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Evaluation of Sample Preparation Methods for the Analysis of Papaya Leaf Proteins through Two-dimensional Gel Electrophoresis

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ABSTRACT:

Introduction – A variety of sample preparation protocols for plant proteomic analysis using two-dimensional gel electrophoresis (2-DE) have been reported. However, they usually have to be adapted and further optimised for the analysis of plant species not previously studied.

Objective – This work aimed to evaluate different sample preparation protocols for analysing *Carica papaya* L. leaf proteins through 2-DE.

Methodology – Four sample preparation methods were tested: (1) phenol extraction and methanol–ammonium acetate precipitation; (2) no precipitation fractionation; and the traditional trichloroacetic acid–acetone precipitation either (3) with or (4) without protein fractionation. The samples were analysed for their compatibility with SDS–PAGE (1-DE) and 2-DE. Fifteen selected protein spots were trypsinised and analysed by matrix-assisted laser desorption/ionisation time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS), followed by a protein search using the NCBInr database to accurately identify all proteins.

Results – Methods number 3 and 4 resulted in large quantities of protein with good 1-DE separation and were chosen for 2-DE analysis. However, only the TCA method without fractionation (no. 4) proved to be useful. Spot number and resolution advances were achieved, which included having an additional solubilisation step in the conventional TCA method. Moreover, most of the theoretical and experimental protein molecular weight and p*I* data had similar values, suggesting good focusing and, most importantly, limited protein degradation.

Conclusion – The described sample preparation method allows the proteomic analysis of papaya leaves by 2-DE and mass spectrometry (MALDI-TOF-MS/MS). The methods presented can be a starting point for the optimisation of sample preparation protocols for other plant species. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: 2-DE; *Carica papaya*; plant proteomics; protein extraction method; mass spectrometry

Introduction

Papaya, *Carica papaya* L. (*Caricaceae* family), is cultivated worldwide in tropical and subtropical climates, mainly for its fruit. The commercially reported production of papaya in 2006 reached 1.57 million metric tons in Brazil, which is considered to be the major producer (Food and Agriculture Organization of the United Nations, 2007). By contrast, papaya suffers economic losses as a result of both biotic and abiotic stresses. Papaya diseases stand out because their presence can cause severe economic losses in production, sales and exportation of fresh fruit that may reach 100% loss in some cases.

The papaya genome sequence offered a new perspective (Ming *et al*., 2008). Nowadays, papaya nucleotide sequences are available in the NCBInr database (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=3649), allowing utilisation of molecular biology techniques to evaluate the papaya genome and gene expression patterns (Ming *et al*., 2008).

Experimental protein analyses in papaya have been restricted to latex samples. Different papaya proteins, such as chymopapain, caricain, papaya proteinase IV and papain (Balls *et al*., 1937) have been purified from latex and are known for their proteolytic activities. For most of these, structural and regulatory details are already known (Golan *et al*., 2000), but no large scale protein experimental analyses have been published for the papaya plant to date.

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Two-dimensional polyacrylamide gel electrophoresis (2-DE) is one of the most powerful tools for large-scale protein separation and quantification (Weiss and Görg, 2008). The separated proteins can be accurately characterised through other complimentary proteomic techniques, mainly mass spectrometry. Unfortunately, there is no single method of sample preparation that can be universally applied to all kinds of samples analysed by 2-DE. In plants, a variety of 'standard' protocols and sample solubilisation buffers have been reported (Weiss and Görg, 2008), and four protocols are commonly used: (1) trichloroacetic acid (TCA)–acetone precipitation; (2) TCA–acetone precipitation with fractionation; (3) no protein precipitation; and (4) phenol extraction methanol–ammonium acetate precipitation (Carpentier *et al*., 2005). These methods were applied for different plants (Natarajan *et al*., 2005; Cho *et al*., 2006), but they usually have to be adapted and further optimised for the proteomics analysis of plant species not studied before.

In this work, papaya leaf proteins were prepared using four sample preparation methods. They were evaluated for their compatibility with SDS–PAGE (1-DE) and 2-DE. The original TCA method was also optimised by including an additional protein solubilisation step. Fifteen spots distributed along the gel with different molecular weights, p*I*s and intensity were chosen for further identification by MALDI-TOF-MS/MS. The analysis presented here is a starting point for additional large-scale studies of the papaya plant, such as plant development, acquired pathogens resistance, differential expression during stress and pathological states of the papaya plant (Rossignol *et al*., 2006). The method described can be further tested and adjusted for other plant species. It is important to stress that proteomic analysis allow the study of proteins of biosynthetic pathways leading to secondary metabolites (Jacobs *et al*., 2000).

Experimental

Chemicals and reagents

Tris, urea and thiurea were purchased from Sigma (St Louis, MO, USA). Iodoacetamide, acrylamide, bis-acrylamide, standard molecular weight and carrier ampholyte were obtained from GE Healthcare (GE Healthcare, Uppsala, Sweden). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), sodium dodecyl sulfate (SDS), glycerol and dithiothreitol (DTT) were purchased from USB (USB, Cleveland, OH, USA). Ultra-pure water was used to prepare the protein extracts, 2-DE and MALDI-TOF-MS/MS solutions.

Plant material

Carica papaya cv. Golden (a commercial variety widely cultivated in Brazil) plants were cultured under experimental conditions at the INCAPER Experimental Farm located in the north of the State of Espírito Santo, Brazil. Three different plants had their leaves collected (three completely expanded leaves per plant, mainly from the limb part of the leaf tip). This part of the leaf was weighted and pooled together in equal amounts. The samples were frozen in liquid nitrogen and remained at –70°C until use. The frozen tissues were subsequently ground into fine powders in liquid nitrogen using a pre-cooled mortar and pestle. Samples from three biological replicates were submitted to protein extraction as described below. For each method, 150 mg of powdered tissues was used. A scheme of all sample preparation methods tested is presented in Fig. 1.

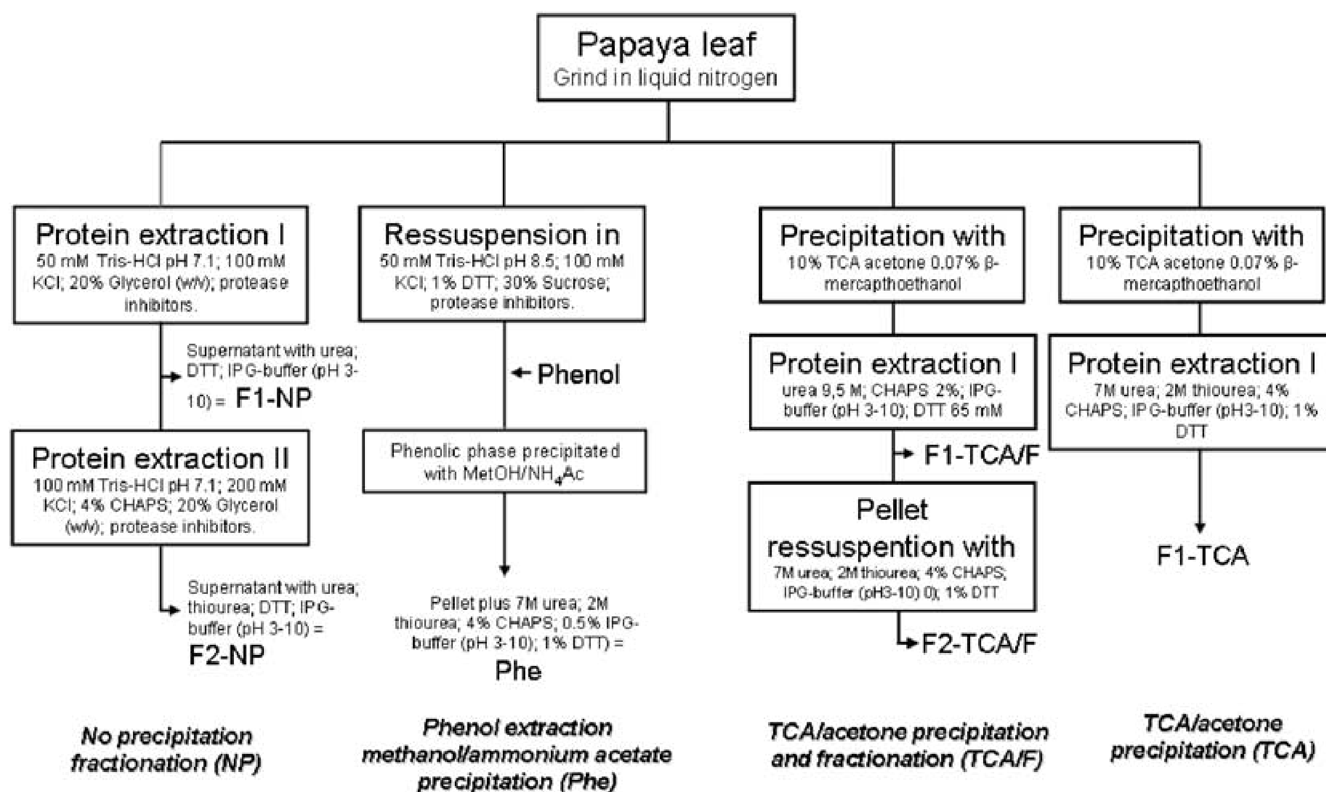

Figure 1. Schematic representation of the four sample preparation methods evaluated in this work.

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Protein extraction methods

No precipitation fractionation (NP). The extraction was performed according to Giavalisco *et al*. (2003) with several modifications. A volume (150 μL) of extraction buffer I (50 mM Tris–HCl pH 7.1, 100 mM KCl, 20% w/v glycerol, 3 mM benzamidine, 1 mM PMSF, 1 mM EDTA) was added (w/v) to the powdered tissues. After mixing, the samples were centrifuged at 16000g for 30 min at 4°C. The supernatant (50 μL) was collected, mixed with 54 mg urea and 10 μL 10% w/v DTT, and named F1-NP. The remaining supernatant was eliminated to avoid contamination between fractions 1 and 2. Next, 200 μL of buffer II (100 mM Tris–HCl pH 7.1, 200 mM KCl, 4% CHAPS, 20% v/v glycerol, 3 mM benzamidine, 1 mM PMSF, 1 mM EDTA) were added to the pellet and mixed, which was then followed by the addition of 70 μL of denaturating buffer (7 M urea, 2 M thiourea, 700 mM DTT). The samples were vortexed (10 min) and centrifuged (16000*g*, 30 min at 4°C). The supernatant was collected and is referred to as F2-NP.

TCA–acetone precipitation (TCA). TCA was performed as described previously (Damerval *et al*., 1986) with some modifications. The powdered tissue was allowed to precipitate overnight with 1.5 mL acetone–10% TCA–0.07% 2-mercaptoethanol at −20°C. The supernatant was discarded after centrifugation (16000*g*, 30 min at 4°C). The resulting pellet was washed twice in ice-cold acetone–0.07% 2-mercaptoethanol. The pellet was vortexed (10 min) and sonicated (10 min) at 4°C to properly remove papaya pigments and other interfering compounds. The samples were kept 1 h at −20°C between each wash. The vacuum-dried pellets were resuspended in 100 μL resolubilisation buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT). The samples were sonicated (30 min) and vortexed (30 min) at 25°C. When necessary, they were cooled on ice to prevent sample heating. The supernatant was collected after centrifugation (16000*g*, 30 min at 4°C) and is referred to as F1-TCA. In this work, an additional solubilisation step was used. The resolubilisation buffer (100 μL) was again added to the remaining pellet. The sample was sonicated/vortexed and centrifuged as described above. The supernatant was collected and is referred to as F2-TCA. To obtain a single sample, the F1-TCA and F2-TCA were combined (2 : 1 v/v).

TCA–acetone precipitation and fractionation (TCA/F). The sample precipitation and pellet wash were conducted as described in the section above. The vacuum-dried pellets were resuspended in 200 μL solution containing 9.5 M urea, 2% CHAPS and 1% DTT. The samples were sonicated/vortexed and centrifuged as described above. The supernatant was collected and is referred to as F1-TCA/F. The remaining pellet was resuspended in resolubilisation buffer (section above). Again, the samples were sonicated/vortexed and centrifuged as described above. The supernatant was collected and is referred to as F2-TCA/F (Carpentier *et al*., 2005).

Phenol extraction methanol–ammonium acetate precipitation (Phe). A volume (500 μL) of extraction buffer (50 mM Tris–HCl pH 8.5, 100 mM KCl, 1% DTT, 30% w/v sucrose, 5 mM EDTA, 3 mM benzamidine, 1 mM PMSF) was added to powdered papaya tissue and vortexed (30 s). Ice-cold Tris-buffered (pH 8.0) phenol (500 μL) was added to the samples. They were vortexed 15 min at 4°C and centrifuged 6000*g* for 3 min at 4°C. The phenolic phase was collected and re-extracted with 500 μL extraction buffer. The phenolic phase after centrifugation was allowed to precipitate overnight with 1 mL 100 mM ammonium acetate in methanol at −20°C. The vacuum-dried pellets were resuspended and treated as described (section above) (Carpentier *et al*., 2005).

Protein measurement

Protein concentration was determined as previously described (Peterson, 1983) with modifications. In the precipitation step, 1.5% w/v DOC (sodium deoxycholate) was used. The standard curve (10–50 μg BSA) was plotted using BSA diluted in the same solution used for the papaya leaf proteins solubilisation.

Isoelectric focusing (IEF)

The proteins were diluted (2 : 1 v/v) with a rehydration buffer (8 M urea, 2% w/v CHAPS, 0.2% w/v DTT, 0.5% v/v IPG buffer, pH 3–10, 0.002% bromophenol blue) and aliquoted into either 0.2 or 1.6 mg samples. The proteins were subsequently loaded onto an IPG strip holder with 7 or 18 cm, pH 3–10 linear gradient pre-cast IPG strips (GE Healthcare) and rehydrated for 20 h. The IPG strips with 7 or 18 cm used 0.2 mg or 1.6 mg samples, respectively. IEF was carried out on the IPGphor system according to the manufacturer's instructions (2-DE Manual, GE Heathcare).

SDS–PAGE

After IEF, the strips were first equilibrated with an equilibrium solution (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) containing 1% w/v DTT and subsequently with 4% w/v iodoacetamide for 15 min for each equilibration step. The second dimension separation on the Mini-Protean (Bio-Rad) or Ettan DALT-Six (GE Healthcare) systems was realised with lab-cast 1.5 mm SDS polyacrylamide gels according to the manufacturer's instructions (2-DE Manual, GE Heathcare).

Gel staining, imaging and data analysis

Proteins were visualised by colloidal Coomassie brilliant blue (CBB) staining (Neuhoff *et al*., 1988). The gels were fixed for 1 h in a fixing solution (2% *o*-phosphoric acid, 30% ethanol), washed for 30 min in 2% *o*-phosphoric acid solution and stained overnight in a staining solution (0.02% w/v CBB G-250, 2% *o*-phosphoric acid, 18% ethanol, 15% ammonium sulfate). The background staining was removed with distilled water. The gel images were obtained and analysed with Labscan software and ImageMaster 2D Platinum software (GE Healthcare), respectively, following the user manual's instructions.

Protein identification by MALDI-TOF-MS-MS

Protein spots of interest were excised out of the gel. They were washed three times with a wash solution (1 : 1 v/v 25 mM ammonium carbonate–acetonitrile) at 25°C for 2 h for each step. After removal of the wash solution, they were covered with acetronitrile for 20 min at 25°C. The acetonitrile was removed, and the samples were allowed to dry. Then, the dehydrated gel particles were rehydrated for 10 min with 10 μL digest buffer (25 mM ammonium carbonate) containing 20 ng trypsin (modified porcine trypsin sequencing grade, Promega, Madison, WI, USA). Next, 20 μL of digest buffer was added, and the samples were incubated for 20 h at 37°C. The resulting tryptic peptides were extracted with 50 μL of 0.1% trifluoroacetic acid (TFA) in 50% v/v acetonitrile twice with 20 min sonication. The products from the two extractions were combined, vacuum dried and then dissolved in 0.1% TFA in 50% v/v acetonitrile.

Matrix (10 mg/mL CHCA (α-ciano-4-hydroxycinnamic acid) in 0.1% TFA and 50% v/v acetonitrile) and sample (1 : 1 v/v) were spotted and cocrystallised on a target plate. MALDI-TOF-MS/MS peptide sequencing was performed by precursor ion fragmentation, using N_2 gas in the collision cell at 2.8×10^{-6} torr in a 4700 Explorer Proteomics Analyser (Applied Biosystem). Trypsin autolysis peptides masses 842.5 and 2211.1 and calibration mixture 1 or 2 (Sequazyme Peptide Mass Standard kit, PerSeptive Biosystems, Foster City, CA, USA) were used, respectively, as internal and external standards in both MS and MS/MS procedures.

For database searching, ppw files were submitted to the Mascot search engine using Daemon 2.1.0 (Matrix Science; http://www.matrixscience.com) on a mascot server version 2.2.1. The data was searched against the latest version of the public non-redundant protein database of the

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National Center for Biotechnology Information (NCBI) downloaded on august 2008 with a mass accuracy of 15 ppm for the parent ion (MS) and 0.2 Da for the fragment ions (MS/MS). The peptides were constrained to be tryptic with a maximum of one missed cleavage. Carbamidomethylation of cysteine was considered a fixed modification, whereas oxidation of methionine residues was considered as a variable modification. An initial list of proteins was generated and formed the basis on which further analysis was performed. A 'positive list' was generated by considering only proteins containing at least one unique peptide (minimum 10 aa) with a Mascot score above 67 (p-value < 0.05) in the dataset.

Results and Discussion

In this work, four different methods to prepare papaya protein extract samples compatible for 1-DE and 2-DE analysis were evaluated. This is a crucial step prior to any proteomic analysis, as all plant species have specific interfering compounds. Plant tissues often possess salts, polyquinones, carbohydrates and phenols that can bind to proteins, causing charge heterogeneity and gel streaking (Carpentier *et al*., 2005). This problem is especially important to *C. papaya* samples, whose tissues are rich in laticifers. This specialised cell type produces elevated amounts of phenols, terpens and alkaloids (Chow *et al*., 2007). In addition, papaya laticifers are well-known for possessing a complex protease mixture that is activated immediately after tissue damage (Moutim *et al*., 1999) and will change any protein profile if the preparative conditions are not well-established.

Measurement of papaya proteins obtained by different methods

Different sample preparation protocols often result in chemically complex samples (containing Tris, urea, thiurea and others) that overestimate protein concentration through traditional Lowry's protein assay. In this work, we used the method described by Peterson (1983), in which a TCA/DOC precipitation is performed prior to protein quantification by Lowry´s method. The commercially available 2-D Quant Kit (GE Healthcare) was also used as a control (data not shown), but consistent results were obtained using the Peterson method. As listed in Table 1, similar protein yields, 5.6, 4.9 and 4.9 mg/g fresh weight (FW), were obtained from the same sample using the F1-TCA, TCA/F (F1-TCA/F plus F2-TCA/F) and Phe methods, respectively. These data are consistent with previous reports in which no difference was observed using the TCA or Phe method (Carpentier *et al*., 2005). In general, TCA-precipitated proteins are difficult to redissolve (Nandakumar *et al*., 2003). However, this did not appear to be a problem for the papaya proteins, as the TCA method resulted in the highest protein yield.

Inferior protein yield was obtained using the NP method (Table 1). The protein amounts measured for F1-NP (0.5 mg/g FW) and F2-NP (1.2 mg/g FW) are not consistent with the data presented in Fig. 1 (lanes 1 and 2), in which no protein band was observed. It is likely that some interfering compounds remained in those samples, thus leading to incorrect dosage even for the Peterson method. Indeed, the NP method does not efficiently remove carbohydrates, phenols and other interfering compounds (Carpentier *et al*., 2005).

Table 1. Evaluation of protein yield from papaya leaves extracted by different sample preparation methods[a]

Method		Protein yield (μg/g FW)
TCA–acetone precipitation (TCA)	F1-TCA	5666 ± 273.6
	F2-TCA	840 ± 92.0
TCA–acetone precipitation and fractionation (TCA/F)	F1-TCA/F	3432 ± 185.6
	F2-TCA/F	1555 ± 132.6
No precipitation fractionation (NP)	F1-NP	584 ± 85.3
	F2-NP	1213 ± 212.3
Phenol extraction methanol–ammonium acetate precipitation (Phe)	Phe	4995 ± 361.3

[a] Each sample was extracted at least three times. The values are mean ± SD. F1, fraction 1. F2, fraction 2. FW, fresh weight.

Qualitative evaluation of proteins extracted by different methods

Proteins extracted using the NP, TCA, TCA/F and Phe methods were compared in 1-DE (Fig. 2). The NP method was originally proposed for the model plant *A. thaliana* to limit protein loss associated with precipitation and fractionation (Giavalisco *et al*., 2003). However, it did not provide a high yield of protein extraction from papaya tissue (Fig. 2, lanes 1 and 2). Conversely, the use of TCA/F, TCA and Phe methods led to a richer protein profile, as shown in Fig. 2, lanes 3–6. The Phe method resulted in fewer protein bands (Fig. 2, lane 6) compared with TCA/F and TCA (Fig. 2, lanes 3–5). In the Phe extraction, the aqueous phase preferentially dissolves nucleic acids, carbohydrates and cell

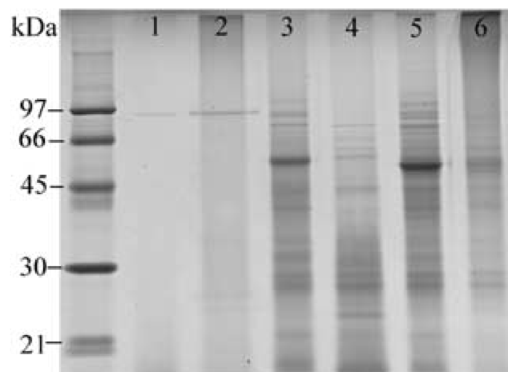

Figure 2. 1-DE profile of proteins extracted from papaya leaves by different methods. Samples F1-NP (1) and F2-NP (2) from the no precipitation fractionation method. Samples F1-TCA/F (3) and F2-TCA/F (4) from the TCA precipitation and fractionation methods (TCA/F). Protein extract (F1-TCA) from the TCA method (5). Protein extract (6) from the phenol extraction methanol–ammonium acetate precipitation method (Phe). Twenty micrograms of protein were loaded per lane. M, molecular weight marker.

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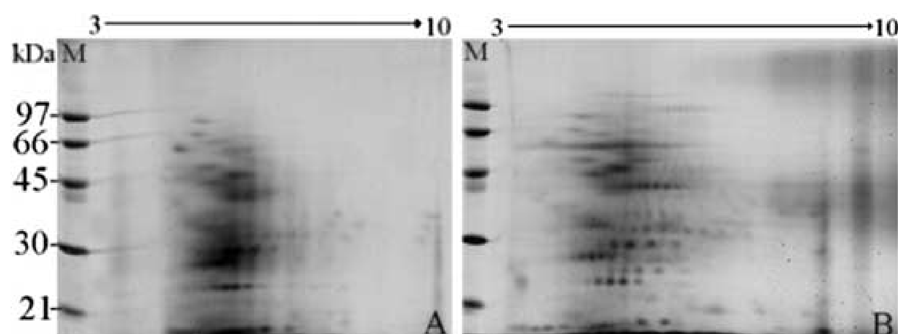

Figure 3. 2-DE gels of proteins from papaya leaves extracted using the TCA/F method. Protein (200 μg) from F1-TCA/F (A) and F2-TCA/F (B) first-dimension separation using 7 cm IPG strips, pH 3–10. The second-dimension run used 12.5% SDS–PAGE. Gels were stained with colloidal CBB G-250. M, molecular weight marker.

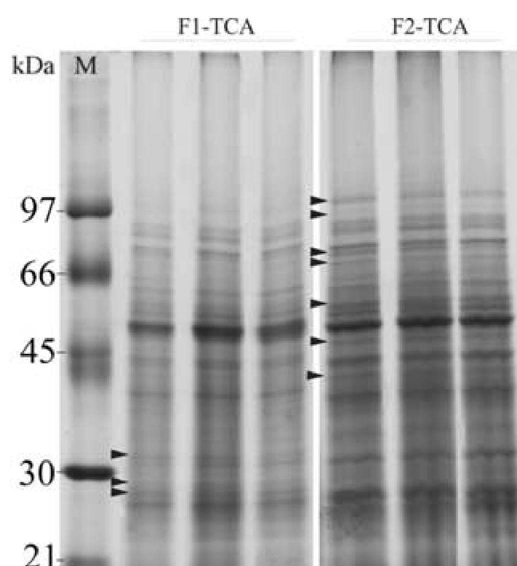

Figure 4. 1-DE profile of protein extracted from papaya leaves by the TCA method. After precipitation of proteins from papaya leaves, the sample was solubilised with resolubilisation buffer (F1-TCA). An additional solubilisation step was included in which the remaining residual pellet was again resuspended using resolubilisation buffer (F2-TCA). Arrows indicate some protein bands that showed differences in intensity or resolution. Twenty micrograms of protein were loaded per lane. M, molecular weight marker.

debris, while the phenolic phase carries membrane and cytosolic proteins and lipids. The method was shown to be useful for removing interfering compounds from banana, another latex-containing plant, and from potato and apple (Carpentier *et al*., 2005). Nevertheless, papaya-interfering compounds did not appear to be completely removed by the Phe method, as a streaking in the high molecular weight region of the gel was observed (Fig. 2, lane 6).

The TCA method is a very effective precipitation procedure that eliminates interfering compounds and limits proteolysis (Damerval *et al*., 1986). Papaya protein extract prepared using the TCA method resulted in numerous protein bands in 1-DE (Fig. 2, lane 5). The TCA/F was established to simplify the protein profile from TCA extraction (Carpentier *et al*., 2005). In the TCA/F method, the first solubilisation is performed using a urea solution (9.5 M), which carries predominantly hydrophilic proteins (F1-TCA/F). Then, the second solubilisation uses a resolubilisation buffer containing thiurea–urea, which makes easier the hydrophobic proteins solubilisation (F2-TCA/F) (Weiss and Görg, 2008). Such a fractionation is confirmed on the TCA/F papaya samples because different protein bands can be observed in the F1- and F2-TCA/F samples (Fig. 2, lanes 3 and 4; Wang *et al*., 2003; 2007). Rubisco large subunit (RLU) is the most abundant plant protein, and it accounts to for up 50% of the soluble proteins in green tissues (Damerval *et al*., 1986; Kim *et al*., 2001; Rossignol *et al*., 2006). In this work, the prominent protein band near 50 kDa region was also observed on 1- and 2-DE gels (Figs. 2–6). The protein spot was further excised from 2-DE gel (Fig. 6, spot 4), analysed by MALDI-TOF-MS/MS and identified as RLU (accession number: gi|167391813). The same prominent protein band was probably observed for those samples whose preparation protocol extracted mainly soluble proteins such as TCA (Fig. 2, lane 5) and Phe (Fig. 2, lane 6) methods. In the Phe method, the proteins are carried in the phenolic phase. For that reason, it was less effective in extracting 50 kDa proteins from papaya leaves compared with the TCA method. The fractionation method could be used when the objective is to analyse hydrophobic or membrane proteins. Such proteins are most present in F2-TCA/F, where 50 kDa appeared as a discrete band (Fig. 2, lane 4). The problems previously reported to be associated with 50 kDa, such as cloud spots and failure to detect other proteins at similar positions (Shaw and Riederer, 2003), are reduced in the F2-TCA/F extraction.

2-DE analysis of papaya proteins obtained using TCA and TCA/F methods

TCA and TCA/F methods were selected for further analysis because they were shown to result in a more complex 1-DE profile than the Phe and NP methods. F1- and F2- TCA/F samples were analysed by 2-DE. Poorly resolved spots were observed for both samples [Fig. 3(A, B)] and were mainly concentrated in the acidic gel region near the pH 4–6 interval. These results might be

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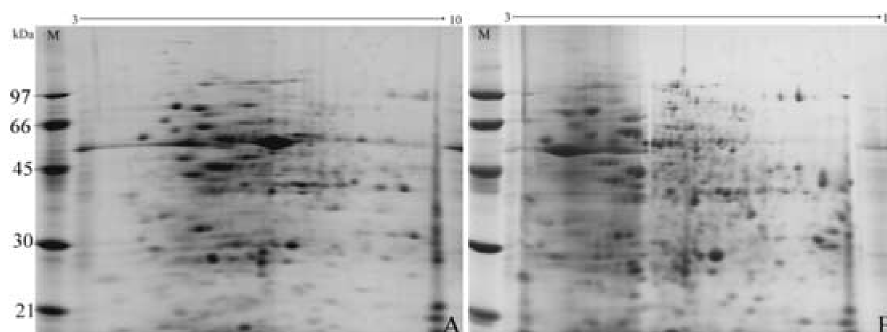

Figure 5. 2-DE gels of proteins from papaya leaves extracted using the TCA method. Protein extract obtained through the original TCA precipitation method was referred to as F1-TCA (A). Protein extract obtained after the additional solubilisation step proposed in this work was referred to as F2-TCA (B). Both extracts (0.2 mg) were submitted to first-dimension separation using 7 cm IPG strips, pH 3–10. The second-dimension run used 12.5% SDS–PAGE. Gels were stained with colloidal CBB G-250. M, molecular weight marker.

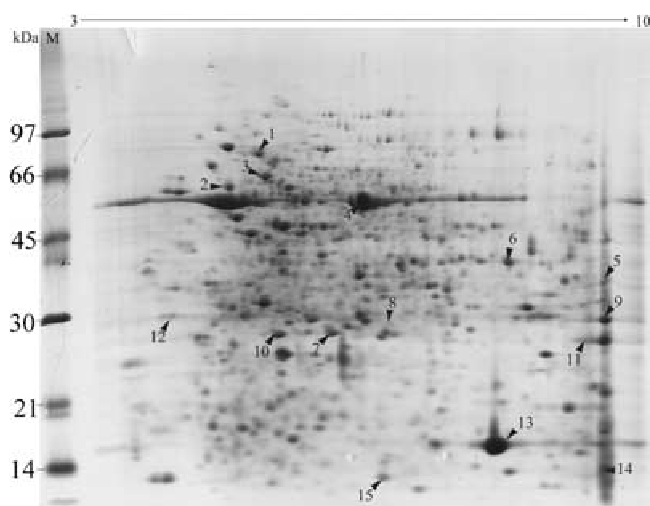

Figure 6. 2-DE gel of proteins extracted using the TCA precipitation method adjusted for papaya leaves. In order to obtain increased protein coverage on 2-DE gel, F1-TCA and F2-TCA protein extracts were combined. The protein mixture (1.6 mg) was submitted to first-dimension separation using 18 cm IPG strips, pH 3–10. The second-dimension run used 12.5% SDS–PAGE. Gels were stained with colloidal CBB G-250. Arrows indicate some protein spots constant in papaya protein extract. M, molecular weight marker.

associated with the IEF step because F1- and F2- TCA/F showed a good molecular weight-based separation (Fig. 2, lanes 3 and 4). Solubilisation using a 'weak solution' (urea) followed by a 'strong solution' (resolubilisation buffer) possibly allowed proteins and interfering compounds to be carried together. Alternatively, the results could be explained by the buffers that were used to produce both fractions. As a result, a typical gel streaking in the acid region was observed [Fig. 3(A, B)], a problem previously reported for some samples prepared by TCA–acetone precipitation (Carpentier *et al*., 2005).

Solubilisation using solubilisation buffer is the last sample preparation step in the TCA method (F1-TCA). Afterwards, residual tissues are normally discarded. Some proteins, however, could still remain in this residue. Therefore, it was further submitted to solubilisation (F2-TCA) using solubilisation buffer. Surprisingly, proteins obtained by F2-TCA resulted in a more complex 1-DE profile compared with proteins obtained for F1-TCA (Fig. 4). They represented a small fraction (0.8 mg/g FW) of the total papaya leaf protein, as the major fraction (5.6 mg/g FW) was extracted in the first solubilisation. The 1-DE gel better resolved low molecular weight proteins (about 30 kDa) from F1-TCA (Fig. 4, arrows). This datum is supported by data presented for soybean seeds (Natarajan *et al*., 2005) and banana (Carpentier *et al*., 2005), for which proteins prepared by the TCA method were resolved preferentially near the 25 kDa region. Intermediate or high molecular weight proteins were more intense or better resolved in the F2-TCA extract (Fig. 4, arrows). Therefore, the additional solubilisation step proposed in this work for papaya samples improved high and middle molecular weight protein extraction compared with the original TCA method. The first extraction of the more abundant and/or more soluble proteins perhaps improves the efficiency of the second extraction. Considering the analysis of other plant species, similar additional solubilisation using a unique buffer type should be tested. This procedure might reduce the protein lost and increase the proteomic array coverage.

As shown in Fig. 5(A, B), 2-DE from F1- and F2-TCA samples generated hundreds of proteins spots with a broad distribution in both dimensions. There were approximately 300 detectable protein spots in the F1-TCA gel [Fig. 5(A)] and 530 in the F2-TCA gel [Fig. 5(B)]. Here, spot detection was performed to easily allow their identification and subsequent manual processing. Spots with insufficient intensity were excluded. Supporting data are presented in Fig. 4; the F2-TCA samples presented major spots around the 2-DE gel middle and high molecular weight region [Fig. 5(B)]. This gel also presented more spots below 30 kDa, although the original TCA method preferentially showed well-resolved low molecular weight proteins in 1-DE [Fig. 4(A)] and 2-DE (Carpentier *et al*., 2005; Natarajan *et al*., 2005). Despite these differences, it is difficult to exclude the use of proteins

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Table 2. Evaluation of 2-DE gel matching for papaya proteins extracted by the TCA precipitation method adjusted for papaya leaves[a]

Gel number[b]	No. of spots	Match number[c]	Match (%)
1	865	—	—
2	818	559	66.4
3	643	495	65.6
4	672	542	70.5
5	832	370	49.2
6	588	465	73.8
mean	736	486	65.1

[a] 2-DE gels obtained as described in Fig. 5. [b] Numbers 1–6 were gel replicates of equivalent papaya leaf samples. [c] Gels 2–6 had their spots matched to gel 1.

obtained in F1-TCA because it also provided many well-resolved spots and, most importantly, less gel streaking in the acidic pH region [Fig. 5(A)]. Therefore, either F1- or F2-TCA protein extracts may be adequate according to the specific objectives of different studies.

2-DE-separated samples require numerous gel replicates to decrease experimental error. A preparation method that results in two final samples will require additional effort for gel preparation and analysis; especially in the beginning of some proteomic studies, a single sample is very attractive. Whole protein extracts obtained after F1- and F2-TCA were mixed and analysed in 2-DE. A mean of 736 protein spots was well-resolved in both dimensions (Fig. 6). Contrary to results shown in Fig. 5(B), gel streaking was almost absent. RLU was the most abundant protein (Fig. 6, spot 4). The shapes of spots around the RLU region, and also at both the cathode and anode points, appeared round or elliptical (Fig. 6), suggesting that focusing was adequate. Six independent gels were matched, presenting 65.1% similarity with 486 spots matched (Table 2). These results suggest that the spots on 2-DE had an acceptable reproducibility. The traditional TCA method with a further solubilisation step described here has the potential to be used in other papaya tissues or even for tissues of other plant species. The method requires a small amount of tissue, is easy and fast.

Protein identification by MALDI-TOF-MS/MS

Using the 2-DE approach on the TCA method prepared sample (F1 plus F2), 15 spots were selected (Fig. 6, arrows) and analysed to check their quality with MALDI-TOF-MS/MS. The proteins were chosen from different gel regions as acidic, basic, high and low molecular weight and also with different intensities. Protein identities are listed in Table 3 and the Supporting Information.

Plant heat shock proteins (HSPs) are present in more than one cellular compartment under both optimal and stress conditions (Wang *et al*., 2008). They function as molecular chaperones in processes such as protein folding, translocation and degradation; they are also involved in membrane stabilisation. Four HSPs (HSP70, spot 1; chaperonin 60 alpha-subunit, spot 2; chaperonin 60 beta-subunit, spot 3; chaperonin 20, spot 10) were identified on papaya 2-DE gels. Similar results were reported from the bryophyta plant (*Physcomitrella patens*) proteome analysis using 2-DE (Wang *et al*., 2008).

NADPH–protochlorophyllide oxidoreductase (spot 5) is a plastid-localised enzyme that catalyses light-dependent chlorophyll biosynthesis (Melkozernov *et al*., 2006). Once synthesised, the chlorophyll molecules are organised in the light-harvesting antennas (LHAs) coupled with chlorophyll a/b binding protein (spot 7) (Melkozernov *et al*., 2006). LHAs drain energy to photosystems (photosystem I subunit VII, spot 15) during the light reactions of photosynthesis, which provide reducing power for the biochemical reactions of photosynthetic CO_2 fixation. In agreement with previous reports (Hancock *et al*., 2005), proteins involved with CO_2 fixation (RLU, spot 4; glyceraldehyde-3-phosphate dehydrogenase, spot 6; triosephosphate isomerase, spot 8; ribulose bisphosphate carboxylase small chain, spot 13) were also identified in this study.

Chitinases (endochitinase, spot 9) are involved with the plant response to pathogenic microorganisms (El Moussaoui *et al*., 2001). Proteins related to RNA translation (40S ribosomal protein, spot 14; component of the nascent polypeptide-associated complex, spot 12) and protein degradation (chymopapain isoform V, spot 11) were also identified. Both chymopapain and chitinase were expected to be present in the papaya sample because they are present in the plant laticifers (El Moussaoui *et al*., 2001). They were identified specifically as *C. papaya* proteins, with high MASCOT scores values (610 for endochitinase; 112 for chymopapain isoform V), suggesting a high-quality identification.

Overall, the presented results indicate the quality of the spots obtained through TCA method adjusted for papaya leaves to be used with MALDI-TOF-MS/MS. The complete analysis of papaya leaves proteome in different stress condition is under progress in our laboratory.

In conclusion, the method reported here allowed papaya proteins to be efficiently extracted and separated through 2-DE. The resulting spots were compatible with MALDI-TOF-MS/MS and suitable for papaya proteomic analysis. The protocols described can be used as a starting point for the optimisation of sample preparation protocols for other plant species. The idea of submitting the plant tissue to additional solubilisation steps using the same solubilisation solution can be helpful not only for papaya leaf tissues, but also for tissues of other plants.

Supporting information

Supporting information can be found in the online version of this article.

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Table 3. Identification of protein spots from papaya leaves using MALDI-TOF-MS/MS[a]

Spot no.	Theoretical p*I*/M_r (kDa)	Experimental p*I*/M_r (kDa)	*M* score	*M* no.	SC (%)	Peptides sequences	Accession no.	Plant species	Protein Identification
1	5.2/71	5.1/79	371	13	29	R.VEIIANDQGNR.T/R.TTPSYVAFTDTER.L/ K.NAVVTVPAYFNDSQR.Q/R.FEELNMDLFR.K/ K.STVHDVVLVGGSTR.I/ K.EQVFSTYSDNQPGVLIQVYEGER.T	6911553	*Cucumis sativus*	Heat shock protein (HSP) 70
2	4.7/60.0	5.2/61	492	11	23	K.EIAFDQSSR.A/K.LADAVGLTLGPR.G/ R.NVVLDEFGSPK.V/R.AIELPDAMENAGAALIR.E/ K.DSTTIIADAASKDELQAR.V	3790441	*Canavalia lineata*	Chaperonin 60 alpha-subunit
3	5.2/64	6.2/64	311	7	16	K.LADLVGVTLGPK.G/K.VVAAGANPVLITR.G/ K.SAENNLYVVEGMQFDR.G/ R.GYISPYFVTDSEK.M/K.AAVEEGIVVGGGCTLLR.L	15222729	*A. thaliana*	Chaperonin 60 beta-subunit
4	8.5/55	6.1/52	290	9	31	K.LTYYTPDYQTK.D/K.DTDILAAFR.V/ K.TFQGPPHGIQVER.D/ R.GGLDFTKDDENVNSQPFMR.W/R.FLFCAEAIYK.A	167391813	*C. papaya*	Rubisco large subunit (RLU)
5	9.5/63	9.0/43	253	6	20	K.ENYTVMHLDLASLDSVR.Q/ R.LIIVGSITGNTNTLAGNVPPK.A/R.EHIPLFR.I/ R.ILFPPFQK.F	10720220	*Cucimis sativus*	NADPH-protochlorophyllide oxireductase
6	8.3/40	8.3/36	404	5	22	K.DAPMFVVGVNEK.E/R.VPTVDVSVVDLTVR.L/ K.GILGYTEDDVVSTDFVGDSR.S	120666	*Antirrhinum majus*	Glyceraldehyde-3-phosphate dehydrogenase
7	6.0/28	8.6/29	167	3	13	R.YAMLGVAGAIAPEILGK.L/K.QYFLGFEK.F	116519121	*Lycoris aurea*	Chlorophyll a/b-binding protein
8	6.8/28	6.6/27	155	6	23	K.FFVGGNWK.C/K.VIACVGETLEQR.E/ K.VATPDQAQEVHDGLR.K	553107	*O. sativa*	Triosephosphate isomerase
9	8.8/32	9.1/27	610	8	44	R.SMFDQMLK.H/K.GFYTYDAFLAAAK.S/ K.SFPSFGTTGSTDVR.K/ R.GPLQLSWNYNYGPCGEALR.V/ R.VNLLGNPDLVATDR.V/R.WQPSAADTAAGR.L/ K.GPNPQVADR.L	146286071	*C. papaya*	Endochitinase
10	5.3/28	8.8/26	182	3	11	K.YTSIKPLGDR.V/K.DLKPLNDR.V/K.GKDGSNYIALR.A	15242045	*A. thaliana*	Chaperonin 20
11	9.5/27	9.1/25	112	4	23	–.YPQSIDWR.A/K.IVTGNLLELSEQELVDCDK.H	4469159	*C. papaya*	Chymopapain isoform V
12	4.0/31	4.3/21	172	3	20	K.LGMKPIPGVSR.V/K.NILFVISKPDVFK.S/ K.IEDLSSQLQTQAAEQFK.A	124484511	*Nicotiana benthamiana*	Nascent polypeptide associated complex
13	9.0/20.4	8.5/17.0	129	5	25	K.EVDYLLR.K /R.SPGYYDGR.Y	132145	*Helianthus annuus*	Ribulose bisphosphate carboxylase small chain
14	9.5/14	10.7/14	92	3	27	R.VNQAIYLLTTGAR.E	108864390	*Oryza sativa*	40S ribosomal protein
15	5.6/9.3	6.6/10.0	112	2	27	K.IYDTCIGCTQCVR.A/R.VYLGPETTR.S	108796706	*Zygnema circumcarinatum*	Photosystem I subunit VII

[a] Spot no., protein spot numbers as shown in Fig. 6. *M* score, MASCOT search score. *M* no., matched peptides numbers. SC, sequence coverage. Accession no., NCBInr accession number.

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