Two-dimensional profiling of proteins from *Curculigo latifolia* fruit by three different extraction protocols

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Lemba, *Curculigo latifolia* (family Hypoxidaceae) is an indigenous plant of Malaysia, found mainly in swampy areas of tropical Asia and Australia. This study was designed to compare the efficiency of three protocols employed for the extraction of proteins from *C. latifolia* fruit and to find the best compatible with proteomic analysis of fruit on the basis of profiling using two-dimensional gel electrophoresis. Phenol, trichloroacetic acid–acetone and trichloroacetic acid–acetone/phenol-based extraction protocols were evaluated by examining the quantitative and qualitative characteristics of the extracted proteins. A few modifications were introduced to the phenol and the combination of phenol and trichloroacetic acid–acetone protocols in order to improve the two-dimensional gel electrophoresis analysis results. With the exception of trichloroacetic acid–acetone method, the two other protocols were found to extract proteins efficiently and reproducibly. The protein yields from the phenol (3.5 ± 0.12 mg/g) and trichloroacetic acid–acetone/phenol-based (3.7 ± 0.11 mg/g) protocols as well as and the two-dimensional gel electrophoresis patterns showed no appreciable differences. Since using phenol protocol is considerably more time consuming and laborious than the trichloroacetic acid–acetone/phenol-based protocol, therefore trichloroacetic acid–acetone/phenol-based protocol was considered to be a superior protocol for total proteins extraction of *C. latifolia* fruit.

**Key words:** *Curculigo latifolia*, fruit, plant proteomics, protein extraction, two-dimensional gel electrophoresis.

**INTRODUCTION**

Proteins are known as the functional molecules and play fundamental roles in the cells (Ingaver Eidhammer, 2007). As proteins are the main structural and functional molecules, molecular characterization of proteomes is the cornerstone for any comprehensive understanding of any biological system (BeraNova-GiorGianni, 2003). The study of proteins is far more complicated than nucleic acids due to the three-dimensional structures of proteins, various post-transcriptional and post-translational processing (Yun et al., 2010). Furthermore, no amplification method equivalent to polymerase chain reaction (PCR) is available for proteins to aid the studying of those with low copy number (Rose et al., 2004). Two dimensional gel electrophoresis (2-DE) has proven to be one of the most efficient and robust methods (Xie et al., 2007) for profiling large sets of complex protein mixtures, and permits comparative studies between pairs of samples (Song et al., 2006). The most problematic step in any protein profiling research is protein extraction and preparation, especially, for plant tissues containing high levels of interfering compounds. This step is found to be more crucial in plant samples compared to other organisms (Saravanan and Rose, 2004a). Preparation of high-quality protein samples from plant tissues for descriptive
analysis of protein patterns represents a great challenge (Isaacson et al., 2006). In addition to having relatively low protein concentrations, plant tissues are often rich in proteases and materials that severely interfere with subsequent protein separation and analysis, including cell wall and vacuolar components as well as storage polysaccharides, lipids, pigments, phenolic compounds and a broad array of other secondary metabolites (Jorrin-Novó et al., 2009). These contaminants can produce smearing, horizontal and vertical streaking and a notable decrease in the number of well resolved protein spots in 2-DE (Saravanan and Rose, 2004a).

Lemba, Curculigo latifolia (Hypoxidaceae family) is one of the indigenous plants of Malaysia. It is found mainly in swampy areas of tropical Asia and Australia. C. latifolia is a rarely cultivated plant (Shaari, 2005). Despite the relatively recently identified proteins named curculin and neoculin which possess both sweet and taste modifying properties (Barre et al., 1997; Nakajima et al., 2006), no large scale protein experimental analyses have been published for this plant to date. The only reported commercial product of C. latifolia is a chewing gum, which contains a component containing curculin with a coating agent (Kurhara et al., 1994).

To our knowledge, no reports on total protein extraction of this plant have been reported. Unfortunately there is no commonly used protocol of sample preparation and extraction that can be applied to all kinds of samples (Rodrigues et al., 2009). Therefore, different plant tissues are subjected to different protocols to optimize protein extraction for each tissue. A commonly used protein extraction method is based on the principle of precipitating proteins from homogenized tissues with trichloroacetic acid (TCA) in acetone (Méchin et al., 2006).

This protocol is based on protein denaturation under acidic condition in particular, TCA. It has been proven in previous report that TCA or acetone alone would be less effective than the combination of TCA/acetone (Wang et al., 2008). An alternative method is based on the solubilization of proteins in phenol, followed by their precipitation with ammonium acetate in methanol. Phenol extraction was initially served to remove proteins from carbohydrates based sample as well as nucleic acids. Nowadays, it is the standard technique for protein extraction for recalcitrant plant tissues (Faurobert et al., 2006). The third alternative method combines TCA-acetone precipitation, methanol washes and phenol extraction. The basic methanol wash was found to be useful in removing phenolic compounds (Wang et al., 2006). In this study we compared the above mentioned extraction methods for C. latifolia fruit. A considerable portion of this fruit comprises of seeds. They contain large quantities of polyphenols, which interfere with the protein extraction. The efficiency of applied methods was evaluated by the resulting protein quality (appearance of resolved spots, spot intensity, unique spots detected and reproducibility), and quantity through 2-DE electrophoresis and a 2-DE quantification kit respectively.

MATERIALS AND METHODS

Plant material

C. latifolia fruits were collected from the Universiti Putra Malaysia farm from different plants at different times for technical replication purposes. The fruit tissue was separated and washed with water, then dried with tissue paper. The fruit tissue was quick frozen in liquid nitrogen and then freeze-dried. The tissue was subsequently ground into a fine powder using a laboratory blender, followed by final grinding with a standard ceramic mortar and pestle. The fruit samples were subjected to various protein extraction protocols as described below.

Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich, GE-Healthcare, Merck, Bio-Rad and were of molecular biology grade.

Protocols for total protein extraction

TCA-acetone protocol

The TCA-acetone protocol was performed as described previously by Damerval et al. (1986) with slight modifications. Two hundred milligrams of fruit powder was thoroughly mixed, using a vortex mixer, with 1.8 mL of chilled (20°C) precipitation solution containing acetone, 10% TCA, 0.07% 2-mercaptoethanol (2ME) and placed for 1 h at −20°C. Following subsequent centrifugation (13000 g, 20 min at 4°C) the supernatants were discarded. The resulting pellets were washed with 1.8 mL of rinsing solution containing ice-cold acetone, 0.07% 2-mercaptoethanol and stored at −20°C for 1 h and centrifuged at 13000 g, for 20 min at 4°C. This rinsing step was repeated for a total of 3 times. The pellets were dried in a freeze drier for 15 min to eliminate the residual acetone, and then stored at -80°C until analysis (Méchin et al., 2006).

Phenol based protocol

The phenol/SDS protocol was performed as described by Vincent et al. (2006), Saravanan and Rose (2004b) with a few modifications. Two hundred milligrams of fruit powders were added to 0.8 ml of cold (2-4°C) sucrose extraction buffer [0.7 M sucrose, 0.5 M Tris-HCl pH 7.5, 50 mM EDTA, 0.1 M potassium chloride (KCl), 2 mM PMSF, 2% 2-beta-mercaptoethanol (2ME), 1% cross-linked polyvinylpyrrolidone (PVPP)].

The tube was kept on ice with frequent vortexing for 20 min. Subsequently, an equal volume of the equilibrated phenol was added to the tube and vigorously vortexed. The tube was placed at 4°C for 35 min. During this time, the tubes were taken out and vortexed briefly every 10 min. The phases were separated by a 15 min spin at 13,000 g. The upper (phenol) phase of each tube was removed and re-extracted twice with an equal volume of the sucrose extraction buffer (the above -20°C incubation with vortexing every 10 min was repeated three times). The final phenol layer of
each tube was transferred into a tube large enough to accommodate 5 volumes of the chilled precipitation solution (0.1 M ammonium acetate in methanol). The phenol phase of each tube was mixed thoroughly with the precipitation solution and kept at -20°C overnight. After overnight precipitation, the tubes were spun to bring down the protein precipitate (13,000 g, 20 min at 4°C). The pellets were washed 3 times with about the same volume of cold precipitation buffer as was used in the previous step. The final pellets were washed with the same volume of cold 100% acetone and dried under vacuum for 15 min. The dried pellets were stored at -80°C until analysis (Saravanan and Rose, 2004a; Vincent et al., 2006).

TCA/acetone precipitation/phenol protocol

This protocol was performed as described previously by Wei Wang et al. (2006) with some modifications. Two hundred milligrams of fruit powder were thoroughly mixed, (using a vortex mixer) with 1.8 mL of chilled (-20°C) precipitation solution containing acetonitrile, 10% TCA, 0.07% 2-mercaptoethanol and centrifuged (13000g, 10 min at 4°C). Following the centrifugation the supernatant was discarded and the tube was filled with cold 80% methanol containing 0.1 M ammonium acetate and vortexed. The centrifugation was repeated and the supernatant was discarded. The resulting pellet was washed with 80% acetone, vigorously vortexed and centrifuged (13000g, 10 min at 4°C). The supernatant was discarded and the resulting pellet was dried by nitrogen gas. Eight hundred microlitres methanol containing 0.1 M ammonium acetate. The tube’s contents were mixed well and placed at -20°C for 10 min over night. After overnight precipitation the tubes were spun to bring down the protein precipitate (13,000 g, 20 min at 4°C). The supernatant was collected and subjected to protein quantification. The protein concentration was expressed here as µg/µL. Each analysis was done in duplicates.

SDS-PAGE

The purity and overall quality of protein extracts were evaluated using the standard Laemmli buffer SDS-PAGE protocol. Thirty microgram of protein was dissolved in 2.5 µL of 4X loading buffer and loaded onto a 1.0 mm thick 12% acrylamide gel (Bio-Rad Laboratories).

Electrophoresis was conducted in a Bio-Rad mini-Protean™ II apparatus (Bio-Rad Laboratories) at 80 V for 15 min then at 120 V for 1 h 45 min. Subsequently the gel was stained as described below with manually prepared Colloidal Coomassie G-250. All SDS-PAGE gel evaluations were repeated in triplicate.

2DE

IEF

The protein pellets were redissolved in solubilisation buffer as described above. IPG strips (7 cm, 3–10 pH, non-linear ReadyStrip™ IPG Strip; Bio-Rad) were rehydrated with 125 µL rehydration buffer (containing 60 µg of protein) in a Protean IEF Cell (BioRad), at 20°C actively for 12 h at a low voltage (50 V). IEF was performed at 250 V for 20 min, 4000 V for 2 h, 4000 V for 10 000 V-hour. After IEF, strips were transferred to equilibration trays to be prepared for performing the second dimension.

SDS-PAGE

Prior to running the second dimension it is necessary to equilibrate the IPG in SDS-containing buffers. The proteins in the strip were reduced in 2.5 mL of equilibration/solubilisation buffer (6 M urea, 20% w/v glycerol, 4% SDS, and 375 M Tris-HCl, pH 8.8, 0.001 Bromophenol blue) for 25 min and subsequently alkylated with 2.5% w/v iodoacetamide in 2.5 mL of equilibration buffer for another 25 min (Bio-Rad instruction manual).

The separation of proteins in the second dimension was performed on 12% linear (1.00 mm) gels in the Mini-Protean (Bio-Rad) apparatus at a constant voltage of 80 V for 15 min and then 120 V for 1 h 45 min according to the manufacturer’s recommendation (ReadyStrip™ IPG Strip Instruction Manual). All 2-DE gel separations were repeated three times.

Gel Staining, imagining and data analysis

The gels were washed three times with Milli-Q water (each for 10 minutes) on a horizontal shaker and stained with Colloidal Coomassie G-250 (5 % (w/v) aluminum sulfate (16)-hydrate, 10% (v/v) ethanol, 0.02% (w/v) CBB G-250, and 2% (v/v) orthophosphoric acid 85%) (Dybalba and Metzger, 2010). The washed gels were incubated with the Coomassie solution by agitation on a shaker for 12 h. After staining, the Coomassie solution was removed and the gels were rinsed twice with Milli-Q water. Next, the gels were destained for 60 min with 10 % (v/v) ethanol (96%), 2% (v/v) orthophosphoric acid (85 %). 2-DE gel images were obtained on a GS-700 Imaging Densitometer (Bio-Rad Laboratories) and image analysis was performed using PDQUEST 2-DE analysis software (Bio-Rad Laboratories) according to the instruction manual. Figure 1 summarizes the steps mentioned above.
sources of protein degradation (Wang et al., 2006). TCA/acetone is a very efficient precipitation method, which eliminates proteolytic and other modifying enzymes. The disadvantage of this method is the prolonged washing step with the TCA-acetone solution, which is time-consuming. Moreover, prolonged exposure to low pH may cause protein degradation or modifications (Wang et al., 2006). TCA and acetone denature and precipitate the proteins and this solution inactivates the oxidases and phenol oxidases, thus blocking phenol oxidation into quinones etc., and indirectly prevents proteins binding to insoluble compounds. Acetone solubilizes the pigments, lipids, and terpenoids routinely present in the tissue. 2ME prevents the formation of disulfide bonds during precipitation (Méchin et al., 2006). In case of C. latifolia fruit, the main disadvantages of TCA precipitated proteins are, 1-they are very difficult to be resolubilized (Carpentier et al., 2005) and 2-this method is not suitable for protein extraction because of the co-precipitation of gums with the proteins (Awang et al., 2010). These are the main reasons for the deficiencies of this protocol for C. latifolia fruit which is plagued by high amounts of polysaccharide, presumably pectins. The C. latifolia fruit protein extracted by this protocol could not be resolubilized and therefore was excluded from further analysis.

An alternative method to TCA-acetone extraction is a phenol-based method that proved to be a suitable method for recalcitrant plant tissues. Phenol is an effective protein solvent and that can considerably reduce molecular interaction between proteins and other compounds that inhibit electrophoresis (Wang et al., 2007). Phenol dissolves proteins and lipids while leaving water-soluble substances such as polysaccharides, nucleic acids, etc in the aqueous phase. Subsequently the proteins can be separated from lipids by adding precipitation with 100% methanol containing 0.1 M ammonium acetate. The slightly alkaline pH of the buffered phenol helps to reduce protein degradation (Zheng et al., 2007). Although the phenol protocol could effectively extract the proteins from C. latifolia fruit but the resulting protein pellets were dark yellowish, which indicated the co-precipitation of phenolic compounds and other contaminants. To overcome this problem, fruit powder was pulverized with PVPP present, in addition to the original protocol (Rodrigues et al., 2009). This helped to minimize the impact of phenolic compounds. Furthermore, a methanol wash was also added as an aid to extract residual phenolic compound (Wang et al., 2006). The phenol protocol for banana mesocarp, avocado, tomato and orange peel represented higher protein yields and better 2-DE results than the TCA-acetone protocol due to the abundance of interfering compounds (Saravanan and Rose, 2004a). Phenol based protocols also found to be an efficient method for extracting protein from mature grape berry clusters which contained interfering compounds such as high sugar.

RESULTS AND DISCUSSION

Plants in general are recognized as recalcitrant subjects when used for proteomic studies. Establishing a protein extraction protocol is an even bigger challenge when proteomic analysis of fruit tissues are involved (Xie et al., 2007; Zheng et al., 2007). The current study was performed with a plant species (C. latifolia) for fruit in which no proteomic 2-DE studies have been published. As mentioned above, there is no single extraction protocol that can efficiently and universally extract the total plant proteins (Rose et al., 2004). The most obvious obstacle is differential solubility and extraction due to the varying biochemical characteristics and properties of the individual proteins (Saravanan and Rose, 2004a) different tissues. The efficiency of protein extraction from any tissue depends strongly on the efficiency of sample disruption. In this study, the fruit tissue was frozen in liquid nitrogen and then freeze dried. The freeze dried fruits were pulverized to a fine powder in a mortar and pestle in an effort to minimize proteolysis and other
concentrations (Vincent et al., 2006). The low yield of proteins extracted by TCA-acetone method and the difficulty in solubilizing the pellets generated by this method makes it a poor alternative comparing to phenol protocol in extracting total proteins from citrus flesh (Muccilli et al., 2009).

The TCA-acetone-phenol protocol is a combination of two individual protocols and therefore most of the components used for extraction are similar. Here, the impact of the new steps and chemicals are discussed. The methanol wash in the presence of ammonium acetate neutralizes residual TCA and thus raises the pH to about 7, which improves the subsequent extraction of proteins by phenol. SDS, which is used in the SDS/phenol extraction medium, is an efficient solubilizing reagent for proteins and in particular enhances the solubilization of membrane proteins (Zheng et al., 2007). This modified protocol only has a brief TCA/acetone wash step, which was followed by washing with methanol and acetone to remove residual TCA and other contaminants. The main advantage of this protocol is the considerably reduced time of sample preparation which in turn improves sample quality. As well the shortened exposure to low pH reduces protein degradation or modification. Despite these apparent advantages, there were no appreciable differences between protein yields extracted by phenol (3.5 ± 0.12 mg/g) and TCA-acetone-phenol (3.7 ± 0.11 mg/g) based protocols as well as 1-DE and 2-DE patterns. Although the addition of 2% w/v SDS to the extraction buffer has been reported to improve the protein solubility in olive leaf (Wang et al., 2003) here in Curculigo latifolia fruit, SDS did not enhance the protein solubility. Carpentier et al. (2005) also found that adding SDS did not improve protein solubility in banana extractions (Carpentier et al., 2005). We also increased the incubation time of the fruit powder with the TCA-acetone solution to 1 h but observed no differences in protein quality and quantity (data not shown). The TCA-acetone-phenol protocol gave as good a 2-DE result for banana and apple fruits as did the phenol protocol (Carpentier et al., 2005). Wang et al. (2006) demonstrated that the combination of TCA-acetone and phenol method generates satisfactory results in extracting protein from grape, kiwi and orange. But TCA-acetone and phenol combination method was not successful at extracting proteins from citrus flesh due to the formation of precipitates which interferes with further proteomics analysis (Muccilli et al., 2009). In contrast, TCA-acetone-phenol, TCA-acetone, phenol protocols gave similar protein yields in Arabidopsis thaliana but the TCA-acetone-phenol method yielded the best 2-DE result (Maldonado et al., 2008).

The qualitative characteristics of protein extracts obtained using the more efficient two protocols were evaluated using both SDS-PAGE and 2-DE, and proteins visualized with colloidal Coomassie Blue stain. As mentioned above, because of the high concentration of polysaccharides, TCA-acetone protocol could not extract the proteins in a form that permitted efficient resolubilization and so was excluded from further protein analysis. As shown in Figures 3 and 4, 60 µg proteins extracted by phenol and TCA-acetone-phenol protocols showed similar 1-DE and 2-DE patterns. The most abundant proteins had pl's of 5 to 8 and their molecular weights were around 30 kDa. In Figure 2, more sample (110 µg) was loaded to show the less abundant proteins with the molecular weights in the range of 44-66 kDa. The additional protein loaded, however, produced an overloading of abundant proteins. The concentration of individual proteins in fruit tissue prevents analysis of low abundant proteins. Some proteins have several million copies in a single cell to a few copies for low abundant proteins. This explains the major obstacles for detecting, quantifying and analyzing a wide range of proteins (Šamaj and Thelen, 2007).

**Conclusion**

Curculigo latifolia fruit proteins extracted by TCA-acetone protocol could not be resolubilized due to inefficiency of this protocol to remove high levels of polysaccharides present in the fruit. The phenol and TCA-acetone-phenol protocols were found to have similar results in terms of protein yield, 1-DE pattern, resolution, spot focusing, spot intensity, number of spots and reproducibility. These two methods were able to resolve low abundant proteins although at the expense of overloading the abundant proteins but performing phenol-based protocol is more laborious and requires about 1.5 h more time than trichloroacetic acid–acetone/phenol-based protocol. Therefore, trichloroacetic acid–acetone/phenol-based protocol was recommended as the better choice in extracting total proteins from Curculigo latifolia fruit.
REFERENCES


