Analytical Biochemistry 509 (2016) 100–103

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Purification of plant complex protein extracts in non-denaturing conditions by in-solution isoelectric focusing

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ARTICLE INFO

Article history:
Received 26 May 2016
Received in revised form
30 June 2016
Accepted 4 July 2016
Available online 7 July 2016

Keywords:
In-solution IEF
Non-denaturing conditions
OFFGEL electrophoresis
pI
Phragmites sp.
Plant protein purification

ABSTRACT

An alternative approach for plant complex protein extracts pre-purification by in-solution isoelectric focusing in non-denaturing conditions is presented. The separation of biologically active proteins, in narrow ranges of isoelectric point (pI) was obtained by a modified OFFGEL electrophoresis. Two different water-soluble protein extracts from *Phragmites* leaves were fractionated into 24 fractions within a 3–10 pI range at 10 °C in the absence of denaturing/reducing agents. One-dimensional electrophoretic analysis revealed different protein distribution patterns and the effective fractionation of both protein extracts. Peroxidase activity of each fraction confirmed that proteins remained active and pre-purification occurred. Biological triplicates assured the needed reproducibility.

Plant protein extracts have a relatively low protein/volume ratio when compared with tissues from bacterial or animal and are often rich in compounds that interfere with protein stability, separation and analysis [1]. Over the years, different plant protein extraction and purification protocols have been reported [2]. OFFGEL electrophoresis (OGE) is the isoelectric point (pI) based fractionation technique, that enables the separation of proteins and peptides by its native pI, firstly reported by Ros et al. [3] and improved and adapted to a multiwell device by Michel et al. [4]. This technique uses a set of wells placed over an immobilized pH gradient (IPG) gel strip. After rehydration of IPG strip wells are filled up with the protein extract diluted in appropriate solution and covered. A variable electrical field is then applied and charged proteins migrate to and through the IPG gel strip, from well to well (pI protein ≠ pH gel) until they reach the well where proteins pI is equal to the pH of the gel. At this point, proteins return into solution, and, therefore, can be easily recovered and used or combined with a broad range of downstream analysis. Length and pH gradient range of the selected IPG strip determines the separation resolution. OGE has been used as a reproducible alternative approach for protein purification and identification in different types of samples [4–8] including proteins extracted from plant tissues in reducing/denaturing conditions [9]. However, if the research objective comprises, e.g., enzymatic activity characterisation, purified proteins need to be in their native conditions and thus, the use of OGE in non-denaturing conditions seemed crucial and a step forward in the fractionation of plant protein extracts.

Phragmites sp. already proved to hardly develop signs of phytotoxicity or abnormal development, indicating that *Phragmites* is able to biochemically self-engineer [10,11]. Albeit, its role in phytoremediation is still in the initial stage of investigation due to the specific challenges that plants protein approaches face. Hence, and aiming the subsequent development of a methodology for the identification and characterization of *Phragmites* sp. enzymes involved in the phytoremediation of xenobiotic molecules, OGE functioning was evaluated in non-denaturing conditions by focusing *Phragmites* leaf water-soluble proteins at low temperature (10 °C) and in the absence of any denaturing/reducing agents. All materials were autoclaved at 121 °C for 15 min prior to use. The chemicals used were of high chemical grade. Solutions and buffers were prepared with ultra-pure water (Milli-Q, Millipore) and all assays were done in triplicate.

Phragmites leaf samples were collected from a pilot constructed wetland being fed with tap water, immediately frozen in liquid nitrogen and ground to powder in a pre-cooled mortar and pestle

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http://dx.doi.org/10.1016/j.ab.2016.07.004
0003-2697/

under liquid nitrogen [12]. Two different protein crude extracts (CE) were prepared: CE_I by a modified plant tissues peroxidases (POD) extraction [13] and CE_{II} following a buffer based procedure. To obtain CE_I, 8 mL of an ice-cold 0.5 mM calcium chloride ($CaCl_2$) solution was added to approximately 1 g of fresh ground tissue. Tissue suspension was mixed with a vortex and centrifuged for 10 min at 4000 rpm and 4 °C. Supernatant was collected into a new test tube and stored on ice. The cell wall pellet remaining in the centrifuge tube was re-suspended with 2.5 mL of $CaCl_2$ solution and centrifuged as before. The supernatant was added to the first collected supernatant and stored on ice for 2 h. The final supernatant was collected and stored at −80 °C in aliquots. CE_{II} was prepared by adding 8 mL of extraction buffer (50 mM phosphate buffer (pH 7.5) containing 10 mM potassium chloride, 1 mM ethylenediamine tetraacetic acid, 5 mM dithiothreitol, 0.5 mM Pefabloc and 25% (w/w) polyvinylpolypyrrolidone) to approximately 1 g of ground tissue. The suspension was centrifuged for 20 min at 4000 rpm and 4 °C. The remaining supernatant was given a second centrifugation at 24,000 rpm and 4 °C, for 90 min. The extract was passed through a 0.2 μm sterile and low protein binding syringe filter (Acrodisc® Syringe Filters with Supor® Membrane, Pall Corporation) and desalted with PD-10 columns (GE Healthcare) using 0.05 M tris-acetate buffer (pH 7.0) as equilibration and elution buffer. The protein extract was stored at −80 °C in aliquots. Total protein concentration was determined using the bicinchoninic acid assay (Pierce) with bovine serum albumin as standard.

CE_I and CE_{II} were fractionated using a 24-well setup and the OFFGEL Kit pH 3–10 (Agilent Technologies) on a 3100 OFFGEL Fractionator. Contrary to manufacturer's instructions, proteins denaturing and reducing agents (thiourea/dithiothreitol) were not added to OGE stock solution. Therefore a non-denaturing stock solution (SS) was used. Fifty mL of SS were prepared by dissolving in water 6 mL of glycerol (50%) plus 600 μL of the ampholytes solution pH 3–10 (Agilent Technologies), aliquoted and stored at −20 °C until use. Fifteen minutes prior to sample loading, 24 cm pH 3–10 IPG strips were rehydrated in the assembled device with 40 μL of SS. Afterwards, each of the 24 wells was loaded with 150 μL of sample (30 μL of CE plus 120 μL of SS) and focused at 10 °C. CE_I samples were focused with typical voltages ranging from 150 to 4500 V for 32 h (total of 40 kVh) and CE_{II} with voltages ranging from 150 to 2750 V for 47 h (total of 50 kVh). In both cases a maximum current of 50 μA per strip and 200 mW of power were

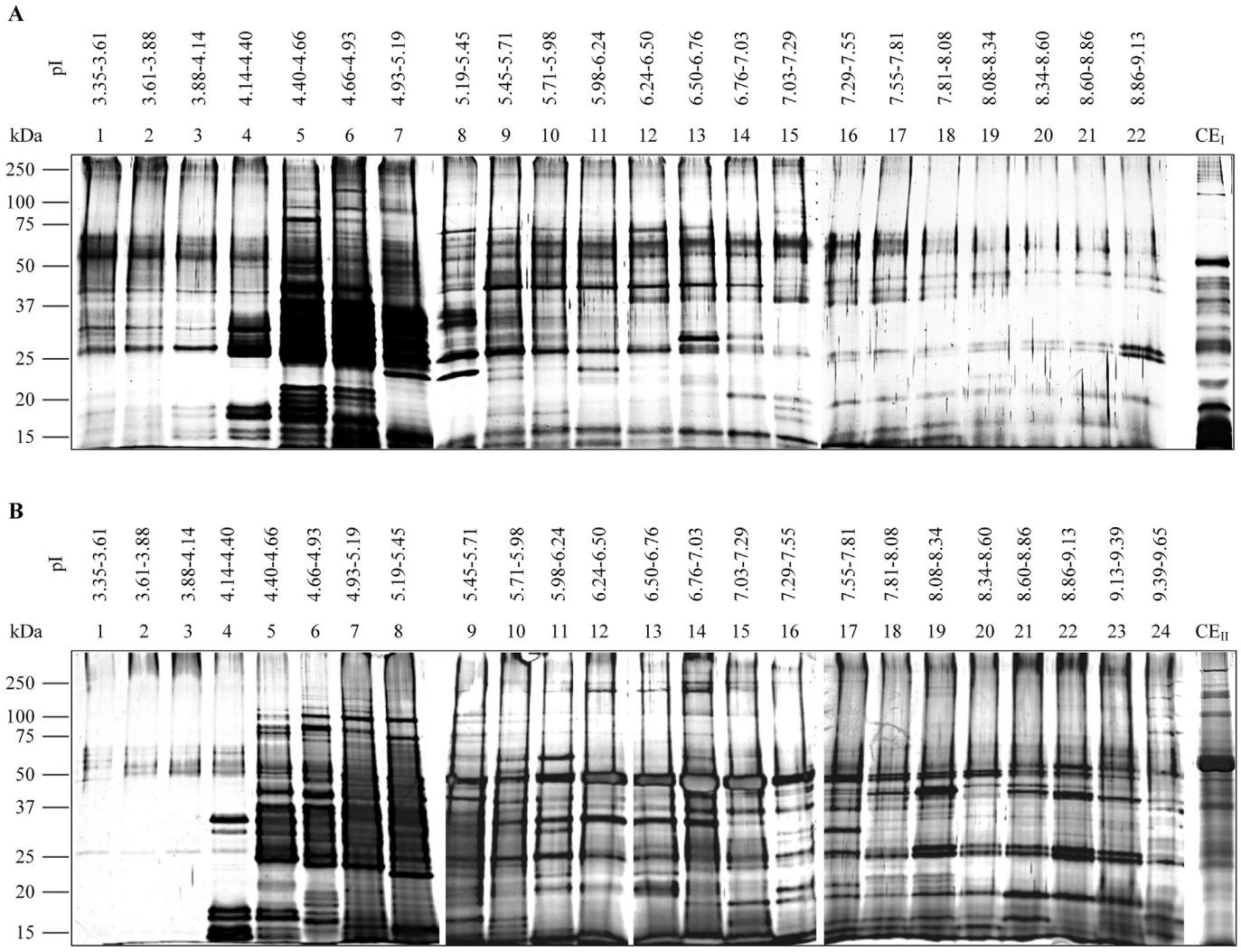

Fig. 1. Representative SDS-PAGE (12%) gels of *Phragmites* leaf soluble proteins crude extracts CE_I (A) and CE_{II} (B) and their 1–24 pI fractions obtained by OGE in non-denaturing conditions using 24 cm IPG strips with a pH range of 3–10. CE_I (A) corresponds to a POD saline extraction and CE_{II} (B) to a phosphate buffer based extraction. pI range of each fraction (as supplied by the manufacture) is represented above the lane fraction number. Each gel lane contains 4 μg of protein sample. Proteins were detected by silver staining and gels were digitalised with a calibrated densitometer (GS-800, Bio-Rad). Precision Plus Protein Dual Color Standard (250–15 kDa; Bio-Rad) was used as molecular weight marker.