



Research Article

Quinetides: diverse posttranslational modified peptides of ribonuclease-like storage protein from *Panax quinquefolius* as markers for differentiating ginseng species

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ABSTRACT

Background: Peptides have diverse and important physiological roles in plants and are ideal markers for species identification. It is unclear whether there are specific peptides in *Panax quinquefolius* L. (PQ). The aims of this study were to identify Quinetides, a series of diverse posttranslational modified native peptides of the ribonuclease-like storage protein (ginseng major protein), from PQ to explore novel peptide markers and develop a new method to distinguish PQ from *Panax ginseng*.

Methods: We used different fragmentation modes in the LTQ Orbitrap analysis to identify the enriched Quinetide targets of PQ, and we discovered Quinetide markers of PQ and *P. ginseng* using ultrahigh-performance liquid chromatography–quadrupole time-of-flight mass spectrometry analysis. These “peptide markers” were validated by simultaneously monitoring Rf and F11 as standard ginsenosides.

Results: We discovered 100 Quinetides of PQ with various post-translational modifications (PTMs), including a series of glycopeptides, all of which originated from the protein ginseng major protein. We effectively distinguished PQ from *P. ginseng* using new “peptide markers.” Four unique peptides (Quinetides TP6 and TP7 as markers of PQ and Quinetides TP8 and TP9 as markers of *P. ginseng*) and their associated glycosylation products were discovered in PQ and *P. ginseng*.

Conclusion: We provide specific information on PQ peptides and propose the clinical application of peptide markers to distinguish PQ from *P. ginseng*.

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1. Introduction

Panax quinquefolius (PQ) exhibits antitumor, antioxidant, and neuroprotective effects [1,2]. PQ and *Panax ginseng* are very similar morphologically and pharmacologically but show subtle differences in terms of bioactivities and chemical compositions. *P. ginseng* is a good invigorator, whereas PQ is capable of heat-clearing and refreshing [3–5]. Ginsenosides are considered to be the main active ingredients in these species [6–8]. Previous studies have revealed that more than 600 compounds have been identified by MS [9], and approximately 70 ginsenosides have been isolated from *P. ginseng* and PQ [10]. Approximately 26,000 expressed sequence tags of *P. ginseng* have also been determined [11], and more than 100 proteins were identified [12]. A

proteomics research study [13] on wild and cultivated *P. ginseng* has been published in *Journal of Ginseng Research*, and another study has aimed to reveal the recent progress in proteomics studies of ginseng [11]. A report has revealed that the proteins isolated from *P. ginseng* and PQ possess antifatigue, antifungal, and antiviral activities [2]. Ginseng major protein (GMP) is a major protein present in ginseng [14–16], and it may function as a storage protein [11,17]. GMP exhibits anticomplementary activity during hemolysis of red blood cells [18] and plays a role in plant defense against salt stress [19]. Peptidomics can be defined as studies of low-molecular-weight fraction of proteins, including endogenous peptides, protein fragments, and protein degradation products [20,21]. For research on native peptides, our research team has identified a total of 308 peptides in *P. ginseng* [22], some

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of which originated from GMP. Peptides are involved in a variety of physiological processes [23], such as the regulation of growth and immunity [24,25] and estimation of genetic variability. However, little is known about the peptides in PQ as its entire genome has not been sequenced.

Furthermore, the chemical differences between the two ginseng species have not been fully clarified [26,27]. Many analytical approaches have been used to identify *Panax* species including HPLC and MS. F11 and Rf are two reference compounds used for the identification [28–30], but because they share the same molecular weight, they often coelute. Only trace amounts of F11 are present in *P. ginseng* [31]; therefore, it cannot be used to fully depict the chemical differences between the two species. A great demand thus exists to establish reliable markers to differentiate the *Panax* species. Markers used to distinguish different species should be easily detectable with high sensitivity and specificity [32–34]. A comparative peptidomic study highlighted the utility of identifying peptides as potential biomarkers [35]. Peptides appear to be promising and ideal candidates as they can be effectively used to study the dynamic expression of genes. To date, there have been no reports on peptide biomarkers for PQ and *P. ginseng*.

Ultrahigh-performance liquid chromatography (UHPLC)–MS and nano–LC–MS allow detection at high resolution, sensitivity, and throughput for the discovery of novel peptides [36]. Because most peptides are multiple charged ($z > 1$) in the electrospray ionization (ESI) program [37], their charge state can be used to speculate whether the ions present are peptide candidates.

Besides proteomics research using digested peptides, we performed peptidomics research following our previous work [22]. By combining the three aforementioned factors, we discovered and characterized Quinetides as markers abundantly present in PQ. To our knowledge, this is the first report on peptide markers used for distinguishing between PQ and *P. ginseng* species.

2. Materials and methods

2.1. Chemical reagents and reference standards

HPLC-grade acetonitrile, methanol, and Tris were purchased from Sigma-Aldrich, Germany. Formic acid, ethanol, ethyl acetate, *n*-butanol, urea, and NH_4HCO_3 were purchased from Tianjin Kemiou Chemical Reagent Co, Ltd, China, and distilled water was obtained by purification using a Milli-Q gradient system (MilliPore Ltd, France). Dithiothreitol (DTT, 99% purity), iodoacetamide (IAM, 98% purity), urea, Tris buffer, and reference ginsenosides Rf (98% purity) and F11 (98% purity) all were obtained from Aladdin Industrial Corporation, China.

2.2. Plant materials

Dried samples of PQ ($n = 84$) and *P. ginseng* ($n = 82$) roots were collected from various provinces in China. Detailed information for the samples is provided in [Supplementary Table S1](#). Samples of PQ ($n = 84$) and *P. ginseng* ($n = 82$) were each used for two types: for Sample Type 1, 10 PQ and 10 *P. ginseng* samples were used to identify Quinetides and identify peptide markers and for Type 2, 74 PQ and 72 *P. ginseng* samples were used to authenticate PQ and *P. ginseng* based on peptide markers. Sample identification was performed by Professor Daixian Chen of the Dalian Institute of Drug Administration.

2.3. Sample preparation

2.3.1. PQ extract for nontargeted component analysis

Dried PQ powder (100 mg, $n = 10$) of Type 1 was weighed and treated with 1 mL of 50% aqueous ethanol and shaken vigorously. The tube containing this mixture was then placed in an ultrasonic cleaner (1130 W, 37 kHz) for 30 min. The solution was then dried under nitrogen and reconstituted in 0.2 mL of water. The resulting solution was centrifuged at 15000 rpm for 10 min at 4°C to obtain the test solution that was used to identify peptides in PQ for UHPLC–quadrupole time-of-flight (Q-TOF)–MS analysis.

2.3.2. Preparation of enriched Quinetides

Dried PQ powder (100 g) of one of the Type 1 samples was extracted with 800 mL of 50% aqueous ethanol and shaken vigorously. The tube was then placed in an ultrasonic cleaner (1130 W, 37 kHz) for 30 min, and the obtained residue was repeatedly extracted. The two obtained solutions were pooled and concentrated; the solvent was recovered and then reconstituted in 100 mL of water. The extract was added to ethanol (300 mL) to remove small polar substances, and the precipitate of the suspected peptide candidate was dissolved in 40 mL of water. Ethyl acetate (120 mL) and *n*-butanol (120 mL) were used separately to remove other components; this removal process was repeated three times. Quinetides were found in the aqueous layer using UHPLC–Q-TOF–MS. An Amicon Ultra-4 centrifugal filter device (Millipore Corporation, USA) with a molecular weight cutoff of 3 kDa was then used to separate the different fractions, and the upper layer was fractionated using a preparative column. The preparative separation system consisted of a pump and a 996 photodiode array detector. The peptides were primarily separated using an Innova C18-Preparative column (5 μm , 4.6×250 mm; Agela Technologies, China) and monitored at 190 nm and 200 nm. The flow rate was $1 \text{ mL} \cdot \text{min}^{-1}$ at 25°C. The binary elution system contained 0.5% formic acid in water (A) and acetonitrile (B). Separation was achieved using the following gradient conditions: 0 min, 10% B, flow rate = $1 \text{ mL}/\text{min}$; 5 min, 20% B, $1 \text{ mL}/\text{min}$; 10 min, 30% B, $1 \text{ mL}/\text{min}$; 15 min, 40% B, $1 \text{ mL}/\text{min}$; and 17 min, 100% B, $1 \text{ mL}/\text{min}$. The target peptide enrichment product was concentrated in the 10–12.5 min fraction and subjected to the conventional LTQ Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany) and nano–LC–MS analyses.

2.3.3. Reduction and alkylation of the enriched Quinetide product

Reduction and alkylation of the enriched Quinetide products were performed by the classical DTT/IAM reaction. First, 1 mg of the enriched product was dissolved in 100 μL of 6 M urea and 100 mM Tris buffer (pH = 7.8). A 20- μL volume of the reducing agent (800 mM DTT in 100 mM Tris buffer, pH = 7.8) was added to the mixture, and the reaction was performed in the dark at room temperature for 4 h. An alkylating agent (80 μL , 200 mM IAM in 100 mM Tris buffer, pH = 7.8) was added to further hydrolyze the mixture placed in the dark at room temperature for 4 h. Thereafter, an additional 30 μL of the reducing agent was added to consume any unreacted IAM. The sample was then desalted using an Amicon Ultra-4 centrifugal filter device with a molecular weight cutoff of 3 kDa, followed by nano–LC–MS analysis.

2.3.4. Preparation of PQ and *P. ginseng* for comparison and authentication

The stock solutions of the standard ginsenosides, Rf (1 mg/mL) and F11 (1 mg/mL), were prepared in methanol. A total of 72 *P. ginseng* and 74 PQ samples of Type 2 extracts were obtained,

using the sample preparation method described in section 2.3.1, for UHPLC–Q-TOF-MS analysis.

2.4. UHPLC–Q-TOF-MS and LTQ Orbitrap conditions

2.4.1. UHPLC–Q-TOF-MS data acquisition conditions

Analysis of the PQ and *P. ginseng* extracts was performed using an Agilent 1290 LC system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6520 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed using a ZORBAX Eclipse plus RR HD C18 column (2.1 mm × 50 mm, 2.7 μm; Agilent, USA). The column temperature was maintained at 60°C. A two-component mobile phase composed of 0.5% formic acid in water (A) and acetonitrile (B) was used. Separation was achieved using a 5 μL sample injection volume and the following gradient conditions: 0 min, 5% B, flow rate = 0.3 mL/min; 10 min, 100% B, 0.3 mL/min.

The mass spectra were obtained using the following conditions: ESI source operated in both negative and positive ion modes, desolvation gas flow rate = 8.0 L/min at 350°C, capillary voltage = –2.5 kV, cone voltage = 120 V, and source offset voltage = 80 V. Nitrogen was used as the atomizing and drying gas, and argon, as the collision gas. The acquisition time for each scan was 970.8 ms, with data acquisition performed using Mass Hunter software (Agilent Technologies Inc, USA). Centroid data were acquired for each sample ranging from 100–3000 Da. The experimental data were calibrated using real-time reference ions m/z 121.0508 and 922.0098 in the positive mode and m/z 112.9855 and 1033.9888 in the negative mode.

2.4.2. Conventional LTQ Orbitrap data acquisition conditions for peptide characterization

The enriched Quinotide product used for peptide sequencing was analyzed using the Thermo LTQ Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany) system. The total run time per injection was 11 min, with the injection volume set to 5 μL. The conditions for LC–MS analysis included linear gradient elution of solvent A (0.5% formic acid) and solvent B (100% acetonitrile), with the following stepped gradient program: 0 min, 5% B, flow rate = 0.3 mL/min; 7 min, 100% B, 0.3 mL/min; and 9 min, 100% B, 0.3 mL/min.

Data acquisition on the LTQ Orbitrap Elite system included a full Fourier transform mass spectrometry scan event, with an m/z range of 500–1700 and a resolution (full width at half maximum) of 6,000. The peptides detected in full-scan MS were selected for collision-induced dissociation and higher-energy collisional dissociation (HCD) fragmentation. For data-related acquisitions, the method was set to analyze the five strongest ions with the normalized collision energy set to 22%, 28%, and 30%.

2.4.3. Nano–LC–MS data acquisition conditions for the characterization of glycopeptides

Reduction/alkylation of the enriched Quinotide product was analyzed for disulfide bond formation and to determine glycosylation sites for electron transfer dissociation (ETD), using the Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific, Massachusetts, USA). An Ultimate 3000 nano-LC pump and a self-packed C18 column (75 μm × 15 cm) in which the packing material was ReproSilPur C18 AQ particles (3 μm, 100 Å; Dr. Maisch GmbH, Germany) were coupled to the mass spectrometer through a nanospray ion source.

The total run time per injection was 90 min, and the sample injection volume was set to 5 μL. For LC–MS analysis, linear gradient elution using solvent A (0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid) was performed using the following step gradient procedure: 0–3 min, 3–8% B, 0.4 μL/min; 3–73 min,

8–35% B, 0.4 μL/min; 73–85 min, 35–60% B, 0.4 μL/min; 85–88 min, 60–90% B, 0.4 μL/min; and 88–90 min, 90% B, 0.4 μL/min. Mandate acquisition was performed with an m/z range of 500–1700. Peptides detected in full-scan MS were selected for HCD and ETD fragmentation. The method was configured to analyze, with the normalized collision energy set to 35%.

2.5. Data processing and sequence analysis

2.5.1. Data extraction

We used Progenesis QI software (Water Company, USA) to obtain accurate masses. During data analysis, retention times (t_R) and m/z values were used to identify each peak, and a list of detected peak intensities was generated. For screening, the filter was set to 0.3. The data (including sample information and variables) were subsequently exported in a list for analysis.

2.5.2. Sequence analysis

All raw data obtained by conventional nano–LC–MS/MS analysis were uploaded in Peaks Studio 7 (BSI, Canada) software to determine the primary structures of these Quinetides. Peaks Studio allows data refinement, automatic de novo sequencing and peak search. Data refinement allows the calibration of the m/z value and charge states of the parent ion, providing more accurate monoisotopic m/z values. Peak centroiding and charge deconvolution and depolarization were also performed with strict control in the error tolerance (precursor: 10.0 ppm; the main fragments observed in the MS/MS spectra should also satisfy the result with an error less than 0.5 Da). Filters with a mass value > 0.3 were saved, with no enzymes specified for cleavage.

Variable PTMs were selected including methylation (Δ_{mass} , +14.02), deoxygenation (Δ_{mass} , –15.99), dehydration (Δ_{mass} , –18.01), hexose addition (Δ_{mass} , +162.052), addition of 2 hexoses (Δ_{mass} , +324.11), oxidation (Δ_{mass} , +15.99); the fixed PTM was disulfide bond formation (Δ_{mass} , –1.0078). For alkylated samples, fixed PTM, carbamidomethylation (Δ_{mass} , +57.02), at the cysteine site was selected. Data retrieval was from a specific ginseng protein database downloaded from the www.uniprot.org website in the SwissProt database.

3. Results

3.1. Discovery of high-abundance Quinetides including a series of glycopeptides in PQ

We first analyzed the extract from the PQ sample by UHPLC–Q-TOF-MS analysis, which was performed in the positive ion mode. The data (m/z , t_R , intensity, and charge) for each ion was then aligned using Progenesis QI software and exported in the CSV file format. Because most peptides are multiple charged ($z > 1$) in the ESI program [37], their charge state can be used to speculate whether the ions present are peptide candidates. Many of the components detected in the research were multiple charged ($z = 4–7$) in this research, with a retention time of 2.8–3.4 min; these compounds were speculated as the peptide candidates. For the 4+, 5+, 6+, and 7+ ions formed by ESI, their corresponding m/z values were the same after calculation. The base peak ion chromatograms of PQ were recorded, as shown in Fig. 1A. Fig. 1B shows the representative MS peaks of PQ (peak 1 and peak 2 in Fig. 1A give examples of the location of the 6-charged ions selected). Owing to the natural abundance of the ^{13}C isotope, the isotopic peaks for each ion were observed at 0.1667 atomic mass unit (amu) intervals for the 6-fold charged ions. Most peptide candidates were of high polarity and were essentially eluted during the first phase of the entire elution process.

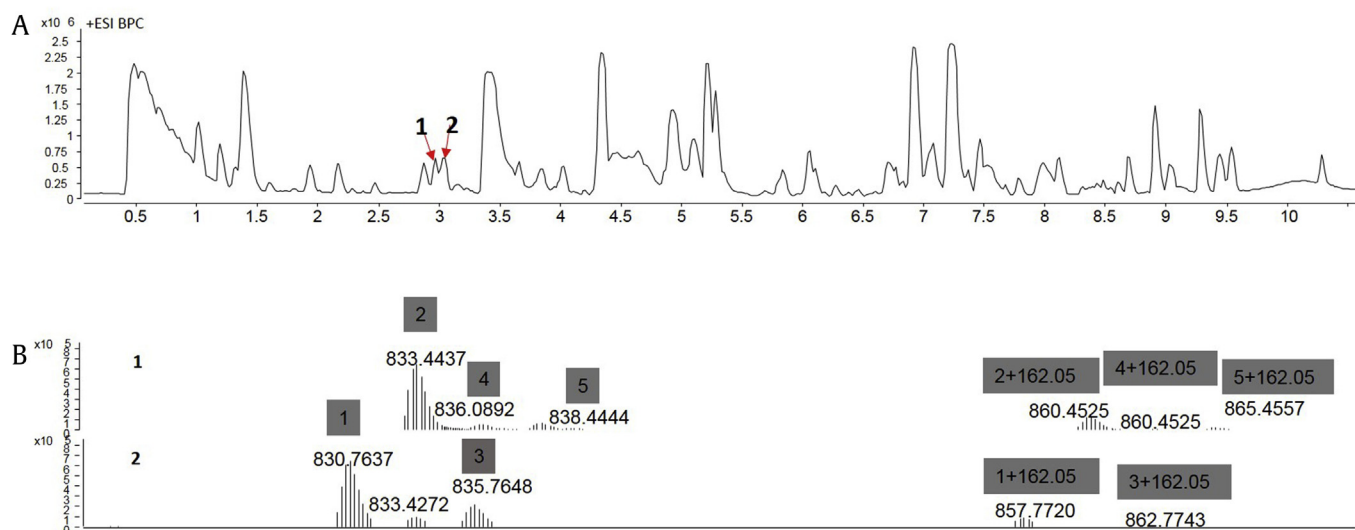


Fig. 1. Chromatogram and mass spectrum of PQ. (A) Base peak ion chromatograms (BPC) of the PQ sample were recorded over an analysis time of 10 min. (B) MS peaks of two representative chromatograms (Peak 1 and Peak 2, take 6-charged ions as an example) of PQ. ESI, electrospray ionization; PQ, *Panax quinquefolius*.

These promising representative PQ peptides are listed in [Table 1](#) (the 6-charged ions are an example). The molecular weights of ions 1, 2, 3, 4, and 5(M) were 4,975.4770, 4,991.4678, 5,005.4825, 5,007.4250, and 5,021.4693, respectively, as shown by the “M” in [Table 1](#). The molecular weight differences of ions 1, 3, 4, and 5 were -15.9900 , $+14.0147$, $+15.9660$, and $+30.0015$, respectively, compared with 2 (M1). The molecular weight difference among ions 1', 2', 3', 4', and 5' (as shown by the “M'” in [Table 2](#)) was $+162.05$ compared with 1, 2, 3, 4, and 5 listed in [Table 2](#). These peptides [referred to as glycopeptide (GP)] exhibited glycosylation characteristics. In addition, some unlisted ions had a mass difference of $n \times 162.05$ ($n > 1$). The molecular weight of the PQ peptide candidates was mainly distributed in the 4000–6000 Da range; they were temporarily referred to as high-concentration peptides because of their high abundance.

Previous studies have provided little information on these peptides. Thus, to further investigate the properties of these PQ Quinetides, such as their primary structures, the raw LTQ-Orbitrap MS/MS data were analyzed.

3.2. Enrichment and analysis of the Quinetides including GPs in PQ

As various types of compounds are found in PQ, it is necessary to establish an efficient method to extract peptides by combining the ultrasonic cleaner process with organic solvent extraction, high-concentration alcohol precipitation, and other solvent extraction techniques. An Amicon Ultra-4 centrifugal filter device was used with a molecular weight cutoff of 3 kDa. After several rounds of primary separation as described previously, the fractions were separated using a preparative column. The results of the peptide enrichments were then obtained ([Fig. 2](#)) and analyzed using nano-

LC-MS. All raw data of the PQ peptides were subjected to Peaks Studio 7 (BSI, Canada) to determine the primary structures. We tested the accuracy of the method by comparing the peptide sequences identified by the database search and the results of de novo sequencing; manual inspection was performed thereafter.

Based on these results, we concluded that 100 peptides named Quinetides as shown in [Supplementary TableS2](#) originated from the following fragments “SEYVLTDINVC (-1.01), VNQQATRFVDC (-1.01), PTDDATDDYRLK FVRLPSKMK ($186-228$).” The peptide sequence of ion 2 in [Table 1](#) was obtained from the related precursor protein, the GMP. These 100 peptides were cataloged based on their peptide sequences and comprised 8 to 43 amino acid residues with molecular weights ranging from 973.5742 to 5345.54 Da.

The results of this sequence formation suggest the possibility of truncation in the C-terminal regions of the GMP proteins. The molecular weight, sequence, PTMs, precursor mass error, and $-10 \log P$ of the identified peptides are summarized in [Supplementary TableS2](#). Most of the PTMs that occurred in these peptides were disulfide bond formation, deoxygenation, methylation, dehydration, oxidation, and addition of hexoses. The relatively high abundance of acidic amino acids of these peptides may confer a specific function as they may play a physical role in the interaction with carbohydrates [16]. Based on the BLAST search performed, the peptide sequence of ion 2 in [Table 1](#) had significant sequence homology to the peptide of the RNase-like major storage protein of *Panax notoginseng*, with an 81% identity. Proline in PQ is replaced by leucine in *P. notoginseng*. Using the online Swiss-Model website, we conducted a search of the SWISS-MODEL template library with BLAST and HHblits to predict the 3D structure of the peptide

Table 1
Detailed information on promising representative peptides (1, 2, 3, 4, and 5) of PQ

ID	Mass (M, Da)	t_R (min)	m/z (Z = 6)	M-M1 (Da)
1	4975.4770	3.32	830.2535	-15.99
2	4991.4678	3.26	832.9186	0
3	5005.4825	3.32	835.2538	14.0147
4	5007.4250	3.18	835.5781	15.966
5	5021.4693	3.27	837.9188	30.0015

PQ, *Panax quinquefolius*

Table 2
Detailed information on promising representative peptides (1', 2', 3', 4', and 5') of PQ

ID	Mass (M', Da)	t_R (min)	m/z (Z = 6)	M' = M+162.05 (Da)
1'	5137.5182	3.32	857.2606	1 + 162.05
2'	5153.5174	3.26	859.9268	2 + 162.05
3'	5167.5241	3.32	862.2613	3 + 162.05
4'	5169.5352	3.18	862.5965	4 + 162.05
5'	5183.5281	3.27	864.9286	5 + 162.05

PQ, *Panax quinquefolius*

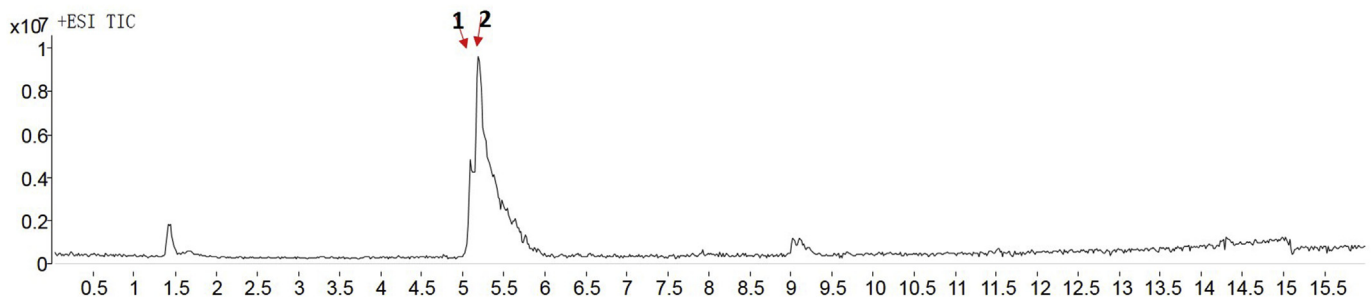


Fig. 2. The total ion chromatogram of the enriched peptides. ESI, electrospray ionization.

sequence (Supplementary Fig.S1). Because the template's detected Global Model Quality Estimation score was 0.43 and 28% sequence identity relative to the template, some of the alignments were deemed reliable.

One of the Quinetides identified is shown in Fig. 3, and its spectral identification details are provided. The most common peptide fragments observed in HCD collisions were the b and y ions. The b ion extended from the N-terminus, and the y ion, from the C-terminus. As shown in the annotated spectrum in Fig. 3, the peptide bond between two adjacent amino acids was cleaved from the C- and N-termini at several sites. Evidently, most of the peaks in the mass spectrum were automatically interpreted by the software; however, the few peaks that were not assigned values required manual assignments to their origins to improve the identification rate. For example, the 939.5385 Da peak was a product ion from y_8 -NH₃ Da that lost an H₃O⁺ during fragmentation.

3.3. Analysis of GPs of Quinetides

Although many GPs have been described in previous research [38,39], glycosylation of Quinetides from PQ is yet to be explored. Determining peptide-modified glycosylation sites is important. As a consensus sequence for "N"-glycosylation sites (N-X-S/T) was not found using the amino acid sequence, this GP might be an "O"-glycopeptide.

The reduction and alkylation of peptide extracts including a series of GPs from the enriched peptides were performed using the classical DTT/IAM reaction. Nano-LC-MS data acquisition conditions for HCD and ETD analysis were used to confirm the sites of GP glycation. As the condensation of a hexose molecule leads to an increased mass of 162 Da, we calculated the number of hexose residues in the peptides. The glycation level for the peptides of interest was determined by comparing their molecular weights.

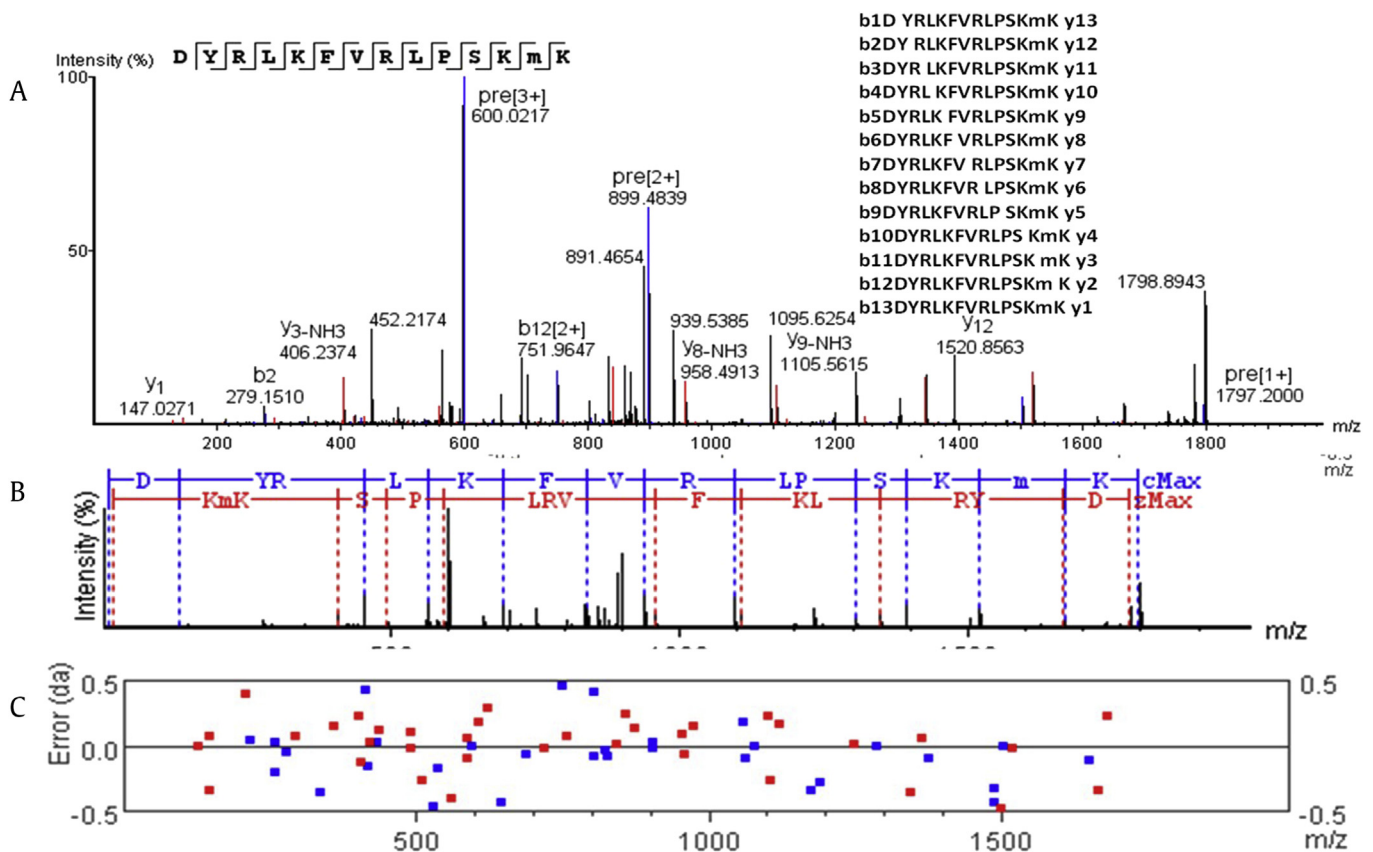


Fig. 3. The peptide (sequence = DYRLK FVRLPSK mK) has been sequenced using the database search. (A) MS/MS spectrum. (B) Spectrum alignment. (C) Error map. Tag length = 14; $-10 \lg P = 76.57$; $m/z = 599.6810$; $z = 3$; ppm = 4.6.

These values existed within the estimated range of 900–5500 Da, which corresponds to the condensation of different numbers (ranging from 1 to 5) of hexose molecules in the peptides. Based on the data obtained from nano-LC-MS/MS for GPs, the major sites linked to a hexose or two hexoses were Y31, K34, and S40 of SEYVLTIDINVC (−1.01), VNQQATRFVDC (−1.01), and PTDDATD-DYRLKFVRLPSKMK (186–228), respectively.

One of the identified GPs is shown in Fig. 4, and its spectral identification details are provided. The most common peptide fragments observed in ETD were the c and z ions. As shown in the annotated spectrum in Fig. 4, most of the peaks in the mass spectrum were automatically interpreted by the software; A few peaks required manual assignments to their origins. For example, for “c10,” the 1,439.7893 Da peak was produced by glycosylation on the serine residue (S40), and the 720.3955 Da peak was produced by the 22-charged ion form of “C10.”

3.4. Discovery of unique quinetide markers between PQ and *P. ginseng*

We also discovered a series of Quinetides in *P. ginseng*, which are found in PQ; these peptides were less prominent in the *P. ginseng* samples. We compared 10 PQ and 10 *P. ginseng* samples to determine peptide markers using UHPLC–Q-TOF. A comparative analysis of the two relatives indicated that the number of Quinetides in *P. ginseng* was similar to that in PQ, and some peptides such as Quinetides TP1–TP5 (Their molecular weight is expressed by M)

Table 3
Detailed information on common peptides to both PQ and *P. ginseng*

ID	Common peptide	Mass (Da)	t_R (min)	m/z ($Z = 6$)	M-M1 (Da)	PTM
TP1	RS/XYS	4746.389	3.40	792.0721	14.0147	M2+CH ₃
TP2	RS/XYS	4991.468	3.26	832.9186	0	M1
TP3	RS/XYS	5005.483	3.32	835.2538	14.0147	M1+CH ₃
TP4	RS/XYS	5007.434	3.18	835.579	15.966	M1+O
TP5	RS/XYS	5021.469	3.27	837.9188	30.0015	M1+CH ₃ +O

PQ, *Panax quinquefolius*; PTM, posttranslational modified native peptide

and their glycosylation products were common to both species (Table 3). We confirmed the identities of Quinetides TP1–TP5 as M2+CH₃, M1, M1+CH₃, M1+O, and M1+CH₃+O, respectively. Among these common Quinetides, the PTMs were characterized as methylation, oxidation, and hexose addition products, with 4,991.4678 and 4,732.3741 as the molecular weights of M1 and M2, respectively. The fragment sequences of M1 and M2 were SEYVLTIDINVC (−1.01) VNQQATRFVDC (−1.01) PTDDATD-DYRLKFVRLPSKMK (186–228) and SEYVLTIDINVC (−1.01) VNQQATRFVDC (−1.01) PTDDATDDYRLKF VRLPSK (186–226), respectively.

In addition to these common peptides, those peptides with specialized PTMs (used as markers to identify PQ and *P. ginseng*, Quinetides TP6 and TP7 and their glycosylation products were found to be specific for PQ. As shown in Table 4, TP6 and TP7 (Their molecular weight is expressed by M) were very prominent in PQ but absent in *P. ginseng*; thereby functioning as two potential PQ

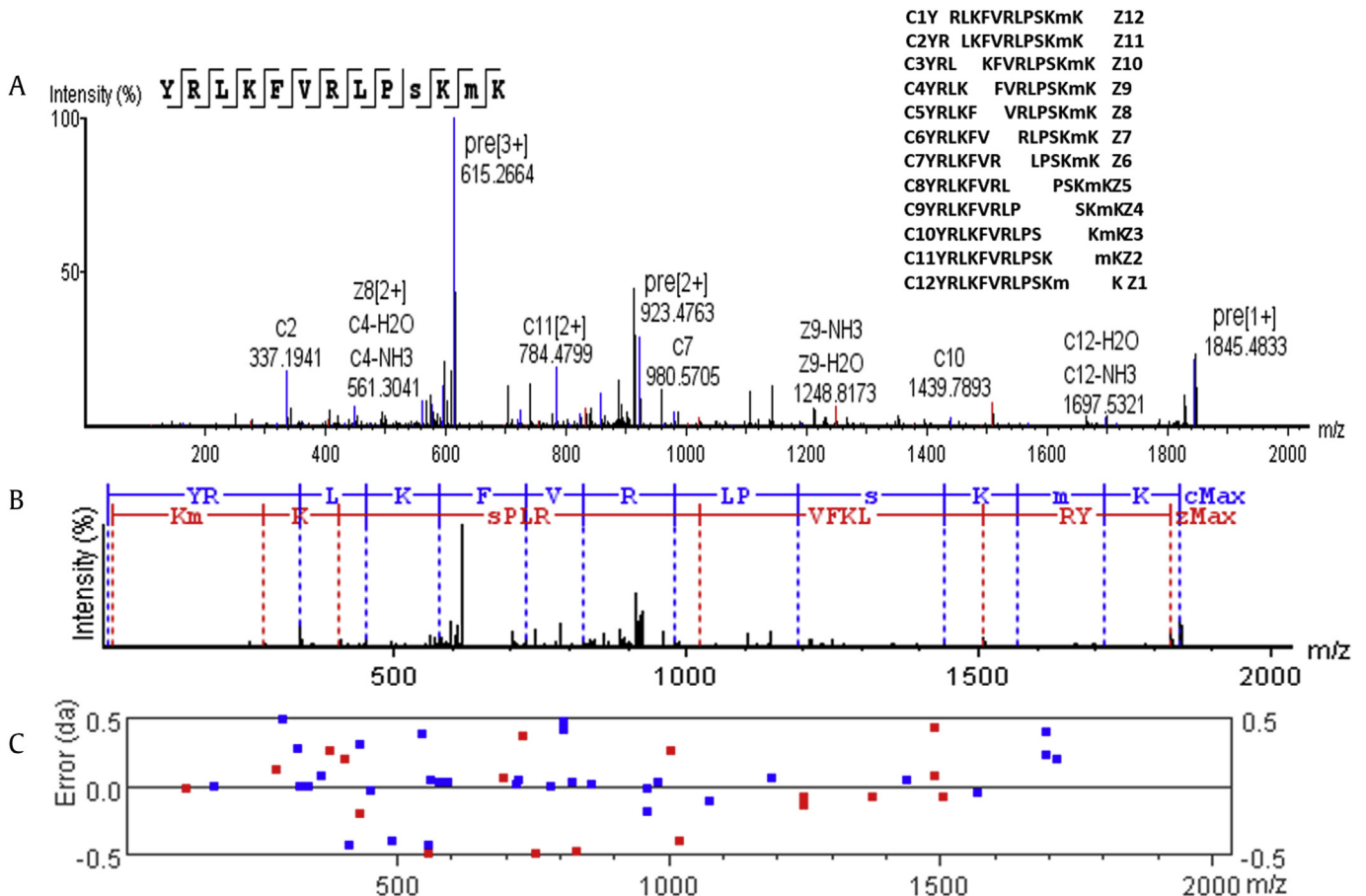


Fig. 4. The peptide (sequence = YRLKFVRLPSKMK) has been sequenced using the database search. (A) MS/MS spectrum. (B) Spectrum alignment. (C) Error map. Tag length = 13; $-10 \lg P = 57.55$; $m/z = 615.3564$; $z = 3$; ppm = 4.6.

Table 4
Detailed information on specific peptides of PQ

ID	Specific peptide (existence)	Mass (Da)	t_R (min)	m/z (Z = 6)	M-M1 (Da)	PTM
TP6	XYS	4716.427	3.40	787.0785	-15.99	M2-O
TP7	XYS	4975.477	3.32	830.2535	-15.99	M1-O

PQ, *Panax quinquefolius*; PTM, posttranslational modified native peptide

biomarkers, with the peak at m/z 830.2535 (TP7, M = 4975.48) as one such example. Based on the extracted ion chromatograms of TP7 for the 5 batches of PQ and 5 batches of *P. ginseng* samples in Fig. 5A, TP7 which is considered a biomarker of PQ was not present in the *P. ginseng* samples. TP6 and TP7 were identified as M2-O and M1-O, with masses of 4,716.4272 and 4,975.4770 Da, respectively. The PTM of both markers was characterized as deoxygenation. Analogously, it was found that TP8 and TP9 (Their molecular weight is expressed by M) with masses of 4968.3710 and 4984.3639 Da,

Table 5
Detailed information on specific peptides of *P. ginseng*

ID	Specific peptide (existence)	Mass (Da)	t_R (min)	m/z (Z = 6)	M-M1 (Da)	PTM
TP8	RS	4968.371	3.4	829.0691	-23.0968	M1-23
TP9	RS	4984.364	3.34	831.7346	-7.1039	M1-7

PTM, posttranslational modified native peptide

respectively, and their glycosylation products were specific to *P. ginseng* (Table 5). Based on the extracted ion chromatograms for 5 batches of the PQ and 5 batches of the *P. ginseng* samples in Fig. 5B, the feature with m/z 831.7346 (TP9, z = 6, M = 4,984.3639) used as a biomarker of *P. ginseng* was absent in the PQ samples. The molecular weight differences between ions TP8 and TP9 were 23.0968 and 7.1039, respectively, when compared with M1.

We analyzed the maps of the aforementioned peptides for PQ and *P. ginseng*, as shown in Figs. 5C, 5D, respectively. TP1–TP5

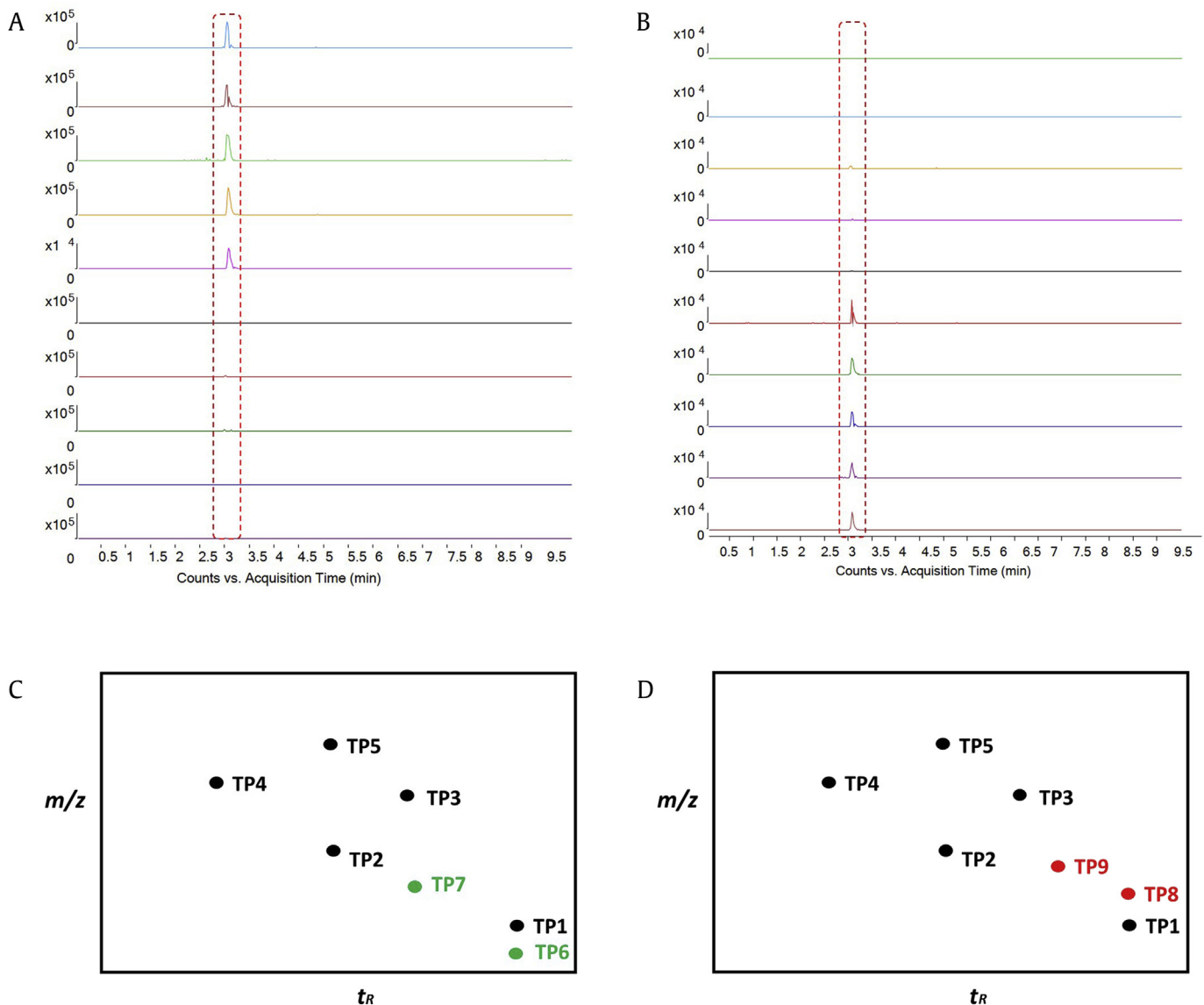


Fig. 5. The presence of marker peptides in PQ and *P. ginseng*. (A) and (B) represent the extracted chromatograms of TP7 and TP9, respectively, with the first and last five representing PQ and *P. ginseng*, respectively. (C) and (D) represent peptide maps of PQ and *P. ginseng*, respectively, and black circles represent the peptides they share. Green circles and red circles represent PQ- and *P. ginseng*-specific peptides, respectively.

represents common peptides between the two. TP6–TP7 and TP8–TP9 represent PQ- and *P. ginseng*-specific peptides, respectively. The differences between the two were evident, thereby, allowing ease of differentiation using TP6–TP9 and their glycosylation products.

3.5. Authentication of PQ and *P. ginseng* based on F11 and Rf and the quinetide markers

In the past, the two major marker compounds that could be used to discriminate between the two species were the ginsenosides, Rf and F11 [31]. To validate the peptide markers that we developed, our present method provided two independent types of biomarkers, F11 and Rf, and specific peptides Quinetide TP6–TP9 to identify PQ and *P. ginseng*. It was predicted that combining the two types of markers would achieve the same results. We analyzed 146 commercial ginseng products including 74 PQ and 72 *P. ginseng* samples to confirm the effectiveness of the unique peptide markers.

The ginsenosides, Rf and F11, were separated at baseline, with a retention time of 4.33 and 4.40 min, respectively. Their purities were >95% by UHPLC–MS analysis, as shown in [Supplementary Fig.S2](#). [Table 6](#) summarizes the number of samples used for positive identification of the two types of markers. As shown in [Table 6](#), among these products, 69 samples that appeared as PQ were found to contain TP7 and F11, which were derived from PQ. TP7 was extremely evident in 100% of the 69 PQ samples; however, they were either undetectable or absent in all the *P. ginseng* samples. This confirmed the robustness of the TP7 biomarker of PQ. TP6 was clearly indicated in 91.13% of the 69 PQ samples and was either undetectable or absent in all *P. ginseng* samples. We speculate that the peak intensity of TP6 of some of the PQ samples was weak and could not be detected. The other five samples that appeared as PQ contained Rf, TP8, or TP9, with two brands derived from *P. ginseng*, as F11, TP6, and TP7 were absent. Another three brands were however made from a mixture of PQ and *P. ginseng*, where F11, TP6, or TP7 was present. Therefore, TP7 is a robust marker of PQ as F11.

Although the Quinetides were mostly similar to PQ, they were not detected in several of the *P. ginseng* samples. However, the developed method was however still applied to the analysis of *P. ginseng*. Rf, TP8, and TP9 are the characteristic constituents of *P. ginseng*. If the sample contains TP8 and TP9 of the peptides or Rf, it is a *P. ginseng* sample. The amounts of Rf, TP8, and TP9 in *P. ginseng* from different geographic origins are shown in [Table 7](#). The 69 samples were speculated to be *P. ginseng* samples. TP8 and TP9 that are considered biomarkers of *P. ginseng* were clearly indicated in 100% of the 56 *P. ginseng* (69 *P. ginseng*, 56 *P. ginseng* with peptides) samples and were undetectable in all PQ samples. One brand was derived from PQ, whereas two were made from a mixture of both PQ and *P. ginseng*.

Based on the aforementioned analyses, we developed the following summary: (1) all PQ samples are rich in Quinetides, with nonexistence in a small amount of *P. ginseng* samples (i.e., no Quinetides exist, if the sample containing Rf is identified as

Table 7
Summary of the number of samples used for identification of *P. ginseng*

Number	Markers (PQ)			Markers (<i>P. ginseng</i>)			Authentication
	F11	TP6	TP7	Rf	TP8	TP9	
55	×	×	×	√	√	√	<i>P. ginseng</i>
1	×	×	×	√	√	×	<i>P. ginseng</i>
13	×	×	×	√	×	×	<i>P. ginseng</i>
2	√	×	√	√	√	×	PQ mixed with more <i>P. ginseng</i>
1	√	×	√	×	×	×	PQ

PQ, *Panax quinquefolius*

P. ginseng); (2) the presence of F11, TP7, or TP6 is diagnostic of PQ; and (3) the presence of Rf, TP8, or TP9 may allow the identification of *P. ginseng*.

Taking the sample number as the abscissa and the peak intensity of the markers TP7 and F11 as the ordinate, the peak intensity of 80 PQ samples could be plotted. Although F11 and TP7 occur in all PQ samples, the peak intensity of TP7 in PQ is 14 times higher than that of F11 found in some PQ roots (e.g., number 33, 65, and 79), based on the peak areas of F11 and TP7 in the chromatograms ([Fig. 6](#)). The main features of using peptide markers is that the content of peptides differs greatly in different samples. In addition to distinguishing between PQ and *P. ginseng*, the markers may also indicate the quality of PQ as they can effectively be used to study the dynamic expression of genes.

4. Discussion

The Orbitrap MS analysis serves as the optimal analytical platform as it displays its ions with different fragmentation patterns (HCD, collision-induced dissociation, and ETD); Peaks software can be used in database searches. The main advantage of using mass spectroscopy and the database search is the ability to study hundreds of peptides simultaneously, while attaining an overview of different PTMs. We have discovered and characterized a total of 100 Quinetides of PQ, with a variety of PTMs originating from a related precursor protein (GMP, P83618), including a series of GPs. Most PTMs of these peptides are disulfide bond formation, deoxygenation, methylation, oxidation, and hexose addition products ($n \geq 1$). Polypeptides containing disulfide bonds can experience an increase in specificity by derivatization. Increasing saccharification increases the formation of higher charged forms. Therefore, it is important to consider all detected charged forms of each peptide. However, our data for the sequence alignment and secondary structure prediction are insufficient to interpret the many variations observed.

Over the course of our investigation, we identified several common and specific peptides in PQ and *P. ginseng*. The most significantly differential components (TP6–TP9 and their glycosylation products) were selected as new markers to discriminate between *P. ginseng* and PQ. TP6 and TP7 were identified as M2-O and M1-O, respectively. It is speculated that there is a specific

Table 6
Summary of the number of samples used for identification of PQ

Number	Markers (PQ)			Markers (<i>P. ginseng</i>)			Authentication
	F11	TP6	TP7	Rf	TP8	TP9	
63	√	√	√	×	×	×	PQ
6	√	×	√	×	×	×	PQ
2	√	√	√	√	×	×	PQ mixed with a small amount of <i>P. ginseng</i>
1	√	√	√	√	√	√	PQ mixed with more <i>P. ginseng</i>
2	×	×	×	√	√	√	<i>P. ginseng</i>

PQ, *Panax quinquefolius*

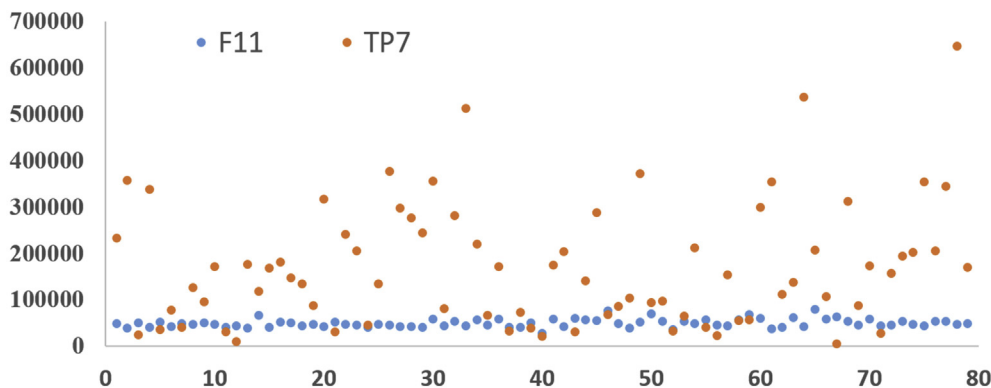


Fig. 6. Comparison of the content of TP7 and F11 that was found in 80 PQ samples on the basis of the peak areas in the UHPLC–MS–MS chromatograms. PQ, *Panax quinquefolius*; UHPLC, ultrahigh-performance liquid chromatography.

enzyme in PQ for deoxygenation. Presently, characterization of TP8 and TP9 is underway as our data for the sequence alignment and secondary structure prediction are insufficient to accurately perform this activity. Our present method provides the following two independent types of markers: the specific peptides TP6–TP9 and the unique saponins, F11 and Rf, for identification of PQ and *P. ginseng*. PQ and *P. ginseng* were correctly identified by using multiple chemical markers. TP7 is a robust biomarker of PQ. The presence of F11, TP7, or TP6 is indicative of PQ, whereas the presence of Rf, TP8, or TP9 may allow the identification of *P. ginseng*. Furthermore, our results demonstrated that the Quinetides existed in almost all PQ samples but were nonexistent in a small number of *P. ginseng* samples. Considering it is difficult to forecast the cultivated conditions of the *P. ginseng* samples, it is rather difficult to clarify the lack of Quinetides in a small percentage of the *P. ginseng* herbs; therefore, this will be explored in a future study. Essentially, the use of unique peptides as markers is indicative of them belonging to either PQ or *P. ginseng*.

The 28-kDa protein (GMP) is a major storage protein [11,17] present in PQ and *P. ginseng* [3,14–16]. It exhibits anti-complementary activity during hemolysis of red blood cells [18] and plays a role in plant defense against salt stress [19]. RNase with anti-HIV-reverse transcription, ribonuclease, and cell-free translation-inhibitory activities has been reported [40–44]; however, GMP does not exhibit RNase activity. Gintonin, a novel ginseng-derived exogenous G protein-coupled lysobisphosphatidic acids (LPA) receptor ligand [45], was extracted from PQ and *P. ginseng*, containing major latex-like protein 151 and GMP [46]. Glycosylation is one of the most significant and widespread posttranslational activities, which not only affects the biological activity but also plays a role in signal transduction [47]. Ginseng GPs also show good biological activity. It has a significant inhibitory effect on inflammation and obviously reduces malondialdehyde in inflammatory foot tissue [48]. The biological significance of these peptide markers also remains unclear. Nonetheless, information regarding the pharmacology of the various Quinetides will be useful to understand the expression of GMP.

5. Conclusion

Herein, we report for the first time the diverse PTMs of native Quinetides, including a series of GPs. These peptides originated from the fragments, SEYVLTIDINVC (–1.01), VNQQATRFDVC (–1.01), and PTDDATDDYRLKFLPSKMK (186–228), of the related precursor protein, GMP. Most PTMs of these peptides included were disulfide, deoxygenation, methylation, oxidation, and addition of hexoses ($n \geq 1$). We also demonstrated that these peptides, such as

deoxygenation-modified peptides (TP6 and TP7), can be undoubtedly used as novel markers to construct a new method for distinguishing between PQ and *P. ginseng*. We have combined and added the specific peptide markers “Quinetides TP6–TP9” to the unique ginsenosides, F11 and Rf, for PQ and *P. ginseng* identification. This report will be of interest to the readers in the field of peptidomics, proteomics, and quality control of ginseng.

Conflicts of interest

All authors have no conflicts of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jgr.2019.05.010>.

References

- [1] Vuksan V, Sievenpiper JL, Koo VY, Francis T, Beljan-Zdravkovic U, Xu Z, Vidgen E. American ginseng (*Panax quinquefolius* L) reduces postprandial glycemia in nondiabetic subjects and subjects with type 2 diabetes mellitus. *Arch Intern Med* 2000;160:1009–13.
- [2] Qi B, Wang S, Wang Q, Zhang H, Bai XY, He HN, Sun WJ, Liu L, Zhao DQ. Characterization and immunostimulating effects on murine peritoneal macrophages of a novel protein isolated from *Panax quinquefolius* L. *J Ethnopharmacol* 2016;193:700–5.
- [3] Lum JH-K, Fung K-L, Cheung P-Y, Wong M-S, Lee C-H, Kwok FS-L, Leung MC-P, Hui P-K, Lo SC-L. Proteome of Oriental ginseng *Panax ginseng* C. A. Meyer and the potential to use it as an identification tool. *Proteomics* 2002;2:1123–30.
- [4] Yang W, Qiao X, Li K, Fan J, Bo T, Guo DA, Ye M. Identification and differentiation of *Panax ginseng*, *Panax quinquefolium*, and *Panax notoginseng* by monitoring multiple diagnostic chemical markers. *Acta Pharm Sin B* 2016;6:568–75.
- [5] Zhu W, Han B, Sun Y, Wang Z, Yang X. Immunoregulatory effects of a glucogalactan from the root of *Panax quinquefolium* L. *Carbohydr Polym* 2012;87:2725–9.
- [6] Zhang K, Wang X, Ding L, Li J, Qu C-I, Chen L-g, Jin H-y, Zhang H-q. Determination of seven major ginsenosides in different parts of *Panax quinquefolius* L. (*American ginseng*) with different ages. *Chem Res Chin Univ.* 2008;24:707–11.

- [7] Qu C, Bai Y, Jin X, Wang Y, Zhang K, You J, Zhang H. Study on ginsenosides in different parts and ages of *Panax quinquefolius* L. *Food Chem* 2009;115:340–6.
- [8] Wang CZ, Xie JT, Fishbein A, Aung HH, He H, Mehendale SR, He TC, Du W, Yuan CS. Antiproliferative effects of different plant parts of *Panax notoginseng* on SW480 human colorectal cancer cells. *Phytother Res: PTR* 2009;23:6–13.
- [9] Qiu S, Yang WZ, Shi XJ, Yao CL, Yang M, Liu X, Jiang BH, Wu WY, Guo DA. A green protocol for efficient discovery of novel natural compounds: characterization of new ginsenosides from the stems and leaves of *Panax ginseng* as a case study. *Analytica Chim Acta* 2015;893:65–76.
- [10] Li K-k, Yang X-w. Research progress of chemical components of stems and leaves of *Panax ginseng*. *Zhongguo Xiandai Zhongyao* 2012;14:47–50.
- [11] Nam MH, Kim SI, Liu JR, Yang DC, Lim YP, Kwon KH, Yoo JS, Park YM. Proteomic analysis of Korean ginseng (*Panax ginseng* C.A. Meyer). *J. Chromatogr B, Anal Technol Biomed Life Sci* 2005;815:147–55.
- [12] Kim SI, Kim JY, Kim EA, Kwon KH, Kim KW, Cho K, Lee JH, Nam MH, Yang DC, Yoo JS, et al. Proteomic analysis of hairy root from *Panax ginseng* C.A. Meyer using peptide fingerprinting, internal sequencing and expressed sequence tag data. *Proteomics* 2003;3:2379–92.
- [13] Sun H, Liu F, Sun L, Liu J, Wang M, Chen X, Xu X, Ma R, Feng K, Jiang R. Proteomic analysis of amino acid metabolism differences between wild and cultivated *Panax ginseng*. *J Ginseng Res* 2016;40:113–20.
- [14] Liu S, Wang S, Liu M, Yang F, Zhang H, Liu S, Wang Q, Zhao Y. De novo sequencing and analysis of the transcriptome of *Panax ginseng* in the leaf-expansion period. *Mol Med Rep* 2016;14:1404–12.
- [15] Liwei S. Two-dimensional gel electrophoresis analysis of different parts of *Panax quinquefolius* L. root. *Afr J Biotechnol* 2011;10.
- [16] Yoon JY, Ha BH, Woo JS, Lim YH, Kim KH. Purification and characterization of a 28-kDa major protein from ginseng root. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* 2002;132B:551–7.
- [17] Kim SI, Kweon SM, Kim EA, Kim JY, Kim S, Yoo JS, Park YM. Characterization of RNase-like major storage protein from the ginseng root by proteomic approach. *J Plant Physiol* 2004;161:837–45.
- [18] Kwon TH, Oh SR, Park H, Kim KH. Purification of a major protein with physiological activities from *Panax ginseng* C.A. Meyer. *Han'guk Nonghwa Hakhoechi* 1998;41:410–3.
- [19] Sun Tae Kim D-WB, Lee Kyunghye, Hwang Jung Eun, Bang Kyong-Hwan. Proteomic analysis of Korean ginseng (*Panax ginseng* C. A.Meyer) following exposure to salt stress. 2008.
- [20] Jahn H, Wittke S, Zurbig P, Raedler TJ, Arlt S, Kellmann M, Mullen W, Eichenlaub M, Mischak H, Wiedemann K. Peptide fingerprinting of Alzheimer's disease in cerebrospinal fluid: identification and prospective evaluation of new synaptic biomarkers. *PLoS One* 2011;6:12.
- [21] Hui L, Cunningham R, Zhang Z, Cao W, Jia C, Li L. Discovery and characterization of the Crustacean hyperglycemic hormone precursor related peptides (CPRP) and orcoxinin neuropeptides in the sinus glands of the blue crab *Callinectes sapidus* using multiple tandem mass spectrometry techniques. *J Proteome Res* 2011;10:4219–29.
- [22] Ye X, Zhao N, Yu X, Han X, Gao H, Zhang X. Extensive characterization of peptides from *Panax ginseng* C. A. Meyer using mass spectrometric approach. *Proteomics* 2016;16:2788–91.
- [23] Marmioli N, Maestri E. Plant peptides in defense and signaling. *Peptides (N. Y., NY, U. S.)* 2014;56:30–44.
- [24] Zhan C, Li S, Zhong Q, Zhou D. Structure-based grafting, mutation, and optimization of peptide inhibitors to fit in the active pocket of human secreted phospholipase A2: find new use of old Peptide agents with anti-inflammatory activity. *Chem Biol Drug Des* 2015;85:418–26.
- [25] Chavan SG, Deobagkar DD. An in silico insight into novel therapeutic interaction of LTNF peptide-LT10 and design of structure based peptidomimetics for putative anti-diabetic activity. *PLoS One* 2015;10:e0121860.
- [26] Chen CF, Chiou WF, Zhang JT. Comparison of the pharmacological effects of *Panax ginseng* and *Panax quinquefolium*. *Acta Pharmacol Sin* 2008;29:1103–8.
- [27] Li L, Luo GA, Liang QL, Hu P, Wang YM. Rapid qualitative and quantitative analyses of Asian ginseng in adulterated American ginseng preparations by UPLC/Q-TOF-MS. *J Pharm Biomed Anal* 2010;52:66–72.
- [28] Lee TM, Marderosian AD. Two-Dimensional TLC analysis of ginsenosides from root of dwarf ginseng (*Panax trifolius* L.) Araliaceae. *J Pharmaceut Sci* 1981;70:89–91.
- [29] Fuzziati N, Gabetta B, Jayakar K, Pace R, Peterlongo F. Liquid chromatography-electrospray mass spectrometric identification of ginsenosides in *Panax ginseng* roots. *J Chromatogr A* 1999;854:69–79.
- [30] Li W, Gu C, Zhang H, Awang DVC, Fitzloff JF, Fong HHS, van Breemen RB. Use of high-performance liquid chromatography-tandem mass spectrometry to distinguish *Panax ginseng* C. A. Meyer (Asian ginseng) and *Panax quinquefolius* L. (North American ginseng). *Anal Chem* 2000;72:5417–22.
- [31] Li W, Gu C, Zhang H, Awang DVC, Fitzloff JF, Fong HHS, van Breemen RB. Use of high-performance liquid Chromatography-Tandem mass spectrometry to distinguish *Panax ginseng* C. A. Meyer (Asian ginseng) and *Panax quinquefolius* L. (North American ginseng). *Anal Chem* 2000;72:5417–22.
- [32] Puntmann VO. How-to guide on biomarkers: biomarker definitions, validation and applications with examples from cardiovascular disease. *Postgrad Med J* 2009;85:538–45.
- [33] Pepe MS, Thompson ML. Combining diagnostic test results to increase accuracy. *Biostatistics* 2000;1:123–40.
- [34] Zhao Q, Zhao N, Ye X, He M, Yang Y, Gao H, Zhang X. Rapid discrimination between red and white ginseng based on unique mass-spectrometric features. *J Pharm Biomed Anal* 2019;164:202–10.
- [35] Gelman JS, Sironi J, Castro LM, Ferro ES, Fricker LD. Peptidomic analysis of human cell lines. *J Proteome Res* 2011;10:1583–92.
- [36] Bai Y, Zhao Q, He M, Ye X, Zhang X. Extensive characterization and differential analysis of endogenous peptides from *Bombyx batryticatus* using mass spectrometric approach. *J Pharm Biomed Anal* 2018;163:78–87.
- [37] Qian MG, Zhang Y, Lubman DM. Collision-induced dissociation of multiply charged peptides in an ion-trap storage/reflection time-of-flight mass spectrometer. *Rapid Commun Mass Spectrom* 1995;9:1275–82.
- [38] Levery SB, Steentoft C, Halim A, Narimatsu Y, Clausen H, Vakhshushev SY. Advances in mass spectrometry driven O-glycoproteomics. *Biochim Biophys Acta* 2015;1850:33–42.
- [39] Pasing Y, Sickmann A, Lewandrowski U. N-glycoproteomics: mass spectrometry-based glycosylation site annotation. *Biol Chem* 2012;393:249–58.
- [40] Wang HX, Ng TB. Quinqueginsin, a novel protein with anti-human immunodeficiency virus, antifungal, ribonuclease and cell-free translation-inhibitory activities from American ginseng roots. *Biochem Biophys Res Commun* 2000;269:203–8.
- [41] Ng TB, Wang H. Panaxagin, a new protein from Chinese ginseng possesses anti-fungal, anti-viral, translation-inhibiting and ribonuclease activities. *Life Sci* 2001;68:739–49.
- [42] Lam SK, Ng TB. Isolation of a novel thermolabile heterodimeric ribonuclease with antifungal and Antiproliferative activities from roots of the sanchi ginseng *Panax notoginseng*. *Biochem Biophys Res Commun* 2001;285:419–23.
- [43] Wang HX, Ng TB. A ribonuclease from Chinese ginseng (*Panax ginseng*) flowers. *Protein Expr Purif* 2004;33:195–9.
- [44] Wang HX, Ng TB. Isolation of a ribonuclease from sanchi ginseng (*Panax pseudoginseng*) flowers distinct from other ginseng ribonucleases. *Biochem Biophys Res Commun* 2006;343:198–202.
- [45] Choi SH, Hong MK, Kim HJ, Ryoo N, Rhim H, Nah SY, Kang LW. Structure of ginseng major latex-like protein 151 and its proposed lysophosphatidic acid-binding mechanism. *Acta Crystallogr D Biol Crystallogr* 2015;71:1039–50.
- [46] Choi S-H, Kim H-J, Cho H-J, Park S-D, Lee N-E, Hwang S-H, Cho I-H, Hwang H, Rhim H, Kim H-C, et al. Gintonin, a ginseng-derived exogenous lysophosphatidic acid receptor ligand, protects Astrocytes from hypoxic and Reoxygenation stresses through stimulation of Astrocytic glycogenolysis. *Mol Neurobiol* 2018.
- [47] Ohtsubo K, Marth JD. Glycosylation in cellular mechanisms of health and disease. *Cell* 2006;126:855–67.
- [48] Luo H, Zhu D, Wang Y, Chen Y, Jiang R, Yu P, Qiu Z. Study on the structure of ginseng glycopeptides with anti-inflammatory and Analgesic activity. *Molecules* 2018;23.