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Full Length Research Paper

Critical evaluation of proteomic protocols for passion fruit (*Passiflora edulis* Sims) leaves, a crop with juice market benefits

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Passion fruit grows practically all over Brazilian territory; its production is largely destined to juice industry and expanding to overseas markets. The suitability of four protein extraction protocols for plant proteome was investigated to determine the best choice for studies concerning passion fruit leaf proteins. Trichloroacetic acid (TCA)/acetone extraction; isoelectric focusing (IEF) buffer extraction; phenol (Phe) extraction and Phe-SDS extraction were tested. The Phe method produced the best results, showing higher reproducibility of resolved protein spots and clearer 2D gel background staining. In comparison, the Phe-SDS method presented fewer spots and lower reproducibility. The TCA/acetone method produced the fewest identifiable spots and the IEF buffer produced the poorest results, displaying fewer reproducibly detected spots, more vertical streaks and darker 2D staining. Selected spots, obtained with Phe method, were identified by spectrometric analysis (MALDI-TOF-TOF) to exemplify the viability to perform more comprehensive proteomic studies with passion fruit leaves and, therefore increase information about stress-related and developmental responses in this fruit crop.

Key words: Passion fruit, proteomic, protein extraction, juice industry.

INTRODUCTION

Currently, proteomics approach is acknowledged as a powerful strategy to analyze protein complexity and therefore, gain a better understanding of physiological responses to developmental and environmental cues in a target living organism. As emphasized by Remmerie et al. (2011) advances in bioinformatics are contributing to

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proteomic studies in non-genomic model species. On the other hand, more information are becoming available about passion fruit for instance, a floral expressed sequence tag (EST) sequence data base was reported (Cutri and Dornelas, 2012) and more recently, efforts were made to have its genome elucidated (Santos et al., 2014). Nevertheless, sample extraction and preparation is of pivotal importance in any proteomic research. However, as "plant scientists" already know, proteomic analysis of vegetal tissues and organs can be very frustrating. The obstacles come mainly from the severe interference of intrinsic molecules such as, pigments, cell wall compounds, carbohydrates, lipids, (poly)phenolic compounds and a myriad of secondary metabolites (Wang et al., 2003; Jamet et al., 2008). Thus, the removal of these contaminants in order to increase the resolution of protein spots and to obtain reproducibility between 2D gels patterns from independent extraction experiments by reducing artifacts and minimizing protein losses remains a challenge.

To this end, a number of protein extraction protocols were developed or modified on the basis of the tissue sample peculiarities (Saravanan and Rose, 2004; Rodrigues et al., 2009; Lee et al., 2010). The Trichloroacetic acid (TCA)/acetone method is largely, if not most, used method in plant proteomic studies and has been reported for several plant species, that is, *Arabidopsis*, barley, Mexican lime, *Withania somnifera*, *Populus cathayana*, common bean and wheat (Guo et al., 2012; Fatehi et al., 2012; Taheri et al., 2011; Dhar et al., 2012; Zhang et al., 2010; Salvati et al., 2012; Xu et al., 2013). A different type of extraction is accomplished by direct solubilization of proteins with IEF buffer (Kang et al., 2007; Afroz et al., 2010), once it contains the detergent CHAPS and chaotropic agents (for example, urea and thiourea). The attractiveness of this method relies on the reduction of protein losses due to the absence of precipitations and washing steps, as well as its simplicity and speed of operation.

Another extraction method originally described by Hurkman and Tanaka (1986), is based on the solubilization of proteins in Phe and subsequently precipitation with methanol and ammonium acetate, followed by resolubilization in IEF buffer. Although being applied to proteomic studies with model plants, as *Arabidopsis*, (Mooney et al., 2006) this technique is frequently the choice for resistant tissues. For instance, seeds (Hajduch et al., 2005; Hajduch et al., 2006; Hajduch et al., 2007; Houston et al., 2009), autumn olive fruit (Wu et al., 2011), *Vitis vinifera* and *Gmelina arborea* Linn. Roxb leaves (Jellouli et al., 2010; Rasinemi et al., 2010) as well as rice seedlings (Chi et al., 2010). The inclusion of SDS in phenol (Phe) based extraction procedure was positively correlated with 2D gel quality (Wang et al., 2003).

Passion fruit is a tropical crop that presents great potential for industrialized juice production, based on its distinctive and exotic aroma, with Brazil being one of the most prominent producers, having plantations spread over practically the entire territory. The pattern of juice production principally for domestic consumption is changing, and expanding into international markets (Bernacci et al., 2008; Oliveira et al., 2012). In spite of its prospect as a tropical fruit crop, there is a lack of information regarding proteomic approaches using passion fruit tissues. Therefore, this study compared the effectiveness of four distinct protein extraction methods for passion fruit leaves suitable for proteomic studies. A methodology was sought that combined reproducibility between several experiments with a high number of well resolved protein spots.

MATERIALS AND METHODS

Plant material

Passion fruits (*Passiflora edulis* Sims) were purchased at the local market of Campos dos Goytacazes, a city located in the northern region of Rio de Janeiro State, Brazil. The seeds were collected, dried at room temperature, and stored at 4ºC in the dark. Plants were grown in vermiculite pots and maintained in environmental chambers for 17 h under 300 mE m^{-2} s^{-1} light at 28ºC and for 7 h in the dark at 18ºC and 62% relative humidity. Four-week-old plants (with 3-4 developed leaves) were used for all experiments. T

hree different plants had their leaves collected and subsequently ground into fine powder in liquid nitrogen using a pre-cooled mortar and pestle. For all samples, protein extraction (with individual buffers) was performed in a cold room at 4ºC to avoid protease degradation by intrinsic protease activity, as described below.

TCA/acetone extraction

This method is based on precipitation of proteins by TCA/acetone according to Hajheidari et al. (2005), and subsequent resolubilization in IEF buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v DTT and 2% v/v ampholytes, pH 4-7, Pharmalyte, Amersham). Leaves were ground to a fine powder in liquid N_2, 10% (w/w) insoluble polyvinylpolypyrolidone (PVPP) was added and proteins extracted in an ice-bath with 5 mL of ice-cold extraction solution (10% (w/v) TCA and 0.07% (v/v) β-mercaptoethanol in acetone) for each g of powdered leaves. After 1 h incubation at -20ºC, samples were centrifuged at 10,000 *g* (4ºC) for 15 min. The pellets were incubated again in 10% w/v TCA/acetone with 0.07% β-mercaptoethanol v/v at -20ºC for 1 h and then centrifuged at 10,000 *g* (4ºC) for 15 min. The washing step was performed twice with ice-cold acetone with 0.07% β-mercapthoetanol. The pellets were air dried and resolubilized in IEF buffer, while insoluble material was removed by centrifugation as describe above. The supernatant was stored at -20ºC until analysis.

IEF buffer extraction

Based on the work of Kang et al. (2007), leaves were ground to a fine powder in liquid N_2, 10% (w/w) insoluble PVPP was added and proteins extracted in an ice-bath with 2 mL of ice-cold IEF buffer for each gram of powdered leaves. After centrifugation at 10,000*g* (4ºC) for 15 min, the resulting supernatant was ready for protein quantification and 2D electrophoresis.

Phe extraction

In this protocol, leaves were ground to a fine powder in liquid N_2, 10% (w/w) PVPP was added and proteins extracted in an ice-bath with 3 mL of ice-cold extraction buffer (0.5 M Tris-HCl pH 7.5, 50 mM EDTA, 2% β-mercapthoetanol and 0.7 M sucrose) for each gram of powdered leaves. After centrifugation at 10,000*g* (4ºC) for 15 min the supernatant was collected and an equal volume of water-saturated phenol was added. After homogenization, samples were centrifuged at 10.000 *g* (4ºC) and the upper phenol layer was collected. Next, the Phe phase was re-extracted twice with extraction buffer as above. The protein precipitation was achieved by adding 5 volumes (v/v) of 0.1 M ammonium acetate in methanol and 16 h incubation at -20ºC. After centrifugation at 10,000 *g* (4ºC) for 5 min, pellets were washed three times with 0.1 M ammonium acetate in methanol and once with acetone 100%, (Schuster and Davies, 1983; Wu et al., 2011). Pellets were air dried and proteins resolubilized in IEF buffer, while insoluble material removed by centrifugation. The supernatant was collected for protein quantification and 2D analysis.

Phe-SDS extraction

This methodology (Wang et al., 2003) is similar to the Phe extraction with the main difference being the inclusion of SDS (2%) in the extraction buffer. Protein purification was carried out as described for Phe procedure.

Protein quantification

Protein concentration in all extracts was determined using 2-D Quant kit (GE Healthcare) following manufacturer's instruction.

Gel electrophoresis analysis, staining and image analysis

To compare the effectiveness of all extraction methods by 2D analysis, equal amount of protein (500 µg) were loaded on the first dimension. IEF (IPG strips, 18 cm length, pH 4-7, Immobiline ™ DryStrip GE Healthcare) was performed using IPGfor (GE Healthcare) as follow: (1) step to 50 V (600 Vh); (2) step to 200 V (200 Vh); (3) 500 V (500 Vh); (4) step to 1000 V (1000 Vh); (5) gradient to 4000 V (5000 Vh); (6) gradient to 8000 V (6000 Vh); (7) Step to 8000 V (54000 Vh); (8) step to 100 V (600 Vh). After focusing, proteins were reduced with 1% w/v DTT for 15 min and alkylated with 2.5% w/v iodoacetamide in 10 mL of equilibration buffer (6 M urea, 30% glycerol, 2% SDS, and 50 mM Tris-HCl, pH 8.8) for 15 min. The strips were transferred to 10% SDS-PAGE gels for second dimension electrophoresis with the Protean II xi Cell (Bio-rad, Hercules, CA, USA), using SDS electrophoresis buffer (250 mM Tris pH 8.4, 1.92 M glycine and 1% SDS) with 25 mA per gel for 6 h. The gels were stained with Colloidal Coomassie Blue (Neuhoff et al., 1985). 2D gels were scanned with ImageScanner (GE Healthcare) and the data were analyzed using the ImageMaster 2D-Platinum, Version 7.0 software (GE Healthcare) to discriminate the protein spots. The mean ± SD of 3 independent extraction experiments for each methodology was used to produce the results. To check for reproducibility of tested protein extraction methodologies, a coefficient of variation (sd/mean)*100 was calculated. For analytical replicates to be reliable, a coefficient of variation below 10% is expected. 1D analysis from proteins extracted by Phe methodology were analyzed by SDS-PAGE (10%) and stained with Coomassie Brilliant Blue R-250.

Protein identification by MALDI-TOF-TOF

The spots were collected and incubated in 25 mM ammonium bicarbonate containing 50% (v/v) acetonitrile for 16 h. After discoloration, the spots were washed with deionized water and covered with acetonitrile 100%; subsequently they were dried in speed-vac for 15 min. Later, the spots were rehydrated in 50 mM ammonium bicarbonate with trypsin 33 ng/µL for 1 h in ice. Excess of protease solution was removed and the samples were incubated at 58ºC for 30 min. The reaction was stopped by addition of 1 µL of formic acid 5%. Afterward, peptides were extracted with 30 µL of formic acid 5%, acetonitrile 50% solution and sonicated for 10 min. This process was repeated twice, and the samples were concentrated in a SpeedVac (Savant Instruments, Inc., Farmingdale,NY) to about 10 µL and desalted using Zip-Tip (C18 resin:P10 Millipore Corporation, Bedford, MA). Peptides were eluted from the column with 60% acetonitrile 0.1% trifluoroacetic acid. The sample solution (0.3 µL) was mixed with an equal volume of a matrix solution (R-cyano- 4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) in 50% acetonitrile 0.1% trifluoroacetic acid) on the target plate and allowed to dry at room temperature. The MS/MS data were acquired with a neodymium-doped yttrium aluminum garnet (Nd:YAG) laser with a 200-Hz repetition rate. Typically, 1600 shots were accumulated for spectra in the S mode, while 2400 shots were accumulated for spectra in the MS/MS mode. Six of the most intense ion signals with a signal-to-noise ratio above 30 were selected as precursors for MS/MS acquisition, with the exclusion of common trypsin autolysis peaks and matrix ion signals. External calibration in MS mode was performed using a mixture of four peptides: des-Arg1-Bradykinin (m/z 904.468); angiotensin I (m/z 1,296.685); Glu1-fibrinopeptide B (m/z 1,570.677); and ACTH (18_39) (m/z 2,465.199). MS/MS spectra were externally calibrated using known fragment ion masses observed in the MS/MS spectrum of angiotensin I. The Mascot MS/MS Ion Search (www.matrixscience.com) was used to blast sequences against the NCBInr databank. Combined MS-MS/MS searches were conducted with parent ion mass tolerance at 50 ppm, MS/MS mass tolerance of 0.2 Da, carbamidomethylation of cysteine (fixed modification) and methionine oxidation (variable modification). According to MASCOT probability analysis, only hits significant at $P<0.05$ were accepted.

RESULTS AND DISCUSSION

Figure 1A shows the 2D protein profile when leaf proteins were obtained by TCA/acetone extraction. Protein yield produced by this method was 21±1.6 mg/g of total fresh leaves. In this case, despite a reasonable stained background only 249 protein spots were resolved, with considerable variation in recognizable proteins between the independent experiments (±44). When leaf proteins were directly extracted with IEF buffer (Figure 1B), the 2D protein profile quality was significantly reduced due to vertical streaks, darker stained background, and above all the highest variation of discerned protein spots between the individual experiments was observed, that is, 478±138, while protein yield for this method was 8.7±2.3 mg/g.

Figure 1C shows the protein profile obtained with Phe extraction method, given a protein yield of 9.7±0.2 mg/g, of which appears to be the best option to study passion fruit leaf proteins. This conclusion was not only due to the number of resolved protein spots (~400) and clearer stained background, but most importantly because of the highest reproducibility of observed spots between independent extractions (393±14). Even though Phe-SDS

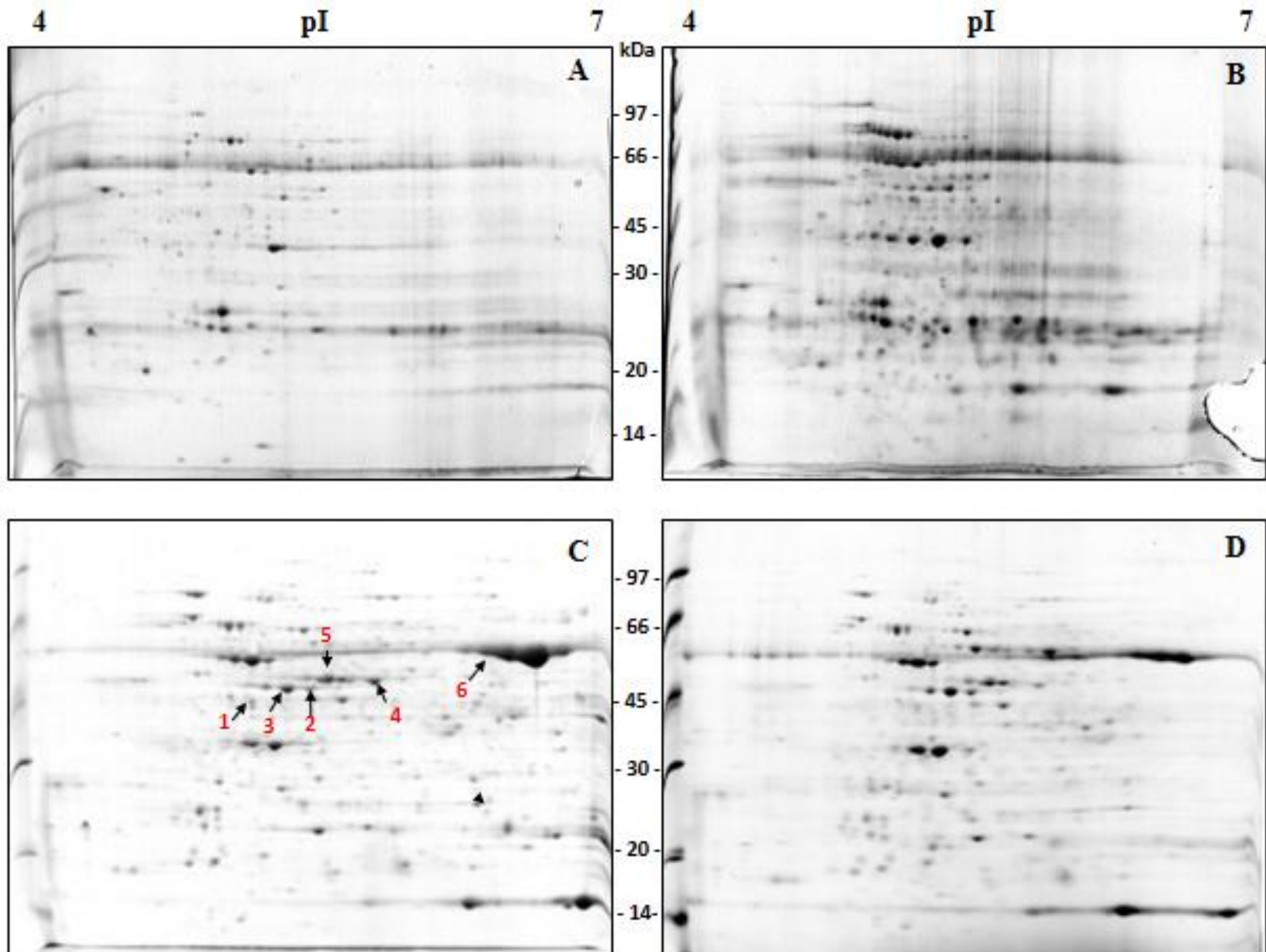

Figure 1. Representative 2D gel analysis of proteins extracted from passion fruit leaves. **A**, TCA/acetone method; **B**, IEF buffer method; **C**, Phe method. **D**, Phe-SDS method. IEF was carried on 18 cm strips pH 4-7 (500 μg of protein/strip). Protein spots were visualized in 2D gels by Colloidal Coomassie Blue and molecular weight markers are in kDa. In all cases, leaves from 3 plants were pooled for each extraction procedure to obtain an average pattern.

methodology (rendering protein yield of 6.6±2.4 mg/g) presented a quite clean staining background, in comparison with the Phe method it produced considerably fewer protein spots and a higher variation between the individual experiments (338±67; Figure 1D).

Extreme care was taken to optimize each extraction procedure and standardize the electrophoretic and staining conditions throughout the entire comparative study. Thus, it was reasoned that differences among the 2D gels patterns (compare Figures 1A-D) might be reflecting artifacts provoked by the interference of substances remaining from each extraction method. With regards to protein yield among different extraction procedures, the TCA/acetone methodology provided higher amounts of extracted proteins while the other 3 tested methods produced quite similar protein yields. Evaluation of sample preparation protocols for plant tissues suitable for 2D gel presented clear variation on protein yielding among tested procedures. For instance, in the work described by Saravanan and Rose (2004), it was the Phe extraction procedure that gave higher protein yield (in a similar range to our data) over methods based on TCA extraction using tomato green fruit and tomato root. While Jellouli et al. (2010) showed much higher variation among tested protocols using roots from grapevine when comparing with what has been obtained with passion fruit leaves.

In this work, it is believed that the improvements in the 2D gel quality offered by the Phe protocol, which allowed more protein spots to be unambiguously noted, compensates for the choice of a laborious extraction method. Moreover, it was the only protocol rendering identified protein spots with a coefficient of variation below 10% (data not shown), reinforcing its suitability for

Table 1. Examples of proteins identified from *Passiflora edulis Sims* leaf bye MALDI-TOF-TOF.

Spot nº	Protein name [species]	Th. Mr/pI	Ex. Mr/pI	Score	PM	AccN
1	Chloroplast sedoheptulose-1,7-bisphosphatase [Solanum lycopersicum]	43,017/6.07	42,000/5.14	89	1	gi|350538149
2	Rubisco activase precursor [Spinacia oleracea]	51,737/6.28	44,666/5.44	272	2	gi|170129
3	Rubisco activase precursor [Spinacia oleracea]	51,737/6.28	44,666/5.32	303	2	gi|170129
4	Hypothetical protein OsI_20474 [Oryza sativa Indica Group]	30,521/6.86	45,666/5.77	391	3	gi|125552851
5	Hypothetical protein VITISV_014296 [Vitis vinifera]	49,185/6.54	46,666/5.48	59	1	gi|147784261
6	Ribulose 1,5-bisphosphate carboxylase [Canarium ovatum]	52,695/5.86	53,000/6.33	429	4	gi|7259805

Th. Mr/pI, theoretical; Ex. Mr/pI, experimental; Score, more than 50; PM , the number of unique peptides matched; AccN, accession number. The assigned protein that best matched has been given with the species in which it has been identified and its accession number.

2D gel analysis. Table 1 shows the identification 6 protein spots selected from leaf proteins extracted with Phe procedure (Figure 1C) by MALDI-TOF-TOF analysis. Such results illustrate the appropriateness for further research, such as studies of stress-related responses (or even developmental) in passion fruit by combining 2D analysis and mass spectrometry techniques to identify differentially regulated/expressed proteins. Moreover, as leaf proteins extracted by Phe procedure also rendered good quality 1D gels, that is, SDS-PAGE analysis (Figure 2), additional proteomic strategy, namely shotgun (Mirzaei et al., 2012; Monavarfeshani et al., 2013) is potentially promising. The results described here can certainly facilitate and stimulate proteomic studies with passion fruit in Brazil and internationally, especially with advances of ESTs, genomic and suppression subtractive hybridization strategies (Cutri and Dornelas, 2012; Santos et al., 2014; Munhoz et al., 2015).

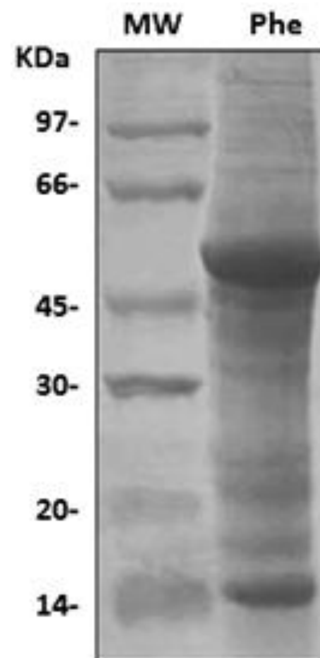

Figure 2. Electrophoretic analysis (10%) SDS–PAGE. MW: Molecular weight markers; Phe, leaf proteins extracted by Phe procedure (60 µg). The gel was stained with Coomassie Brilliant Blue R-250.

Conclusions

The Phe extraction procedure provided the best results to analyze passion fruit leaf proteins via 2D gels for proteomic analyzes. Therefore, our results provide framework for more comprehensive studies on this model plant addressing responses to different stimulus, in order to better understand the physiology of a tropical crop having strong possibility in the fruit juice industry, not only in Brazil but also abroad. Additionally, with the wider recognition of its potential for processed juice market, this crop can become an attractive option for even small farmers.

Conflict of interests

The authors did not declare any conflict of interest.

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