Resolution of *N*-linked oligosaccharides in glycoproteins based upon transglycosylation reaction by CE-TOF-MS

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The resolution of asparagine-type oligosaccharides in glycoproteins was carried out by combination of the transglycosylation reaction and CE-TOF-MS. The oligosaccharides enzymatically transferred to a fluorescent acceptor (NDA-Asn-GlcNAc) with Endo-M. The resulting fluorescent-oligosaccharides were separated by CE and detected by TOF-MS. Disialo-Asn was successfully identified by the proposed procedure. Application to oligosaccharides in ovalbumin was also described in this communication.

The important roles of carbohydrate chains in glycoproteins in biological processes have recently been recognized. Information on the correlation of the carbohydrate structure to biological function is significantly increasing. Both the complete elucidation of the carbohydrate structure and the carbohydrate distribution in the peptide core are very important for the total understanding of the correlation. Much research has already been carried out for the resolution of carbohydrate structures. High-performance liquid chromatography (HPLC) is an attractive tool for the analysis of carbohydrate chains.[1–4] Although the separation of carbohydrate chains is possible by capillary electrophoresis (CE) and capillary electrochromatography (CEC), such research is still minimal.[5–7] Since HPLC itself does not provide information about the structures, the elucidation of the structures is usually performed by comparison with reference carbohydrates whose structures have been established by other means. In general, carbohydrates do have not effective absorption and fluorescence for sensitive detection. Furthermore, the available amounts in most analyses are minute, and hence, the direct resolution of carbohydrate chains in real samples is fairly difficult. Thus, derivatization is a key step in the HPLC analysis of carbohydrate chains. Various tagging reagents, *e.g.*, 2-aminopyridine (AP),[8–10] 4-aminobenzoic acid ethyl ester (ABEE)[11] and 1-phenyl-3-methyl-5-pyrazolone (PMP),[12] have been proposed for the precolumn derivatization of mono- and oligosaccharides. A number of methods utilizing these reagents have been reported for the precolumn derivatization of carbohydrates. Among them, the AP method has been accepted as a standard method for the resolution of oligosaccharide sequencing. All of these derivatizations are based upon reductive amination, and essentially require a two-step reaction, *i.e.*, Schiff-base formation and reduction of the base. Furthermore, tedious steps are necessary for the purification of the labeled oligosaccharides.[13] Since sialic acid residues are easily hydrolyzed under acidic conditions, the derivatization requires long reaction periods at low temperature.[14,15] In spite of such limitations, the AP method is still used for the resolution of carbohydrate chains, because no reliable method has been reported until now. However, the drawback should be avoidable by enzyme labeling.

Endo-β-*N*-acetylglucosaminidase (Endo-M), found in the culture fluid of *Mucor heimalis*, cleaves not only the high-mannose type and hybrid type of asparagine-linked oligosaccharides, but also the complex type of biantennary oligosaccharides by hydrolysis of the diacetylchitobiose core of the oligosaccharide chain in the glycoproteins.[16–18] Endo-M could transfer the intact complex oligosaccharide in the glycopeptide to suitable acceptors during hydrolysis of the glycopeptide. The oligosaccharide transferring activity can be useful for the remodeling of carbohydrate chains, and thus various oligosaccharides could be synthesized by the enzyme reaction.[19] The transglycosylation reaction with Endo-M seems to be applicable not only for the enzyme synthesis of oligosaccharides, but also for the resolution of carbohydrate chains.

In this communication, we describe the enzyme labeling of glycopeptides using the transglycosylation activity of Endo-M and the resolution by CE-TOF-MS. The resolution of the asparagine-type oligosaccharide in ovalbumin by the proposed CE-MS method was also attempted.

Endo-M possesses not only hydrolysis activity toward the glycosidic bond in the *N*,*N*′-diacetylchitobiose moiety of the *N*-linked oligosaccharides in glycopeptides, but also transglycosylation activity to transfer both the complex-type and high mannose-type oligosaccharides of the *N*-linked sugar chains from the glycopeptides to suitable acceptors having an *N*-acetylglucosamine (GlcNAc) residue. The transglycosylation activity is important for the addition of an oligosacchride to an acceptor. Using the transglycosylation activity of Endo-M, a few oligopeptides were enzymatically synthesized from the peptides having an *N*-acetylglucosaminyl-asparagine or -glutamine residue. The transglycosylation activity seems to be useful for the resolution of carbohydrate chains in the oligopeptides using a suitable acceptor having an *N*-acetylglucosamine residue, because the fluorescent oligosaccharides are easily obtained from the oligopeptides by the enzyme reaction of Endo-M in the presence of the fluorescent-GlcNAc residue. Based on this strategy, several fluorescent-Asn-GlcNAcs (*i.e.*, DBD-Asn-GlcNAc, NBD-Asn-GlcNAc, NDA-Asn-GlcNAc, PS-Asn-GlcNAc, FITC-Asn-GlcNAc, DMEQ-Asn-GlcNAc and DBD-PZ-Boc-Asn-GlcNAc) were synthesized as the acceptor, and the transglycosylation efficiency of the fluorescent-Asn-GlcNAc using Endo-M was studied in our laboratory.

The synthesized acceptors showed a high fluorescence intensity, although the degree was different for each acceptor. The maximal

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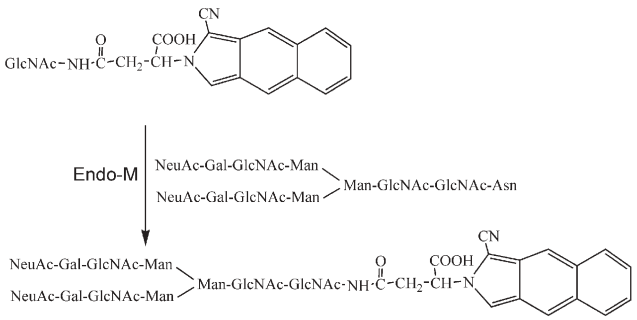

Fig. 1 Transglycosylation reaction between Disialo-Asn (a donor) and NDA-Asn-GlcNAc (an acceptor) with Endo-M.

wavelengths of excitation and emission were based upon the fluorescent moiety of the tagging reagents. Thus, the selective detection of oligosaccharides must be possible after the enzyme reaction of real samples.

Initially, the transglycosylation reaction of Disialo-Asn (a donor) to NDA-Asn-GlcNAc (an acceptor) with Endo-M was studied. The Disialo-Asn was immediately transferred to NDA-Asn-GlcNAc in phosphate buffer (pH 6.0) (Fig. 1), and the amounts of the transglycosylation product reached a maximum at approximate 30 min. Similar results were also obtained from the other fluorescent acceptors. Thus, the resolution of the oligosaccharides with NDA-Asn-GlcNAc was only described in this paper. The enzyme reaction solution was separated by CE and detected with TOF-MS. Fig. 2 shows the total ion chromatogram (TIC) and MS spectrum, obtained from the reaction of Disialo-Asn and NDA-Asn-GlcNAc. The Disialo-Asn-NDA derived from the transglycosylation reaction appeared at approximate 20 min. The peaks of the fluorescent acceptor (NDA-Asn-GlcNAc) and unreacted Disialo-Asn migrated next to Disialo-Asn-NDA. Judging from the MS spectrum (Fig. 2(B)), Disialo-Asn-NDA was detected as a bivalent ion, $[M + 2H]^{2+}$, because 0.5 mass unit different peaks appeared at before and after the *m*/*z* 1257.6 peak. The MS data suggested that Disialo-Asn was completely assigned by the proposed CE-MS method combined with the enzyme reaction. These results encouraged us to try a real sample analysis. Thus, the CE-TOF-MS method after enzyme labeling with Endo-M was applied to the resolution of the asparagine-type oligosaccharides, obtained from the pronase treatment of the 43 kDa glycoprotein of hen egg yolk. Fig. 3 shows the TIC of the reaction solution of the asparagine-type oligosaccharides in ovalbumin by CE-TOF-MS. NDA-Asn-GlcNAc and the unreacted oligosaccharides appeared at 21.5 and 30.5 min, respectively. The peak at 24.5 min, which showed *m*/*z* 1930.5 as a monovalent ion, seemed to be $(Man)_5(GlcNAc)_4$-Asn-NDA. Although the other oligosaccharides might be included in the peaks at around 34–38 min on the CE chromatogram, they could not be currently identified. However, all the structures of the asparagine-type oligosaccharides will be resolved by improvement of the CE separation conditions.

In the present research, we have described a rapid method for the resolution of *N*-glycans in a glycopeptide using a fluorescent acceptor and Endo-M by CE-TOF-MS. The proposed one-pot reaction method is simple and reliable for the resolution of asparagine-type oligosaccharides. Since the proposed method does

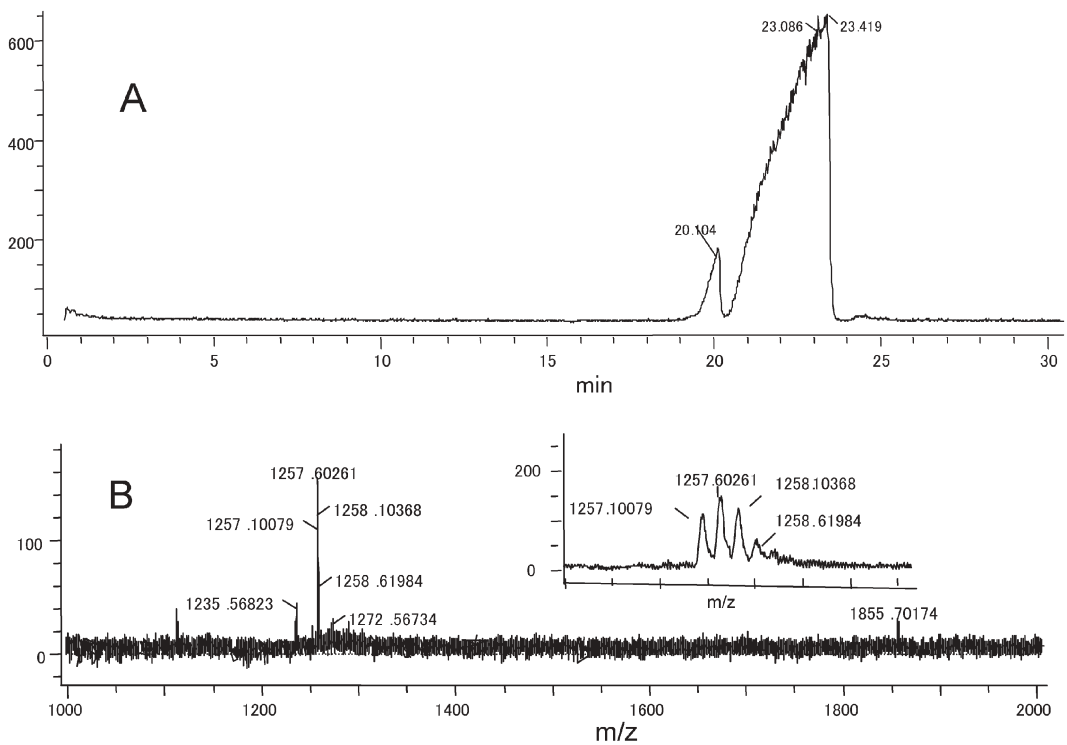

Fig. 2 CE-TOF-MS of the reaction solution obtained from the transglycosylation reaction of Disialo-Asn and NDA-Asn-GlcNAc using Endo-M. (A) TIC, (B) MS spectrum of peak at 20 min. CE conditions: instrument, Hewlett-Packard ^{3D}CE; capillary, fused silica (75 cm × 50 μm); eluent: 50 mM ammonium acetate (pH 4.5); Electrode: inlet (−); voltage: 20 kV; pressure, 10 mbar. MS conditions: instrument, JEOL JMS-T100LC AccuTOF; ion mode, ESI+; capillary voltage, 3500 V; nebulizer gas, 0.75 bar; sheath solution, 2% acetic acid–MeOH (1:1), flow rate, 8 μL min^{-1}; detector, 2600 V.

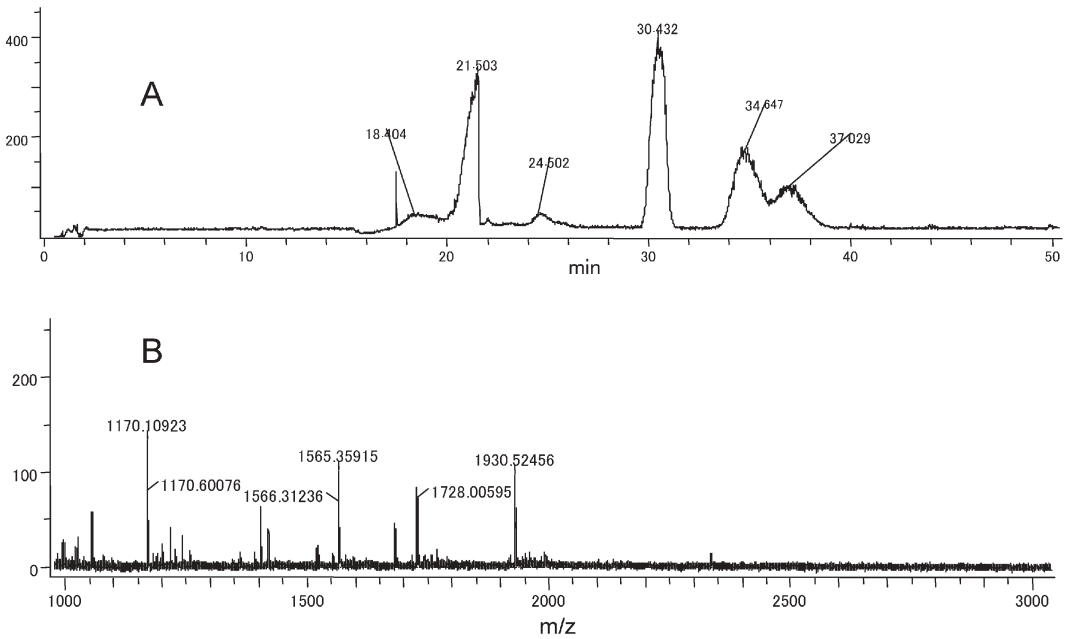

Fig. 3 CE-TOF-MS of the transglycosylation reaction solution of asparagine-type oligosaccharides in ovalbumin. (A) TIC, (B) MS spectrum of peak at 24.5 min. The CE and MS conditions are the same as those in Fig. 2.

not include a chemical reaction, but enzyme derivatization, the possible degradation of unstable residues, such as sialic acid is avoidable. Furthermore, the original oligosaccharide structure is maintained after the derivatization. These characteristics seem to be important advantages for the resolution of the oligosaccharide structure. All the structures of the asparagine-type oligosaccharides in ovalbumin could not be assigned until now. However, the structures will be resolved by improvement of the CE-MS conditions. The improvement of the separation and detection conditions of CE-MS are accompanied by optimization of the transglycosylation reaction conditions. MS detection after separation by CE is only described in this paper. Since the oligosaccharides, derived from the enzyme transglycosylation reaction, fluoresce, the resulting oligosaccharides are selectively identified from the other nonfluorescent substances. The resolution of the oligosaccharides by two-dimensional LC combined with FL and MS detectors are currently being investigated, and the results will be reported elsewhere in the near future.

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