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## Preparation of Protein Samples for 2-DE from Different Cotton Tissues

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### 1. Introduction

Cotton today is one of the most important economic crops. Cotton fiber is the most used material for the textile industry, and takes an important strategic status in the world economy. Proteomics is one of the most important techniques in the post-genome era, and two-dimensional electrophoresis (2-DE) is one key technology for proteomics. Protein extraction and sample preparation are of prime importance for optimal 2-DE results (Isaacson et al., 2006). The cotton is a highly recalcitrant plant material and rich in compounds such as polysaccharides, polyphenols, nucleic acids, cellulose, and other secondary metabolites, which interfere with protein extraction, produce highly diluted protein extracts, and affect protein migration in 2-DE (Görg et al., 2000). Moreover, modified or different protein extraction method should be used for different cotton tissues (e.g., leaves, roots, seeds, and stems), which contain different secondary metabolites.

Here, several protocols to extract total proteins for different cotton tissues are described based on methods routinely used in our laboratory.

### 2. Materials

Ultrapure water (doubly distilled, deionized, > 18 MΩ) is used for all reagent preparation. Reagent grades should be of the highest quality.

1. 1 M Tris-saturated phenol (pH 8.0)
2. Extraction buffer: 0.1 M Tris-HCl, pH 8.0, containing 30% w/v sucrose, 2% w/v SDS, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2% v/v thioglycol 2-mercapitoethanol (2-ME).
3. Lysis buffer: 7 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM DTT, and 0.5% v/v carrier ampholytes.
4. Equilibration buffer: 6 M urea, 20% w/v glycerol, 2% w/v SDS, and 50 mM Tris-HCl, pH 8.8.
5. Staining solution: 0.12% w/v Coomassie brilliant blue G-250, 10% w/v ammonium sulfate, 10% w/v phosphoric acid, 20% v/v methanol.

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### 3. Methods

All plant tissue samples should be ground to fine powder with a pre-chilled mortar and pestle in liquid nitrogen. Before grinding, silicon dioxide (SiO<sub>2</sub>) and polyvinylpolypyrrolidone (PVPP, 10% w/w of sample weight) were added into mortar. The finely ground powder (ca. 0.5 g per tube) was immediately transferred into a 10-mL centrifuge tube (**see Note 1**) precooled in liquid nitrogen. The powder sample can be immediately used or stored in a -80 °C freezer until protein extraction.

#### 3.1 Extraction from leaves

- The powder sample was resuspended in 4 mL 10% v/v trichloroacetic acid (TCA) in acetone (**see Note 2**) and extensively homogenized.
- Centrifuge (12, 000g at 4 °C) for 5 min, the pellet of proteins was washed once with 5 mL 0.1 M ammonium acetate in 80% v/v methanol and once with cold 80% v/v acetone.
- The pellet was dried in vacuum (**see Note 3**).
- It was resuspended in 3 mL of extraction buffer.
- An equal volume of 1 M Tris-saturated phenol (pH 8.0) was added, and then the mixture was homogenized on ice for 5 min. The upper phenol phase was collected after centrifuge (12, 000g at 4 °C) for 5 min, and this extraction step was repeated once (**see Note 4**).
- The total phenol phase was transferred into a new tube, and an equal volume of extraction buffer was added into the total phenol phase, and then the mixture was homogenized on ice for 5 min. The upper phenol phase was collected after centrifuge (12, 000g at 4 °C) for 5 min (**see Note 4**).
- The proteins were precipitated with five volumes of 0.1 M ammonium acetate in methanol overnight at -20 °C.
- Centrifuge (12, 000g at 4 °C) for 10 min. The collected protein pellets were washed once with 3 mL methanol, and then washed once with 3 mL cold 80% v/v acetone in water. The pellets were dried in a freeze vacuum dryer for 10 min and stored at -80 °C.

#### 3.2 Extraction from roots (**see Note 5**)

- The powder sample was resuspended in 3 mL of extraction buffer.
- An equal volume of 1 M Tris-saturated phenol (pH 8.0) was added, and then the mixture was homogenized on ice for 5 min. The upper phenol phase was collected after centrifuge (12, 000g at 4°C) for 5 min, and this extraction step was repeated once (**see Note 4**).
- The total phenol phase was transferred into a new tube, and an equal volume of extraction buffer was added into it. The mixture was homogenized on ice for 5 min, and the upper phenol phase was collected after centrifuge (12, 000g at 4°C) for 5 min (**see Note 4**).
- Precipitation the phenol phase with 5 volumes of 0.1 M ammonium acetate in methanol overnight at -20°C.
- Centrifuge (12, 000 g at 4°C) for 10 min. The protein pellets were washed once with 3 mL methanol, and then washed once with 3 mL 80% v/v acetone in water. The pellets were dried in a freeze vacuum dryer for 10 min and stored at -80 °C.

### 3.3 Extraction from fibers (see Note 6)

- The ground powder was resuspended in 4 mL acetone and extensively homogenized. The sample was kept at -20 °C overnight.
- Centrifuge (8,000g at 4 °C) for 5 min (see Note 6). Discard the supernatant and the pellets were washed once with 4 mL acetone containing 2% v/v 2-ME.
- Centrifuge (10,000 g at 4°C) for 5 min, and the wash was repeated once.
- Discard the supernatant and the pellet was dried in vacuum.
- Resuspend the pellet with 4 mL of extraction buffer.
- An equal volume of 1 M Tris-saturated phenol (pH 8.0) was added and homogenized on ice for 5 min. Centrifuge (12,000g at 4 °C) for 5 min.
- The upper phenol phase was collected. The phenol extraction procedure was repeated once.
- The collected phenol phase was precipitated with 5 volumes of 0.1 M ammonium acetate in methanol overnight at -20 °C.
- Centrifuge (10,000g at 4 °C) for 10 min. Discard the supernatant and the pellet was washed twice with cold 0.1 M ammonium acetate in methanol. Wash twice with cold 80% acetone in water. The pellet was dried in a freeze vacuum dryer and stored at -80 °C.

### 3.4 Proteins pellet resuspension

Proteins pellet was resuspended in lysis buffer and shaken for 1 h (IKA Vortex Genius 3, Staufen, Germany). After centrifugation at 15,000g for 20 min to remove debris, the supernatant could be used immediately for first-dimensional IEF gels. Protein concentration was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

### 3.5 Two-dimensional gel electrophoresis

The 2-D gel electrophoresis (2-DE) protocol was adapted by O'Farrell (1975). The first electrophoresis was performed using immobilized pH gradient (IPG) strips on an IPGphor isoelectric focusing (IEF) system (Amersham Pharmacia, San Francisco, CA). For example in our experiment, the IPG strips (13 cm, 3–10 nonlinear pH gradient; GE Healthcare, Piscataway, NJ) were rehydrated with 250 µl of rehydration buffer (containing 370 µg proteins). Focusing was then performed at 20 °C as follows: active rehydration at 30 V for 12 h, 200 V for 2 h, 500 V for 3 h, 1,000 V for 4 h, 8,000 V for 5 h, with a gradient increase in voltage between 8,000 V and 40,000 V. After IEF, the proteins in the strips were reduced with 1% w/v DTT in 10 ml of equilibration buffer for 15 min and alkylated with 2.5% w/v iodoacetamide in 10 ml of equilibration buffer for 15 min. The strips were transferred onto vertical 10.5% w/v SDS-PAGE selfcast gels. The second electrophoresis (SDS-PAGE) was performed on an Amersham Hoefer SE 600 system (Amersham Pharmacia) at 10 mA for 1 h and 20 mA for 6 h at 15 °C.

### 3.6 Protein visualization

The 2-DE gel was stained with blue silver (Candiano et al., 1975). The gel was fixed in a solution of 40% v/v methanol and 10% v/v acetic acid for 30 min, washed in distilled water 4 times for 15 min, and finally incubated in a staining solution (see Note 7) overnight with gentle shaking. The gel was decolorized in distilled water.

As shown in Figure 1, a great many of protein spots were detected on the 2-DE image. The crude protein yield and the number of protein on 2-DE gels are also summarized in Table 1.

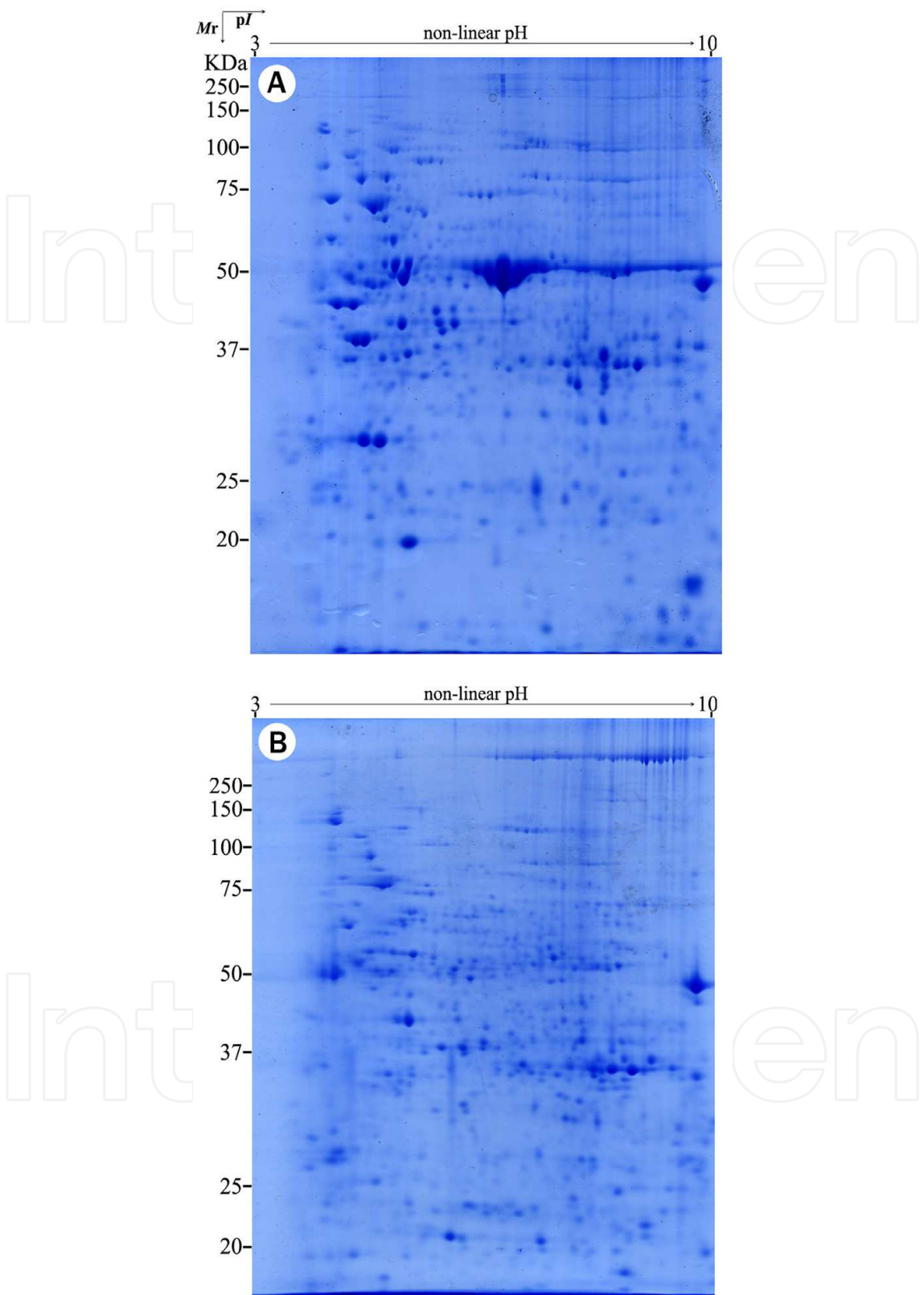


Fig. 1. Two-dimensional gel electrophoresis gel of proteins extracted from cotton leaves (A) and roots (B). Proteins (370  $\mu$ g) were separated on a 13-cm pH 3–10 nonlinear gradient immobilized pH gradient strip and on 10.5% SDS-PAGE gel. The gels were stained using the blue-silver method. Mr, molecular mass; pI, isoelectric point.



Tissues	Protein yield (mg/ g; crude protein/ powdered tissue)	The number of protein on 2-DE gels
Leaves	8.7	About 900
Roots	5.1	About 830
Fibers	4.6	About 850

Table 1. The crude protein yield and the number of protein on 2-DE gels for different extraction methods. Proteins (370 µg) were separation on 13cm pH 3-10 non-linear gradient IPG strip and 10% SDS-PAGE gel. The gel was stained using Blue silver [4]. The above results are from different experiments and represent initial results. It can provide preliminary reference for selecting extraction method.

These results demonstrate that above protein extraction methods could be compatible with cotton different tissues.

4. Notes

1. Small plant samples can yield a sufficient amount of protein for 2-DE. Furthermore, using small plant samples easily extract the high purity of protein.
2. These mentioned solutions except for lysis buffer were stored at 4 °C and cooled on ice in advance for use.
3. A good principle for drying sample is that the edge of protein pellets turned white, and too dry samples is bad for resuspension.
4. To reduce loss of protein, this phenol extraction step was repeated once. However, some water soluble organic (such as polysaccharides and nucleic acids) was involved in collected total phenol phase and resulted in horizontal streaking visible on two dimensional gels. Once more wash step with an equal volume of extraction buffer was performed to remove water soluble organic in phenol phase.
5. TCA-acetone precipitation can effectively remove pigments (Xie et al., 1975). There are not too many pigments such as chlorophyll in cotton roots, so we could get the high purity protein samples if step 1-3 of “**Extraction from leaves**” was removed.
6. The method described here was adapted from published paper with minor modifications. Centrifugation at lower speeds (step 2) is beneficial to resuspend protein pellets. It, sometimes, is the necessary to use auxiliary tool for scraping pellets. Using TCA-acetone instead of acetone (step 1) is helpful for pellets’ resuspension, although TCA-acetone precipitation could increase the loss of protein (Görg et al., 2004).
7. To prepare blue silver staining solution, phosphoric acid and distilled water (10% of the final volume) firstly were mixed, and then ammonium sulfate fine powder was added and completely dissolved on magnetic stirrer. Next, 0.12% (w/v) coomassie brilliant blue G-250 was added and stirred at least 2 hour. Fill to 80% final volume with distilled water, sequentially add 20% volume methanol and mix thoughly. These undissolved particles in staining solution were not needed to filter out and they can increase sensitivity of staining solution.

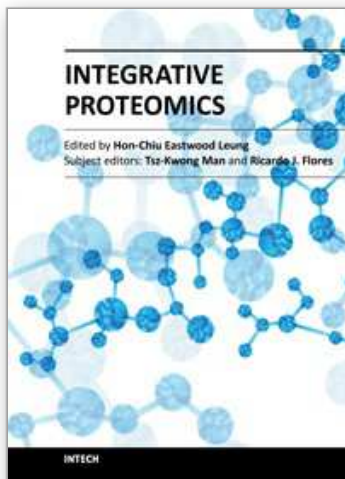
5. Acknowledgments

“Extraction from leaves” was adapted from Wang et al. method with modifications (Wang et al., 2003, 2006) and “Extraction from fibers” was based on the method previously

described by Yao et al. (2006), with slight modifications. Furthermore, this work was supported by the National Natural Science Foundation of China (Grant no. 30771388), the Natural Science Foundation Project of CQ CSTC (Grant no. 2009BB1123) and the Fundamental Research Funds for the Central Universities (Grant no. XDJK2011C016).

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