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

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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.

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

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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under liquid nitrogen [12]. Two different protein crude extracts (CE) were prepared: CEI by a modified plant tissues peroxidases (POD) extraction [13] and CEII following a buffer based procedure. To obtain CEI, 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. CEII 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 mm 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 bicincho- ninic acid assay (Pierce) with bovine serum albumin as standard.

CEI and CEII 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 ofthe 24 wells was loaded with 150 µL of sample (30 mL of CE plus 120 mL of SS) and focused at 10 °C. CEI samples were focused with typical voltages ranging from 150 to 4500 V for 32 h (total of 40 kVh) and CEII 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

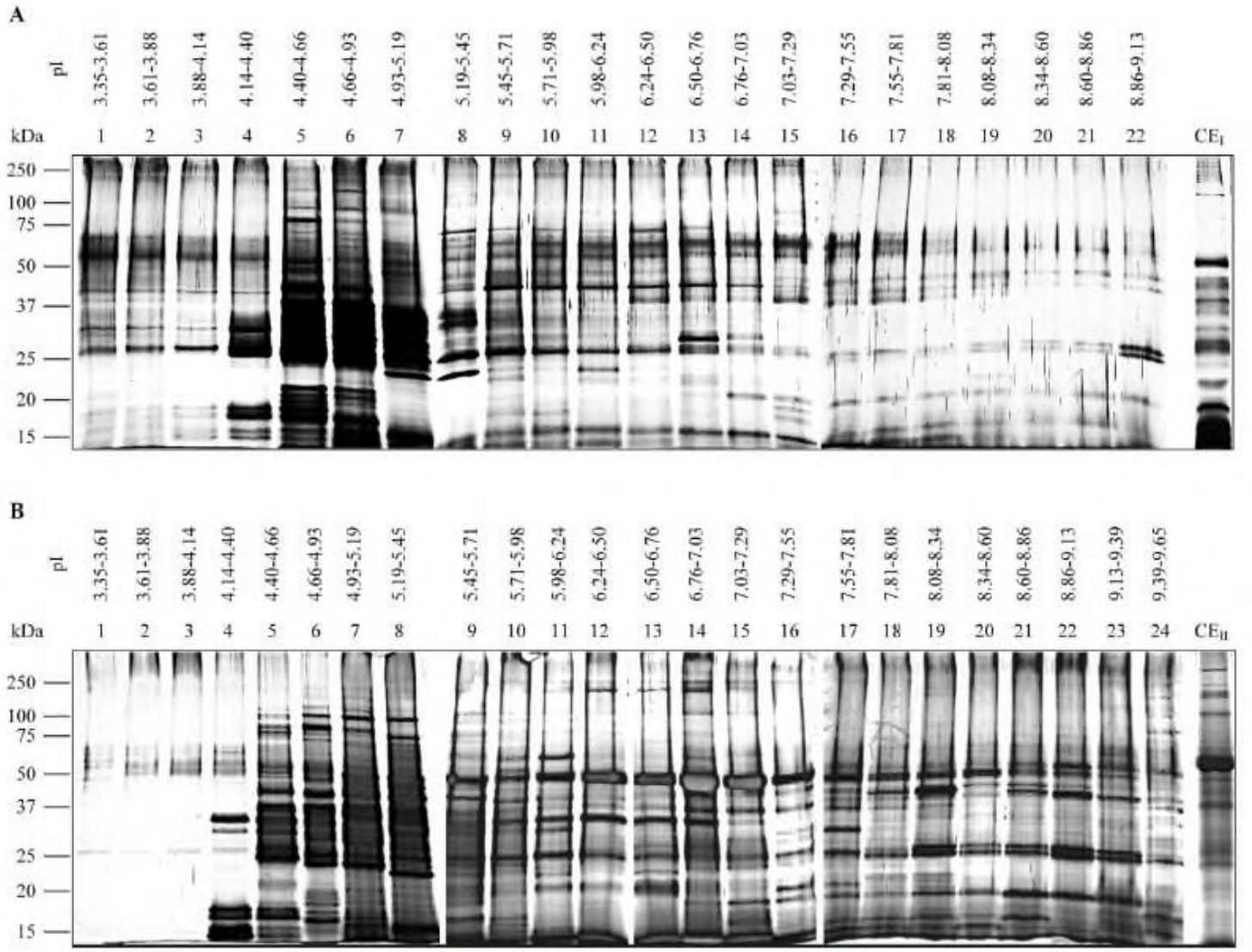

Fig. 1. Representative SDS-PAGE (12%) gels of *Phragmites* leaf soluble proteins crude extracts CE_I (A) and CE_{II} (B) and their 1–24 pI fractions obtained by OGE in non-denaturing conditions using 24 cm IPG strips with a pH range of 3–10. CE_I (A) corresponds to a POD saline extraction and CE_{II} (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.

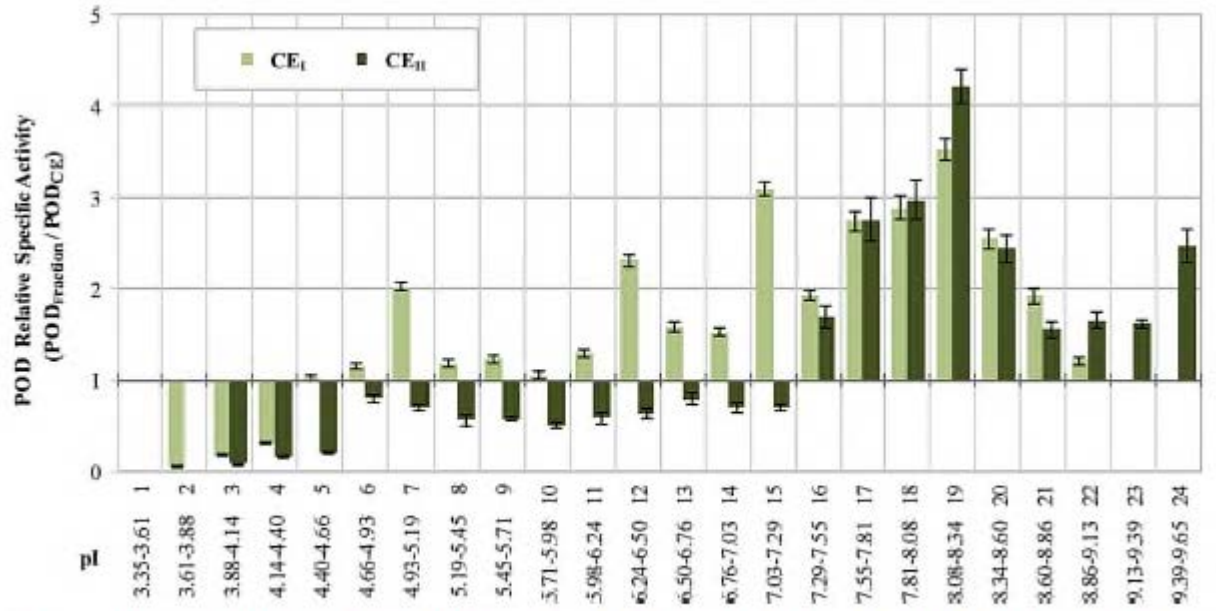

Fig. 2. POD relative specific activity obtained after low temperature (10 °C) and non-denaturing OGE fractionation of two *Phragmites* leaf soluble proteins crude extracts; CE_I and CE_{II}. CE_I was obtained by a POD saline extraction method and CE_{II} by a phosphate buffer based extraction. POD assays were carried out at 25 °C using o-dianisidine (0.17 mM) and H_2O_2 (3 mM) as substrates. 90 mM citrate-phosphate buffer (pH 5.2) ensured the pH stability during POD assays. Error bars represent standard deviations of three independent OGE runs.

used. A minimum of two IPG strips per sample were run in parallel. Each protein CE was fractionated by OGE as per obtained by the extraction procedure. On average a total of 2.3 and 0.55 mg of protein was loaded onto each IPG strip for CEI and CEII, respectively. After each run, the 24 fractions were collected and transferred into individual sterile 1.5 mL conical tubes placed on ice. Volume and total protein concentration of each fraction were determined and their distribution pattern across the pI fractions were similar for each run of the same CE (results not shown).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 12% polyacrylamide gel, as previously described [14] and 4 µg of protein per sample/lane. SDS-PAGE separation was carried out on a Mini-Protean® Tetra Cell (BioRad) at 70 V for the first 30 min and at 90 V until the end of the run (nearby 2 h). Subsequently, proteins were detected by silver staining, as described [15] with modifications. Briefly, gels were fixed (30% v/v ethanol, 10% v/v acetic acid) from to 2 h to overnight and then washed following 10 min cycles (1 x ethanol (30% v/v), 2 x water); sensitized for 1 min (0.02% w/v sodium thiosulfate), washed with water (3 x 30 s) and stained (0.15% w/v silver nitrate) at room temperature for 30 min. Afterwards gels were gently washed with water (30 s) and developed in a freshly prepared solution (3% w/v sodium carbonate, 0.05% v/v formaldehyde) till the protein bands became visible. Acetic acid (5% v/v) was added as stopping solution. In the end, gels were transferred to water (3 x 2 min) and kept in water.

CEI and CEII one-dimensional profiles, before and after OGE, revealed different electrophoretic patterns, as expected, displaying well-resolved bands even within the most complex fractions (Fig. 1). High abundant proteins such as RuBisCO are known to present lower resolving power and therefore, appear within several OGE compartments [9]. Hence the presence of RuBisCO large subunit (~56 KDa) in fractions 12 to 15 of CEII was not surprising and did not compromised OGE pre-purification efficiency. Moreover, proteins with a pI near by the limit of a fraction pI range might actually be split onto two adjacent wells [16] and it should be further checked.

The key point of this study was to assure the preservation of protein samples integrity. This feature was evaluated by quantification of Peroxidase (POD) activity as described by Ref. [17] with slight changes: 10 µL of each CE fraction was added to 300 µL of 90 mM citrate-phosphate buffer (pH 5.2), containing 0.17 mM *o*-dianisidine and 3 mM H_2O_2 as substrates. The absorbance change at 420 nm was measured at 25 °C, for 60 s, using a UVeVis spectrophotometer (Cary 50 Bio, VARIAN) equipped with a microplate reader with temperature controller. One unit of specific activity (U/ mg) was defined as the amount of enzyme that catalysed the oxidation of 1 µmol of *o*-dianisidine per minute per mg of protein ($\varepsilon_{420} = 30\,000\ M^{-1}\ cm^{-1}$). Prior to POD quantification the pH of the reaction mixture was measured and the selected buffer proved to be adequate. POD relative specific activity of each pI fraction, was defined as the ratio between POD specific activity of a fraction and POD specific activity of the respective CE (2.64 ± 0.03 U/mg in CEI and 3.69 ± 0.08 U/mg in CEII). After OGE, significant fold changes in POD specific activity could be noticed, Fig. 2.

The obtained results proved that it is possible to separate/purify plant complex protein extracts in non-denaturing conditions without loss of protein biological activity. Independent runs led to similar results demonstrating good reproducibility. Indeed this insolution IEF procedure does not eliminate proteins-proteins complexes neither multimers but enables a pre-purification based on plant proteins native pI that can head a broad range ofdownstream processes as an alternative/complementary to other processes used for separation/purification and identification of proteins.

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