**Full Length Research Paper**

**An efficient protein preparation method compatible with 2-DE analysis of *Panax quinquefolius* root - a tissue riches in interfering compounds**

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Extraction of high-quality proteins from tissue like *Panax quinquefolius* root is difficult due to high levels of interfering compounds, mainly viscous polysaccharide, which obstructs the extraction of low abundant proteins. To establish an efficient method for the application of proteomic analysis to *P. quinquefolius* root, 4 sample preparation methods were tested: Acetone precipitation, ethanol precipitation, chloroform/isoamyl alcohol extraction and phenol/chloroform/isoamyl alcohol extraction. These methods were evaluated based on the quality of SDS-PAGE and two-dimensional electrophoresis (2-DE) patterns. In chloroform/isoamyl alcohol extraction, due to the different polarity, proteins were dispersed in water-phase and inter-phase. There were abundant water-soluble proteins in water-phase and fat-soluble proteins in inter-phase. In order to acquire an optimized 2-DE pattern containing as many protein spots as it can be, the both phase proteins were mixed together, and the results of SDS-PAGE and 2-DE showed well-resolved low-abundant proteins with low background. Some significantly changed spots on 2-DE gel of optimized method were identified and their potential relationships to root development were discussed. These results showed that optimized method gives satisfactory and reproductive 2-DE protein patterns. It is expected that this method can also be applied to other plant root tissues that are rich in interfering ingredients.

**Key words:** 2-DE, MALDI-TOF/TOF MS, *Panax quinquefolius* root, protein extraction, proteomics.

**INTRODUCTION**

*Panax quinquefolius* (American ginseng) root is a traditional herbal medicine that belongs to Araliaceae family. It is wildly used in many East Asian and North American countries for its extensive medicinal bioactivities such as anti-diabetic, anti-carcinogenic, anti-pyretic, analgesic, anti-aging, anti-stress, and anti-fatigue effects (Wang et al., 2008a; Lin et al., 2008; Xie et al., 2009; Luo and Luo, 2006). Since 1980s, many studies have been focused on the extraction of secondary metabolism (ginsenoside, flavone, and essential oil) from American ginseng which was thought to be the main efficacy ingredients of ginseng (Wang et al., 2007; Richtera et al., 2005; Leala et al., 2010). However, little is known about their biosynthetic pathway and metabolic regulation mechanism in plant until some putative coding genes (SQS, SQE, FPS, DS, AS etc) of ginsenoside biosynthesis were found by Liang and Zhao (2008). Even though, due to the complexity of anabolism regulation in plant, it is still difficult to study this complex process only using the limited putative sequences information obtained at gene transcription level.

2-DE is one of the most efficient and powerful methods used to study the gene expression at protein molecule level (Gygi et al., 2000). In *Panax* plant research, proteomic analysis has been employed for *Panax ginseng* (Korea ginseng) root, leaf, fruit and callus. However, the application of proteomics to *P. quinquefolius* is still limited. Lum et al. (2002) did 2-DE research on main root (at least 0.5 cm from the surface of...
Figure 1. Flow chart of the protocols for protein extraction from *P. quinquefolius* root.

MATERIALS AND METHODS

Fresh 4-year-old *P. quinquefolius* roots were purchased in Fusong, Jilin province. Samples were washed clean and transferred to a mortar. Liquid nitrogen was added immediately, and then ground to fine powder using a pestle. The powder was stored at -80°C.

Protein extraction

Protein was extracted using several protocols as summarized in Figure 1.

Acetone extraction

2 g of ground powder was suspended in four volumes of lysis buffer containing 7M Urea, 2M Thiourea, 2% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 1% phenylmethanesulfonyl fluoride (PMSF), 1% Protease inhibitors. The mixture was repeatedly frozen in liquid nitrogen and thawed at 37°C for five times and then shaken on ice for 2 h, finally centrifuged at 15000 rpm 4°C for 15 min. Then the supernatant was removed to a new tube and four volumes of ice-cold acetone were added in. The mixture was stored at -20°C for 2 h, then centrifuged as described above and washed by five volumes of absolute alcohol two times followed by ice-cold acetone until the acetone became colorless. The powder was dried at room temperature and redissolved in rehydration buffer.

Ethanol extraction

The crude supernatant obtained as described in acetone extraction, then homogenized in eight volumes of ice-cold ethanol and stored at -20°C for 2 h, then centrifuged at 15000 rpm 4°C for 15 min, and washed by five volumes of ice-cold ethanol until the ethanol became colorless. The powder was dried at room temperature and

the skin and free from lateral roots) of *P. quinquefolius*, direct extraction method was used then for sample preparation and 212 protein spots were obtained with 17 cm pH 3 to 10 linear gradient immobilized pH gradient (IPG) strip. To the best of our knowledge, this is the only report regarded to proteomic analysis of *P. quinquefolius* and few studies have focused on the proteome of whole body of *P. quinquefolius* root.

As we know, preparation of high-quality protein samples for 2-DE is the most critical for any proteomic analysis. However, proteins extracted from plant tissues are more difficult than those from other organisms due to the existence of various interference compounds. The commonly applied procedure of sample preparation for 2-DE analysis of *Panax* tissues is homogenizing the tissue in different extraction buffer for 2h, freeze thawing the mixture three times, then followed by precipitating proteins with trichloroacetic acid (TCA), acetone or TCA/acetone (Giavalisco et al., 2003; Wang et al., 2003). Nevertheless, they became insufficient when used to extract proteins from *P. quinquefolius* root which is perennial plant organ rich in interfering substances, particular saccharides such as polysaccharose and soluble starch. When preparing samples, these saccharides that have similar water-solubility with proteins are easily co-extracted, and seriously affecting the process of 2-DE.

To overcome this problem, in this study, we developed a new protocol that attempt to minimize the influence of interfering compounds especially water-solubility saccharides. In addition, other methods were compared and evaluated with the new protocol, which produced high-quality proteins were suitable for both SDS-PAGE and 2-DE studies of *P. quinquefolius* root.
reddissolved in rehydration buffer.

**Chloroform/isooamyl alcohol extraction**

2 g of ground powder was added into two volumes of Tris buffer containing 30 mM Tris-HCl at pH 7.5, 2% CHAPS, 2% 2-mercaptoethanol, 1% PMSF, 1% Protein inhibitors. The mixture was repeatedly frozen thawing five times and shaken on ice-cold for 2 h, followed by centrifuged at 15000 rpm 4°C for 15 min. The supernatant was transferred to a new tube and an equal volume of chloroform/isooamyl alcohol (24:1 v/v) was added. The mixture was shaken on ice for 40 min, and then stood at 4°C for at least 10 min until different layers appeared. After centrifugation at 12000 rpm 4°C for 5 min the mixture was divided into three phases, removed the water-phase (upper phase) and inter-phase (middle phase) to new tubes separately, and the bottom phase was discarded.

The water-phase was homogenized with an equal volume of Tris-saturated phenol solution pH 7.5, shaken on ice for 30 min followed by centrifugation at 15000 rpm 4°C for 30 min. The phenol phase was transferred to a new tube and precipitated with five volumes of methanol solution containing 0.1 M ammonium acetate. Then the mixture was stood at -20°C overnight. The pellet rediscovered by centrifugation at 15000 rpm 4°C for 15 min (Xu et al., 2008) and washed three times with cold methanol and twice with ice-cold 80% acetone. After drying at room temperature, the final pellet was re-dissolved in rehydration buffer.

The inter-phase was washed with four volumes of 80% ice-cold acetone three times, for each time the mixture was allowed stored at -20°C for 30 min, then dried the pellet at room temperature before was re-dissolved in rehydration buffer.

**Phenol/chloroform/isooamyl alcohol extraction**

The crude supernatant obtained as described in chloroform/isooamyl alcohol extraction and was then homogenized with an equal volume of phenol/chloroform/isooamyl alcohol (25:24:1 v/v). Then the mixture was shaken on ice for 30 min. After centrifugation at 15000 rpm 4°C for 30 min the mixture was divided into three phases, removed the interphase (middle phase) and organic phase (bottom phase) to new tubes separately, and the upper phase was discarded. And then the interphase and organic phase were treated as the interphase of chloroform/isooamyl alcohol extraction, respectively.

**Protein quantification**

Protein was quantified by Bradford protein assay, with bovine serum albumin as a standard. Total protein yield was calculated according to the absorbance at 595 nm (Bradford, 1976).

**Electrophoresis**

SDS-PAGE was carried out using a 12.5% polyacrylamide gel. We performed 30 μg solubilized protein per lane and equal volume of 2× sample buffer were added (volume up to 30 μl). At the beginning of electrophoresis, 10 mA/gel current was used, when bromophenol blue indicator goes across the spacer gel, transferred the current to 20mA/gel until the electrophoresis finished.

For the first-dimensional electrophoresis isoelectric focusing (IEF), the protein supernatant (containing 250 μg of protein per 125 μl) was subsequently loaded onto an IPG strip holder and put a 7 cm pH 3-10 linear gradient IPG strip (GE Healthcare) onto the holder and then rehydration for 12 h at 20°C. IEF was carried out on Ettan IPGPhor (Amersham Biosciences) system at 50 μA per strip and focusing was performed under the following conditions: a rapid gradient increase from 30 to 100 V for 1 h, 100 to 500 V for 1 h and then a linear increase from 500 to 1000 V for 2 h, 5000 V for 4000 V.h, and at last a rapid gradient of 5000 V until 6000 V.h. After IEF, the IPG strip was immediately equilibrated for 15 min with an equilibration buffer (75 mM Tris-HCl, pH 8.8, 6 M urea, 2 M thiourea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% w/v dithiothreitol [DTT]) followed for 15 min with the same buffer but containing 2.5% iodoacetamide replaced 1% DTT. The second dimension SDS-PAGE was performed on a 10×11 cm, 12.5% linear gel in a Hoffer MiniVE (Amersham Biosciences) gel system at a current of 5mA per gel for 15 min and 10 mA for 3 h. After 2-DE, gel was stained with coomassie brilliant blue R-250.

**Image analysis**

Gel image was digitalized with Image scanner 6.0 (Amersham Biosciences) and analyzed with the Image Master 2D Platinum 6.0 software (Amersham Biosciences) using a semiautomatic method, with manual correction and edition of spot features created by automatic default spot analysis settings. Total numbers of protein spots were determined.

**Protein Identification by MALDI-TOF-TOF**

Protein spots were manually excised from the preparative gel, digested with trypsin and analyzed using MALDI-TOF/TOF MS with a 4700 Proteomics Analyzer (Applied Biosystems, USA) as described by Mathesius et al. (2010). Peptides mass fingerprint was analyzed with GPS (Applied Biosystems, USA)-MASCOT (Matrix Science, UK) against the viridiplantae protein database from NCBI nr. Functional classes for the identified proteins were determined using the UniProtKB keywords, a controlled vocabulary used to index UniProtKB entries.

**RESULTS AND DISCUSSION**

Sample preparation is one of the most critical steps for acquiring high quality resolution proteins in 2-DE patterns (Yao et al., 2006). Plant roots, especially perennial roots, are rich in compounds that interfere with 2-DE. These interfering compounds such as polyphenols, nucleic acids, terpenes, especially polysaccharose and soluble starch mainly exist in water solution in various soluble forms. So, when preparing proteins they could be easily co-extracted and seriously affect the voltage of IEF, finally result in the failure of 2-DE. Therefore, as a representative of P. quinquefolius root, plant protein preparation for proteomic analysis is optimized.

**Comparison of the different methods based on SDS-PAGE patterns**

Proteins prepared using four different extraction methods from P. quinquefolius roots were first compared by SDS-PAGE (Figure 2). The remarkable differences between each method mainly focused on the high-abundance (arrow showed) and high-molecular weight protein bands.
Figure 2. SDS-PAGE separation of proteins extracted with different methods from Panax quinquefolius root. 30μg proteins/lane was separated using 12.5% polyacrylamide gel and stained with CBB-R250. Lane 1: Acetone extraction, Lane 2: Ethanol extraction, Lane 3: the waterphase of Chloroform/isoamyl alcohol extraction, Lane 4: the interphase of Chloroform/isoamyl alcohol extraction, Lane 5: the interphase of Phenol/chloroform/isoamyl alcohol extraction, Lane 6: the organic phase of Phenol/chloroform/isoamyl alcohol extraction.

In chloroform/isoamyl alcohol extraction, the high abundance protein bands were mainly enriched in waterphase (lane 3) furthermore, they also existed in low abundant ways in interphase (lane 4). Compared the interphase (lane 5) and organic phase (lane 6) bands of phenol/chloroform/isoamyl alcohol extraction, we found that the resolution and sharpness of the low abundance proteins in lane 6 were less than in lane 5. Although the high molecular weight protein bands displayed in chloroform/isoamyl alcohol extraction and phenol/chloroform/isoamyl alcohol extraction were not found in acetone extraction (lane 1) and ethanol extraction (lane 2), the other bands were also got a good resolution.

Comparison of the different methods based on 2-DE patterns

Proteins prepared were then evaluated by 2-DE patterns (Figure 3). The 2-DE patterns of acetone (Figure 3A) and ethanol (Figure 3B) extraction were similar. Meanwhile, due to the high abundance proteins in 15 to 30 kDa region, many important low abundance protein spots around them were hided, and serious horizontal streaking was caused in their 2-DE patterns. Using phenol/chloroform/isoamyl alcohol extraction, relatively clean 2-DE patterns (Figure 3E to F) were obtained but the resolution of protein spots was low and horizontal streaking still appeared in organic phase (Figure 3F).

The chloroform/isoamyl alcohol extraction displayed a good resolution (Figure 3C to D) and given more spots (Table 1). In its waterphase (Figure 3C), there were too abundant water-soluble nonprotein interference substances. This procedure is based on phenol, is a powerful protein denaturant, when added into sample supernate, proteins are denatured and dissolved in, Thereby, isolated with other water-soluble non-protein ingredients particular polysaccharose and soluble starch. To insure the maximum proteins are extracted, we repeat this procedure three times. Then the further 2-DE analysis was proceeded, though trifling horizontal streaking still appeared in the 2-DE pattern, the resolution of proteins...
Figure 3. Two-dimensional electrophoresis separation of proteins extracted with different methods from *P. quinquefolius* root. 250 µg proteins were loaded in a pH 3 to 10, 7 cm linear IPG strip. SDS-PAGE was separated using 12.5% polyacrylamide gel and stained with CBB-R250. A: Acetone extraction; B: Ethanol extraction; C: the waterphase of Chloroform/isoamyl alcohol extraction; D: The interphase of Chloroform/isoamyl alcohol extraction; E: The interphase of Phenol/chloroform/isoamyl alcohol extraction; F: The organic of Phenol/chloroform/isoamyl alcohol extraction. Some significant differences during different phases of chloroform/isoamyl alcohol extraction were showed in rectangle and ellipsoid regions.

much better than the other methods, and the background was also cleaner.

Its inter-phase (Figure 3D) is a solid phase where riches in protein pellets. Meanwhile, many solid impurities are also involved in this phase. To overcome this problem, we first washed these pellets three times with isometric water and chloroform/isoamyl alcohol, and then further washed them with acetone. This procedure had lower loss for low abundance proteins, which performance important functions in organism. So, the interphase proteins were used to do 2-DE analysis. 282 protein spots were detected in this 2-DE pattern.

Moreover, the 2-DE patterns obtained from different phases of chloroform/isoamyl alcohol extraction had
Table 1. Total number of protein spots using different extractions.

<table>
<thead>
<tr>
<th>Extractions</th>
<th>Phases</th>
<th>Number of spots</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone extraction</td>
<td>-</td>
<td>157 ±8</td>
<td>2.3</td>
</tr>
<tr>
<td>Ethanol extraction</td>
<td>-</td>
<td>159 ±6</td>
<td>1.7</td>
</tr>
<tr>
<td>Chloroform/isoamyl alcohol extraction</td>
<td>Waterphase</td>
<td>239 ±10</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>282 ±12</td>
<td>2.4</td>
</tr>
<tr>
<td>Phenol/chloroform/isoamyl alcohol</td>
<td>Interphase</td>
<td>198 ±8</td>
<td>3.2</td>
</tr>
<tr>
<td>extraction</td>
<td>Organic phase</td>
<td>187 ±10</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Figure 4. 2-DE pattern of all proteins extracted by chloroform/isoamyl alcohol method. 1.3 mg proteins were loaded in a pH 3 to 10, 24 cm IPG strip. SDS-PAGE was separated using 12.5% polyclarlamide gel and stained with CBB-R250. Arrows indicate some protein spots identified constant in *Panax quinquefolius* root protein extract.

many significant differences. To further explore these differences clearly, some significant different spots were labeled with rectangle and ellipsoid as shown in Figure 3C and D.

In order to acquire a 2-DE pattern that containing as many of protein spots existed in the waterphase and interphases of the chloroform/isoamyl alcohol extraction, we mixed the proteins of the both phase together, and used a pH 3-10, 24 cm IPG strip to do further study, finally a high resolution, good repeatability 2-DE patterns of *P. quinquefolius* root was established (Figure 4). Total 848 ± 23 protein spots were found in this 2-DE pattern.

Identification of proteins

The 24 changed significantly low abundance protein spots (Figure 4, arrows), which were on the 2-DE gel of chloroform/isoamyl alcohol method from different regions as acidic, basic and different molecular weights, were
chosen and identified by MALDI-TOF/TOF. Among them, 15 protein spots were accurately identified using the NCBInr database and listed in Table 2. So this method was proved to increase the protein identification rate.

Nucleoside diphosphate kinase (spot 1) could maintain intracellular NTP concentration of organisms may regulate cell proliferation, differentiation, development and apoptosis. RuBisCO (spot 14) is a key enzyme of photosynthesis in plants (Martel et al., 1990). Proteins related to glycol-metabolism (PFK, spot 13; ATPPC4, PFK, spot 24) were also identified in this study.

Glycine-rich RNA-binding protein (spot 6) is a cold-induced mRNA-binding protein. It can prevent the inhibition of cell proliferation and protect the cells under stress at low temperatures (Hisada et al., 1999). Catalase (CAT, spot 15) is a major antioxidant to protect cells from the toxic effects of hydrogen peroxide. The enzyme can increase the resistance of plants to oxidative stress (Tuskan et al., 2006). Luminal binding protein (BiP, spot 19) is present in more than one cellular compartment under both optimal and stress conditions (Wang et al., 2008b). It is necessary to further study for such these proteins associated with the defence on plant growth and development.

Fructose-bisphosphate aldolase (spot 12) mainly exists in the chloroplast (starch synthesis) and cytoplasm (sucrose biosynthesis), which is able to catalyze triose phosphate into 1,6 – diphosphate fructose. Konishi et al. (2005) studied found increased fructose-bisphosphate aldolase activity stimulate the glycolytic pathway to accelerate root growth.

Overall, the presented results indicated that the high resolution 2-DE pattern of total proteins obtained by chloroform/isoamyl alcohol extraction for P. quinquefolius root was established. And the optimized method to enrich the low-abundant proteins is helpful to improve the protein identification rate. Therefore the qualities of the proteins extracted by chloroform/isoamyl alcohol extraction were suitable for ginseng proteomic analysis, thereby laying the foundation to expound the growth and development and pharmacodynamic mechanisms of Panax quinquefolius root. We expect that this method can also be applied to other plant root tissues that are rich in interference ingredients.

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