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Analysis of phospholipase A_2 glycosylation patterns from venom of individual bees by capillary electrophoresis/electrospray ionization mass spectrometry using an ion trap mass spectrometer

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A method based on tryptic digestion, ultrafiltration and capillary electrophoresis/mass spectrometry (CE/MS) has been developed for the analysis of the glycosylation pattern in the phospholipase A_2 (PLA) of individual honeybees. Without reducing the disulfide bonds, PLA was digested with trypsin and filtered with a 3 kDa molecular weight (MW) cut-off membrane. With this procedure, the glycopeptides could be isolated from the nonglycosylated peptides. After tryptic digestion and ultrafiltration, the disulfide bonds were reduced before analysis by CE. To reduce the adsorption, CE separation was performed on successive multiple ionic-polymer (SMIL) polybrene (PB) coated capillary columns. The SMIL-PB columns allowed partial separation of the glycopeptides and eight glycopeptides were identified by on-line coupling of CE with electrospray ionization (ESI) mass spectrometry. The analysis of phospholipase A_2 from the venom of individual bees indicated that the variation and relative abundances of different glycopeptides were similar between the younger and the older bees.

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Bee venom contains a complex mixture of biologically active agents, such as peptides, enzymes, biogenic amines, etc.[1,2] The main components of honeybee (*Apis mellifera*) venom are the enzymes phospholipase A_2 (PLA) and hyaluronidase, and the low-molecular-weight proteins melittin and apamine. Bee venom exhibits bacteriostatic and bactericidal properties,[3,4] and a slight direct hemolytic effect.[5]

PLA, the major protein constituent of honeybee venom (12–14% of the dry weight of bee venom),[1] is an allergenic, basic glycoprotein[6] 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.[7–9] Numerous biochemical and pharmacological studies have been carried out with PLA.[10–14]

During the past three decades, the sugar moieties of glycoproteins have been found to be involved in important interactions with the immunological specificity of antigens, and to participate in a variety of cellular functions. There are several reports which indicate a contribution of covalently linked carbohydrate to the allergenicity of a glycoprotein PLA allergen.[11–13,15,16] Since different glycoforms of a given glycoprotein may exhibit different biological properties,[17,18] characterization and monitoring of glycosylation patterns are of growing importance.

Many methods have been developed for the characterization of honeybee PLA. Conventional preparative and analytical methods, such as ion-exchange chromatography, gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), are relatively time consuming and therefore, are often replaced by high-performance liquid chromatography (HPLC).[19–21] Characterization of the oligosaccharide structures on PLA has been reported and fourteen different structures of heterogeneous *N*-glycans from honeybee venom PLA have been elucidated by HPLC in combination with composition analysis, methylation analysis and NMR spectroscopy.[22]

In comparison with HPLC, CE offers extraordinary resolving power and speed of analysis. Proteins and peptides, having chromophores and electric charges under most pH conditions, are appropriate analytes for analysis by CE/UV and CE/MS. On major problem in the application of CE to proteins and peptides is their inherent tendency to adsorption on capillary walls owing to the electrostatic attractive forces between the silanol groups on the wall surface and the positively charged protein molecules.[23,24] Among the approaches used for wall modification, capillaries modified by polybrene (PB) using successive multiple ionic-polymer (SMIL) coating[25] have proved to be stable and efficient for the separation of proteins.

CE has been applied previously to the analysis of honeybee venom.[26,27] These reports mainly focused on the analysis of the major component melittin, and the separation of melittin and PLA in bee venom was the goal of these studies. There was no report on the separation and analysis of the different glycoforms of PLA.

Because of its low detection limit, high specificity and, more importantly, ability to provide abundant structural information, ESI mass spectrometry (MS) and related techniques have been considered one of the ideal devices

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for analysis of proteins and peptides. One advantage of using MS instead of UV as the detector is that baseline separation may not be needed for a clear identification.

CE/UV and CE/MS were used to investigate the glycosylation patterns of PLA. The objective of this work was to develop an analytical technique that permited the glycosylation patterns in the PLA of individual bees to be compared. The results of the analysis, and the merits and pitfalls of these techniques, are discussed.

EXPERIMENTAL

Chemicals

Polybrene (PB) and dextran sulfate (Sigma, St. Louis, MO, USA) were used as SMIL coating reagents. Melittin, phospholipase A_2 (PLA), trypsin, dithiothreitol (DTT), cetyltrimethylammonium bromide (CTAB), β-cyclodextran (β-CD) and formic acid were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol was obtained from LAB-SCAN Analytical Science (Labscan Ltd. Dublin, Ireland). Ammonium acetate was purchased from J. T. Baker (Phillipsburg, NJ, USA). Deionized (18 MΩ) water (Milli-Q water system, Millipore Inc., 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).

Collection of bee venom sample

Honeybees (*Apis mellifera*) were collected near a hive entrance with an insect net. The sting was removed directly from each individual bee without anesthetizing. Each sting was placed immediately in buffer (50 mM NH_4OAc, pH 7.2, 200 μL) and was removed after vortexing for 10 min.

Ultrafiltration

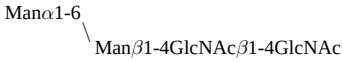
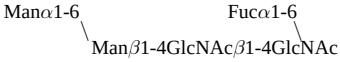
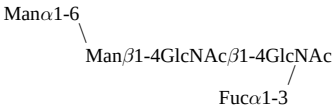
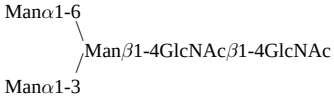
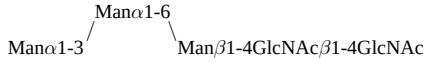
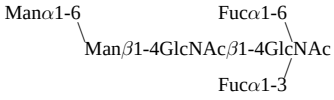
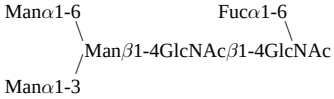
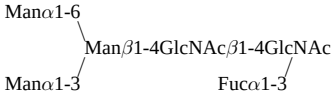
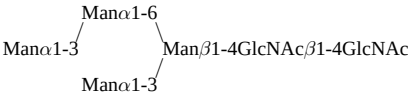
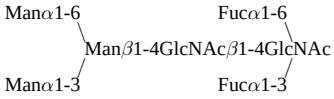
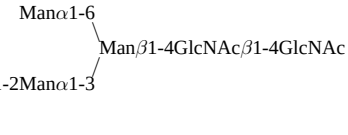
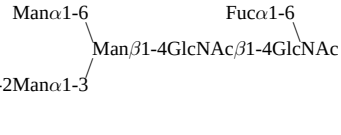
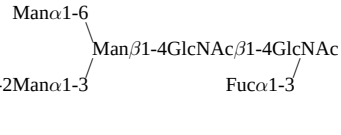
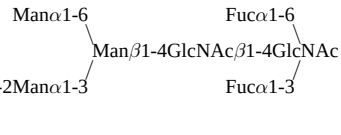
Two ultrafiltration filters were used for desalting, buffer exchange, and purification of PLA or its digests. The Ultrafree-0.5 filter with a 5 kDa MW cut-off membrane (Millipore Corporation, Bedford, MA) was used to purify and desalt bee venom before enzyme digestion. The Microcon-0.3 microconcentrator with a MW cut-off of 3 kDa (Millipore Corporation, Bedford, MA) was used to purify and concentrate the digests, and for buffer exchange before reducing the disulfide bonds.

Preparation of PLA and digests

For the nanospray analysis of intact PLA, PLA was dissolved in a water/methanol/formic acid (49:49:2 v/v/v) solution at a concentration of 1 mg/mL. For the digestion experiments, PLA was digested by trypsin with a substrate to enzyme ratio of 30:1 in 50 mM NH_4OAc buffer solution at pH 7.2 for 3 h at 37 °C. For the single bee venom analysis, the matrix plus the low MW proteins (melittin and apamine) in the bee venom solution were removed with an Ultrafree-0.5 filter, before digestion.

Concentration and buffer exchange (50 mM NH_4OAc, pH 7.2 to 8.0) were achieved with a Microcon-0.3 filter. The reduction of the digests was carried out by adding DTT to the digests at a 100:1 molar ratio (DTT/PLA) and incubating the mixture at 37 °C for 1 h. All ultrafiltration procedures were processed with a desktop centrifuge (HERMLE Z 231 M, BHG, F. R., Germany).

Table 1. Structures of *N*-glycans from PLA of honeybee venom[22]

Glycoforms	Glycans
1	
2	
3A	
3B	
3C	
4A	
4B	
5A	
5B	
6	
7	
8	
9	
10	

CE/UV system

The CE system was constructed in-house and has been

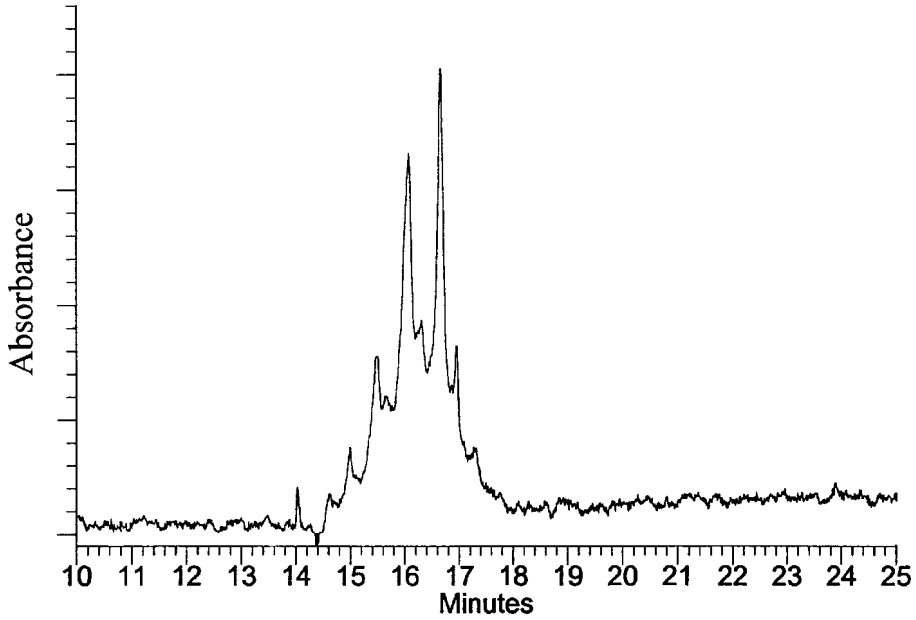


Figure 1. CE/UV electrophorogram of intact PLA standard. Electrophoresis was carried out with a 1.1 m × 50 μm i.d. (1 m effective length) SMIL coated capillary in the presence of 10 mM β-CD (pH 6.7) in 50 mM ammonium acetate. The potential drop was −15 kV and the detection wavelength was 205 nm.

described elsewhere.[28] CE columns were fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA), 50 μm i.d., 375 μm o.d. and 1 m length (0.9 m to detector). A small area of the polyimide coating was burned off to form a window for UV detection. On-column detection was performed with a UV detector (UV-C Rainin, Emeryville, CA, USA) operated at 205 nm. The SMIL coating procedure was based on a literature method.[25] The capillary column was rinsed with 1 M NaOH for 15 min, 0.1 M NaOH for 15 min, and then deionized water for 15 min. After this preconditioning, the capillary was rinsed with 10% PB solution for 15 min to form the first cationic layer and 3% dextran sulfate for 15 min to form the second. Finally, 10% PB solution was applied to rinse over the anionic layer for 15 min. The capillary to which PB was attached as a third layer was named the SMIL-PB capillary. The capillary column was equilibrated with running buffer for 20 min before each CE experiment.

Electrospray and mass spectrometry

Mass spectrometry was performed using a Finnigan LCQ quadrupole ion trap mass spectrometer (Finnigan Corp., San Jose, CA, USA). Two different electrospray sources were used: (1) the pneumatically assisted electrospray ionization source for CE/MS with a sheath gas of nitrogen, and (2) a commercial nanospray ESI source using pulled glass capillaries (Protana, Odense, Denmark), which were positioned with the tip at a distance of <1mm from the entrance hole of the heated transfer capillary with the help of a stereomicroscope. The capillaries were gold-coated for electrical contact. The glass capillaries were filled by gel loader tips with 1–3 μL of analyte solution. Nebulization gas was not necessary in this spray mode.

The mass spectrometer was operated in 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 in the ESI source 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 selected ion monitoring (SIM) mode, and 1×10^7 for ZoomScan mode. Helium was used as the damping gas at a pressure of 10^{-3} Torr. In SIM analysis, the maximum ion collection time was 0.06 s for each step and 3 scans were added for each spectrum.

RESULTS AND DISCUSSION

Nanospray of intact PLA

PLA exists as glycosylated and unglycosylated variants. The glycosylated PLA consists of fourteen glycoforms. Table 1 lists the structures[22] of *N*-glycans from honeybee venom PLA. There are four isobaric pairs (2 and 3A, 3B and 3C, 4B and 5A, 8 and 9) in the PLA glycoforms. For proteins or glycoproteins, nanospray-MS produces a series of multiply charged molecular ions with different charge states. The mass resolution is sometimes insufficient to reveal the full pattern of ions generated by ESI because the molecular weight (15.7 kDa) of the protein is significantly larger than the differences between masses of carbohydrate moieties. For example, the mass difference between the isobaric sugar chain (2, 3A) and (3B, 3C) was ∼16 u. Since the PLA glycoforms carried multiple charges (10+–13+), a mass resolution of more than 10000 was needed to differentiate these two isobaric pairs. To study the potential for direct analysis of PLA by ESI, intact PLA was analyzed by nanospray. The results indicated that the mass resolution of our instrument was insufficient to differentiate (2, 3A) and (3B, 3C) glycoforms (data not shown). A similar result was also observed for (4B, 5A) and 5B glycoforms. Moreover, some minor glycoforms were not detected. These nanospray data suggested that the ion trap mass spectrometer alone was not capable of supplying sufficient information for the analysis of PLA glycoforms.

Table 2. Tryptic peptides of PLA

No.	From–To	Mass (Da) (Monoisotopic mass)	Sequence
T1	1–14	1557.78	IIYPGTLWCGHGNK
T2	15–23	915.44	SSGPNELGR
T3	24–25	293.17	FK
T4	26–32	804.30	HTDACCR
T5	33–47	1606.64	THDMCPDVMSAGESK
T6	48–58	1193.59	HGLTNTASHTR
T7	59–66	897.32	LSCDCDDK
T8	67–72	787.36	FYDCLK
T9	73–85	1387.66	NSADTISSYFVGK
T10	86–94	1143.56	MYFNLIDTK
T11	95–97	412.18	CYK
T12	98–108	1196.56	LEHPVTGCGER
T13	109–112	461.22	TEGR
T14	113–120	977.46	CLHYTVDK
T15	121–124	458.29	SKPK
T16	125–132	1125.56	VYQWFDLR
T17	133–133	146.11	K
T18	134–134	181.07	Y

CE/UV analysis of intact PLA

In order to detect different glycoforms, CE separation was performed prior to analysis by MS. In analysis of proteins by CE, strong analyte-capillary wall interaction is the major cause for the loss of efficiency and poor reproducibility of migration times. In order to reduce the adsorption of PLA, at first the electrophoresis was carried out using dynamic coating with the cationic surfactant CTAB (0.5 mM) as an additive in the separation buffer. The use of CTAB as buffer additive brought partial separation of glycoforms. However, the suppression of ion signal by CTAB made the detection of glycoforms by CE/ESI-MS impossible.

Another approach to reduce the absorption is modification of the capillary wall by SMIL coating. Unlike a dynamic coating, the additive does not flow into the mass spectrometer continuously, and therefore there is less ion suppression in CE/MS applications. In the analysis of PLA glycoforms using the SMIL-PB capillary, it was found that the presence of β-CD in the CE buffer enhanced the resolution significantly. The best result was obtained with the addition of 10 mM β-CD (Fig. 1). There were about nine partially resolved peaks. Although CE buffer containing β-CD has been successfully coupled with ESI-MS in negative ion mode,[29,30] on-line coupling of CE with ESI-MS was not successful for the analysis of PLA glycoforms. This was because the PLA was detected in positive ion mode, and the CD interference in positive ion mode ESI was found to be more serious than in negative ion mode.

Digestion of PLA

Due to the difficulty in the analysis of intact PLA glycoforms by CE/MS, PLA was digested to peptides before the analysis. Table 2 lists all the theoretical peptides of PLA hydrolyzed by trypsin. In general, tryptic digestions of glycoproteins are usually carried out after reducing the disulfide bonds. A different tactic was used in this study; PLA was digested without reducing the disulfide bonds. Without reducing the disulfide bonds the PLA glycopeptides should be significantly larger than other peptides, and

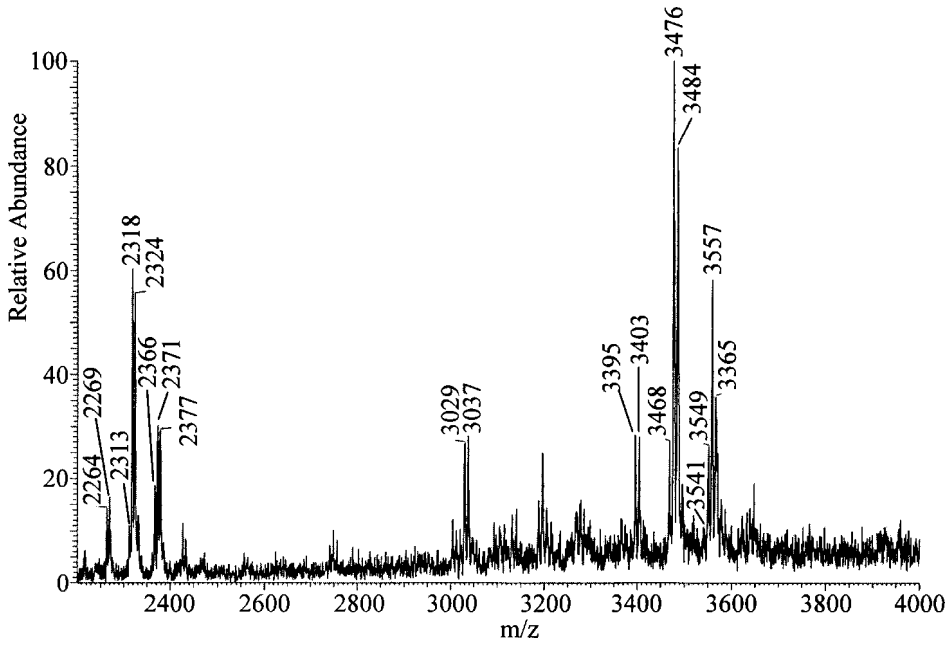
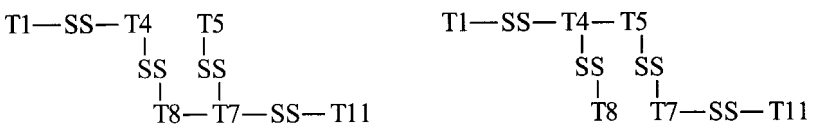


Figure 2. Positive ion nanospray mass spectrum of a tryptic digest of PLA standard.



Scheme 1. Possible structures of glycopeptides resulting from incomplete digestion.

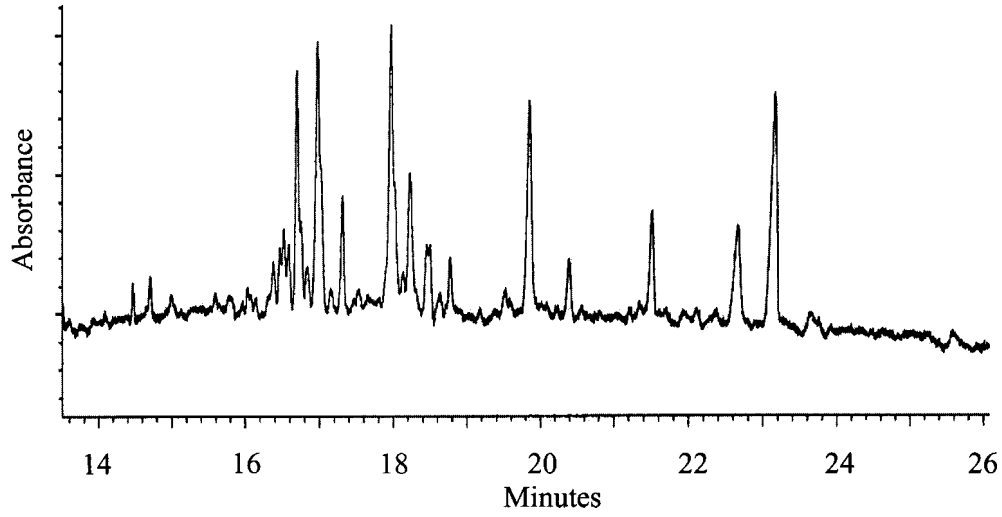


Figure 3. CE/UV electrophorogram of the tryptic digest of PLA standard which had been purified with an ultrafiltration filter and reduced with DTT. Electrophoresis was carried out with a 1.1 m × 50 μm i.d. (0.9 m effective length) SMIL coated capillary in the presence of 0.5% formic acid. The potential drop was −20 kV and the detection wavelength was 205 nm.

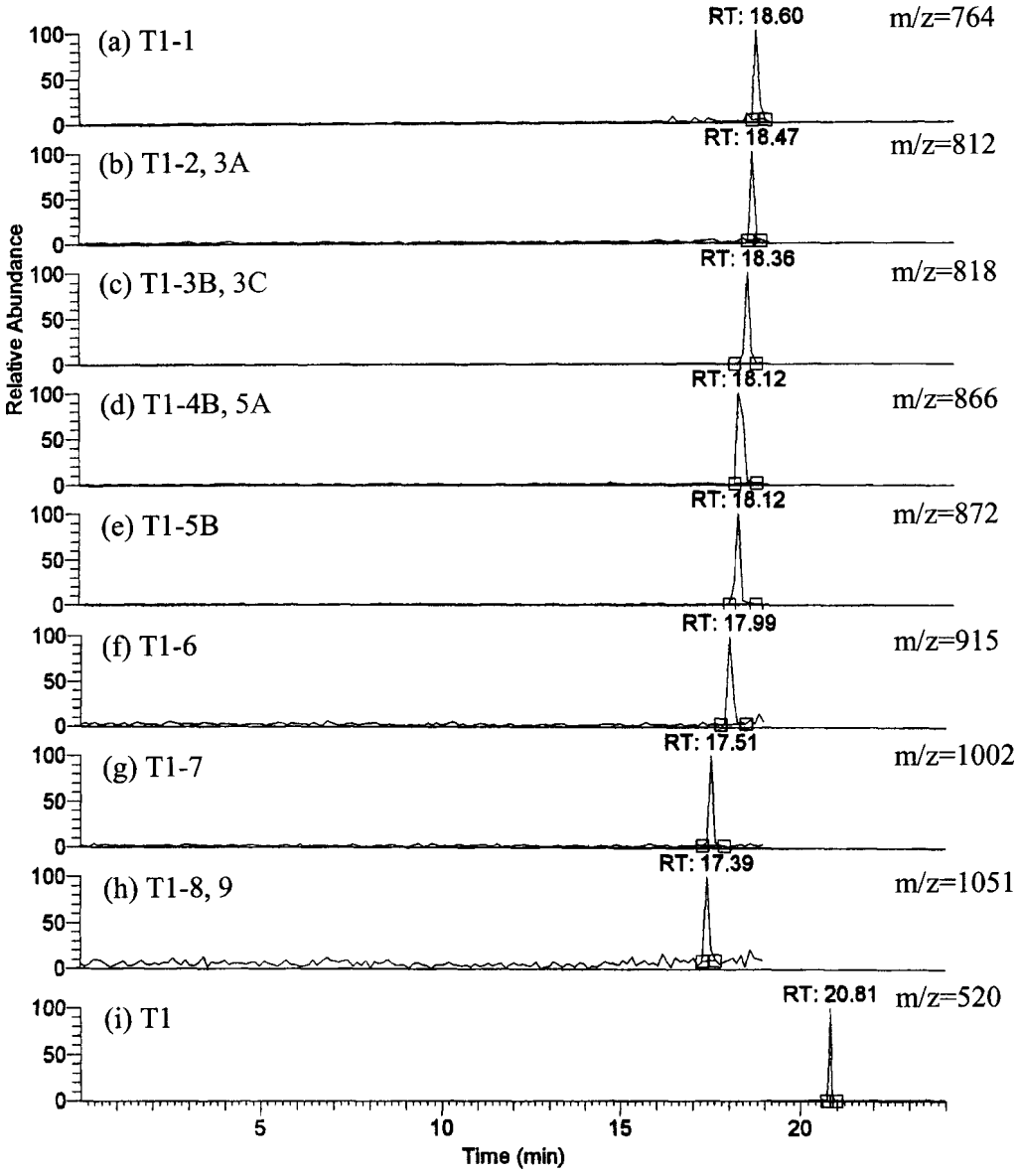


Figure 4. CE/MS electrophorograms of the purified and reduced glycopeptides of PLA standard in SIM mode. Sheath liquid composition was methanol/water 80:20 containing 0.5% formic acid. The potential drop used for CE separation was −15 kV and the ESI voltage was 4.5 kV.

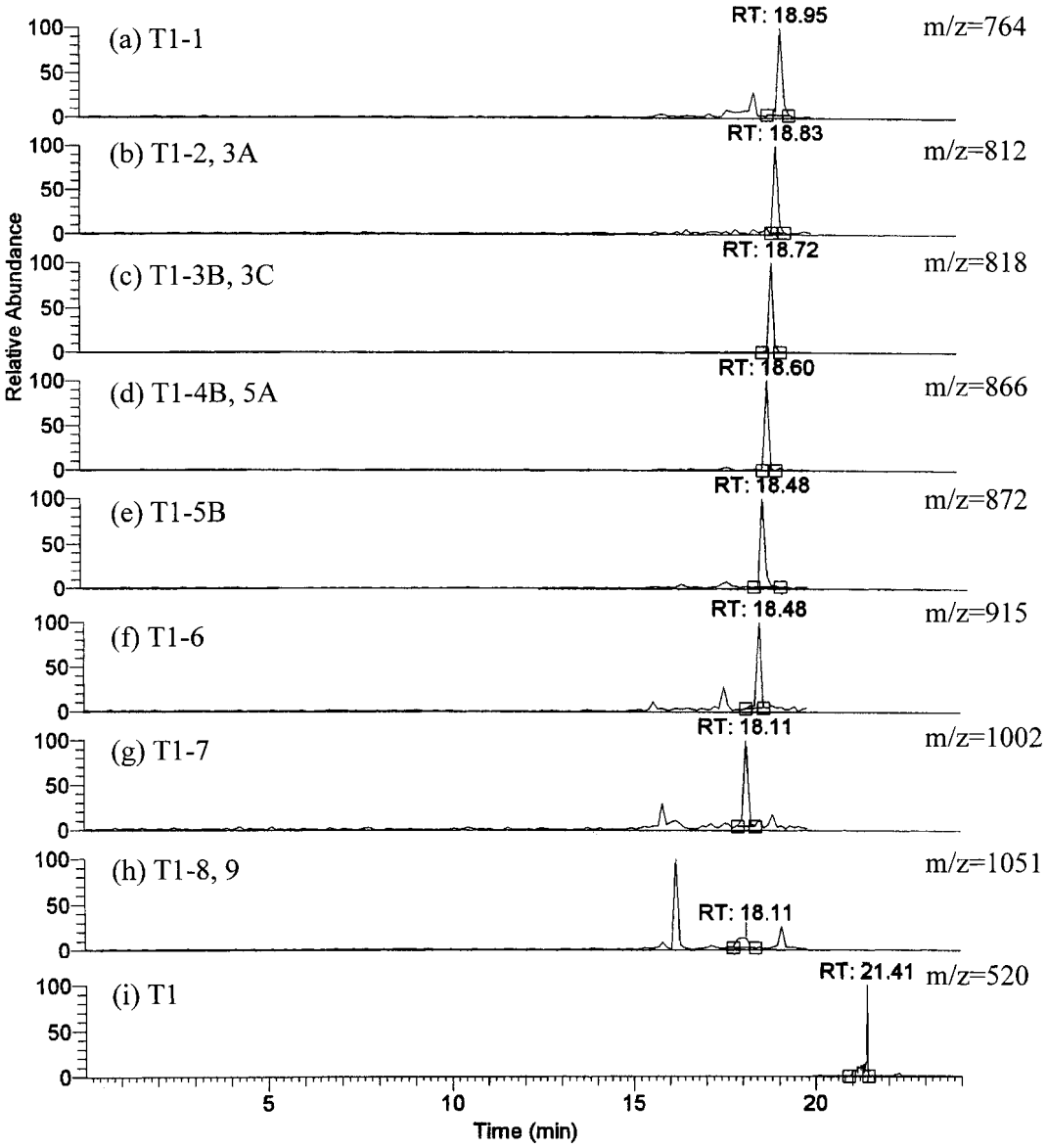


Figure 5. CE/MS electrophorograms of the purified and reduced glycopeptides of PLA from the venom of a single bee. Other conditions were the same as in Fig. 4.

therefore the glycopeptides should be easily isolated from other peptides by ultrafiltration. The glycan moiety is located at Asn13. The glycopeptide T1 is linked with peptide T4 which is also linked with peptide T8 by disulfide bonds. Without reduction of the disulfide bonds, the tryptic glycopeptide ought to be the peptide T1-T4-T8. The predicted molecular weights are about 4–5 kDa for the glycopeptides and 0.2–2.2 kDa for other tryptic peptides (Table 2). This difference made it possible to purify and concentrate glycopeptides using a 3000 Da MW cut-off ultrafiltration filter.

To check the results of digestion, the digests were analyzed by nanospray prior to ultrafiltration and analysis by CE (Fig. 2). The *m/z* values corresponding to the glycopeptides were found to be significantly larger than the expected *m/z* values. Incomplete digestion was believed to be the reason for the mass shifts. The masses of the ions suggested that the Arg32-Thr33 or Lys66-Phe67 peptide bonds in PLA were not cleaved by trypsin (Scheme 1). The glycopeptides were observed as two groups of ions. For example, the ions at *m/z* 3468 and 2313 were assigned as the doubly and triply protonated glycopeptide with the glycans 3B and 3C. The molecular weights of the glycopeptides were about 7 kDa, and this made it convenient to isolate them from other peptides by ultrafiltration.

Table 3. Relative abundance and CV (n = 5) of PLA glycopeptides of older (30 days after eclosion) and younger (10 days) honeybees

	Relative abundance in PLA glycopeptides (%)		CV(%)	
Glycopeptides	Older	Younger	Older	Younger
T1-1	13.1	12.8	4.3	4.2
T1-(2, 3A)	7.2	7.1	3.3	4.2
T1-(3B, 3C)	57.3	58.6	2.7	2.4
T1-(4B, 5A)	7.7	7.5	5.4	5.6
T1-5B	7.5	7.4	5.9	5.6
T1-6	2.9	3.0	5.8	5.9
T1-7	2.2	2.1	5.9	6.8
T1-(8, 9)	1.6	1.5	6.9	6.7

Several modified ions of the type $[M + 2H + 16]^{+2}$ and $[M + 3H + 16]^{+3}$ were observed with much higher abundances (Fig. 2) than those of the unmodified peptides. For example, *m/z* 2318 and 2324 were the modified molecular ions $[M + 3H + 16]^{+3}$ and $[M + 3H + 32]^{+3}$ for the glycopeptides with the glycans 3B and 3C. These modifications are attributed to the selective oxidation of methionyl residues in the glycopeptide (Met36 and Met41 residues in T5). Oxidation of peptides during electrospray ionization has been reported for peptides containing methionyl residues.[31] To eliminate the complexity resulting from oxidation, the disulfide bonds were reduced before analysis by CE but after ultrafiltration, so that the T5 peptide was no longer connected to the glycopeptide T1.

Analysis of PLA digests by CE/UV and CE/MS

The glycopeptides were analyzed using a PB-SMIL capillary column and the best result obtained is shown in Fig. 3. After this CE/UV analysis, the glycopeptides were further analyzed by CE/MS and numerous glycopeptides could be identified even though they were only partially separated (Fig. 4). Glycopeptides were observed as $[M + 3H]^{+3}$ ions in the acidic buffer. Apart from the two low abundance glycopeptides, T1-4A and T1-10, eight glycopeptides including four isobaric pairs [T1-(2, 3A), T1-(3B, 3C), T1-(4B, 5A) and T1-(8, 9)] could be identified (Fig. 4).

Analysis of PLA glycopeptides in venom of individual single bees

As mentioned earlier, there are many components in bee venom. To reduce the interference of other components, PLA in bee venom was separated using a 5 kDa MW cut-off filter. Digestion and ultrafiltration without reducing the disulfide bonds, followed by DTT reduction before analysis, by CE were performed as on the standard PLA sample.

The CE/MS electropherogram obtained from the analysis of glycopeptides from single bee venom is similar to Fig. 4 except that there were more interferences in the traces of T 1-6, T 1-7, and T 1-8,9 (Fig. 5). This technique was used to investigate the PLA glycopeptides of individual honeybees. The relative abundances and coefficient of variance (CV) of each glycopeptide for older (30 days after eclosion) and younger (10 days after eclosion) honeybees are listed in Table 3. The relative abundances of each glycopeptide were similar for younger and older honeybees. The CV values were below 7%.

CONCLUSIONS

Baseline separation of all PLA glycoforms was difficult to achieve in CE/UV analysis. Under optimized conditions, up to nine peaks were observed. The analysis of different glycoforms by CE/MS was even more difficult because of the incompatibility of nonvolatile additives such as β-cyclodextran in ESI analysis. Partial separation was achieved if the PLA was digested to glycopeptides before analysis by CE and CE/MS. Under optimized CE/MS conditions, eight glycopeptides could be identified in SIM mode. Several steps were critical to the isolation of glycopeptides before analysis by CE and CE/MS. These steps include isolation of the PLA with ultrafiltration, tryptic digestion and ultrafiltration without reducing the disulfide bridges, and DTT reduction before analysis by CE and CE/MS. This method was found to be useful to estimate the relative concentration of each glycoform in the venom of single honeybees. In order to quantify all the different glycoforms, including the isobaric pairs, baseline or near baseline resolution is needed. Work is in progress to investigate the possibility of better separation if the glycans are removed from PLA before analysis by CE and CE/MS.

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