Analysis of N-glycosylation of phospholipase A_2 from venom of individual bees by microbore high-performance liquid chromatography–electrospray mass spectrometry using an ion trap mass spectrometer

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Abstract

The N-linked oligosaccharides were released from the phospholipase A_2 (PLA) with glycopeptidases and reductively aminated with the chromophore, *p*-aminobenzoic acid ethyl ester (ABEE). The ABEE-labeled oligosaccharides were separated by microbore high-performance liquid chromatography (μ-HPLC) using a reversed-phase column and analyzed by electrospray mass spectrometry. Differentiation between α-1,3 and α-1,6 core-fucosylated glycans was achieved by comparison the glycans released by glycopeptidases peptide-*N*-glycanase A (PNase A) and peptide-*N*-glycanase F (PNase F). All N-linked oligosaccharides except 3B and 3C could be identified in this approach. The analysis of PLA oligosaccharides from the venom of individual bees indicated that glycosylation patterns between the younger and the older bees were similar. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Phospholipase A_2 (PLA), the major protein constituent of honeybee venom (12–14% of the dry weight of bee venom) [1], is an allergenic, basic glycoprotein [2] with a molecular mass of 15.7 kDa. The protein consists of 134 amino acids with a single glycosylation site at Asn13 and is cross-linked by five disulfide bridges [3–5]. Numerous biochemical and pharmacological studies have been carried out with PLA [6–9]. Sera of some bee venom-allergic individuals were shown to react with the carbohydrate moiety of PLA [6] and α1,3-fucosylation at the reducing terminal *N*-acetylglucosamine residue of asparagine-bound oligosaccharide was found as a predominant immunogenic carbohydrate determinate of honeybee PLA [7,8]. Epitope-specific T cell tolerance to PLA in bee venom immunotherapy has also been studied [9]. PLA cleaves the sn-2 ester bond in glycerophospholipids and thereby releases free arachidonic acid. The enzymatically oxidized metabolites of arachidonic acid (eicosanoids), which include prostaglandins, thromboxane, prostacyclin and leukotrienes, are a structure diverse family of lipid molecules that possess profound biological

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activities and disease processes such as inflammation [10,11]. Several new drugs were found as inhibitors of secretory PLA in vitro and in vivo, with anti-inflammatory properties in acute and chronic inflammation [12,13].

During the past two decades, the sugar moiety of glycoprotein has been found to be involved in important interactions with the immunological specificity of antigens and to participate in a variety of cellular functions. Different glycoforms of the same glycoprotein can have different biological properties [14,15]. Moreover, knowledge of the distribution of the glycoforms covalently attached to a particular protein recovered from a biological fluid or tissue can be important as this can be diagnostic of a disease process or of exposure to a toxin or drug substance [16]. There are several reports which indicate a contribution of covalently linked carbohydrate to the allergenicity of a glycoprotein PLA allergen [6–8].

Age-related changes of human IgG N-linked oligosaccharides have been investigated [17,18]. These findings may contribute the understanding of autoimmune disease such as rheumatoid arthritis in which glycosylation is involved. Age-related changes in the amount of PLA have been measured in studies of bee venom [19]. PLA activity was presented at low level in the first week to 10 days of a worker bee's life and then increased rapidly, reach a maxima and fall to lower level in very old (more than 6 weeks) worker bees. But there were no report about the age-related changes of PLA glycoforms in individual bee. Different PLA glycoforms may have different biological properties such as allergenicity.

One common practice of analyzing glycoconjugates is to release carbohydrate by chemical or enzymatic methods and then analyze by HPLC [20]. As most glycans lack a suitable chromophore, the most appropriate method for converting these glycans into a derivative with enhanced detector properties is pre-column derivatization. Because underivatized sugars are hydrophilic and have closely related structures, there are limitations in the ways they can differentially interact with the stationary and mobile phases of reverse HPLC. Tagging of sugars with aromatic groups renders glycans hydrophobic and chromophoric, thus permitting the use of reversed-phase HPLC, which enables separation of glycan mixtures [18,21]. Sensitive methodology has involved derivatization of released glycans with chromophores such as *p*-aminobenzoic acid ethyl ester (ABEE) [18] and 2-aminoacridone (2-AMAC) [21] using reductive amination. These labeling reagents allowed complete derivatization of the carbohydrates within 2 h.

Mass spectrometry (MS) has been recognized to be a very powerful technique and has been used successfully for the determination of molecular masses and structural analysis for a variety of carbohydrates. Recently, electrospray ionization has rapidly emerged as a very promising technique for the analysis of oligosaccharides. Unfortunately, because of the low proton affinity and ionizability of free oligosaccharides, the mass sensitivity of free oligosaccharides, especially neutral oligosaccharides, are generally poor. Reductive amination was often used to solve this problem. Reductive amination led to higher proton affinity and ionizability [22–25].

The combination of HPLC with MS has become a powerful tool in the characterization of biomolecules and structural elucidation. One advantage of using MS instead of UV as the detector is that baseline separation may not be needed for a clear identification, and the relative concentration of each compound can be calculated based on their peak areas. HPLC–ESI–MS has been applied to the analysis of oligosaccharides from various glycoproteins [18,26–28].

Recently, We have reported the analysis of the glycosylation pattern in the PLA of individual honeybee based on tryptic digestion, ultrafiltration and CE–MS [29]. Under optimized CE–MS conditions, only eight of 14 glycopeptides were identified. One major goal of this study is to investigate the difference in glycosylation pattern between young and older bees. Therefore, there is a need to develop a method capable of identify all the fourteen different oligosaccharides.

In this report, a method based on ultrafiltration, enzymatic deglycosylation, derivatization, SPE purification, and microbore HPLC/MS was developed for the analysis of glycosylation pattern of glycoproteins. This method is simple, effective and particularly suitable to the detection and assessment the glycosylation pattern of glycoproteins with limited quantities. The potential of this methodology was demonstrated in the analysis of PLA from the venom of individual bees.

2. Experimental

2.1. Chemicals

Phospholipase A_2 (PLA), pepsin, β-mercapto-ethanol, formic acid, maltooligosaccharides (maltose linear homologs G_4 through G_{10}), *p*-aminobenzoic ethyl ester (ABEE), sodium cyanoborohydride, tri-fluoroacetic acid (TFA), glacial acetic acid, sodium carbonate, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Glycopeptidases, peptide-*N*-glycanase A (PNase A) and peptide-*N*-glycanase F (PNase F) were purchased from Seikagaku Kogyo (Tokyo, Japan). HPLC grade methanol and acetonitrile were obtained from Lab-Scan Analytical Science (Labscan, Dublin, Ireland). Deionized (18 MΩ) water (Milli-Q water system; Millipore, Bedford, MA, USA) was used in the preparation of the samples and buffer solution. Prior to use, the buffer solution was filtered through a 0.45-μm membrane filter (Gelman Sciences, Michigan, USA).

2.2. Collection of bee venom sample

Honeybees (*Apis mellifera*) were collected near a hive entrance with an insect net. Sting was removed directly from each individual bee without anesthetizing. Each sting was placed immediately in the deionized water and was removed after vortex for 10 min.

2.3. Ultrafiltration

The Ultrafree-0.5 filter with a MW cut-off 5 kDa membrane (Millipore, Bedford, MA, USA) was used for purifying and desalting of bee venom before enzyme digestion.

2.4. PNase A release of oligosaccharides

PLA (6 nmol) was digested by pepsin with a substrate to enzyme ratio of 30:1 in 0.5% (v/v) formic acid at pH 2.0 for 16 h at 37°C. The pepsin digest was neutralized and heated at 95°C for 10 min. The digest was then placed in an evaporator (Techne, Cambridge, UK) and evaporated to dryness under a stream of dry nitrogen. The residue containing glycopeptides was digested with 0.02 mU of

PNase A in 25 μl of 0.1 *M* citrate–phosphate buffer (pH 5.0) at 37°C for 16 h. Blank without PLA went through the same experimental steps to be a negative control.

2.5. PNase F release of oligosaccharides

The protocol used for the enzymatic cleavage of oligosaccharides from PLA with PNase F were as follows: 2 μl of 2-mercaptoethanol was added into 25 μl of an aqueous solution containing 6 nmol of PLA in a 200-μl polypropylene PCR tube. The reaction mixture was then heated at 100°C for 15 min; after cooling, the solution was diluted with 150 μl of 50 m*M* phosphate buffer (pH 7.2) prior to the addition of 3 μl of PNase F solution (containing 3 Units). The final solution was incubated at 37°C for 16 h.

2.6. Purification of carbohydrates with a graphitized carbon solid-phase extraction (SPE) column [30,31]

Non-porous graphitized carbon black (Carbograph) SPE columns were purchased from Alltech. Carbograph SPE packing is a homogenous, nonporous, graphitized carbon black (GCB) with a surface area of 100 m^2/g and a particle size range of 38–125 μm. Prior to use, the column was washed with 80% (v/v) acetonitrile in 0.1% (v/v) TFA (three column volumes) followed by three column volumes of water. The sample to be desalted and purified is applied to the carbon SPE columns. Salts are washed off with three column volumes of water, while the glycans are adsorbed to the carbon. The glycans were eluted with 1 ml of 25% acetonitrile without elution of protein and peptides. These glycans are evaporated to dryness with evaporator prior to derivatization with ABEE.

2.7. Derivatization of carbohydrates

Typical experiment involved the using of glycans residue from PLA standard or from single bee venom. The glycans were added to 5 μl of 0.7 *M* ABEE solution (dissolved in a 3:7 (v/v) mixture of glacial acetic acid and DMSO). The mixture was agitated for 30 s before the addition of an aliquot (10 μl) of a freshly prepared 1.0 *M* solution of

$NaBH_3CN$ (dissolved in a 3:7 (v/v) mixture of glacial acetic acid and DMSO). The solution was incubated at 80°C for 60 min in the dark.

2.8. Purification of ABEE labeled carbohydrates with Oasis SPE column (Waters) [20]

Oasis extraction cartridges in 1 ml with 30 mg HLB (hydrophilic–lipophilic balance) sorbents (30 μm, 84 Å) were used for purification of ABEE labeled carbohydrates. The Oasis HLB sorbent is a macroporous copolymer made from a balanced ratio of two monomers, the lipophilic divinylbenzene and the hydrophilic N-vinylpyrrolidone. Prior to use, the Oasis SPE column was primed with 2 ml of acetonitrile followed by 2 ml of water. The ABEE-labeled PLA glycan were loaded onto the Oasis SPE column and washed with 1 ml of water. This was followed by 1 ml of 5% acetonitrile, 1 ml of 10% acetonitrile, 1 ml of 15% acetonitrile, 1 ml of 20% acetonitrile and 25% acetonitrile. Each of these fractions was collected and analyzed by nanospray MS. It was found that no ABEE–glycans were eluted during the water wash and 20% acetonitrile would elute all the PLA glycans without eluting the excess ABEE reagent. These derivatized glycans were evaporated to dryness and reconstituted in 20 μl mobile phase for HPLC–MS analysis

2.9. HPLC–UV system

The chromatographic system consisted of two model LC-10AD pumps (Shimadzu, Kyoto, Japan), an Acurate microflow mixer (LC Packings, USA), a Rheodyne model 7125 injection valve (Rheodyne, USA) fitted with a 1-μl loop, and a Rainin Dynamax UV-C UV detector set at 305 nm (Rainin, MA, USA). Chromatograms were recorded on a Pentium-PC using SISC PC Integrator software package (Scientific Information Service Corporation, Taipei, Taiwan).

HPLC analysis was performed on a reversed-phase Symmetry C_{18} column (1 mm I.D.×15 cm; particle size: 3.5 μm; Waters). The mobile phases were deionized water and methanol. The mobile phase was started with 20% methanol and then increased to 38% in 40 min. The flow-rate was set at 40 μl/min.

2.10. Electrospray and mass spectrometry

Mass spectrometry was performed on a Finnigan LCQ quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA, USA). Two different electrospray sources were used: (1) the pneumatically assisted electrospray ionization source for HPLC–MS, and (2) a commercial nanospray ESI source using gold coated pulled glass capillary (Protein Analysis, Odense, Denmark). The capillary was positioned at a distance of <1 mm from the entrance hole of the heated transfer capillary. The glass capillary was filled with 1–3 μl of analyte solution.

The mass spectrometer was operated in the positive ion mode by applying a voltage of 4.5 kV to the ESI needle and 800 V to the nanospray needle. The temperature of the heated capillary was set at 200°C. To avoid space charge effects, the number of ions stored in the trap was regulated by the automatic gain control, which was set at 4×10^7 ions for full scan mode, 2×10^7 for SIM mode, and 1×10^7 for ZoomScan mode. Helium was used as the damping gas at a pressure of 10^{-3} Torr. In selected ion monitoring (SIM) analysis, the maximum ion collection time was 0.08 s for each step and three scans were added for each spectrum.

3. Results and discussion

3.1. Purification and derivatization of the PLA oligosaccharides

PLA exists in a glycosylated and an unglycosylated variant. The glycosylated PLA consists of 14 glycoforms. The structures of the N-glycans from honeybee venom are shown in Table 1. There are four isobaric pairs (2 and 3A, 3B and 3C, 4B and 5A, 8 and 9) in PLA glycoforms. Following pepsin and PNase A treatment, the salts, deglycosylated peptides and proteins (pepsin and PNase A) were removed with a graphitized carbon (Carbograph) SPE columns. The efficiency of the separation was confirmed with the positive ion nanospray mass spectrum (data not shown). The removing of salts and proteins was necessary because the yield of the labeling reaction was poor in the presence of salts, peptides and proteins. After the derivatization, the

Table 1
Structures of *N*-glycans from PLA of honeybee venom [32]

Glycoforms	Glycans
1	Manα1-6 　　　　Manβ1-4GlcNAcβ1-4GlcNAc
2	Fucα1-6 Manα1-6 　　　　Manβ1-4GlcNAcβ1-4GlcNAc
3A	Manα1-6 　　　　Manβ1-4GlcNAcβ1-4GlcNAc 　　　　　　　　Fucα1-3
3B	Manα1-6 　　　　Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3
3C	Manα1-3—Manα1-6 　　　　　　　Manβ1-4GlcNAcβ1-4GlcNAc
4A	Fucα1-6 Manα1-6 　　　　Manβ1-4GlcNAcβ1-4GlcNAc 　　　　　　　　Fucα1-3
4B	Fucα1-6 Manα1-6 　　　　Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3
5A	Manα1-6 　　　　Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3　　　　Fucα1-3
5B	Manα1-3—Manα1-6 　　　　　　　Manβ1-4GlcNAcβ1-4GlcNAc 　　　　　Manα1-3
6	Fucα1-6 Manα1-6 　　　　Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3　　　　Fucα1-3
7	Manα1-6 　　　　　　　　Manβ1-4GlcNAcβ1-4GlcNAc GalNAcβ1-4GlcNAcβ1-2Manα1-3 Fucα1-3
8	Manα1-6　　　　Fucα1-6 　　　　　　　　Manβ1-4GlcNAcβ1-4GlcNAc GalNAcβ1-4GlcNAcβ1-2Manα1-3 Fucα1-3
9	Manα1-6 　　　　　　　　Manβ1-4GlcNAcβ1-4GlcNAc GalNAcβ1-4GlcNAcβ1-2Manα1-3　　　　Fucα1-3 Fucα1-3
10	Manα1-6　　　　Fucα1-6 　　　　　　　　Manβ1-4GlcNAcβ1-4GlcNAc GalNAcβ1-4GlcNAcβ1-2Manα1-3　　　　Fucα1-3 Fucα1-3

labeled glycans were purified using an Oasis SPE column. The conditions and recoveries for loading and eluting free glycans and ABEE-labeled glycans on Carbograph and Oasis SPE columns were investigated in order to establish a cleanup strategy. Since we do not have the authentic PLA glycoform standards, maltooligosaccharides (maltose linear homologs G_4 through G_{10}) were used for method development. The efficiency of the Carbograph SPE technique was about 95%. Good recovery (about 97%) of the Oasis SPE step for variant malto-

oligosaccharides was also obtained. These data were all judged by the LC–MS before and after the SPE columns cleanup. These results suggest that the two SPE methods are suitable for purification of PLA glycoforms.

3.2. *μ-HPLC–UV and μ-HPLC–MS analysis of ABEE-labeled PLA glycans*

In order to differentiate and detect the four isobaric pairs (2 and 3A, 3B and 3C, 4B and 5A, 8 and 9), ABEE glycans were separated by reverse phase μ-HPLC before MS analysis. For better ESI ionization efficiency, deionized water and methanol were selected as the mobile phase in the separation of ABEE–glycans. Under the optimal condition, more than ten peaks were observed (Fig. 1). Since we do not have the authentic PLA glycoform standards, identification of these peaks by retention time was not feasible.

In order to identify the PLA glycoforms, the ABEE–glycans were analyzed by μ-HPLC–ESI–MS and the mass chromatograms were shown in Fig. 2. Peaks detected at 40.81, (28.67, 47.00), 37.87, 32.82, (25.92, 44.81), 36.03, 30.79, 31.62, (20.37, 38.81) and 25.99 min correspond to protonated molecular
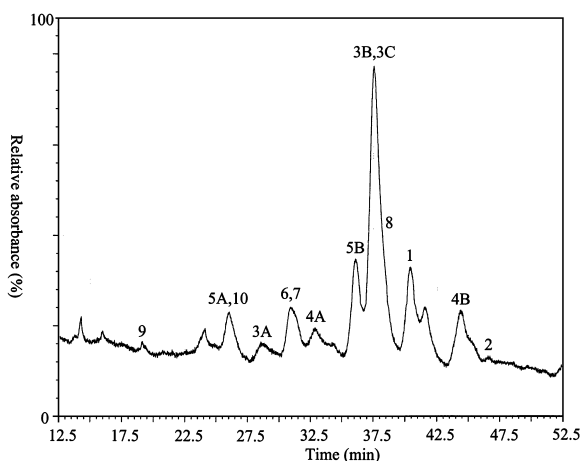

Fig. 1. LC–UV chromatogram of ABEE-labeled oligosaccharides from PLA standard. The mobile phases were deionized water (solvent A) and methanol (solvent B). The mobile phase was started with 20% methanol and then increased to 38% in 40 min. The flow-rate was set at 40 μl/min. The detection wavelength was 305 nm.
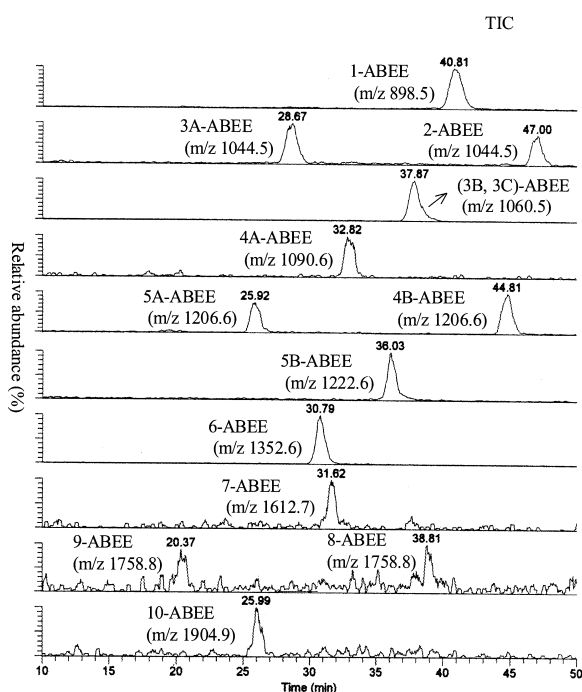
Fig. 2. Mass chromatograms of ABEE-labeled oligosaccharides obtained under selected ion monitoring (SIM) operation. These oligosaccharides were released with PNase A from PLA standard. The chromatographic conditions were the same as in Fig. 1.

ions with m/z values of 898.5, 1044.5, 1060.5, 1090.6, 1206.6, 1222.6, 1352.6, 1612.7, 1758.8 and 1904.9, respectively. Based on the molecular mass, the peaks detected at 40.81, 37.87, 32.82, 36.03, 30.79, 31.62 and 25.99 min were identified as 1-ABEE, 3B/3C-ABEE, 4A-ABEE, 5B-ABEE, 6-ABEE, 7-ABEE and 10-ABEE. The elution order of the glycans appears to be related to their size. One advantage of using MS instead of UV as the detector is that baseline separation may not be needed for a clear identification. For example, 6-ABEE (30.79 min) could be identified although it was partially separated from 7-ABEE (31.62 min) (Fig. 2).

As mentioned earlier, there are four isobaric pairs. These isobaric pairs were different in the core structure with either α-1,3 fucosylation or α-1,6 fucosylation. Among the isobaric pairs, except the isobaric glycans, 3B and 3C (m/z 1060.6 at 37.87 min), the other three isobaric pairs (2 and 3A, 4B and 5A, 8 and 9) were separated completely. Because of the lack of authentic standards, it is not

possible to differentiate these isobaric pairs based on their retention time. The differentiation of the isobaric pairs was achieved based on the different specificity of PNase A and PNase F. It is known that α-1,3 core fucosylation prevents the cleavage of oligosaccharide by PNase F [15]. In contrast, the specificity of PNase A is broader than the PNase F; PNase A is able to cleave oligosaccharides from substrates containing core fucose at C-3 of the *N*-acetylglucosamine (GlcNAc) residue. The mass chromatogram of ABEE–glycans released by PNase F followed by reversed-phase μ-HPLC analysis is shown in Fig. 3. The comparison of oligosaccharides cleavaged by PNase A (Fig. 2) and PNase F (Fig. 3) suggested that α-1,3 core fucosylated glycans had a shorter retention time than the corresponding α-1,6 analogs. Therefore the peaks detected at 20.37, 25.92, 28.67, 38.81, 44.81 and 47.00 min in Fig. 2 were identified as 9-ABEE, 5A-ABEE, 3A-ABEE, 8-ABEE, 4B-ABEE and 2-ABEE, respectively.

The differences in the retention behavior for these isobaric oligosaccharides are most likely resulting
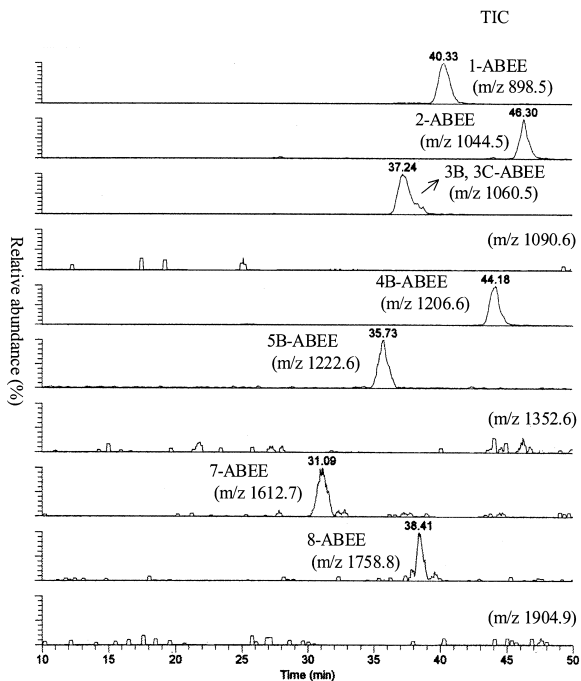

Fig. 3. Mass chromatograms of ABEE-labeled oligosaccharides obtained under SIM operation. These oligosaccharides were released with PNase F from PLA standard. The chromatographic conditions were the same as in Fig. 1.

from the differences in their hydrophobicity. The
degree of the hydrophobic nature in these ABEE-
labeled glycans is reduced when the 3-hydroxy group
of the GlcNAc at the reducing end of the oligo-
saccharide is substituted by a fucose residue, e.g.
3A-ABEE, 5A-ABEE and 9-ABEE. In contrast, the
hydrophobicity of the 1,6 core-fucosylated glycans is
increased and therefore eluted later than the non
core-fucosylated glycans, e.g. 2-ABEE, 4B-ABEE
and 8-ABEE. The retention behavior and hydro-
phobic nature of α-1,3 and 1,6 core-difucosylated
glycans is between the 1–3 and 1–6 mono-
fucosylated glycans, e.g. 4A-ABEE, 6-ABEE and
10-ABEE.

3.3. Analysis of PLA glycans in venom of individual single bees

This technique was used to investigate the PLA
glycoforms of individual honeybee. In addition to
PLA, there are many other components in bee
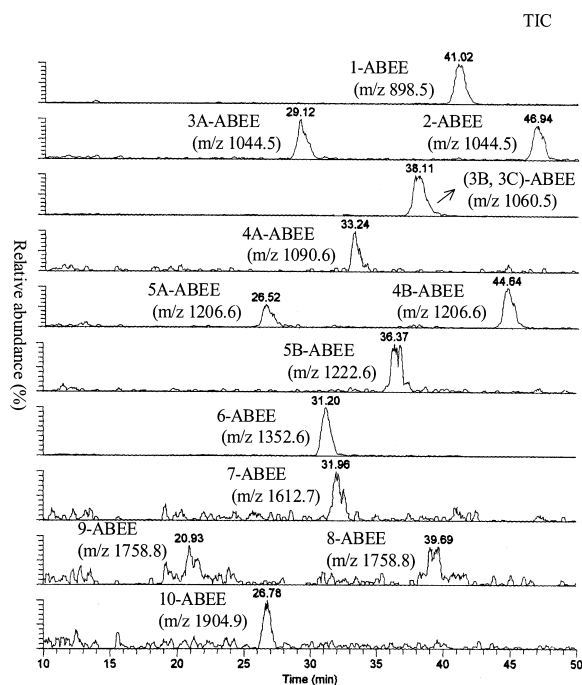venom. To reduce the interference of these com-

Fig. 4. Mass chromatograms of ABEE-labeled oligosaccharides
obtained under SIM operation. These PLA oligosaccharides were
released with PNase A from the venom of a honeybee. The
chromatographic conditions were the same as in Fig. 1.

Table 2
Relative abundance and C.V. ($n=3$) of PLA N-glycans of older (30
days after eclosion) and younger (10 days) honeybees

N-glycans	Relative abundance in PLA (%)		C.V. (%)	
	Older	Younger	Older	Younger
1	13.72	13.91	2.6	2.5
2	3.14	3.75	8.8	9.4
3A	2.92	3.28	7.4	5.2
3B, 3C	45.31	44.22	3.5	2.1
4A	2.47	2.21	9.4	10.2
4B	9.88	9.23	2.5	4.1
5A	3.56	3.04	10.4	6.5
5B	10.38	10.87	4.2	3.6
6	7.31	7.72	6.1	7.2
7	0.83	0.98	10.1	10.4
8	0.39	0.32	9.8	9.5
9	0.31	0.33	11.0	9.4
10	0.23	0.21	12.3	7.9

ponents, PLA in bee venom was separated using a
MW 5-kDa cut-off filter before digestion, derivatiza-
tion and μ-HPLC–MS analysis. The recovery of the
ultrafiltration technique was 94% for PLA standards,
as judged by the LC–MS with and without the
ultrafiltration step. A typical LC–MS chromatogram
obtained from the analysis of glycans from single
bee venom is shown in Fig. 4. The relative abun-
dances and C.V. of each glycoforms for older (30
days after eclosion) and younger (10 days after
eclosion) honeybees were listed in Table 2. The
relative abundances of each glycoforms were similar
for younger and older honeybees. The C.V. values
were below 13%.

4. Conclusion

A method for the analysis of PLA oligosaccha-
rides from the venom of single honeybee was
developed. Several procedures were critical to the
isolation of glycans and ABEE labeled glycans
before HPLC–MS analysis. These steps include
isolation of the PLA with ultrafiltration, enzymatic
deglycosylation and isolation of the glycans with
Carbograph SPE, derivatization and purification of
ABEE-labeled glycans with Oasis SPE column. This
methodology is expected to be suitable in the
analysis of oligosaccharides released from picomole

level of glycoproteins. In previous CE–MS study [25], only eight glycopeptides could be identified under SIM mode. However, 13 glycans could be identified in this HPLC–MS study. The identification of each glycoform was based on the use of HPLC–ESI–MS and glycanases of different specificity. The analysis of ABEE-labeled oligosaccharides from the PLA of individual bees venom indicated that glycosylation patterns of younger and older honeybees were similar.

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