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Full Length Research Paper

Optimization of two-dimensional gel electrophoresis protocols for *Boesenbergia rotunda in vitro* suspension culture

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Boesenbergia rotunda **belongs to the Zingiberaceae family and is abundant in the Southeast Asia. It is widely used as food ingredient and traditional medicine. Biologically, the plant extract contains pharmaceutically important bioactive compounds that exhibit anti-HIV protease, antibacterial, antifungal, anti-inflammatory, anti-tumor and antioxidant activities. Proteomics approaches to study the proteins and/or enzymes involved in the biosynthesis of these compounds are challenging due to the complexity of plant samples and the presence of interfering substances. Here, we describe the development a highly robust and reproducible two-dimensional gel electrophoresis (2DE) protocols for resolving the proteome of** ***B. rotunda*** **suspension cultures.**

Key words: Two dimensional electrophoresis, plant proteomics, *Boesenbergia rotunda*, Temu kunci, callus.

INTRODUCTION

Boesenbergia rotunda (formerly known as *Kaempferia pandurata Roxb.* or *Boesenbergia pandurata (Roxb. Schltr.*) is a small perennial monocotyledonous plant belonging to the Zingiberaceae family. It is traditionally known as Chinese keys, Temu Kunci or Fingerroot ginger. *B. rotunda* is well-known as folk medicine and food ingredient (either as a spice or condiment) in South East Asia. Tubers of *B. rotunda* are used as a traditional remedy for tumours, swellings, wounds as well as for treating colic disorder such as diarrhea and worms, removing blood clots and as an aphrodisiac. A number of bioactive compounds have been identified from the rhizome extract of *B. rotunda*. These include boesenbergin, cardamonin, pinostrobin, pinocembrin, panduratin A and 4-hydroxypanduratin A (Jaipetch et al., 1982) that exhibit antioxidant, antibacterial, antifungal, anti-inflammatory, antitumor and anti-tuberculosis activities. The antiviral activity of Panduratin A and 4-hydroxypanduratin A in *B. rotunda* has been demonstrated (Kiat et al., 2006). These compounds have been shown to possess significant inhibitory activities against NS2B/NS3 Dengue viral protease which is required for viral replication. However, their presence in *B. rotunda* is scarce and the synthesis is likely to involve many proteins and complex pathways. Plant tissue culture and metabolite engineering offer means for exploiting important metabolites and their biosynthesis so that they can be produced in a sustainable fashion.

An understanding of the biosynthesis pathways involved can be explored using proteomics. Plants at different developmental stage exhibit temporal and spatial regulation of protein expression (Barbier-Brygoo and Joyard 2004; Hennig, 2007; Klein and Thongboonkerd, 2004; Qureshi et al., 2007). The expression of proteins changes when cells are exposed to different environments (Zang and Komatsu, 2007). There are limited numbers of studies on plant proteomics (rice, *Arabidopsis*, barley and etc.) (Ferreira et al., 2006;

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Finnie et al., 2002; Gallardo et al., 2001). Currently, most plant proteomics studies are focused on crops rather than medicinal plants. Furthermore, studies on medicinal plants are more focused on the extraction of bioactive compounds and analysis for distinct activity without looking into the biosynthesis of the compounds concerned, or gene regulations for the production of the enzymes involved in the biosynthesis pathway (Bolwell et al., 2004; Cash, 2002). In addition, the complexity of plant samples makes it difficult to analyze them by two-dimensional gel electrophoresis (2DE). In this study, efficient protein extraction and 2DE protocol have been developed and optimized for resolving the proteome of *B. rotunda* suspension cultures.

MATERIALS AND METHODS

Total protein extraction

Total protein was extracted from fresh and frozen *B. rotunda* suspension cultures. Explants of *B. rotunda* used were obtained from a field in Temerloh, Pahang and Malaysia. Meristems from sterilized sprouts were cultured in suspension as previously described (Tan et al., 2005). Approximately, 2 g of frozen cells were ground to fine powder in a pre-chilled mortar with a small amount of liquid nitrogen.

Extraction buffer (4 ml), containing 160 µl of tris-HCl (pH 9.5, 40 mM), 100 mg Polyvinyl polypyrolidone (PVPP), 40 µl protease inhibitor mix and 60-63 µl (approximately 1200 units) of nuclease mix in distilled water, was added and the mixture was thoroughly homogenized. 500 µl of the homogenate was then placed into a 2 ml microcentrifuge tube, vortexed vigorously for 1 min and sonicated. This was repeated three times. The homogenate was then centrifuged twice at 5000 g for 5 min and about 350 to 450 µL of the supernatant was recovered.

Protein precipitation

Protein extracts were concentrated by trichloroacetic acid (TCA) precipitation. Briefly, 37.5% TCA (w/v) with 1% β-mercaptoethanol (v/v) was added to 400 µl of sample to achieve a final volume of 2 ml. The mixture was then incubated at -20°C for at least 1 h. This was followed by centrifugation at 13, 000 g (4°C for 15 min). The resulting protein pellet was washed three times in 80% acetone (v/v) containing 0.05% β-mercaptoethanol (v/v) and then air-dried in a laminar flow hood at room temperature.

The pellet was then solubilized in isoelectric focusing buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer (GE Healthcare), 1 M Thiourea and trace amounts of Orange G) and incubated at room temperature for at least 30 min. The solubilized protein was then centrifuged (10,000 g at 4°C for 20 min) and either used immediately or stored at -80°C (Franceschetti et al., 2004; Fukuda et al., 2007; Gorg et al., 2004).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were routinely size-separated by SDS-PAGE (10% v/v acrylamide) according to methods previously described (Laemmli, 1970). The samples were electrophoresed on gels containing 0.1% (w/v) SDS in Tris-glycine buffer under standard conditions (Sambrook, 2001) using the BioRad Protean II mini-gel system and proteins were visualized by Coomasie staining.

2-Dimensional electrophoresis

First-dimension isoelectric focusing (IEF)

First-dimension IEF was performed on an IPGPhor III IEF system (GE Healthcare). Protein concentration was determined using 2-D Quant kit (GE Healthcare) using BSA as standards. Protein samples (equivalent to 20 µg protein) were diluted in buffer containing 8 M urea, 2% CHAPS, 0.5% IPG buffer (GE Healthcare), 1 M Thiourea and trace amounts of Orange G. In-gel rehydration of Immobiline DryStrip gels (GE Healthcare) was then performed and the proteins separated by IEF up to 35.5 kVh.

Second-dimension SDS PAGE

Focused IPG strips were equilibrated for 15 mins in equilibration solution (6 M urea, 50 mM Tris-HCl pH 8.8, 30% [w/v] glycerol, 2% [w/v] SDS, trace of bromophenol blue) containing 0.1% DTT and then alkylated for a further 15 mins in equilibration solution containing 0.45% iodoacetamide. Electrophoresis of reduced and alkylated samples was then carried out using 13 cm 10% SDS–PAGE gels (Ruby 600, GE Healthcare).

Silver staining and image analysis

Protein spots were visualized using the protocols described in the PlusOne™ Silver staining kit (GE Healthcare). Silver-stained gels were scanned (Image scanner, GE Healthcare) and protein profiles analyzed (Image Master Platinum software, GE Healthcare) for gel quality, number of spots and gel resolution.

RESULTS AND DISCUSSION

Friable yellowish callus (Figure 1A) was obtained from meristematic cultures of *B. rotunda* and used in protein extractions according to several conditions shown in Table 1. Protein profiles on SDS-PAGE were shown to be highly consistent and reproducible among different protein extractions and different culture batches. Representative results are shown in Figure 1B.

Optimization of 2DE performed on pI 3-10 gels showed that conditions No. 6 in Table 1 resulted in the best gel resolution (Figure 2). An equivalent of 20 µg of protein was focused for 35.5 kVhrs during IEF. Lower focusing times resulted in less focused gels. Protein was extracted using a buffer containing 40 µL protease inhibitor, 60 µL nuclease mix and 0.1 g PVPP. Lower quantities of protease inhibitor, nuclease mix and PVPP resulted in gels that were not completely focused with higher background staining. Analysis of the gel images using the Image Master Platinum Version 7 software showed that with pH 3-10 gels a total of 1047 protein spots were resolved (Figure 2) whereas for pH 4-7 a total of 2313 of protein spots were resolved (data not shown).

Plant tissue contains a high proportion of metabolites, especially phenolic compounds, which can interfere with 2DE. Removal of these compounds is therefore necessary before any meaningful 2DE analysis can be performed. In our experiments, it was found that the

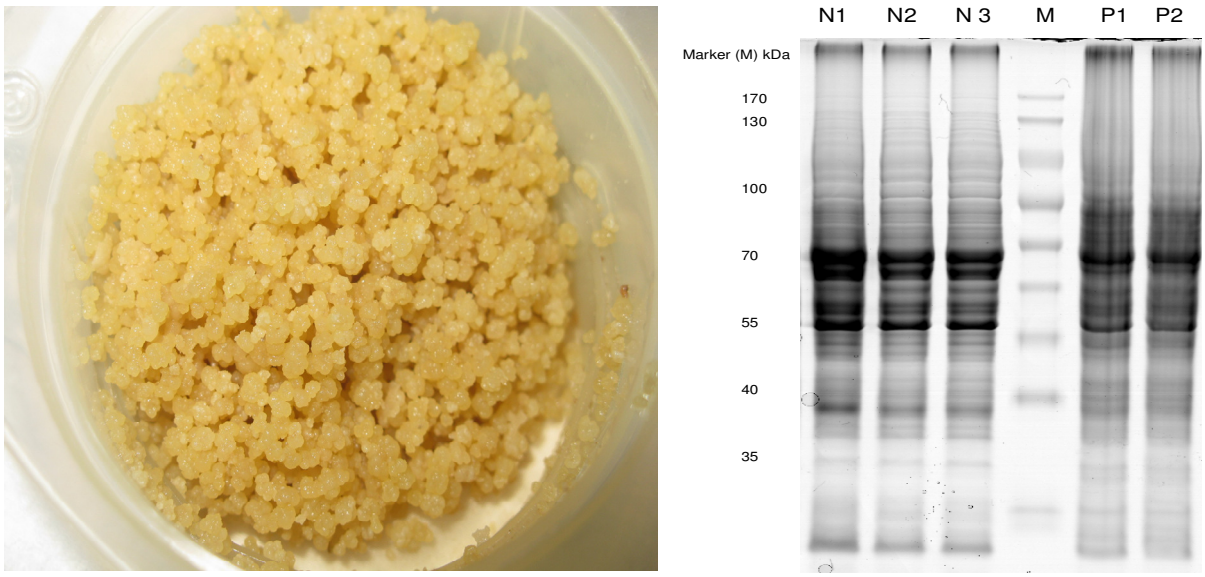

Figure 1. (A) Photographs of the *B. rotunda* friable yellowish callus. (B) SDS-PAGE of Random Total Protein Samples of *B. rotunda* callus. N1 to N3; Randomly-picked normal protein samples, lane M; pre-stained molecular weight marker; P1 and P2; insoluble pellet.

Table 1. Optimization of several parameters in the protein extraction protocol.

No	Protein loaded (µg)	Protease inhibitor (µL)	Nuclease mix (µL)	PVPP (g)	TCA volume (µL)	Incubation	IEF Vhrs
1	20	20	30	0.06	500	No	16.5k
2	30	20	30	0.06	500	No	16.5k
3	10	40	60	0.08	500	No	35.5k
4	20	40	60	0.08	750	Yes	35.5k
5	10	40	60	0.1	750	Yes	35.5k
6	20	40	60	0.1	750	Yes	35.5k
7	25	40	60	0.1	750	Yes	35.5k
8	20	40	60	0.1	750	Yes	35.5k
9	30	40	60	0.1	750	Yes	35.5k

addition of 0.10 g of PVPP during sample preparations was able to remove these phenolic compounds sufficiently. Phenolic compounds bound to PVPP molecules were precipitated with plant debris during centrifugation. In addition, PVPP was also able to remove excess small molecules which could affect IEF and modify peptide functional groups.

The presence of proteases would result in sample degradation, loss of resolution and interferes with the resolution of IEF, resulting in high background staining of 2DE gels. These macromolecules were eliminated with the use of a nuclease mix. Further clean-up and concentration of samples were found to be necessary. This was achieved by TCA-acetone precipitation that eliminated ionic molecules and lipids within the sample. Prior to IEF, the pellet resulting from TCA-acetone precipitation was dissolved in rehydration buffer containing urea and thiourea. Urea is a non-charged chaotrope which disrupts non-covalent bonds within proteins leading to protein denaturation. Thiourea was used to solubilize proteins especially basic and hydrophobic proteins.

In conclusion, we have described an efficient protein extraction protocol for *B. rotunda* suspension cultures samples using TCA/Acetone precipitation for use in 2DE analysis. These protocols have been successfully optimized to generate high resolution and reproducible 2DE gels of the *B. rotunda* proteome. This facilitates

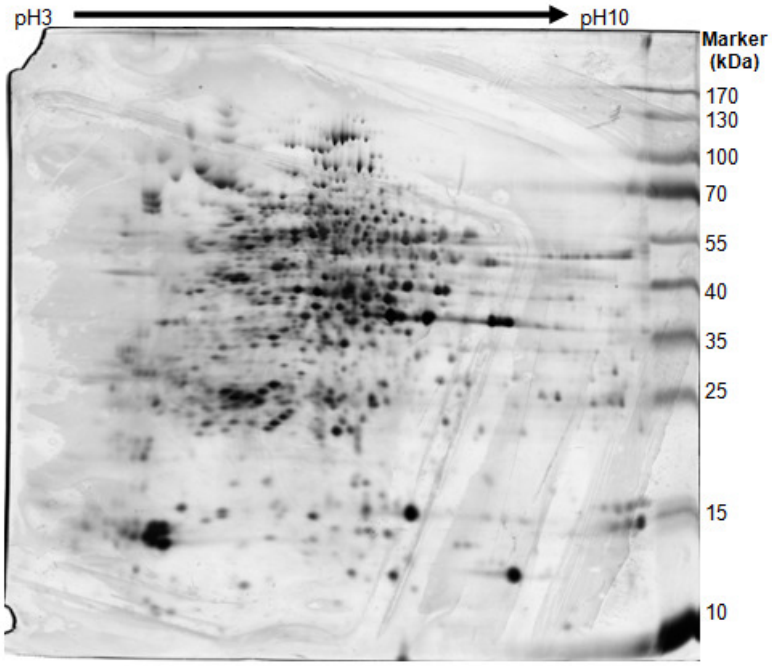

Figure 2. 2DE gel of *B. rotunda* protein extract obtained under condition No. 6 as shown in Table 1, performed on a pH 3-10 strip.

further exploration of the metabolites and biosynthesis pathway analysis of pharmaceutically important compounds in *B. rotunda.*

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