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Research

Evaluation of Six Protocols for Protein Extraction from Rice Young Panicles by Two-Dimensional Electrophoresis

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ABSTRACT

For most reported proteomics approaches, protein extraction and sample preparation are of crucial importance for optimal results. However, extraction of protein from crop plant tissues remains a great challenge due to low protein content and abundant secondary metabolites that prominently interfere with isoelectric focusing and subsequent proteomic analysis. Up until now, no attempts are focused on comparison of protein extraction procedures from rice young panicles. To establish a high-efficiency protein extraction protocol suitable for two-dimensional electrophoresis (2-DE) in rice young panicles, six protocols for protein preparation were evaluated: (1) Phenol extraction; (2) Mg/ Nonidet P-40 (NP-40) extraction; (3) Tris-Base/acetone precipitation; (4) SDS extraction; (5) trichloroacetic acid (TCA)/acetone/phenol extraction; (6) TCA/acetone precipitation. The study explicitly demonstrates that TCA/ acetone/phenol method provides a high-enhanced protein extraction efficacy from rice young panicles than other protocols in terms of the higher quality of 1-DE gel, the maximum number (450) of well-resolved protein spots, greater resolution and spot abundance. In addition, these methods also generated remarkably different two-dimensional gel electrophoresis protein spot patterns. Twenty-nine of 30 visible differential extracted proteins were identified by MALDI-TOF-MS/MS analysis and were divided into eight categories according to molecular function. Accumulated data suggested that different extraction methods respectively necessitate certain special plant tissues due to different physicochemical properties of each protocol. Overall, this study, which is presented in this paper, will facilitate to providing a cornerstone of comparative proteomic analysis from rice young panicles, including other complicated plant tissues.

Keywords: Phenol; Proteomics; Rice young panicles; Two-dimensional electrophoresis

INTRODUCTION

Over the last few decades, rapid advancements in high-resolution protein separation, mass spectrometry techniques and bioinformatics knowledge have led to an increasing application of proteomics to elucidate the underlying biology mechanisms [1]. Now, proteomics approaches integrated two-dimensional electrophoresis (2-DE) with mass spectrometry (MS) has still proved to be the predominant technique [2-8], which was widely used in comparative proteomics studies, post-translational modifications and protein interaction network analysis [9-11].

Protein quality is unquestionably a crucial importance factor for better proteomics results involving the number of well-separated protein spots, reproducibility, resolution [12,13], so protein extraction procedure is of extremely essential. The perfect protocol of protein extraction should reproducibly capture all most unprejudiced and comprehensive proteins from various tissues of different species while reducing contamination and minimizing degradation and modification. However, considering the diversity and specificity of sample tissues or species, no general and high-efficiency extraction protocol can capture the full proteome [1,10,11]. To date, tremendous efforts are more devoted to developing sample preparation protocols that could

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enhance plant proteomic analysis [14-17]. Nevertheless, plant proteomics analysis remains to be more troublesome with challenges that are associated with large amounts of co-extraction of non-protein constituents, and comprehensive proteins are notoriously difficult to extract from plants, mostly due to having relatively low protein content and interfering compounds such as phenolic compounds, lipids, polysaccharides, proteolytic enzymes, oxidative enzymes and other secondary materials [18-22].

Currently, several reviews related to protein sample preparation have been published [23,24]. The most classical extraction tactics is TCA/acetone precipitation method followed by re-solubilization [25-27]. This protocol is suitable for various types of diverse plant tissues, including maize leaves [28,29], tomato [30] and lucid ganoderma [31], but it was found to remain the inherent drawback that proteins may not completely re-solubilize after precipitation. The merit of this protocol is that a precipitation procedure was proposed to concentrate proteins and eliminate interfering elements. An alternative phenol extraction protocol followed by ammonium acetate precipitation in methanol has been developed and was used successfully with grape [32], barely [33], olive leaf [34]. Furthermore, a combination of TCA/acetone and phenol method is also similarly confirmed to be very effective that provides enhanced 2-DE based proteomics analysis [12]. In addition, SDS extraction [10], Tris-Base/acetone precipitation [35-39] and Mg/Nonidet P-40 (NP-40) extraction [40] have also been reported in different tissues while not as frequently as the method mentioned above. Notoriously, how to select an appropriate protein extraction protocol mainly depends on the nature of plant tissues and on the downstream application. Considering this, there is an extremely urgent requirement for establishment of protein extraction protocols aimed at different tissues and species to facilitate comparative proteomics analyses.

Rice (*Oryza sativa* L.) is one of the major foods consumed by more than half of the world's population. However, rice production was negatively influenced by various environmental stresses, including high temperature [41-43]. It was reported that exposure to high temperatures induced sterility, and rice was more sensitive to excessive heat especially in the young microspore period of booting stage [44]. To study changes at the proteomics level in rice young panicles in response to high temperature stress, the current work was performed to screen a suitable protein extraction protocol for subsequent proteomics analysis.

Thus, in this paper presented here, six protocols of protein extraction (phenol extraction, Mg/NP-40 extraction, Tris-Base/acetone precipitation, SDS extraction, TCA/acetone precipitation, TCA/acetone/phenol extraction) were evaluated based on 1-DE maps, 2-DE maps and MS/MS analysis. To our best knowledge, this is the first study on comparison of protein extraction protocols from rice young panicles. Our results indicated that TCA/acetone/phenol method has the better efficacy of protein extraction and this evaluation will provide useful information for other rice tissues.

MATERIALS AND METHODS

Plant material

Rice (Oryza sativa L ssp. indica) was used in experiment. Seeds were sterilized, soaked, germinated, sown, the strains were transplanted in an open field condition in April, 2017, and were cultured in a traditional management way. The field temperature was monitored by using RR-9100A Farmland environment automatic detection system (RainRoot scientific, Beijing). Rice young panicles were harvested (Figure 1). All harvested sample tissues were packed in aluminum foil, flash-frozen in liquid nitrogen, and stored at a temperature of -80°C prior to protein extraction.

Figure 1: Harvested rice young panicles. The lengths of all harvested sample materials were approximately 7 cm.

Protein extraction protocols

Three grams of each frozen plant tissues (rice young panicles) of each protocol was finely pulverized in a pre-chilled mortar with liquid nitrogen. Three replicates were performed for every protocol. The main differences of each extraction protocol were described in Table 1.

Protocol 1: Phenol extraction was performed according to Hurkman et al. [33]. Briefly, the powder was homogenized in 25 mL protein extraction buffer containing 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl (pH 7.5), 50 mM EDTA, 2% v/v 2-mercaptoethanol (2-ME), 1% w/v PVPP. The homogenate was thoroughly vortexed for 5 minutes, and then incubated on ice, sharply shaken every 10 minutes for 30 minutes. An equal volume of Tris-saturated phenol (pH 8.0) was added and the mixture was re-homogenized for 30 minutes. After centrifugation at 12500 × g for 30 minutes at 4°C, the upper phenolic phase was collected and transferred into new eppendorf tubes followed by centrifugation again. The collected phenolic phase was precipitated with five volumes of ammonium acetate in methanol at -20°C overnight and centrifuged as above. The precipitate was washed three times with cold acetone before centrifugation circularly. The final pellet was lyophilized, re-dissolved, and stored at -80°C until analyzed.

Protocol 2: Mg/NP-40 extraction procedure was described by Kim et al. [45]. The powder sample was transferred to a 50 mL centrifuge tube and pre-chilled protein extraction buffer (25 mL)

containing 0.5 M Tris-HCl (pH 8.3), 2% v/v NP-40, 20 mM $MgSO_4$, 2% v/v 2-mercaptoethanol (2-ME), 1% w/v PVPP was added. The homogenate was fully vortexed for 5 minutes and ultra-sonicated in an ice bath for 30 minutes before the mixture was incubated at 4°C for one hour. After centrifugation at 12500 × g for 30 minutes at 4°C, the supernatant was precipitated by adding four volumes of cold acetone containing 10% v/v TCA and 0.07% v/v 2-mercaptoethanol (2-ME), vortexed, incubated at -20°C overnight. Subsequently, pellet was re-suspended in acetone with 0.07% v/v 2-mercaptoethanol (2-ME) and incubated at -20°C for one hour, and then centrifuged at 12500 × g for 30 minutes at 4°C. The purification step was repeated three times and finally the pellet was lyophilized, re-dissolved, and stored at -80°C until analyzed.

Protocol 3: Tris-Base/acetone extraction was based on a previously method published by Zhang et al. [36]. The powder was mixed with extraction buffer (25 mL) consisting of 50 mM Tris-HCl (pH 7.5), 50 mM EDTA, 100 mM KCl, 20 mM DTT, 1% w/v PVPP. The homogenate was fully vortexed for 5 minutes and ultra-sonicated in an ice bath for 30 minutes before the mixture was incubated at 4°C for one hour. After centrifugation (12500 × g, 30 minutes, 4°C) the supernatant was manipulated identically to the Mg/NP-40 precipitation method.

Protocol 4: SDS extraction was adapted from Zhen et al. [10]. Powder was re-suspended in 25 mL of lysis buffer (2% w/v SDS, 5% w/v sucrose, 0.6% w/v PVPP, 0.3% w/v DTT, 20 mM sodium phosphate, pH 7.0) and incubated at 65°C for 20 minutes. The mixture was cooled on ice prior to centrifugation at 12500 × g at 4°C for 45 minutes. The precipitate was treated as the procedure of Mg/NP-40 precipitation method.

Protocol 5: The protein extraction protocol was a classical TCA/acetone precipitation procedure adapted by Damerval et al. [25] with some modifications. The powder samples were re-suspended in 25 mL of 10% w/v TCA in acetone containing 0.07% v/v 2-mercaptoethanol (2-ME) at -20°C for overnight, followed by centrifugation at 12500 × g at 4°C for 45 minutes. The pellet was rinsed thrice with acetone supplemented with 0.07% v/v 2-mercaptoethanol (2-ME), subsequently was lyophilized, re-dissolved, and stored at -80°C until analyzed.

Protocol 6: The protocol is the combination of TCA/acetone precipitation and phenol extraction method referred to Isaacson et al. [1]. The powder was manipulated as TCA/acetone precipitation method until obtaining pellets, followed by treated in the same way as phenol extraction method.

Protocols	Main extraction buffer	Precipitant	Features
Phenol extraction	0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl (pH 7.5), 50 mM EDTA, Tris-buffered Phenol 8.0	ammonium acetate/ methanol	Time consuming; Toxic
Mg/NP-40 extraction	0.5 M Tris-HCl (pH 8.3), 2% v/v NP-40, 20 mM $MgSO_4$	TCA/acetone	Relatively Simple; Ice bath; Ultra-sonication
Tris-Base/ acetone precipitation	50 mM Tris-HCl (pH 7.5), 50 mM EDTA, 100 mM KCl, 20 mM DTT,	TCA/acetone	Relatively Simple; Ice bath; Ultra-sonication
SDS extraction	2% w/v SDS, 5% w/v sucrose, 0.3% w/v DTT, 20 mM sodium phosphate, pH 7.0	TCA/acetone	Heat dissolution
TCA/acetone/ phenol extraction	0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl (pH 7.5), 50 mM EDTA, Tris-buffered Phenol 8.0	TCA/acetone; ammonium acetate/ methanol	Relatively complicated and time consuming; Toxic
TCA/acetone precipitation	10% w/v TCA, 0.07% v/v 2-ME in acetone	TCA/acetone	Proteins are difficult to fully re-solubilize

Table 1: The main differences between each extraction protocol

Total protein quantification

The total protein concentration was assessed by Bradford assay [46] with bovine serum albumin as a protein calibration standard. Protein content of original sample (μg/μL)=dilution multiple × actual value.

One-dimensional SDS-PAGE

To validate quantified total protein concentration and evaluate the quality of obtained young panicle protein, one dimensional SDS-PAGE was conducted. Briefly, 36 μg of each sample protein and 2 μL of each sample protein was respectively loaded into each well, and then separated on 12.5% w/v polyacrylamide gel and 5% w/v stacking gel. The run was performed at 160 V for 2.5 hours using DYCZ-24A double vertical electrophoresis system (Beijing LiuYi Biotechnology CO, China). Proteins were visualized by then staining with Coomassie Brilliant Blue G-250 protocol and Silver nitrate dyeing protocol according to the previous method [47].

Two-dimensional electrophoresis

A total of 250 μg each protein sample was mixed with corresponding volume rehydration buffer (7 M urea, 2 M Thiourea, 1.2% w/v CHAPS, 0.005% w/v Bromophenol blue, 20 mM DTT, 0.25% v/v Ampholytes, pH 3-10) and dissolved at room temperature for at least 1 hour. After centrifugated at 13000 × g at 4°C for 15 minutes, the immobilized linear pH

gradient strips (24 cm, pH 3-10, Bio-Rad Ready Strip; Bio-Rad) were rehydrated for 14 hours in 450 μL rehydration buffer, and isoelectric focusing was carried out using PROTEAN i12 IEF cell system (Bio-Rad, USA) at 20°C with current limit 50 μA per strip applying the following procedure: 100 V rapid for 1.5 hours, 200 V rapid for 1.5 hours, 500 V rapid for 1.5 hours, 1000 V rapid for 1.5 hours, 5000 V rapid for 1 hours, 10000 V gradual for 1 hour, and to 135000 VH with a maximum voltage of 10000 V. Prior to second dimension analysis, the strips were equilibrated for 15 minutes in 10 mL equilibration buffer (6 M urea, 2% w/v SDS, 20% v/v glycerol, 50 mM Tris, pH 8.8) containing 2% w/v DTT and then replaced with 10 mL equilibration buffer containing 2.5% w/v iodoacetamide for 15 minutes. The equilibrated strips were then transferred onto 12% SDS polyacrylamide gels and sealed with 1% w/v low-melting agarose solution. The second dimension electrophoresis was performed using PROTEAN Plus Dodeca cell system (Bio-Rad, USA) at 80 V for 40 minutes followed by constant voltage of 160 V for 4.5 hours until the bromophenol blue front reached the bottom of the gel and then the gels were stained as established by Liao et al. [47,48].

Gel images analysis

All stained gel images were captured and digitalized with transmittance mode at 600 dpi by GS-800 calibrated imaging densitometer (Bio-Rad, USA). Gel images analyses were performed with Image Master 2-D PDQuest™ analysis software (version 8.0, Bio-Rad, USA) and Quantity One software (Bio-Rad, USA). Protein spots were automatically detected by image editing, background subtraction without spots editing. The protein preparation and 2-DE experiments were performed in triplicate for each protocol. Statistical analysis was performed using the SPSS version 17.0, One-way analysis of variance and least significant differences test were used to determine the significant differences among extraction methods, with $p<0.05$ considered as statistically different. Visible protein spots absented in multiple protocols (not all protocols) were considered to be differentially extracted proteins.

Protein identification by MALDI-TOF-MS/MS

Selected differentially extracted protein spots were excised out of the gels, transferred into individual 1.5 mL centrifugate tubes, and then digested with trypsin (modified porcine trypsin sequencing grade, Promega) follow the previous method reported by Liao et al. [48,49].

The gel plugs were washed twice and then were de-stained by incubation with 15 mM K3Fe(CN)6 and 50 mM Na2S2O3 in 40% v/v acetonitrile (ACN) at 37°C. After removal of the de-staining solution, then were covered with buffer containing 25 mM ammonium bicarbonate and 40% v/v ACN at room temperature for 30 minutes. Next, the sample was dried at room temperature for 15 minutes. Subsequently, 20 ng of trypsin (modified porcine trypsin sequencing grade, Promega), at a final concentration of 0.02 μg/μL in 10% v/v ACN and 25 mM ammonium bicarbonate was added into the dry gel masses and the digestion process was conducted at 37 °C overnight. The resulting tryptic peptides were extracted with 0.5% trifluoroacetic acid (TFA) in 50% v/v ACN for 15 minutes of incubation.

Matrix (α-cyano hydroxycinnamic acid, at a concentration of 67% v/v ACN/0.1% v/v TFA), mixtured with sample (1:1v/v), which was spotted on a stainless steel sample target plate. Peptide MS and MS/MS were further performed using the ABI 5800 MALDI-TOF/TOF Plus mass spectrometer, a matrix-assisted laser desorption ionization time of flight mass spectrometer (Applied Biosystems, USA). Mass spectra data were recorded in positive MS reflector mode, with an accelerating voltage of 20 kV, over a mass range of 800-3500 m/z, calibration with peptides from trypsin autolysis. Both the MS and MS/MS data were integrated and processed by using the GPS Explorer V3.6 software (Applied Biosystems, USA).

A peptide mass fingerprinting search was performed against the NCBInr database and UniProt database using the Mascot program (http://www.matrixscience.com/). The following parameters were used for database searches: (1) a maximum of one missed tryptic cleavage; (2) allowed a fixed modification of cysteine carbamidomethylation; (3) possible variable modification of oxidation at methionine; (4) 100 ppm for precursor ion tolerance; (5) MS/MS tolerance of 0.3 Da; (6) taxonomy restrictions to rice. Spots were considered to be identified accurately according to MASCOT score.

Prediction methods

The grand average of hydropathy index (GRAVY) was obtained using the ProtParam Tool (https://web.expasy.org/protparam/). Predictions for protein subcellular localization were performed by UniProtKB and WoLF PSORT Prediction (https://www.genscript.com/tools/wolf-psort).

RESULTS AND DISCUSSION

To date, a variety of extraction protocols from diverse tissues of different species for plant proteomics analysis based on two-dimensional electrophoresis have been published while few attempts were focused on the protein preparation of rice young panicles.

In this work, we quantitatively and qualitatively evaluated six protocols based on precipitation: TCA/acetone precipitation, Mg/NP-40 extraction, Tris-Base/acetone precipitation, SDS extraction, and Phenol extraction, a combination method of TCA/acetone precipitation and Phenol extraction method (TCA/acetone/phenol extraction).

Quantitative comparison of protein content

Quantitative comparisons of protein extracted from rice young panicles revealed that phenol extraction showed no significant difference compared with that Mg/NP-40 extraction while the two methods above gave statistically significantly greater concentrations than that with other methods by SPSS analysis (Table 2).

Lane	Extraction methods	Protein concentra	Number of detected bands	Number of protein spots

		tions (µg/ µL)	CCB G-250	silver nitrate	
1	Phenol extraction	13.59 ± 0.057	21	13	400 ± 89
2	Mg/ NP-40 extraction	13.02 ± 0.61	13	9	422 ± 37
3	Tris-Base/ acetone precipitation	10.89 ± 0.46	18	16	424 ± 66
4	SDS extraction	10.76 ± 0.17	11	8	287 ± 31
5	TCA/ acetone/ phenol extraction	9.79 ± 0.23	26	17	450 ± 53
6	TCA/ acetone precipitation	5.93 ± 0.22	7	13	321 ± 17

* Protein concentrations and number of protein bands were described by mean value of triplicates ± standard deviation (SD).

Table 2: Protein content and number of detected bands from rice young panicles using six protocols.

Exhilaratingly, a giant result was obtained from rice young panicles than other plant tissues by using TCA/acetone precipitation (5.93 ± 0.22 µg/µL) and Tris-Base/acetone precipitation (10.8 ± 0.46 µg/µL) that greatly facilitate to gaining better 2-DE patterns [50,51]. Furthermore, significant changes in the protein content of each protocol were discernible by SDS-PAGE (Figure 2).

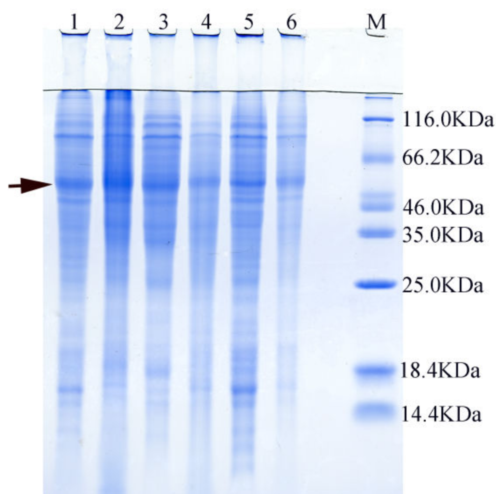

Figure 2: SDS-PAGE gels of protein extracted from rice young panicles using six protocols: (1) Phenol extraction; (2) Mg/NP-40 extraction; (3) Tris-Base/acetone precipitation; (4) SDS extraction; (5) TCA/acetone/phenol extraction; (6) TCA/acetone precipitation. Two microlitres of protein were loaded per lane. M is molecular weight marker. Gels were stained using Coomassie brilliant blue G-250 protocol.

One dimensional SDS-PAGE evaluation of six protocols

The bands of proteins were separated by one dimensional SDS-PAGE, then gels were stained using Coomassie brilliant blue G-250 staining protocol and silver staining protocol, respectively. Representative gel images from replicated experiments were shown in Figure 3.

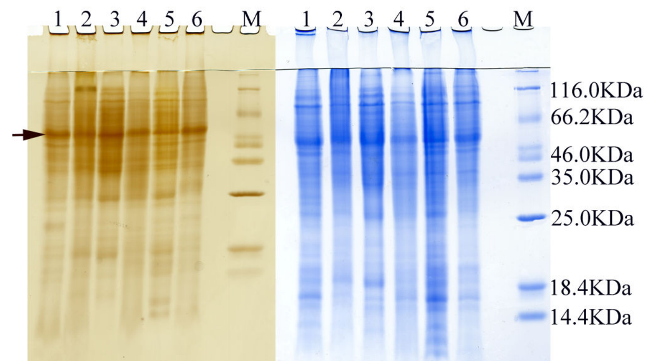

Figure 3: SDS-PAGE gels of protein extracted from rice young panicles using six different protocols: (1) Phenol extraction; (2) Mg/NP-40 extraction; (3) Optimized Tris-Base/Acetone precipitation; (4) SDS extraction; (5) TCA/acetone/phenol extraction; (6) TCA/acetone precipitation. Thirty-six micrograms of protein were loaded per lane. M is molecular weight marker. Gels were stained using Coomassie brilliant blue G-250 protocol and silver staining protocol, respectively.

Firstly, comparisons of two staining protocols revealed that G-250 staining protocol has better image resolution, more detected bands (Table 2). In present study despite higher sensitivity of silver staining, the grouped proteins were resolved into clear bands varied from 10 KDa to more than 150 KDa by Quantity One software analysis (Figure 3). Then, quality characteristics and resolution of protein bands per lane presumably reflected contaminants contents from each protocol of protein extraction that as assessed by smearing, evaluated background staining. Both Mg/NP-40 extraction sample and TCA/acetone/phenol method sample commonly showed a greater extent of smearing in the higher molecular weight (MW) regions of the gels that may be caused by the presence of nucleic acid, because viscous and stringy loading protein sample added into the well were observed in our experiments. The Tris-Base/acetone precipitation typically contained fewest higher MW proteins similar to that SDS extraction method while exceptionally TCA/acetone precipitation has a maximum number of high MW protein bands. The Phenol extraction and TCA/acetone precipitation generated apparently greater quality protein samples in terms of well-resolved bands spanned a broad range of MW, no smearing, better background, no redundant materials in the wells.

Many protein determination assay that are generally used in Bio-Lab: Bradford assay, Lowry assay and UV spectroscopy to assess protein concentration, nevertheless no single protein assay that could yield absolutely accurate results [52]. Accordingly,

opposite SDS-PAGE gel patterns results between TCA/acetone precipitation and SDS extraction method suggested the existence of some contaminant that will skew accurate sample loading.

Obvious differences were visualized in the pattern of bands, especially in the matter of TCA/acetone/phenol extraction method which provided comprehensive molecular weight polypeptides comparing with other protocols. Interestingly, approximately 55KDa of the large subunit of certain protein in current study similar to ribulose bisphosphate decarboxylase/ oxygenase (Rubisco) was appeared here indicated by an arrow at each protocol. It is all known to that ribulose bisphosphate decarboxylase/oxygenase is arguably considered to be the most abundant protein and can consist the majority of total leaf protein, and many efforts have been developed to remove Rubisco because itself will greatly decrease the number of detectable protein spots during 2-DE [45,53]. Up until now, no extraction protocols have focused on how to avoid this problem, but phenol-based method could acquire high quality protein and simultaneously reduce contents of Rubisco described by Saravanan [12].

Two-dimensional electrophoresis evaluation of six protocols

The proteins of rice young panicles extracted using six preparation protocols were separated by two dimensional electrophoresis and all gels were stained with silver nitrate (Figure 4). All six extraction protocols gave many hundreds of protein spots (Table 2) and different resolution per gel, which was discussed in details below.

Methods of protein extraction based on phenol have been widely utilized, especially TCA/acetone/phenol extraction as well as phenol extraction. Based on phenol of original method was developed in the 1980's described by Hurkman et al. and Meyer et al. [33,54]. Further, a tremendous progress involving modification of phenol extraction methods have been made [14,19,34,55]. For example, some methodologies added sucrose into aqueous buffer containing nucleic acids and carbohydrates to create aqueous phase with higher density than phenol phase which includes lipids and pigments [33]. To some extent, the best merit of phenol method is very efficient for producing superior protein sample by allowing removal of polysaccharides and nucleic acids with the enzyme inactivation than other methods. Nonetheless, some limitations still exist here that it is toxic, time-consuming and not able to produce high-quality 2-DE profiles in special recalcitrant tissues from various species.

Besides, classical TCA/acetone precipitation produce few protein spots and poor resolution including streaking and smearing, mainly because the precipitated pellets are difficult to dissolve and multiple washing step caused protein losses, which is not considered to be favored in complex plant tissues.

Considering this potential challenge, a protocol integrated TCA/acetone precipitation method with phenol extraction, which is used for protein sample preparation from recalcitrant plant tissues described in details by Wang et al. [18,19]. Originally, this protocol was developed to extract proteins from the adult evergreen olive leaves containing abundant phenolic substances [14]. All the published paper also confirmed that TCA/acetone/phenol protocol is sufficient to produce higher quality profiles than either TCA/acetone precipitation or phenol extraction alone, and could provide an enhanced 2-DE based proteomic analysis [9,21].

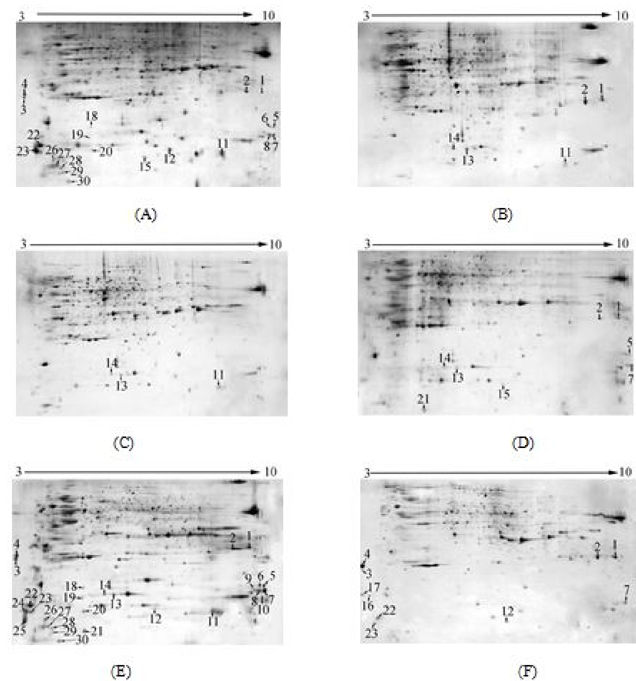

Figure 4: Representative 2-DE profiles of protein extracted from rice young panicles using six protocols. A: Phenol extraction; B: Mg/NP-40 extraction; C: Tris-base/acetone precipitation; D: SDS/acetone extraction; E: TCA/acetone/phenol extraction; F: TCA/acetone precipitation. Equal protein (250 µg) was separated on 24 cm nonlinear immobilized pH gradient pH 3-10 and 12.5% SDS-polyacrylamide gel. The gels were stained with silver nitrate. Differential extracted proteins absented in multiple extraction protocols (not all protocols) were indicated by arrows.

In the present study, TCA/acetone/phenol protocol gave satisfactory results involving the maximum number of well-resolved protein spots (450 ± 53 SD) than phenol extraction (400 ± 89 SD), TCA/acetone precipitation (321 ± 17 SD) and other three methods not commonly used. Especially, obvious difference was observed in the spot patterns that the abundance of same protein spots extracted by TCA/acetone/phenol based method was higher than the others (Figure 5).

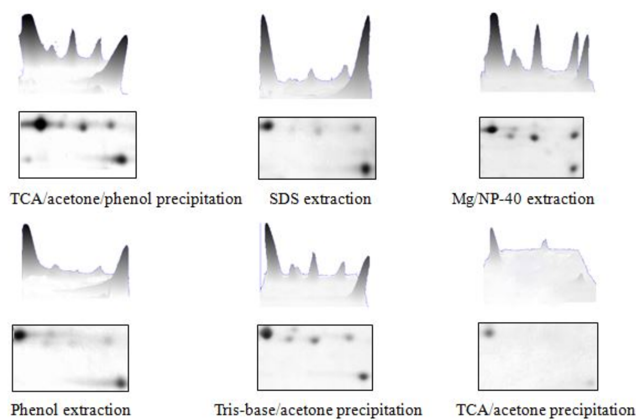

Figure 5: Differential abundance of selected same protein spots extracted by six protocols. The thicker and blacker peaks indicate the higher abundance.

The confused fact is that three methods of SDS extraction, Mg/NP-40 extraction, Tris-Base/acetone precipitation created fairish effect whereas few reports were published. SDS extraction is designed for total protein preparation similar to TCA/acetone precipitation, which big difference between both methods is that the use of SDS anionic detergent. It has been reported that SDS possesses an ability to highly bind protein at an increased temperature of 95°C, which can improve the solubilization of membrane proteins [3].

In this paper, SDS extraction produces the fewest number of protein spots (287 ± 31 SD). We speculated that heating may cause protein degradation and then the utilization of anionic surfactants may cause proteins to precipitate in IEF gels [3,56]. In contrast to the poor result of SDS extraction, Mg/NP-40 extraction gave more well-separated protein spots (422 ± 37 SD) with application of nonionic surfactant NP-40 which may reduce extraction of ribulose bisphosphate decarboxylase/oxygenase [57]. However, membrane proteins may be sparingly soluble, especially in the NP-40 detergent used in the isoelectric focusing gels [58], therefore showed a slightly inferior resolution and high background staining. The last protocol described is Tris-Base/acetone method (424 ± 66 SD) which exert remarkable difference in the number of protein spots whereas no significant difference in protein concentration compared with SDS extraction method. Adding KCl into Tris-Base extraction buffer may facilitate the extraction of proteins due to salting in effect. Furthermore, we opted to keep pH 7.5 extraction buffer inhibiting proteases to assure that large amounts of phenolic substances are mainly ionized and H not binding with proteins, which was confirmed to be very effective [59]

Overall, the TCA/acetone/phenol protocol has the incomparable efficacy with holding the merits of both TCA/acetone precipitation and phenol extraction.

Protein identification

Obvious qualitative differences were visualized that same differential extracted protein spots absented in multiple extracts (not all protocols) due to the property of each extraction protocol. All the differential extracted protein spots existed in each gel were indicated by arrows (Figure 4) and were subjected to MALDI-TOF-MS/MS analysis. Protein identification was accomplished by searching against the NCBInr database and Uniport database with MASCOT software. The identification results were shown in Table 3.

Spot	**Protein Accession**	**Gene Symbol**	**Protein identification**	MW(Da)/pI	Score	Coverage	GRAVY	Protein subcellular localization	Matched Peptides
Oxidation									
1	gi|29367429	LOC_Os03g10510	Mitochondrial outer membrane protein porin 5	29456/9.17	689	38%	-0.146	Mitochondrion, Mitochondrion outer membrane	9 (8)
2	gi|18076158	LOC_Os05g45950	Mitochondrial outer membrane protein porin 5	29584/8.56	836	43%	-0.105	Mitochondrion, Mitochondrion outer membrane	10(8)
30	gi|28564644	LOC_Os08g06550	putative Acyl-CoA binding protein (ACBP)	10137/5.16	99	32%	-0.673	Cytoplasm	2(1)
19	gi|46805895	LOC_Os02g52430	4-hydroxy-4-methyl-2-oxoglutara	18323/5.6	216	25%	0.056	Cytoplasm	4(4)

			tealdolase						
29	gi|47497145	LOC_Os02g55060	Cytochrome b5 domain-containing protein-like	10819/5.42	270	50%	-0.295	Cytoplasm	4(2)
21	gi|485953	LOC_Os04g42930	Glutaredoxin-C6	11866/6.56	445	58%	0.087	Cytoplasm	4(4)
Photosynthesis									
4	gi|11955	rbcL	Ribulose bisphosphate carboxylas large chain	53421/6.13	107	7%	-0.283	chloroplast	3(1)
Biosynthesis									
6	gi|2058273	LOC_Os09g31180	60S ribosomal protein L9	21406/9.62	547	35%	-0.217	Cytoplasm	7(7)
7	gi|39545864	LOC_Os04g50990	OSJNba0093F12.16 protein	17851/9.23	335	25%	-0.228	Cytoplasm	3(3)
27	gi|55296630	LOC_Os06g05880	putative profilin	14352/4.73	282	32%	-0.086	Cytoplasm, Cytoskeleton	4(4)
22	gi|50725625	LOC_Os08g02340	putative acidic ribosomal protein P1a	11133/4.43	334	68%	0.181	Cytoplasm	4(4)
10	gi|55296170	LOC_Os06g04290	40S subunit ribosomal protein	14151/9.48	152	18%	-0.303	Cytoplasm	3(3)
Unknown protein									
11	gi|125537087	OsI_38785	hypothetical protein OsI_38785	16976/8.26	230	36%	-0.147	Vacuoles; Cytoplasm	4(4)
Translation									
12	gi|18461185	LOC_Os01g48280	Putative ubiquitin-conjugating enzyme E2	17206/6.74	215	33%	-0.286	Nucleus	5(4)
13	gi|108711192	LOC_Os03g55150	Eukaryotic translation initiation factor 5A	17930/5.87	602	48%	-0.463	Cytoplasm	7(7)
17	gi|303835	LOC_Os11g43900	Translationally-controlled	18991/4.51	593	60%	-0.383	Cytoplasm	8(8)

			tumor protein homolog						
14	gi|77555893	LOC_Os12g32240	Eukaryotic translation initiation factor 5A	17774/5.6	868	57%	-0.497	Cytoplasm	8(8)
Transcriptional regulation									
15	gi|108707099	LOC_Os03g13800	NHP2-like protein 1, putative, expressed	14037/6.56	82	14%	-0.013	Nucleus	2(0)
20	gi|22831338	LOC_Os07g30090	Actin-depolymerizing factor 9	16280/5.52	49	6%	-0.525	Cytoplasm, Cytoskeleton	2(1)
Defense response									
16	gi|21741225	LOC_Os04g39150	OSJNBb0048E02.12 protein	17256/4.75	563	63%	-0.25	Cytoplasm	9(8)
24	gi|3603473	OSIGBa0125M19.3	Elicitor-responsive protein 3	15973/4.22	51	9%	-0.468	Cytoplasm	1(1)
18	gi|29467522	LOC_Os08g03520	Cold shock domain protein 2	19024/6.28	119	9%	-0.724	Mitochondrion	1(1)
Transportation and signal transduction									
23	gi|20188	CAM1-1	calmodulin	16878/4.11	587	51%	-0.619	Nucleus	1(1)
26	gi|29367559	LOC_Os08g02420	putative serine/threonine kinase	16672/4.89	581	56%	-0.411	Cytoplasm	8(7)
28	gi|5360221	LOC_Os08g42000	nuclear transport factor 2 (NTF2)	13459/5.33	65	6%	-0.129	Cytoplasm	1(1)

Table 3: Identification of differential extracted protein spots in rice young panicles

Twenty-nine of thirty selected differential extracted protein spots were identified. However, identified protein spot 6 (gi|2058273), spot 7 (gi|39545864), spot 23 (gi|20188) and the corresponding spot 9, spot 8, spot 25 are the same protein spots, respectively. Interestingly, we found a cold shock domain protein 2, spot 18 (gi|29467522) only presented in phenol based protocol, which differed from always detecting 18.1 KD heat shock protein in our previous study [48,49]. A hypothesis of this novel phenomenon was determined that it may be caused by the low temperature stimulation, because we recorded the weather data in panicle primordium differentiation stage by using RR-9100A Farmland environment automatic detection system (RainRoot scientific, Beijing). Spot 24 (gi|3603473), only presented in TCA/acetone/phenol protocol, was an elicitor-responsive gene, may be play an important role in responding to environmental stress. Furthermore, two mitochondrial outer membrane protein porin 5, spot 1(gi|29367429) and spot 2 (gi|18076158) was only just not present in Tris-base/acetone extraction protocol.

Each protocol of protein extraction has its preference for extracting special proteins [60]. Therefore we speculated that a possible explanation for the difference is some cellular substances are more effectively disrupted by other multiple protocols and then were released. To validate this idea, predictions for protein subcellular localization were performed by UniProtKB and WoLF PSORT Prediction. Prediction results indicated that spot 1 (gi|29367429), spot 2 (gi|18076158), spot 18 (gi|29467522) were located in mitochondria, spot 12 (gi|18461185), spot 15 (gi|108707099), spot 23 (gi|20188) was located in nucleus, spot 4 (gi|11955) was located in chloroplast; other 22 protein spots were located in cytoplasm, so no conclusion can be made.

The second explanation for the difference is hydropathicity of proteins, because hydropathicity plays a critical role in the solubilization of proteins. A widely used method for predicting hydropathicity is calculation of GRAVY score [34]. The GRAVY value of the identified proteins ranges from -0.724 to 0.181 (Table 3) and only three positive GRAVY value (spot 19, gi|46805895; spot 21, gi|485953; spot 22, gi|50725625) were obtained. Thus, we concluded that phenol-based protocols more easily extracted hydrophobic proteins.

CONCLUSION

In summary, the results showing on evaluation of six extraction protocols in this study presented here suggested that TCA/acetone/phenol provides a better enhanced efficacy on protein extraction of rice young panicles. It depends on the fact that TCA/acetone/phenol protocol integrates the merits of both TCA/acetone precipitation, which actually eliminates non-protein substance, and phenol extraction, which purposefully dissolves water-soluble proteins and non-water-soluble proteins, to facilitate effective purification for crop plants. And it's worth noting that visible value of detecting greater amounts of protein spots far outweighed the little extra labor investment in sample preparation, although it was sometimes described as toxic and with more time consuming nature. Finally, we expected that this paper will facilitate to providing useful information for protein extraction of other tissues from rice, including other complicated species.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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