Acoustic Technology for High-Performance Disruption and Extraction of Plant Proteins

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Abstract: Acoustic technology shows the capability of protein pellet homogenization from different tissue samples of soybean and rice in a manner comparable to the ordinary mortar/pestle method and far better than the vortex/ultrasonic method with respect to the resolution of the protein pattern through two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). With acoustic technology, noncontact tissue disruption and protein pellet homogenization can be carried out in a computer-controlled manner, which ultimately increases the efficiency of the process for a large number of samples. A lysis buffer termed the T-buffer containing TBP, thiourea, and CHAPS yields an excellent result for the 2D-PAGE separation of soybean plasma membrane proteins followed by the 2D-PAGE separation of crude protein of soybean and rice tissues. For this technology, the T-buffer is preferred because protein quantification is possible by eliminating the interfering compound 2-mercaptoethanol and because of the high reproducibility of 2D-PAGE separation.

Keywords: adaptive focused acoustics • lysis buffer • plant tissues • proteomics • rice • soybean • 2D-PAGE

Introduction

High-throughput screening of plant materials is a key factor in proteomics, and it requires an alternate technology for the rapid and uniform homogenization of tissue as well as protein pellets. Handling complex biological molecules such as proteins is very critical and requires more attention in order to avoid contamination with undesirable compounds, particularly when it is required to determine the protein structure. Physical contaminations also should not be ignored. In addition, a comparative screening of biological materials such as plant and animal tissues under different growing conditions or treatments requires a precise and standard method to obtain highly accurate and comparable results. This important task primarily starts from initial tissue processing, which has already been standardized for some cases such as extracting plasmid DNA from *Escherichia coli* and other Gram-negative bacteria through automatic DNA isolation systems.

Proteins are very sensitive to unsuitable handling. Furthermore, preliminary expression analyses of protein spots comprise their comprehensive comparison by an image analysis system, which is in fact the comparison of corresponding protein spots across different treatments or growing stages. Therefore, it is important to control the deconstruction of proteins while maintaining individual target protein spots. Typically, in proteome experiments, the initial tissue disruption and final pellet homogenization are based on manual, laborious, and uncontrolled tissue grinding with an extraction buffer/ liquid nitrogen or pellet homogenization using a lysis buffer. This procedure is time-consuming and can occasionally be hazardous. In recent years, some researchers have used the ultrasonic method for protein pellet homogenization with energy produced by an acoustic transducer that is coupled with the pellet in the microtube, which is carried out manually in small batches, and processing parameters are determined empirically. Therefore, the adaptation of a time-saving method along with the elimination of variation related to operators appears to be important for high-throughput proteome experiments.

The introduction of a high-performance, single-tube sample preparation device (Covaris, Covaris Inc., Woburn, MA) has enabled noncontact tissue disruption and protein pellet homogenization, thereby avoiding contamination and degradation. The Covaris process using adaptive focused acoustics produces a controlled acoustic field inside a sealed vessel for sample preparation. Walsh-Haney and Coticone[1] have used Covaris-CryoPrep and Covaris E200 for DNA extraction from human bones. They have found that acoustic technology is a rapid and effective method of DNA extraction that simultaneously preserves the integrity of the DNA since there is minimal heating or foaming during the automated extraction process. They have achieved minimal handling of DNA through the extraction using this instrument as compared to the current mortar/pestle method, which prevents its contamination from other sources such as technicians and anthropologists. Automated acoustic energy obtained by Covaris E200 has also been satisfactorily used for the tissue preparation of the heart, liver, and muscle tissue sections of animals;[2,3] however, the preparation of plant tissue samples has not yet been attempted.

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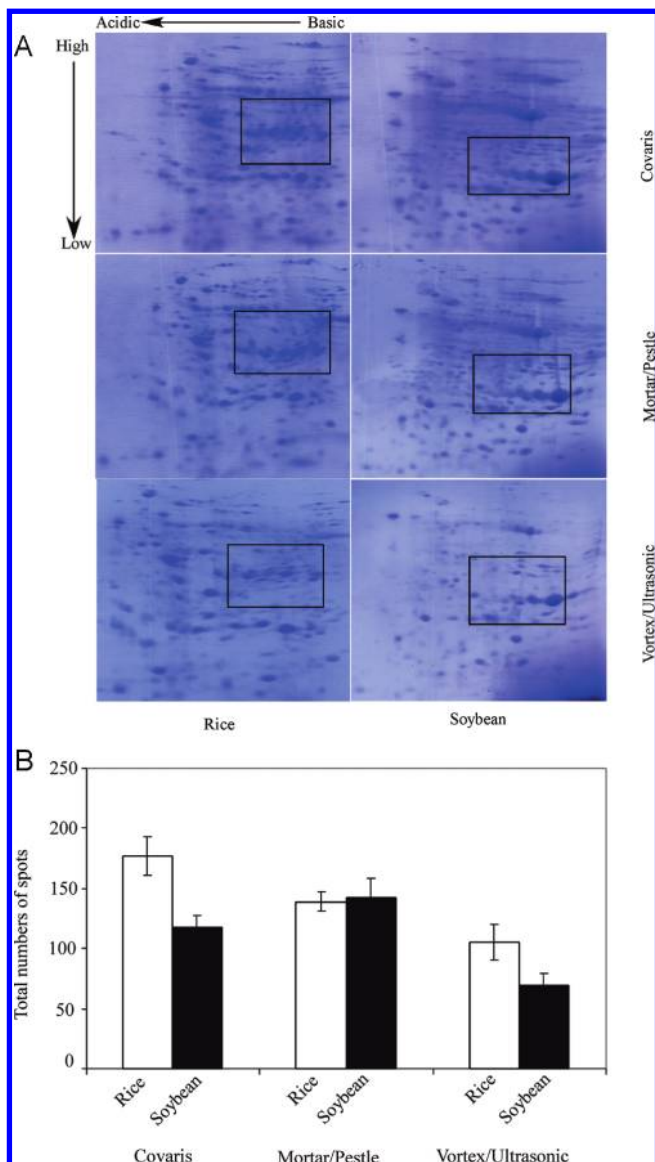 § University of Tsukuba.
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Figure 1. Comparison of Covaris, mortar/pestle, and vortex/ultrasonic methods for protein pellet homogenization in soybean and rice. One- and 2-week-old seedlings from soybean and rice, respectively, are sampled for the experiment. The leaf, hypocotyl, and root of soybean and the leaf blade, leaf sheath, and root of rice are sampled individually. Proteins are extracted using the Covaris, mortar/pestle, and vortex/ultrasonic methods. The extracted proteins are separated by 2D-PAGE and stained by CBB (A). The rectangle frames show the changed areas among Covaris, mortar/pestle, and vortex/ultrasonic methods for protein pellet homogenization. The total number of main protein spots detected in soybean and rice using different tissues and both lysis buffers is given by the mean values ± standard deviation (B).

Figure 2. Comparison of root, leaf sheath/hypocotyl, and leaf tissue samples from soybean and rice used for protein pellet homogenization using Covaris, mortar/pestle, and vortex/ultrasonic methods. One- and 2-week-old seedlings from soybean and rice, respectively, are sampled for the experiment. The leaf, hypocotyl, and root of soybean and the leaf blade, leaf sheath, and root of rice are sampled individually. Proteins are extracted using the Covaris, mortar/pestle, and vortex/ultrasonic methods. The extracted proteins are separated by 2D-PAGE and stained by CBB. The total number of main protein spots detected in different tissues from soybean/rice using both lysis buffers is given by the mean values ± standard deviation.

Experimental Procedures

Plant Material and Growth Condition. Soybean (*Glycine max* cv. Sachiyutaka) and rice (*Oryza sativa* L. cv. Nipponbare) were used to examine the possibility of using acoustic technology for protein pellet homogenization. Soybean seeds were surface sterilized in 1.25% sodium hypochlorite solution (antiformin) for 2 min followed by rinsing 3 times with water and grown in plastic tubs containing 400 mL of sand under white fluorescent light (600 μmol m^{-2} s^{-1} and 12-h light/12-h dark photoperiod) at 25 °C and 75% relative humidity in a growth chamber. In the case of rice, the seedlings were grown in seedling cases containing soil with stand water under controlled condition as soybean at 28 °C in growth chamber. One and 2 weeks old seedlings from soybean and rice were sampled for the experiment, respectively. The soybean leaf, hypocotyl, and root as well as leaf blade, leaf sheath, and root from rice were individually sampled, and after weighing, soaked in liquid nitrogen, and kept at −30 °C until use.

Protein Extraction. For both soybean and rice, 0.5 g of root and 0.25 g of other tissue samples were homogenized with 2 and 1 mL of phosphate buffer, respectively, (pH 7.6) containing 65 mM K_2HPO_4, 2.6 mM KH_2PO_4, 400 mM NaCl, and 3 mM NaN_3 using glass mortar and pestle on ice. The homogenate was centrifuged two times each at 15 000g for 10 min at 4 °C. The supernatants after the second centrifugation were incubated on ice by adding trichloroacetic acid to a final concentration of 10% to precipitate the proteins. After 30 min, the solution was centrifuged at 15 000g for 10 min. The resultant precipitate was washed with prechilled ethanol twice and was suspended in lysis buffer through three different methods, viz. Covaris instrument (S-series), mortar/pestle, and vortex/ultrasonic. In the case of Covaris instrument, the pellets were

In this study, an experiment is conducted to determine the possibility of plant tissue disruption and pellet homogenization by the Covaris instrument and its comparison with the commonly used mortar/pestle and ultrasonic methods. Soybean and rice plants are selected, and three different plant tissues from soybean (root, hypocotyl, and leaf) and rice (root, leaf sheath, and leaf) are separately used as tissue materials for protein extraction. In addition, two different lysis buffers are evaluated for protein pellet homogenization.
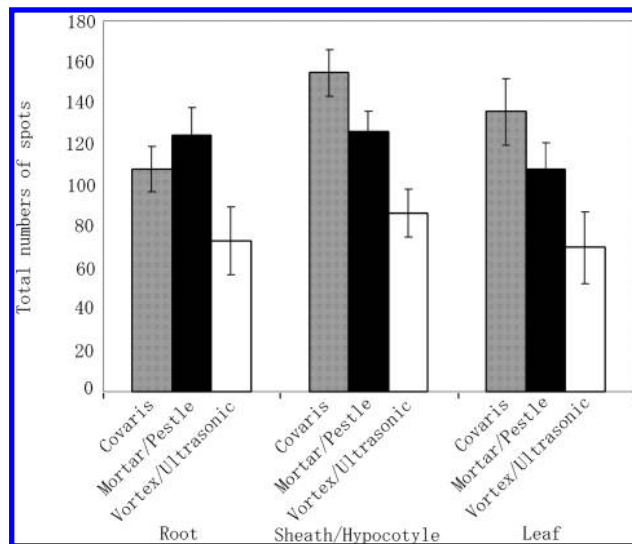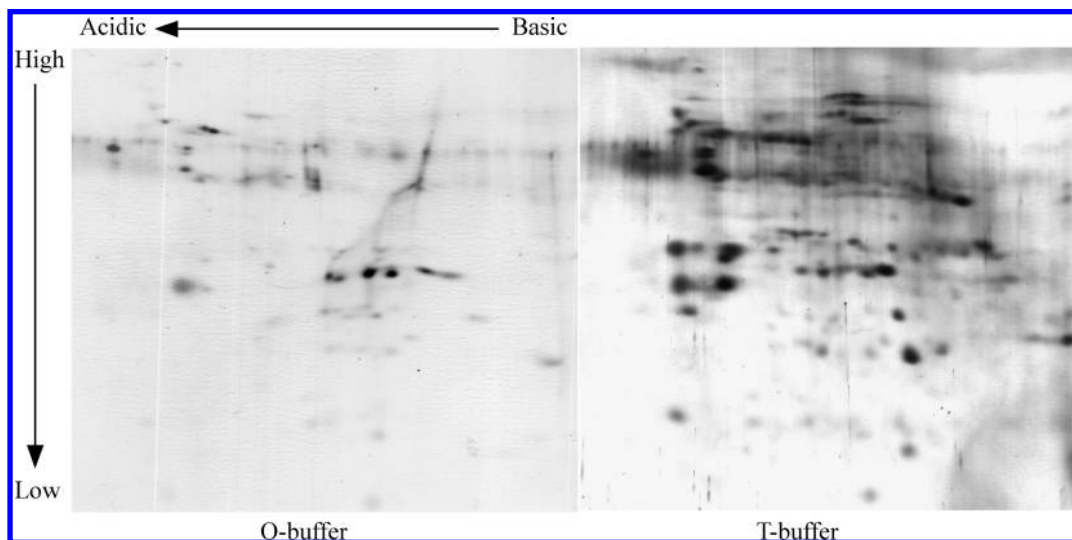
Figure 3. Evaluation of the O-buffer and T-buffer used for homogenization of plasma membrane protein pellets purified from soybean root tissue samples using Covaris instrument. One-week-old seedlings from soybean are sampled for the experiment. Plasma membranes are purified from roots by the two-phase partitioning method, and proteins are homogenized with the O-buffer and T-buffer. The extracted proteins are separated by 2D-PAGE and stained by silver nitrate.
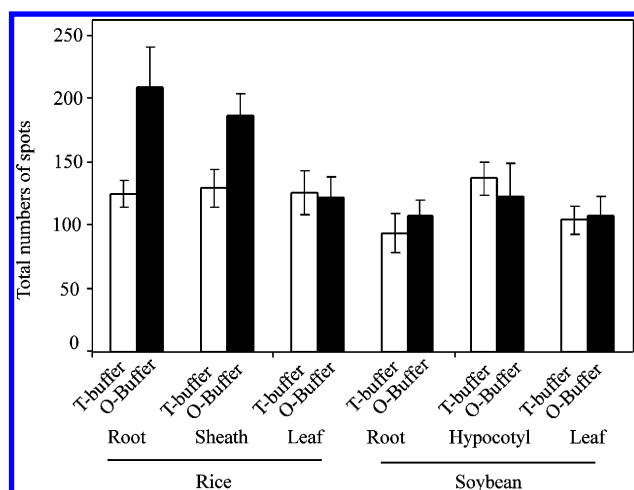

Figure 4. Comparison of O-buffer and T-buffer used for homogenization of protein pellets extracted from the root, leaf sheath/ hypocotyl, and leaf tissue samples of rice and soybean. One- and 2-week-old seedlings from soybean and rice, respectively, are sampled for the experiment. The leaf, hypocotyl, and root of soybean and the leaf blade, leaf sheath, and root of rice are sampled individually. Proteins are extracted in the O-buffer and T-buffer. The extracted proteins are separated by 2D-PAGE and stained by CBB. The total number of main protein spots is given by the mean values of triplicate gels ± standard deviation.

homogenized using the saved treatments setting as duty cycle, 10%; intensity, 6−8; cycles/burst, 100−500 for a period of 15 s each.

Two types of lysis buffer were tested. The first lysis buffer (O-buffer) was prepared following O'Farrell[4] containing 8 M urea, 2% Nonidet P-40, 2% Ampholine (pH 3.5−10.0, GE Healthcare, Piscataway, NJ), 5% 2-mercaptoethanol, and 5% polyvinylpyrrolidone (PVP)-40. The second lysis buffer (T-buffer) was prepared as per our experience on proteome analysis of different crop tissues and protein from purified plant organelles like plasma membrane with 7 M urea, 0.2 M thiourea, 0.2 mM tributylphosphine (TBP), 0.4% 3-([3-. cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS),

0.2% Biolyte (pH 3−10, Bio-Rad, Hercules, CA), and 5% PVP-40. The volume of lysis buffer used for protein pellet extraction was 300 μL for leaves and 200 μL for the sheath/hypocotyl and leaf tissue samples.

For soybean plasma membrane purification, 20 g of fresh root samples was chopped and ground in 210 mL of extraction buffer containing 0.4 M sucrose, 75 mM MOPS/KOH (pH 7.6), 5 mM EDTA/KOH (pH 7.5), 5 mM EGTA/KOH (pH 8.2), 10 mM KF, 1 mM dithiothreitol, and 2% polyvinylpyrrolidone-40 using a mortar and pestle on ice. These homogenates were filtered through four layers of Miracloth (Calbiochem, San Diego, CA). The filtrates were centrifuged at 10 000g for 15 min and 200 000g for 30 min at 4 °C. The plasma membrane-enriched fractions were obtained using a two-phase partitioning method.[5] Two-phase partitioning was repeated four times for root tissue samples.

The membrane specific H^+-ATPase activity assay was adapted to investigate the quality of purified plasma membrane. A reaction solution contained 0.3 M MES-Tris (pH 6.5), 0.5 M KCl, 0.1 M MgSO$_4$ and 0.1 M ATP with 1 mM Na$_3$VO$_4$, 0.5 M KNO$_3$ and 0.1 M NaN$_3$, three inhibitors of H^+-ATPase for plasma membrane, tonoplast and mithocondrial membranes, respectively, and without inhibitors as control. After 15 min incubation of solution in 30 °C, the reactions were stopped by addition of 0.5% ammonium molybdate, 1% SDS and 1.96% H$_2$SO$_4$, followed by adjusting the ascorbate concentration to 0.3%. The absorbance at 750 nm was measured after incubation of the reaction mixtures at room temperature for 30 min. Products of H^+-ATPase were calculated from the standard curve and the purity of plasma membrane fractions was measured by the inhibitory efficiency of inhibitors.

Protein quantification was performed[6] and 300 μg of extracted proteins was applied to IEF tube gels.

Two-Dimensional Polyacrylamide Gel Electrophoresis. The crude protein (100 μL) samples were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)[4] in the first dimension by isoelectric focusing (IEF) tube gels and in the second dimension by SDS-PAGE. A prepared IEF tube gel of 11 cm length and 3 mm diameter consisted of 8 M urea, 3.5% acrylamide, 2% NP-40, 2% Ampholines (pH 3.5−10.0 and

5.0−8.0), ammonium persulfate and *N,N,N′,N′*-tetraethyleth-ylenediamine. Electrophoresis was carried out at 200 V for 30 min, followed by 400 V for 16 h, and 600 V for 1 h. After IEF, SDS-PAGE was performed in the second dimension using 15% polyacrylamide gels with 5% stacking gels, followed by Coomassie brilliant blue (CBB) and silver (in the case of soybean root plasma membrane proteins) staining before drying of the gels. Three replicates from four to five independent experiments were selected for statistical analysis.

Image Acquisition and Data Analysis. The 2D-PAGE gels were scanned with densitometer GS-800 calibrated densitometer (Bio-Rad) and analyzed with PDQuest ver. 7.0 (Bio-Rad). Image analysis included spot detection, spot measurement and background subtraction. To correct the variability due to CBB or silver staining and to reflect the accurate quality of the protein spots, the spot volumes were normalized as a percentage of the total density in all of the spots in the gels group. *F*-tests through factorial analysis of variance were adapted to find the significant differences between treatments including three homogenization methods, two lysis buffer, two crops and three plant tissues from each crop. To obtain the precision of the procedure, coefficient of variation was calculated as the standard deviation divided by the mean of total number of protein spots found on 2D-PAGE gels.

Results and Discussion

Current protein extraction methods, apart from being extremely time-consuming and labor-intensive, carry additional risks of degradation and contamination. The efficiencies of three different methods—acoustic technology, pestle/mortar, and vortex/ultrasonic methods—for plant tissue and protein pellet homogenization are compared in this experiment. There is a significant difference between these three methods with respect to the total numbers of detected protein spots on 2D gels, as revealed by the analysis of variance. Despite the total number of main protein spots, the Covaris instrument provides a clearer protein pattern than the other conventional methods (Figure 1A). Acoustic technology and the mortar/pestle method are better than the vortex/ultrasonic method for protein pellet homogenization, and they produce more number of spots in both soybean and rice (Figure 1B). Although there is no significant difference between the Covaris and mortar/pestle methods in the case of soybean, the former method can resolve more number of spots in rice. The Covaris instrument is excellent for the homogenization of plasma membrane protein pellets. In our experiment, just a few seconds are required to dissolve the protein pellets by the Covaris method without losing the lysis buffer, which is a common occurrence during the homogenization by the pestle/mortar method.

Apart from the discrepancy between soybean and rice in response to acoustic technology, different tissue samples including root, sheath/hypocotyle and leaf show contrasting results. The number of protein spots produced from the root tissue samples by the Covaris method is less than that by the mortar/pestle method in both soybean and rice, and significantly more than the number of spots produced from the root tissue samples by the vortex/ultrasonic method (Figure 2). In addition, among different tissue samples, the protein pellet of the root tissue samples dissolves in the lysis buffer faster than the pellets of both the leaf and sheath/hypocotyl using all three methods. The pellets of crude proteins extracted from leaf tissues are thick and difficult to dissolve in a 200 μL lysis buffer used to dissolve the pellets of all other tissue types. Therefore,

we use an additional 100 μL of lysis buffer to dissolve the protein pellets extracted from leaves. In the case of the soybean hypocotyl and rice sheath tissue samples, the Covaris method produces a significantly higher number of total protein spots than the mortar/pestle and vortex/ultrasonic methods in both soybean and rice, ignoring the type of lysis buffer. Furthermore, the repeatabilities of the procedures are investigated by calculation of the coefficient of variability. This value for the Covaris and Mortar/pestle methods was 8.23 and 8.57%, respectively. So, the repeatability of the procedures for these two methods is more or less the same; however, these coefficients of variabilities are less than that by the vortex/ultrasonic method with 14.38%, indicating less repeatability of the vortex/ultrasonic method and that this method is more erroneous in different experiments.

For homogenizing the pellets of all tissue samples, water bath sonication requires more time than the Covaris method and even the mortar/pestle method; however, this is more obvious in the case of the leaf tissue with almost 20−25 min required to dissolve the entire protein pellet. The Covaris method has an advantage over the conventional ultrasonic method as the former does not increase the temperature because it comprises a cooling system and also because it reduces the period of sample processing. Although sonication also utilizes the acoustics-based process, it is intrinsically distinct from the Covaris method for a number of reasons. The Covaris device functions by transmitting high-frequency acoustic energy waves from a dish-shaped transducer to a small localized area, thereby causing intense mixing. The Covaris acoustic transducer operates at 500 KHz with a wavelength of ∼1 mm, unlike low-frequency conventional sonics (typically 20 KHz) with a wavelength of ∼100 mm.[7] This enables the sound waves to be focused on samples in a vessel in a noncontact and isothermal mode to effectively disrupt and homogenize the tissue/pellet, thereby avoiding contamination and degradation. Adaptive focused acoustics for tissue homogenization result in improved extraction efficiency from animal tissue samples together with a simplified workflow, ease of use, and greater potential for increasing the throughput due to automated sample processing.[2]

We have also attempted to use the Tissue CryoPrep device (Covaris) for the noncontact disruption of different tissue samples of rice and soybean, followed by the homogenization of the resultant tissue powder by the Covaris instrument using a phosphate extraction buffer; however, we have been unsuccessful at obtaining good 2D gels from samples other than the leaf samples of both soybean and rice (data not shown). For almost all tissues, the results are better when the tissue samples are ground with liquid nitrogen before employing the Covaris unit; however, the resolution of the 2D gels is better for the leaf and hypocotyl/sheath tissue samples. The homogenization of tissue samples, of course, requires a higher level of treatment in the Covaris instrument for a vigorous homogenization effect. In our experiment, 3−5 min are required to disrupt the tissue samples using the Covaris method. The tissue CryoPrep device is successfully utilized for human bone tissue disruption; however, we have been unable to utilize this device in our experiment for the preparation of plant tissues such as thin and branched roots as it cannot receive the total force provided by the instrument.

Since extraction of the protein pellet by O-buffer is not good in some of the tissue samples, T-buffer is prepared for better solubilization of protein pellets. The purity of soybean
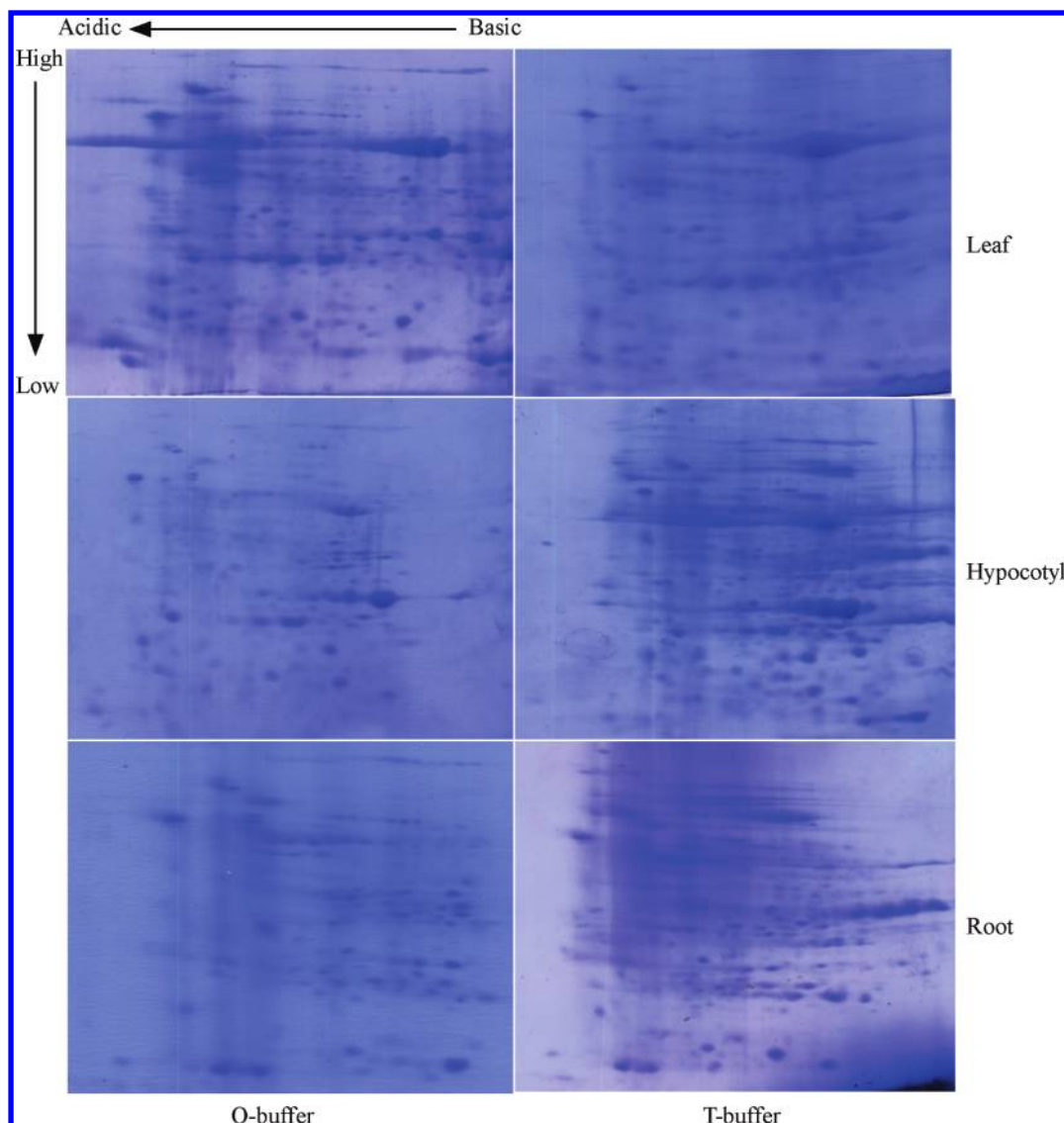
Figure 5. Evaluation of O-buffer and T-buffer used for homogenization of protein pellets extracted from soybean leaf, hypocotyl, and root tissue samples using Covaris instrument. One-week-old seedlings of soybean are used, and the leaf, hypocotyl, and root are sampled individually. Proteins are extracted in the O-buffer and T-buffer using the Covaris instrument. The extracted proteins are separated by 2D-PAGE and stained by CBB.

plasma membrane is tested by assaying membrane specific H^+-ATPase activity. The sensitivity of H^+-ATPase activity to vanadate, nitrate and azide is used to distinguish between the plasma membrane, vacuolar and mitochondrial membranes, respectively.[8] Proportions of the total activity sensitive to vanadate, nitrate and azide amounted to 1.8%, 67.7% and 72.9%, respectively, indicating a high purity of plasma membrane. The T-buffer is certainly better than the O-buffer[4] for plasma membrane purified protein in soybean (Figure 3). The total number of main protein spots for the O-buffer and T-buffer are 69 ± 32 and 128 ± 25 (mean values of triplicate gels $\pm$ standard deviation), respectively. However, the reverse is true for both the rice root and sheath crude protein, in which the total number of protein spots produced by the O-buffer is significantly more (Figure 4). Although less hydrophobic proteins like crude proteins from leaves and roots are expected to allow better resolution on 2D-PAGE than plasma membrane proteins, contamination with the components such as lipids, in the case of total proteins, interfered in getting such a result. This is concluded

because the plasma membrane pellet was very soft after TCA precipitation, whereas the pellet extracted of crude protein was rough and hard confirming its contamination with other cellular components. In addition, the combination of TBP with thiourea and CHAPS can provide a better resolution of the plasma membrane protein on 2D gels. It has been reported that TBP enhances the protein solubility during IEF, resulting in better focusing and resolution.[9] Furthermore, the inclusion of thiourea and Tris in the lysis buffer increases the solubilization of proteins from the rice leaves, and the solubilization improves further when the total protein is precipitated with trichloroacetic acid/acetone.[10]

In the case of crude protein, the T-buffer performs better in soybean than rice (Figure 5). Both lysis buffers produce the same number of total protein spots in the soybean and rice leaf tissue samples; however, the T-buffer is preferred because of elimination of 2-mercaptoethanol, an interfering agent in the Bradford method[6] for protein quantification. In addition, the T- buffer shows less discrepancy between different biological experiments, as indicated by a small value of the coefficient
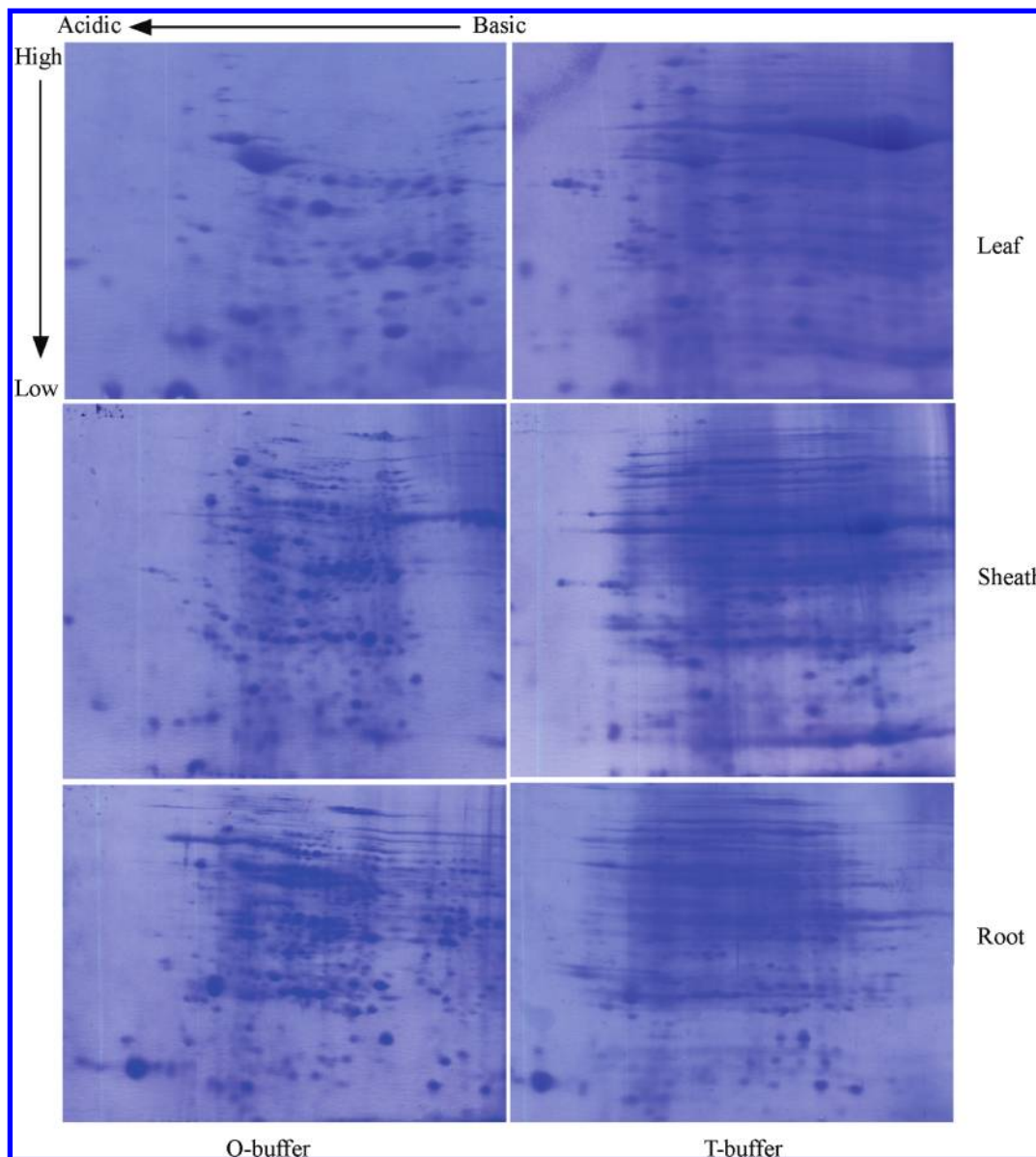
Figure 6. Evaluation of O-buffer and T-buffer used for homogenization of protein pellets extracted from rice leaf, leaf sheath, and root tissue samples using Covaris instrument. Two-week-old seedlings of rice are used, and the leaf, leaf sheath, and root are sampled individually. Proteins are extracted in the O-buffer and T-buffer using the Covaris instrument. The extracted proteins are separated by 2D-PAGE and stained by CBB.

of variation (33.22% for T-buffer vs 39.82% for O-buffer). The problem with the T-buffer in rice is high horizontal streaking on the 2D gels (Figure 6). Delaplace et al.[11] have investigated SDS- and phenol-based extraction buffers for the 2D-PAGE of a potato tuber and found the same type of horizontal streaking on the gels. They believe that the SDS extraction buffer is better than the phenol extraction buffer in reducing such streaking lines. Reducing TBP to 0.10−0.15 mM in the T-buffer may aid in obtaining a better resolution of 2D gels for rice tissues.

Concluding Remarks

Tissue and pellet homogenization is a preliminary yet important step in high-throughput proteomics and other omic-related sciences. This study provides new insights into the methods available at the laboratory scale—the mortar/pestle and water bath sonication methods—which are widely employed; however, both methods have their inherent limitations.

We have attempted to use a high-performance, single-tube sample preparation device (Covaris) for the noncontact disruption and uniform preparation of plant tissue and for protein pellet homogenization. The results show a comparable capability of acoustic technology to the mortar/pestle method, with regard to both plants and tissue, by providing reproducible and acceptable results. This technology performs far better than water bath sonication by producing high-quality 2D gels and minimizing the processing time required for high-throughput proteomics research. The T-buffer can produce a high-quality 2D protein pattern particularly for pure protein extracts such as soybean plasma membrane; however, it has some advantages over the O-buffer in the case of plant crude proteins.

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