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Establishment and Optimization of Two-dimensional Electrophoresis (2-DE) Technology for Proteomic Analysis of Ramie

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Abstract

This research aimed at establishment of a two-dimensional electrophoresis that is suitable for the proteomic analysis of ramie's various parts by optimizing the traditional TCA/acetone protein extraction method: protein purification (4 times volume of acetone), and IPG strips (pH 4–7, 17 cm). This technology is applied to the roots, stems and leaves of ramie. Compared with traditional method, this technology can effectively remove the non-protein impurities from ramie, the protein spots obtained from it (823±15) significantly higher than the traditional (320±8) method, and it is suitable for the different parts of ramie and the requirement of protein identification by Mass Spectrum; The number of protein in the stem, roots and leaves were 280±6, 1093±20 and 765±13, respectively. The method used provides reliable technical support to the proteomic study of ramie that will aid the understanding of higher yield, higher quality, stress tolerance, and disease resistance. © 2013 Friends Science Publishers

Keywords: Ramie; Proteomics; Protein extraction; Two-dimensional gel electrophoresis; TCA/acetone method
Abbreviations: 2-DE, two-dimensional gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl) dimethylam-monio] propanesulfonic acid; DTT, dithiothreitol; IAA, Iodoacetamide; IEF, isoelectric focusing; IPG, immobilized pH gradient; PMSF, phenylmethanesulfonyl fluoride; pI, isoelectric point; Rubisco, ribulose bisphosphate carboxylase/oxygenase; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

Introduction

Ramie (*Boehmeria nivea* L.) is a member of Urticaceae from the genus *Bochmeria*. It is a perennial bast fiber crop originating from China, and known as "China grass". Ramie is mainly planted in China and other Asian countries, such as the Philippines, India, South Korea and Thailand (Liu *et al*., 2001). The current research focus is how to improve ramie's yield, quality, stress tolerance and disease resistance. These issues have been researched from the genomic perspective (Liu *et al*., 2008, 2009; Wang *et al*., 2009, 2010; Li *et al*., 2010). Genomics divides into structural genomics and functional genomics, both of which study cellular mRNA based on the assumption that the level of mRNA in the cells represents the level of protein expression. To date the genome of many species has been sequenced (such as human and rice), but only 1–2% of the genome encodes protein, and more than half of them currently have unknown functions (The International Human Genome Sequencing Consortium, 2001; Francis *et al*., 2003), and the effect and number of minor genes are also unclear today (Rahman *et al*., 2002). However, it has been verified by research that there are mistakes in the genome sequencing. For instance, 8% of the *Mycoplasma genitalium* genome proved to be incorrectly sequenced (Brenner, 1999). Also, there is no significant correlation between mRNA abundance and protein abundance, especially for low abundance proteins (Anderson and Anderson, 1998; Gygi *et al*., 1999; Ideker *et al*., 2001). Moreover, different proteins may be produced by the same gene with their abundance varying greatly under different life activities due to the complex process of mRNA processing, protein hydrolysis and post-interpretation modification. The abundance of housekeeping proteins, which are responsible for signal transmission may change by 10^6 (Pandey and Mann, 2000). Proteomic analysis detects the gene expression during different life activities (e.g. disease resistance and stress resistance) according to the variation in protein expression level (Anderson and Anderson, 1998). Proteomics can give a more accurate understanding than genomics on cell metabolism, enzymes, etc. (Rose *et al*., 2004). However, other aspects can only be

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studied by proteomic analysis, such as subisoform changes, post-interpretation modification, protein-protein interactions, and molecular formation of organelles (Pandey and Mann, 2000).

Proteomics studies the relevant properties according to the difference in protein expression levels and post-interpretation modification by means of protein separation and identification. Two dimensional electrophoresis (2-DE) is the most commonly used technology to separate hundreds of proteins from plant tissues (Görg *et al*., 2004). For proteomic analysis, protein extraction and preparation are key steps for the success of 2-DE (Isaacson *et al*., 2006). Protein extraction and preparation methods mainly include: TCA/acetone, phenol, chloroform/acetone, phenol/ ammonium acetate, and Tris base/acetone (Wang *et al*., 2008; Jesús *et al*., 2009; Xie *et al*., 2009). Protein movement in the second phase of electrophoresis can be seriously affected due to contamination by non-protein substances present after protein extraction (Görg *et al*., 2000). These non-protein substances include complex compounds from the plant cells, such as polysaccharides, nucleic acids, and lipids; secondary metabolites, such as phenols, flavonoids, lignins, and tannins (Shaw and Riederer, 2003; Görg *et al*., 2004; Rose *et al*., 2004; Saravanan *et al*., 2004; Vâlcu and Schlink, 2006). Different species or organs of the same species may contain different amounts of non-protein substances. Organs from ramie contain a large amount of polysaccharides and secondary metabolites. The polysaccharide content of ramie (dry weight) is 47.16–50.17% (Li *et al*., 2004), the flavonoids content 2.74–6.62% (He *et al*., 2010), the average chlorogenic acid content in leaves 0.35% (Zhao e*t al*., 2003), the total phenolic acids content 0.05% (Liu *et al*, 2011), the tannin content 2.93–6.02 mg/g, and the oxalic acid content 3.80–4.34 mg/g (Zeng *et al*., 2011). The presence of these compounds will affect the results of the 2-DE. Therefore, the optimum protein extraction method is required to effectively remove the non-protein substances from the tissues and different methods are applicable to different plant parts (such as roots, stems and leaves) (Isaacson *et al*., 2006). This paper firstly establishes an optimized protein extraction method and a 2-DE technology based on the study of protein extraction and purification in ramie's various parts. This study will provide technical support to proteomic analysis of ramie.

Materials and Methods

Experimental Material Preparation

The experimental material was Hua Zhu #5 (biennial ramie from experimental base of Hua Zhong Agricultural University).

The experiment used pot plants. Each pot was 40 cm in height and 30 cm in diameter, containing the same volume of fine sand 5 cm below the pot surface. One plant of ramie was grown in each of the 10 pots. The pots were placed in a greenhouse with natural lighting at 25–30°C. The pot management was the same as in the field. The young leaves and stem samples were harvested (phloem fibers) when the ramie plants had grown to s height of 15–20 cm. Hua Zhu #5's young shoots were subjected to hydroponic culture in the same greenhouse. The young root samples were harvested for the experiments when the roots grew to the length of 3–5 cm.

Reagents

DTT, SDS, 30% polyacrylamide solution and two types of IPG strips (pH 3–10, 17 cm, non-linear; pH 4–7, 17 cm, linear) were purchased from Bio-Rad. All other reagents were purchased from Sigma.

Protein Extraction and Preparation

The most commonly or traditionally used TCA/acetone protein extraction method was adopted (Vâlcu and Schlink, 2006; Xu *et al*., 2008). For each of the plant sections (roots, stems and leaves) 1 g of the experimental materials was fully ground into powder in liquid nitrogen, and 10 mL of extract, cold (–20°C) 10% TCA-0.07% β-mercaptoethanol -0.01% of 100 mmol/L PMSF in acetone. The mixture was left to stand overnight at –20°C. The mixture was centrifuged at 12000 rpm, 30 min, 4°C and the supernatant discarded; the precipitate was washed with 10 mL of cold 80% acetone containing 0.01% of 100 mmol/L PMSF before being placed in the freezer at –20°C for 1 h. The tube was centrifuged again at 12000 rpm, 30 min, 4°C and the supernatant was discarded. The procedure was repeated three times until the precipitate turned white or green. After vacuum drying, the precipitate was made into dry protein powder and weighed.

Cleavage method as following, Protein lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT) was added to the dry protein powder at 20 μL/mg, and kept at room temperature for 2 h. The tube was put into the flume of ultrasound cleaner, which was run for 10 min at temperature 20–25°C. The tube was removed and centrifuged at 17500 rpm, 20°C, 30 min and the precipitate discarded. The supernatant contained the protein sample solution. The samples were divided into two groups, of which one group was subjected to cleavage following the above traditional method (Vâlcu and Schlink, 2006; Xu *et al*., 2008); for the other group, we added an extra protein purification step by adding four fold volume of 100% acetone (–20°C) in the sample solution, and left the mixture stand 1.5 h at –20°C. After centrifuged at 12000 rpm, 15 min, 4°C and the supernatant discarded, about 1/2 volume of the former protein sample solution using protein lysis buffer was added to dissolve the precipitate (protein). The Bradford method was adopted for the determination of protein concentration (Bradford, 1976).

Two-dimensional Electrophoresis (2-DE)

The loading amount of the sample was 150 μg/strip (silver stain), 600 μg/strip (Coomassie brilliant blue stain) and lysis buffer was added until reaching the volume of 300 μL. After passive hydration for 12–14 h at room temperature, isoelectric focusing program was: 250 V 10 min, 500 V 30 min, 1000 V 1 h, 9000V 5h, 50000 VH. The strips were put into 5 mL of equilibrium buffer (6 M Urea, 2% SDS, 0.375 M, pH 8.8, Tris-HCl, 20% Glycerol); 0.05 g of DTT was added with gentle shaking on a shaker for 15 min in order to reach the first equilibrium; the strips were placed in the equilibrium buffer again; 0.255 g of IAA was added with gentle shaking for 15 min to reach the second equilibrium. A 12% polyacrylamide gel was used for the second phase of electrophoresis, according to the following program: 10 mA, 1 h; 30 mA, 3.5 h. The gels were visualized using the Silver and Coomassie brilliant blue staining method referred to in Yan *et al.* (2000) and Giovanni *et al.* (2004) respectively. SDS-PAGE two-dimensional electrophoresis was repeated for three times.

Results and Discussion

Protein Extraction and Preparation

Protein extraction and preparation are key steps for the success of the 2-DE (Isaacson *et al*., 2006). A large amount of complex macromolecules and secondary metabolites are usually contained in the plant tissues, placing certain limitations on the establishment of 2-DE (Shaw and Riederer, 2003; Görg *et al*., 2004; Rose *et al*., 2004; Saravanan *et al*., 2004; Vâlcu and Schlink, 2006). In particular, secondary metabolites have greater impact on 2-DE, such as phenols, which are easy to be oxidized during protein sample preparation resulting in irregular protein dots and streaks in the 2-D electrophoretogram image (Vâlcu and Schlink, 2006). The presence of pigments, polysaccharides and lipids also has an effect on the results of 2-DE (Damerval, 1986). As can be seen from Fig. 1 the optimized TCA/acetone extraction method was better at removing these impurities. However, in the absence of purification, there were still many impurities in the protein sample solution (traditional method) (Fig. 1A). This is because ramie contains a large amount of substances such phenols, and organic acids (Kong *et al*., 2011), which require protein purification. After cleavage reaction, a four-fold volume of pre-cooled acetone was added for protein purification, to obtain a clear and impurity-free electrophoretogram (Fig. 1B). The greatly obscure background of the electrophoretogram is obtained by traditional method, which indicated a large amount of substances were not wiped out in the sample. Meanwhile, compared with the number of protein spots of traditional and optimized method, more protein spots obtained from the optimized (823±15) than the traditional(320±8) method. In addition, ramie proteins were mainly distributed in the pH 4–7 region (Fig. 1). Therefore, pH 4–7 linear strips were selected in the technology to increase the protein resolution.

Results of Two-dimensional Electrophoresis of Proteins in Ramie's Different Organs

The optimized two-dimensional electrophoresis technology used in this experiment can be applied to ramie's roots (A), stems (C), and leaves (B), to obtain clear electrophoresis patterns. The number of protein spots were 1093±20, 280±6and 765±13, respectively (Fig. 2). The protein spots for the stem sample were detected in significantly small quantities compared with the root and leaf samples. This is because ramie is perennial bast fiber crop, and the phloem is the major transport tissue and less involved in other physiological functions. Higher-abundance proteins were found in the leaves (oval area of Fig. 1 and Fig. 2B), which affected the resolution of lower-abundance proteins to some extent. The reason is that the presence of high-abundance photosynthetic proteins in leaves (e.g., Rubisco) affects the separation and detection of low-abundance proteins (Wang *et al*., 2008).

To be better suitable for the requirement of protein spot identification by Mass Spectrum, the loading quantity used was 600 μg and Coomassie Brilliant Blue procedure was used to stain 2-DE gels too, where a clear protein 2-DE electrophoretogram also can be obtained. As showed in Fig. 3, about 780±17 protein spots were detected on 2-DE image. Therefore, the optimized 2-DE technology for proteomic analysis of Ramie established in this paper can be used to the future study of proteomic researches.

Conclusion

The optimized two-dimensional electrophoresis technology for ramie consists of the optimized TCA/acetone extraction method, protein purification using a four-fold volume of pre-cooled acetone after the cleavage reaction; isoelectric focusing for 50000 VH. Compared with traditional method, this optimized technology can effectively remove the non-protein substances from the samples, and is applicable to ramie's different organs (roots, stems and leaves), as well as the requirement of protein spot identification by Mass Spectrum. Proteins occurred in the least amount in stems, while the roots had the most protein spots. It should provide the reliable technical support to study ramie for high yield, good quality, stress tolerance, and disease resistance plants from a proteomics perspective after this technology was established.

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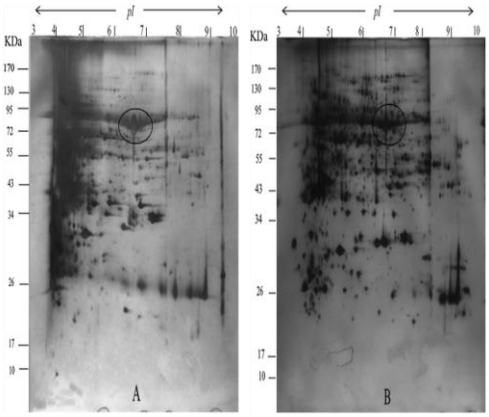

Fig. 1: Two-dimensional electrophoretogram of proteins from leaves of ramie by the traditional and optimized method
TCA/acetone extraction method was applied; sample loading amount was 150 μg/strip; IPG strip used was pH 3–10, 17 cm, non-linear; 12% polyacrylamide gel, silver staining
A. Traditional method; B. Optimized method. Oval area shows the high-abundance protein

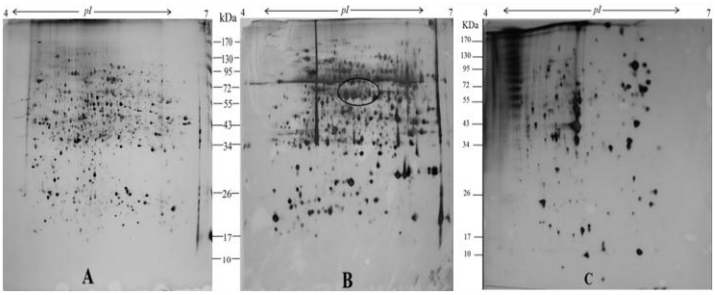

Fig. 2: 2-DE pattern of proteins in ramie's roots, stems and leaves using optimized 2-DE technical system
An optimized TCA/acetone extraction method was used; sample loading amount, 150 μg/strip, pH 4–7, 17 cm, linear, 12% polyacrylamide gel, silver staining. (A) roots, (B) leaves, (C) stems. Oval area shows the high-abundance protein

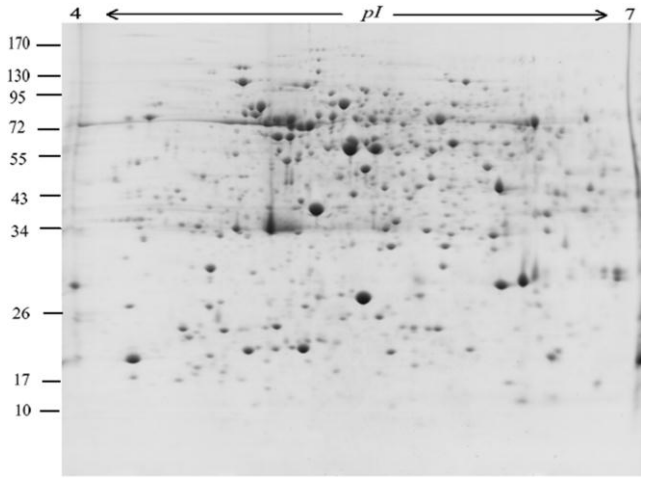

Fig. 3: 2-DE electrophoretogram of ramie's leaves using Coomassie Brilliant Blue staining
An optimized TCA/acetone extraction method was applied; sample loading amount was 600 μg/strip; IPG strip used was pH 4–7, 17 cm, linear; 12% polyacrylamide gel, Coomassie Brilliant Blue staining

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