A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis

Sample preparation is one of the most crucial steps in obtaining high-quality resolution of proteins in proteomic analysis, yet it can be problematic [1]. Proteins isolated from plant tissues are often difficult to resolve by 2-DE due to the abundance of secondary metabolites. In particular, recalcitrant plant tissues such as aged evergreen leaves and mature fruits often contain high levels of materials that strongly interfere with 2-DE, resulting in horizontal and vertical streaking, smearing, and reduced numbers of distinctly resolved protein spots [2, 3]. An excellent review on the practical issues associated with plant proteomic analysis has recently been published [4].

Many sample preparation and protein separation methods to obtain well-resolved 2-DE maps have been reported, e.g. [5–7]. These methods usually involve TCA/acetone wash or precipitation steps. We recently reported a protocol based on phenol extraction that is highly effective for the production of high-quality protein samples from recalcitrant tissues [3]. Furthermore, a combination of TCA/acetone and phenol methods provides enhanced 2-DE-based proteomic analysis of most plant tissues [2]. To date, no common and simple protocol for protein extraction that can be used on a large scale for tissues of various plant species has been reported. There is a critical need for such a rapid and universal protocol, especially for projects in which many samples must be analyzed and when comparative proteomic analysis is carried out with different tissues or species. Here, we describe a rapid and universally applicable protocol for protein extraction from recalcitrant tissues. The protocol is effective for the extraction of proteins from a variety of plant species.

Aged leaves used in this study were collected from plants in the botanical garden at Siena University, Italy: bamboo (Bambusa vulgaris), grape (Vitis vinifera), iris (Iris pseudacorus), olive (Olea europea), lemon (Citrus limonum), pine (Pinus nigra), redwood (Sequoia sempervirens), sugarcane (Saccharum officinarum), and tobacco (Nicotiana tabacum). Tissues were also collected from posidonia grass (Posidonia oceanica) plants growing underwater in the Mediterranean Sea. Apple, banana, grape, kiwi, olive, orange, pear, and tomato fruits used in this study were bought at local fruit shops.

An outline of the protocol is shown below. The protocol was designed to process small amounts of tissues in microtubes within 1 h, but can be scaled up for larger samples (ca. 1–5 g tissue in 30–50 mL centrifugation tubes). All wash solutions were prechilled for at least 1 h at 2–20°C.

SDS-PAGE was performed in 12.5% polyacrylamide gels [8] using a BioRad Mini-Protein II equipment. For 2-DE, samples of 100 μg were applied by overnight rehydration into IPG strips of pH 4–7 or 3–10 (Amersham Biosciences), subjected to IEF as previously described [3], and then resolved in 12.5% polyacrylamide gels. Following electrophoresis, protein gels were visualized with CBB R, or colloidal CBB G, or using the Silver Staining Kit from Amersham Biosciences. Protein was quantified by the BioRad protein assay with BSA as standard [9].

Keywords: Plant tissue / Proteomics / Sample preparation / Two-dimensional electrophoresis

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The protocol we present here combines TCA/acetone and methanol washes and a phenol extraction. It is greatly simplified, with minimal sample handling and fewer wash steps, and much quicker than the protocol that we used previously to extract proteins from olive leaves [3]. However, it still results in good electrophoretic separation of proteins from various recalcitrant plant tissues. The worth of protocol was demonstrated using pine needle leaves. As shown in Fig. 1 (left panel, lanes 1–4), conventional protein extraction method followed by acetone, TCA, or methanol precipitation did not give good results of electrophoretic separation even in 1-D gel, due to the presence of interfering compounds. After sequential washes by acetone/TCA and basic methanol, the quality of protein patterns was highly improved (Fig. 1, left panel, lanes 5–7). Importantly, the protocol is compatible with silver staining, which visualizes more bands compared to CBB R staining (Fig. 1, right panel). Furthermore, this protocol can be applicable to samples from aged leaves and mature fruits from a variety of sources (Fig. 2), resulting in sharp and well-resolved protein bands in the SDS-PAGE gel.

To validate the protocol on a large scale, about 5.0 g pine leaves were subjected to the protocol reported here, but the wash steps were carried out in 35 mL tubes (Rotor SS34, Sorval Superspeed Centrifuge). The resolved spots on 2-DE gel were visualized using silver staining, and the background of the whole gel was very low, indicating the high quality of the protein preparation (Fig. 3). The protocol was used for a wide range of leaves containing high levels of polyphenols (olive and pine leaves) and fruits with low protein contents (apple and pear), high sugar content (banana), high acidity (grape

| Tissue powder | Grind sample (leaf or fruit flesh) into a fine powder in a mortar and pestle under liquid nitrogen. Note: a fine powder is important for effective contaminant removal and protein extraction. |
| TCA/acetone wash | Transfer the powder (0.1-0.3 g) into a 2-ml tube. Fill the tube with 10% TCA/acetone. Mix well by vortexing or inversion. Centrifuge at 16,000 × g for 3 min (4°C). Remove the supernatant by decanting or careful pipetting. |
| Methanol wash | Fill the tube with 80% methanol plus 0.1 M ammonium acetate. Mix well by vortexing or inversion. Centrifuge at 16,000 × g for 3 min (4°C). Discard the supernatant. |
| Acetone wash | Fill the tube with 80% acetone. Vortex until the pellet is fully dispersed. Centrifuge at 16,000 × g for 3 min (4°C). Discard the supernatant. |
| Dry | Air-dry at room temperature or incubate at 50°C for at least 10 min to remove residual acetone. |
| Protein extraction & precipitation | Add 0.4-0.8 ml/0.1 g starting material of 1:1 phenol (pH 8.0, Sigma) / SDS buffer [3]. Mix thoroughly and incubate the mixture for 5 min. Centrifuge at 16,000 × g for 3 min. Transfer the upper phenol phase (0.2-0.4 ml) into a new 2-ml tube. Fill the tube with methanol containing 0.1 M ammonium acetate and store at -20°C for 10 min to overnight, and centrifuge at 16,000 × g for 5 min (4°C). Carefully discard the supernatant; a white pellet should be visible. Note: If no phase separation occurs, add more phenol (100 µl) to the mixture, mix well, and centrifuge again. |
| Wash and air-dry the pellet | Wash the pellet once with 100% methanol and once with 80% acetone. During each wash step, mix well by vortexing and centrifuge as above. Allow the proteins to air dry briefly. Dissolve the proteins in a buffer of choice (e.g., SDS sample buffer or IEF rehydration buffer). |
| Protein analysis | (quantification, PAGE or 1EF etc.) |
Figure 1. SDS-PAGE separation of pine leaf proteins prepared using different methods. Left panel: lanes 1–4, aged pine leaves were pulverized in a mortar with liquid nitrogen and then extracted with 2x SDS sample buffer [8]. After centrifugation at 16000 x g for 3 min, the supernatant was directly used for electrophoresis (lane 1), or followed by acetone (lane 2), 10% TCA (lane 3), and basic methanol precipitation (lane 4). Lanes 5–7, pine aged leaves were pulverized in a mortar with liquid nitrogen, and the resultant tissue powders were washed with only acetone (lane 5), 10% TCA/acetone and then acetone (lane 6), and 10% TCA/acetone, basic methanol and acetone (lane 7), followed by phenol extraction. Electrophoresis analysis was carried out on the basis of equal amounts of starting materials. Right panel: pine proteins prepared by the protocol reported here. Lane 1, 35 μg protein loaded, CBB R-stained; 2, 7 μg protein loaded, silver-stained. Protein molecular mass standards with sizes (in kDa) are indicated on the left.

Figure 2. SDS-PAGE separation of proteins from a range of leaves and fruits. A 12.5% CBB-stained gel is shown. 1–9, aged leaves; 10–16, mature fruits; 1, olive, one TCA/acetone wash; 2, olive, two TCA/acetone washes; 3, bamboo; 4, orange; 5, sugar-cane; 6, redwood; 7, pine; 8, tobacco; 9, iris; 10, apple; 11, banana; 12, grape; 13, kiwi; 14, orange; 15, pear; 16, tomato. M, protein mass markers.

Figure 3. Silver-stained 2-DE maps of proteins from pine leaves. Protein was extracted by the protocol reported here and 20 μg was used for 2-DE on 7-cm IPG strip (pH 4–7) and silver-stained. Left panel: original silver-stained gel; Right panel, the gel after removal of backgrounds with the software PDQUEST (BioRad).
Figure 4. Representative 2-DE maps of proteins from recalcitrant plant tissues. Protein samples were prepared by the protocol here and approximately 100 μg were separated by 2-DE, with the first dimension, IEF, on 7-cm IPG strips, and the second dimension on 12.5% polyacrylamide gels. Gels were stained with CBB G-250 and thoroughly destained with water.
A, aged olive leaf; B, olive flesh; C, posidonia leaf; D, apple flesh.

Table 1. Protein yield of the protocol reported here

<table>
<thead>
<tr>
<th>Plant tissues</th>
<th>Protein yield (mg/g fresh weight)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Bamboo leaf</td>
<td>9.20</td>
<td>Iris leaf</td>
<td>4.12</td>
</tr>
<tr>
<td>Lemon leaf</td>
<td>7.80</td>
<td>Olive young leaf</td>
<td>1.30</td>
</tr>
<tr>
<td>Olive aged leaf</td>
<td>2.52</td>
<td>Pine leaf</td>
<td>1.86</td>
</tr>
<tr>
<td>Redwood leaf</td>
<td>4.08</td>
<td>Apple flesh</td>
<td>0.291</td>
</tr>
<tr>
<td>Olive flesh</td>
<td>1.47</td>
<td>Pear flesh</td>
<td>0.387</td>
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Results presented above are the average of two extrac-
tions.

and orange), and high contents of pigment (olive and
tomato). In all cases, use of this protocol facilitated the
extraction of high-quality protein samples suitable for
electrophoretic analysis, and the overall quality of the
protein profiles was good, with less vertical and horizontal
streaking and smearing (Fig. 4).

The protein yield of this protocol is summarized in Table 1. For plant leaves tested, the average yield of proteins is
1.30–9.2 mg/g. It means that using the protocol on a small
scale, e.g., in 2 mL tubes, starting with 1.0 g leaf, enough
proteins can be obtained for several routine 1-DE and
2-DE analyses. For example, with this protocol, the aver-
age protein yield from apple flesh, a low protein source,
was approximately 291 μg/g of fresh weight.

The efficient extraction of proteins from a tissue sample
depends on the quality of the sample disruption. In this
protocol, the plant tissues are pulverized to a fine powder
in a mortar and pestle under liquid nitrogen to minimize
proteolysis and other modes of protein degradation. The
combination of TCA and acetone is commonly used to precipitate proteins and remove contaminants during preparation for 2-DE. However, these TCA/acetone-based preparations typically require washing until the tissue powder becomes colorless, which is time-consuming [3]. Furthermore, extended exposure to low pH may lead to protein degradation or modifications. Instead, the present protocol includes only a brief TCA/acetone wash step, followed by basic methanol and acetone wash steps to remove residual TCA and contaminants. The main advantage of the new protocol is the greatly shortened time of sample preparation that avoids producing samples of reduced quality. In fact, there was no visible difference between samples subjected to one or two TCA/acetone wash steps (compare lanes 1 and 2, Fig. 2).

Another important step that was introduced is the basic methanol wash. In fruit and vegetable analysis, an aqueous methanol solution is often used to extract (poly)-phenolic compounds [10, 11]. In addition, a methanol wash in the presence of ammonium acetate can neutralize residual TCA and increase the pH above 7, facilitating the subsequent extraction of proteins by phenol. It may be noted that the protein pellet from phenol extraction usually looks white, and a yellowish pellet indicates coprecipitation of phenolic compounds. In the case of tissues containing high levels of phenolic compounds, such as olive and pine aged leaves, pulverizing plant tissues with polyvinylpolypyrrolidone (PVPP, 0.05 g/g tissue) can help to remove phenolic compounds (not shown).

Finally, we used a phenol/SDS mixture to extract proteins from dry pellets. Phenol extraction has proven useful for plant samples that contain high levels of interfering substances [2–5]. Our recent work showed that phenol/SDS extraction of proteins is more efficient than either phenol or SDS buffer alone [3].

In summary, the protocol was used with a wide range of leaves and fruits, and all of the extracted proteins separated well during electrophoresis. Since the protocol is universal and rapid, requiring less than 1 h, it can be used for routine protein extraction from recalcitrant plant tissues for proteomic analysis.

References