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ISOLATION AND IDENTIFICATION OF PROTEINS FROM POTATO LEAVES

ABSTRACT

Potato leaves are a very convenient material to compare the differences in potato proteomes. Proteins isolation depends on the type of analysis. The method of isolation of proteins from potato leaves, 2D electrophoresis and liquid chromatography–tandem mass spectrometry (LC-MS/MS) were described in detail.

Key words: leaf proteomes, potato, protein profiles

INTRODUCTION

Proteomics is a branch of science which is focused on identification of proteins, their functions and interactions in organisms. Proteome analysis has usually complimented the cytological and biochemical characterization and confirmed transcriptome analysis observations. The proteomes of potato are compared at two different states of plants, for example in biotic or abiotic stress conditions versus untreated plants (Szajko *et al.*, 2017). Proteins isolation is the first, important step in proteomic research, as it affects the result of the subsequent analyses. These procedures have to be compatible with posterior analyses by two-dimensional electrophoresis (2DE) and/or liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Martínez-Maqueda *et al.*, 2012). In this manual a procedure for isolation of proteins from potato leaves is described. For the measuring of the protein

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content in the sample the Bradford method is applied (Bradford, 1976). The amount of protein in a solution can be quantified by measuring the absorbance value and interpolating the protein concentration from the plot prepared with protein solutions of known concentration (a standard curve).

MATERIALS AND REAGENTS

Plant material

1) Potato leaves

Reagents

1) Liquid nitrogen
2) SafeSeal tube, 1.5 ml (Sarstedt, cat. No 72.706)
3) SafeSeal tube, 2 ml (Sarstedt, cat. No 72.695.500)
4) Eppendorf® micropestle for 1.2 - 2 mL tubes (Eppendorf®, cat. No 0030120.973)
5) Tips DL10 (Gilson, cat. No F161451)
6) Tips DF100ST (Gilson, cat. No F171403)
7) Tips D1000 (Gilson, cat. No F161670)
8) Semi-micro cuvette PMMA (BRAND, cat. No 759115)
9) Glass tube 250 mm, ø12
10) Cellophane Membrane Backing (Bio-Rad, cat. No 1650963)
11) Filter Paper Backing (Bio-Rad, cat. No 1650962)
12) Sequencing Gel Filter Paper (Bio-Rad, cat. No 1650959)
13) DryStrip pH 4-7, 18cm (GE Healthcare, cat. No 17-1233-01)
14) Sucrose (Sigma-Aldrich, cat. No S7903)
15) Trizma (Sigma-Aldrich, cat. No T1504)
16) Hydrochloric acid 30% (HCl(aq)) (POCH, cat. No 575235832)
17) Ethylenediaminetetraacetic acid disodium salt (EDTA) (Sigma-Aldrich, cat. No E5134)
18) Potassium chloride (Sigma-Aldrich, cat. No P9333)
19) Proteinase Inhibitor Coctail (Sigma-Aldrich, cat. No P9599)
20) 1,4-Ditiotreithol (DTT) (A&A Biotechnology, cat. No 2010-25)
21) Roti®-Aqua-Phenol (Carl Roth, cat. No A980.3)
22) Ammonium acetate (Sigma-Aldrich, cat. No O9688)
23) Methanol (POCH, cat. No 621990110-66-1)
24) Acetone (Sigma-Aldrich, cat. No 270725)
25) Urea (Sigma-Aldrich, cat. No U6504)
26) Tiourea (GE Healthcare, cat No RPN6301)
27) 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma-Aldrich, cat. No C3023)
28) Bromophenol blue (Sigma-Aldrich, cat. No B5525)
29) Ammonium bicarbonate (Chempur, cat. No 111403608)

30) Bradford reagent (Sigma-Aldrich, cat. No B6916)
31) Protein Standard 200 mg x ml-1 BSA (Sigma-Aldrich, cat. No P5369)
32) IPG buffer pH 4-7 (GE Healthcare, cat. No 17-6000-86)
33) Mineral oil (Sigma-Aldrich, cat. No M5904)
34) Sodium dodecyl sulfate (Sigma-Aldrich, cat. No L3771)
35) Glicerol (Sigma-Aldrich, cat. No G5516)
36) Iodoacetamide (Sigma-Aldrich, cat. No I6125)
37) Acrylamide:bis-acrylamide 37.1:1(w/w) 40% (Sigma-Aldrich, cat. No O1709)
38) Ammonium persulfate (APS) (Sigma-Aldrich, cat. No A3678)
39) N,N,N',N-Tetramethylethylenediamine (TEMED)(Sigma-Aldrich, cat. No T9281)
40) n-Butanol (Sigma-Aldrich, cat. No B7906)
41) Glycine (Sigma-Aldrich, cat. No G8898)
42) Pink Protein Ladder (NipponGenetics, cat. No MWP02)
43) Glacial acetic acid (Sigma-Aldrich, cat. No A6283)
44) Formaldehyde solution 36.5-38% (Sigma-Aldrich, cat. No F8775)
45) Ethanol (POCH, cat. No 396420113-666-1)
46) Sodium thiosulfate (Sigma-Aldrich, cat. No 217263)
47) Silver nitrate (Sigma-Aldrich, cat. No S6506)
48) Sodium carbonate (Sigma-Aldrich, cat. No S7795)
49) Endoproteinase Lys-C, (Pierce™ Lys-C Protease, cat. No 90307)
50) Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) (Pierce™, cat. No 20490)
51) MS-methyl methanethiosulfonate (MMTS) (Pierce™, cat. No 23011)
52) Sequencing Grade Modified Trypsin, (Promega, cat. No V5117)
53) Trifluoroacetic acid (TFA) (Sigma-Aldrich, cat. No 299537)

EQUIPMENT

1) Pipette P10 0.1 µl – 10µl (Gilson, cat. No F144802)
2) Pipette P100 10 µl – 100 µl (Gilson, cat. No F123615)
3) Pipette P1000 100 µl – 1000 µl (Gilson, cat. No F123602)
4) Vortex (IKA, LabDancer)
5) Centrifuge (Hettich, Micro 200R)
6) IPGBox (GE Healthcare, cat. No 28-9334-92)
7) Ettan IPGphor Manifold, complete (GE Healthcare, cat. No 80-6498-38)
8) Ettan IPGphor 3 (GE Healthcare, cat. No 11-0033-64)
9) Gel cast 1.0 mm Ettan DALT twelve (GE Healthcare, cat. No 80-6466-84)
10) Gel caster Ettan DALT six (GE Healthcare, cat. No 80-6485-46)
11) Ettan DALT six (GE Healthcare, cat. No 80-6485-27)
12) MultiTemp IV (GE Healthcare, cat. No 28-9941-71)
13) PowerPac HV (Bio-Rad, cat. No 1645056)

14) PTFE boxes to silver staining 200×200×50
15) Model 543 Gel Dryer (Bio-Rad, cat. No 1651746)
16) HydroTech Vaccum Pump (Bio-Rad, cat. No 1651782)
17) Epson Expression 11000XL Pro scanner with transparency unit (Seiko Epson Co)
18) Image Master 2D Platinum Software 7.0 (Swiss Institute of Bioinformatics)
19) Thermo EASY-nLC 1000 (Thermo Fisher Scientific, cat. No LC120)
20) Thermo Orbitrap Elite (Thermo Fisher Scientific, cat. No IQLAAEGA-APFADBMAZQ)

PROCEDURE

Protein extraction

1) Homogenize a minimum of three leaf samples (100 mg) collected individually from treated plants in liquid nitrogen,
2) Suspend the powdered tissue in 350 µl of the extraction buffer [0.7 M sucrose, 0.5 M Trizma, 30 mM HCl, 50 mM EDTA, 100 mM KCl, Proteinase Inhibitor Cocktail, 2% 1,4-Dithiothreitol (DTT) (w/v)],
3) Incubate the homogenate at 4°C for 30 min,
4) Add 500 µl of phenol solution (Roti®-Aqua-Phenol) and incubate at room temperature for 10 min,
5) Recover the phenol phase by centrifugation at 5000 × g for 6 min at 4°C,
6) Transfer the phenol phase to new tubes with 400 µl of the extraction buffer,
7) Recover the phenol phase, as in step 5), and transfer to new, empty tubes,
8) Precipitate the proteins from the upper phenol phase by adding four volumes of cold methanol containing 0.1 M ammonium acetate,
9) Incubate the mixture overnight at –20°C,
10) Centrifuge at 9 000 × g for 6 min at 20°C,
11) Wash the resultant pellet once with 100% methanol pre-chilled to -20° C, centrifuge (6 min, 10 000 × g, 4°C) and the last one with 80% acetone, centrifuge (6 min, 11 000 × g, 20°C),
12) Air-dry the final pellet of proteins and dissolve in 200 µl of 7 M urea, 2 M thiourea for the 2D electrophoresis.
13) Air-dry the final pellet of proteins and dissolve in 200 µl of 25 mM NH_4CO_2 for the LC-MS analysis (Szajko *et al.*, 2018),
14) Determine the samples' protein content according to the method described by Bradford (1976), using bovine serum albumin (BSA) as a standard.

Protein assay

1) Prepare BSA standard solutions from primary dilution (2 mg×ml^{-1}) at a concentration of 0; 25; 125; 250; 500; 750; 1,000; 1,500 and 2,000 µg×ml^{-1} (Table 1),
2) Add 20 µl of samples, standards or buffer (blank) to 600 µl of Bradford

reagent, mix and incubate 10 min at room temperature,

3) Measure the absorbance (A) of samples and calibration standards at a wavelenght of 595 nm,
4) Prepare a calibration plot by graphing the A595 values for the standards versus standards concentration. Determine the protein concentration of the samples by interpolation from the plot.

Table 1

The dilution of the reference solution of the protein to determine the calibration curve.

No	The volume of the protein suspension buffer [µl]	Volume of BSA [µl]	The final concentration of BSA [µg × ml^{-1}]
1.	0	300	2000
2.	125	375	1500
3.	325	325	1000
4.	175	175 from 2	750
5.	325	325 from 3	500
6.	325	325 from 5	250
7.	325	325 from 6	125
8.	400	100 from 7	25
9.	400	0	0

Two-dimensional electrophoresis (2-DE)

The protocol is developed basing on the manufacturer manual (GE Healthcare, 2010) for GE Healthcare (Uppsala, Sweden) equipment.

1) Dilute the samples containing 60 µg of the total protein fraction to 340 µl mixture [7 M urea, 2 M thiourea, 4% 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (w/v), 0.5% IPG buffer pH 4-7 (v/v), 40 mM DTT and 0.002% bromophenol blue (w/v)],
2) Carry out the first dimension isoelectric focusing (IEF) on 18 cm immobilized pH gradient (IPG) strips pH 4-7 (GE Healthcare) with 500 V for 1 h, followed by 1 000 V for 1 h and 8 000 V for 3 h, and then focusing on 8 000 V until 21 200 V/h,
3) Reduce the focused proteins on the strip 15 min in 10 ml of equilibration buffer [6 M urea, 75 mM Tris-HCl, 29% glycerol(w/v), 2% sodium dodecylsulfate (SDS) (w/v) and 0.002% bromophenol blue (w/v)] with 100 mg DTT,
4) Subsequently alkylate in 10 ml equilibration buffer with 250 mg iodoacetamide for 15 min,
5) Place the IPG strips on top of vertical slaps of 12.5% polyacrylamide gels and seal by a layer of 0.5% agarose gels in an electrophoresis buffer [25 mM Tris, 192 mM glycine, 0.1% SDS (w/v), 0.002% bro-

mophenol blue (w/v)],
6) Perform the electrophoretic migration along the second dimension using Ettan DALT six (GE Healthcare) under a current of 10 mA for 1 h, 40 mA for 5 h, and 12 mA for 12 h per gel,
7) Perform the silver-staining of the SDS-polyacrylamide gels according to the method described by Blum *et al.* (1987).
8) Incubate each gel in 3% glycerol (w/v), 40% methanol (v/v) and 10% acetic acid (v/v) buffer for 2 h,
9) Dry the gel at 68°C for the next 2 h using Gel Dryer 543 (Bio-Rad, Hercules, CA, USA) (Szajko *et al.*, 2018).

Image Analysis

All dried gels are scanned using the Epson Expression 11,000XL Pro scanner with a transparency unit (Seiko Epson Co, Shinjuku, Tokyo, Japan). The image analysis is conducted using the Image Master 2D Platinum Software 7.0 (Swiss Institute of Bioinformatics, Geneva, Switzerland). Protein spot detection is performed automatically by the software used with the following parameters: smooth, minimum area and saliency set to 2, 15 and 8, respectively, follow by manual spot editing. The gels are matched to the reference gel in automated mode with Image Master 2D Platinum 7.0. The molecular weight (MW) values of the spots are calculated in comparison with the protein standard markers. The isoelectric point (pI) values are determined to using a linear scale over the total dimension of the IPG strip (GE Healthcare Bio-Sciences AB, 2012).

Protein identification by the liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The protein spots are detected in three biological repetitions of a given group are considered for mass spectrometry (MS) analysis. The selected spots are excised manually from the dried SDS-PAGE, rehydrated in water and send to the Mass Spectrometry Laboratory (MSL). The reduction, alkylation and tryptic digestion are performed as described in the MSL manual (www.ibb.waw.pl/en). The peptides are analyzed by the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system using liquid chromatography Thermo EASY-nLC 1,000 (Thermo Fisher Scientific, San Jose, California, USA) coupled with the mass spectrometer Thermo Orbitrap Elite (Thermo Fisher Scientific). Raw data files are pre-processed with Mascot Distiller Software 2.4.2.0 (Matrix Science, London, UK). The obtained peptide masses and fragmentation spectra are matched to the National Center Biotechnology Information (NCBI) non-redundant database with a Viridiplantae filter (4,026,621 sequences) and S. tuberosum filter (35,393 sequences) using the Mascot search engine - Mascot Daemon v. 2.4.0, Mascot Server v. 2.4.1 (Matrix Science, London, UK).

Differential proteomics

Go back to point l.2 of Protein extraction part and measure protein concentrations in each sample:

1) Take 50 µg of proteins from the pellets diluted in 25 mM ammonium bicarbonate and remove to new tubes and fill to 50 µl,
2) Add 20 µl of 100 mM ammonium bicarbonate with 0.1 µg endoproteinase Lys-C, the amount of enzyme adjust to 50 µg of the total protein content in the samples for digestion of the peptide bonds carboxyterminal to lysine and incubate at room temperature for 90 min,
3) For reduction of disulphide bridges: add 1 µl of 0.5 M tris (2-carboxyethyl), phosphine hydrochloride (TCEP) and incubate at 60°C for 20 min,
4) For alkylation to block the re-creation of disulfide bridges in proteins: add 3 µl of 0.2 M S-methyl methanethiosulfonate (MMTS),
5) For tryptic digestion: add 20 µl of trypsin, the amount of enzyme adjust to 50 µg of the total protein content in the samples and incubate the sample overnight at 37°C,
6) For termination of the tryptic digestion: add 2 µl of 1 % Trifluoroacetic acid (TFA) and keep refrigerated before the use of LC-MS/MS,
7) Analyze the peptides on the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system using liquid chromatography Thermo EASY-nLC 1000 (Thermo Fisher Scientific, San Jose, California, USA) coupled with the mass spectrometer Thermo Orbitrap Elite (Thermo Fisher Scientific),
8) Pre-process the raw data files with Mascot Distiller Software 2.4.2.0 (Matrix Science, London, UK). The obtained peptide masses and fragmentation spectra match the National Center Biotechnology Information (NCBI) non-redundant database with a Viridiplantae filter (4 026 621 sequences) and S. tuberosum filter (35 393 sequences) using the Mascot search engine (Mascot Daemon v. 2.4.0, Mascot Server v. 2.4.1 (Matrix Science, London, UK),
9) Perform quantitative statistical analysis by Diffprot software (MSL, Warsaw, Poland) (Malinowska *et al.*, 2012).

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