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Comparative and optimized dabsyl-amino acid analysis of synthetic phosphopeptides and glycopeptides

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Abstract

The optimal conditions for amino acid analysis of phosphopeptides and N-acetylglucosamine- and N-acetylgalactosamine-containing glycopeptides were investigated by dabsyl-Cl derivatization and reversed-phase high-performance liquid chromatographic separation. By comparing the chromatographic behaviour of the dabsylated phosphoamino acids and dabsylated aminosugars on three different columns, it appears that the mechanism of binding to the column is different for the two modified dabsyl derivatives. The acid sensitivities of sugar and phosphate groups were also investigated. We found that while the optimal hydrolysis conditions for phosphopeptide analysis are peptide sequence-dependent, there is generally an applicable condition for the highest recovery of glycopeptides. A 1-h gas-phase hydrolysis time seems to be appropriate for the majority of glycopeptides and 1.5 h is suitable for the majority of phosphopeptides. The analysis was extended to the successful verification of the presence of the N-acetylglucosamine and the N-acetylgalactosamine moieties when these sugars were incorporated as parts of a disaccharide side chain of glycopeptides.

1. Introduction

Although codons for only 20 amino acids are found in the genome (and are used in protein synthesis), 140 modified amino acids exist in various proteins [1]. These posttranslationally modified proteins are known to play a crucial role in many recognition processes. The two most frequent forms of posttranslational modifications are the phosphorylation of hydroxy-amino acids and the glycosylation of the asparagine, serine and threonine residues. Both the oligosaccharide antennae and the negatively charged phosphoryl groups are located on the surface of the proteins and, therefore, can easily participate in numerous recognition processes. The structure and the biological significance of glycosylated and phosphorylated proteins have been discussed in a great variety of review articles [2–8].

Small- and medium-sized glycopeptides and phosphopeptides are appropriate models for biological studies so the improvement of their synthesis has become the focus of interest. Since the basic methodology of glycopeptide synthesis has been developed, the current study addresses the incorporation of more complex sugar systems [9]. As a result of this improvement longer oligosaccharide chains can be attached to a model peptide, where the first sugar moiety is N-acetylglucosamine (GlcNAc, 2-acetamido-2-deoxy-D-glucose) or N-acetylgalactosamine (GalNAc, 2-acetamido-2-deoxy-D-galactose) in almost all cases [10].

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Recognition of the increasing importance of model glycopeptides and phosphopeptides demanded the development of appropriate analytical methods [11,12]. Among the standard procedures of amino acid analysis the use of 4-dimethylaminoazobenzene-4′-sulphonyl chloride (dabsyl-Cl) is the most appropriate for analysis of acid-sensitive phosphopeptides and glycopeptides [13,14]. Partial hydrolysis, the stability of dabsyl-phosphoamino acids and dabsyl-aminosugars, and the usage of visible wavelength are the advantages of this analysis method. Recently, we reported the application of the dabsyl-Cl amino acid analysis for the compositional study of synthetic glycopeptides [13] and phosphopeptides [14].

In this paper we further investigate the optimal hydrolysis and chromatographic conditions of the similarly acid-sensitive glycopeptides and phosphopeptides and extend the amino acid analysis of glycopeptides to those disaccharide-containing units of the natural glycoprotein antennae.

2. Experimental

2.1. Chemicals

The unmodified and modified peptides were synthesized in our laboratory [15,16] and their composition was verified (including mass spectroscopy). 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), O-phospho-L-tyrosine [L-3-(4-hydroxyphenyl)alanine 4′-phosphate], were purchased from Sigma (St. Louis, MO, USA). Sugar standards glucosamine (2-amino-2-deoxy-D-glucopyranose), galactosamine (2-amino-2-deoxy-D-galactopyranose), mannosamine (2-amino-2-deoxy-D-mannopyranose), GlcNAc and GalNAc were purchased from Sigma. Gal(β1–3)GalNAc (Gal = galactose) standard was purchased from Bachem (Torrance, CA, USA), Fmoc-Asn(O-tBu)-Gal(β1-3)GlcNAc (Fmoc = 9-fluorenylmethoxycarbonyl; tBu = *tert.*-butyl) was synthesized in our laboratory [17]. Hydrolysis and dabsylating reagents were purchased from Beckman (Fullerton, CA, USA), HPLC solvents and all the rest of the chemicals were from Aldrich (Milwaukee, WI, USA).

2.2. Gas-phase hydrolysis

Lyophilized samples (twelve in 600-μl vials) and 700 μl of 6 *M* HCl were placed in a hydrolysis vessel (provided by Beckman; volume 113 ml), and then flushed with argon and evacuated at 0.1 mbar for 1–2 min. The vessels were placed in a drying oven at 110°C for 1 or more h (see details in Results and discussion section).

2.3. Dabsylation

The amino acid mixture —the result of hydrolysis of 2–5 nmol peptide or phosphoamino acid standards— was dissolved in 20 μl $NaHCO_3$–NaOH buffer (pH 8.3) and to that mixture 40 μl dabsyl-Cl solution (40 μg in 40 μl acetonitrile) was added [18–20]. The vials were closed and placed in a drying oven at 70°C for 12–14 min. After derivatization, samples were diluted with 440 μl of ethanol–water (1:1), and 8% of the diluted sample was injected for HPLC analysis.

2.4. HPLC

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, a Rheodyne 7725i injector, and a C_{18} column. Three different columns were used: a Beckman Ultrasphere-dabsyl C_{18} column (250 × 4.6 mm), a Merck LiChrospher 100 RP-18 column (Gibbstown, NJ, USA) (5 μm; 250 × 4.0 mm) and an Alltech Econosphere analytical column (Deerfield, IL, USA) (5 μm; 250 × 4.6 mm). The columns were installed as follows: the columns were rinsed with 30% acetonitrile in water for 30 min at 1 ml/min then rinsed with an installation buffer for 30 min at 1 ml/min. The installation buffer was a

Table 1
Solvent composition during RP-HPLC

Gradient	Time (min)	Solvent		Duration (min)
		A (%)	B (%)	
1	Start	71	29	
	0	49	51	24
	24	14	86	10
	40	0	100	1
	47	71	29	0.25
	55			End of run
2	Start	71	29	
	0	49	51	12
	12	14	86	5
	20	0	100	1
	27	71	29	0.25
	35			End of run

solution of 100 m*M* citric acid, pH 3.5 containing 20% N,N-dimethylformamide (DMF). After this procedure the columns were rinsed with solution A and solution B (see below) until the baseline stabilized.

The HPLC 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: solution 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 DMF. Solution B contained 300 ml solution A, 672 ml acetonitrile and 28 ml DMF. The flow-rate was 1.4 ml/min. The gradient conditions were as listed in Table 1. The solution vessels were continuously flushed with argon. All runs were done at room temperature.

3. Results and discussion

The main goal of our study was to investigate the influence of the identity of the chromatographic column, the influence of the extension of sugar chain, and the influence of changes of the partial hydrolysis conditions on our analysis method.

3.1. Chromatographic behaviour of dabsylated phosphoamino acids and aminosugars

Three different columns were used in order to compare the elution properties of dabsylated phosphoamino acids and aminosugars. (2-Aminosugars were found as the only derivatizable species after hydrolysis of GlcNAc and GalNAc [13].) The columns used were manufactured by Beckman, Merck and Alltech. Using the same elution conditions we observed slight differences among the resolution of the three columns. The three phosphoamino acids (phosphoserine, phosphothreonine and phosphotyrosine) eluted in the same order on all columns (Table 2). Comparison of the retention times show that on the Alltech column the hydrophilic amino acids eluted very early. Dabsyl-aminosugars were eluted from all three columns in the following order: galactosamine, glucosamine and mannosamine (Fig. 1A). The absolute retention times of the aminosugars were very similar on each column (Table 2). The retention times of the other peaks (phosphoamino acids and dabsyl-OH) however, were shorter on the Alltech column than on the other two indicating the possibility that dabsyl-aminosugars bound to the Alltech column by a differ-

Table 2
Comparison of retention time of dabsyl-phosphoamino acids and dabsyl-amino sugar standards

Column	Retention time (min)					
	Phosphoserine	Phosphothreonine	Phosphotyrosine	Galactosamine	Glucosamine	Mannosamine
Beckman	7.48	8.26	9.10	19.39	20.08	20.75
Merck	7.37	8.11	9.09	20.36	21.11	21.85
Alltech	5.62	6.20	6.80	19.94	20.65	21.38

ent mechanism than the other derivatized species.

When a dabsyl-amino acid standard mixture was injected into each column we found that the separation properties of Beckman and Merck columns were similar, while on the Alltech column the peaks eluted much earlier.

Chromatograms made after coinjection of the amino acid standards, phosphoamino acids and aminosugars provided the best comparison of the elution properties from the different columns (Fig. 1C). Phosphopeptides eluted separately from the hydrophilic amino acids (aspartic, glutamic) on the Beckman and Merck columns, while they could not be separated on the Alltech column. Glucosamine eluted between the proline and valine peaks, galactosamine coeluted with the proline, and mannosamine coeluted with the valine. (In our previous study [13] using gradient 2 we could separate proline from galactosamine.)

In summary, the strongly hydrophilic feature of the phosphate group equally shifted the retention time of the corresponding unmodified dabsyl-amino acids as was expected and did not change the order of the peaks. In addition, no major difference was found in the elution profile of phosphoamino acids from the different columns. Good separation of phosphoamino acids from the other amino acids can be achieved by modifying the gradient or the pH of the eluents in each of the columns tested. Derivatized aminosugars were eluted later than it was predicted by their hydrophilic properties. While the reversed-phase chromatographic behaviour of the glycopeptides was dependent upon the conformation of both the peptide and the sugar, the incorporation of phosphate group decreased the retention time of the non-phosphorylated analogues independent of the peptide sequence [21]. Our findings concern both the separation of

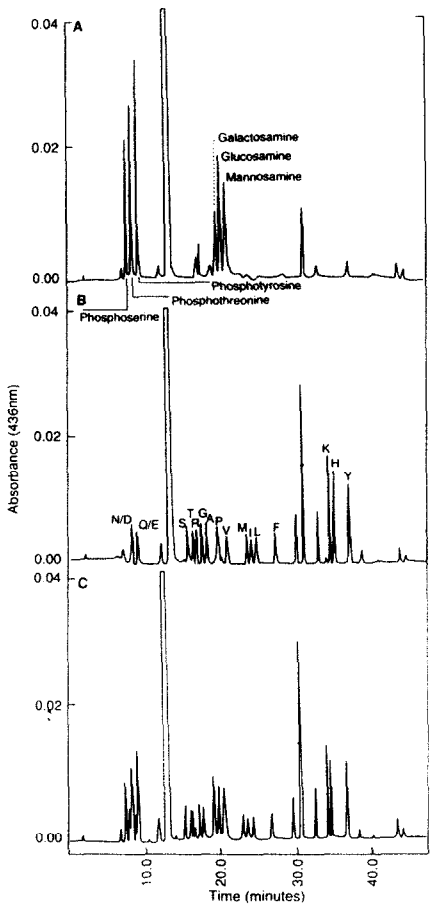

Fig. 1. Chromatographic separation of dabsyl-phosphoamino acids and dabsyl-aminosugars (A), dabsyl-amino acids (B) and a mixture of dabsyl-phosphoamino acids, amino acids and aminosugars (C). The separation was carried on a Beckman column using gradient 1 (Table 1).

phosphopeptides/glycopeptides, and the chromatographic behaviour of dabsyl-phosphoamino acid standards or dabsyl-aminosugars. These results emphasize the importance of not only the hydrolysis or derivatization conditions, but also the identity of chromatographic columns for finding appropriate conditions for successful aminosugar or phosphate analysis.

3.2. *Analysis of disaccharide-containing glycopeptides*

The ultimate goal of our study was to extend the analysis of glycopeptides from monosaccharide- to oligosaccharide-containing glycopeptides. GlcNAc and GalNAc were the first sugar moieties found in natural glycoproteins, therefore their presence in protein hydrolysates is a clear indication of the presence of glycosylated protein backbone. Most of the synthetic glycopeptide models also contain these sugar residues, so that analysis of GlcNAc and GalNAc can indicate the success of glycopeptide synthesis. In our previous study [13] we reported the development of a dabsyl-amino acid analysis method to verify the presence of the GlcNAc and GalNAc in glycopeptides. These sugar residues can be hydrolyzed by selective hydrolysis to glucosamine and galactosamine, and these intermediate products can be derivatized in a manner similar to that used for amino acids. These glycopeptides contained only monosaccharide units. In the current study we extended our analysis method to glycopeptides in which the sugar moiety is a disaccharide.

First, we investigated if our method could be used for disaccharide standards. We hydrolysed a Gal(β1–3)GalNAc standard for 1 h, dabsylated it, and analyzed it with gradient 2 (Table 1) using the Beckman column. Fig. 2A shows the chromatogram of this analysis. The peak at 14.22 min (labeled with an asterisk) eluted at the same position as the previously studied galactosamine from the GalNAc monosaccharide standard. This result indicated that the presence of a GalNAc moiety can be detected not only for the monosaccharide, but also as a part of a disaccharide. The utility of the strategy was tested

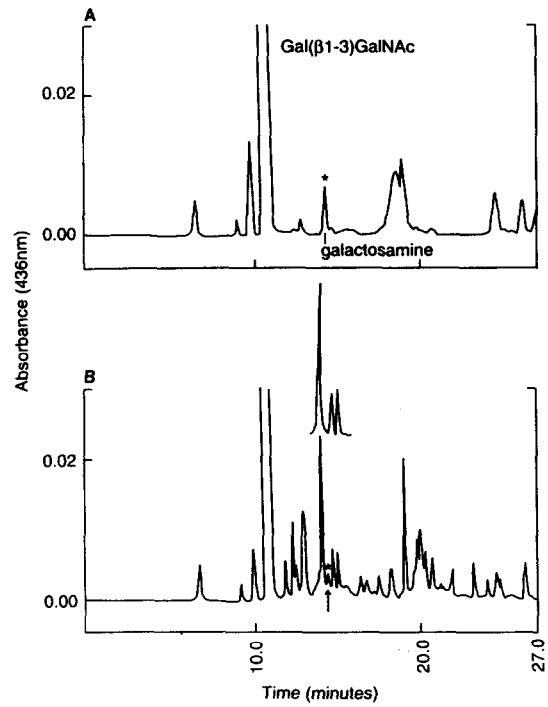

Fig. 2. Chromatograms of the analysis of Gal(β1–3)GalNAc standard (A) and a *Drosophila*-originated O-glycosylated glycopeptide (B), GKPRPYSPRPT[Gal(β1–3)GalNAc]SHPRPIRV after 1 h hydrolysis and derivatization. For chromatographic analysis we used gradient 2 (Table 1).

on two O-glycosylated peptides. Using the same conditions for the hydrolysis and for the analysis we could easily detect the presence of sugar on peptide 31DS {sequence: T[Gal(β1–3)GalNAc] RIMMNGGR}. This sequence does not contain proline or valine residues (neighbouring dabsyl derivatives) so that the separation of dabsyl-galactosamine from the dabsyl-amino acid peaks was unambiguous. An extra peak at the position of dabsyl-galactosamine verified the presence of GalNAc (data not shown). We tested our method on a *Drosophila*-originated peptide [22], GKPRPYSPRPT[Gal(β1 – 3)GalNAc]SHPRPIRV. The difficulty level of this analysis was particularly high, since the sequence contained six prolines and one valine. Fig. 2B illustrates the result of the analysis. An extra peak was found at 14.20 min (labeled with an asterisk). The chromatogram of the non-glycosylated pep-

tide (processed exactly the same way) did not contain this peak (see insert), indicating that the extra peak on the chromatogram of the glycosylated peptide can be assigned to the sugar (GalNAc), and that the peak was not an intermediate of the partial hydrolysis of the peptide bonds.

To test whether the presence of GlcNAc in a disaccharide can be detected, we synthesized an Fmoc-Asn(OtBu)-[Gal(β1–3)GlcNAc] standard [17]. After a 1-h acidic hydrolysis and derivatization we analyzed it with gradient 1 (Table 1) on a Beckman column. Fig. 3A shows the chromatogram of this analysis where we found an extra peak at the position of the dabsyl-glucosamine peak (20.70 min). This result indicates that the presence of GlcNAc as a part of a disaccharide side chain can be analyzed in the same way as a monosaccharide GlcNAc linked to a peptide chain. The utility of the method was tested on an N-glycosylated peptide, N[Gal(β1–3)GlcNAc]HSGKRELSAEK. Fig. 3B shows the chromatogram of the analysis of this peptide. The peak at 20.12 min (labeled with an asterisk) coeluting with the GlcNAc standard verified the presence of the sugar. (The shift in the retention times compared to those in Fig. 3A is similarly observable for the other peaks and reflects aging of the column [23].)

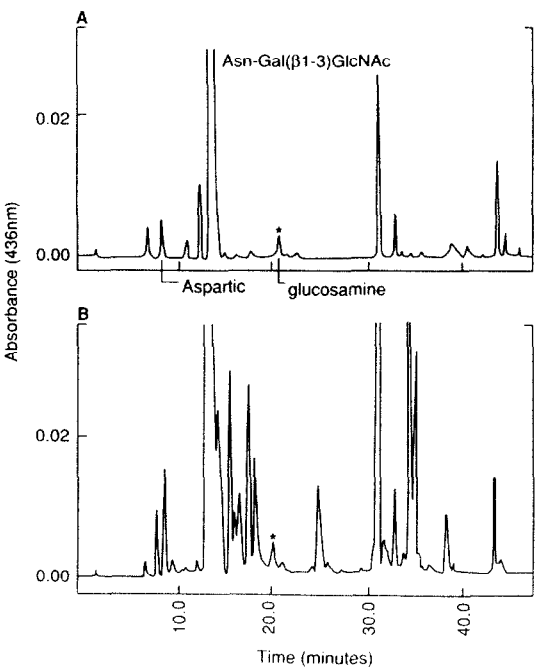

Fig. 3. Chromatograms of the analysis of Fmoc-Asn(OtBu)Gal(β1–3)GlcNAc standard (A) and a N-glycosylated glycopeptide (B), N[Gal(β1–3)GlcNAc]HSGKRELSAEK after 1 h hydrolysis and derivatization. For chromatographic analysis we used gradient 1 (Table 1).

In summary, our previously developed analysis method, using standard dabsyl-Cl amino acid analysis for verifying the presence of GalNAc or GlcNAc on synthetic glycopeptides, is suitable when a disaccharide is attached to the peptide chain with either O- or N-glycosydic linkages. GlcNAc and GalNAc are the first sugar residues in natural glycoproteins, and thus, verification of their presence is a clear indication of a successful glycosylation not only in synthetic glycopeptides, but in protein hydrolysates as well.

3.3. Comparison of partial hydrolysis conditions of glycopeptides and phosphopeptides

Both sugars and phosphoamino acids are labile during standard acidic hydrolysis, but partial hydrolysis can offer a viable alternative for their analysis [13,14,24]. Partial hydrolysis conditions are, however, found to be inappropriate for qualitative analysis of both amino acids and modified derivatives. Nevertheless, verification of the presence of phosphate groups, as well as sugars, is an important task for peptide synthesis laboratories. Instrumentation for a standard amino acid analysis is available in almost all peptide or protein laboratories; therefore, partial hydrolysis combined with a standard derivatization procedure offers a quick, inexpensive, and unambiguous checking method.

In order to study partial hydrolysis conditions, a phosphoserine standard, two phosphopeptides, a GlcNAc standard and two glycopeptides were partially hydrolysed using different hydrolysis times. Our goal was to find optimal hydrolysis times for these acid-sensitive residues. The sequence of these peptides were: GDS(Ph)KG, GDRS(Ph)G, N(GlcNAc)TTNYT, and DELLQKEQN(GlcNAc)YSDDVLA.

Table 3 illustrates that less glucosamine was

Table 3
Yield (recovery) of glucosamine or phosphoserine hydrolyzing GlcNAc or phosphoserine standards as well as glycopeptides or phosphopeptides

Sample	Yield (%) after hydrolysis time of			
	1 h	2 h	3 h	4 h
GlcNAc	17	8	5	
N(GlcNAc)TTNYT	22	12	10	
DELLQKEQN(GlcNAc)YSDDVLA	24	20	11	
Phosphoserine	77	53	21	15
GDS(phospho)KG	7	12	4	4
GDRS(phospho)G	10	9	3	2

recovered from the GlcNAc standard as the hydrolysis time increased. A 1-h hydrolysis gave the highest recovery of glucosamine from GlcNAc. As we reported earlier [13], less than 1 h hydrolysis decreases the recovery of glucosamine from GlcNAc, because the hydrolysis of the acetamido group requires some time. Similarly, a 1-h hydrolysis was found to be the optimum for glycopeptides (Table 3). As the hydrolysis time increased the recovery of the sugar slowly decreased. It is important to note that 1 h hydrolysis was found reliable not only for these two glycopeptides, but for several other glycopeptides that were analyzed earlier [13]. In our experience, the analysis of glycopeptides with partial acid hydrolysis does not seem to be peptide sequence-dependent.

Similar experiments with phosphoserine and phosphopeptides (Table 3) demonstrated that a longer period of hydrolysis resulted in a dramatic decrease in the recovery of phosphoserine. A 1-h hydrolysis gave the best results. Under the same conditions, using phosphopeptides, 2 h hydrolysis increased, or at least did not decrease the recovery of phosphoserine. The recovery of phosphoserine is far less from the phosphopeptides than from the phosphoserine standard clearly indicating that analysis of phosphoserine from phosphopeptides is much more difficult. Furthermore, our results indicated that general hydrolysis conditions are not applicable to phosphopeptides because the recovery of phosphoserine is sequence-dependent. This is in good agreement with our previous phosphopeptide analysis experience [14]. Other laboratories using liquid-phase hydrolysis have found the detection of phosphoserine to be similarly peptide sequence-dependent [24]. We expected that the milder conditions of gas-phase hydrolysis might offer a generally more reliable hydrolysis condition, but the acid sensitivity of the phosphoserine is higher than we expected. The result that the recovery of phosphoserine from phosphopeptides is high enough when the hydrolysis period is more than 1 h and less than 2 h, combined with our previous study [14], which shows that the 1.5 h hydrolysis time was found to be appropriate for most of the analyzed phosphopeptides [14], indicates that a 1.5-h hydrolysis time is suitable for the majority of phosphopeptides.

4. Acknowledgements

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5. References

[1] F. Wold, *Ann. Rev. Biochem.*, 50 (1981) 783.
[2] A. Varki, *Glycobiology*, 3 (1993) 97.
[3] R. Kornfeld and S. Kornfeld, *Annu. Rev. Biochem.*, 54 (1985) 631.

[4] P.L. Mollison, in C.P. Engelfriet and M. Contreras (Editors), *Blood Transfusion in Clinical Medicine*, Blackwell, Oxford, 1987, p. 269.
[5] E.A. Kabat, in J.D. Ebert, A.G. Loewy and H.A. Schneiderman (Editors), *Structural Concepts in Immunology and Immunochemistry*, Holt, Rinehart & Winston, New York, 1987, p. 269.
[6] G. Thomas, E.J. Podesta and J. Gordon (Editors), *Protein Phosphorylation and Bio-Regulation*, S. Karger, Basel, 1980.
[7] S. Shenolikar, *J. Cyclic Nucleotide Protein Phosphorylation Res.*, 11 (1986–1987) 531.
[8] A.W. Frank, *CRC Crit. Rev. Biochem.*, 16 (1984) 51.
[9] K.B. Reimer, M. Meldal, S. Kusumoto, K. Fukase and K. Bock, *J. Chem. Soc.*, 1 (1993) 925.
[10] A. Kobata, in V. Ginsburg and P. Robbins (Editors), *Biology of Carbohydrates*, Vol. 2, Wiley, New York, 1984, p. 87.
[11] D.E.H. Palladino, R.M. House and K.A. Cohen, *J. Chromatogr.*, 599 (1992) 3.
[12] F. Altmann, *Anal. Biochem.*, 204 (1992) 215.
[13] L. Gorbics, L. Urge, E. Otvos-Papp and L. Otvos, Jr., *J. Chromatogr.*, 637 (1993) 43.
[14] L. Gorbics, L. Urge, E. Lang, G.I. Szendrei and L. Otvos, Jr., *J. Liq. Chromatogr.*, 17 (1994) 175.
[15] L. Otvos, Jr., I. Elekes and V.M.-Y. Lee, *Int. J. Peptide Protein Res.*, 34 (1989) 129.
[16] L. Urge, E. Kollat, M. Hollosi, I. Laczko, K. Wroblewski, J. Thurin and L. Otvos, Jr., *Tetrahedron Lett.*, 32 (1991) 3445.
[17] L. Urge, L. Otvos, Jr., E. Lang, K. Wroblewski, I. Laczko and M. Hollosi, *Carbohydr. Res.*, 235 (1992) 83.
[18] J.-Y. Chang, R. Knecht and D.G. Braun, *Biochem. J.*, 199 (1981) 547.
[19] J.-Y. Chang, R. Knecht and D.G. Braun, *Biochem. J.*, 203 (1982) 803.
[20] R. Knecht and J.-Y. Chang, *Anal. Chem.*, 58 (1986) 2375.
[21] L. Otvos, L. Urge and J. Thurin, *J. Chromatogr.*, 599 (1992) 43.
[22] P. Bulet, J.L. Dimarcq, C. Hetru, M. Lagueux, M. Charlet, G. Hegy, A. Van Dorsselaer and J.A. Hoffmann, *J. Biol. Chem.*, 268 (1993) 14 893.
[23] C.T. Mant and R.S. Hodges (Editors), *High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation*, CRC Press, Boca Raton, FL, 1991, p. 289.
[24] D.B. Bylund and T.-S. Huang, *Anal. Biochem.*, 73 (1991) 477.