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ISOLATION OF PROTEINS FROM POTATO TUBERS

ABSTRACT

Here we optimized an efficient and reproducible method for proteins isolation from potato tubers for quantitative proteomic analysis, aimed at detection of differentially expressed proteins upon various experimental conditions.

Key words: bicinchoninic acid assay, mass spectrometry, tuber proteomes

INTRODUCTION

Proteomics aims at identifying and quantifying of proteins, elucidating their functions and interactions in living organisms. Reproducible sample preparation protocols that minimize technical variability are of greatest importance. We present a procedure for reproducible isolation of proteins from potato tuber tissue for proteomic investigation. An integral part of this workflow is measuring the total protein content in the sample using the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985), that allows for protein amount normalization across the samples, thus reducing the technical variability. The protein concentration is estimated twice – after proteins extraction from homogenised tissue, to minimize variations resulting from material isolation, tissue homogenization, etc., and after acetone precipitation. The second quantitative evaluation of proteins with BCA is useful, as the efficiency of protein resolubilization may vary in the samples. BCA has a greater tolerance for detergents used in protein extraction (for example, BCA tolerates 5% sodium dodecyl sulphate, which efficiently

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solubilizes proteins, including membrane proteins, inhibits the enzymatic activity by denaturizing the proteins and reduces protein-protein interactions) and buffers (Smith *et al.*, 1985) as compared for example with the method of assessing the protein concentration according to Lowry (Lowry *et al.*, 1951**;** Brown *et al.,* 1989) and Bradford (Bradford, 1976).

MATERIALS AND REAGENTS

1) Potato tubers
2) Liquid nitrogen
3) Eppendorf® micropestle for 1.2 - 2 mL tubes (Eppendorf®, cat. No 0030120.973)
4) Eppendorf Tubes® 3810X, 1.5 mL (Eppendorf®, cat. No 0030125.215)
5) SafeSeal tube, 2 ml (Sarstedt, cat. No 72.695.500)
6) Microtest Plate 96 Well,F (Sarstedt, cat. No 82.1581)
7) Parafilm
8) epT.I.P.S.® 2 – 200 µL (Eppendorf®, cat. No 0030000.870)
9) epT.I.P.S.® 50 – 1000 µL (Eppendorf®, cat. No 0030000.919)
10) Sodium dodecyl sulfate (Sigma-Aldrich, cat. No L3771)
11) Ammonium bicarbonate (Chempur, cat. No 111403700)
12) Pierce™ BCA Protein Assay Kit (Pierce, cat. No 23225)
13) Bovine Serum Albumin Standard (Pierce, cat. No 23209)
14) Urea (Sigma-Aldrich, cat. No U6504)
15) Endoproteinase Lys-C, (Pierce™ Lys-C Protease, cat. No 90307)
16) Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) (Pierce™, cat. No 20490)
17) MS-methyl methanethiosulfonate (MMTS) (Pierce™, cat. No catalog number: 23011)
18) Sequencing Grade Modified Trypsin, (Promega, cat. No V5117)
19) Trifluoroacetic acid (TFA) (Sigma-Aldrich, cat. No 299537)

EQUIPMENT

1) Thin corkborer ø4
2) ULT cabinet (Vest Frost Solution, cat. No VTS258)
3) Freeze-drier (Labconco, Freezone 18)
4) Eppendorf Research® plus pipette 2 µl – 20 µl (Eppendorf®, cat. No 3120000.038)
5) Eppendorf Research® plus pipette 20 µl – 200 µl (Eppendorf®, cat. No 3120000.054)
6) Eppendorf Research® plus pipette 100 µl – 1000 µl (Eppendorf®, cat. No 3120000.062)
7) Vortex (IKA, LabDancer)
8) Ultrasonic bath Sonic-6 (Polsonic)
9) Centrifuge 5424R (Eppendorf®, cat. No 5404000.014)
10) ELISA plate reader with 560 nm filter (Tecan, Infinite F50)

11) Thermo EASY-nLC 1000 (Thermo Fisher Scientific, cat.No LC120)
12) Thermo Orbitrap Elite (Thermo Fisher Scientific, cat. No IQLAAE-GAAPFADBMAZQ)

PROCEDURE

Preparation of the standard curve according to Pierce™ BCA Protein Assay Kit, 23225

Two standard curves, according to the buffer applied, are prepared to determine the protein concentration in an unknown sample (Fig. 1). There are two buffers: (a) 4% sodium dodecyl sulfate (SDS) with 25 mM ammonium bicarbonate, (b) 8 M urea with 100 mM ammonium bicarbonate.

Prepare BSA standard solutions (Bovine Serum Albumin Standard, Pierce, 23209) at a concentration of 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 $\mu g \times ml^{-1}$ are prepared and measure with the use of a spectrophotometer at 560 nm.

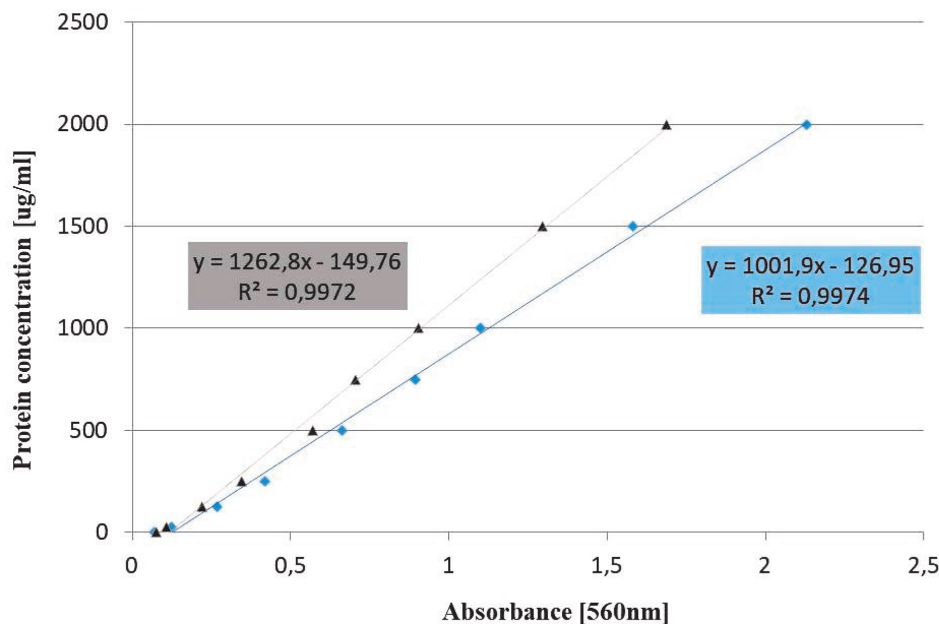

Fig. 1. The exemplary *BCA assay* standard curves for the buffer containing 4% SDS with 25 mM ammonium bicarbonate (the blue line), for the buffer of 8 M urea with 100 mM ammonium bicarbonate buffer (the gray line).

Isolation of proteins from potato tuber tissue

1) Cut out tuber tissue fragment up to 250 mg using thin corkborer. Collect the samples into 2 ml tubes, quickly frost in a liquid nitrogen and then kept in a freezer at -80°C before lyophilisation.
2) Open the tubes and cover with a parafilm. In each make small holes with a toothpick and then freeze-dry for two days.
3) Open the tubes and homogenise using micro pestles.
4) Add 15 µl 4 % SDS and 135 µl 25 mM ammonium bicarbonate.
5) To lead the releasing proteins associated with the cell structures (like cell membranes or chromatin), sonificate the samples for 15 min.
6) Add 100 µl 25 mM ammonium bicarbonate, vortex and centrifuge for 10 min at 12 000 ×g
7) Collect the supernatant into a new tube.
8) Measure protein content:
   a) take 5 µl of the examined supernatant to a new tube,
   b) add 45 µl 25 mM ammonium bicarbonate,
   c) mix reagent A with reagent B (50:1) (BSA standard solutions),
   d) pipette 20 µl of the supernatant with 25 mM ammonium bicarbonate into ELISA plate and add 200 µl of the AB reagent mix,
   e) incubate the plate for 30 min at 37°C,
   f) read the absorbance at 560 nm with the ELISA plate reader.
9) Take an equivalent amount of 200 µg of protein from the samples and fill with 25 mM ammonium bicarbonate to 100 µl.
10) Add 1 ml of cold acetone and incubate for 30 min at 20°C, to precipitate proteins and purify them from SDS, which is incompatible with mass spectrometry by its inhibiting activity of proteolytic enzymes as well as suppressing MS signals.
11) Centrifuge at 12 000 ×g for 10 min.
12) Remove the acetone and rinse the pellet with 20 µl of 8 M urea with 100 mM ammonium, which solubilizes the proteins.
13) Mix and centrifuge at 12 000 ×g for 10 min.
14) Add 60 µl of water.
15) Measure the protein content:
   a) add 5 µl of supernatant to new tubes,
   b) add 45 µl 8 M urea with 100 mM ammonium bicarbonate,
   c) follow as in step 8c, 8d, 8e, 8f.
16) Take an equivalent amount of 50 µg of protein from the samples and fill with 25 mM ammonium bicarbonate to 50 µl.
17) For digestion of peptide bonds carboxyterminal to lysine at high molar urea, adjust the amount of enzyme to 50 µg total protein content in the samples with 20 µl of 100 mM ammonium bicarbonate with 0.1 µg endoproteinase Lys-C, (Pierce™ Lys-C Protease, MS-grade, 90307).
18) Incubate for 90 min at room temperature**.**
19) Add 1 µl 0.5 M Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and incubate for 20 min at 60°C. Note that peptides connected by disulphide bond are not identified by MS and TCEP disulphide bridges in

proteins.

20) Add 3 µl 0.2 MS-methyl methanethiosulfonate (MMTS), which blocks re-creation of disulphide bridges in proteins.
21) To hydrolyze peptide bonds between lysine or arginine on the carbonyl side of basic amino acid, add 20 µl trypsin adjusted to 50 µg total protein content in the samples (Sequencing Grade Modified Trypsin, Promega, V5117), and incubate overnight at 37°C.
22) Add 2 µl 1 % TFA (Trifluoroacetic acid) and keep refrigerated before LC-MS analysis.

Protein differential analysis by mass spectrometry (MS)

The samples are submitted to a mass spectrometry laboratory followed by a bioinformatic analysis. As the result of the analysis, a list of the proteins identified in every sample with the corresponding signal intensities for individual peptides was obtained. Pearson correlation analysis revealed low variability within the experimental groups, proving the reproducibility of the protocol. The correlation of the intensity of signals (Label Free Quantification, LFQ) in 12 samples collected from 3 different tubers of the same potato cultivar (4 samples per each of the three tubers) ranged from 0.95 to 0.96, and from 0.93 to 0.94 among tubers. The average number of proteins identified using this method in 39 different samples of potato tubers was 1225 per sample, and ranged from 871 to 1720. Obtained reproducibility of samples preparation and number of identified proteins indicated that the proposed methodology is appropriate for proteomic analysis.

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