Evaluation of six different protocols for protein extraction from rice young panicles by two-dimensional electrophoresis

Xiaoping Huang (✉ xphuang940812@163.com)

Jiangxi Agricultural University

Hui-wen Zhou

Jiujiang University

Research

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Abstract

Background: For most reported proteomics approaches, protein extraction are of crucial importance for optimal results. However, extraction of protein from plant tissues still exist great challenges due to low protein content and many secondary metabolites that prominently interfering with isoelectric focusing. Up to now, no attempts are focused on comparison of protein extraction from rice young panicles.

Methods: To establish a higher efficiency protein extraction protocol suited for two-dimensional electrophoresis in rice young panicles, six protocols for protein preparation were evaluated in terms of protein concentration, the molecular weight range of protein, gel image resolution, the number of protein spots: 1) Phenol extraction; 2) Mg/Nonidet P-40 (NP-40) extraction; 3) Tris-Base/acetone extraction; 4) SDS extraction; 5) trichloroacetic acid (TCA)/acetone/phenol extraction; 6) TCA/acetone precipitation.

Results: The result explicitly demonstrated that TCA/acetone/phenol method provided a high-enhanced protein extraction efficacy from rice young panicles than other protocols in terms of the protein concentration (9.79 ± 0.23 SD), the most comprehensive proteins (10 KDa to 150 KDa), the maximum number of protein spots (450 ± 53 SD), the greater gel image resolution and spot abundance. In addition, these methods also generated remarkably differential 2-DE protein patterns. Twenty-nine of 30 visible differentially extracted proteins were identified by MS analysis and were divided into eight categories. Prediction for protein subcellular localization and grand average of hydropathy (GRAVY) analysis showed that certain special proteins respectively necessitate different extraction methods due to different physicochemical properties of each protocol.

Conclusions: Overall, this paper will facilitate to provide a cornerstone of comparative proteomic analysis from rice young panicles, including other complicated plant tissues.

1 Background

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 enhance plant proteomic analysis [14–17]. Nevertheless, plant proteomics analysis remains to be more troublesome 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 resolubilize 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 food 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 extraction, 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.

2 Materials And Methods

2.1 Plant material

Rice (*Oryza sativa* L. *subsp. indica*) was grown in paddy field at Jiangxi Agricultural University. Rice cultivation was essentially conducted by normal management way.

Rice young panicles in 7 cm length were sampled at booting stage (Fig. 1), packed in aluminum foil, flash-frozen in liquid nitrogen and stored at -80°C for further proteomic protocols' evaluation.

2.2 Six protocols for protein extraction

Total protein from rice young panicles were extracted by six protocols including Phenol extraction, Mg/NP-40 extraction, Tris-Base/acetone extraction, SDS extraction, TCA/acetone/phenol extraction, TCA/acetone precipitation. Extraction procedure of each protocol was repeated three times. Frozen rice young panicles were pulverized to a fine powder in a pre-chilled mortar with liquid nitrogen. Later, the finely ground powder (three grams) was used for protein extraction. The six procedures for protein extraction as follows:

A) Phenol extraction in this study was performed according to Hurkman *et al* [33]. The powder was mixed with 15 mL protein extraction buffer (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 incubated for 30 min at 4°C with sharply vortexing every 10 min. Then an equal volume of Tris-saturated phenol (pH 8.0) was added into the above homogenate, and the formed mixture was incubated for 30 min at 4°C. The formed mixture was cleared by centrifuged at 15000 × g for 30 min at 4°C, the upper phenolic phase was collected and transferred into a clean 50 mL centrifuged tube followed by centrifugation. The collected phenolic phase was precipitated with five volumes of 0.1 M ammonium acetate in methanol at -20°C overnight and centrifuged as above. The precipitate was washed with cold acetone for three times. After each wash, the precipitate was centrifuged at 15000 × g for 30 min at 4°C. The final precipitate was lyophilized, then fully dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 50 mM DTT, 0.25% v/v carrier ampholytes, pH 3-10) by water-bath heating at 28°C and stored at -80°C.

B) Mg/NP-40 extraction procedure in this paper was described by Kim *et al* [45]. The sample powder was transferred into a clean 50 mL centrifuge tube and then mixed with 15 mL pre-chilled protein extraction buffer (0.5 M Tris-HCl (pH 8.3), 2% v/v NP-40, 20 mM MgSO₄, 2% v/v 2-ME, 1% w/v PVPP). The homogenate was ultra-sonicated for 30 min at 4°C by Ultrasonic Cleaner (NingBo Scientz Biotech Co, China) before incubation for 1 h at 4°C, and the homogenate was centrifuged at 15000 × g for 30 min at 4°C. After centrifugation, the supernatant was collected, and then precipitated by adding four volumes of cold acetone (10% v/v TCA, 0.07% v/v 2-ME) at -20°C overnight. Subsequently, protein pellet was obtained after centrifugation, the obtained protein pellet was resuspended in acetone with 0.07% v/v 2-ME and incubated at -20°C for 1 h, and then centrifuged at 15000 × g for 30 min at 4°C. Later, the protein pellet was repeatedly washed with cold acetone (0.07% v/v 2-ME) for three times. After each wash, the protein pellet was centrifuged at 15000 × g for 30 min at 4°C. The final protein pellet was lyophilized, then fully dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 50 mM DTT, 0.25% v/v carrier ampholytes, pH 3-10) by water-bath heating at 28°C and stored at -80°C.

C) Tris-Base/acetone extraction was based on a previously method published by Zhang *et al* [36]. The powder was transferred into a clean 50 mL centrifuge tube and mixed with 15 mL extraction buffer (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 1 min and ultra-sonicated for 30 min at 4°C by Ultrasonic Cleaner (NingBo Scientz Biotech Co, China) before incubation for 1 h at 4°C. After centrifugation (15000 × g, 30 min, 4°C), the supernatant was treated as described above in "Mg/NP-40 extraction".

D) SDS extraction was adapted from Zhen *et al* [10]. The powder was homogenized in 15 mL 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 min. The homogenate was cooled at 4°C prior to centrifugation at 15000 × g at 4°C for 30 min. Then supernatant was treated as described above in "Mg/NP-40 extraction".

E) The new protocol integrating TCA/acetone precipitation with phenol extraction was adapted from Isaacson *et al* [1]. The sample powder was homogenized in 15 mL cold acetone (10% w/v TCA, 0.07% v/v 2-ME) at -20°C overnight, followed by centrifugation at 15000 × g at 4°C for 30 min. The obtained precipitate was rinsed with acetone and incubated for 1 h at -20°C for three times. After each wash, the precipitate was centrifuged at 15000 × g for 30 min at 4°C. Subsequently, the final protein precipitate was lyophilized and treated as described above in "phenol extraction".

F) The protein extraction protocol was a classical TCA/acetone precipitation procedure adapted from Damerval *et al* [25] with some modifications. The sample powder was homogenized in 15 mL cold acetone (10% w/v TCA, 0.07% v/v 2-ME) at -20°C overnight, followed by centrifugation at 15000 × g at 4°C for 30 min. The obtained precipitate was rinsed with acetone and incubated for 1 h at -20°C, then was filtered by medical gauze and centrifuged. The purification step above was repeated for three times until white protein pellet was obtained and supernatant was colorless. After each wash, the precipitate was centrifuged at 15000 × g for 30 min at 4°C. Finally, the white protein pellet was lyophilized, then fully dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 50 mM DTT, 0.25% v/v carrier ampholytes, pH 3-10) by water-bath heating at 28°C and stored at -80°C.

2.3 Total protein quantification

The lyophilized protein pellet was fully dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 50 mM DTT, 0.25% v/v carrier ampholytes, pH 3-10) by water-bath heating at 28°C and the concentration of total protein was determined by Bradford assay [45], using bovine serum albumin as calibration standard protein.

2.4 One dimensional SDS-PAGE

To evaluate the integrity of total protein and visible differences of protein concentrations, 36 µg and 2µL of each protein sample extracted by six protocols was separated by one dimensional SDS-PAGE, respectively. Briefly, the total protein was separated on 12.5% w/v SDS-PAGE gel and 5% w/v stacking gel. Electrophoresis was performed at 80 V until the dye traversed the stacking gel, followed by 120 V until the

dye front reached the bottom of the SDS-PAGE gel. Gels were stained with Coomassie brilliant blue G-250 (CBB G-250) and silver stained as previously described [46], then gels were kept in 30% glycerol.

2.5 Two-dimensional electrophoresis

Firstly, 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 carrier ampholytes, pH 3-10) and dissolved at room temperature for at least 1 h. The mixed buffer (including rehydration buffer and 250 µg total protein sample) was centrifuged at 15000 × g at 4°C for 15 min and the immobilized linear pH gradient (IPG) strips (24 cm, pH 3-10) thawed at room temperature for 15 min. Then the IPG strips were rehydrated for 14 h in 450 µL mixed buffer, and isoelectric focusing was carried out using PROTEAN i12 IEF cell system (Bio-Rad, USA) at 20°C, applying the following procedure: 100 V rapid for 1.5 h, 200 V rapid for 1.5 h, 500 V rapid for 1.5 h, 1000 V rapid for 1.5 h, 5000 V rapid for 1 h, 10000 V gradual for 1 h, and until 135000 VH with a maximum voltage of 10000 V. Prior to second dimension analysis, the strips were reduced for 15 min 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 subsequently alkylated for 15 min in 10 mL equilibration buffer containing 2.5% w/v iodoacetamide. Finally, the strips were transferred to 12.5% w/v SDS-PAGE gels and further sealed with 1% w/v low-melting agarose solution. The second dimension electrophoresis was performed at 100 V using PROTEAN Plus Dodeca cell system (Bio-Rad, USA) until the dye began enter into gel, followed by constant voltage of 170 V until the dye front reached the bottom of the gel. Then the gels were silver stained as established by Liao *et al* [46, 47], and gels were kept in 30% glycerol.

2.6 Gel images acquisition and analysis

All stained gel images were captured by GS-800 calibrated imaging densitometer (Bio-Rad, USA) with transmission-mode at 600 dpi and saved as "TIFF" format. The gel images were analyzed by Quantity One software and Image Master 2D PDQuest 8.0 software (Bio-Rad; USA), respectively. Briefly, the number of protein bands per lane and molecular weight were detected by Quantity One software, and the number of protein spots and abundance automatically detected by PDQuest software. At the same time, in order to compare the types of proteins extracted by various procedures, visible proteins which existed in either one protocol or less than six protocols were considered to be differentially extracted proteins and marked in the gel images by arrows. Then, the data including protein concentrations and the number of protein spots, was statistically evaluated by one-way analyses of variance (ANOVA) to determine the significant difference among extraction methods using SPSS version 17.0 software. In all cases, significance was defined as $p < 0.05$. The repeated measurement was given as means $\pm$ standard deviation (SD).

2.7 Protein digestion

For protein digestion, visible differentially extracted protein spots were excised out of the gels and transferred into clean 1.5 mL centrifuge tubes. Protein spots were repeatedly washed twice with deionized water, and then destained with 30% v/v acetonitrile solution (15 mM $K_3Fe(CN)_6$, 50 mM $Na_2S_2O_3$) at 37°C

for 30 min. After removal of the destaining solution, protein spots were washed twice again, and dehydrated with acetonitrile (ACN) for 30 min until protein spots completely appeared white. Subsequently, protein spots were digested for 16 h at 37°C in 20 µL of 0.02 µg/µL trypsin solution (10% v/v ACN, 25 mM NH₄HCO₃) as previously described by Liao et al[47, 48]. After centrifugation, the supernatant were transferred into another tube, and the undissolved gel particles were further extracted by extraction solution (5% v/v trifluoroacetic acid (TFA), 67% v/v ACN) at 37°C for 30 min. Finally, the extracts and the supernatant were pooled and then lyophilized. The lyophilized protein samples were kept at -80°C until MS analysis.

2.8 Protein identification by MS analysis

For MALDI-TOF-MS/MS analysis, the lyophilized protein samples were dissolved in 5µL of 0.1% v/v TFA solution, and mixed (1:1, v/v) with Matrix solution (α-cyano-4-hydroxycinnamic-acid in 50% v/v ACN and 0.1% v/v TFA). Then 1µL of the mixture samples were spotted on a stainless steel sample target plate, the peptide 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).

MS data was acquired over a mass range of 800-3500 Da in positive ion reflector mode and data independent acquisition mode, with an accelerating voltage of 20 kV. MS was used a CalMix5 standard to calibrate the instrument (ABI 5800 Calibration Mixture). For MS data accuracy, autolysis peaks of trypsin were used as internal calibration. And for MS/MS data acquisition, up to 10 of the most intense ion signals were selected as precursors in positive ion mode, excluding the trypsin autolysis peaks and the matrix ion signals. Both the MS and MS/MS data were integrated and processed by using the GPS Explorer V3.6 software (Applied Biosystems, USA) with default parameters. Peptides were identified in the NCBI nr database/UniProtKB database using MASCOT Version 2.3 search engine (Matrix Science, London, UK).

The following parameters were used for searching: 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) precursor ion tolerance of 100 ppm; 5) MS/MS tolerance of 0.3 Da; 6) taxonomy restrictions to rice. Protein spots were considered to be successfully identified based on 95% or higher confidence interval of MS/MS data score (score > 40). Finally, the identified proteins were classified according to its annotated functions.

2.9 Prediction methods

To evaluate solubilization of proteins, 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>).

3 Results And Discussion

3.1 Quantitative comparison of protein concentrations

The concentrations of total proteins ($\mu\text{g}/\mu\text{L}$) were shown in Table 1 and were 13.59 ± 0.057 for phenol extraction, 13.02 ± 0.61 for Mg/NP-40 extraction, 10.89 ± 0.46 for Tris-Base/acetone extraction, 10.76 ± 0.17 for SDS extraction, 9.79 ± 0.23 for TCA/acetone/phenol extraction, 5.93 ± 0.22 for TCA/acetone precipitation. Quantitative comparisons of proteins extracted from rice young panicles revealed that phenol extraction showed no statistically significant total protein concentration difference compared with Mg/NP-40 extraction, but the two methods above gave statistically significantly greater concentrations than other methods.

Exhilaratingly, a giant result was obtained from rice young panicles than other plants tissue by using TCA/acetone precipitation ($5.93 \pm 0.22 \mu\text{g}/\mu\text{L}$) and Tris-Base/acetone precipitation ($10.89 \pm 0.46 \mu\text{g}/\mu\text{L}$), which greatly facilitate to gaining better 2-DE patterns [49, 50].

3.2 One dimensional SDS-PAGE evaluation of six protocols

The total proteins were separated by one dimensional SDS-PAGE, gels were stained with CBB G-250 and silver stained, respectively. Representative gel images from replicated experiments were shown in Fig 2.

Firstly, comparison of two staining protocols revealed that G-250 staining protocol has better image resolution, more detected bands (Table 1), despite higher sensitivity in silver staining. Then, quality characteristics of protein bands per lane presumably reflected contaminants contents from each protocol of protein extraction that as assessed by resolution. And the total proteins were resolved into clear bands varying from 10 KDa to more than 150 KDa by Quantity One software analysis (Fig 2). Both the sample of Mg/NP-40 extraction and TCA/acetone/phenol extraction commonly showed a greater extent of smearing in the higher molecular weight (MW) regions of the gels that may cause by the presence of nucleic acid, because viscous and stringy protein sample were observed to load into the well in our experiments. The sample of Tris-Base/acetone extraction typically contained fewest higher MW proteins similar to that SDS extraction. Exceptionally, the sample of TCA/acetone precipitation has a maximum number of high MW protein bands by Quantity One software analysis. The phenol extraction and TCA/acetone precipitation generated apparently greater quality protein samples in terms of well-resolved bands, no smearing, better background, no redundant materials in the wells.

Many protein determination methods including Bradford assay, Lowry assay and UV spectroscopy, which were used to assess protein concentration, nevertheless no single assay could yield absolutely accurate results [51]. In this study, accordingly, contrary results of SDS-PAGE gel patterns between TCA/acetone precipitation and SDS extraction suggested that the existence of some contaminants would skew the accuracy of sample loading.

Finally, the most obvious difference was visualized that the sample of TCA/acetone/phenol extraction provided the most comprehensive polypeptides comparing with other samples. Interestingly, approximately 55 KDa of the large subunit of certain protein in current study similar to ribulose

bisphosphate decarboxylase/oxygenase (Rubisco) was appeared here indicated by an arrow. It is all known to that Rubisco is arguably considered to be the most abundant protein and can make up 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 [52, 53]. Up to now, no extraction protocol has focused on how to avoid this problem, but phenol-based method could acquire high quality protein sample and simultaneously reduce contents of Rubisco described by Saravanan [12].

3.3 Two-dimensional electrophoresis evaluation of six protocols

The total proteins respectively extracted by six protocols from rice young panicles were separated by 2-DE and all gels were silver stained (Fig 3). The six protocols for protein extraction gave hundreds of protein spots (Table 1) and different resolution, which were discussed in details below.

In this study, TCA/acetone/phenol extraction gave satisfactory results with the maximum number of protein spots (450 ± 53 SD) than that phenol extraction (400 ± 89 SD), Mg/NP-40 extraction (422 ± 37 SD), Tris-Base/acetone extraction (424 ± 66 SD), SDS extraction (287 ± 31 SD) and TCA/acetone precipitation (321 ± 17 SD). Especially, TCA/acetone/phenol extraction showed the best image resolution including well-resolved protein spots, minimum background staining and horizontal stripes. Obvious differences were also observed in the spot patterns that the abundance of same protein spots extracted by TCA/acetone/phenol protocol was higher than the others (Fig 4).

First, the three commonly used methods: phenol extraction, TCA/acetone/phenol extraction and TCA/acetone precipitation were analyzed in details. Phenol extraction method originally described by Meyer and Hurkman [33, 54] was further developed with making some modifications [14, 19, 34, 55]. For example, some methodologys added sucrose into aqueous buffer to create that aqueous phase has greater density than phenol phase [33]. To some extent, the best merit of phenol extraction was very efficient for producing superior protein sample by allowing removal of polysaccharides and nucleic acids with the enzyme inactivation than other methods due to the utilization of phenol. 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, in this paper, classical TCA/acetone precipitation produce few protein spots (321 ± 17 SD) and poor resolution including streaking and smearing, mainly because the precipitated pellets were difficult to dissolve, and multiple washing step caused protein losses, which was not considered to be favored in complex plant tissues.

Considering this potential challenge, a new protocol integrated TCA/acetone precipitation with phenol extraction, which was used for protein sample preparation from recalcitrant plant tissues described by Wang [18, 19]. Our results including the number of protein spots (450 ± 53 SD) and image resolution were similar to the majority of previously reported papers, which showed TCA/acetone/phenol protocol was 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].

The confused fact was that the three methods of SDS extraction, Mg/NP-40 extraction, Tris-Base/acetone extraction created fairish effect whereas few reports were published. SDS extraction was designed for total protein preparation similar to TCA/acetone precipitation, which the biggest difference between both methods was that the use of SDS anionic detergent. It has been reported that SDS possessed to an ability of 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, 55]. 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 biphosphate decarboxylase/oxygenase [56]. However, membrane proteins might be sparingly soluble, especially the existence of NP-40 detergent [57]. Therefore, in this study, the gel image of Mg/NP-40 extraction showed a slightly inferior resolution including high background staining (Fig. 4B). The last protocol to describe was Tris-Base/acetone extraction, which existed remarkable difference in the number of protein spots (424 ± 66 SD) whereas no significant difference in protein concentration compared with SDS extraction. We kept extraction buffer pH 7.5 to assure that large amounts of phenolic substances are mainly ionized and H not binding with proteins. Finally, we obtained a better image resolution and more protein spots, which were confirmed to be very effective compared with previously published paper [58].

3.4 Protein identification

Thirty differential extracted protein spots were excised out of the gels and were subjected to MALDI-TOF-MS/MS analysis. Protein identification was accomplished by searching against the NCBI nr database and Uniport database with MASCOT software. Twenty-nine of 30 selected differential extracted protein spots were identified and the identification results were shown in Table 2.

However, the identified protein spot 6 (gi|2058273, 60S ribosomal protein L9), spot 7 (gi|39545864, OSJNb0093F12.16 protein), spot 23 (gi|20188, calmodulin) and the corresponding spot 9, spot 8, spot 25 were the same protein spots, respectively. Interestingly, spot 18 (gi|29467522, cold shock domain protein 2), spot 19 (gi|46805895, 4-hydroxy-4-methyl-2-oxoglutarate aldolase), spot 20 (gi|22831338, actin-depolymerizing factor 9), spot 22 (gi|50725625, putative acidic ribosomal protein P1a), spot 26 (gi|29367559, putative serine/threonine kinase), spot 27 (gi|55296630, putative profilin), spot 28 (gi|5360221, nuclear transport factor 2), spot 29 (gi|47497145, cytochrome b5 domain-containing protein-like) and spot 30 (gi|28564644, putative Acyl-CoA binding protein) were both presented in phenol extraction protocol and TCA/acetone/phenol extraction protocol. Spot 21 (gi|485953, Glutaredoxin-C6), and spot 24 (gi|3603473, Elicitor-responsive protein 3) only presented in TCA/acetone/phenol protocol. And spot 24 was an elicitor-responsive gene, which might play an important role in responding to environment stress. Furthermore, two mitochondrial outer membrane protein porin5, spot 1 (gi|29367429) and spot 2 (gi|18076158) were only just not presented in Tris-base/acetone extraction protocol. Finally,

functions of the identified proteins were annotated and classified into eight categories involving in oxidation, defense response, photosynthesis, biosynthesis, transcriptional regulation, transportation and signal transduction, translation, unknown protein (Table 2).

3.5 Explanation for differential extraction

Each protocol of protein extraction had its preference for extracting special proteins [59]. Therefore we speculated that a possible explanation for the differences were some cellular substances were 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) were located in nucleus, spot 4 (gi|11955) was located in chloroplast, other 22 protein spots were located in cytoplasm, so the conclusion was made that proteins presented in all organelles was almost easily extracted by phenol-based method.

The second explanation for the differences was hydropathicity of proteins, because hydropathicity played a critical role in the solubilization of proteins. A widely used method for predicting hydropathicity was the calculation of GRAVY [34]. The GRAVY value of the identified proteins ranges from -0.724 to 0.181 (Table 2) and only three positive GRAVY values (spot 19, gi|46805895; spot 21, gi|485953; spot 22, gi|50725625) were obtained. Simultaneously, the three proteins with positive GRAVY value were obtained by phenol extraction and TCA/acetone/phenol extraction. Thus, we concluded that phenol-based protocols more simply extracted hydrophobic proteins.

4 Conclusions

In summary, we evaluated six protocols for protein extraction based on the protein concentrations, the molecular weight range of proteins, image resolution and the number of protein spots. The accumulated data showed in this study presented here that TCA/acetone/phenol protocol provided the best extraction efficacy from rice young panicles.

It depended on the fact that TCA/acetone/phenol protocol integrated the merits of both TCA/acetone precipitation, which actually could eliminate non-protein substance, and phenol extraction, which purposefully could dissolve water-soluble proteins and non-water-soluble proteins, to facilitate effective purification for crop plants. And it was 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 rice tissues.

Declarations

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Author information

Affiliations

Key Laboratory of Crop Physiology, Ecology and Genetic Breeding (Jiangxi Agricultural University), Ministry of Education of the P.R. China, Nanchang 330045, Jiangxi Province, China

Xiaoping Huang

2 College of Pharmacy and Life Science, Jiujiang University, Jiujiang City, Jiangxi Province, 332005, China

Huiwen Zhou

Contributions

XH finished the experiment and draft the manuscript. HZ participated in the discussions.

Corresponding author

Correspondence to Xiaoping Huang

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Total protein concentrations and the number of protein spots

Lane	Extraction methods	Total protein concentrations	Number of protein spots
A	Phenol extraction	13.59±0.057	400±89
B	Mg/NP-40 extraction	13.02±0.61	422±37
C	Tris-Base/acetone extraction	10.89±0.46	424±66
D	SDS extraction	10.76±0.17	287±31
E	TCA/acetone/phenol extraction	9.79±0.23	450±53
F	TCA/acetone precipitation	5.93±0.22	321±17

* Total protein concentrations and the number of protein spots were described by mean value of triplicates ± standard deviation (SD).

Table 2 Identification of differential extracted protein spots in rice young panicles

Spot	Protein Accession	Gene Symbol	Protein identification	MW(Da)/pI	Score	Coverage	GRAVY	Protein subcellular localization	Matched Peptides
Oxidation									
1	gi 29367429	<i>LOC_Os03g10510</i>	Mitochondrial outer membrane protein porin 5	29456/9.17	689	38%	-0.146	Mitochondrion, Mitochondrion membrane	9 (8)
2	gi 18076158	<i>LOC_Os05g45950</i>	Mitochondrial outer membrane protein porin 5	29584/8.56	836	43%	-0.105	Mitochondrion, Mitochondrion membrane	10(8)
30	gi 28564644	<i>LOC_Os08g06550</i>	putative Acyl-CoA binding protein (ACBP)	10137/5.16	99	32%	-0.673	Cytoplasm	2(1)
19	gi 46805895	<i>LOC_Os02g52430</i>	4-hydroxy-4-methyl-2-oxoglutaratealdolase	18323/5.6	216	25%	0.056	Cytoplasm	4(4)
29	gi 47497145	<i>LOC_Os02g55060</i>	Cytochrome b5 domain - containing protein-like	10819/5.42	270	50%	-0.295	Cytoplasm	4(2)
21	gi 485953	<i>LOC_Os04g42930</i>	Glutaredoxin-C6	11866/6.56	445	58%	0.087	Cytoplasm	4(4)
Photosynthesis									
4	gi 11955	<i>rbcL</i>	Ribulose biphosphate carboxylas large chain	53421/6.13	107	7%	-0.283	chloroplast	3(1)
Biosynthesis									
6	gi 2058273	<i>LOC_Os09g31180</i>	60S ribosomal protein L9	21406/9.62	547	35%	-0.217	Cytoplasm	7(7)
7	gi 39545864	<i>LOC_Os04g50990</i>	OSJNba0093F12.16 protein	17851/9.23	335	25%	-0.228	Cytoplasm	3(3)
27	gi 55296630	<i>LOC_Os06g05880</i>	putative profilin	14352/4.73	282	32%	-0.086	Cytoplasm, Cytoskeleton	4(4)
22	gi 50725625	<i>LOC_Os08g02340</i>	putative acidic ribosomal protein P1a	11133/4.43	334	68%	0.181	Cytoplasm	4(4)
10	gi 55296170	<i>LOC_Os06g04290</i>	40S subunit ribosomal protein	14151/9.48	152	18%	-0.303	Cytoplasm	3(3)
Unknown protein									
11	gi 125537087	<i>OsI_38785</i>	hypothetical protein OsI_38785	16976/8.26	230	36%	-0.147	Vacuoles; Cytoplasm	4(4)
Translation									
12	gi 18461185	<i>LOC_Os01g48280</i>	Putative ubiquitin-conjugating enzyme E2	17206/6.74	215	33%	-0.286	Nucleus	5(4)
13	gi 108711192	<i>LOC_Os03g55150</i>	Eukaryotic translation initiation factor 5A	17930/5.87	602	48%	-0.463	Cytoplasm	7(7)
17	gi 303835	<i>LOC_Os11g43900</i>	Translationally-controlled tumor protein homolog	18991/4.51	593	60%	-0.383	Cytoplasm	8(8)
14	gi 77555893	<i>LOC_Os12g32240</i>	Eukaryotic translation initiation factor 5A	17774/5.6	868	57%	-0.497	Cytoplasm	8(8)

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

Figures

Figure 1

Rice young panicles in 7 cm.

Figure 2

SDS-PAGE gel images of protein extracted from rice young panicles using six protocols. (A) Phenol extraction; (B) Mg/NP-40 extraction; (C) Tris-Base/acetone extraction; (D) SDS extraction; (E) TCA/acetone/phenol extraction; (F) TCA/acetone precipitation, M: marker. 36 micrograms of total protein each protocol were loaded per lane. Gels were stained with CBB G-250 (right) and silver stained (left), respectively.

Figure 3

Representative 2-DE gel images of protein respectively extracted from rice young panicles by six protocols. (A) Phenol extraction; (B) Mg/NP-40 extraction; (C) Tris-Base/acetone extraction; (D) SDS extraction; (E) TCA/acetone/phenol extraction; (F) TCA/acetone precipitation. 250 μ g protein of each protocol was separated on 12.5% SDS-PAGE gel with 24 cm nonlinear IPG strips pH 3-10. The gels were

silver stained. Differential extracted proteins presented in either one protocol or less than six protocols were indicated by arrows.

Figure 4

The abundance of same protein respectively extracted by six protocols. (A) Phenol extraction; (B) Mg/NP-40 extraction; (C) Tris-Base/acetone extraction; (D) SDS extraction; (E) TCA/acetone/phenol extraction; (F) TCA/acetone precipitation. The larger and darker of protein spots indicated the higher abundance.