A New Highly Efficient Protein Extraction Technique for Proteomic Study in Litchi *(Litchi chinensis* Sonn.)

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ABSTRACT

Proteomic study in litchi (*Litchi chinensis* Sonn.) has been of increasing interest in recent years. Agriculturists expect to gain better knowledge for solving the problem of fruit production caused by climate changes by proteomic studies. However, very limited information has been available due lack of a proper technique for protein extraction. Litchi plants contain a high level of secondary compounds (phenolics), which strongly reduce the purity of protein yields. This study aimed to develop a new highly efficient protein extraction technique by improving the available methods: (A) Trichloroactic acid/acetone; (B) Homogenization buffer/phenol; and (C) Phenol/SDS buffer. The results showed that method C gave the highest protein yield and resolution of protein separation using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and two−dimensional gel electrophoresis (2−DE), while the method A could not detect any protein and method B gave low protein separation.

Keywords: 2−DE, litchi, plant protein extraction, proteomics, recalcitrant plant

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INTRODUCTION

Litchi (*Litchi chinensis* Sonn.) is an economically important fruit crop widely grown in subtropical climates of Asia, South America, and Africa. Fruits are freshly consumed or sold as industrial products worldwide. Presently litchi crops are facing severe problems due to climate change and global warming. Many scientists have been working on how to manipulate flowering physiology (Chattrakul, 2005; Charoenkit *et al*., 2015), but so far with uncertain success. Therefore, a new biotechnological research trend in litchi is the study of proteomics. Agriculturists expect that this technology will allow the possibility for real time follow−up on physiological changes of plants during their developmental stages, at a given time and under specific environmental conditions. Through an efficient proteome technique, change of protein expression of plants can be evaluated qualitatively and quantitatively (Barbier−Brygoo and Joyard, 2004; Chen and Harmon, 2006). For example, Liu *et al.* (2017) had been studied protein expression in litchi fruit during early fruit development for deeper understanding of pollination and parthenocarpy fruit to establish proper crop management.

However, litchi is a chemically recalcitrant plant containing various secondary compounds (e.g. natural antioxidant phenolic compounds, pigments etc.), which complicates the first steps of protein extraction and separation (Prasad *et al*., 2009; Ahmad *et al*., 2015). So far, according to the available public NCBI database on April 20, 2017, only around 276 litchi protein sequences were registered.

Among the available protein extraction methods which have been employed in big fruit trees and recalcitrant plants, the original and classical one is called the "Lysis buffer technique". This is a well−known standard technique and the fastest protocol based on 2−D electrophoresis principles and a methods handbook (Gorg *et al*., 2004), but can be effectively used only with a limited number of plant tissues (O'Farrell, 1975). Recently, an alternative extraction protocol has been developed, called the "Trichloroacetic acid (TCA) and acetone technique". This method has been applied successfully with more types of plant tissues. This technique is also quick and easy to perform, but usually leads to the problem of irreversible protein denaturation (Copeland, 1994). However, the technique has a big advantage in increasing protein concentration and minimizing the contamination of interfering compounds (Damerval *et al*., 1986; Gorg *et al*., 1997; Chen and Harmon, 2006; Wang *et al*., 2008). The combination of TCA and acetone is usually more effective than either TCA or acetone alone. Gorg *et al*. (2004) reported that 10% (w/v) TCA with 0.3% (w/v) Dithiothreitol (DTT) in acetone could significantly remove the interfering substances from difficult protein source tissues and produce protein solutions substantially free of salts, nucleic acids and other contaminants. Thereafter, Hurkman and Tanaka (1986) developed the "Phenol extraction technique". This method was introduced for isolating protein from plant membranes by solubilizing protein in the phenol. This technique can minimize proteolysis during protein precipitation in methanol and ammonium acetate and also gave high quality protein (Hurkman aha Tanaka, 1986; Saravanan and Rose, 2004; Wang *et al*., 2008). However, the weak point of the technique is of a limited capability to extract low molecular weight proteins. To enhance its efficiency, the phenol extraction usually required addition of an organic buffer such as homogenization buffer or SDS buffer (Wang *et al*., 2003, 2006; You *et al*., 2012). Many papers reported successful protocols involving "SDS buffer extraction" for apple and banana, which inhibited protease activity during cell disruption and extraction (Gorg *et al*., 2004; Song *et al*., 2006). Wang *et al*. (2006) also reported the successful use of this technique with a wide range of leaves containing high levels of polyphenols (e.g. olive and pine leaves), fruits with low protein contents (e.g. apple and pear), high sugar content plants (e.g. banana), high acidity plants (e.g. grape and orange) and plants with high contents of pigments (e.g. olive and tomato). Currently, the "homogenization buffer" was also reported to be successful in protein extraction from longan bud tissues and gave high quality protein resolution on 2−DE gel (You *et al*., 2012).

Our research focused on developing a new highly efficient technique for extracting protein from leaves and apical buds of litchi. The new extraction protocol should help remove non−proteinaceous contaminants, significantly increase protein yield and quality especially the separation potential, protein banding, and clearer protein spotting for better sequencing steps.

MATERIALS AND METHODS

Plant Materials

Fifteen litchi trees cv. Hong Huay at the age of 15 y were randomly selected from a highland orchard (1,200 masl) in Mae−rim District, Chiang Mai Province, Thailand. Leaves and apical buds at the mature leaf stage were individually collected from each plant at the amount of ten leaves and ten buds, and immediately frozen in liquid nitrogen (N_2) before storage at –80ºC until analysis.

Tissue Powder Preparation

Frozen litchi leaves and apical buds were firstly crushed and ground in N_2 using a mortar and

pestle. The fine powdered tissues (0.1 g) were placed in 1.5 – 2.0 mL microtubes and stored at –80ºC until the protein extraction step.

Protein Extraction Methods

The frozen litchi powder samples were extracted using three different protocols as comparatively described in Figure 1.

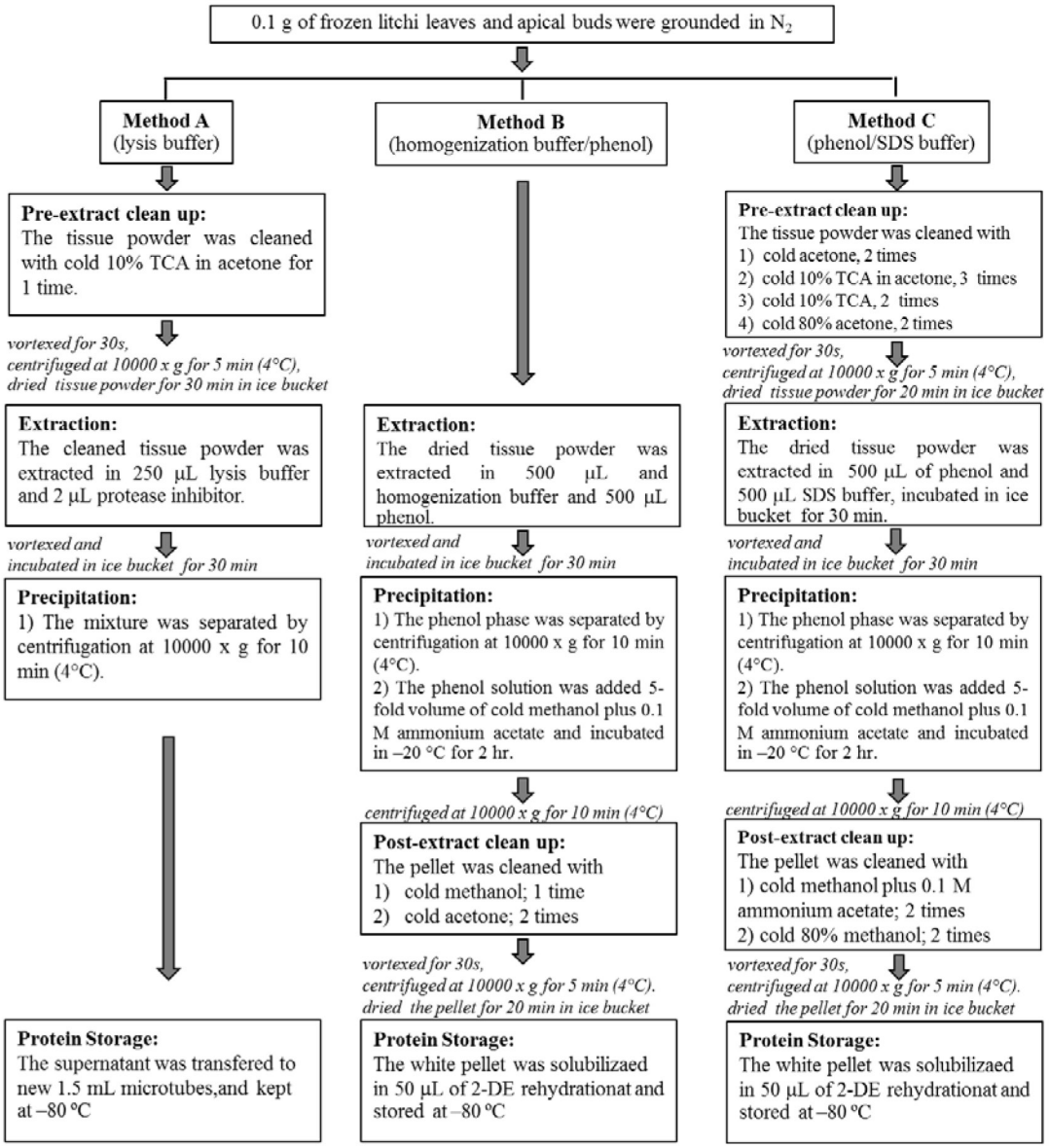

Figure 1 Comparison details of protein extraction methods according to "Lysis buffer method" (Method A), and those adapted from "Homogenization buffer/phenol method" (Method B) and "Phenol/SDS buffer method" (Method C)

Method A (control treatment): The "Lysis buffer method", was performed according to 2−D electrophoresis principles and a methods handbook (Gorg *et al*., 2004). The powdered tissues were firstly cleaned by rinsing with cold 10% (w/v) TCA in acetone and vortexed thoroughly for 30 s, then the tubes were centrifuged at 10,000 x *g* for 5 min (4°C), and then dried for 30 min in an ice bucket. For the protein extraction step, cleaned tissue powder was suspended in 250 µL lysis buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) IPG buffer, 40 mM DTT and the buffer was adjusted to pH 8.0] and 2 µL protease inhibitor were added, vortexed thoroughly and incubated in ice bucket for 30 min. For protein precipitation, the mixture solution was separated by centrifugation at 10,000 x *g* for 10 min (4°C). The supernatant was collected and transfered to new 1.5 mL microtubes and stored at –80ºC until used for the protein separation step.

Method B: The "Homogenization buffer/ phenol method" was improved from the protocol recommended by You *et al*. (2012), which reported a high efficiency in giving high resolution protein spots from longan (*Dimocarpus longan* Lour.) floral buds on 2−DE gel. In method B, there was no recommendation for pre−extract clean up but only a post−extract clean up step. So the frozen powdered tissues were directly extracted with 500 µL of homogenization buffer [100 mM Tris−HCl; pH 8.0, 50 mM L−ascorbic acid, 100 mM KCl, 50 mM disodium tetraborate decahydrate, 1% (v/v) Triton X−100, 2% (v/v) β−mercaptoethanol, 1 mM PMSF] and 500 µL phenol (saturated, pH 7.9), before being vortexed thoroughly and incubated in an ice bucket for 30 min. The homogenate was centrifuged at 10,000 x *g* for 10 min (4°C). For the precipitation step, 250 µL of the upper yellowish phenol phase were pipetted into new microtubes. A five−fold volume of cold methanol plus 0.1 M ammonium acetate were added, then samples were kept at –20°C for 2 hr. In the post−extraction clean up step, the protein mixture was centrifuged at 10,000 x *g* for 10 min (4°C) and washed once with cold methanol and twice with cold acetone. Finally, the pellet was dried in an ice bucket for 20 min and solubilized in 50 µL of 2−DE rehydration solution [7 M Urea, 2 M Thiourea, 2% (w/v) CHAPS. 0.5% (v/v) IPG buffer, 20 mM DTT, 0.002% (w/v) bromophenol blue]. The protein solution was lastly stored at –80ºC until the next step of protein separation.

Method C: The "Phenol/SDS buffer method", a new protocol developed by improving the protocol recommended by Wang *et al*. (2003). Major improvement was focused on both pre−extraction and post−extraction clean up steps of the sample with the expectation of achieving purer protein and a higher possibility of protein spot separation. Plant tissues were cleaned four times at the pre−extraction clean up step (Figure 1). The powdered tissues were twice suspended in 1.0 mL of cold acetone, before vortexing thoroughly for 30s and centrifuged at 10,000 x *g* for 3 min (4°C). The powdered tissue was repeatedly washed three times with cold 10% (w/v) TCA in acetone, vortexed thoroughly for 30s and centrifuged at 10,000 x *g* for 3 min (4°C). Then, the tissue powder was again washed twice with cold 10% (w/v) TCA and then twice with cold 80% (v/v) acetone, before drying in an ice bucket for 20 min.

For protein extraction, the dried tissue powder was suspended in 500 µL of phenol (saturated, pH 7.9) and 500 µL of SDS buffer [30% (w/v) sucrose, 2% (w/v) SDS, 0.1 M Tris−HCl; pH 8.0, 5% (v/v) β−mercaptoethanol and 1% (w/v) polyvinlypolypyrrolidone (PVPP)], mixed well by vortexing thoroughly and incubated in ice bucket for 30 min before passing to the precipitation step. At this step, the phenol supernatant was separated by centrifugation at 10,000 x *g* for 10 min (4°C). 250 µL of the upper phenol phase was pipetted to new microtubes and a five−fold volume of cold 0.1 M ammonium acetate in methanol was added to the phenol supernatant, then mixed thoroughly before storage at –20°C for 2 hr. The protein solution was centrifuged at 10,000 x *g* for 5 min (4°C). Only the pellet was collected and passed to the post−extraction clean up step by washing twice with cold 0.1 M ammonium acetate in methanol and two times with cold 80% (v/v) methanol. The final pellet was dried in an ice bucket for 20 min and suspended

in 50 µL of 2−DE rehydration solution then stored at –80°C until used for protein separation.

Protein Quantification

The concentration of extracted protein was determined using the 2D Quant kit (GE Healthcare Bio−Sciences Corp., USA) and compensating for the interfering compounds according to the manufacturer's protocol. Bovine serum albumin (BSA) was employed as a standard. The protein yield was calculated as micrograms per 0.1 g of dried litchi powder. Three replicates were used, and protein yields are presented as means.

Protein Separation

To compare the quality of extracted protein from the three methods examined protein from litchi leaves and from apical buds were analyzed using SDS−PAGE and 2−DE procedures.

SDS−PAGE

Protein separation by an SDS−PAGE technique (Laemmli, 1970) was carried out by running the protein solution on a 15% (w/v) SDS polyacrylamide gel. The solubilized protein of each sample was loaded at 10 µg per lane together with sample buffer, and denatured at 95°C for 3 min. The proteins were separated under a constant voltage of 50V in a Bio−Rad mini−Protean II apparatus for around 5 hr. After finishing the electrophoresis step, the gel was transferred into a fixing solution (50% (v/v) methanol, 10% (v/v) acetic acid) for 30 min, and then stained in 0.25% (w/v) Coomassie Blue R250 with gentle shaking for 2 hr. After staining, the gel was decolorized 2−3 times with destaining solution [45% (v/v) methanol and 10% (v/v) acetic acid] for 15 min and then transferred to distilled water with gentle shaking until the gel background became transparent.

2−DE

Isoelectric focusing (IEF) was performed using an Ettan IPGphor II, IEF system (Amersham Biosciences, Sweden). Sample solution (pellet) of 100 µg protein from leaves or 200 µg protein from apical buds were separately mixed with rehydration buffer, and each sample was loaded in IPG strip gels (7 cm, pH 3−10, GE Healthcare). The strips were rehydrated at 20°C, 50 A/strip for 12 hr, then the electric current was stepped up (at a constant 20°C) in a stepwise manner and with different exposure times as follows: 150V for 2 hr, 300V for 30 min, 30 min gradient 1,000V, 1.20 hr gradient 5,000V and for 25 min at 5,000V.

After IEF operation, the strips were equilibrated in 5 ml of equilibration buffer I [6 M urea, 75 mM Tris−HCl; pH 8.8, 29.3% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue and 1% (w/v) DTT] for 15 min, then transferred into 5 ml of equilibration buffer II [6 M urea, 75 mM Tris−HCl; pH 8.8, 29.3% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue and 2.5% (w/v) iodoacetamide (IAA)] for another 15 min with gentle shaking. After equilibration, strips were loaded in 15% (w/v) SDS polyacrylamide gels and sealed with agarose sealing solution [0.5% (w/v) agarose in SDS buffer plus 0.1% (w/v) bromophenol blue]. The electrophoresis was conducted at 10 mA/gel for 15 min and 20 mA/gel until the bromophenol blue dye reached the bottom of the gel. Before drying, gels were fixed with 50% (v/v) methanol plus 10% (v/v) acetic acid for 30 min, then transferred into the staining solution (0.25% (w/v) Coomassie Blue R250),and left overnight with gentle shaking. After staining, the gel was decolorized 2−3 times with destaining solution [45% (v/v) methanol, 10% (v/v) acetic acid] for 15 min and then transferred to distilled water with gentle shaking until the gel background became clear.

Image Analysis

Digital images of SDS−PAGE gels and 2−DE gels were scanned using Epson Expression 1680 Pro. The intensity of protein spots from 2−DE gels was processed and analyzed using Dymension Revolutionary 2DE software version 2.05a (Syngene, UK).

Statistical Analyses

Data analysis was conducted using the Statistix for Windows version 8 (Stat Soft INC., Tulsa, OK, USA). Least Significant Difference (LSD) test was applied at $P \leq 0.05$ and conducted to determine the significance probability between the protein yields from the three protocols.

RESULTS AND DISCUSSION

Increase of Protein Yield

Total protein yield extracted from litchi leaves and from apical buds are compared among three different extraction protocols in Table 1. Method A gave a high protein yield from leaves (503.00 ug/0.1 g dried leaf powder) which was equivalent to method C but higher than method B, and the highest yield from apical buds (931.88 ug/0.1 g dried bud powder). The second best procedure was method C, which also gave high protein yield from leaves powder (570.75 ug/0.1 g dried leaf powder), but an intermediate protein yield between methods A and B from apical buds (524 ug/0.1 g dried bud powder). Method B showed the lowest yields of protein from both litchi leaves and apical buds.

Table 1 Protein yields from leaves and apical buds of litchi tissues using three different extraction methods

Extraction method	Protein yield (µg/0.1 g frozen lychee powder weight)	
	Leaves	Apical buds
Method A	503.00 ± 21.61[a]	931.88 ± 22.21[a]
Method B	227.50 ± 26.55[b]	375.33 ± 25.64[c]
Method C	570.75 ± 26.55[a]	524.00 ± 31.40[b]

Note: Values are the mean of three independent replicates; the different letters in the same column indicate a statistically significant difference by LSD at $P \leq 0.05$

The results of protein yield experiments suggest that the most efficient extraction method is the control method (Method A) followed by Method C. However, the protein quality from both methods needed further confirmation steps especially regarding their separation potential, banding, and spot clarity.

Confirmation of Protein Quality

Protein bands from SDS−PAGE analysis

To validate the analysis system, protein ladders of different molecular sizes between 1−120 kDa, the normal range of protein molecules found in plant cells, were parallel loaded and run on SDS−Polyacrylamide gel electrophoresis (PAGE) to confirm the efficiency of the running phase and the ability of the PAGE in protein separation. As shown in Figure 2, a good protein separation was achieved confirming the acceptability of the protein band analysis system used in this study.

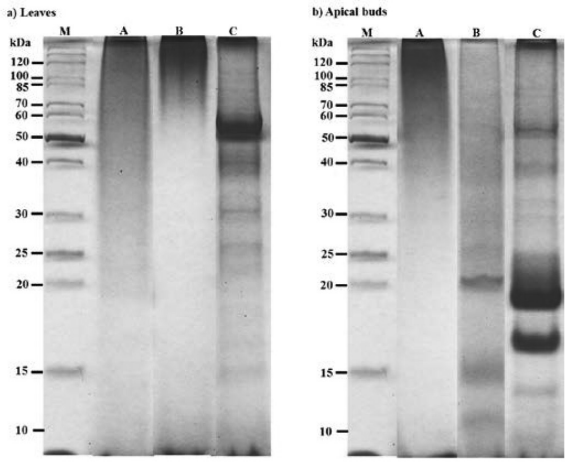

Figure 2 15% SDS polyacrylamide gel electrophoresis profiles of total proteins extracted from (a) litchi leaves and (b) apical buds following method A, B and C. The gels were stained with Coomassie Blue R250. Lane M; protein molecular mass standards

Comparing the three extraction methods, it can be seen that the protein solution obtained from method A could not be separated into bands, both those extracted from leaves and from apical buds due to possibly degrade after extraction and storage. Method B could produce a protein band separation only with extraction from apical buds, but band images were not clear enough. Method C was the only protein extraction method that showed a relatively clear protein band separation based on different molecular sizes ranging from high−molecular masses (M_r) of 40−120 kDa (Figure 4, region I), to those with 15−25 kDa (Figure 4, region II), and also produced prominent protein bands with low−molecular masses (M_r) of below 15 kDa (Figure 4, region III). Interestingly, Figures 2 and 3 were represented the enrichment of Rubisco in method C which is the remarkable protein and comprise more than half of total protein in plant, especially in leaf tissue (Saravanan and Rose, 2004). This is a considerable advantage of method C in giving the potential for extraction of a broad spectrum of protein molecule sizes.

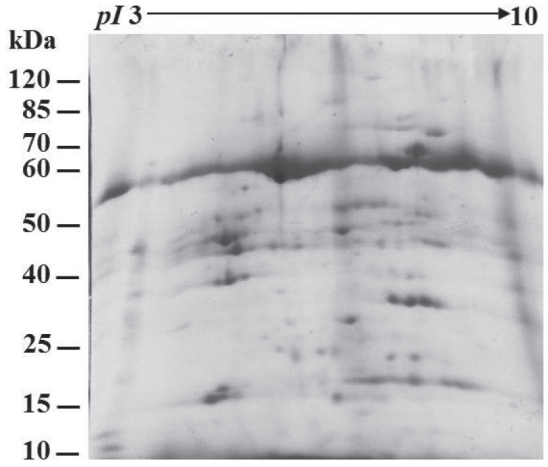

Figure 3 Protein pattern from litchi leaves using method C. Protein 100 µg were separated on 3−10 linear gradients in first dimension, and 15% polyacrylamide−SDS gel in second dimension, and visualized in Coomassie Blue R250

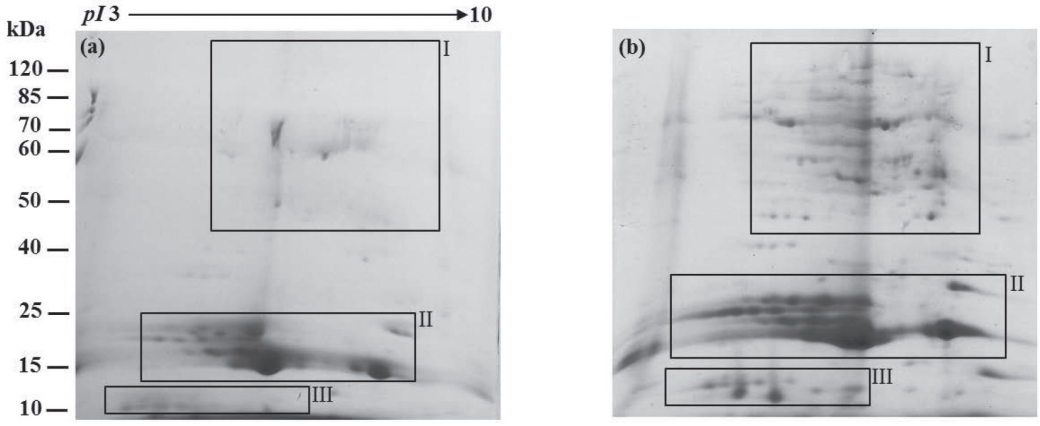

Figure 4 Comparison of 2−DE gels protein pattern from litchi apical bud using (a) method B and (b) method C. Protein 200 µg were separated on 3−10 linear gradients in the first dimension, and 15% polyacrylamide−SDS gel in the second dimension, and visualized by Coomassie Blue R250

Protein quality by 2−DE analysis

Through a 2−Dimension Electrophoresis method, the quality of the protein was further evaluated in terms of protein separation at the spot level. This criterion is important as a basic step required for protein sequencing step in proteomic studies. The best protein extraction method should not only produce more protein yield but also more countable protein spots with 2−DE analysis. Method A was excluded from this study due to its inability to separate protein bands. Only methods B and C were compared. The spot resolution from 2−DE analysis is shown in Figures 3, 4a and 4b, in which method C gave a high−resolution of protein separation and displayed clearer protein profiles than method B. By protein spot counting, method C also gave a higher number of spots, up to 90 for leaf protein and 80 for apical bud protein. Method B gave almost 50% less spots from apical bud protein when compared with method C (Table 2).

Table 2 Number of protein spots using three different extraction methods

Extraction method	Number of protein spots (µg/0.1 g frozen litchi powder weight)	
	Leaves	**Apical buds**
Method A	Not test	Not test
Method B	Not test	49
Method C	90	80

Special Techniques of the New Highly Efficient Protein Extraction Protocol

Emphasizing cleaning up in pre− and post−extraction steps

Method C gave for a significant increase in protein extraction from leaf and apical buds with high protein separation. Especially noteworthy was the cleanliness of protein band separation and clarity of spot patterns. Method A could extract large amount of protein but gave rather poor protein separation and is therefore, not appropriate for litchi. Method B was also not applicable for litchi. Two weak points of method B include: 1) no pre− and post−extraction clean up step and 2) inefficient protein extraction of the homogenization buffer plus phenol. The efficiency of the two clean up steps of Method C in producing clean protein extracts from litchi confirmed this two−fold cleanup recommendation of Wang *et al*. (2003) for gaining a higher and purer protein yield by removing contaminant compounds.

This study clearly showed the advantage of the two stages clean−up for litchi protein extraction from leaves and buds. For the first pre−extraction clean up step, plant tissue should be repeatedly washed with cold TCA and acetone as shown in method C in Figure 1. TCA and acetone solvents are very effective in inhibiting protease activity (e.g. phenol oxidase and peroxidase) and promoting enrichment of alkaline protein (e.g. ribosomal proteins) from the total cell lysates (Damerval *et al*., 1986; Granier, 1988; Gorg *et al*., 2004; Saravanan and Rose, 2004). Moreover, those organic solvents have a high efficiency for removal of contaminant compounds, especially the phenolics in litchi tissue Wang *et al*. (2003). The combination of TCA and acetone is usually more effective than either alone (Chen and Harmon, 2006). The second clean up step, post−extraction clean up, was employed at the pellet level. Phenol phase containing protein from extraction step was firstly precipitated with cold methanol plus

ammonium acetate and centrifuged to separate the pellet, which was lastly washed again four times with cold methanol plus ammonium acetate. The overall results of the protein separation on SDS−PAGE and 2−DE gel in this study revealed that the second clean up step increased spot separation in the 2−DE analysis.

The critical phenol and SDS plus PVPP extraction buffer

Another noteworthy finding was the effect of the extraction buffer of method C which contained a mixture of phenol and SDS. Many papers confirmed the advantage of phenol in being: 1) highly efficient for protein extraction from tissues containing small amounts of protein, 2) low protein degradation which is often encountered during the extraction step, 3) effectiveness in dissolving protein in plant tissue (including membrane proteins), and 4) efficiency in eradicating lipids and various water−soluble substances (polysaccarides, nucleic acids etc.) (Schuster and Davies, 1983; Gorg *et al*., 2004; Carpentier *et al*., 2005; Wang *et al*., 2003; 2008). Phenol extraction procedures are also well known to decrease the interaction between protein and other materials (Carpentier *et al*., 2005). Moreover, in method C 1% (w/v) PVPP was also added in the SDS buffer which increased its effectiveness when combined with phenol. PVPP is an effective agent for removal of phenolic compounds from plant extracts (Toth and Pavia, 2001; Wang *et al*., 2008). Two percentage (w/v) of SDS buffer is an excellent solubilizing agent which is very effective for maintaining protein solubility (Wang *et al*., 2003; 2006; 2008).

Method C was found to be the most effective of the methods tested based on the yield and purity of the protein extracts from litchi leaves and apical buds. This allowed for clearer electrophoretic separation of proteins and more numerous protein spots that encompassed a wide range of molecular weights.

CONCLUSIONS

Protein extraction is important as it is the first step for protein analysis in proteomic studies. This study aimed to establish the most suitable method for protein extraction from the litchi leaves and apical buds. The results confirmed the benefit of method C (Phenol/SDS buffer method). This method gave the highest protein yields with a broad spectrum of molecular sizes and produced clear protein bands and a higher number of protein spots using SDS−PAGE gel and 2−DE gel separation.

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