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Improved capillary electrophoretic separation of glycosylated oligopeptides through addition of poly(vinyl alcohol), and analysis by electrospray mass spectrometry

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Abstract

A method for the analysis of O-glycosylation of peptides has been developed, combining capillary electrophoretic (CE) separation and electrospray ionization mass spectrometry. Synthetic peptides with apomucin 'tandem repeat' sequences which present potential O-glycosylation sites on threonine and serine residues were used as model system. In vitro O-glycosylated peptide samples were obtained by incubation of the peptides with human gastric microsomal homogenates containing N-acetylgalactosamine transferase activity in the presence of uridyl diphosphate N-acetylgalactosamine (UDP-GalNAc). CE was carried out in the presence of the linear polymer poly(vinyl alcohol) in the electrophoresis solvent, resulting in a greatly improved separation of the up to five different glycoforms of peptides with lengths of 8, 16 or 23 amino acids, and the unglycosylated peptides. After separation and peak collection, the number of modifications with N-acetyl galactosamine (GalNAc) could be determined by electrospray ionization mass spectrometry. The glycosylation pattern was shown to depend on the amino acid sequence of the peptides. © 1998 Elsevier Science B.V.

Keywords: Buffer composition; Peptides; Poly(vinyl alcohol)

1. Introduction

There is a recent resurgence of interest in the post-translational modification of protein serine and threonine residues by glycosylation. The resulting O-linked oligosaccharide chains tend to be clustered over short stretches of peptides, and they can present multivalent carbohydrate antigenic or functional determinants for antibody recognition, mammalian cell adhesion and microorganism binding [1,2]. The main O-glycosylation pathway in animal cells involves the synthesis of oligosaccharides linked via N-acetylgalactosamine (GalNAc) to serine or threonine in mucine type glycoproteins or their analogues [3]. In mucins, several cores have been described having GalNAc as a key motive [4]. Directly linked to the protein, the number and position of GalNAc has an important influence on the glycosylation pattern. Through sugar nucleotide donors, one by one, the O-oligosaccharide chain is elongated, and elongation is thought to depend on the flanking amino acid sequences. This process is

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often perturbed in pathological situations such as cancer at lung, colon or stomach or inflammatory pathologies of digestive tissues. In order to evaluate GalNAc transferase activities from different tissue homogenates and to appreciate their affinity for different peptide sequences, synthetic peptides (from 8 to 23 amino acids) with the tandem repeat sequences found in mucins were used as a model system. A simple and efficient method is needed to analyze the first step of the O-glycosylation reaction. In previous work HPLC, high-performance anion-exchange chromatography (HPAEC) and capillary electrophoresis (CE) have been used to separate O-glycosylated peptides. The results were satisfactory considering eight-residue peptides substrates [5,6] but these methods failed with peptides longer than eight amino acids. CE appears to be well adapted for the analysis of peptide glycosylation owing to the very low quantity of sample required and its high separation power. The aim of the present work was to extend useful purpose of the CE separation method developed in [6] to larger glycosylated and nonglycosylated model peptides, while keeping the possibility of direct electrospray ionisation (ESI) MS analysis of the glycosylation products. This was accomplished by addition of hydrosoluble linear polymers to the CE run solvent.

2. Experimental

2.1. Reagents

Poly(ethylene glycol) 500 (PEG 500) and 20 000 (PEG 20 000), poly(vinyl alcohol) (PVA) 30 000–70 000 were purchased from Sigma (St. Louis, MO, USA). PVA 15 000 and 49 000 were purchased from Fluka. All other reagents were of analytical grade. The synthetic peptides were from Neosytem (Strasbourg, France). All solutions were passed through a 0.22-μm membrane filter prior to use.

Microsome preparation and in vitro O-glycosylation were performed as described in [5].

2.2. Capillary electrophoresis

CE experiments were performed using a Beckman P/ACE system model 2200 (Beckman, Fullerton, CA, USA). Beckman n-CAP fused-silica capillaries 57 cm (effective length of 50 cm)×75 μm I.D. were used in all experiments. CE was performed at 23°C, at an electrical field strength of 263 V/cm, with the cathode at the detector end. Detection was by UV absorption at 200 nm. After each run the capillary was regenerated by flushing with 0.1 *M* NaOH for 2 min, followed by ultrapure water for 2 min. Peptide samples with a concentration of 2 mg/ml were introduced at the anode side by pressure of nitrogen.

2.3. Mass spectrometry

ESI-MS analysis of the in vitro glycosylated products was done using a Perkin-Elmer mass spectrometer (Perkin-Elmer SCIEX, Toronto, Canada) with an electrospray ion source operating at atmospheric pressure, followed by a quadrupole mass analyzer with an upper mass limit of m/z 2400. The samples (0.1 mg/ml in 20% acetonitrile in water containing 0.1% formic acid) were introduced into the source at a flow-rate of 3 μl/min. The sampling cone voltage was adjusted to 90 V.

The sample collection for this analysis was carried out according to [7]. Briefly, the peaks corresponding to specific precise migration times, based on the excellent reproducibility of the CE runs, were collected and pooled and subjected to ESI-MS analysis and Edman degradation to check the peptide sequences. The amounts of available glycopeptides varied between 1 and 3 nmol and 10–15 runs were performed to collect each fraction in a sufficient amount.

3. Results

3.1. Study of glycosylated model peptides by conventional methods

Model peptides with 8, 16 or 23 amino acids as shown in Fig. 1 were incubated with gastric microsomal preparations to evaluate O-glycosylation by GalNAc transferase and the influence of the amino acid sequence of the peptide. An important issue is the number of potential O-glycosylation sites. Other parameters, e.g., the presence of proline (possible influence of a *cis*/*trans* isomerization on glycosyla-

1- TTSTTSAP (tandem repeat unit MUC 5 AC)
2- TTSAPTTS
3- TTSSPTTS
4- GTTPSPVP
5- TTSAPTTSTTSAPTTS
6- TTSGPGTTPSPVPTTS
7- TTSSPTTSTTSAPTTS
8- PTTTPITTTTTVTPTPTPTGTQT (tandem repeat unit MUC 2)
9- TTAAPPTPSATTPAPPSSSAPPE (tandem repeat unit MUC 7)

Fig. 1. Synthetic peptide models representing the most common peptide sequences in mucin.

tion) and a sterical hindrance generated by GalNAc linkage may also have an influence on the O-glycosylation pattern. In the case of octapeptides, separation by HPLC followed by ESI-MS analysis was studied to determinate the number of GalNAc motifs [5]. Both HPLC and conventional high-performance capillary electrophoretic (HPCE) methods yielded poor results for longer peptides in preliminary experiments (16 or 23 amino acids) due to insufficient resolution. As an alternative method, capillary zone electrophoresis was tested. Fig. 2a shows a typical electropherogram obtained with the hexadecapeptide 6, glycosylated by in vitro reaction as described under experimental. Only three peaks are visible on this electropherogram, with a poor resolution of the second and the third peak. Whereas the second peak was identified as the nonglycosylated peptide by ESI-MS, the third peak seemed to be heterogeneous and could not be analyzed by ESI-MS. Additionally to the GalNAc transferase reaction, side reactions can occur due to dipeptidyl transferase and proteolytic activities, resulting in a mixture of products with the same mobilities under the electrophoretic conditions employed.

3.2. Soluble polymers as separation matrix

Improvement of separations through the suppression of electroendoosmotic flow and zone stabilisation in ‘free zone electrophoresis’ by the addition of polymers to the migration buffer had already been reported by Hjertén in 1958 [8]. Following this, several polymers have been used (dextran, hydroxyethyl cellulose, poly(ethylene glycol), linear polyacrylamide, poly(vinyl alcohol)) to separate different molecules, e.g., DNA strands, proteins, small compounds [9–11]. Initially we chose PEG and PVA as

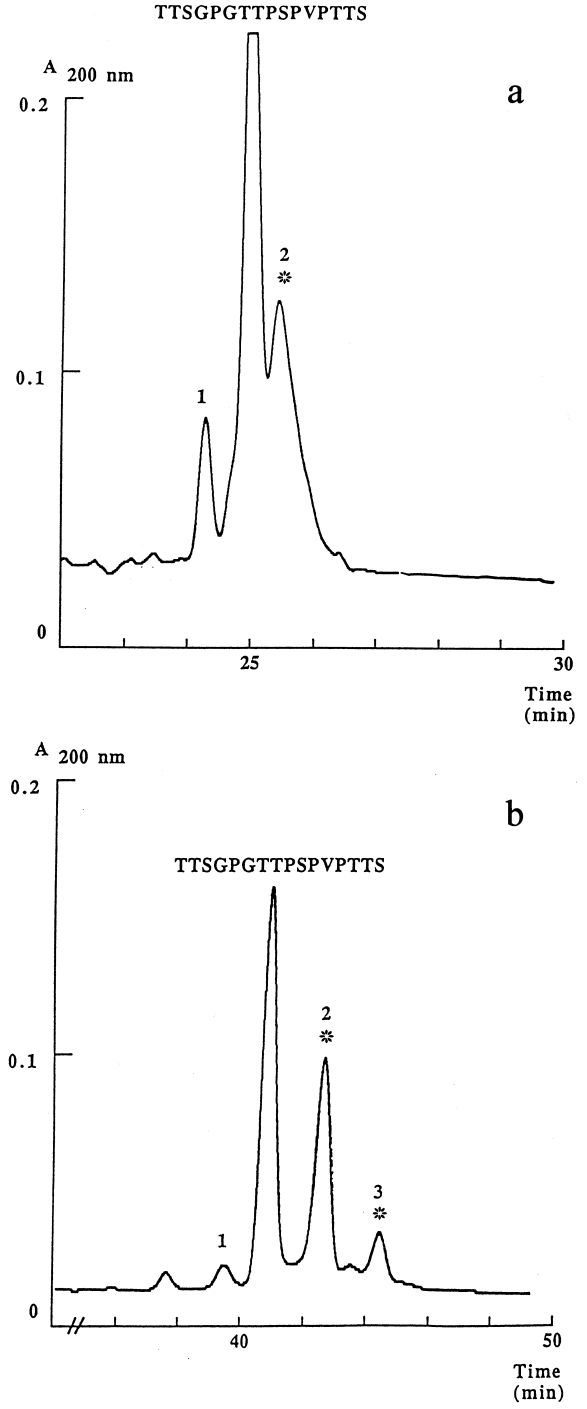

Fig. 2. Electropherograms of the hexadecapeptide 6 (TTSGPGTTPSPVPTTS) after incubation with human gastric microsomes in presence of UDP-GalNAc. Electrophoresis solvent: (a) 2 *M* formic acid; (b) 2 *M* formic acid containing 2.5% (w/w) PVA 15 000. * Glycosylated peptide.

solvent additives in our CE system in an attempt to improve the separation of our glycosylated model peptides. After preliminary experiments, PVA turned out to be the better choice, as PEG (5000 or 20 000 molecular mass) did not improve the resolution when added to the electrophoresis solvent at concentrations between 0 and 3%, and due to its rather high absorbance in lower UV, the signal of the peptide peaks was reduced. Advantages of PVA as buffer additive in CE are its low viscosity at low concentrations, its hydrosolubility and its UV transparency. First tests realized with PVA 30 000–70 000 showed a good separation of glycopeptides at concentrations higher than 1%, with a good peak symmetry (not illustrated). However, the run to run reproducibility was poor, which may be due to the high polydispersity of this polymer preparation. Therefore, narrow-range polymers (PVA 15 000 and 49 000) were tested and yielded better results. In the presence of PVA 49 000, peak resolution and run-to-run reproducibility were good, however, migration times were very long (ca. 58 min at a polymer concentration of 2.5%). The best results were obtained with PVA 15 000 (Fig. 2b). Separation improved considerably in presence of 2.5% of the polymer, as compared to the run without addition of PVA (Fig. 2a). Four symmetric baseline-separated peaks were obtained and the run-to-run reproducibility was excellent (±5.0% of the migration time), allowing for preparative CE with multiple injections and peak collection for further studies.

However, the quality of separation and the migration times were dependent on the concentration of PVA 15 000 in the electrophoresis solvent. Fig. 3 shows a plot of the migration times of two different hexadecapeptides, 6 and 7, polymer concentrations from 0 to 3% (At higher concentrations, PVA 15 000 was not fully soluble). Migration times increased slightly between 0 and 1% but significantly at higher concentrations. At concentrations below 1%, addition of PVA 15 000 to the electrophoresis solvent had no effect on separation, whereas the best resolution was obtained at 2.5%. As shown in Fig. 3, concentration of polymer up to 1% did not significantly change the

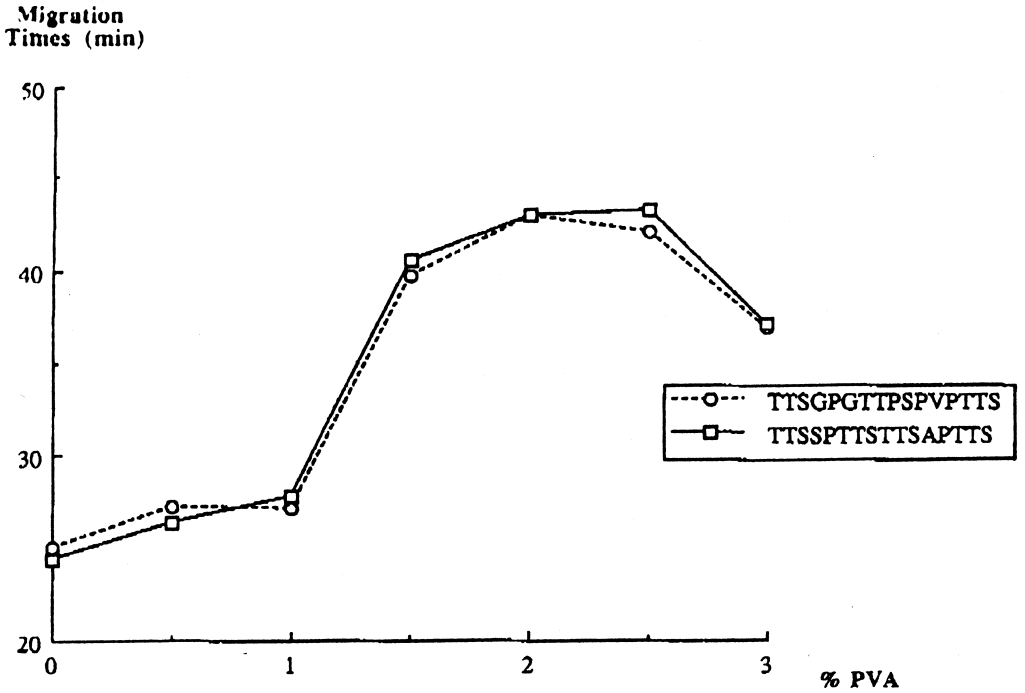

Fig. 3. Migration times of hexadecapeptides 6 and 7 (TTSSPTTSTTSAPTTS and TTSGPGTTPSPVPTTS) in 2 *M* formic acid as a function of PVA 15 000 concentration.

migration times nor the separation of peptides. Above this concentration, the migration times of the tested peptides increased almost exponentially to attain a plateau at 2–2.5%. Fig. 4 shows the migration times for a set of five model peptides (prior to any glycosylation), two octamers, two hexadecamers and one 23-residue peptide of variable sequences. The migration times for hexadecamers and for 23-residue peptide were significantly higher as compared to those of the octamers, irrespective of their peptide sequence. This data implies that a separation based on size and not on the peptide sequence is taking place in the presence of 2.5% PVA added to the electrophoretic buffer (2 *M* formic acid).

3.3. Analysis of glycosylated peptides

Hexadecamers (e.g. peptide No. 6) and 23-residues peptides (e.g. peptide No. 8) were subjected to in vitro glycosylation as described in Section 2. HPCE in the presence of 2.5% PVA were run for these glycosylated peptides along with appropriate blank controls. The data obtained with the 23-residue peptide No. 8, representing the tandem repeat unit MUC 2 is chosen to illustrate the efficiency of the separation (Fig. 5A–E). Fig. 5C and E show the clear separation of glycosylated species, as identified by ESI-MS analysis. They represent about 37.8% (Fig. 5E) and 28.9% (Fig. 5C) of total glycosylated peptides.In the case of glycosylation in the presence of protease inhibitor E64, 28.5% of peptide with one GalNAc, 6.9% of peptide with two residues of GalNAc, 1.5% of peptide with three residues of GalNAc and a minor peak of $<0.5\%$ of tetra-glycosylated species could be quantified (Fig. 5E). However, in the absence of E64, slightly higher percentages of the four glycosylated species were calculated. Nevertheless, as this calculation is based on relative percentages with reference to the total peptides, this difference is not very significative. Fig. 5B and A show respectively the products of the same hexadecapeptide subjected to microsomal enzymatic activity in the absence of UDP-GalNAc and the blank of the native peptide not subjected to any enzymatic reaction. In Fig. 5B, small peaks, corresponding to peptide degradation products are seen. The presence of protease inhibitor E 64 added in the reaction medium does not seem much to interfere in the migration of either the glycosylated peptide (Fig. 5E) or the blank run with peptide subjected to enzymatic reaction in the absence of the sugar (UDP-GalNAc) substrate (Fig. 5D) as its migration time in all the cases is around 24 min, which is much earlier than the glycosylated/unglycosylated peptides migration time (between 48 and 57 min). Note that the concentration of injected sample is much higher in Fig. 5C, as compared to that in Fig. 5E. This does not seem to influence neither the retention time of E64 nor the relative migrations of the glycosylated/unglycosylated peptides.

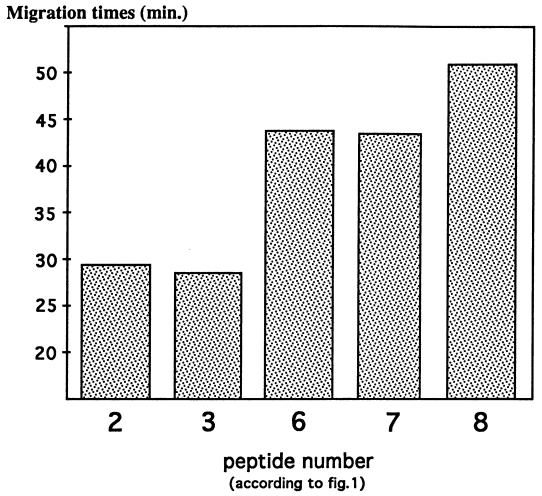

Fig. 4. Influence of peptide length on migration times in 2 *M* formic acid containing 2.5% (w/w) PVA 15 000 (see Section 2 for details)

3.4. ESI-MS analysis

After separation of the glycosylation reaction mixture by capillary electrophoresis with PVA 15 000 as buffer additive, and peaks were collected from different runs (the runs were reproducible), the number of GalNAc motifs on the reaction products has been determined by ESI-MS (Fig. 6). Direct analysis without exchange of buffer etc.of the collected peaks was possible owing to the electrophoresis solvent used (2 *M* formic acid), which avoided the need of solvent exchange and loss of sample. In the case of the tested hexadecapeptide 6 and the 23-mer peptide 8, mass spectra revealed distinct products with addition of one GalNAc

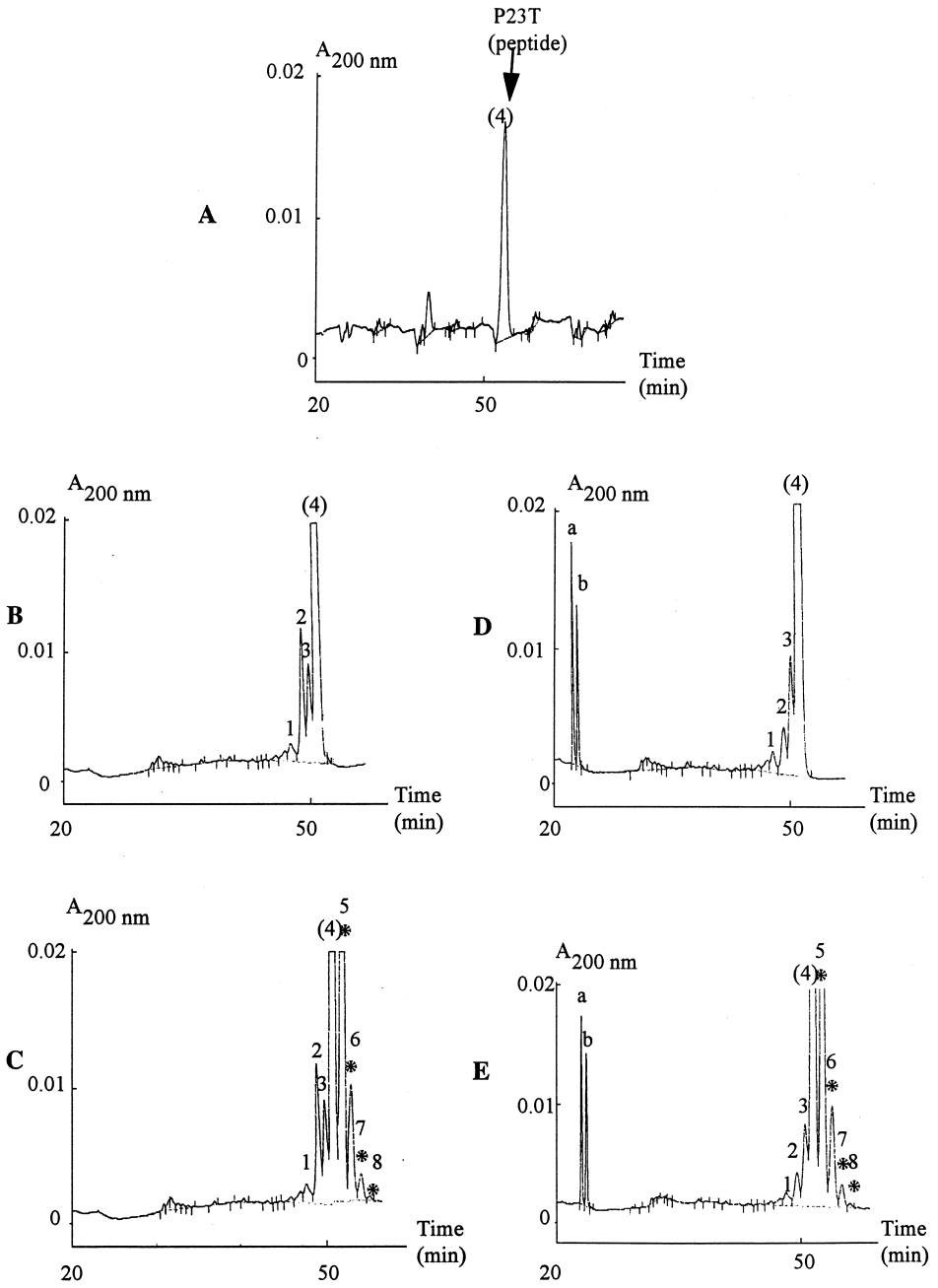

Fig. 5. HPCE patterns of peptide 8 subjected to in vitro glycosylation by microsomal extract: (A) blank without incubation with microsomal extract; (B) without UDP-GalNAc without thiol protease inhibitor E64 but subjected to microsomal enzymatic action; (C) in presence of sugar substrate (UDP-GalNAc) without thiol protease inhibitor E64; (D) in presence of sugar substrate (UDP-GalNAc) and thiol protease inhibitor E64; (E) without sugar substrate (UDP-GalNac) in presence of thiol protease inhibitor E64; a and b peaks representing E64 products; * indicates glycosylated species tested by radioactive marking.

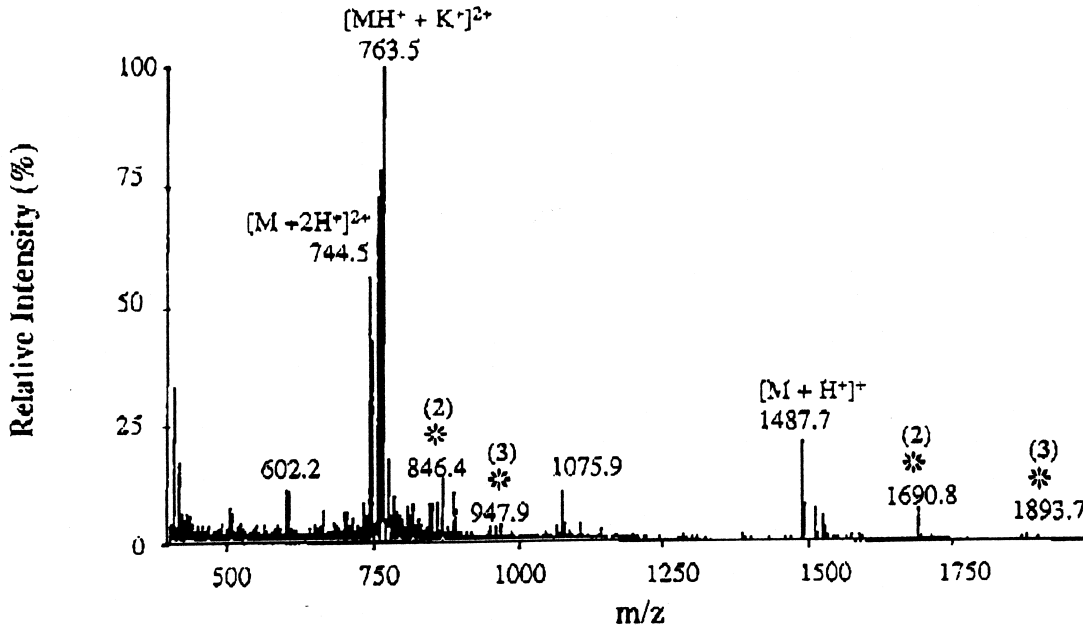

Fig. 6. ESI mass spectra of hexadecapeptide 6 (TTSGPGTTPSPVPTTS) after incubation with human gastric microsomes in presence of labelled UDP-GalNAc; * indicates glycosylated peptide.

(m/z 1690.8) or two GalNAc (m/z 1893.7) to the original peptide No. 6 (m/z 1487.7) (Fig. 6) and addition species of 3 GalNac and 4 GalNac for peptide 8. Based on these results, the peaks in Fig. 2b and Fig. 5A could be identified; for example, Peaks 2 and 3 in Fig. 2b corresponded to glycopeptides carrying one GalNAc and two GalNAc, respectively. Contrary to the findings reported by Tetaert et al. [5], in our case, only the capture of one potassium ion by the peptide has been detected after UDP-GalNAc incorporation, corresponding to a mass increase of 19.5 of the double charged form of the peptides.

3.5. Influence of the amino acid sequence on the glycosylation pattern

Different hexadecapeptides were incubated with human gastric microsomal preparations in presence of UDP-GalNAc. Peptides and glycopeptides were separated and analyzed by ESI MS. Table 1 shows the number of GalNAc incorporated in each peptide.

Table 1
Relative migration times of glycosylated and nonglycosylated peptides by HPCE in the presence of 2.5% PVA-15 000

MUC peptide motifs	Rel. migration times of peptide (min)	No. of O-linked positions	Rel. migration times of related glycopeptides (min)
TTSAPTTSTTSAPTTS (5)	39.9	2	41.7; 42.3
TTSGPGTTPSPVPTTS (6)	42.0	2	44.0; 45.5
TTSSPTTSTTSAPTTS (7)	41.1	2	42.7; 44.6
PTTTPITTTTVTPTPTPTGTQT (8)	51.6	4	52.9; 54.2; 55.4; 56.6

The results clearly demonstrate the influence of the peptide amino acid sequence on the glycosylation pattern.

4. Discussion

It has been shown that the physical properties of the electrophoresis buffer changed above a critical concentration of added soluble polymer [12,13]. In dilute solution, polymer chains are separated from each other, but dynamic coating of the capillary walls is obtained. By increasing the polymer concentration above a critical threshold, overlapping and interactions between the polymer chains occur, which is called the 'entanglement effect'. Dynamic coating of capillaries with PVA allowing for the separation of protein glycoforms (e.g., of RNase B) has been reported by Gilges et al. [10]. By addition of PVA at concentrations above the entanglement threshold, Simo-Alfonso et al. [11] have shown that it was possible to obtain a good separation of protein mixtures. They attribute the separation of proteins (M_r 14 000–94 000) to molecular sieving in the in situ formed pores of PVA due to 'entanglement effect' occurring at concentration above the entanglement threshold. In our work, addition of PVA 15 000 at concentrations below 1% did not result in an improved separation of glycopeptides, even though the electrophoresis solvent used (2 *M* formic acid, pH 1.7) provided favourable conditions for a dynamic coating of the silica capillary walls [10]. The critical concentration of PVA 15 000 being 0.95% (calculated according to [14]), it seems that separation occurred due to the polymer being in the entangled state.

The concentration of PVA at 2.5%, used in this study, is surely above the entanglement threshold concentration and hence a size based separation could be expected. Nevertheless, a precise size based discrimination of monoglycosylated from unglycosylated peptides may be quite difficult to imagine. However, Jentoft [1], in his review quotes that a simple O-glycosylation can tremendously increase the molecular radius. He notes that the mucin core peptides with substituted GalNac residues can assume up to 145 Å as compared to 10 Å for unsubstituted peptide. This can partly explain a molecular sieving effect, able to discriminate between the unsubstituted and mono/di/tri-substituted peptides. Another possible explanation could be the interaction of the glycosylated peptides with the PVA via hydrogen bonds. It has been reported by other authors that model analytes (benzoic acids and derivatives) interact with polyether (PEG) matrices used in CE [15]. Contrarily to PEG which is only hydrogen bond acceptor, PVA can also act as hydrogen bond donor thus allowing an 'interactive molecular sieving effect'.

5. Conclusion

Separation of different glycoforms of O-glycosylated peptides with lengths of 16 amino acids and longer was obtained through addition of 2.5% PVA 15 000 to the electrophoresis solvent. Peak collection was possible owing to a very good run-to-run reproducibility, and the peaks could directly be analyzed by ESI MS due to the compatibility of the solvent used in CE with the latter technique. A varying degree of glycosylation was observed for peptides with different amino acid sequences. Glycosylation patterns were also dependent on the origin of the microsomal preparations containing GalNAc transferase activity that were used for glycosylation. This paves the path for an elaborate study of the target amino acid/sequence specificities of the different isoforms of the N-acetylgalactosaminyltransferase from different sources. As an extension, microsomal preparations (pathological or normal, from colon, lung or stomach) are being tested with different peptides and seem to show different glycosylation patterns with identified specificities of glycosylation sites. This information could be important if characteristic O-glycosylation patterns in pathological situations such as cancer are to be determined.

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