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Characterization of saccharide using high fluorescent 5-(((2-(carbohydrazino)methyl)thio)acetyl)-aminofluorescein tag by Capillary-HPLC-LIF and MALDI-TOF-MS

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ABSTRACT

The new approach to one-step derivatization of saccharide with 5-(((2-(carbohydrazino)methyl)thio) acetyl)-aminofluorescein (C356) was described. In this approach, high fluorescent C356 was applied to label saccharide to enhance the response of derivative saccharide and high sensitive capillary high performance liquid chromatography with laser-induced fluorescence (Capillary-HPLC-LIF) associated with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to characterize C356 labeled saccharide. The effect of derivatization conditions was evaluated and discussed. The limit of detection (LOD) of neutral saccharide in our method attained the level of femtomolar. As a result, this method could be successfully applied to determine the structure of N-glycans of glycoprotein.

1. Introduction

Glycosylation is one of the most important post-translational modifications of proteins found in nature. Glycoproteins are well known to exhibit multiple biological functions in living organisms (molecular recognition, protein folding, fertilization, canceration and immune response, etc.) [1,2]. Several researches have reported the close relationship between the disease states and unusual forms of glycosylation [3–5]. Consequently, the analysis of saccharides in glycoprotein poses significant analytical challenges, because of their features of the structural complexity, limited quantities and diversity.

Due to the lack of chromophores in saccharide, derivatization of it with fluorescent reagent was usually applied to improve both the detection and the separation selectivity in HPLC [6,7]. Numerous labeling reagents such as 2-aminopyridine (2-AP) [8,9], 2-aminobenzamide (2-AB) [10], 2-aminobenzoic acid (2-AA) [11,12] and many others have been used for the derivatization of saccharide based on the reaction of reductive amination [13]. This kind of labeling reaction usually needs two steps. Firstly, Schiff base was formed from the aldehyde of reducing carbohydrates and amino group of labeling reagent. Secondly, the obtained Schiff base was reduced to a stable amine to increase the stability of labeled product. But, compared with amino group, hydrazide group in labeling reaction had a higher reactivity caused by the α-effect [14]. So, the derivative reaction could be carried out in only one step. The tags containing hydrazide group were thus widely used in labeling saccharide [15,16].

Many fluorescent labeling reagents have been used in the labeling reaction, including aminopyrene-3,6,8-trisulfonate (APTS) [17], 5-aminofluorescein [18], and so on. But some of them were not suitable for analysis in HPLC due to their own property or complicated labeling reaction. Therefore, the development of the derivative method applied to HPLC fluorescence detection using the suitable fluorescent reagent has an urgent need for improving the sensitivity of the analysis of saccharide.

High performance liquid chromatography (HPLC) was commonly used in the analysis of fluorescently labeled glycans for oligosaccharide mapping and quantification of glycans. But, compared with traditional HPLC, the Capillary-HPLC-LIF system has advantages of low sample quantity, high resolution and high sensitivity. So it was well suited for the micro-analysis of saccharide.

In order to develop suitable labeling reagents for the analysis of saccharides by Capillary-HPLC-LIF, high fluorescent C356 carried both high sensitive fluorescent group and high reactive hydrazide group came to be of great interest [19]. Importantly, the fluorescent property of high fluorescent C356 was very suitable for LIF detection. According to the reaction between aldehyde of reducing carbohydrates and hydrazide group of C356, the hydrazone linkage was formed with the covalent bond (in Scheme 1). Due to the hydrazone linkage was stable in most cases, C356 was used in the direct derivatization of saccharide in only one-step reaction.

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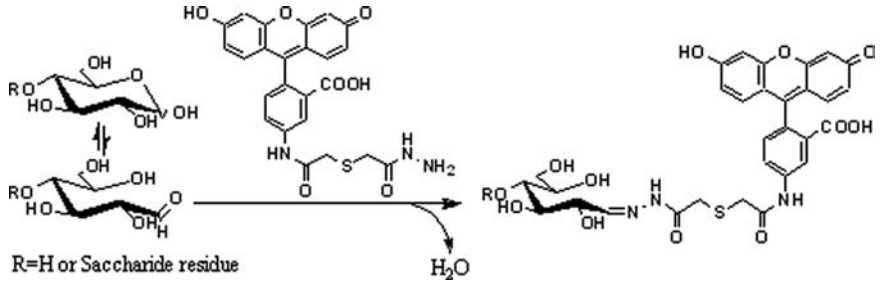


Scheme 1. Derivatization reaction of saccharide with C356.

In this research, using high fluorescent C356, the derivative saccharides were separated by high sensitive Capillary-HPLC-LIF and characterized by MALDI-TOF-MS. As a result, the micro-analysis method for trace amounts of saccharide was developed and then successfully used in the analysis of oligosaccharides in glycoprotein.

2. Experimental

2.1. Chemicals and reagents

Fused-silica capillaries (250 μm I.D. × 375 μm O.D.) were purchased from Yongnian Optical Fiber Factory (Hebei, China). Spherical silica gel (5 μm, 300 Å) was purchased from Suzhou nanomicro technology Co., Ltd (Suzhou, China). Packing material of C18 particles (Kromasil, 5 μm, 300 Å) was obtained from Eka Nobel (Switzerland). Methyltriethoxysilane (MTES), Ribonuclease B (RNase B), 2,5-dihydroxybenzoic acid (DHB), peptide N-glycosidase F (PNGase F) and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO). Maltoheptaose and 5-(((2-(carbohydrazino) methyl)thio)acetyl)-aminofluorescein (C356) were purchased from Supelco (USA) and Invitrogeno (USA) respectively. D-mannose was purchased from Sinopharm chemical reagent Co., Ltd (Shanghai, China). HPLC grade acetonitrile (ACN) was purchased from Dikma (Dikma Technologies Inc, USA). Water was purified by a Milli-Q system (Millipore, Molsheim, France). And other chemicals were of analytical-reagent grade.

2.2. Optimization of derivative procedure

The reaction scheme was demonstrated in Scheme 1. 1 nmol of maltoheptaose, 5 μL of 0.01–2.0% aqueous acetic acid, 5 μL of ethanol and 1 μL of the solution of 2 mM–0.1 M C356 in methanol were added into an eppendorf tube. The reaction mixture was maintained at defined temperature between 37 °C and 80 °C for 20–180 min under dark. After cooling to the ambient temperature, the saccharide derivatives were lyophilized and diluted with 50 mM aqueous NH_4HCO_3 followed by mixing vigorously on a vortex mixer. Finally, the C356-labeled maltoheptaose was detected with Capillary-HPLC-LIF.

2.3. Derivatization of the partial acid hydrolysis of maltoheptaose

10 nmol of maltoheptaose was hydrolyzed by 10 μL of 1 mol/L aqueous TFA at 100 °C for 12 min. After the obtained solution was dried by vacuum centrifugation, 5 μL of 1% acetic acid solution, 5 μL of ethanol and 1 μL of the solution of 1 M C356 in methanol were added. The reaction was performed for 1 h at 70 °C under dark. After cooling to ambient temperature, the solution of C356-labeled hydrolyzed saccharides was diluted to $1{:}4.0 \times 10^5$ with 50 mM aqueous NH_4HCO_3 followed by detection.

2.4. Analysis of N-glycans

2.4.1. Preparation of micro C18 SPE

The micro C18 SPE was home-made by employing a slurry-packing procedure as previously described [20,21]. Briefly, the on-column frit was fabricated in 250 μm I.D. capillary by sol–gel technology. Then, 5 μm C18 particles slurry was driven by pressure and packed into the capillary to form 15 mm column bed.

2.4.2. Preparation of free N-glycans from glycoprotein

Free N-glycans were released from 15 μL of 2 μg/μL aqueous RNase B (1.97 nmol) by digestion with 5 μL of 500 units/mL peptide N-glycosidase F in 15 μL of 2 mM PBS at 37 °C overnight. After cooling to ambient temperature, the digested solution was neutralized with 1% TFA solution and then treated by micro C18 SPE. Micro C18 SPE was initialized by drawing with 50 μL of 100% ACN and 50 μL of 0.1%TFA solution. Then the resulting solution was pumped slowly through the micro C18 SPE to collect the eluate. After that, the free N-glycans loading on SPE column bed were eluted by 10 μL of 0.1% TFA solution and combined with the former collecting solution. Finally, the resulting solution was dried followed by adding 20 μL H_2O.

2.4.3. Derivatization of free N-glycans

The solution of N-glycans was prepared from RNase B according to the above method. 3 μL of N-glycans solution was labeled with C356 by adding 3 μL of 1% aqueous acetic acid, 3 μL of ethanol and 0.2 μL of the solution of 10 mM C356 in methanol. The reaction was performed for 1 h at 70 °C under dark. After cooling to ambient temperature, the solution of C356-labeled hydrolyzed saccharides was dried and diluted with 10 μL of 50 mM aqueous NH_4HCO_3 followed by detection.

2.5. Capillary-HPLC-LIF analysis

The Capillary-HPLC-LIF included a pump system, a 6 port injection valve with a 300 nL loop, a home-made normal column of 25 cm × 250 μm I.D. packed with 5 μm amide-80 particles, and a home-made DPSS laser-induced fluorescence detector as reported before [22]. Fluorescence was monitored at an emission wavelength of 520 nm by an excitation with a solid blue laser at 473 nm (20 mW).

The Shimadzu LC-10Ai pumping system was used for mobile phase delivery. Binary solvents of A (5% ACN) and B (90% ACN) were used as the elution. The flow rate was adjusted to 1.8 μL/min by splitting.

Gradient elution for maltoheptaose labeled with C356 was as follows: 100% B maintained for 20 min, 10 min linear gradient from 100% to 60% B, maintained for 5 min, and back to 100% B in 2 min, maintained for 10 min.

Gradient elution for D-mannose labeled with C356 was as follows: 4 min linear gradient from 100% to 96% B, 16 min linear gradient from 96% to 89% B, then back to 100% B in 2 min.

Gradient elution for C356-labeled saccharides after hydrolysis of the maltoheptaose was as follows: 4 min linear gradient from 100% to 96% B, 14 min linear gradient from 96% to 89% B, then 12 min linear gradient from 89% to 83% B, 17 min linear gradient from 83% to 78% B, 5 min linear gradient from 78% to 70% B, and back to 100% B in 2 min.

Gradient elution for free N-glycans labeled with C356 was as follows: 100% B maintained for 10 min, 15 min linear gradient from 100% to 95% B, then 20 min linear gradient from 95% to 90% B, 10 min linear gradient from 90% to 88% B, 10 min linear gradient from 88% to 80% B, then 10 min linear gradient from 80% to 60% B, and back to 100% B in 2 min.

2.6. MALDI-TOF-MS analysis

Positive ion MALDI-TOF mass spectrometry spectra was acquired on AB SCIEX TOF/TOF 5800 (Applied Biosystems). The sample was excited using Nd: YAG laser (355 nm) operated at a repetition rate of 400 Hz and acceleration voltage of 20 kV. Before identifying the samples, the MS instrument was calibrated with tryptic peptides of myoglobin.

As far as sample preparation was concerned, DHB (the solution of 12.5 mg/mL 2,5-dihydroxybenzoic acid in 20% aqueous acetonitrile and 0.1% aqueous TFA) was used as the matrix. 0.5 μL of analyte solution was deposited on the stainless steel target plate and allowed to dry. Then, 0.5 μL of the matrix solution was used to cover the sample solution on the target plate and allowed to dry.

3. Results and discussion

3.1. Optimization of derivatization conditions

Reduced saccharides could be labeled with C356 in one-step reaction, and the derivatization conditions were optimized, including concentration of acetic acid, reaction temperature, reaction time and molar ratio of C356 to saccharide. Maltoheptaose was used as model saccharide in the optimization of derivatization conditions.

Firstly, the optimized concentration of acetic acid was confirmed. As shown in Fig. 1a, the strongest fluorescent signal was observed when the concentration of acetic acid was 1.00%. Subsequently, from Fig. 1b, it was shown that the fluorescent signal was increased with the increase of the reaction temperature between 37 °C and 70 °C and the strongest fluorescent signal was achieved at 70 °C. Then the reaction time was discussed. As observed in Fig. 1c, it was found that the reaction time of 60 min was enough for derivative reaction and increasing the reaction time could not further enhance the fluorescent signal. Finally, the molar ratio of C356 to saccharide was researched. According to Fig. 1d,

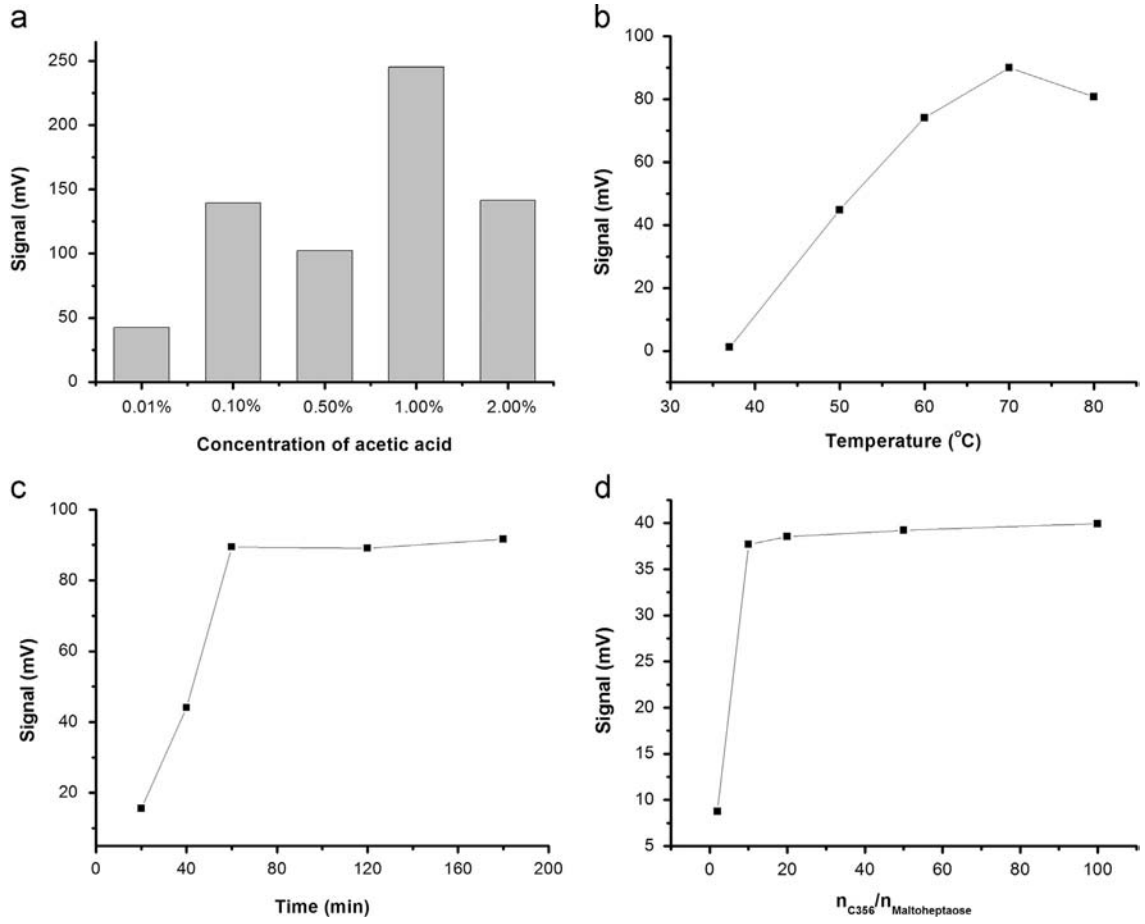


Fig. 1. Effect of the concentration of acetic acid, reaction temperature, reaction time and molar ratio of C356 to maltoheptaose on derivatization of maltoheptaose with C356. Derivatization conditions: (a) 1 μL of 1 mM maltoheptaose was reacted with 1 μL of the solution of 0.1 M C356 in methanol in 5 μL of ethanol and 5 μL of the concentration of aqueous acetic acid between 0.01% and 2.0% at 70 °C for 180 min under dark; (b) 1 μL of 1 mM maltoheptaose was reacted with 1 μL of the solution of 0.1 M C356 in methanol in 5 μL of ethanol and 5 μL of 1% aqueous acetic acid at the temperature between 37 °C and 80 °C for 180 min under dark; (c) 1 μL of 1 mM maltoheptaose was reacted with 1 μL of the solution of 0.1 M C356 in methanol in 5 μL of ethanol and 5 μL of 1% aqueous acetic acid at 70 °C for the time between 20 min and 180 min under dark; (d) 1 μL of 1 mM maltoheptaose was reacted with 1 μL of the solution of 2 mM–0.1 M C356 in methanol in 5 μL of ethanol and 5 μL of 1% aqueous acetic acid at 70 °C for 60 min under dark.

the optimal fluorescent signal was obtained when the molar ratio of C356 to saccharide was 10:1 and the fluorescent signal was almost same with the increase of the molar ratio of C356 to saccharide between 10:1 and 100:1.

3.2. Limitation of C356 labeled maltoheptaose detected by Capillary-HPLC-LIF

After labeling with high fluorescent C356, saccharide could be detected by high sensitive Capillary-HPLC-LIF. In order to evaluate the sensitivity of C356-labeled oligosaccharide, the LOD for the derivatizated oligosaccharide in Capillary-HPLC-LIF was determined by two ways. There were one way to determine the lowest concentration of the C356-labeled oligosaccharide detected by the fluorescent detector and the other way to determine the lowest oligosaccharide concentration that could be labeled satisfactorily with C356 [23].

The LOD of C356-labeled maltoheptaose in Capillary-HPLC-LIF was evaluated. 1.17×10^{-4} M aqueous maltoheptaose was derivatized and then diluted to different orders of magnitude of the concentration with 50 mM aqueous NH_4HCO_3 followed by analysis by Capillary-HPLC-LIF. Good linearity was observed in the range of orders of magnitude of the concentration of C356-labeled maltoheptaose between 10^{-4} M and 10^{-7} M. The R^2 value from a linear fit of the data was 0.996; the slope of the linear regression was 0.933. The LOD estimated at signal-to-noise (S/N) ratio = 3 was found to be 9.7 fmol for maltoheptaose.

The LOD of the lower oligosaccharide concentration that could be derivatized satisfactorily with C356 was also determined by Capillary-HPLC-LIF. When the concentration of maltoheptaose was diluted to 2.5×10^{-7} M, the fluorescent signal could be observed by Capillary-HPLC-LIF after maltoheptaose was labeled by C356, as shown in Fig. 2. So 15 fmol of maltoheptaose still could be detected with the high sensitive laser-induced fluorescence detection. And, by the same method, when the concentration of D-mannose was diluted to 2.0×10^{-7} M, 12 fmol of D-mannose could also be detected, as shown in Fig. 3.

3.3. Analysis of the partial acid hydrolysate of maltoheptaose by Capillary-HPLC-LIF and MALDI-TOF-MS

In order to study the C356 derivatization on saccharide having different number of monosaccharides, the method of mild acid

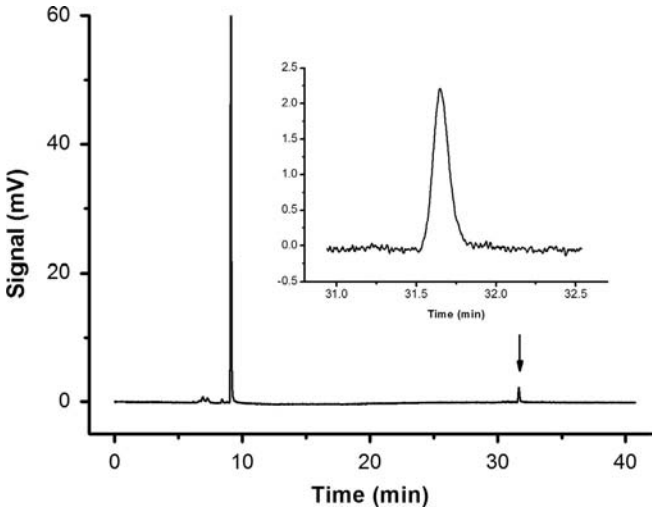


Fig. 2. Chromatogram of C356-labeled maltoheptaose at low concentration. Arrow indicated C356 labeled maltoheptaose. 1 μL of 2.5×10^{-7} M aqueous maltoheptaose was reacted with 1 μL of the solution of 1 mM C356 in methanol in 5 μL of ethanol and 5 μL of 1% aqueous acetic acid at 70 °C for 60 min in the dark; sample was dried and then diluted with 5 μL of 50 mM aqueous NH_4HCO_3 prior to injection.

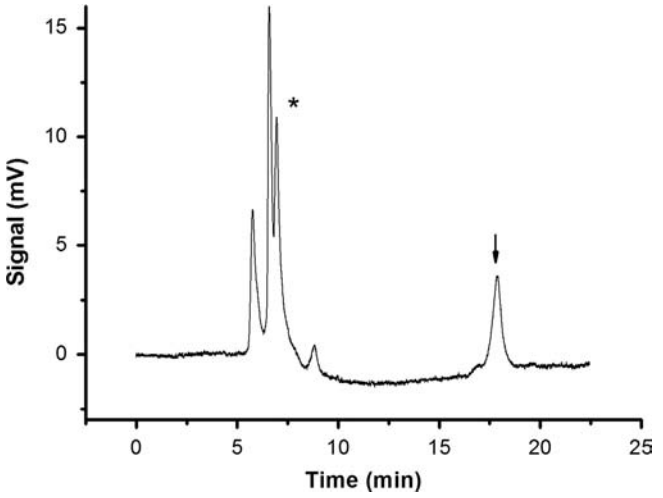


Fig. 3. Chromatogram of C356-labeled D-mannose at low concentration. Peak marked with "*" represents the excess fluorescent reagent. Arrow indicated C356 labeled D-mannose. 1 μL of 2.0×10^{-7} M aqueous D-mannose was reacted with 1 μL of the solution of 1 mM C356 in methanol in 5 μL of ethanol and 5 μL of 1% aqueous acetic acid at 70 °C for 60 min in the dark; sample was dried and then diluted with 5 μL of 50 mM aqueous NH_4HCO_3 prior to injection.

hydrolysis of saccharide was used to generate a ladder of saccharides. Maltoheptaose was used as a model herein. Under the optimum conditions of glycosyl partial hydrolysis, the composed units of maltoheptaose were produced and then labeled with C356. C356 labeled saccharides could be direct analyzed by MALDI-TOF-MS and Capillary-HPLC-LIF without further purification, so that the loss of the prepared glycan sample could be minimized. After partial acid hydrolysis of maltoheptaose, the MALDI-TOF-MS profile of C356-labeled saccharides was given in Fig. 4. The $[M+Na]^+$ ions (m/z 678, m/z 840, m/z 1002, m/z 1164, m/z 1326, m/z 1488 and m/z 1650) for C356 labeled glycans were observed after hydrolysis. So, comparing with the original molecular ion of each saccharide, each partial acid hydrolyzed saccharide could be successfully derivatized with the fluorescent molecule of C356.

From Fig. 5, C356-labeled hydrolyzed maltoheptaose was successfully analyzed by Capillary-HPLC-LIF. Seven peaks of saccharides having different number of glycan units were observed using home-made capillary column. To confirm the compositions of these saccharides, each peak in chromatogram was collected and its structure was identified by MALDI-TOF-MS. The retention time of the C356-labeled saccharides was increased with the increase of the number of monosaccharides on the corresponding saccharide. As a result, the use of Capillary-HPLC-LIF for high-resolution separation of C356-labeled saccharides was achieved in the separation of a ladder of maltooligosaccharides.

3.4. The analysis of N-glycans of glycoprotein by Capillary-HPLC-LIF and MALDI-TOF-MS

In order to elucidate the feasibility of the analysis of glycans of glycoprotein by C356 derivatization and the detection of Capillary-HPLC-LIF and MALDI-TOF-MS, glycoprotein RNase B was used as a model sample. The complete structural characterization of the oligosaccharides from RNase B had been reported [24]. And these oligosaccharide structures were reported as high-mannose type of $Man_{5-9}GlcNAc_2$ [25]. In our approach, PNGase F enzyme was used to release N-glycans from RNase B so as to obtain free oligosaccharides from glycoprotein. After enzymatic digestion, the oligosaccharides were purified by a home-made micro C18 SPE.

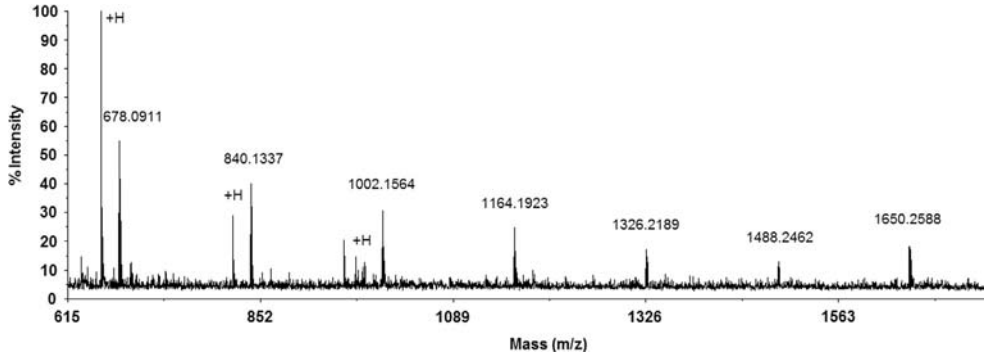


Fig. 4. MALDI-TOF mass spectrum of C356-derivatizated saccharides after hydrolysis of the maltoheptaose.

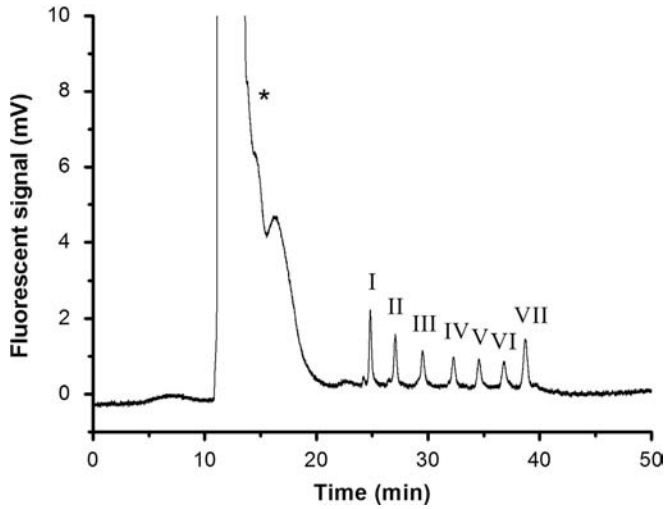


Fig. 5. Chromatogram of C356-derivatizated saccharides after hydrolysis of the maltoheptaose by Capillary-HPLC-LIF. Peak marked with "*" represents the excess fluorescent reagent. Peak I, Hexose-C356; Peak II, $Hexose_2$-C356; Peak III, $Hexose_3$-C356; Peak IV, $Hexose_4$-C356; Peak V, $Hexose_5$-C356; Peak VI, $Hexose_6$-C356; Peak VII, $Hexose_7$-C356.

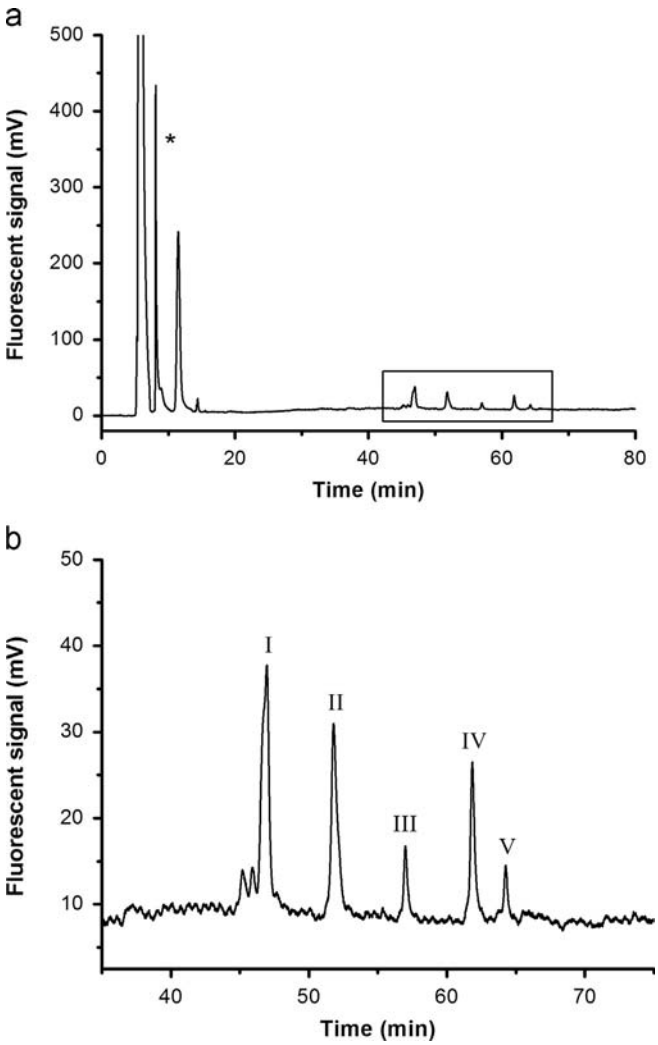


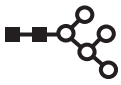
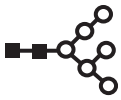
Fig. 6. (a) Analysis of C356-labeled N-glycans of RNase B with Capillary-HPLC-LIF. (b) Zoom of C356-labeled N-glycans region. Peak marked with "*" represents the excess fluorescent reagent.

The collected oligosaccharide solution was labeled with C356 and then directly detected by Capillary-HPLC-LIF and MALDI-TOF-MS. As seen in Fig. 6a, because of C356's weak retention in capillary column, the excess of it was firstly eluted, which made it easily separated from the mixture of C356-labeled saccharides. From Fig. 6b, five structural isomers of $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ from RNase B were separated to baseline resolution by Capillary-HPLC-LIF. And each of separated C356-labeled oligosaccharides was detected by MALDI-TOF-MS to identify their compositions, and the result was shown in Table 1. According to the result of MALDI-TOF-MS, C356-labeled high-mannose type oligosaccharides were eluted in order of their size, $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ with the retention behavior of capillary column. As a result, the oligosaccharides from glycoprotein were characterized successfully according to this method.

4. Conclusions

In this work, a new method to analyze carbohydrate including C356 derivatization and detection by Capillary-HPLC-LIF and MALDI-TOF-MS was realized. This new technique offers a high sensitive analytic approach for neutral carbohydrates in natural oligosaccharide and the LOD attained the level of femtomolar. In addition, this analytical approach seems powerful for the structural characterization of the saccharides in glycoproteins. Further study to apply this method to analyze real sample was in progress now.

Table 1
Identification of N-glycan peaks by MALDI-TOF-MS.

Peak	Name	Obs before $[M+Na]^+$	Obs after $[M+Na]^+$	Structure
I	$Man_5GlcNAc_2$	1257.6108	1732.7815	
II	$Man_6GlcNAc_2$	1419.6879	1894.8485	
III	$Man_7GlcNAc_2$	1581.8181	2057.0293	
IV	$Man_8GlcNAc_2$	1743.8088	2218.9841	
V	$Man_9GlcNAc_2$	1905.9539	2381.1145	

Note: ■, N-acetylglucosamine; ○, Mannose; Obs before, observed molecular weight of glycan before derivatization with C356; Obs after, observed molecular weight of glycan after derivatization with C356.

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