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Short communication

Extraction methods for analysis of *Citrus* leaf proteins by two-dimensional gel electrophoresis

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Abstract

General procedures for the extraction of *Citrus* leaf proteins for analysis by two-dimensional electrophoresis (2-DE) were developed through the evaluation and modification of existing methods. Among the methods evaluated, the best results were obtained when Tris–HCl, KCl, and phenol extractions were followed by precipitation with organic solvents to purify and concentrate the samples. The utility of the Tris–HCl extraction method was demonstrated on the leaves of six genetically different *Citrus* varieties. The methods described are versatile and result in high resolution 2-DE gels of *Citrus* leaf proteins.
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Keywords: Two-dimensional electrophoresis; Leaf tissue; *Citrus*; Protein extraction

1. Introduction

Citrus is grown as a combination tree composed of the fruit-producing scion variety that has been bud-grafted onto a rootstock variety adapted to the soil and environment of the local production area. The large field acreage and years required to adequately evaluate field performance makes rootstock and scion variety development inherently costly and a conservative estimate of 30 years to bring a new selection to commercial cultivation. Human encroachment on land traditionally used for citrus cultivation, environmental changes, emerging diseases and pests, in addition to changing consumer preferences have driven both researchers and commercial producers of citrus to search for methodologies to reduce the time and financial costs in generating new varieties. A proteomics approach using two-dimensional electrophoresis analysis in combination with mass spectrometry has the potential to be a powerful tool in the selection and evaluation of new varieties. This approach permits simultaneous separation and identification of hundreds of proteins. Because proteins can be quantified, this approach also can be used for analyzing changes in the proteome, such as responses to biotic and abiotic stresses at various pathological states of an organism. However, realization of the full potential of two-dimensional electrophoresis (2-DE) is dependent on good sample preparation [1].

Protocols for the extraction of *Citrus* proteins from leaves for polyacrylamide gel electrophoresis (PAGE) [2] and from the albedo, the white internal part of the peel, for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [3] have been reported. Because the leaf method was optimized for enzyme activity analysis and the albedo method for Western blot analysis, they are of limited applicability to 2-DE analysis. The extraction and 2-DE analysis of lemon fruit tissue proteins [4] was reported but lacked specific experimental details or evidence that supported the efficacy of the method. With the exception of *Arabidopsis*, the majority of sample preparation protocols described in the literature have focused on the extraction of proteins from protein rich sources (bacteria, soft tissue, plant seeds, etc.). Leaf tissue is difficult to work with because protein content is low and high levels of non-protein components (e.g. lipids, organic acids, phenols, etc.) [2,5] can interfere with 2-DE separations

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requiring development of uniquely optimized experimental strategies for each plant [1]. In the course of this study, single step extractions (Tris–HCl, KCl, Phenol) [5–8], sequential extractions [9,10], and direct extraction into a lysis buffer [11] were evaluated.

2. Experimental

2.1. Chemicals

Iso-Dalt Servalyt 40% was obtained from Crescent Chemical Co. (Islandia, NY), 10% NP-40 from Roche Diagnostics Co. (Indianapolis, IN), dithiothreitol (DTT) from Boehringer Mannheim Co. (Indianapolis, IN), trichloroacetic acid (TCA) and glycerol from Sigma (St. Louis, MO). Electrophoresis grade urea, Tris base, and Tris–HCl were purchased from Fisher Biotech (Fair Lawn, NJ), sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and glycine from Bio-Rad Laboratories (Hercules, CA).

2.2. Plant material

Plant leaves [*Citrus aurantium*, cv. Bouquet des fleurs (sour orange); *Citrus tangerina*, cv. Dancy (mandarin); *Citrus medica*, cv. Diamante (citron); *Citrus limon*, cv. Frost eureka (lemon); *Citrus sinensis*, cv. Powell (navel orange); *Citrus paradisi*, Redblush (grapefruit) were obtained from the USDA-ARS National Germplasm Repository for Citrus and Dates (Riverside, CA). *Citrus grandis*, cv. Chandler (pummelo) seedlings were grown under greenhouse conditions and leaves were harvested as needed. The age of seedlings ranged from 1 to 2 years. Leaves (50 g) were ground in a cold mortar with liquid nitrogen until a fine powder was obtained. Samples were stored at −20 °C.

2.3. Tris–HCl extraction

Ground leaves (0.5 g) were suspended in a Tris–HCl buffer (2.5 ml, 50 mM, pH 8.8) and homogenized (25 K rpm, 1 min, Polytron PT 3100, Brinkmann Instruments, Inc., Westbury, NY) on ice. The homogenate was centrifuged (26 000 × *g*, 10 min, +4 °C) and the pellet discarded. Three volumes of cold TCA (10%, w/v, −20 °C) in acetone were added to the supernatant, and after overnight incubation (−20 °C), the precipitate was collected by centrifugation (16 000 × *g*, 10 min, room temperature). The pellet was resuspended in cold 10% TCA in acetone (1 ml) and sonicated (1 min, output control 2, constant duty cycle, Branson Sonifier 450, Branson Ultrasonic Corp., Danbury, CT) on ice. The resuspension was centrifuged (16 000 × *g*, 10 min, room temperature) and the supernatant discarded. After two more rinses with cold 10% TCA in acetone (1 ml) and one with acetone (1 ml) the resulting pellet was air-dried at room temperature. The protein pellet was resuspended in a sample buffer (0.4 ml, 9.5 M urea, 2% (v/v) NP-40, 1% (w/v) DTT, and 2% (v/v) 3–10 Iso-Dalt Servalyt) using a micro-pestle and centrifuged (16 000 × *g*, 10 min, room temperature) to remove insoluble material. The supernatant was collected and stored at −20 °C.

2.4. KCl extraction

Ground pummelo leaves (0.5 g) were suspended in a KCl solution (2.5 ml, 3%, w/v) and homogenized (25 K rpm, 1 min, Polytron PT 3100, Brinkmann Instruments, Inc., Westbury, NY) on ice. The homogenate was centrifuged (26 000 × *g*, 10 min, +4 °C) and the pellet discarded. Protein precipitation and subsequent steps were conducted as outlined in Section 2.3.

2.5. Phenol extraction

The method of Hurkman et al. [6] was followed with some modification. Ground pummelo leaves (0.5 g) were homogenized on ice (2 min) in an extraction solution (5.0 ml) consisting of equal volumes of PCI (49% phenol, 49% chloroform, 2% isoamyl alcohol) and NTES (10 mM NaCl, 1 mM EDTA, 1% SDS, 10 mM Tris–HCl, pH 8.5). The phenol phase was recovered by centrifugation (16 000 × *g*, 10 min, room temperature) and extracted with an equal volume of NTES. The phenol phase was collected, and diluted with 5 volumes of 0.1 M ammonium acetate in methanol. After overnight incubation (−20 °C), the precipitate was collected by centrifugation (16 000 × *g*, 10 min, room temperature). The resulting pellet was washed by resuspension in 0.1 M ammonium acetate in methanol (1 ml) and sonication (1 min) on ice. Following centrifugation (16 000 × *g*, 10 min, room temperature), the supernatant was discarded. After two more rinses with 0.1 M ammonium acetate in methanol (1 ml) and one with acetone (1 ml), the resulting pellet was air-dried. The protein pellet was resuspended in the sample buffer, centrifuged, and supernatant was collected and stored as above.

2.6. Two-dimensional gel electrophoresis

2-DE technique was adapted from literature [6,12] with modifications. The isoelectric focusing (IEF) gels were cast in glass capillary tubes (75 mm × 1 mm i.d.) and contained 9.2 M urea, 4% acrylamide/bis-acrylamide (28.38% (w/v) acrylamide and 1.62% bis-acrylamide), 2% (v/v) NP-40, 2% (v/v) 3–10 Iso-Dalt Servalyt, 0.015% (w/v) ammonium persulfate and 0.125% (v/v) TEMED. Electrophoresis experiments were conducted utilizing a Bio-Rad Mini-Protean 2-D Cell and Bio-Rad 3000 power supply (Bio-Rad Laboratories, Hercules, CA). For first dimension IEF the cathode buffer was 0.5% (v/v) ethanolamine and anode buffer was 0.2% (v/v) H_2SO_4. The IEF gels were prefocused at 200 V for 10 min, 300 V for 15 min, and 400 V for 15 min. Samples were loaded (5 μl) at the cathode end of the IEF gels and overlaid with 5 M urea (5 μl). Samples were focused at 500 V for 10 min and 750 V for 5 h. Focusing was stopped overnight

for 14 h after which focusing was resumed at 750 V for 2 h. We did not observed any significant differences between the gels that were run continuously for 7 h and the gels that were run for 5 h, stopped for 14 h, and continued for 2 h. Following IEF, gels were extruded into micro-centrifuge tubes, overlaid with 0.5 ml of overlay solution (2.3% (w/v) SDS, 10% (v/v) glycerol, 0.05% (w/v) DTT, 62.5 mM Tris–HCl pH 6.8) and frozen immediately (dry ice/methanol) without equilibration. Gels were stored at −20 °C.

The IEF gels were thawed, immediately loaded onto the SDS-PAGE gels (50 mm 10% separating gel, 15 mm 4% stacking gel, 1 mm thick), and overlaid with 50 μl overlay solution. The gels were run with an electrophoresis buffer (25 mM Tris base, 192 mM glycine, 3.5 mM SDS). Following electrophoresis (200 V, 40 min), gels were removed from the apparatus, washed briefly with deionized water and fixed for 1 h in 40% (v/v) methanol and 10% (v/v) acetic acid. Gels were silver stained as described in the product's manual (Silver Staining Kit, Protein; Amersham Biosciences, Uppsala, Sweden). The total number of spots visualized was determined manually.

3. Results and discussion

The objective of this study was to generate standard methods for the 2-DE analysis of *Citrus* leaf proteins. A number of extraction and sample processing methods were evaluated, modified, and adapted for their application to the 2-DE analysis of *Citrus* leaf proteins. Because neither the direct extraction with lysis buffer [11] method nor sequential extraction [9,10] methods yielded satisfactory results in our laboratory they were abandoned. The direct extraction method with a lysis buffer gave 2-DE gels that had very poor resolution, high background, and small number of spots (data not shown). In the case of the sequential extractions (data not shown), we found that 2-DE analysis of the initial aqueous extractions (Tris–HCl or KCl) gave low intensity gels. Subsequent extractions with organic solvents following aqueous extraction did not produce any protein bands on silver stained one-dimensional gels.

Prior to IEF, protein extracts are usually treated to remove non-protein components and concentrated. Sample clean-up and concentration are necessary to obtain well resolved, high intensity 2-DE gels. Common methods for removing non-protein components before concentration include dialysis, gel filtration, or the addition of polyvinyl polypyrrolidone [5,13–15]. Concentration may be accomplished through precipitation or lyophilization. In the case of Tris–HCl and KCl extractions of *Citrus* leaf proteins, precipitation with 10% TCA in acetone yielded a white precipitate while the acetone and ammonium sulfate precipitations yielded discolored precipitates. The resulting 2-DE gels from the discolored precipitates were plagued with streaking. The discoloration and streaking were likely due to residual phenol oxidation products [5]. No significant increase or decrease in spot number or quality was obtained from the gel filtration, dialysis, or addition of polyvinyl polypyrrolidone to extracts prior to precipitation (data not shown) and we therefore did not include any of these as components of the extraction. The 10% TCA precipitation and washing steps described in Section 2.3 were deemed sufficient to both purify and concentrate the extracted proteins.

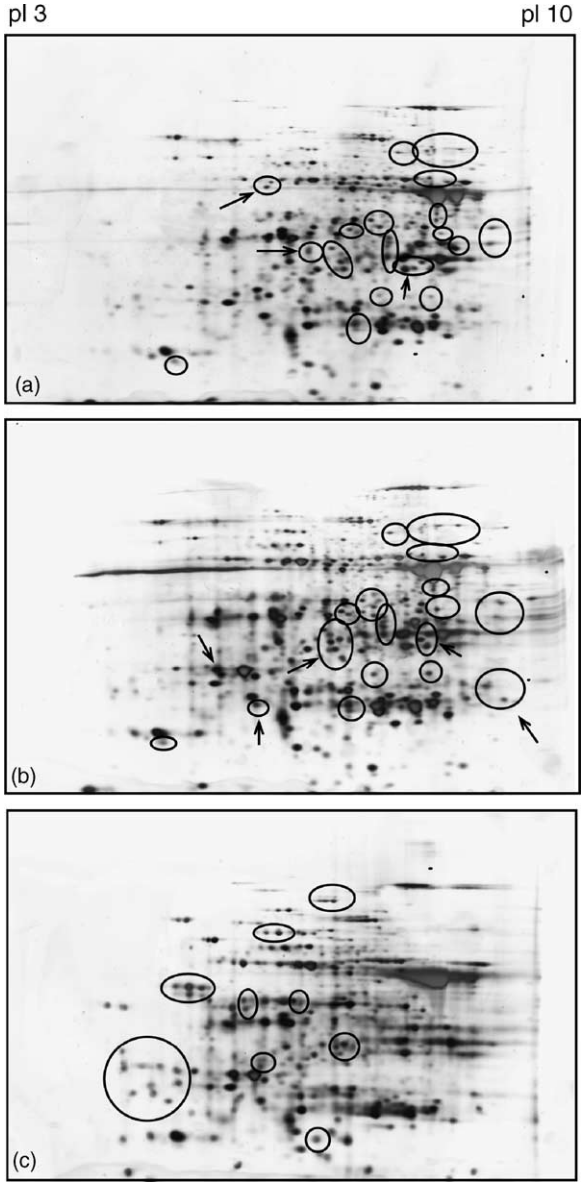

Fig. 1. 2-DE patterns of pummelo leaf proteins. IEF pH 3–10, 2-D PAGE (10% T) silver stained. Proteins extracted with (a) Tris–HCl buffer, (b) 3% KCl and (c) phenol. Arrows indicate differences between (a) and (b). Circles correspond to differences between phenol (c) and aqueous extractions (a and b).

Fig. 1 shows the silver-stained 2-DE gel images of the optimized extraction protocols (Tris–HCl, KCl, and phenol) obtained with approximately 50 μg of protein precipitate extracted from pummelo leaves. The gels displayed good resolution, low background staining, and very little streaking.

The extractions primarily contained proteins in a pH range of 4–8 and displayed 411, 379 and 310 spots for the Tris–HCl, KCl, and phenol methods, respectively. In the absence of other reports for the 2-DE analysis of citrus leaves or without the knowledge of the total number of proteins present in the leaves it is difficult to assess what percentage of the total proteins these extractions represent. However, the 411 spots visually counted for the Tris–HCl extraction of pummelo leaves is within the range of other reports for the 2-DE analysis of various samples (130–750 spots) [16–21].

In Fig. 1, protein spots present on the gels from the Tris–HCl (a) and KCl (b) extracts that are absent on the phenol extract gel (c) are circled. Circled regions on the phenol gel (Fig. 1c) indicate spots not present in the Tris–HCl (Fig. 1a) and KCl (Fig. 1b) gels. Differences between the Tris–HCl (Fig. 1a) and KCl (Fig. 1b) gels are identified with arrows. Although the overall protein patterns of the gels were similar, the results suggest that the phenol method preferentially extracts proteins with lower p*I* values whereas the aqueous based methods (Tris–HCl and KCl) extract proteins with more basic p*I* values.

Because the Tris–HCl method resulted in the greatest number of spots and had fewer manipulations than the phenol method, it was tested on six genetically different *Citrus* varieties to evaluate the applicability of the method. Fig. 2 shows the silver-stained 2-DE gel images obtained with approximately 50 μg of protein precipitate extracted from the leaves of (a) sour orange, (b) mandarin, (c) citron, (d) lemon, (e) navel orange and (f) grapefruit. As with the pummelo sample (Fig. 1a), the extraction method yielded gels with good resolution, low background staining, and very little streaking.

Recent reports of the 2-DE comparison of species have been made for wheat [16], *Brassicaceae* [17], ginseng [18], puffer fish [19], seafood [20], and for the comparison of ecotypes in *Arabidopsis* [21]. Within each of these reports, for all samples under investigation, a single extraction method was

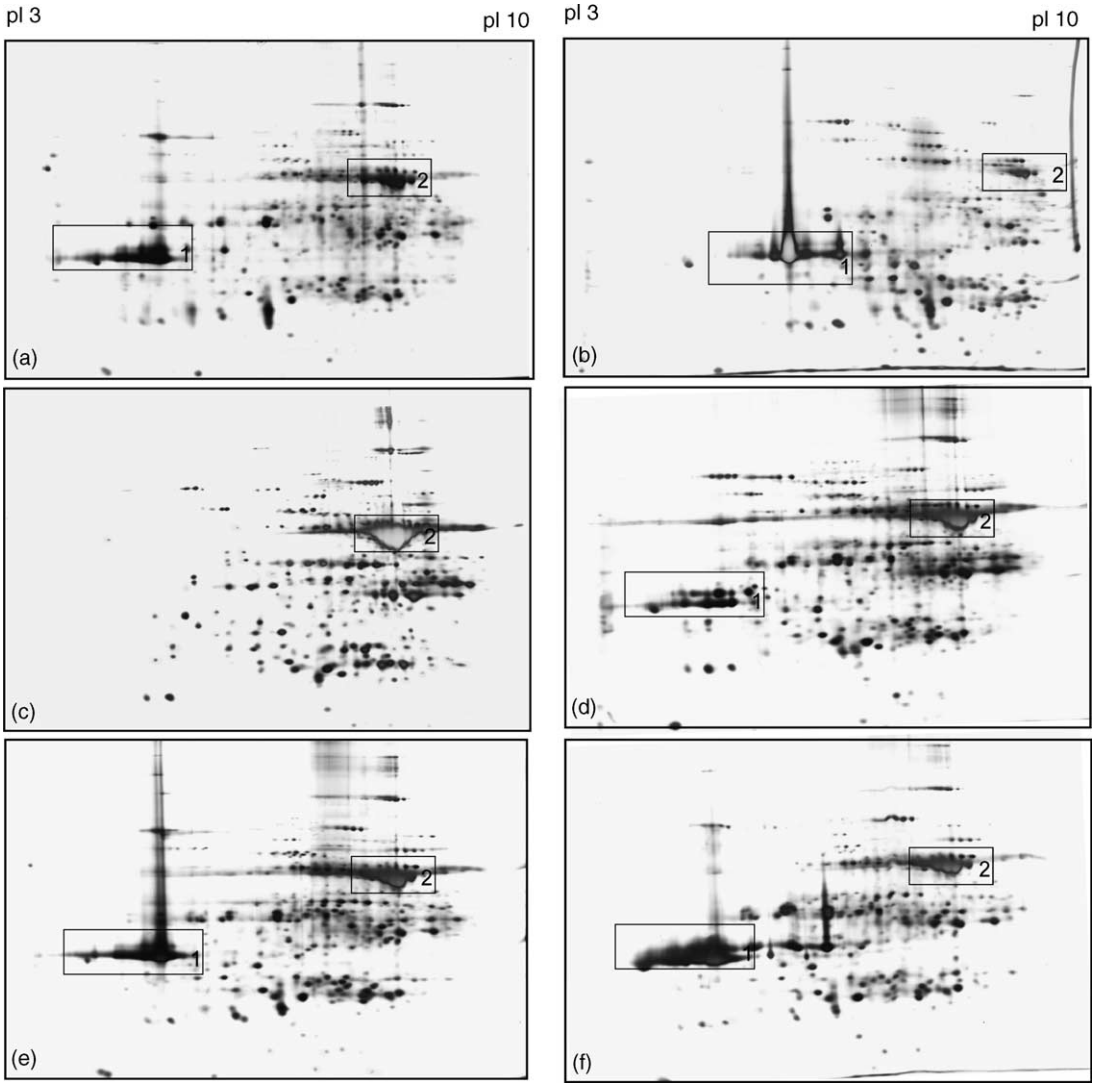

Fig. 2. 2-DE patterns of *Citrus* leaf proteins. IEF pH 3–10, 2-D PAGE (10% T) silver stained. (a) Sour orange, (b) mandarin, (c) citron, (d) lemon, (e) navel orange and (f) grapefruit. Proteins extracted with a Tris–HCl buffer. Potential diagnostic regions 1 and 2 are indicated.

used without modification and without the addition of either internal or external standards. These reports, although not quantitative, have suggested that 2-DE may be used to make gross comparisons between related and unrelated species for the identification of uniquely expressed proteins, the adulteration of materials or to determine the genetic origins of a sample.

In the case of the citrus samples (Fig. 2), we found that the overall spot number and pattern was similar among the samples with the exception of two regions identified as 1 and 2 in Fig. 2. Region 1 found in the acidic p*I* range was visualized on the 2-DE gels of all the samples, except citron and pummelo. Region 2 was located in the basic p*I* range and was visualized in all samples at varying intensity. The majority of citrus varieties are considered to be hybrids derived from crosses between pummelo, citron and mandarin species. Because the citrus hybrid species analyzed (sour orange, naval orange, grapefruit and lemon) all exhibited regions 1 and 2, whereas region 1 was not found in pummelo and citron samples suggests that regions 1 and 2 have the potential to serve as diagnostic markers in establishing the genetic origin of citrus samples. However, this will require further analysis and validation.

4. Conclusion

The need to develop uniquely optimized extraction methods for individual plant and tissue types [1] has been an obstacle to comparing genetically different samples by 2-DE and proteomic methods. In the case of *Citrus* leaf proteins, we found that extraction by Tris–HCl, KCl, or phenol followed by precipitation to purify and concentrate extracted leaf proteins prior to electrophoresis yielded 2-DE gels with the highest number of protein spots and the best resolution. The described methods are relatively easy to perform and will provide a resource for those desiring to study a broad range of *Citrus* leaves by 2-DE and proteomic methods. In addition, the utility of the Tris–HCl extraction as a general method was demonstrated through the extraction and 2-DE analysis of leaf proteins from six genetically different cultivars of *Citrus*. The overall spot number was similar among the samples. However, there were differences among the varieties, and further investigations will be needed to identify these proteins and determine their biological relevance.

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