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Determination of amino sugars in synthetic glycopeptides during the conditions of amino acid analysis utilizing precolumn derivatization and high-performance liquid chromatographic analysis

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ABSTRACT

The growing number of synthetic glycopeptides required an in-house method for the analysis of the final products. A reversed-phase high-performance liquid chromatographic protocol was developed to verify the presence of N-acetylglucosamine and N-acetylgalactosamine in synthetic glycopeptides after hydrolysis and derivatization with 4-dimethylaminoazobenzene-4′-sulphonyl chloride. Different sugar and glycoamino acid standards were used to separate the two carbohydrate moieties, and the location of the derivatized 2-aminoglucose was established by the use of radiolabeled sugar. The utility of the approach is demonstrated by the amino acid analysis of N- and O-glycosylated synthetic peptides and the method could provide an alternative for sugar analysis of glycoproteins.

INTRODUCTION

Many proteins contain modified amino acids for which no codons exist in the genome. To understand the biological function of post-translationally modified proteins and protein fragments, the chemical synthesis of glycosylated and phosphorylated peptides has become the central interest of biotechnology sector. In contrast to the single phosphate group on the phosphoproteins, both the N- and O-glycoslyated proteins carry complex oligosaccharide antennae [1]. The carbohydrate chain often carries a highly specific biological recognition structure [2], and extensive studies have been performed on the chromatographic analysis of these oligosaccharide systems [3–5]. As far as the peptide part is concerned, the removal of the first one or two monosaccharide units of the complex carbohydrate antennae seems to result in a much more dramatic conformational [6] and immunological [7] change than the removal of the remainder of the carbohydrate residues. In fact, we used

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synthetic non-glycosylated and glycosylated peptides to demonstrate that incorporation of single N-acetylglucosamine (2-acetamido-2-deoxy-D-glucopyranose) (GlcNAc) moieties (at natural glycosylation sites) into T-cell epitopic peptides resulted in a break in the characteristic α-helical structures; the formation of β-turns was also observed [8]. Elongation of the carbohydrate only slightly enhanced this effect [8].

N-Acetylglucosamine and N-acetylgalactosamine (2-acetamido-2-deoxy-D-galactopyranose) (GalNAc) are the first sugar moieties attached to asparagines and serines/threonines for almost all N- and O-glycoproteins [9]. The elution time of the glycopeptides compared with their non-glycosylated analogs in reversed-phase high-performance liquid chromatography (RP-HPLC) is usually reduced owing to the incorporation of the hydrophilic carbohydrate [10]. Increased retention times compared with the predicted values (owing to induced conformational orientation on the surface of the bonded phase) were observed when synthetic glycopeptides exhibited more ordered secondary structures [11]. As an extreme, glycosylated analogs of peptide T, a pharmaceutically promising candidate against human immunodeficiency virus type-1 infection [12], assumed a β-turn conformation so stabilized that some of the glycopeptides could not be distinguished from their parent, non-glycosylated analog by RP-HPLC [13].

Amino acid analysis of synthetic peptides is historically accomplished by post- or precolumn derivatization after acidic hydrolysis [14–17]. Precolumn derivatization offers a sensitivity range at the low-picomole level [18], and a combination of the two more common precolumn techniques, using 4-dimethylaminoazobenzene-4′-sulphonyl chloride (DABS-Cl) and phenyl isothiocyanate, has been reported [19,20]. In addition to high sensitivity, the popularity of the DABS-Cl method also arises from use of a visible wavelength detection range and the stability of the derivatized amino acids [21,22]. As mono- and oligosaccharides are more sensitive than amino acids to acid hydrolysis [23,24], they are expected to be recovered in a lesser amount than regular amino acids. In contrast to the existing techniques based on analysis of the intact sugar moiety, we decided to derivatize the intermediate product of the hydrolysis featuring a free amino group. The procedure is similar to the derivatization of regular amino acids. Most synthetic peptide laboratories are equipped for amino acid analysis as the first screening of peptide integrity, offering a useful addition to the currently used mass spectrometry [25] for the verification of the integrity of the rapidly growing number of synthetic glycopeptides carrying a limited number of sugar residues. A rapid, in-house analysis of synthetic glycopeptides is further justified by the appearance of techniques to prepare them on automated peptide synthesizers [26]. Most recent reports from several laboratories have been aimed at the development of glycopeptide analytical strategies closer to the practice in biochemistry laboratories [27,28], but uncommon handling of the sugar derivatives could not be fully eliminated. We report here the application of DABS-Cl amino acid analysis in synthetic glycopeptides, N- and O-glycosylated analogs of peptide T5 and RGD analogs, conformationally and pharmacologically interesting fragments of the glycoprotein of the human immunodeficiency virus 1 and fibronectin.

EXPERIMENTAL

Chemicals

Unmodified and glycosylated T5 peptides were synthesized and purified as described previously [13]. The following peptides were investigated:

T5: H-TTNYT-NH_2;
NGlcNAcT5: H-N(GlcNAc)-TTNYT-NH_2;
T5GlcNAc: H-TTN (GlcNAc)-YT-NH_2;
TGalNAcT5: H-T(GalNAc)-TTNYT-NH_2;
RGD: Ac-GRGDSPK-NH_2;
N(GlcNAc)RGD: Ac-N(GlcNAc)GRGDSPK-NH_2;
N(GlcNAc)RGDN(GlcNAc): Ac-N(GlcNAc)-GRGDSPKN(GlcNAc)-NH_2;
RGD-O-glycoside: Ac-GRGDS(GlcNAc)PK-NH_2;
N(GlcNAc)PV: H-N(GlcNAc)PV-NH_2.

Sugar standards, glucosamine hydrochloride (2-amino-2-deoxy-D-glucopyranose hydrochloride), galactosamine hydrochloride (2-amino-

2-deoxy-D-galactopyranose hydrochloride), GlcNAc, GalNAc and 1-amino-glucose (1-amino-1-deoxy-β-D-glucose) were purchased from Sigma (St. Louis, MO, USA), H-Asn(GlcNAc)-OH from Oxford Glycosystems (Rosedale, NY, USA) and radiolabeled GlcNAc ([6-^{3}H]-2-acetamido-2-deoxy-β-D-glucopyranose) from American Radiolabeled Chemicals (St. Louis, MO, USA). Hydrolysis and dabsylating reagents and amino acid standards were purchased from Beckman (San Ramon, CA, USA); HPLC solvents and all other chemicals were obtained from Aldrich (Milwaukee, WI, USA).

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 cm^3), followed by flushing with argon and evacuation at 0.1 mbar (0.00145 p.s.i.; 1 p.s.i. = 6894.76 Pa) for 1–2 min. The vessel was placed in an oven 110°C for 1 or 10 h.

Dabsylation

$NaHCO_3$ buffer (20 μl) (pH 8.3) provided by Beckman was added to 1.5–3 μg of hydrolyzed peptide or 0.3–2 μg of hydrolyzed sugar standard, followed by the addition of 40 μg of DABS-Cl in 40 μl of acetonitrile. The vials were closed and placed in a drying oven at 70°C for 12–15 min. Ethanol–water (1:1, v/v) (440 μl) was added to the samples and 6–12% of the resulting solution was injected into the HPLC column.

HPLC

The Beckman System Gold HPLC apparatus consisted of a Model 126 programmable solvent-delivery module, a Model 167 scanning ultraviolet–visible detector module operating at 436 nm, an Altex 210A injector and a C_{18} Ultrasphere-DABS column (250 × 4.6 mm I.D.). The system was controlled by an IBM System-2 Model 55 SX using the Beckman System Gold Personal Chromatography software, version 6.0. The chromatographic conditions were as follows: solvent A (final pH 6.50–6.52) contained 115 ml of 0.11 *M* sodium citrate (pH 6.51), 845 ml of water and 40 ml of N,N-dimethylformamide; solvent B contained 300 ml of solvent A, 672 ml of acetonitrile and 28 ml of N,N-dimethylformamide; and the flow-rate was 1.4 ml/min. Table I shows the two different gradients used. The solvent vessels were continuously flushed with argon to keep the pH constant. All runs were carried out at room temperature.

TABLE I

SOLVENT COMPOSITION DURING RP-HPLC

	Time (min)	Solvent		Duration (min)
		A (%)	B (%)	
Gradient 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
Gradient 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

RESULTS AND DISCUSSION

Hydrolysis products and their identification

Fig. 1 outlines the possible cleavage products after acidic hydrolysis of a GlcNAc-coupled asparagine residue. The only final product that can be dabsylated is glucosamine (or similarly galactosamine for the O-glycopeptides). To test the possibility of incomplete hydrolysis, H-Asn-(GlcNAc)-OH and 1-amino-GlcNAc standards were subjected to derivatization and to the chromatographic conditions of amino acid analysis. DABS-Asn(GlcNAc)-OH was eluted just before the broad DABS-ONa peak, and was entirely absent after hydrolysis. Based on this, we disregarded the possibility of obtaining glucosamine-coupled asparagine. No new peak was found for unhydrolyzed 1-amino-GlcNAc (compared with the blank chromatogram), and after hydrolysis (1 h) only one peak, identical with the dabsylated glucosamine standard, was detected. Moreover, our standard procedure [29] to acylate 1-amino

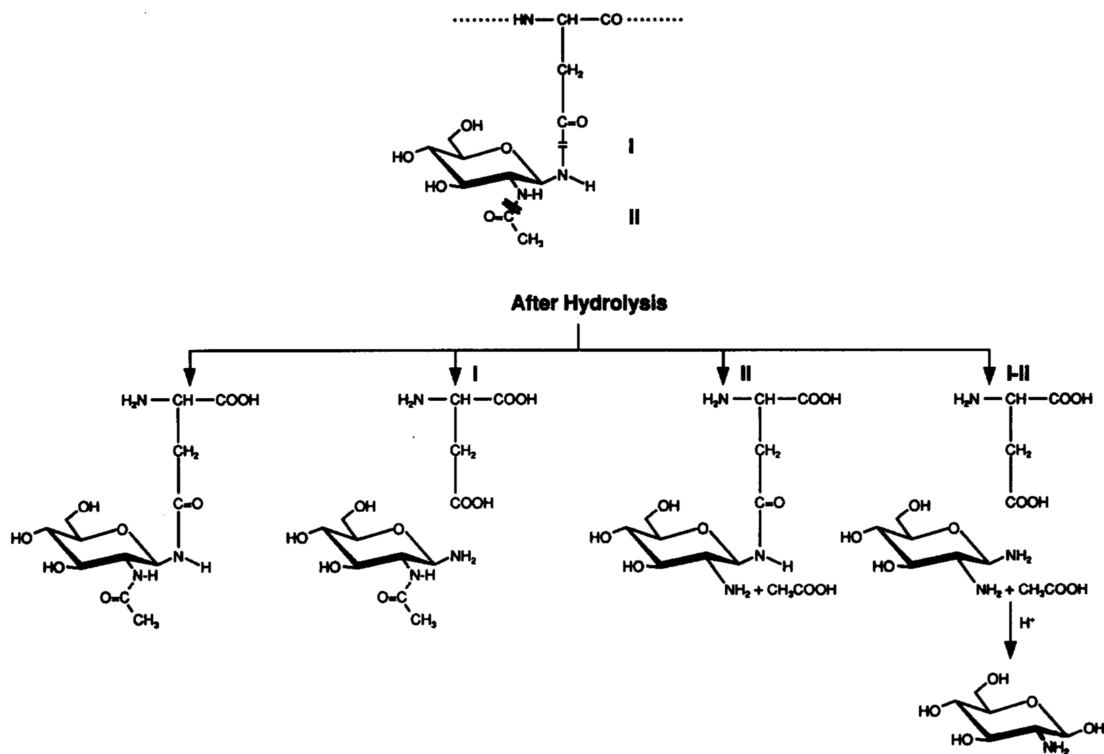

Fig. 1. The possible pathways of acidic hydrolysis of a GlcNAc-conjugated asparagine residue.

sugar derivatives with N-α-fluorenylmethoxycarbonyl-aspartic acid-α-*tert.*-butyl-β-pentafluoro-phenyl ester [Fmoc-Asp(OPfp)-O^{t}Bu] was unsuccessful after hydrolysis of the 1-aminoglucose for 1 h. In contrast, almost quantitative acylation of the same compound was achieved without hydrolysis, indicating the acid lability of the 1-amino sugar. This result is in good agreement with the previously reported lability of 1-amino sugars in an acidic environment [30]. Intact GlcNAc and GalNAc were not considered, as these compounds cannot be derivatized with DABS-Cl at the pH we used [17].

Amounts of 10 nmol of glucosamine hydrochloride and galactosamine hydrochloride standards were dabsylated, and 10% of them (10% of 10 nmol) were subjected to HPLC using gradient 1 (see Experimental). Gradient 1 is our method of choice for the determination of amino acids in peptides, because the mixture of dabsylated amino acid standards is baseline separated. New peaks were detected at 18.50 min for DABS-galactosamine and 19.05 min for DABS-glucosamine. These peaks fall between the DABS-proline (18.27 min) and DABS-valine (19.36 min) peaks. Whereas co-injection of the glucosamine and valine derivatives revealed two separate peaks, the galactosamine and proline derivatives could not be separated by this gradient. Fig. 2 shows the chromatograms obtained after injecting 8% of 5 nmol GlcNAc and GalNAc after hydrolysis for 1 h and dabsylation. The new peaks were detected at the position of the amino sugar standards. (The 1.2-min shift in the retention times of the dabsylated amino sugar peaks compared with the amino sugar standards reflects a column change and also a general shift of the peaks in RP-HPLC.) Moreover, co-injection of hydrolyzed GlcNAc and glucosamine or hydrolyzed GalNAc and galactosamine revealed single peaks. As Fig. 2 shows, the two different sugar derivatives are baseline separated. When the same procedure was applied to H-Asn(GlcNAc)-OH, peaks were detected at both Asx and glucosamine, as expected.

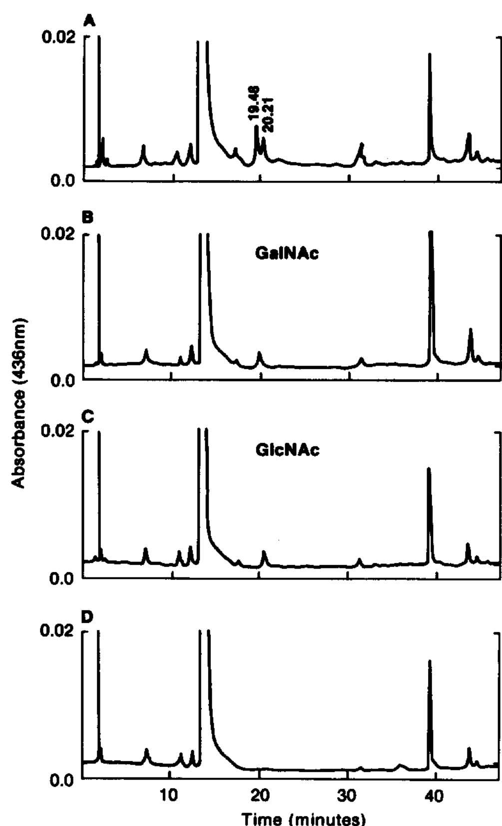

Fig. 2. Reversed-phase chromatography of hydrolyzed and dabsylated (C) GlcNAc, (B) GalNAc and (A) their mixture using gradient 1. Hydrolysis was carried out at 110°C for 1 h; 8% of 5 nmol sugar moieties were injected for the individual runs and 12% of 10 nmol GalNAc and 5 nmol GlcNAc were injected in (A). The detected ratio of the carbohydrates in (A) is 1.77:1 and 1.61:1, calculated on peak areas and peak heights, respectively. (D) blank.

Repeated attempts at fast atom bombardment mass spectrometry on several instruments failed to reveal any molecular ions from DABS-valine or DABS-glucosamine, even in the 100-μg range. Similar unsuccessful attempts were made to obtain molecular ions of phenylthiohydantoin-derivatized Asn(GlcNAc) and Asn(chitobiose) during peptide sequencing. Therefore, we used radioactively labeled GlcNAc (mixed with unlabeled GlcNAc) to verify the origin of the new peak found at 19–20 min. Fig. 3 shows the distribution of the counts after hydrolysis, dabsylation and HPLC separation. The major peak of the radioactivity was detected at the expected time. It should be noted that most of

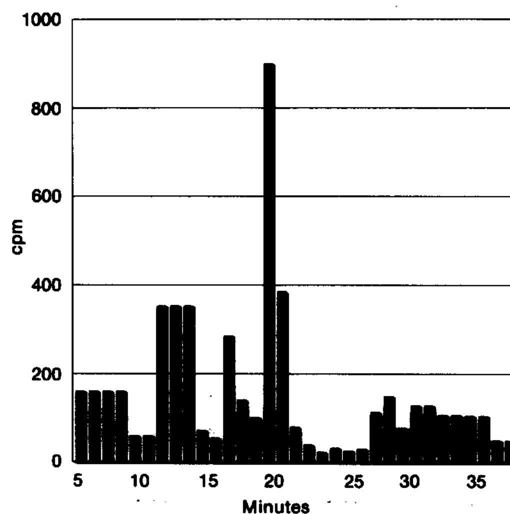

Fig. 3. Recovered radioactivity distribution of hydrolyzed and dabsylated ^{3}H-labeled GlcNAc in the amino acid region of the chromatogram. The total recovered radioactivity in the dabsylated amino acid range of the chromatogram was 3440 cpm, of which 1192 cpm corresponded to the dabsylated sugar at 19–20 min. The recovered radioactivity in the flow-through (corresponding to the underivatized sugar) was 8001 cpm.

the counts were found in the flow-through, indicating a low yield (14–19%) of dabsylation. When the same radiolabeled GlcNAc preparation in a 10 molar excess was hydrolyzed, derivatized and chromatographed together with the amino acid standards, DABS-glucosamine was repeatedly eluted between DABS-proline and DABS-valine, as Fig. 4 shows. As DABS-galac-

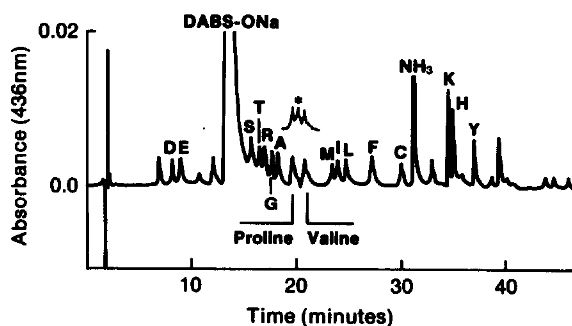

Fig. 4. DABS-glucosamine elutes between DABS-proline and DABS-valine using gradient 1. The full chromatogram corresponds to the dabsylated amino acid standard. The inset corresponds to the sugar region of a mixture of dabsylated amino acid standard, and an excess of hydrolyzed and dabsylated GlcNAc (marked with an asterisk).

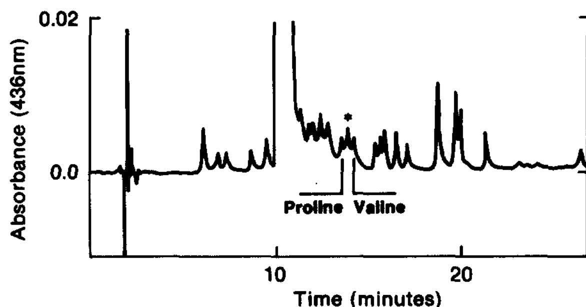

Fig. 5. DABS-galactosamine can be separated from DABS-proline and DABS-valine using gradient 2. The chromatogram was obtained by co-injection of a dabsylated amino acid standard, and an excess of hydrolyzed and dabsylated GalNAc (marked with an asterisk).

tosamine co-elutes with DABS-proline using gradient 1, this gradient (optimum for full amino acid analysis of glycopeptides containing the GlcNAc moiety) needs to be changed when GalNAc-containing peptides are to be analyzed. DABS-galactosamine can be resolved from DABS-proline using gradient 2 (the peak of the sugar derivative falls between the peaks of proline and valine derivatives), as Fig. 5 shows. This gradient, however, does not separate DABS-arginine, DABS-glycine or DABS-threonine, and consequently is used only for the verification of the presence of the GalNAc moiety in synthetic glycopeptides.

Utility of the method

The utility of the strategy outlined above was tested on the amino acid analysis of peptides T5 and RGD and a model tripeptide, and of their glycosylated derivatives. Fig. 6 shows the chromatograms of the analysis of peptide T5 and N-glycopeptide NGlcNAcT5 using gradient 1. The peak at 20.1 min on curve A clearly indicates the presence of the sugar moiety. Fig. 7 shows the chromatograms of the analyses of peptide T5 and O-glycopeptide TGalNAcT5 using gradient 2. The presence of the sugar moiety is shown by the appearance of the peak at 13.8 min on curve A. The N(GlcNAc)PV glycopeptide was prepared and analyzed to verify the baseline separation of DABS-proline, DABS-valine and DABS-glucosamine, not only in the standard but also in a glycopeptide hydro-

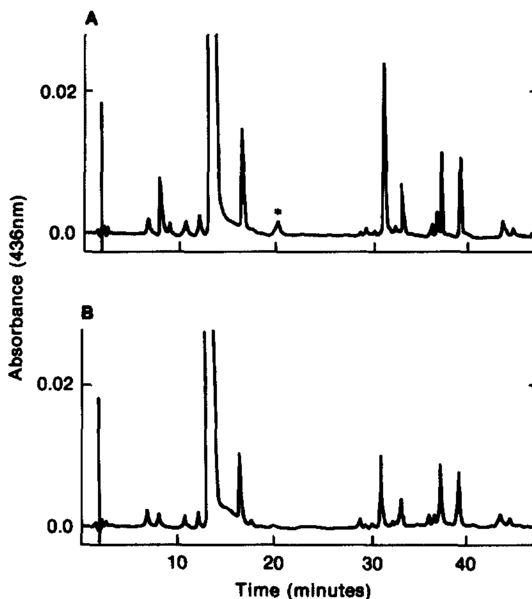

Fig. 6. Chromatograms after HPLC separation of (B) 8% of 1.9 μg of non-glycosylated peptide T5 and (A) 8% of 2.4 μg of N-glycosylated peptide NGlcNAcT5 using gradient 1. The peak marked with asterisk reveals the presence of the sugar moiety.

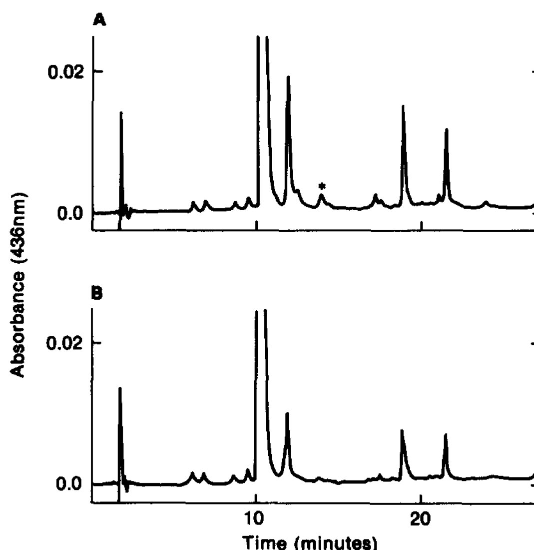

Fig. 7. Chromatograms after the amino acid analysis of (B) 6% of 1.9 μg of non-glycosylated peptide T5 and (A) 6% of 2.2 μg O-glycosylated peptide TGalNAcT5 using gradient 2. The peak marked with an asterisk verifies the presence of the carbohydrate moiety.

lysate that contained all three derivatives in question simultaneously (Figure 8).

Remarkably, both the dabsylated proline and dabsylated sugars could be detected after analysis of the RGD analogs (Table II). Our ability to detect the GlcNAc moiety, even in the O-glycosylated peptide, further justifies the use of this method to analyze glycopeptide structures.

Optimum sugar analysis conditions

During the development of an optimum hydrolysis procedure for the determination of the glycopeptides using amino acid analysis conditions, the following were considered.

The classical methods of protein and peptide hydrolysis feature heating the sample in constant-boiling HCl or 6 *M* HCl for periods of 18–24 h at 110°C to hydrolyze fully all the peptide bonds [31]. Gas-phase hydrolysis has been developed for samples with limited amounts to decrease the background noise caused by contaminants of the 6 *M* HCl [22,32,33]. The effect of raising the temperature on the recovery of amino acids by hydrolysis has also been reported [34–36]. Microwave heating is a viable alternative, and may be a promising method for the hydrolysis of the glycopeptide, but it requires special equipment and consequently its applicability is limited [37,38].

Sugars are less stable than the amino acids to boiling acid, and milder conditions of hydrolysis have to be used for their analysis. This degradation chain gives many intermediate products, and the sugar will be destroyed irrespective of whether the intermediates react with amino acids or whether the degradation proceeds to the final products [24]. It has also been reported that no perfect conditions of hydrolysis by acid can be derived so as to ensure that all glycosidic linkages present in the glycoproteins and glycopep-

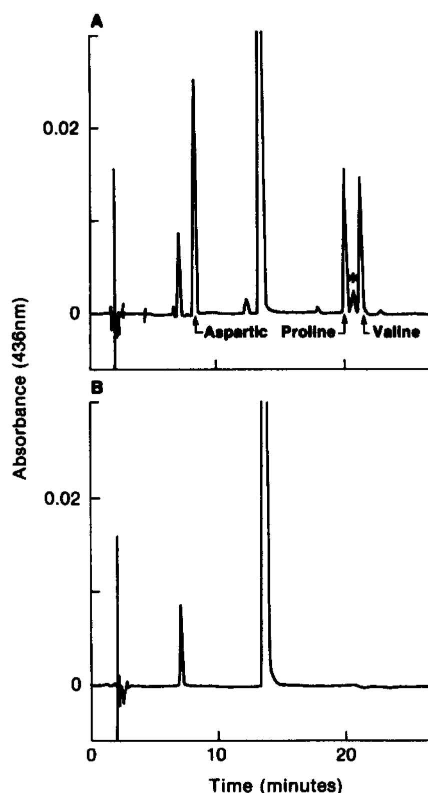

Fig. 8. Chromatogram after hydrolysis and dabsylation of (A) model peptide N(GlcNAc)PV compared with (B) the blank. The peak marked with an asterisk reveals the presence of the sugar.

TABLE II

DETECTION OF THE PRESENCE OF SUGAR IN THE SYNTHETIC RGD PEPTIDE AND OF ITS GLYCOSYLATED ANALOGS USING GRADIENT 1

Sequence	Peak of DABS-proline	Peak of DABS-glucosamine	FAB-MS[a] ($[M+H]^+$, m/z)
Ac-GRGDSPK-NH_2	+	–	758
Ac-N(GlcNAc)GRGDSPK-NH_2	+	+	1075
Ac-N(GlcNAc)GRGDSPKN(GlcNAc)-NH_2	+	+	1392
Ac-GRGDS(GlcNAc)PK-NH_2	+	+	912

[a] Fast atom bombardment mass spectrometry.

tides are cleaved, and at the same time that all monosaccharides are still intact at the end of the acid treatment [39]. Earlier reports of glycopeptide analysis failed to identify and quantify the new peak that was observed after hydrolysis and derivatization by phenyl isothiocyanate [40,41]. We wanted to develop methods to determine the glucosamine and galactosamine as intermediates of acidic hydrolysis of glycopeptides. Preliminary results show that decreasing the degradation of sensitive amino acids by using additives can be successful. A reduction of the degradation products of sensitive amino acids was reported by adding phenol to the hydrolysis mixture during both phenyl isothiocyanate- and DABS-based amino acid analysis [42,43].

Increasing the time of GlcNAc hydrolysis to between 1.5 and 16 h at 110°C or higher temperatures [44] decreased the detected amount of DABS-glucosamine (Table III). On the one hand, it is not recommended to maintain an extended hydrolysis time because the decrease in the detected amount of DABS-glucosamine was not accompanied by higher reproducibility compared with shorter hydrolysis times. On the other hand, it can be expected that the hydrolysis of the acetyl group from GlcNAc takes some time. As Fig. 9 indicates, hydrolysis for 1 h seems to be optimum to obtain the highest amount of DABS-glucosamine. Taken together, the hydrolysis time for the sugar determination should not exceed 1 h. We studied the reproducibility and the determination of DABS-glucosamine in a glucosamine standard and in hydrolyzed and dabsylated GlcNAc using these

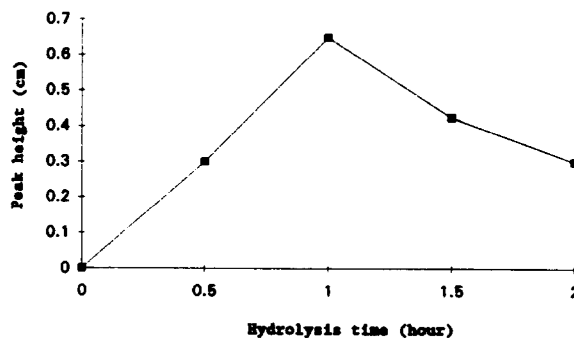

Fig. 9. Amount of dabsylated glucosamine as the function of the hydrolysis time, after acidic hydrolysis of GlcNAc.

conditions. The detected peak area of hydrolyzed and dabsylated GlcNAc equaled that of dabsylated glucosamine (Table IV) in the 2.5–7.5-nmol range (8% of them were injected: 0.2, 0.4 and 0.6 nmol). The detected peak area was directly proportional to the amount of the sugar. The variability of the results was within 10% in the individual experiments. When different sets of experiments were compared, the reproducibility favored the GlcNAc over the glucosamine, and high variability of the detected DABS-glucosamine was found when triplicate samples of glucosamine were subjected to the same hydrolysis conditions. These results show that the underlying events cannot be fully understood when only amino sugar standards are examined. It was also observed that the recovery of the dabsylated glucosamine standard and of the hydrolyzed and dabsylated GlcNAc was 20% of that of the dabsylated asparagine standard. This finding is in strong agreement with our prelimin-

TABLE III

SENSITIVITY OF THE CARBOHYDRATE MOIETY TO ACIDIC HYDROLYSIS

Temperature (°C)	Hydrolysis time (h)	Relative peak height[a]
110	1.5	0.81
	8	0.37
	16	0.22
150	1	0.20

[a] Expressed in 5 nmol GlcNAc / 1 nmol asparagine.

TABLE IV

RECOVERY OF DABSYLATED GLUCOSAMINE AND HYDROLYZED AND DABSYLATED GlcNAc AS A FUNCTION OF THE AMOUNT APPLIED

Injected (units)	One unit area of[a]	
	Dabsylated glucosamine	Hydrolyzed and dabsylated GlcNAc
2.5	0.2208	0.2175
5.0	0.2145	0.1998
7.5	0.2181	0.2175

[a] One unit equals 0.08 nmol of dabsylated sugar.

ary results, which indicate that 8% of 5 nmol of the sugar standards needs to be dabsylated in order to obtain peak heights similar to those for 8% of 1 nmol dabsylated asparagine (8% of the dabsylated amounts were injected). Most of the sugars probably remain undabsylated, as 86 and 81%, respectively, of the radioactivity of GlcNAc was recovered in the flow-through in two replicate experiments. We attempted to improve the efficiency of the dabsylation reaction. An increase in the temperature to 90°C, lengthening of the time of dabsylation from 15 to 25 min or using microwave irradiation were less effective. We also tested whether dabsylation is inferior to the currently partially used phenyl isothiocyanate (PITC) derivatization of glucosamine. The standard derivatization protocol of glucosamine with PITC resulted in 20% of that of phenylthiocarbamyl (PTC)-Aspartic, the same value that was obtained with dabsylation. Moreover, reaction with PITC may lead to the formation of a sugar alcohol [45] and further work-up is required to obtain a single analyzable compound.

Two different possibilities were scrutinized for the determination of sugars in the glycopeptides (using amino acid analysis conditions). First, analysis of the amino sugars cleaved from the glycopeptides was performed. Second, as we demonstrated earlier, a 10–30% loss of the asparagine occurred when peptides containing GlcNAc-conjugated asparagine were subjected to hydrolysis at 150°C for 1 h, followed by PITC amino acid analysis [46]. Different amounts of Asn-GlcNAc and T peptides were hydrolyzed and dabsylated in order to investigate the possibility of obtaining useful information concerning the number of incorporated sugar moieties based on the detected aspartic values. The recovery of dabsylated asparagine from the Asn-GlcNAc standard is reproducibly 57% of that of the asparagine standard, and is constant in the 2.5–5 nmol range. The complete recovery of the sugars remains unsuccessful, as more than 40% variability of the detected carbohydrate was found. Our experiments with the T peptides showed that the detected asparagine is well below the expected amount after hydrolysis at 110°C for 1 h, even when no sugar is attached (Table V), owing to incomplete hydrolysis of the peptide bonds. Extension of the hydrolysis time to 10 h resulted in the recovery of the correct amount of asparagine for the non-glycosylated peptide T5 and the N-terminally N-glycosylated peptide NGlcNAcT5, but remained low for the N-terminally O-glycosylated peptide TGalNAcT5 and especially the peptide T5GlcNAc, which contains the carbohydrate in a mid-chain position (Table V). Unfortunately, these hydrolysis conditions are not compatible with the analysis of the sugar moieties.

TABLE V

RECOVERY OF THE ASPARAGINE RESIDUE AS A FUNCTION OF HYDROLYSIS TIME AND THE PRESENCE AND LOCATION OF THE SUGAR MOIETIES

Peptide	Amount of DABS-ASX[a]		
	Detected after 1 h hydrolysis	Detected after 10 h hydrolysis	Expected
T5	0.66	1.04	1
T5GlcNAc	0.75	0.89	1
GlcNAcNT5	1.71	2.09	2
TGalNAcT5	0.63	0.94	1

[a] The values are normalized to threonines.

The observed reliable lower detection limit of the sugar moieties in the glycopeptides was estimated to be 0.2 nmol and upper detection limit 0.5 nmol. The DABS-sugar peak is well observed in this range, but increasing the amount of peptide results in an increasing number and heights of unidentified peaks. Although no unidentified peak was observed using 0.2 nmol of peptide, two new peaks were observed at the position of DABS-alanine and DABS-valine when 0.5 nmol of peptide was applied. These new, unidentified peaks may originate from other intermediate products of sugar degradation. Many other unidentified peaks are detected on the chromatogram after derivatization with DABS-Cl [21,22], even when blank samples are analyzed. One of these unidentified peaks appears after several hours of continuous chromatography on any given working day, and is located close to the peaks of DABS-glucosamine and DABS-galactosamine.

We attempted to improve the recovery of sugars by using phenol (as a scavenger of oxidant) with 6 *M* HCl during hydrolysis or, alternatively, with changed reaction conditions [42]. Three kinds of hydrolysis conditions were used: (A) 110°C, 1 h, without phenol; (B) 150°C, 1 h, with addition of 3% phenol; and (C) 100°C, 3 h, with addition of 3% phenol (as was suggested by Muramoto and Kamiya [42]). Although the sugar could be detected at 100°C, the addition of phenol did not improve its recovery from Asn-GlcNAc compared with hydrolysis at 110°C without addition of phenol. A lack of all contaminating peaks was observed at 150°C after addition of phenol. The carbohydrate, however, was also missing from the chromatogram (Table VI). The presence of the sugar was detected in 0.5-nmol amounts of the glycosylated T peptides after applying all three hydrolysis conditions, but the determination of the amino acids was still outside the acceptable range.

In conclusion, we have demonstrated that the presence of GlcNAc and GalNAc (the starting sugar structures of N- and O-glycoproteins, respectively) and, consequently, the integrity of synthetic glycopeptides can be verified by DABS-Cl amino acid analysis. The sugars can be recovered in 15–25% overall yields. The enzymatic cleavage of glycoproteins usually results in peptide fragments bearing glycosylated side chains. The protocol discussed above is a viable alternative for the rapid determination of such glycosylated protein fragments.

TABLE VI

EFFECT OF DIFFERENT HYDROLYSIS CONDITIONS ON THE DETERMINATION OF Asn-GlcNAc

Injected (nmol)	Detected area[a]					
	A		B		C	
	Asn	Sugar	Asn	Sugar	Asn	Sugar
0.2	1.36	0.15	1.25	–	1.61	0.15
0.4	2.65	0.33	2.89	–	3.03	0.53

[a] The conditions of hydrolysis were (A) 110°C, 1 h, without phenol; (B) 150°C, 1 h, with addition of 3% phenol; and (C) 100°C, 3 h, with addition of 3% phenol. The recovered amounts of asparagine in A, B and C compared with the Asn standard were 57, 62, and 55%, respectively.

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REFERENCES

1 R. Kornfeld and S. Kornfeld, *Annu. Rev. Biochem.*, 54 (1985) 631.
2 P.L. Mollison, in C.P. Engelfriet and M. Contreras (Editors), *Blood Transfusion in Clinical Medicine*, Blackwell, Oxford, 1987, p. 269.
3 K. Kakehi, S. Suzuki, S. Honda and Y.C. Lee, *Anal. Biochem.*, 199 (1991) 256.
4 R. Mögele, B. Pabel and R. Galensa, *J. Chromatogr.*, 591 (1992) 165.
5 T. Akiyama, *J. Chromatogr.*, 588 (1991) 53.
6 M.C. Rose, W.A. Voter, H. Sage, C.F. Brown and B. Kaufman, *J. Biol. Chem.*, 259 (1984) 3167.
7 E.A. Kabat, in J.D. Ebert, A.G. Loewy and H.A. Schneiderman (Editors), *Structural Concepts in Immunology and Immunochemistry*, Holt, Rinehart, and Winston, New York, 1987, p. 269.
8 L. Otvos, Jr., J. Thurin, E. Kollat, L. Urge, H.H. Mantsch and M. Hollosi, *Int. J. Pept. Protein Res.*, 38 (1991) 476.
9 A. Kobata, in V. Ginsburg and P. Robbins (Editors), *Biology of Carbohydrates*, Vol. 2, Wiley, New York, 1984, p. 87.
10 C.A. Browne, H.P.J. Bennett and S. Solomon, *Anal. Biochem.*, 124 (1982) 201.
11 L. Otvos, Jr., L. Urge and J. Thurin, *J. Chromatogr.*, 599 (1992) 43.
12 C.B. Pert, J.M. Hill, M.R. Ruff, R.M. Berman, G. Robey, L.O. Arthur, F.W. Ruscetti and W.L. Farrar, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) 9254.
13 L. Urge, L. Gorbics and L. Otvos, Jr., *Biochem. Biophys. Res. Commun.*, 184 (1992) 1125.
14 S. Moore and W.H. Stein, *J. Biol. Chem.*, 211 (1954) 893.
15 D.W. Hill, F.H. Waters, T.D. Wilson and J.D. Stuart, *Anal. Chem.*, 51 (1979) 1338.
16 R.L. Heinrikson and S.C. Meredith, *Anal. Biochem.*, 136 (1984) 65.
17 J.-K. Lin and J.-Y. Chang, *Anal. Chem.*, 7 (1975) 1634.
18 J.-Y. Chang, R. Knecht and D.G. Braun, *Biochem. J.*, 199 (1981) 547.
19 J.-Y. Chang, A. Lehmann and B. Wittmann-Liebold, *Anal. Biochem.*, 102 (1980) 380.
20 C.-Y. Yang and S.J. Wakil, *Anal. Biochem.*, 137 (1984) 54.

21 J.-Y. Chang, R. Knecht and D.G. Braun, *Biochem. J.*, 203 (1982) 803.
22 J.-Y. Chang and R. Knecht, *Anal. Chem.*, 58 (1986) 2375.
23 H. Kunz, H. Waldmann and J. März, *Liebigs Ann. Chem.*, 45 (1989) 45.
24 A. Gottschalk, in A. Gottschalk (Editor), *Glycoproteins, Their Composition, Structure, and Function*, Elsevier, Amsterdam, 1972, p. 96.
25 L. Urge, E. Kollat, M. Hollosi, I. Laczko, K. Wroblewski, J. Thurin and L. Otvos, Jr., *Tetrahedron Lett.*, 32 (1991) 3445.
26 L. Otvos, Jr., L. Urge, M. Hollosi, K. Wroblewski, G. Graczyk, J.D. Fasman and J. Thurin, *Tetrahedron Lett.*, 31 (1990) 5889.
27 F. Altman, *Anal. Biochem.*, 204 (1992) 215.
28 M.J. Spiro and R.G. Spiro, *Anal. Biochem.*, 204 (1992) 152.
29 L. Urge, E. Kollat, M. Hollosi, I. Lacko, K. Wroblewski, J. Thurin and L. Otvos, Jr., *Tetrahedron Lett.*, 32 (1991) 3445.
30 A.B. Foster, *Adv. Carbohydr. Chem.*, 14 (1959) 213.
31 D. Moore and W.H. Stein, *Methods Enzymol.*, 6 (1963) 819.
32 N.M. Meltzer, G.I. Tous, S. Gruber and S. Stein, *Anal. Biochem.*, 160 (1987) 356.
33 D.T. Blankenship, M.A. Krivanek, B.L. Ackermann and A.D. Cardin, *Anal. Biochem.*, 178 (1989) 227.
34 F.C. Westhall and H. Hesser, *Anal. Biochem.*, 61 (1974) 610.
35 D. Roach and C.W. Gehrke, *J. Chromatogr.*, 52 (1970) 393.
36 F.E. Kaiser, C.W. Gehrke, R.W. Zumwalt and K.C. Kuo, *J. Chromatogr.*, 94 (1974) 113.
37 L.B. Gilman and C. Woodward, in J.J. Villafanca (Editor), *Current Research in Protein Chemistry: Techniques, Structure and Function*, Academic Press, New York, 1990, Ch. 3, p. 23.
38 S.-H. Chiou and K.-T. Wang, *J. Chromatogr.*, 491 (1989) 424.
39 A. Neuberger and R.D. Marshall, in A. Gottschalk (Editor), *Glycoproteins, Their Composition, Structure, and Function*, Elsevier, Amsterdam, 1972, p. 190.
40 R. Gupta and N. Jentoft, *J. Chromatogr.*, 474 (1989) 411.
41 D.E.H. Palladino, R.M. House and K.A. Cohen, *J. Chromatogr.*, 599 (1992) 3.
42 K. Muramoto and H. Kamiya, *Anal. Biochem.*, 189 (1990) 223.
43 B.A. Bidlingmeyer, S.A. Cohen and T.L. Tarvin, *J. Chromatogr.*, 336 (1984) 93.
44 G. Allen, in R.H. Burdon and P.H. van Knippenberg (Editors), *Laboratory Techniques in Biochemistry and Molecular Biology: Sequencing of Proteins and Peptides*, Vol. 9, Elsevier, Amsterdam, 1989, p. 40.
45 K.R. Anumula and P.B. Taylor, *Anal. Biochem.*, 197 (1991) 113.
46 L. Otvos, Jr., K. Wroblewski, E. Kollat, A. Perczel, M. Hollosi, J.D. Fasman, H.C.J. Ertl and J. Thurin, *Pept. Res.*, 2 (1989) 362.