Modified TCA/acetone precipitation of proteins for proteomic analysis

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Abstract

Protein extracts obtained from cells or tissues often need to remove interfering substances for preparing high-quality protein samples in proteomic analysis. A number of protein extraction methods have been applied to various biological samples, especially TCA/acetone precipitation and phenol extraction. TCA/acetone precipitation, as a common method, is thought to minimize protein degradation and proteases activity as well as reduce contaminants like salts and polyphenols. However, the TCA/acetone precipitation relies on the completely pulverizing and repeatedly rinse of tissue powder to remove the interfering substances, which is laborious and time-consuming. In addition, a prolonged incubation in TCA/acetone or acetone can lead to the modifications and degradation of proteins, and the precipitated proteins are more difficult to re-dissolve. We have described a modified TCA/acetone precipitation of plant proteins for proteomic analysis. Proteins of cells or tissues were extracted using SDS-containing buffer, precipitated with equal volume of 20% TCA/acetone, and washed with acetone. Compared to classical TCA/acetone precipitation, this protocol generates comparable yields, spot numbers, and proteome profiling, but takes less time (ca. 45 min), thus avoiding excess protein modification and degradation after extended-period incubation in TCA/acetone or acetone. The modified TCA/acetone precipitation method is simple, fast, and suitable for various plant tissues in proteomic analysis.

Keywords: Protein extraction, TCA/acetone precipitation, Removal of interfering substances, SDS sample buffer; 2DE; MALDI-TOF mass spectrometry

Background

Protein extracts obtained from cells or tissues often contain interfering substances, which must be removed for preparing high-quality protein samples [1]. In particular, plant tissues contain a diverse group of secondary compounds, such as phenolics, lipids, pigments, organic acids and carbohydrates, which greatly interfere with protein extraction and proteomic analysis [2]. Thus, sample quality is critical for the coverage, reliability, and throughput of proteomic analysis, protein extraction in proteomics remains a challenge, even though advanced detection approaches (especially LC-MS/MS) can greatly enhance the sensitivity and reliability of protein identification [3].We are always interested in developing protein extraction protocols for 2DE-based proteomics. In last decade, Wang and the colleagues have established many such protocols as integrating TCA/acetone precipitation with phenol extraction [4-8]. A protein extraction protocol that can be universally applied to various biological samples with minimal optimization remains essential in current proteomics.

A number of methods are available for concentrating dilute protein solutions and simultaneously removing interfering substances, especially TCA/acetone precipitation and phenol extraction [9-11]. TCA/acetone precipitation is a common method for precipitation and concentration of total proteins, initially developed by Damerval et al. [12] and then modified for use in various tissues [10, 13-19].

TCA/acetone precipitation is thought to minimize protein degradation and proteases activity as well as reduce contaminants such as salts or polyphenols [20]. During acetone/TCA precipitation, organic-soluble substances are rinsed out, leaving proteins and other insoluble substances in the precipitate, and proteins are extracted using a buffer of choice [2, 5, 9]. The success of TCA/acetone precipitation relies on the completely pulverizing and repeatedly rinse of tissue powder to remove the interfering substances, which is laborious and time-consuming.

However, a prolonged incubation of tissue powder in TCA/acetone or acetone can lead to the modifications of proteins by acetone and the proportion of modified peptide increased over time, thus affecting the outcome of MS/MS analysis [21]. Moreover, long exposure to the acidic pH in TCA/acetone probably causes protein degradation [6, 22]. Alternatively, protein extract can be precipitated using aqueous 10% TCA [23], but the TCA precipitated proteins are more difficult to dissolve and need using NaOH to increase their solubilization [24]. Therefore, aqueous TCA precipitation is not commonly used like TCA/acetone precipitation in proteomic analysis.

To overcome the limitations of aqueous TCA precipitation and TCA/acetone precipitation, we here report a modified, rapid method of TCA/acetone precipitation of plant proteins for proteomic analysis. We systematically compared the modified and classical TCA/acetone precipitation regarding protein yields and proteome profiles.

Methods

Materials

Maize (*Zea mays* L.) seeds were soaked in water for 2 h to soften seed coats and endosperms, and the embryos were manually dissected and used for protein extraction. Leaves and roots (root tips, ca. 1 cm) of maize seedlings were sampled and used for protein extraction.

Escherichia coli BL21 was cultivated at 37°C for 12 h with good aeration until 0.8 optical density. The cells were collected by centrifugation (1,000 g, 3 min, 4°C), rinsed with phosphate-buffered saline thrice, and used for protein extraction.

Modified TCA/ Acetone precipitation of proteins

The modified protocol is designed for running in eppendorf tubes within 1 h, and can be reliably adapted to big volumes. It includes protein extraction, precipitation, and dissolving. The detailed steps were given in Fig. 1. Maize tissues and *E. coli* cells were used for evaluating the protocol. Organic solvents were pre-cooled at -20°C and were supplemented with 5 mM dithiothreitol (DTT)

before use. The subsequent steps were carried out at 4°C unless otherwise indicated.

Protein extraction. Maize embryos (0.2g), leaves (0.4g), and roots (0.4g) and *E. coli* cells (0.2g) were homogenized in a pre-cooled mortar (interior diameter 5 cm)on ice in 2.0 ml of the extraction buffercontaining1% SDS, 0.1 M Tris-HCl (pH 6.8), 2 mM EDTA-Na_2, 20 mM DTT and 2 mM PMSF (adding before use). The homogenate was transferred into eppendorf tubes and centrifuged at 15,000 g for 5 min. Then, the supernatant (protein extract) was pipetted into fresh tubes.

Protein precipitation. Add cold 20% TCA/acetone into the protein extract (1: 1, v/v, with a final 10% TCA/50% acetone), place the mixture on ice for 5 min, centrifuge the mixture (15000g, 3 min), and discard the supernatant. Wash protein precipitate with 80% acetone, followed by centrifugation as above. Repeat the wash step once or more.

Protein dissolving. Air-dry the protein precipitates shortly (1- 3 min), and dissolve in a buffer of choice for SDS-PAGE, IEF or iTRAQ analysis. Notably, do not over-dry the precipitates, otherwise they will be more difficult to resolubilize.

As a parallel experiment, the classical TCA/ acetone method was exactly done according to previously described [10].

Protein assay

For SDS-PAGE, protein precipitates were dissolved in a SDS-containing buffer (0.5% SDS, 50mM Tris-HCl, pH 6.8, and 20mM DTT). Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA) [25]. Prior to SDS-PAGE, protein extracts were mixed with appropriate volume 4 x SDS sample buffer [26]. For 2DE, protein precipitates were dissolved in the 2DE rehydration solution without IPG buffer to avoid its interference [27], and protein concentrations were determined by the Bradford Assay. After then, the IPG buffer was supplemented into protein samples to 0.5% concentration.

102 **SDS-PAGE**

103 SDS-PAGE was run using 12.5% gel [28] and protein was visualized using Coomassie brilliant blue
104 (CBB) G250.

105 **2-DE and MS/MS**

106 Isoelectric focusing (IEF) was performed using 11-cm linear IPG strips (pH 4–7, Bio-Rad).
107 Approximately 600 μg of proteins in 200 μl of the rehydration solution was loaded by passive
108 rehydration with the PROTEAN IEF system (Bio-Rad) for 12 h at 20°C. IEF and subsequent SDS-
109 PAGE, and gel staining were performed as previously described [28].Digital 2-DE images were
110 processed and analyzed using PDQUEST 8.0 software (Bio-Rad). Protein samples were analyzed by
111 2DE in three biological replicates.

112 The spots with at least2-fold quantitative variations in abundance among maize embryos,
113 leaves and roots by two methods respectively were selected for mass spectrometry (MS) analysis.
114 One-way ANOVA was performed based on three biological replications.

115 The selected protein spots were extracted, digested and analyzed by the MALDI-TOF/TOF
116 analyzer (AB SCIEX TOF/TOF-5800, USA) as described previously [29]. MALDI-TOF/TOF spectra
117 were acquired in the positive ion mode and automatically submitted to Mascot 2.2
118 (http://www.matrixscience.com) for identification against NCBInr database (version Feb 17, 2017;
119 species, *Zea mays*, 279566 sequences). Only significant scores defined by Mascot probability
120 analysis greater than "identity" were considered for assigning protein identity. All of the positive
121 protein identification scores were significant ($p < 0.05$).

122 **Bioinformatics Analysis**

123 The unidentified proteins were searched by BLAST using Universal Protein
124 (http://www.uniprot.org/) or National Center for Biotechnology Information
125 (http://www.ncbi.nlm.nih.gov/). Grand average of hydropathicity (GRAVY) analyses were

126 performed using ProtParam tool (http://web.expasy.org/protparam/). Subcellular localization
127 information was predicted using the online predictor Plant-mPLoc
128 (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/).

129 Results

130 The development of the modified TCA and acetone precipitation

131 Rather than preparing tissue powder by extensively TCA/acetone rinse in the classical method, we
132 directly extracted proteins in cells or tissues and then precipitated proteins in the extracts with
133 equal volume of 20% TCA/acetone. Various aqueous buffers can be used for protein extraction,
134 but the composition of the extraction buffers will greatly affect the profiles of the extracted
135 proteome. The Laemmli's SDS buffer [24] was used for protein extraction, because SDS-containing
136 buffer can enhance protein extraction and solubility, especially under heating.

137 The ratio of TCA and acetone was optimized in initial tests (Fig. 2). We compared the effect of
138 10% (w/v) TCA in various concentrations (0-80%, v/v) of aqueous acetone and the effect of various
139 TCA concentrations in aqueous 50% acetone on protein extraction and separation. The presence
140 of TCA could improve protein resolution by SDS-PAGE, but no significant differences were
141 observed among 5-20% TCA (Fig. 2A). At a fixed 10% TCA, protein patterns were quite similar
142 among different acetone ratio, but the background was clearer with the increased acetone ratio
143 (Fig. 2B). Based on the quality of protein gels, we choose the low ratio combination of 10% TCA/50%
144 acetone (final concentration) for further testing.

145 Different from aqueous TCA precipitation, proteins precipitated by 10% TCA/50% acetone are
146 easy to dissolve. After incubated on ice for 10 min, protein precipitates are recovered by
147 centrifugation, and washed with 80% acetone thrice to remove residual TCA in the precipitated
148 protein. Finally, the air-dried protein precipitates are dissolved in a buffer of choice for SDS-PAGE,
149 IEF or iTRAQ analysis.

150 **Comparison of the performance in both methods**

151 We made a comprehensive comparison of protein yields and resolving in 2-DE by the modified
152 method and the classical method. Regarding protein yields, the former was slightly higher, but did
153 not significantly differ from the classical method (t-test, $p < 0.05$). Similar results were obtained by
154 comparison of spot numbers in 2D gels between both methods (Table 1). Compared with other
155 separation methods (e.g. SDS-PAGE, LC), 2DE analysis can display visible results regarding the
156 quality of protein samples.

157 For all materials tested, 2D gels obtained with the two methods were generally comparable
158 regarding the number, abundance and distribution of protein spots, without profound deviations
159 (Fig.3-5); however, several protein spots exhibited at least 2-fold differences in abundance. For
160 example, spots 1, 2 and 4 were more abundant in maize roots by the modified method, whereas
161 the classical method resulted in more abundant spot 3. Of the 11 differential abundance proteins
162 (DAPs) selected for MS/MS identification, nine were identified with MS/MS analysis (Table 2). In
163 particular, the modified method selectively depleted globulin-1 in maize embryos (Fig. 4). Our
164 recent studies showed that globulin-1 (also known as vicilin) was the most abundant storage
165 proteins in maize embryos [11, 30], and selective depletion of globulin-1 improved proteome
166 profiling of maize embryos [11]. In addition, the modified protocol also produced good 2DE maps
167 in *E. coli* cells (Fig. 6).

168 It needed to note that the aqueous TCA precipitation cause severely-denatured proteins that
169 were very difficult to dissolve, and was rarely used in proteomic analysis. Thus, we did not
170 compare it with the modified method in the present study.

171 Discussion

172 The classical TCA/acetone precipitation applies a strategy of removal of interfering substances
173 before protein extraction, involving incubation for extended periods (from 45 min to overnight) in

174 TCA/acetone and between the rinsing steps[10,12,13,15,20]. These steps can lead to the
175 modifications of proteins by acetone [21] or possible protein degradation after long exposure to
176 harsh TCA/acetone [6, 23, 31], thus affecting the outcome of MS/MS analysis.

177 In contrast, the modified method here is using a strategy of removing interfering substances
178 after protein extraction, taking less time to avoid protein modifications by acetone, but producing
179 similar or better results regarding protein yields, 2DE spot numbers and proteome profiling. The
180 resultant protein precipitates are easy to wash using acetone compared to tissue powder in the
181 classical TCA/acetone precipitation method.

182 Previously, Wang et al. [29] observed that in oil seed protein extraction uses 10% TCA/ acetone,
183 rather than aqueous TCA, because the former resulted in protein precipitates easily to dissolve in
184 SDS buffer or 2-D rehydration buffer. The combination of TCA and acetone is more effective than
185 either TCA or acetone alone to precipitate proteins [17, 32]. It is noted that some proteins were
186 preferably extracted by the modified or the classical method, but the rationale remains open to
187 question. Recently, we reported a chloroform-assisted phenol extraction method for depletion of
188 abundant storage protein (globulin-1) in monocot seeds (maize) and in dicot (soybean and pea)
189 seeds [8]. The modified method exhibited also highly efficient to deplete globulin-1, suggesting
190 another application of the modified method in proteomic analysis.

191 In the modified method, final protein pellets were dissolved in the same 2DE buffer as in the
192 classical method, so protein profiles highly depends on the extraction efficiency in the SDS buffer
193 and precipitation efficiency by 20% TCA/acetone. We observed some repeatedly, significant
194 differences in abundance of several DAPs between both methods. There were specific DAPs
195 associated with each method in different samples. However, the rationale behind this
196 phenomenon remains unclear. We tried to analyze the hydropathicity, physicochemical property,
197 and Subcellular compartments of these DAPs (Table 2); however, no a clearly conclusion could be

198 drawn. Reasonably, different extract methods can produce protein profiles with substantial or
199 subtle differences [33], but these inherent differences were yet difficult to explain, as discussed
200 at a previous study [15].

201 To summarize, the greatest advantage of the modified method is simple and fast. Despite the
202 similarity in steps to aqueous TCA precipitation, but it overcame the latter's drawback that TCA-
203 precipitated proteins are difficult to dissolve. Moreover, the modified method uses equal volume
204 20% TCA/acetone to precipitate proteins, and can handle bigger volume of protein extracts than
205 acetone precipitation in a microtube. Because the modified method precipitates proteins in
206 aqueous extracts, it is expected to be universally applicable for various plant tissues in proteomic
207 analysis.

Declarations

Authors' contributions

LJN and WW drafted the manuscript. HZ and LN performed the experiments. All authors contributed the experimental design and data analysis.

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Competing interests

The authors declare that they have no competing interests.

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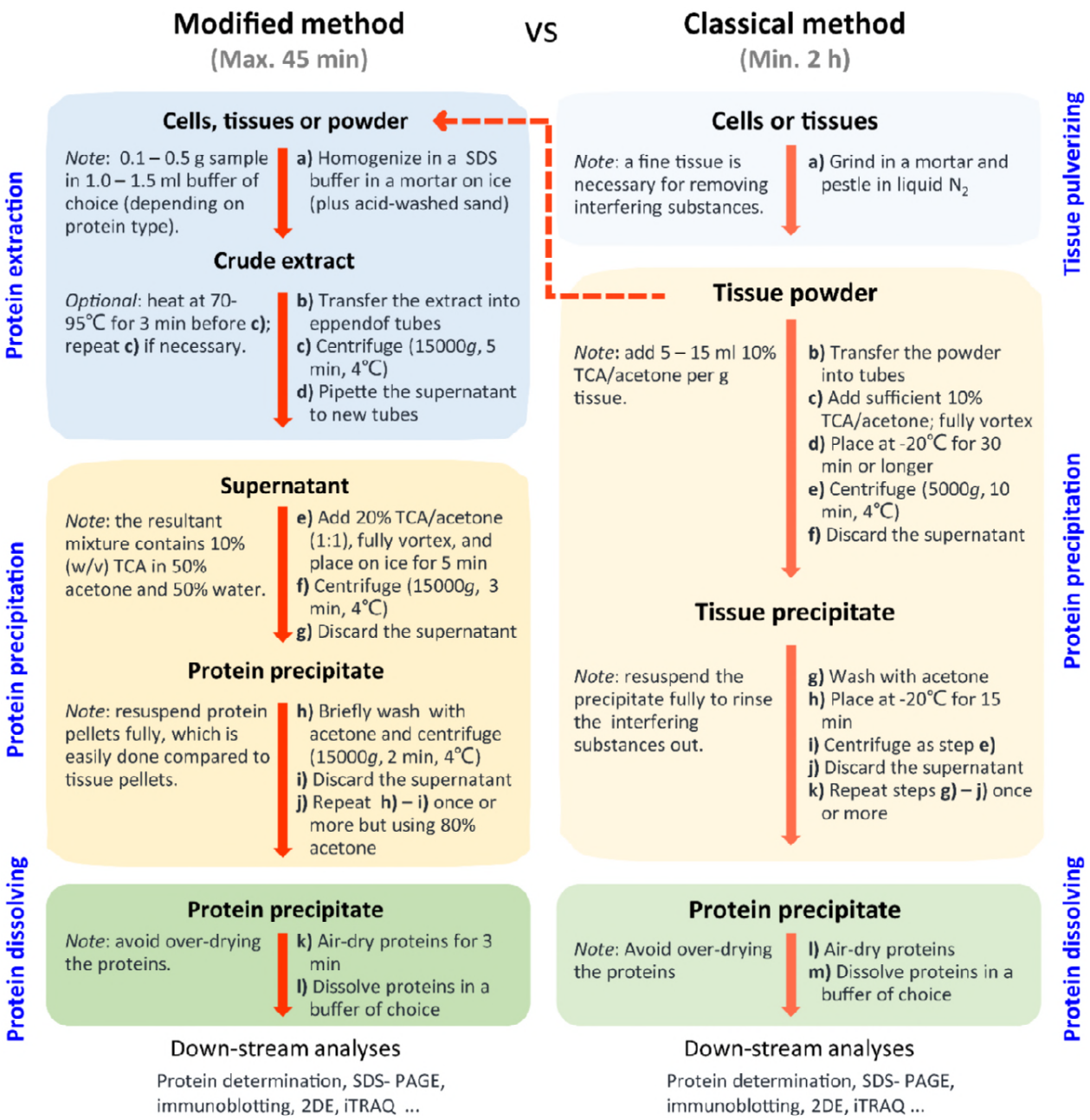

Figure 1. The comparison of the steps between the modified and the classical TCA/acetone precipitation methods. The latter was done according to Isaacson *et al.* [13]. The SDS extraction buffer contains 1% (w/v) SDS, 0.1 M Tris-HCl (pH 6.8), 2 mM EDTA-Na_2, 20 mM DTT and 2 mM PMSF (adding before use). All organic solvents are pre-chilled at -20℃and contain 5 mM DTT (adding before use).

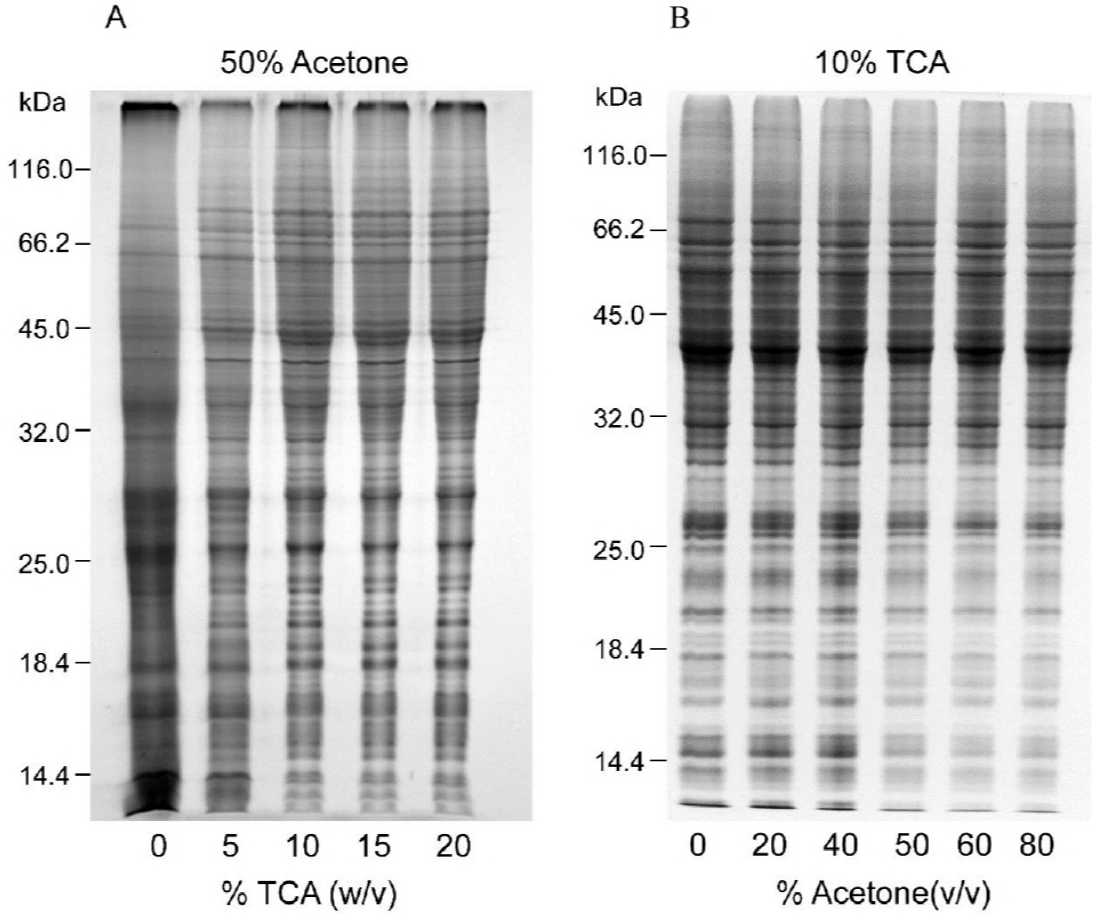

Figure 2 Optimization of TCA/acetone ratio used in the modified method. Equal amounts (ca. 30 µg) of maize root proteins were analyzed by SDS-PAGE (12.5% resolving gel). Protein was stained using CBB. A, final acetone concentration was 50% (v/v), but TCA concentration varied from 0-20% (w/v) in the aqueous mixture. B, final TCA concentration was 10% (w/v), but acetone concentration varied from 0-80% (v/v) in the aqueous mixture.

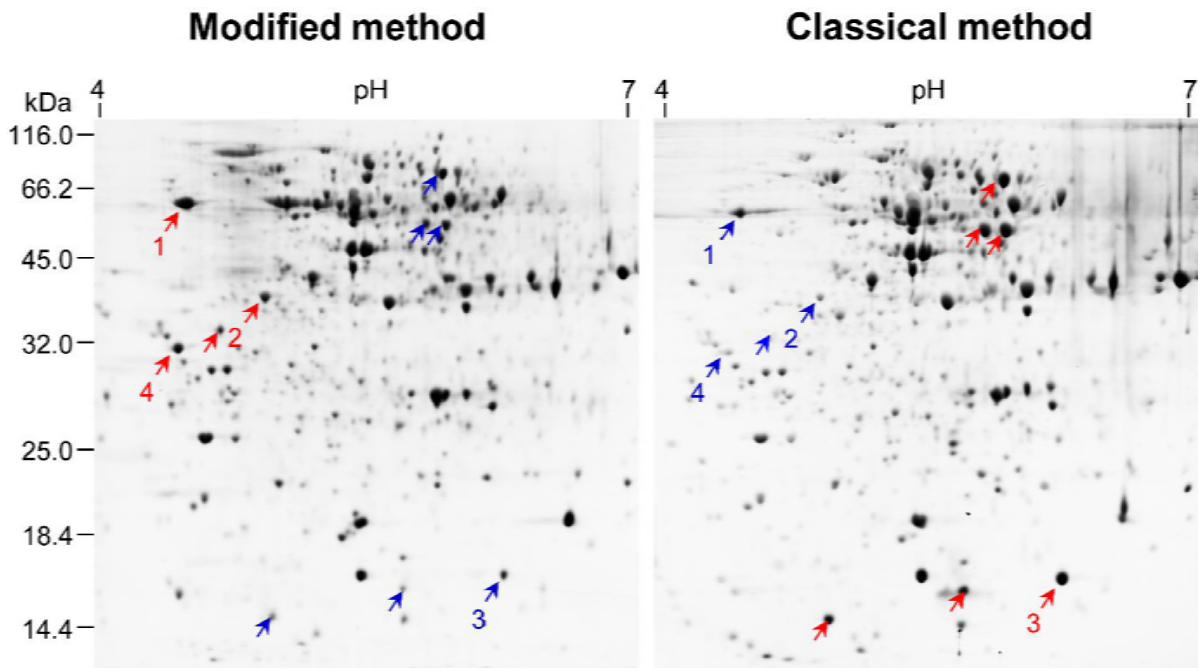

Figure 3 Comparison of 2DE protein profiles of maize root proteins extracted using two methods. Figures were from two independent experiments. *Left panel*: the modified TCA/acetone precipitation. *Right panel*: the classical TCA/acetone precipitation. Spots with increased abundance were indicated in red. About 800 µg of proteins was resolved in pH 4-7 (linear) strip by IEF and then in 12.5% gel by SDS-PAGE. Proteins were visualized using CBB. Differential abundance spots (numbered, >2-fold) were subject to MS/MS analysis.

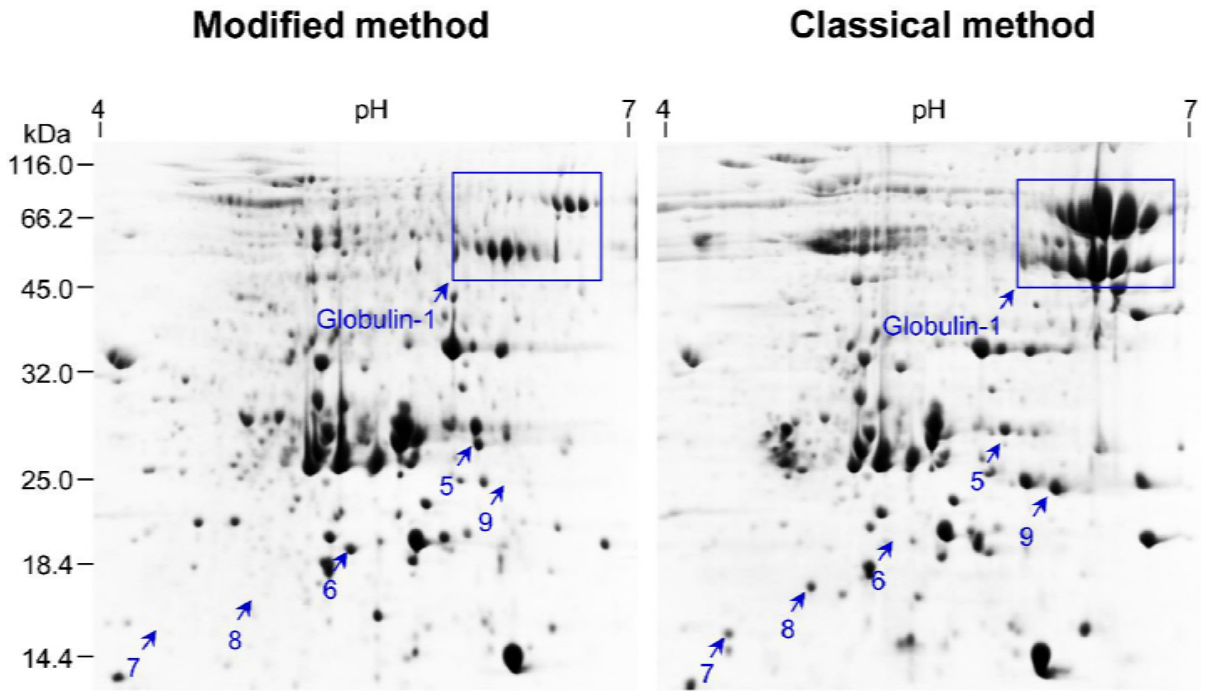

Figure 4 Comparison of 2DE profiles of maize embryo proteins extracted using two methods. Shown were two independent experiments. Left panel: the modified TCA/acetone precipitation. Right panel: the classical TCA/acetone precipitation. About 800 µg of proteins were resolved in pH 4-7 (linear) strip by IEF and then in 12.5% gel by SDS-PAGE. Protein was visualized using CBB. Differential abundance spots (numbered, >2-fold) were subject to MS/MS analysis.

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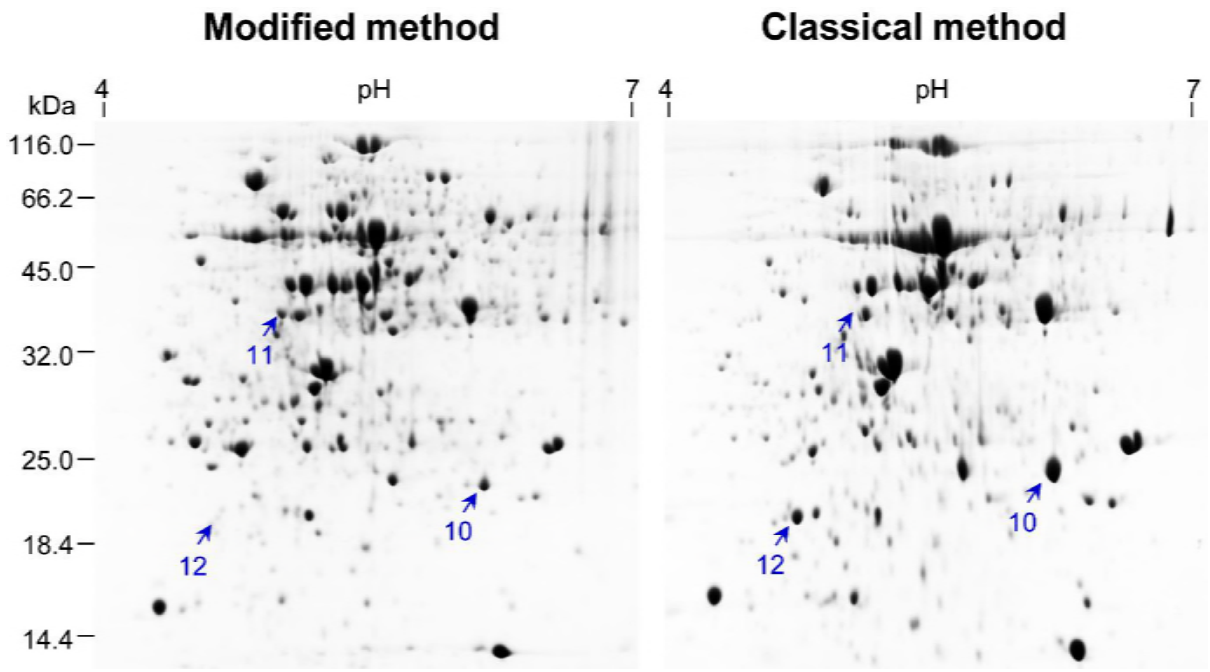
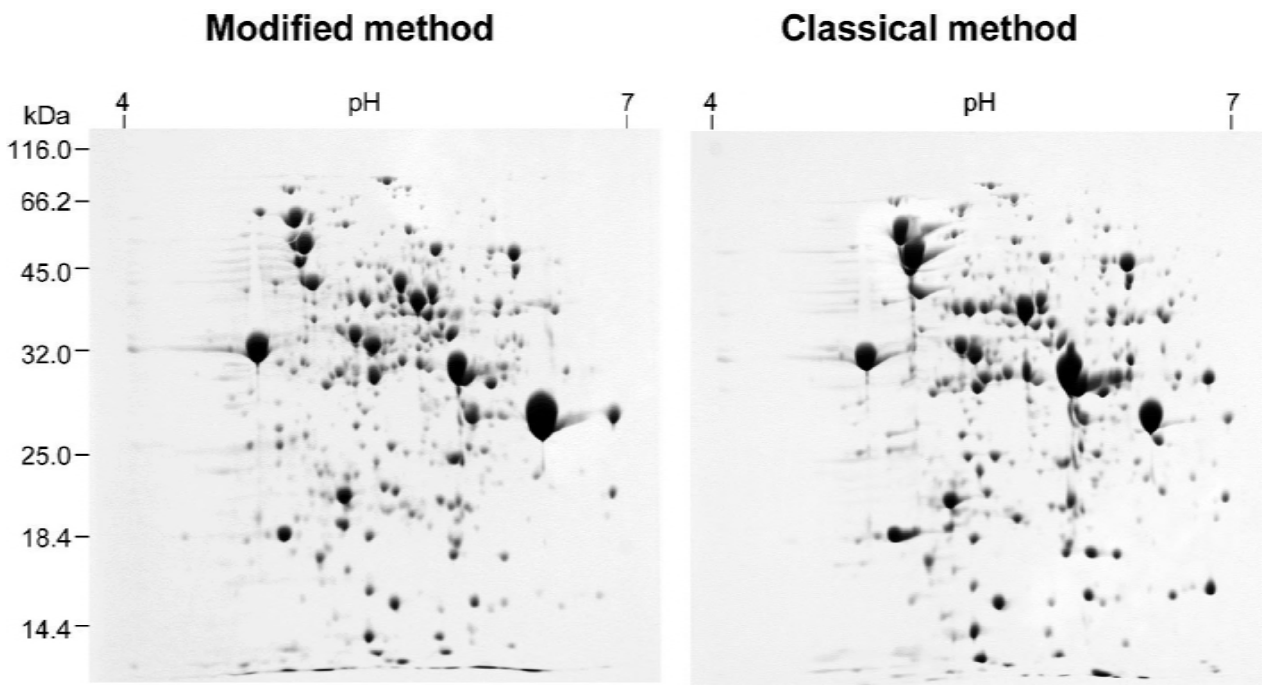


339
340
341 **Figure 5** Comparison of 2DE profiles of maize leaf proteins extracted using two methods. Shown
342 were two independent experiments. Left panel: the modified TCA/acetone precipitation. Right
343 panel: the classical TCA/acetone precipitation. About 800 µg of proteins were resolved in pH 4-
344 7 (linear) strip by IEF and then in 12.5% gel by SDS-PAGE. Protein was visualized using CBB.
345 Differential abundance spots (numbered, >2-fold) were subject to MS/MS analysis.



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348 **Figure 6** Comparison of 2DE profiles of E. coli proteins extracted using two methods. Left panel:

349 the modified TCA/acetone precipitation. Right panel: the classical TCA/acetone precipitation. E.

350 coli BL21 was cultivated at 37°C for 12 h with good aeration until optical density of about 0.8.

351 Cells were collected by centrifugation and washed with phosphate-buffered saline (PBS).

352 Proteins were extracted by using two methods. About 800 µg of proteins were resolved in pH 4-

353 7 (linear) strip by IEF and then in 12.5% gel by SDS-PAGE. Protein was visualized using CBB.

354

Table 1 Comparison of protein yield and spot number in 2DE between both methods

Maize tissues	The modified method		The classical method	
	Yield [a]	Spot No [b]	Yield [a]	Spot No [b]
Roots	4.82±0.07	738±11	4.70±0.08	743±10
Leaves	4.13±0.11	310±9	4.06±0.23	314±4
Embryos	5.80±0.13	314±12	5.56±0.38	304±18

Notes: a, Protein yields were expressed as μg/mg fresh weight. b, Average spot number detected in 2D gels using PDQUEST software (version 8.0, Bio-Rad). All data were from at least three independent experiments. The corresponding data from the two methods did not significantly differ at $p < 0.05$ according to t-test.

Table 2 The identification of the differential extracted proteins between the two methods in maize

Protein name	Accession	MW(kDa)/ p*I*	Protein score	Protein Score C. I. (%)	Coverage (%)	GRAVY	Subcellular localization	Matched Peptides
Calreticulin-2, partial (spot 1)	ONM21219	41.11/4.81	240	100	47.6	-0.870	Endoplasmic reticulum	FEDGWESR;NPNYQGKWK;QTGSIYEHWDILPPK;WKAPMIDNPDFK; FYAISAEYPEFSNK;YIGIELWQVK;KDDNMAGEWNHTSGK; SGTLFDNIIITDDPALAK;WNGDAEDKGIQTSEDYR;KPEGYDDIPKEIPDPDAK;KPEDWDDKEYIPDPEDK;KPEDWDDEEDGEWTAPTIPNPEYK;
Fructokinase-1 (spot 2)	AAP42805	34.67/4.87	86	99.937	39.0	0.139	Chloroplast	MLAAILR;EALWPSR;EFMFYR;LGDDEFGR;DFHGAVPSFK;TAHLRAMEIAK;FANACGAITTTK;DNGVDDGGVVFDSGAR;LGGGAAFVGKLGDDEFGR;AAVFHYGSISLIAEPCR;VSEVELEFLTGIDSVEDDVVMK
Glycine-rich RNA-binding protein 2(spot 3)	ACG28116	15.48/6.1	182	100	34.6	-0.487	Nucleus	NITVNEAQSR;GGGYGNSDGNWR;RDGGGGYGGGGGGYGGGGGYGGGGGGYGGGNR
Elongation factor 1-β (spot 4)	ONM59608	15.67/4.38	134	100	13.2	-0.360	Cell membrane	LDEYLLTR;KLDEYLLTR;LSGITAEGQGVK;WFNHIDALVR
Glutathione transferase (spot 5)	CAA73369	25.10/6.21	132	100	43.8	-0.174	Cytoplasm	GDGQAQAR;NLYPPEK;LYDCGTR;AEMVEILR;KLYDCGTR; VYDFVCGMK;FWADYVDKK;ECPRLAAWAK;GLAYEYEQDLGNK;QGLQLLDFWVSPFGQR;KQGLQLLDFWVSPGQR;QGLQLLDFWVSPFGQRCR;GLAYEYLEQDLGNKSELLLR
Lea14-A (spot 6)	AMY96568	16.08/5.64	514	100	96.7	0.024	Chloroplast. Golgi apparatus. Nucleus.	DGATLAGRVDVR;DWDIDYEMR;SGELKLPTLSSIF;DAGRDWDIDYEMR;LDVPVKVPYDFLVSLAK;TVASGTVPDPGSLAGDGATTR;VGLTVDLPVVGKLTLPLTK;NPYSHAIPVCEVTYTLR;LANIQKPEAELADVTVGHVGR;SAGRTVASGTVPDPGSLAGDGATTR;VDVRNPYSHAIPVCEVTYTLR;GFVADKLANIQKPEAELADVTVGHVGR
Sedoheptulose-1,7-bisphosphatase (spot 11)	ACG31345	41.79/6.8	101	99.998	19.0	-0.136	Chloroplast	EKYTLR;LLICMGEAMR;FEETLYGSSR;GIFTNVTSPTAK;YTGGMVPDVNQIIVK;LTGVTGGDQVAAAMGIYGPR

Note: For MS/MS analysis, differential abundance spots (> 2 folds) were extracted, in-gel digested (trypsin, 37°C, 20 h), and analyzed by the MALDI-TOF/TOF analyzer (AB SCIEX TOF/TOF-5800, USA). MALDI-TOF/TOF spectra were acquired in the positive ion mode and automatically submitted to Mascot 2.2 (http://www.matrixscience.com) for identification against NCBInr database (version Feb 17, 2017; species, Zea mays, 279566 sequences). The search parameters were as follows: type of search: combined (MS + MS/MS); enzyme: trypsin; dynamical modifications: oxidation (M); fixed modifications: carbamidomethyl (C); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ±100 ppm; fragment mass tolerance: ±0.4 Da; peptide charge state: 1+; max missed cleavages: 1. Unambiguous identification was judged by the number of matched peptide sequences, sequence coverage, Mascot score and the quality of MS/MS spectra. All of the positive protein identification scores were significant ($p<0.05$).