides, the observed peak area of phosphoserine originating from the phosphopeptide is far less than the peak area of the phosphoserine standard. Nevertheless, the quantitative analysis is appropriate when the peptide sequences are the same, but the number of incorporated phosphate groups differs. Quantitative analysis may be used to verify mono- or diphosphorylated synthetic peptides.

In conclusion, we have demonstrated our ability to verify the presence of phosphoserine and phosphotyrosine in synthetic phosphopeptides and, consequently, to verify the success of a phosphorylation reaction on synthetic phosphopeptides using DABS-Cl amino acid analysis. This method may not only be applicable for phosphopeptides, but also for analysis of phosphoproteins.

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REFERENCES

1. G. Thomas, E. J. Podesta, J. Gordon, Protein Phosphorylation and Bio-Regulation, S. Karger, Basel, Switzerland, 1980.
2. H. C. Hemmings, Jr., A. C. Nairn, T. L. McGuinness, R. L. Huganir, P. Greengard, FASEB J., 3: 1583-1592 (1989)
3. R. B. Bourret, J. F. Hess, K. A. Borkovich, A. A. Pakula, M. I. Simon, J. Biol. Chem., 264: 7085-7088 (1989)
4. S. Shenolikar, J. Cyclic Nucleotide Protein Phosphor. Res., 11: 531-541 (1986-1987)
5. A. W. Frank, CRC Crit. Rev. Biochem. Mol. Biol., 16: 51-101 (1984)
6. V. M-Y. Lee, B. B. Balin, L. Otvos, Jr., J. Q. Trojanowski, Science, 251: 675-678 (1991)
7. E. Lang, L. Otvos, Jr., Biochem. Biophys. Res. Commun., 188: 162-169 (1992)
8. E. Lang, G. I. Szendrei, V. M-Y. Lee, L. Otvos, Jr., Biochem. Biophys. Res. Commun., 187: 783-790 (1992)
9. E. Lang, G. I. Szendrei, I. Elekes, V. M-Y. Lee, L. Otvos, Jr., Biochem. Biophys. Res. Commun., 182: 63-69 (1992)
10. G. I. Szendrei, V. M-Y. Lee, L. Otvos, Jr., J. Neurosci. Res., 34: 243-249 (1993)
11. L. Otvos, Jr., I. A. Tangoren, K. Wroblewski, M. Hollosi, V. M-Y. Lee, J. Chromatogr., 512: 265-272 (1990)
12. E. Pettersson, B. Luning, H. Mickos, D. Heinegard, Acta Chem. Scand. 45: 604-608 (1991)
13. C. W. I. McClare, Anal. Biochem., 39: 527-530 (1971)
14. J. K. Larson, L. Otvos, Jr., H. C. J. Ertl, J. Virol., 66: 3996-4002 (1992)
15. D. B. Bylund, T-S. Huang, Anal. Biochem., 73: 477-485 (1976)
16. B. Duclos, S. Marcandier, A. J. Cozzone, Methods Enzymol., 201: 10-21 (1991)
17. L. Carlomagno, V. D. Huebner, H. R. Matthews, Anal. Biochem., 149: 344-348 (1985)
18. J. C. Yang, J. M. Fujitaki, R. A. Smith, Anal. Biochem., 122: 360-363 (1982)
19. J.-P. Capony, J. G. Demaille, Anal. Biochem., 128: 206-212 (1983)

20. A. W. Steiner, E. R. Helander, J. M. Fujitaki, L. S. Smith, R. A. Smith, J. Chromatogr., 202: 263-269 (1980)

21. W.-C. Chang, M.-L. Lee, C.-K. Chou, S.-C. Lee, Anal. Biochem., 132: 342-344 (1983)

22. D. P. Ringer, Methods Enzymol., 201: 3-10 (1991)

23. L. R. Murthy, K. Iqbal, Anal. Biochem., 193: 299-305 (1991)

24. J.-Y. Chang, J. Chromatogr., 295: 193-200 (1984)

25. D. A. Malencik, Z. Zhao, S. R. Anderson, Anal. Biochem., 184: 353-359 (1990)

26. L. Otvos, Jr., I. Elekes, V. M.-Y. Lee, Int. J. Peptide Protein Res., 34: 129-133 (1989)

27. J.-Y. Chang, R. Knecht, D. G. Braun, Biochem. J., 199: 547-555 (1981)

28. J.-Y. Chang, R. Knecht, D. G. Braun, Biochem. J., 203: 803-806 (1982)

29. R. Knecht, J.-Y. Chang, Anal. Chem., 58: 2375-2379 (1986)

30. L. Gorbics, L. Urge, E. Otvos-Papp, L. Otvos, Jr., J. Chromatogr., 637: 43-53 (1993)

31. R. E. Williams, Science, 192: 473-474 (1976)

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