

Phenol Extraction of Proteins for Proteomic Studies of Recalcitrant Plant Tissues

Mireille Faurobert, Esther Pelpoir, and Jamila Chaïb

Summary

Phenol extraction of proteins is an alternative method to classical TCA-acetone extraction. It allows efficient protein recovery and removes nonprotein components in the case of plant tissues rich in polysaccharides, lipids, and phenolic compounds. We present here a tried and tested protocol adapted for two dimensional electrophoresis (2-DE) and further proteomic studies. After phenol extraction, proteins are precipitated with ammonium acetate in methanol. The pelleted proteins are then resuspended in isoelectric focusing buffer, and the protein concentration is measured with a modified Bradford assay prior to electrophoresis.

The important points for successful use of this protocol are (1) keeping samples at very low temperature during the first step and (2) careful recovery of the phenolic phase after the centrifugations, which are major features of this protocol.

Key Words: extraction method; proteins; phenol; plant proteomic; membrane proteins; two-dimensional gel electrophoresis; glycoproteins.

1. Introduction

Plant protein extraction is the first step in proteomic studies. Plant tissues contain relatively low levels of proteins whose extraction is often rendered difficult by the presence of other compounds, such as cell wall and storage polysaccharides, lipids, and phenolic compounds. The solubility of plant proteins is closely associated with their intracellular localization, and proteins are classically extracted by either aqueous buffer, detergents, or direct precipitation (1). Besides the most commonly used trichloroacetic acid (TCA)/acetone precipitation method (2), phenol extraction followed by methanol/ammonium acetate precipitation was reported by Hurkman and Tanaka (3) in 1986 for proteomic studies. The authors emphasized the efficiency of the method in removing

nucleic acids, which interact with proteins and give poor resolution and high background in two-dimensional electrophoresis (2-DE).

Phenol extraction was first developed to purify (deproteinize) carbohydrates and then nucleic acids. For molecular biologists, phenol extraction is now the standard and preferred way to remove proteins from nucleic acid solutions.

Phenol is the simplest aromatic alcohol; it contains a polar [OH] group bound to an aromatic ring. It exhibits weak acidic properties and is corrosive and poisonous. Phenol is partially miscible with water: when saturated with water the aqueous layer contains about 7% phenol and the organic layer about 28% water. It interacts with proteins mainly via hydrogen bonding and causes proteins to become denatured and soluble in the organic phase. Then, contrary to widespread belief, proteins are not in the interface but in the phenol phase.

The phenol extraction method is mainly reported for recalcitrant plant tissues or organs such as wood (4) potato and rapeseed seedlings (5); potato, apple, and banana leaves (6); olive leaf (7); and tomato, avocado and banana fruits (8).

Comparison of TCA/acetone and phenol extraction protocols led Carpentier et al. (6) and Saravanan and Rose (8) to the observation that the two methods were efficient in extracting proteins from recalcitrant tissues, but phenol extraction was most efficient in removing interfering substances and resulted in the highest quality gels with less background and less vertical streaking. The two methods minimize the protein degradation often encountered during sample preparation, owing to endogenous proteolytic activity. It was also pointed out that the phenol method yielded a greater number of glycoproteins (8).

The phenol extraction procedure has a high clean-up capacity. It also acts as a dissociating agent decreasing molecular interaction between proteins and other materials (6). The major drawbacks of the protocol are that it is time consuming (at least 6 h) and that phenol and methanol are toxic.

2. Materials

1. Phenol: Tris-HCl saturated, pH 6.6/7.9 (Amresco-Interchim, Biotechnology Grade).
2. Extraction buffer: Prepare a solution of 500 mM Tris-HCl, 50 mM EDTA, 700 mM sucrose, 100 mM KCl and adjust pH to 8.0 with HCl. This solution can be stored for a week at 4°C.
Add just before extraction 2% β -mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF; *see* **Note 1**).
3. Precipitation solution: 0.1 M ammonium acetate in cold methanol. This solution is stored at -20°C.
4. Isoelectric focusing buffer: 9 M urea, 4% CHAPS, 0.5% Triton X-100, 20 mM DTT, 1.2% Pharmalytes pH 3 to 10 (*see* **Note 2**). Triton X-100 is provided as a 10% solution. This solution can be aliquoted and stored at -20°C for months.

5. Determination of protein concentration: protein concentration is evaluated according to a modified Bradford assay using the dye reagent from Bio-Rad (*see Note 3*).

3. Methods

3.1. Protein Extraction

1. Fresh plant tissue is frozen in liquid nitrogen after harvest and ground to a fine powder within precooled steel cylinders of an automatic cryogenic crusher (*see Note 4*).
2. Then 1 g of ground tissue is suspended in 3 mL of extraction buffer in a 15-mL Falcon tube, vortexed, and incubated by shaking for 10 min on ice (*see Note 5*).
3. Afterward, an equal volume of Tris-buffered phenol is added, and the solution is incubated on a shaker for 10 min at room temperature (*see Note 6*).
4. To separate insoluble material (in the pellet), for aqueous and organic phases, the sample is centrifuged for 10 min at 5500g and 4°C. The phenolic phase, which is on the top of the tube (*see Note 7*), is recovered carefully to avoid contact with the interphase and poured into a new tube.
5. This phenol phase is then back-extracted with 3 mL of extraction buffer. The sample is shaken for 3 min again and vortexed. Centrifugation for phase separation is repeated for 10 min at 4°C and 5500g.
6. The phenol phase still on the top of the tube is carefully recovered and poured into a new tube; 4 vol of precipitation solution are added. The tube is shaken by inverting, and the sample is incubated for at least 4 h or overnight at -20°C.
7. Proteins are finally pelleted by centrifugation (10 min, 5500g at 4°C).
8. After centrifugation, the pellet is washed three times with cooled precipitation solution and finally with cooled acetone. After each washing step, the sample is centrifuged for 5 min at 5500g and 4°C.
9. Finally, the pellet is dried under vacuum (*see Note 8*).

The proteins are first extracted in a Tris buffer containing several protecting agents. EDTA inhibits metalloproteases and polyphenol oxidases by chelating metal ions. PMSF irreversibly inhibits serine proteases. β -Mercaptoethanol is a reducing agent that prevents protein oxidation. Moreover, as a precaution against protease activity, the temperature must be kept below 4°C, and samples should be placed on ice during the first step of the extraction process (*see Note 5*). The extraction period should also be minimized. The presence of KCl is related to its “salting in” effect, improving the solubility and then the extraction of proteins.

An alternative method to classical phenol extraction has been proposed by Wang et al. (7). Extraction is carried out in the presence of sodium dodecyl sulfate (SDS) and is termed phenol/SDS extraction. It maximized protein yields in olive leaf tissue, displayed a good 2-DE resolution, and gave more spots

with increased intensity than phenol alone. However, the addition of SDS did not improve extraction in the case of banana, apple, and potato leaves (6).

With sucrose, the Tris buffer is heavier than Tris-buffered phenol. So, during the phase separation the phenol phase is “pushed” on top, which facilitates recovery of the phenol phase (*see Note 7*). The upper phenol phase contains cytosolic and membrane proteins.

Buffering the phenol with Tris to pH 8.0 (*see Note 6*) ensures that nucleic acids are partitioned to the buffer phase and not to the phenol-rich phase (6).

Proteins are usually precipitated by addition of salts or water-miscible organic solvents. Here a combination of both is used. Four volumes of methanol efficiently precipitate most proteins. However, methanol poorly precipitates proteins from acidic solutions. An organic base or a buffer (ammonium acetate) solves this problem.

3.2. Protein Solubilization and Quantification

1. The final pellet is resuspended in IEF buffer. In our conditions, starting with 1 g of fresh tomato fruit tissue, 200 μL of IEF buffer are needed.
2. The sample is incubated for at least 1 h (sometimes more) at room temperature under agitation. Do not heat samples; this would lead to carbamylation of proteins.
3. For quantification, several dilutions of ovalbumin standard are made in IEF buffer (8 dilutions from 0 to 60 $\mu\text{g}/\mu\text{L}$). Then 10 μL of 0.1 *N* HCl are added to every samples. The final volume is adjusted to 100 μL with water, either for standard curve samples or for tissue sample.
4. Then 3.5 mL of diluted dye reagent are added, and the optical density is read at 595 nm.

To estimate the protein concentration in plant samples, the Bradford assay (9) is more appropriate than the Lowry (10) and biuret methods, which are based on the quantification of phenolic compounds (1). However, direct quantification in sample solubilization buffers is not possible owing to interference with IEF buffer components. We therefore use the modified procedure of Ramagli and Rodriguez (11), which is based on acidification of the sample buffer. It allows direct quantitation of protein solubilized in sample buffers containing urea, carrier ampholytes, nonionic detergents, and thiol compounds.

4. Notes

1. **Caution:** β -mercaptoethanol and PMSF are toxic compounds. PMSF can be prepared as a stock solution 200 mM in isopropanol, aliquoted, and stored at -20°C .
2. Don't add too much water to solubilize CHAPS and urea powders; for a 25 mL final volume, add only about 10 mL of water. When preparing this solution, avoid heating above 30°C , to prevent protein carbamylation. Solubilization may take time.

3. The diluted dye reagent is prepared according to the standard macroassay procedure as described in the Bio-Rad instruction manual.
4. It is very important to obtain a fine powder; the finer it is, the more efficient are the protein extraction and the removal of contaminants. The powder should also be homogenous for accurate sample comparison.
5. At this step it is important to work at low temperature to limit protease activity.
6. Tris-buffered phenol is prepared according to the manufacturer's recommendations and is stored at 4°C. In the bottle, the phenol phase is below the Tris phase. Pipet the whole required volume at once to avoid bottle manipulation and ambiguous separation of the two phases.
7. The trick here is to use sucrose in the extraction buffer to invert the phases.
8. It is possible to delay pellet resolubilization by storing well-dried protein pellets at -80°C. Be careful to prevent rehydration of the pellet by placing it in a vacuum chamber while warming up.

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