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Full Length Research Paper

Protein extraction from the stem of *Panax ginseng C. A. Meyer*: A tissue of lower protein extraction efficiency for proteomic analysis

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Ginseng stem, the aerial part of the ginseng is more susceptible to diseases and environmental damages in its long process of growth. Since the molecular mechanism of why ginseng stems are vulnerable remains unclear, the comparison between healthy and pathogen suspicious tissues via proteomics approaches, such as 2-DE, could facilitate the deciphering of the pathogenesis of ginseng and improve ginseng planting industry. A major obstacle for the proteomics study of ginseng stem is the low extraction efficacy of protein due to the properties of its interfering compounds. Here, we tested six different protocols of protein extraction, and identified a protocol that gave us satisfactory yield for 2-DE analysis. The protein extraction was further optimized by chloroform/isoamylol and Tris-saturated phenol extraction that reached the standard of protein purity for 2-DE. Then, using the new extraction protocol, we can efficiently analyze the protein expression patterns of ginseng stem which might provide important information for our understanding of the disease mechanism. Also, our study would lay a foundation for the systematic analysis of the proteomics of ginseng and provide a methodological reference for other similar plant tissues.

Key words: Protein extraction, lower protein extraction efficiency, ginseng stem, two-dimensional electrophoresis.

INTRODUCTION

Ginseng (*Panax ginseng C. A.Meyer*) is a well-known Chinese traditional herbal medicine that has been used for thousands of years in China (Wang et al., 2010; Su et al., 2010; Kaufman et al., 2002; Kim et al., 2002; Keum et al., 2000; Attele et al., 1999). While, only the ginseng root was traditionally used for medical purpose, recent research indicated that the aerial parts of ginseng, such as the stem and leaf, show similar pharmacological effects as the roots (Wang et al., 2009). Since the cost of ginseng stem and leaf is much lower than the roots, their pharmaceutical value has been carefully studied in recent years (Song et al., 2009; Zhang et al., 2008; Cheng, 2000; Hou, 1977; Yip et al., 1985; Yang et al., 2000; Dou et al. 2001; Ma et al., 2005; Jackson et al., 2003). Generally speaking, the growth cycle of cultivated ginseng is four to five years, while the growth cycle of wild ginseng is over ten years. As a result of the length and moisture condition of growth, ginseng stem is susceptible to several diseases such as *Pythium debaryanum*, *Rhizoctonia solani,* Anthracnose, Black spot and *Phytophthora cactorum*, which severely deteriorate the ginseng growth (Sathiyaraj et al., 2010; Cho et al., 2007; Zhao et al., 2006). However, until recently, the prevention and control of these diseases were mainly focused on understanding the pathogen and field management, and the molecular mechanism of why ginseng stems were vulnerable to the disease, remained

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unclear (Zhao et al., 2005; Gorlach et al., 2009; Oliver et al., 2009).

Plant proteomics is a powerful method for the study of plant protein expression patterns (Oeljeklaus et al., 2009; Gygi et al., 2000; Celis et al., 1998). By comparison of two-dimensional polyacrylamide gel electrophoresis (2-DE) pattern between healthy and pathological tissues, proteins that are related to pathogenesis can be systematically analyzed (Sandh et al., 2011). Meanwhile, biosynthetic pathways and rules of the ginseng metabolites can also be assessed by comparison of proteins expression patterns under different growth conditions (Bindschedler et al., 2011). Yet, ginseng stem contains high levels of contaminants that are hard to remove, they include celluloses and pectin, pigment, polyphenols, lipids, flavonoids, etc (Wang et al., 2009). They are well known to reduce the quality and repeat-ability of 2-DE pattern, which is a common problem for proteomics study of other plants as well (Rabilloud, 1996; Rabilloud, 2010; Saravanan et al., 2004; Tsugita et al., 1999).

In this study, we compared several different protocols of protein extraction and identified a protocol that can effectively extract low content protein from ginseng stem with high level of contamination. With our new protocol, a high quality 2-DE analysis of ginseng stem proteomics becomes possible, which would lay the foundation for further study of the molecular mechanism of disease resistant in ginseng stem.

MATERIALS AND METHODS

Plant materials

Fresh ginseng stem of five years was collected in June from Fusong of Jilin Province of China. After rinsed with double distilled water (ddH_2O), they were frozen in liquid nitrogen and transported to the laboratory for storage at -80℃ until used in protein extraction.

Protein extraction preliminarily

Two washing methods (A: acetone containing 0.07% (v/v) β-mercaptoethanol and B: acetone containing 10% (w/v) trichloroacetic acid (TCA) and 0.07% (v/v) β-mercaptoethanol) and three protein extraction buffers (1. Urea lysis buffer (7M Urea, 2M Thiourea, 2% (w/v) dimethylammonio]-1-propanesulfonate (CHPAS), 50 mM dithiothreitol (DTT), 50 mM Tris, and 1% (v/v) plant protease inhibitor (Sigma), pH 8.0); 2. Tris extraction solution (30 mM Tris, 2% (w/v) CHPAS and 1% (v/v) plant protease inhibitor (Sigma), pH 8.0); 3. Sodium dodecyl sulfate (SDS) buffer extraction solution (30% (w/v) sucrose, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 50 mM Tris and 1% (v/v) plant protease inhibitor (Sigma), pH8.0)) were compared (Figure 1), with the protein yields of six protein.

Protein yield measure

Protein yields were measured by the Bradford method (Bradford, 1976) with bull serum albumin as standard, and the best one in term of protein yield for optimization was chosen thereafter.

Optimization of total protein purity

According to the potential contaminants, optimized protocol was designed as follows: 2% (v/v) β-mercaptoethanol was added to the protein sample chosen in Figure 1 in terms of protein yield, shook under 4℃ for 30 min. Equal volume of chloroform/isoamylol (24:1) was then added, shook under 4℃ for 30 min, centrifuged at 10000 rpm/min, 4℃ for 15 min, discarding the chloroform phase. The middle and upper phase were kept and equal volume of Tris-saturated phenol was added, shook under 4℃ for 30 min and centrifuged at 10000 rpm/min, 4℃ for 15 min, discarding the water phase. Four volumes of methanol containing 0.1 M ammonium acetate was added to phenol phase and incubated overnight at -20℃. And then washing solution methanol containing 0.1 M ammonium acetate and acetone was processed to eliminate contaminants for two and three times, respectively. After the complete evaporation of acetate, the proteins were dissolved in appropriate volume of rehydration solution [5 M urea, 2 M thiourea, 2% (w/v) CHPAS, 2% (w/v)N-decyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate (SB3-10)].

Protein separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and 2-DE

Total protein samples of before and after optimization were compared by 12.5% SDS-PAGE (Bio-Rad) (30 μg proteins per well). For 2-DE, the protein samples (1.5 mg) were mixed with 20 mM DTT, 5 mM Tris (2-carboxyethyl) phosphine hydrochloride, 0.5% (v/v) immobilized pH gradient (IPG) buffer (pH range of 3 to10) (Amersham), 0.3% (v/v) IPG buffer (pH range of 4 to 7) (Amersham) and rehydration solution (5 M urea, 2 M thiourea, 2% (w/v) CHPAS, 2% (w/v) SB3-10) to a total volume of 450 μl, and then isoelectric focusing (IEF) was performed at pre-established procedure {Step 30 V for 12 h (rehydration), Grad 200 V for 3 h (demineralization), Grad 500 V for 3 h (demineralization), Grad 1000 V for 3 h, Grad 8000 V for 5 h, Step 8000 V for 72000 Vhr (focusing)} by EttanTM IPGphor IITM (GE Healthcare, Uppsala, Sweden Amersham, UK) using Solid-phase, 24 cm IPG strips (pH range of 3 to 10) (Amersham). Following IEF, the proteins in the strips were reduced with 1% (w/v) DTT in 15 ml of equilibration buffer {6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 70 mM Tris-HCl, pH 8.8} for 15 min and alkylated with 2.5% (w/v) iodoacetamide in 15 ml of equilibration buffer for 20 min. Finally, the second-dimension electrophoresis was performed by vertical 12.5% SDS-PAGE (Amersham) under a constant power of 15 W/Strip for 6 h.

Gel staining and analysis

The gel was stained by Coomassie bright blue R-250 overnight and destained by 10% (v/v) ethanol and 10% (v/v) acetic acid until the background was clear. And then the gel was imaged with Image Scanner (Amersham) and analyzed by Image Master 2D Platinum6.0 software (GE Healthcare, Uppsala, Sweden Amersham, UK).

RESULTS AND DISCUSSION

Protein yields

Protein yields extracted by the six processes are shown

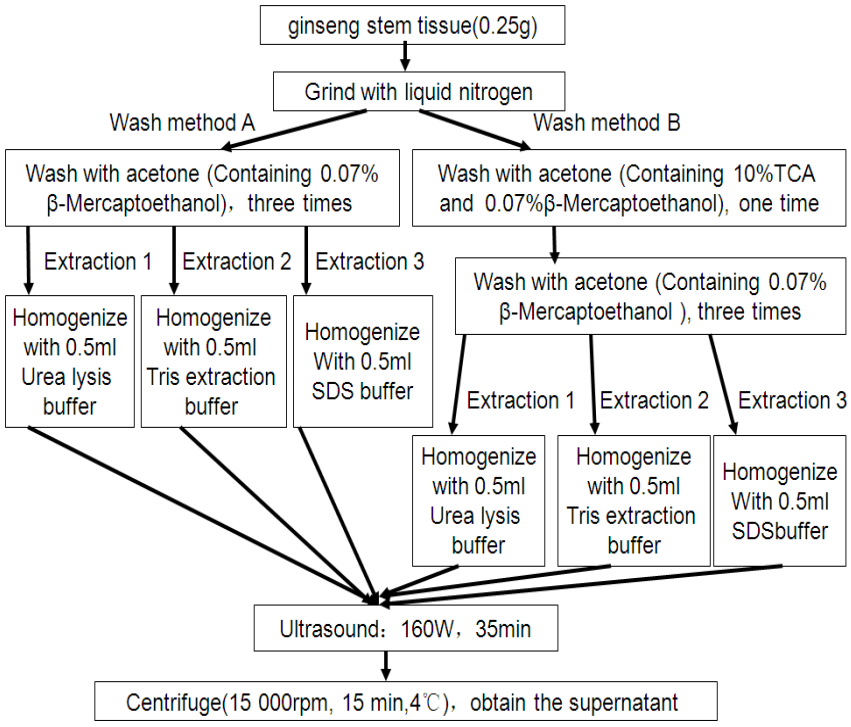

Figure 1. Flow chart of six protein extraction methods.

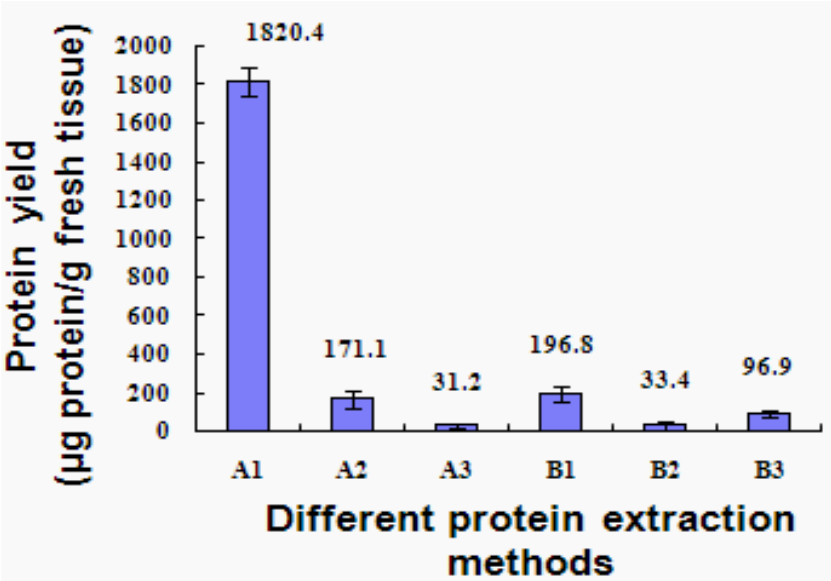

Figure 2. The effects of protein efficiency of six protein extraction methods (named A1, A2, A3, B1, B2 and B3, respectively) were analyzed.

in Figure 2. For washing method B, the protein yields by three protein extraction solutions were all too low, as a result of the higher protein loss ratio induced by TCA, and for the washing method A, urea lysis buffer could obviously dissolve higher protein yield, and others were not suitable for the dissolution of proteins of ginseng stem. So method A1 was suitable for the protein extraction of ginseng stem, and extraction efficiency of other methods were too low (lower than 200 μg protein/g fresh tissue) with possible depressed repetition rate and reduced spots in the 2-DE pattern. Only method A1 was suitable for protein extraction of ginseng stem in terms of

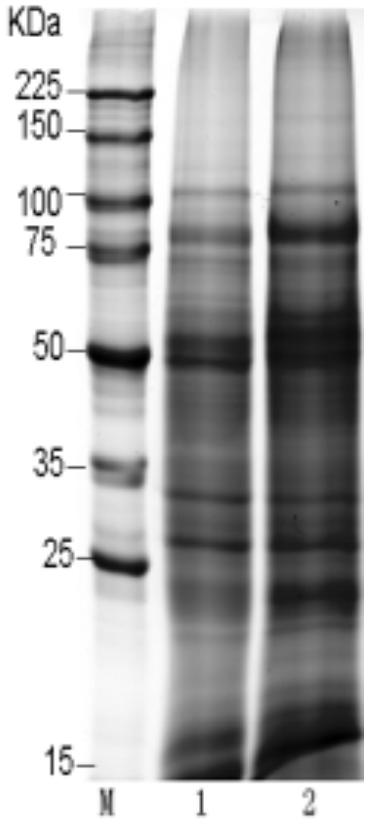

Figure 3. SDS-PAGE pattern of ginseng stem. Lane 1: After optimization; lane 2: before optimization.

extraction efficiency.

SDS-PAGE and 2-DE

The SDS-PAGE pattern of before optimization (lane 2 in Figure 3) showed that not only the bands were less and blurring, but the background was also deep. This indicated that although, the protein yield extracted by method A1 was enough, the elimination of contaminants was inadequate and that the protein sample possibly contained many small-molecules and nucleic acids, polyphenols, lipids, etc. When isoelectric focusing (IEF) was processed by Ettan^{TM} IPGphor II^{TM} (GE Healthcare, Uppsala, Sweden Amersham, UK) using solid-phase, 24 cm strip (pH range of 3 to 10) and 1.5 mg of sample, the desalination process took a long time and the current was too large so that thorough process could not be accomplished. The voltage of IEF could not reach the minimum, which resulted in failure of the IEF procedure. It was possible that the contamination was too high to meet the condition of protein focusing. So, the proteins extracted by A1 should be optimized further. Firstly, the addition of 2% (v/v) β-mercaptoethanol to the protein samples extracted by A1 could break disulfide bonds for better dissolution of proteins. Equal volume of chloroform/isoamylol (24:1) was then added to promote the three phases. The proteins were mainly contained in middle and upper phase, while the chloroform phase containing most fat-soluble components was discarded. But the middle and upper phase also contained some contaminants such as nucleic acids, polyphenols and polysaccharides. In order to eliminate these contaminants, equal volume of Tris-saturated phenol was added to the middle and upper phase. After centrifugation, the water phase possibly containing nucleic acids and also polyphenols, polysaccharides, etc was discarded, and because of the strong degeneration of phenol, the proteins mostly retained in phenol phase resulted to lower protein loss. After optimization, the bands were clearer (lane 1 in Figure 3) than those without optimization (lane 2 in Figure 3) and the back-ground was greatly improved. So the optimization protocol reached expected goal. The protein sample of after optimization could be analyzed by 2-DE thereafter. When IEF was processed by Ettan^{TM} IPGphor II^{TM}, the desalination process of step two and three operated normally and the current was lower to accomplish the process on schedule. From Figure 4, the

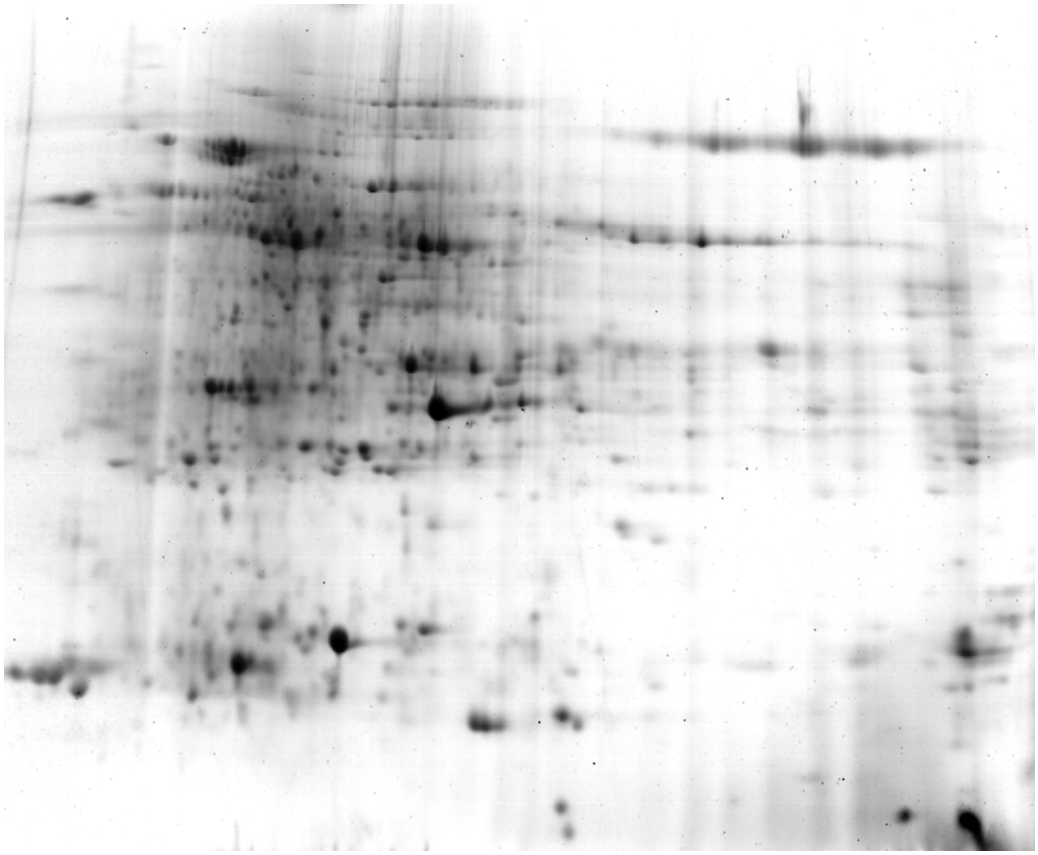

Figure 4. 2-DE pattern of ginseng stem protein after optimization. Horizontal: IEF (24 cm, PH3-10), vertical: SDS-PAGE (12.5%).

2-DE pattern, the background was clean and the protein spots were clear and smooth, which further verified the rationality of protein optimization scheme. By Image Master 2D Platinum6.0 software analysis, the spots reached 1250 ± 26 and mainly focused on acidic region (pH 3 to 7.5). In order to separate more spots that covered all regions, especially the alkaline region that probably contained some special active proteins, the pH range of the strip was appropriate. In comparison with the 2-DE pattern of ginseng leaf obtained by Myung et al. (2003), we did not only improved the background and spots quality, but also obtained more spots on a condition that is more difficult by using much harder ginseng tissue than the leaf. The quality of our 2-DE pattern is fit for further comparative analysis and mass spectrum identification.

Conclusion

Thus, a protein extraction method suitable for lower protein extraction efficiency of plant tissue-ginseng stem was established successfully and higher quality 2-DE pattern was obtained, which could lay foundations of the study of ginseng diseases and make maximum utilization of ginseng resource and booming of ginseng planting industry. Meanwhile, our study would provide large reference for the proteomics research of other similar plant tissues.

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