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Note

Capillary electrophoresis separation of mucin motif MUC 5AC glycopeptides from in vitro *O*-glycosylation reactions

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The initial reaction in *O*-linked oligosaccharide biosynthesis is the transfer of a 2-acetamido-2-deoxy-D-galactose residue from the nucleotide sugar uridine 5′-(2-acetamido-2-deoxy-D-galactose diphosphate) (UDP-GalNAc) to a serine or threonine residue of a protein motif acceptor. This reaction, which occurs post-translationnally, is catalysed by UDP-GalNAc:polypeptide 2-acetamido-2-deoxy-D-galactosyltransferases (GalNAc-transferases, E.C. 2.4.1.41), which are intracellular enzymes believed to be localized in later endoplasmic reticulum compartments and/or in the *cis* region of the Golgi complex [1]. Elongation and termination of *O*-linked oligosaccharides is accomplished by sequential addition of individual monosaccharides by specific transferases [2] and the current data suggest that these reactions are localized primarily in the Golgi apparatus [1]. Concerning the first step of *O*-glycosylation (binding of the initial GalNAc), recent results establish the existence of a family of GalNAc-transferases which would be sequentially expressed in different organs [3,4]. The identification of the acceptor peptides, which can be used to discriminate the GalNAc-transferase(s), is an

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important step towards understanding the molecular basis of GalNAc *O*-linked glycosylation in cells and organs as well as in pathological conditions.

However, the effectiveness and reliability of the separation techniques with different peptide substrates have not been well-documented so far and the comparison of different approaches using mainly chromatographic procedures (such as the anion exchange, the affinity ligand, the reversed-phase high performance liquid chromatography [5,6]) have revealed a number of problems frequently encountered in the purification of the glycopeptides obtained from in vitro reactions.

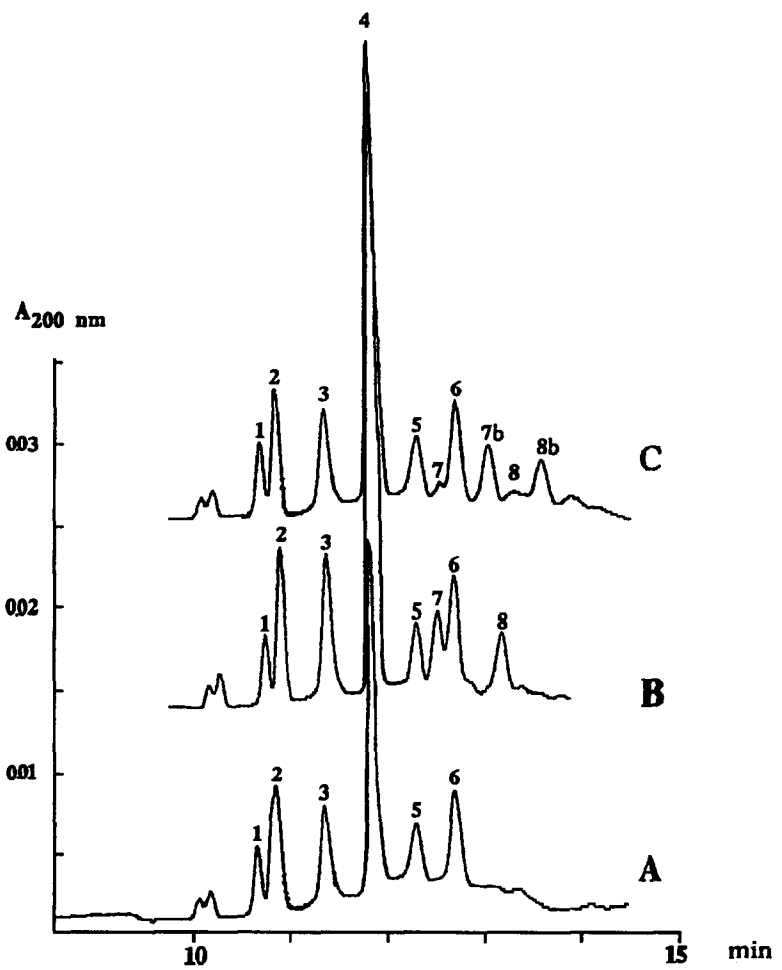

Fig. 1. Capillary zone electrophoresis profiles of the products obtained after reactions using gastric microsomal preparation and the different substrates. (A) Control reaction (incubation was performed in presence of enzyme preparation and TTSAPTTS peptide, without nucleotide–sugar substrate). (B) GalNAc reaction (incubation in presence of enzyme preparation and peptide TTSAPTTS/UDP-GalNAc substrates). (C) Gal-reaction of the GalNAc *O*-glycopeptide products (incubation in presence of enzyme preparation and products obtained from GalNAc-reaction/UDP-Gal substrates).

In the present study, capillary electrophoresis was employed to investigate the *O*-glycosylation products obtained from reactions using (i) as substrate, the octapeptide TTSAPTTS deduced from tandem repeat sequence of MUC 5AC (gene of mucin essentially expressed in the human stomach [7,8]), and (ii) as enzyme source, crude microsomal homogenates from human gastric mucosa. The 8-residue sequence peptide TTSAPTTS is naturally found in the normal as well as in carcinoma-associated gastric mucins (e.g., the tumor cell lines HT29-MTX [9]) and this peptide motif involves a proline residue (inducing the two different conformations *cis / trans* prolyl) inserted between two clusters of potential *O*-glycosylated hydroxy-amino acids. The GalNAc transferase activity is measured by the transfer of radiolabeled [^{3}H]UDP-GalNAc to the peptide following the separation of unincorporated UDP-GalNAc typically removed by passing the reaction products through Dowex 1X8 resin [5,6,10,11]. The human gastric GalNAc-transferase activity rate was determined under our conditions at 148.7 ± 22.2 nmol GalNAc/h/mg of microsomal protein, for three different experiments and mucosa from three distinct patients. Different chromatographic methods have been performed to separate glycosylated peptide(s) from the residual unreacted peptide. However, high performance anion-exchange chromatography (HPAEC) and high-performance liquid chromatography (HPLC using normal or microbore columns) were not thought to be the adequate procedures, especially using an octapeptide rich in hydroxy-amino acids as substrate for the in vitro *O*-glycosylation reactions: the glycopeptides and the unreacted peptide were too closely eluted [12,13].

The capillary zone electrophoresis (CZE), a rapidly evolving technique, has the potential to give rapid separations with high resolution and good quantification [14]. The separation of TTSAPTTS *O*-glycosylated products (obtained after GalNAc-reaction) by CZE has been developed here at analytical level as well as at preparative scale. After desalting, the major fractions migrated predominantly at retention times between 10 and 15 min. The by-products resulting of the reaction between peptide and microsomes (control reaction) were found in Fractions 1, 2, 3, 5, and 7, the unreacted peptide was in Fraction 4 (Fig. 1A), whereas after addition of the second substrate UDP-GalNAc, extra-fractions were found in 6 and 8 (Fig. 1B). After 20 CZE-runs, the *O*-glycosylated products (Fraction 6 as well as Fraction 8) were also obtained at preparative scale, allowing the identification by electrospray mass spectrometry (ESMS) of glycopeptides at m/z 968.5 and 1171.5 respectively, compared to the parent peptide at m/z 765.4. By Edman degradation, the exact location of the GalNAc positions on the peptide remained unsuccessful, because of the heterogeneity of the fractions: some difficulties were encountered in the purification of components that migrated too closely, and/or, glycopeptides and peptidic fragments were found at the same retention time, giving multiple *N*-terminal sequences (for example, the Fraction 8 contained relative amounts of components at m/z 968.5 and 1171.5 and the determination of its sequence by Edman degradation was confused).

To confirm the availability of capillary electrophoresis and the migration of the *O*-glycopeptides, an elongation reaction using UDP D-galactose was performed from the GalNAc-TTSAPTTS preparation, previously obtained, and the crude gastric microsomal homogenate (Gal-reaction). The excess of UDP D-galactose was eliminated passing through Dowex 1X8 column, the salts were removed using Sep-Pak C18 column and the

analysis by ESMS revealed the formation of new components at m/z 1130.6 and 1332.7 (corresponding to the molecular ion of Gal-linked *O*-glycopeptides TTSAPTTS). The Gal-reaction products were introduced into the CZE capillary and the comparison of the two electrophoretic profiles (for GalNAc- and Gal reactions) is shown in Figs. 1B and 1C respectively. Modification of the proportion of Fractions 6 and 8 (the corresponding glycopeptides) was clearly observed: they were almost undetectable in favour of the peaks 6b and 8b at retention times slightly delayed.

In conclusion, the capillary zone electrophoresis was developed to examine the products of the in vitro *O*-glycosylation reactions between the human 8-residue gastric MUC 5AC motif peptide and crude human gastric glycosyltransferase preparations. By capillary electrophoresis technique, the glycopeptides were formely distinguished from the residual unreacted peptide. According to previous reports [1,15], the profiles obtained after addition of D-galactose may account for the activity of GalNAc-3-β-D-galactosyltransferase (namely core 1-β-3-Gal-transferase, E.C. 2.4.1.122): to 90% of [^{14}C]galactose incorporation was associated the 90% of disappearance of the peaks 6 and 8 on behalf of the peaks 6b and 8b in the same ratio. The capillary zone electrophoresis proved thus to be a very efficient analytical tool (the limit of detection at the pmol level), but also for preparative purpose (accumulation of several runs allowed the obtention of substantial amount to proceed ESMS and the Edman degradation approaches). The capillary electrophoresis could be a powerful separation technique that would complement liquid chromatography. Further investigations are now in progress to examine, in human digestive mucosa, the efficiency of transferases involved in sugar elongation process (sialyl-, fucosyl-, 2-acetamido-2-deoxy-D-glucosyltransferases).

1. Experimental

Enzyme preparation.—Microsome suspensions were prepared, as described previously [12] by Potter–Elvehjem homogenization of fundic human gastric (3 g) samples which were obtained from patients after surgery. The microsomal pellet was suspended in the NaCl–sucrose solution to obtain a final protein concentration of 2.8–5.7 mg/mL; the solution was stored at −80°C until use.

Analytical methods.—The protein concentration was determined on the multiparametric automated chemistry Hitachi 717 analyser (Boehringer, Mannheim, Germany) using the Biotrol kit protein assay reagent (Biotrol, Chennevières les Louvres, France). The analysis by electrospray mass spectrometry (ESMS) using the mass spectrometer (Perkin–Elmer SCIEX, Toronto, Canada) has been described in a previous report [13]. The determination of amino acid sequences was performed using an Applied Biosystems gas-phase Sequencer model 477A.

In vitro O-*glycosylation.*—The synthetic peptide substrate NH_2-TTSAPTTS-COOH was from Neosystem (Strasbourg, France): the purity of the peptide was checked by amino acid sequence determination, by high performance liquid chromatography analysis and the mass found by ESMS.

The GalNAc-reaction incubation was made using the peptide TTSAPTTS (3 mM) and 30 μg of microsomal preparations as described earlier [12]. After the addition of the

enzyme, the samples were incubated for 2 h at 37°C. The reaction was stopped and the products were separated on a AG1-X8 anion-exchange resin column by elution with water. To complete the desalting, Sep-Pak C18 reverse-phased cartridges (Millipore, Waters chromatography, Milford, MA, USA) were activated by passage of MeOH 10 mL followed by deionized water 10 mL containing the trifluoroacetic acid (TFA, 0.1%) counterion. Aqueous eluates from the exchanger AG 1X8 were applied and polar materials were washed through twice with water/TFA 5 mL, whereas the glycopeptidic fractions were obtained by elution with acetonitrile 25% in water/TFA (10 mL).

For Gal-reaction, the incubation was performed using the same microsomal preparation (30 μg) in a mixture containing 5 mM D-galactono-1,4-lactone (inhibition of galactosidase) and 1 mM UDP-galactose (Sigma, St Louis, MO) containing [^{14}C]UDP-galactose (40,000–50,000 DPM) (Amersham, France) in the buffer conditions described in [12].

Capillary electrophoresis.—The capillary zone electrophoresis (CZE) was performed on a P/ACE system Model 5000 (Beckman, Fullerton, CA, USA) controlled by the System Gold software V 810 (Beckman). UV Absorbance was monitored by a fixed-wavelength detector at 200 nm. The fused silica capillary was 57 cm long × 75 μm I.D. and fitted in a cartridge with a modified mandrel in order to improve cooling. Migrations were run at 23°C using a 2 N formic acid buffer and the voltage across the capillary was maintained at 20 kV. The injections were carried out by pressure (duration 2–6 s, approximatively 10–20 pmol) and after each separation the capillary was flushed with the appropriate buffer for 1 min.

The preparative scale procedure was the modified method of Biehler and Schwartz [16] with the cumulative collection at the precise migration times corresponding to the two glycopeptidic fractions.

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