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Methods Article

An efficient protocol for the extraction of pigment-free active polyphenol oxidase and soluble proteins from plant cells

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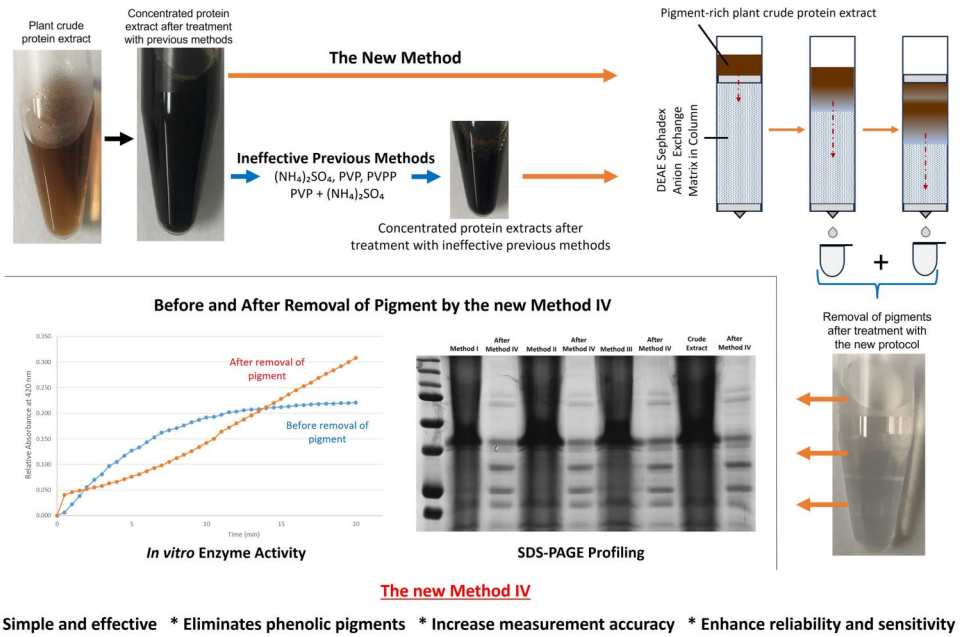
Abstract

The elimination of brownish pigments from plant protein extracts has been a challenge in plant biochemistry studies. Although numerous approaches have been developed to reduce pigments for enzyme assays, none has been able to completely remove pigments from plant protein extracts for biochemical studies. A simple and effective protocol was developed to completely remove pigments from plant protein extracts. Proteins were extracted from red anthocyanin-rich transgenic and greenish wild-type tobacco cells cultured on agar-solidified Murashige and Skoog medium. Protein extracts from these cells were brownish or dark due to the pigments. Four approaches were comparatively tested to show that the diethylaminoethyl (DEAE)-Sephadex anion exchange gel column was effective in completely removing pigments to obtain transparent pigment-free protein extracts. A Millipore Amicon® Ultra 10K cut-off filter unit was used to effectively desalt proteins. Moreover, the removal of pigments significantly improved the measurement accuracy of total soluble proteins. Furthermore, enzymatic assays using catechol as a substrate coupled with high-performance liquid chromatography analysis demonstrated that the pigment-free proteins not only showed polyphenol oxidase (PPO) activity but also enhanced the catalytic activity of PPO. Taken together, this protocol is effective for extracting pigment-free plant proteins for plant biochemistry studies. A simple and effective protocol was successfully developed to not only completely and effectively remove anthocyanin and polyphenolics-derived quinone pigments from plant protein extracts but also to decrease the effects of pigments on the measurement accuracy of total soluble proteins. This robust protocol will enhance plant biochemical studies using pigment-free native proteins, which in turn increase their reliability and sensitivity.

Graphical Abstract



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Keywords: plant protein extraction; anthocyanin; polyphenolics; quinones; pigments; diethylaminoethyl (DEAE)-sephadex anion exchange gel column

Introduction

Plant protein extraction (PPE) is the first essential step in plant biochemistry studies. In the past, a major research effort was focused on the development of extraction methods for high-quality of plant proteins [1–9]. To date, numerous protocols have been reported for protein extraction from different plant species and materials for variable biochemical study purposes, such as enzyme characterization, Krebs cycle and other metabolic pathway analyses, and proteomics [2, 9–19]. Moreover, the past improvements of PPE for native protein research have provided fundamental knowledge to understand biochemical processes from molecular and cellular levels to physiology and ecology [16, 19–23]. In fact, past efforts also indicate that more effective PPE methods will be necessary to refine our current understanding of plant biochemical events that occur in single cells, tissues, development, and different environmental conditions.

One of the main past PPE efforts has been focusing on developing effective techniques to remove plant secondary metabolites from protein extracts [4, 14, 15, 24–26], especially plant polyphenolics. Plant polyphenolics, a large family of plant secondary metabolites, are characterized by diverse structures, such as proanthocyanidins, flavanols, and anthocyanins [27–31]. Plant polyphenolics commonly exist in the plant kingdom. When plant tissues are used to extract proteins, the presence of polyphenolics is a common problem because during enzyme extraction, the easy oxidation of polyphenolics always leads to light to severe browning pigmentation [4, 16, 30, 32–34]. Browning pigmentation can commonly lead to difficulties in enzyme assays because polyphenolics and their derived pigments can bind proteins either reversibly via hydrogen bonding or irreversibly via covalent linkage, thus resulting in extreme difficulty in obtaining pigment-free crude extracts and the inactivation of enzymes [4, 10, 34, 35]. To obtain an appropriate quality of plant enzyme extracts, different chemical reagents and methods have been tested to remove polyphenolics and quinones to overcome this browning pigmentation or other pigmentation problems [10, 34–36]. Common chemical reagent examples tested previously and being used currently include vitamin C, β-mercaptoethanol, dithiothreitol, polyvinylpyrrolidone (PVP), polyvinylpolypyrrolidone (PVPP), and others [10, 15, 37, 38]. Accordingly, numerous promising methods for the reduction of browning pigmentation have been optimized for different purposes. For example, PVPP and PVP are commonly used in protein extractions for enzyme assays, given that both have been reported to remove brownish pigments from protein extracts [4, 18, 32, 34, 35, 37–41]. However, to our knowledge, none of the chemical reagents can completely remove anthocyanins and polyphenolics-derived brownish pigments in plant enzyme extracts.

In this study, we report the development of an effective protocol to obtain pigment-free plant enzyme extracts. We have developed anthocyanin-rich transgenic tobacco calli to characterize metabolism-programmed biochemical processes [42]. Extracts of soluble proteins in these extracts are highly abundant with anthocyanin, oxidized anthocyanin derivatives, and other polyphenolics and quinones, which cause a deeply dark brownish color. To remove all pigments, a quick and effective protocol was developed to obtain high quality pigment-free colorless protein extracts. Further enzyme assays for polyphenol oxidase (PPO) activity demonstrate that this robust protocol extracts active enzymes.

Materials and methods

Reagents and chemicals

Reagents and chemicals used in our experiments are listed in detail. Acetonitrile (LC-MS grade, cat#: 9829-03), glacial acetic acid (HPLC grade, cat#: 9515-03), and methanol (LC-MS grade, cat#: 9830-03) were purchased from Avantor® (Center Valley, PA, USA). Catechol (1, 2-dihydroxybenzene, ≥99% cat#: 135011), polyvinylpolypyrrolidone (PVPP, cross-linked ~110 μm particle size, cat#: 77627), ammonium persulphate (APS, ≥98%, cat#: A3678), and diethylaminoethyl (DEAE)-Sephadex A-25 anion exchange (cat#: A25120) were purchased from Sigma-Aldrich® (St Louis County, MO, USA). Tris powder (≥99.8%, cat#: 17926) and sodium dodecyl sulfate (SDS, cat#: BP166-500) were purchased from Fisher Scientific (Waltham, MA 02451, USA). Acrylamide/bisacrylamide (37.5:1) 30% solvent (cat#: J61505) and ammonium sulphate [$(NH_4)_2SO4$, 99.0%, cat#: A11682] were purchased from Alfa Aesar (Reston, VA, USA). Polyvinylpyrrolidone (PVP, white powder, cat#: 02102787) was purchased from MP Biomedicals (Solon, OH 44139, USA). Ethylenediaminetetraacetic acid (EDTA, cat#: BDH9232) was ordered from VWR Analytical (Radnor, PA, USA). Bovine serum albumin (BSA, 10 mg/mL, cat#: R3961) was ordered from Promega Bio Sciences (San Luis Obispo, CA, USA). Tetramethylethylenediamine (TEMED, cat#:1610801) and R250 Coomassie Brilliant Blue (cat#: 1610400) were purchased from Bio-Rad (Hercules, CA, USA). β-Mercaptoethanol (cat#: 41300000-1) was purchased from bioWORLD (Dublin, OH, USA). Ethyl acetate (cat#: EX0240-3) was ordered from EMD Millipore (Burlington, MA, USA). Bromophenol blue (cat# 151350250) was purchased from Acros Organics (NJ, USA).

Red and green tobacco calli and cells

We have developed different transgenic versus wild-type cell lines to understand anthocyanin biosynthesis in callus and cell cultures [43]. Red cell lines were cloned from *PAP1* (*Production of Anthocyanin Pigment 1* encoding a R2R3-MYB transcription factor) transgenic tobacco (*Nicotiana tabacum* var. Xanthi) plants. One cell line, namely 6R, that overexpresses *PAP1* and yields high production of anthocyanins [43], has been continuously subcultured for different research purposes. This cell line (Fig. 1a) was used for protein extraction in this study. In addition, different cell lines were developed from wild-type tobacco plants as controls [43]. One greenish wild-type cell line, namely P3 (Fig. 1c), was used for protein extraction in this study. Callus subculture and medium components were as described previously [43]. In brief, calli was aseptically inoculated onto phytoagar-solidified 0.8% (w/v) Murashige and Skoog (MS) basal medium (pH 5.7) containing 0.25 mgL^{-1} 2,4-dichlorophenoxyacetic acid, 0.10 mgL^{-1} kinetin, and 30 gL^{-1} sucrose in petri dishes. All petri dishes were placed in a growth chamber maintained at a 22°C temperature and a 16 h/8 h light/dark photoperiod. Calli cultured for 15 days were collected, immediately frozen in liquid nitrogen, ground into a fine powder, and stored at −80°C until use.

Total crude protein extraction

Soluble proteins were extracted from powdered 6R and P3 samples following these steps. The extraction buffer was composed of 0.1 M Tris-Cl (pH 7.0) and 10 mM EDTA. Five grams of fresh powdered sample were suspended in 30-ml of a protein extraction buffer contained in a 50-ml polyethylene centrifuge tube. The tube was vortexed for 1 min thoroughly, sonicated for 15 min in an ultrasonic cleaner (FS60H, Fisher Scientific, Pittsburgh, PA, USA), and then shaken at 100 rpm for 15 min at 4°C. The tube was centrifuged for 15 min at 6500 rpm (5820 rcf) in a high-speed centrifuge with JA-17 rotor (Avanti® JXN-26, Beckman Coulter, Brea, CA, USA). The resulting supernatant with total soluble proteins was transferred to a new tube (Fig. 1b and d). All protein extracts from P3 cells were brownish due to polyphenol oxidation. All protein extracts from 6R cells were deeply dark brownish due to high production of anthocyanins and oxidation of other polyphenolics. One ml of each protein extract was immediately used for PPO assay described below. The remaining 29-ml of each extract was stored at 4°C for removal of pigments and other experiments described below.

Method I: removal of pigment with polyvinylpyrrolidone

Polyvinylpyrrolidone (PVP) is commonly used to reduce pigments in protein extracts [10, 34, 41]. In this experiment, PVP was used to remove pigments from crude protein extracts (Fig. 2a). Three steps, Method I (MI)-1, 2, and 3, were carried out in this method. The MI-1 step was the treatment of protein extracts with PVP. PVP (60, 120, and 180 mg) was added into 9-ml of crude protein extract contained in a 15-ml tube. The tubes were shaken for 15 min at 100 rpm at room temperature and then placed on ice for an additional 30 min. The MI-2 step was the centrifugation of tubes. All tubes were centrifuged at 15 000 rpm (31 000 rcf) for 10 min at 4°C in a high-speed benchtop centrifuge (5810R, 15-amp version, Eppendorf, Hamburg, Germany). The resulting supernatant in each tube was transferred to a new one. The MI-3 step was the reduction of protein extract volume and further removal of pigments using a 15-ml Millipore Amicon® Ultra 10K cut-off filter unit. An approximately 9 ml protein extract was pipetted to a cut-off filter unit. The filter unit was centrifuged at 4000 rpm (3220 rcf) for 30 min at 4°C. Salt and certain pigments were filtered to the bottom of the filter unit and then removed to a waste container. An approximately 1-ml protein extract remained in the upper section of the filter unit. This procedure removed lower molecular weight metabolites including certain pigments and was repeated four to five times by adding fresh buffer until no pigments were filtered to the bottom. The protein extract volume was maintained to 1-ml, transferred to a 1.5-ml Eppendorf tube, and then stored at 4°C until use.

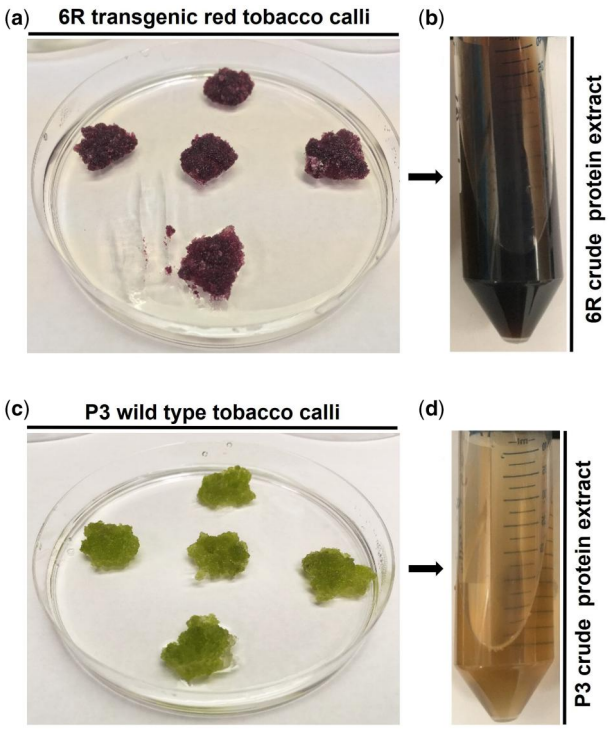

Figure 1. Different calli cultured on agar-solidified medium and colors of crude protein extracts. (**a, b**) transgenic red calli cultured for 15 days (a) and crude protein extract with dark color (b). (**c, d**) Greenish wild-type calli cultured for 15 days (c) and crude protein extract with light brownish color (d)

Methods II and III: removal of pigment with polyvinylpolypyrrolidone

Polyvinylpolypyrrolidone (PVPP) is widely used to remove pigments from plant protein extracts [32, 34, 44]. In this experiment, PVPP was tested to remove pigments in crude protein extractions using methods II (MII) and III (MIII). Method II had three steps, MII-1, 2, and 3 (Fig. 2a). The MII-1 step was addition of PVPP into crude protein extracts. Three amounts of PVPP (60, 120, and 180 mg) were tested. PVPP was added to 9 ml of crude protein extracts as described in the MI-1 step of PVP treatment. The MII-2 was the same as the MI-2 step described above. The MII-3 step was also the same as the MI-3 step described above.

Method III had four steps, MIII-1, 2, 3, and 4 (Fig. 2a). The MIII-1 and 2 steps were the same as the MII-1 and MII-2 described above, respectively. The MIII-3 step was precipitation of crude proteins using ammonium sulfate [$(NH_4)_2SO_4$]. After 9-ml of protein extracts were obtained from the MIII-2 step and transferred to a new tube, three amounts of $(NH_4)_2SO_4$ (2.0, 4.0, and 5.0 g) were tested for precipitation. $(NH_4)_2SO_4$ was added into the tube until completely dissolved with a magnet stirring bar at 4°C. The tube was centrifuged at 15 000 rpm (31 000 rcf) for 10 min at 4°C. After the supernatant was removed, the precipitated proteins were completely dissolved in 9 ml of 0.02 M (pH 7.0) Tris-HCl buffer. The MIII-4 step was to further remove $(NH_4)_2SO_4$ and pigments. The resulting protein extract from the MIII-3 step was gently transferred to a 15-ml 10K cut-off filter unit (Amicon® Ultra, MilliporeSigma, Burlington, MA, USA) to remove salts and certain pigments as described in the MI-3 step above. The resulting upper protein extract was pipetted into a new 1.5-ml Eppendorf tube and then stored at 4°C until use.

Method IV: removal of pigments using DEAE-Sephadex gel

Method IV (MIV) included column preparation and three steps of pigment removal, MIV-1, 2, and 3 (Fig. 2b). DEAE-Sephadex A-25 anion exchange gel was used to prepare pigment removal columns. One gram of gel was fully suspended in 25-ml of 0.02 M Tris-HCl extraction buffer (pH 7.0) and then incubated in boiling water for 1 h. Ten milliliter of suspended gel mix, including 1.0 g of gel, was cooled down to room temperature and then loaded into a new 15-ml polyethylene syringe (height: 8.0 cm and diameter: 1.5 cm), the outlet of which was appropriately plugged with a cotton ball. After most of the buffer had flowed through at a rate of 1.0 ml/min, 30-ml of 0.02 M Tris-Cl (pH 7.0) was added to equilibrate and wash the gel. This flow-through of buffer was not stopped until ~1-ml was left above the top of the gel. No air

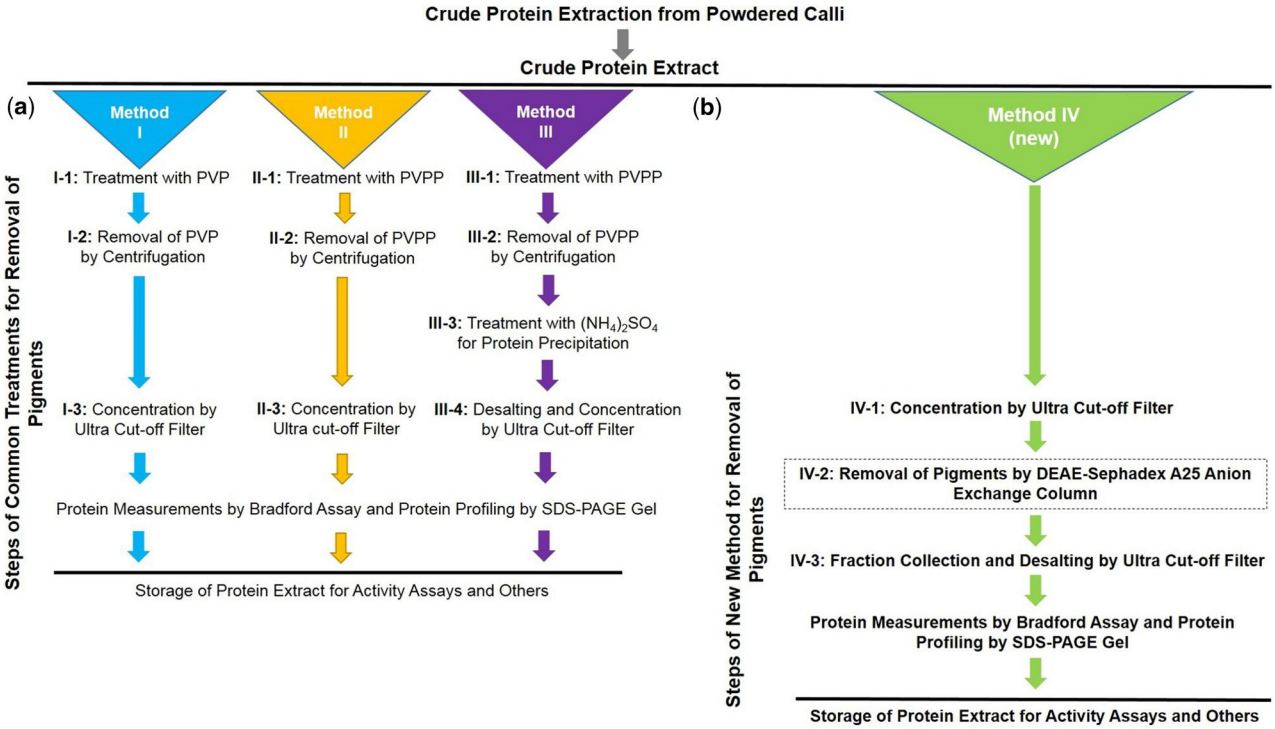

Figure 2. Four methods and steps used to remove pigments from crude protein extracts. (**a**) Step of methods I, II, and III reported in literatures for removal of pigments using PVP and PVPP; (**b**) steps of our new method (IV) for removal of pigments

bubbles were allowed in the gel. The column was ready for the removal of the pigment described below.

The MIV-1 step was the reduction of the protein extract volume. Nine milliliter of protein extract was reduced to 1-ml using a 15-ml 10K cut-off filter unit, the procedure of which was as described above in the MI-3 step. After this step, the volume of protein extract was reduced to approximately 1-ml. The MIV-2 step was the removal of pigments using the prepared DEAE-Sephadex A-25 anion exchange gel column. Concentrated protein extract was loaded on the top of the column and allowed to completely flow into the gel at a rate of 1.0 ml/min. Four elution buffers, 0.0, 0.10 M, 0.20 M and 0.25 M NaCl dissolved in 0.02 M Tris-HCl (pH 7.0), were prepared to elute proteins. For each elution buffer, 30-ml was sequentially used to elute proteins at a flow rate of 1.0 ml/min. The flow-through buffer was collected from each elution and then combined together to obtain approximately 120-ml of collection. The step MIV-3 was that this final protein collection was concentrated and desalted using a 15-ml 10K cut-off filter unit as described above in the MI-3 step. After concentration, the final protein extract volume was adjusted to 1-ml and then stored at 4°C until use.

Measurement of protein concentrations

The total protein concentration was measured using the Bradford protein assay after protein extracts were adjusted to 1-ml in each step for methods I, II, III, and IV. The protein concentration of each sample was calculated using the standard curve equation, with BSA employed as the standard based on the protein assay dye reagent concentrate (Supplementary Results 1). Four µl of protein extract concentrated was added to 76-µl 0.02 M Tris-HCl (pH 7.0) buffer and then thoroughly mixed to obtain an 80-µl mixture, from which 20-µl was added to 980 µl (1×) dye reagent contained in a 1.5-ml Eppendorf tube. The mixture was gently and thoroughly mixed via pipetting and incubated for 5 min at room temperature. The absorbance values of mixtures were recorded at 595 nm on a spectrophotometer (2000C, NanoDrop™, Thermo Fisher Scientific, Waltham, MA, USA). In addition, BSA was used to develop a standard curve to calculate protein concentrations.

Protein analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis and peptide sequencing

Protein extract quality was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE (12.5%) was freshly prepared prior to electrophoresis. The separation gel was prepared using 2.9-ml of 30% acrylamide/bisacrylamide solvent, 1.75-ml of 4X Tris/0.4% SDS (pH 8.8), 2.4-ml of dH_2O, 40-µl of 10% (w/v) ammonium persulfate (APS), and 10-µl of tetramethylethylenediamine (TEMED). The stacking gel was prepared using 312-µl of 30% acrylamide/bisacrylamide solvent, 750-µl of 4× Tris/0.4% SDS (pH 6.8), 1.9-ml of dH_2O, 20-µl of 10% APS, and 8-µl of TEMED. The size of the comb created 40-µl volume wells for sample loading. SDS-PAGE was prepared using a caster (10 × 8 cm). SDS-PAGE was then installed in an electrophoresis apparatus (Mini Protean® Tetra Cell, Bio-Rad, Hercules, CA, USA).

Twenty microgram of the protein sample was mixed with 10-µl of the protein loading buffer, which was composed of 25% glycerol, 10% SDS, 0.5 M Tris-Cl (pH 7.0), 0.15 µM bromophenol blue, dH_2O, and 0.15 mM β-mercaptoethanol. The resulting mixture was incubated for 5 min at 95°C using thermal cycler (6333 Nexus, Mastercycler® PCR, Eppendorf Hamburg, Germany). After samples were cooled down to room temperature, they were loaded to SDS-PAGE. The polyacrylamide gel and 1-L of 1× Tris-Glycine-SDS running buffer including 3.03 g of Tris, 14 g of glycine and 5-mL of 20% SDS were placed in an electrophoresis chamber. In addition, 10-µg of Bio-Rad Precision Plus Protein™ ladder was

loaded into the gel as a molecular weight standard. The voltage of the electrophoresis was set at 70 V for 25 min and then increased to 120 V for 90 min. After the completion of electrophoresis, the gel was rinsed using dH_2O and then placed into in a glass tank containing a protein staining buffer, which was prepared using 450-ml of dH_2O, 450-ml of MeOH, 100-ml of glacial acetic acid (9:9:2, V: V: V) and 1.0 g of Coomassie Brilliant Blue. The staining tank was placed on a shaker with a speed of 100 rpm for overnight gel staining. The stained gel was de-stained with a buffer consisting of 500-ml of dH_2O, 400-ml of MeOH, and 100-ml of glacial acetic acid (5:4:1 V/V/V). After excess dye was removed, the gel was photographed using the 8-megapixel camera of an iPhone (5S, Apple, Cupertino, CA, USA) or placed in a G-Box F3 Gel Doc System (Syngene International Ltd, India) for better images.

Polypeptide chains linked by disulfide bonds were observed in the protein extracts. To understand their amino acid sequence, they were dissociated to obtain each of them by the new method IV. The 12% SDS-PAGE gel was divided into 4 different cutting regions based on the size and separation of the protein bands and labeled as cutting regions #1, #2, #3 and #4 (Supplementary Fig. S1). Each gel cut region containing the different protein bands was transferred to a clean 1.5-ml tube and, sent to the Proteomics & Metabolomics Core Facility, Duke University (Durham, NC, USA) (https://genome.duke.edu/cores-and-services/proteomics-andmetabolomics) for protein sequencing. Sequencing steps followed the Center's protocol and sequencing was performed by the Center. Amino acid sequences from the protein sequencing were used as queries in Blast searches against common tobacco (*Nicotiana tabacum*) sequences in GenBank curated by The National Center for Biotechnology Information (NCBI). All partial sequences of the polypeptides matched forty-seven common tobacco genes (Supplementary Results S2).

Polyphenol oxidase assay and high-performance liquid chromatograph analysis

Polyphenol oxidase (PPO) activity was assayed using catechol, a common *O*-diphenol substrate. Catechol (99.99%) was freshly dissolved in methanol to prepare a 1 M stock solution. An enzymatic reaction was carried out in a 1-ml volume, which was composed of 899-μl of 0.02 M Tris-HCl (pH 7.0) buffer, 100-μl of protein extracts (125 μg) after the treatment of pigment removal from methods I, II, III, and IV, and 1-μl of 1 M catechol (110 μg) contained in a 15-ml polyethylene tube. In addition, reactions without or with catechol, 100-μl buffer, and BSA (125 μg) were performed as controls. Each assay was repeated three times. All reactions were incubated for 45 minutes at room temperature and stopped by adding 3-ml of ethyl acetate (EA). After adding EA, each tube was thoroughly vortexed for two minutes and then centrifuged for five minutes at 8,000 rpm (8,820 rcf) at 22°C in a microcentrifuge (5425 R, Eppendorf, Hamburg, Germany). The resulting upper ethyl acetate phase was gently transferred to a new tube and dried using a savant speed vacuum concentrator. The remaining pellet was dissolved in 200-μl of methanol (LC/MS grade) for high performance liquid chromatography (HPLC) analysis.

HPLC analysis was completed on a liquid chromatography–mass spectrometry (LC-MS) instrument (LCMS-2010 EV, Shimadzu, Kyoto, Kyoto, Japan). Metabolites in samples were separated on an Eclipse XDB-C18 analytical column (250 mm × 4.6 mm, 5.0 μm, Agilent, Santa Clara, CA, USA). The mobile phase solvents used for metabolite elution included 1% acetic acid in water (solvent A, HPLC-grade acetic acid, LC/MS-grade water) and 100% acetonitrile (solvent B, LC/MS-grade). A gradient solvent system, which was developed to separate products, was composed of ratios of solvent A to B: 80:20 (0–5 min), 80:20–70:30 (5–10 min), 70:30–65:35 (10–20 min), 65:35–60:40 (20–30 min), 60:40–55:45 (30–40 min), 55:45–50:50 (40–45 min), 50:50–48:52 (45–50 min), 48:52–45:55 (50–55 min), 45:55–40:60 (55–58 min) and 40:60–10:90 (58–58.5 min). After each elution, the column was washed for 11.5 min with a mixture of elution buffer, including 20% solvent B and 80% solvent A. The flow rate was 0.4 ml/min and the injection volume was 1-μl. Chromatographs of metabolites were recorded from 190 to 800 nm. Prior to and after enzymatic reactions, peak values of catechol were recorded at 280 nm for each reaction and used to evaluate PPO catalytic activity.

PPO assay using ultra-violet spectrophotometer

PPO activity in 6R crude protein extracts from four methods was compared using absorbance values of enzymatic products. 6R crude proteins extracted from methods I, II, III, and IV were used in this assay. Crude proteins extracted using methods I, II, and III and then using method IV were also used in this assay. Crude protein extracts (Fig. 1b and d) after extraction were immediately used to test PPO activity as control. The total volume of incubation reactions for PPO activity was 1-ml including 0.02 M Tris-Cl (pH 7.0) buffer, 20-μg of protein extracts, and 100 mM catechol. The reaction in each tube did not start until each crude protein extract was added. After crude protein was added, each tube was immediately placed in a spectrophotometer (Helios Alpha Beta UV-Visible, Thermo Fisher Scientific, Waltham, MA, USA) to measure the absorbance value (ABS) at 420 nm (A_{420nm}), the wavelength of which has been used to measure PPO activity [45]. ABS was recorded every 30 s for 20 min at 25°C. Dynamic ABS values were used to evaluate PPO activity. This enzymatic assay for each crude protein extract was repeated three times, each with three technical replicates. Meanwhile, negative control reactions, including one combination of catechol and buffer only and the other combination of BSA protein and catechol, were carried out to record absorbance values and repeated three times. One unit of PPO activity was defined as the amount of enzyme necessary to change A_{420nm} in 0.001 per minutes at 25°C.

Statistical analysis

Each pigment-removal experiment with Methods I, II, III and IV were repeated at least five times. Each enzyme assay for PPO was repeated with three biological replicates, each with three technical replicates. Data from the UV spectrometry assay and total protein measurements were expressed as means ± standard deviations and subjected to statistical analysis using the Microsoft Excel software package (2013, Microsoft, Redmond, WA, USA). A one-way analysis of variance (ANOVA) was performed to evaluate the significant difference in PPO activity among eight independent groups, including non-treatment, methods I, II, and III treatments, and four method IV treatments. Based on the F-value being higher than the F_{crit} value (F: $10.11 > F_{crit}$: 2.65), this analysis indicated whether or not the mean difference was statistically significant. Since the ANOVA test compared the overall mean difference among groups, Tukey's Test was selected for its suitability in analyzing data from 10 groups, and controlling the family-wise error rate (FWER), which is the probability of making at least one Type I error (false positive) when performing multiple tests. A Tukey test was performed as a post-hoc test to further evaluate significant difference between groups, and to identify which specific groups'

means (in comparison with one another) exhibited a significant difference, specifically non-treatment vs. Method IV, Method I vs. Method IV, Method II vs. Method IV, and Method III vs. Method IV. Based on degrees of freedom (df) within groups and the number of treatments ($k = 8$), a q-table with a 4.90 value at $\alpha = 0.05$ was generated to calculate the q value for each specific group. The resulting q value for each specific group was used to compare significant differences. When the mean value difference between each specific group was higher than their q value, it indicated significance. In addition, a Student's *t* test was carried out to evaluate statistical significance in comparing soluble protein concentrations and yields prior to and after the treatments with the new method IV.

Results

DEAE-Sephadex gel column produces pigment-free and transparent protein extracts

Crude protein extracts from greenish wild-type P3 calli and red transgenic 6R calli were brownish and extremely dark brownish, respectively (Fig. 1b and d). The dark brownish color in 6R protein extracts resulted from water-soluble anthocyanins and oxidation of other polyphenolics. The brownish pigmentation in P3 protein extracts likely resulted from the oxidation of polyphenolics. To remove these pigments from protein extracts, we compared four methods (Fig. 2). Methods I and II were designed to use PVP and PVPP, respectively. These two reagents have been commonly reported for the removal of pigments from protein extracts. In addition, we used a Millipore Amicon® Ultra 10K cut-off filter unit to remove small molecules, including pigments. The resulting data showed that the combinations of PVP (Method I) and PVPP (Method II) with the Millipore Amicon® Ultra 10K cut-off filter unit were ineffective in removing pigments from protein extracts (Fig. 3a and b). In addition, a combination of PVPP, $(NH_4)_2SO_4$ precipitation, and the Millipore Amicon® Ultra 10K cut-off filter unit was tested in the method III. This method was unable to improve pigment removal from protein extracts either (Fig. 3c).

Method IV is a new approach. A simple DEAE-Sephadex gel A-25 column was prepared to remove pigments. After dark brownish protein extracts from red 6R calli were loaded onto the column, all pigments were bound to a gel matrix (Supplementary Fig. S2a). In addition, brownish pigments in P3 protein extracts were bound to a gel matrix. The resulting protein extracts from both P3 and 6R calli were colorless and transparent (Fig. 3d). This result demonstrated that method IV was effective in removing all pigments from protein extracts from two types of tissues. Furthermore, dark protein extracts obtained from methods I, II, and III treatments (Fig. 3a–c) were loaded onto DEAE-Sephadex gel A-25 gel columns. All pigments in these dark extracts were also completely removed from proteins (Fig. 3e and Supplementary Fig. S2b). These results demonstrated that the new method IV using DEAE-Sephadex gel A-25 was highly

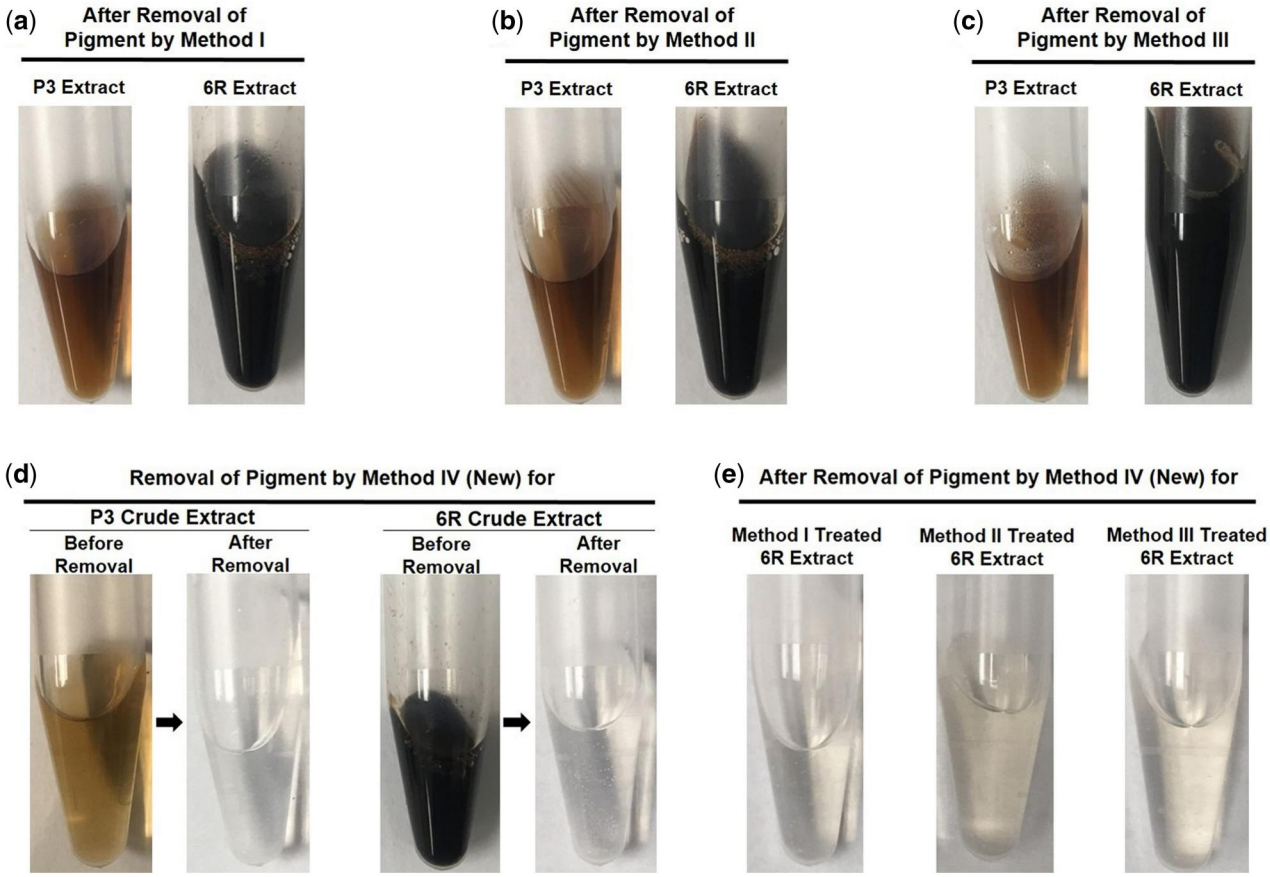

Figure 3. Comparison of pigment removal results obtained with different methods. (**a–c**) Colors of crude protein extracts from P3 and 6R calli after treated with PVP (method I) (a), PVPP (method II) (b), PVPP and $(NH4)_2SO4$ (method III) (c); (**d**) colors of crude protein extracts from P3 and 6R calli after pigment removal using the new method IV; (**e**) pigment-free protein extracts were obtained from crude protein extracts in (a–c) resulted from treatment of PVP, PVPP, and PVPP and $(NH4)_2SO_4$ after re-treated using the new method IV

effective to remove pigments to produce transparent protein extracts (Fig. 3).

Protein quality and production

All protein extracts treated for pigment removal with methods I, II, III, and IV (Fig. 3) were examined using SDS-PAGE to check band profile quality. Gels were photographed to compare protein band profiles. The resulting images showed that apparent protein bands were obtained from protein extracts treated with method IV (Fig. 4). There was no pigment migrating contamination in the gels. By contrast, images showed that proteins from extracts treated with methods I, II, and III were darkly smeared in gels. Pigment migrating contamination was highly severe in gels. After these extracts treated with methods I, II, and III were further re-treated with the new method IV to obtain pigment-free proteins, gel images showed that the severe smearing problem was completely eliminated, resulting in apparent protein band profiles. Accordingly, pigments in the protein extracts from methods I, II, and III caused the smearing problem, which was solved by the new method IV. In conclusion, all results demonstrate that the new method IV using DEAE-Sephadex gel is effective in removing pigments to obtain transparently high-quality proteins.

Protein concentrations were measured to calculate production after pigment removal by the new method IV. It was interesting that after the removal of pigments, the total soluble protein concentrations from both P3 and 6R calli were significantly increased (Table 1). Furthermore, the yield of the total soluble proteins from P3 and 6R calli increased by 16% and 65%, respectively. Moreover, protein productions were measured for those extracts treated with methods I, II, and III and then re-treated with the new method IV (Fig. 3a–c). The removal of pigments with method IV also significantly increased concentration values of total soluble proteins from those extracts treated with methods I, II, and III. Accordingly, these data show that the presence of pigments (binding to proteins) leads to inaccurate measurements of soluble proteins. These results demonstrate that the new method IV using DEAE-Sephadex gel is effective in reducing the effects of pigments bound to soluble proteins on the measurements of concentration and yield.

Polyphenol oxidase activity

Our goal was to remove pigments to obtain high quality soluble proteins with biochemical activities, such as active enzymes. Browning pigmentation occurring during protein extractions has been reported to result from the oxidation of phenolics or polyphenolics catalyzed by polyphenol oxidase (PPO) [33, 46, 47]. We hypothesized that the pigment-free protein extracts had PPO activity. Catechol, an *ortho*-diphenol, is a common substrate that has been used to test PPO activity [47–49]. In our experiments, we used this metabolite to test PPO activity in both dark and pigment-free protein extracts. When an incubation of a pigment-free protein extract listed (Fig. 3) and catechol started at room temperature, a yellowish pigmentation immediately occurred in the reaction. As the incubation continued, the pigmentation apparently changed to deep yellow and then brown. However, these yellowish and brownish pigmentations were not observed in control experiments, in which pigment-free protein extracts were eliminated. To understand the formation of metabolites from the incubation, HPLC was performed to characterize metabolite profiles. The resulting HPLC data showed that in addition to catechol detected at 16:53 min, three different major peaks were detected at 10:17, 17:71, and 24:91 min (Fig. 5a). However, these three peaks were not detected from control incubations. Although we did not have standards for new peaks,

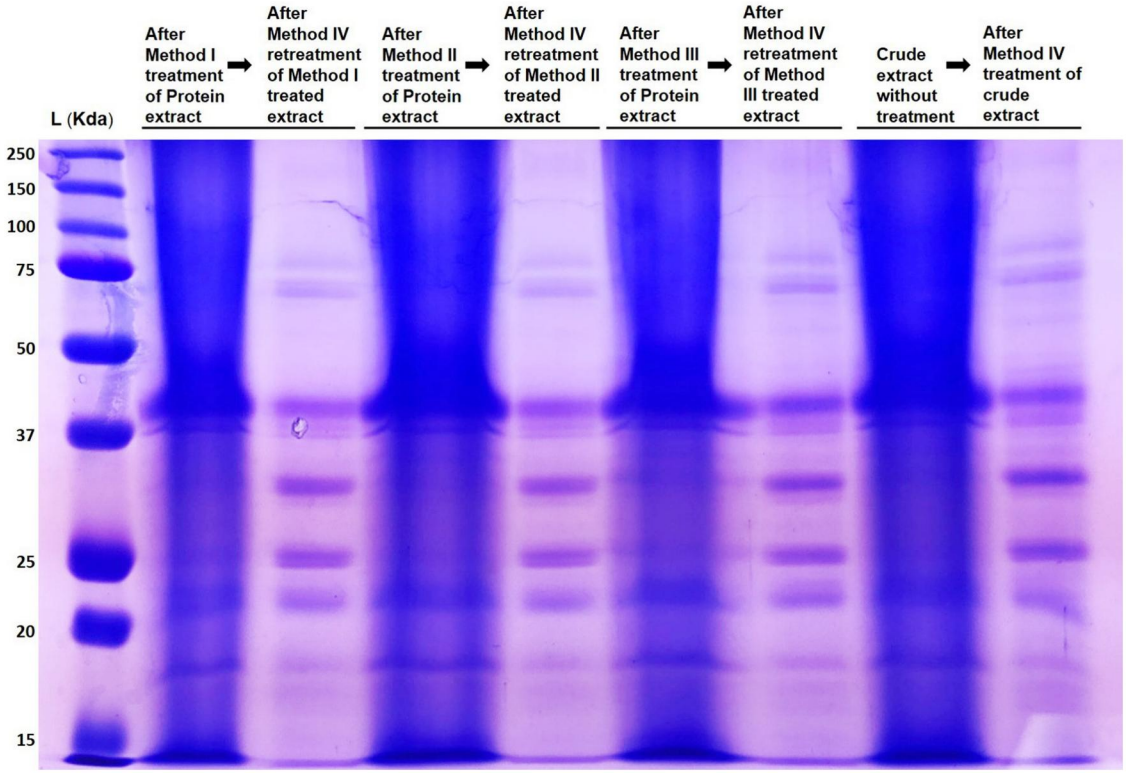

Figure 4. An SDS-PAGE image shows band profiles from protein extracts using four different methods. All crude proteins were extracted from red 6R cells. Protein extracts from methods I, II, and III were retreated with the new method IV. Protein extracts prior to and after treatment using the method IV were loaded gels side by side

Table 1. Protein yields show that the new Method IV decreases the negative effects of pigments on measurement of total soluble proteins.

Calli	Extracts	Total soluble protein concentration (μg/μl)	Total soluble protein production from fresh cell powder (mg/g)	Percentage of total soluble protein in per gram fresh cell powder	Yield increase of total soluble protein by the new method (%)
P3 (green)	Crude extract	0.2792 ± 0.006	1.530[a] ± 0.012	0.153	
	Crude extract after Method I	0.2901 ± 0.001	1.552 ± 0.029	0.155	1
	Crude extract after the new Method IV	5.4776 ± 0.125	1.780[b] ± 0.049	0.178	16
6R (red)	Crude extract	0.2657 ± 0.009	1.541[c] ± 0.07	0.154	
	Crude extract after Method I	0.3029 ± 0.004	1.646 ± 0.037	0.164	6
	Crude extract after Method II	0.3110 ± 0.007	1.679 ± 0.040	0.167	8
	Crude extract After Method III	0.2998 ± 0.008	1.633 ± 0.027	0.163	5
	Crude extract after the new Method IV	7.8236 ± 0.069	2.543[d] ± 0.027	0.254	65

Numbers labeled with "a" and "b" means significant difference. Numbers labeled with "c" and "d" means significant difference (P-value ≤.05, Student's t test).

based on their UV-spectra features, we predict that these peaks are quinones from catechol. Furthermore, peak values of catechol in reactions prior to and after adding PPO were recorded at 280 nm. The resulting data showed that the concentrations of catechol were significantly reduced in the reactions (Fig. 5b). These results demonstrate that pigment-free proteins are active for biochemical analysis.

Enhancement of PPO activity after removal of pigments

PPO activity in original protein extracts and four types of different protein extracts from Methods I, II, III, and IV were compared using product absorbance values recorded at 420 nm on a UV spectrophotometer. After reactions started, the catalytic products' absorbance was recorded every 30 s for 20 min to reflect their initial velocity. The resulting data showed that the products' absorbance values were similar in non-treated original crude protein extracts and three types of protein extracts treated by Method I, II, and III (Fig. 5c). After the removal of pigments using Method IV, PPO activity in all protein extracts was significantly increased (Fig. 5c, Supplementary Results S3 and S4). These results demonstrate that the complete removal of pigments enhances PPO activity.

Discussion

DEAE-Sephadex powder is a type of bead-formed gel that is prepared by cross-linking between dextran and epichlorohydrin and contains a DEAE functional group inside the beads [50]. In the gel, the DEAE functional group is positively charged at the nitrogen (N) atom, and the beads are coated with chloride as a counterion. To date, different types of anion DEAE-Sephadex and other DEAE types have been successfully applied to isolate anion amino acids [50, 51] and proteins for different study purposes [52–56]. However, to our knowledge, no studies have reported the use of the anion DEAE-Sephadex column to remove plant pigments for protein extraction. In our experiments, we compared four different methods, three of which are commonly used to reduce pigment concentrations in extracts, as described in the introduction and results above. In the new method IV, we used DEAE-Sephadex A-25 because this type is an appropriate anion exchange choice to remove small molecules with less than 30 000 Dalton molecular weights. Anthocyanins and most quinones derived from polyphenolics, either via PPO oxidation or spontaneous oxidation, follow this molecular weight range. In the four methods compared, the new method IV using a simple DEAE-Sephadex anion exchange gel column showed the best result in obtaining transparent and pigment-free protein extracts. All brownish pigments were completely removed from all types of extracts (Fig. 3) to result in high-quality protein extracts (Fig. 4). In addition, although we used different buffers (pH 7.0) to heavily wash the column to maximize the release of bound proteins, all pigments remained in the top section of column (Supplementary Fig. S2), indicating that this strong linkage most likely resulted from a covalently cross-linking between pigments and DEAE. Although it is unclear what reaction mechanism is behind this result, we hypothesize that all pigments likely covalently react with DEAE. One indirect piece of evidence is the separation results using sephadex gel G25-100 alone, as tested in our experiments. When brownish protein extracts were loaded onto different sephadex columns, all pigments were co-eluted with proteins. Our results indicated that the sizes of beads used in our experiments did not play a crucial role in the separation of pigments from proteins. Furthermore, when pigmented proteins were loaded onto columns, all pigments stayed on the top (Supplementary Fig. S2). Although different buffers were applied to elute proteins, all pigments stayed on the top. This result suggests that pigments are covalently confined to the outside of beads. Moreover, the past photochemistry studies have provided certain evidence to support this hypothesis. As written in the current textbooks, such as Plant Physiology [57], quinones are acceptors of electrons and play an essential electron-transferring role in plant photosynthesis [58, 59]. Particularly, different photochemistry studies on benzoquinones and their semiquinone structures have demonstrated that quinones exist as anion radical ions in different solvents (including water) [60–65]. This type of anion charge is associated with hydrogen bonding reactions between quinones and other metabolites in chemical and biological processes [61, 66, 67]. Based on these previous reports, we suggest that pigments in protein extracts are negatively charged to form anion ions in our experiments. When brownish protein extracts are loaded to anion DEAE-Sephadex column, DEAE can

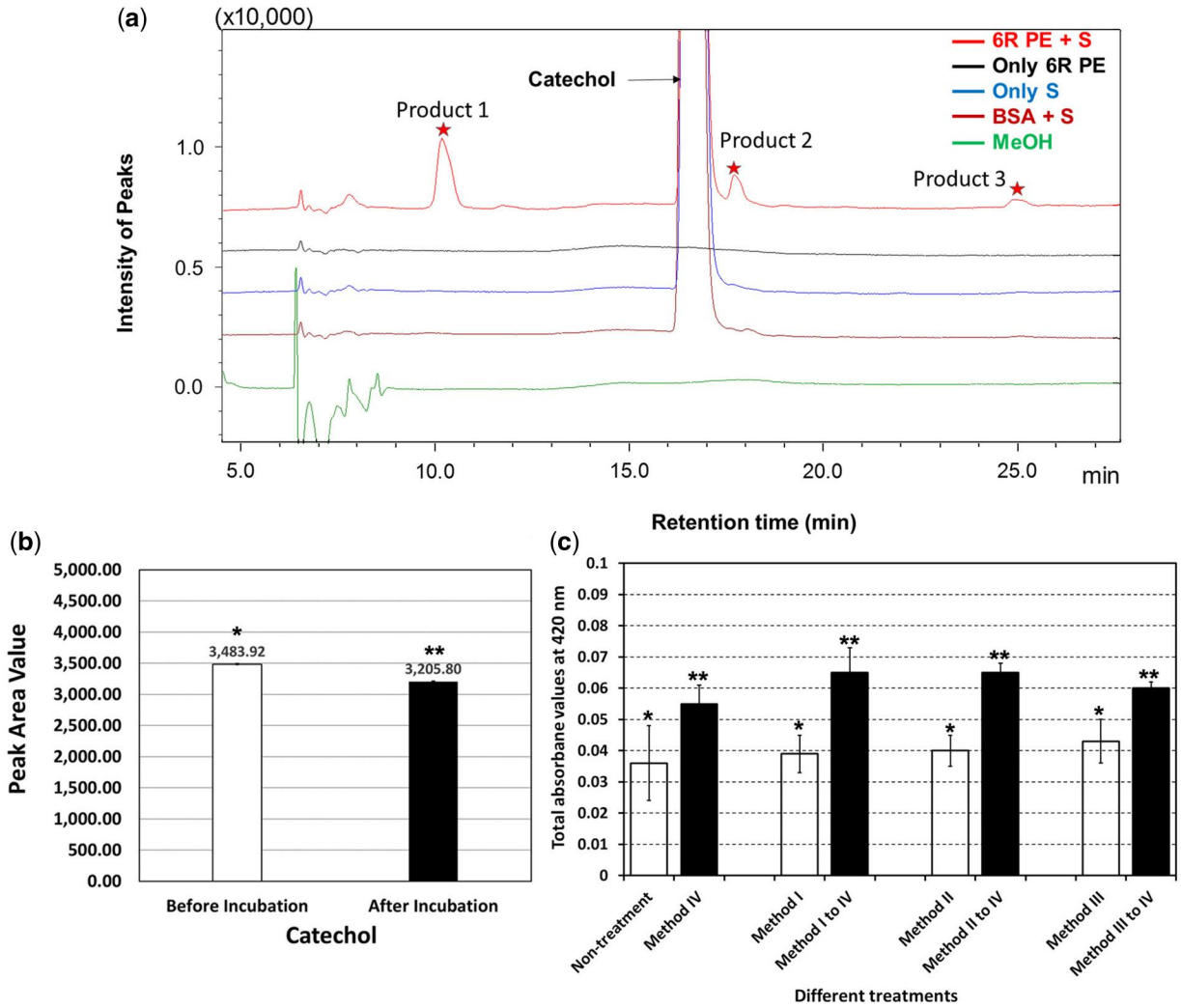

Figure 5. Polyphenol oxidase assay using catechol as substrate. (**a**) HPLC profiles showing catalytic products from incubations of catechol and pigment-free crude protein extracts from the new method IV. Catechol (1.0 mM) was incubated with pigment-free crude protein extracts (PE) and BSA for 45 min, respectively. Catechol (substrate, S) and pigment-free crude protein extract (PE) alone were used as controls, respectively. Absorbance values and chromatographs were recorded at 280 nm. Three new products 1, 2, and 3 are formed from catechol catalyzed by PPO. 6R PE+S: 6R protein extract from method IV was incubated with substrate catechol, 6R PE: 6R protein extract from method IV without substrate, S: substrate alone without protein extract, BSA+S: BSA was incubated with substrate, and MeOH: methanol solvent used as control. (**b**) Comparison of peak area values of catechol before and after incubation ("*" and "**" mean significant difference, *P*-value ≤.05, *t*-value: 2.07). (**c**) Production comparison of reaction products from crude enzyme extracts before and after the pigment removal using the new Method IV. Reaction using catechol was carried out in twenty minutes and absorbance values for products were recorded for each 30 s at 420 nm on a UV spectrophotometry. Four bars labeled "*" or "**" alone mean no significant difference. "Non-treatment" in panel c means that after extraction, crude protein extracts were immediately used to test PPO activity. ANOVA and Tukey's test were performed to evaluate significant differences between non-treatment, methods I, II, and III, and method IV (Supplementary Result 4). In Tukey's test, for non-treatment (n) vs. method IV (IV), $M_{IV\text{-}n}$ (0.0190) > q_1 (0.0154); for method I (I) vs. method IV, $M_{IV\text{-}I}$ (0.0267) > q_2 (0.0217), for method II (II) vs. method IV (IV), $M_{IV\text{-}II}$ (0.0246) > q_3 (0.0200); for method III vs. method IV, $M_{IV\text{-}III}$ (0.0177) > q_4 (0.0144). Bars between labels with "*" and "**" mean significant difference, while bars labeled by the same "*" or "**" mean insignificant difference

covalently link to anion pigments and then substitute those anion proteins and other soluble proteins extracted from plant cells. In the next step, we expect to perform chemical experiments to elucidate the mechanism in the future.

The new method IV reduces the negative effects of pigments on the calculation of the concentration and yield of total soluble proteins. In our experiments, we obtained light and dark brownish protein extracts (Figs 1 and 3), which were completely soluble in buffer. However, pigments could not be removed by PVP, and PVPP, or the Millipore Amicon® Ultra 10K cut-off filter units used in methods I, II, and III (Fig. 3). This result suggests that anion pigments are most likely linked with cation proteins. In addition to our observation, numerous past experiments have demonstrated linkages between brownish quinones and proteins [10, 34, 35]. One linkage is a reversibly cross-linkage via hydrogen binding, and the other is an irreversibly cross-linkage via a covalently binding reaction [4, 34]. Both linkages decrease extraction efficiency for high-quality proteins. Moreover, the covalent linkage not only reduces protein release from pigments but also leads to severe inactivation of enzymes. This was the rationale why different chemical reagents, such as PVP, were tested and identified to partially remove pigments from protein extracts. To date, although PVPP and PVP or other quinone-binding reagents have been used to reduce this problem, to our knowledge, none of them can completely eliminate pigments from protein extracts due to their cross-linking [35, 36, 44, 68]. Our experiments herein

also showed that the use of PVPP, PVP, and other reagents couldn't obtain pigment-free enzyme extracts, indicating that many proteins were bound to pigments. We hypothesized that a breakdown of the cross-linking of proteins and pigments might release more pigment-free soluble proteins. Our data demonstrated that after the DEAE-Sephadex linked pigments, both concentrations and productions of total soluble proteins was significantly increased (Table 1). This result demonstrates that the new method IV eliminated the negative effects of pigments on protein measurement. The difference in the total amount of protein between the extract of wild-type P3 tobacco cells that do not produce anthocyanin pigment and the extract of transgenic anthocyanin-producing 6R cells likely result from the following reasons. The P3 cells are non-transgenic materials that do not produce anthocyanin pigment. As illustrated (Fig. 1b and d), the pigmentation intensity in the P3 extracts is markedly less than that observed in the 6R extracts. The lack of anthocyanins indicates that the P3 cells express different proteins or maybe express less proteins. In contrast, in the 6R cells, the PAP1 transcription factor is overexpressed. As a result, the expression of numerous genes involved in the anthocyanin biosynthesis is increased in the 6R cells. The high production anthocyanins form a hypothesis that the 6R cells likely express more types of proteins and increase the total protein production. The removal of anthocyanins and other pigments by the new Method IV provides evidence to support this hypothesis that the amount of protein present is increased in the 6R cell extracts. In addition, this datum suggests that metabolism-rich cells have more protein complexes and production of important enzymes.

Moreover, we expect that the increase of pigment-free soluble proteins is useful to plant proteomics, which includes extraction, concentration, sequencing, and annotation steps [32, 69, 70]. The protein extraction is the first step obtaining high-quality amino acid sequences. To understand protein profiles in pigment-free extracts, we recently tested proteomics and obtained promising results. Sequence annotation resulted in 47 proteins including candidates of multiple peroxidases, catalase isozyme, glutathione transferase, acetylglucosaminidase, phosphatase, protease and others (Supplementary Results S2). Although these data are preliminary, this result indicates that the new method IV shows a promising potential to extract high-quality proteins for plant proteomics Moreover, it is likely that except for the 47 annotated proteins, other proteins exist but cannot be detected by SDS-PAGE due to the limitation of protein solubility and production of many proteins. In addition, other non-soluble proteins might be more in the red cell.

Furthermore, the new method IV produces active proteins for biochemical studies. Quinones and phenolics can strongly inactivate enzyme activities, such as phenol oxidases [16, 34, 41]. In our experiments, after the removal of pigments, our enzyme assay demonstrated PPO activity to catalyze catechol to quinones (Fig. 5a and b). Moreover, PPO extracted from the new method IV had significantly higher activity than that extracted from other methods (Fig. 5c, Supplementary Results S3 and S4). These results show that the new method IV is effective to reduce or eliminate the inhibitive effects of brownish pigments on enzyme activity and to obtain biochemically active proteins. These results also suggest that many enzyme kinetics crude protein extracts might need to be re-examined with a better purification method like ours described here. The reason is that the presence of pigments in the protein extracts can alter enzymatic activities.

In summary, the new method IV developed herein is effective for extracting active pigment-free soluble proteins with a high yield. This robust protocol will enhance plant biochemistry studies using native proteins.

Conclusion

A new, effective protocol is developed to extract pigment-free, soluble, and desalted plant proteins. Anion DEAE-Sephadex is the main component to covalently bind anthocyanins and polyphenolics-derived quinones, the main components of pigments in plant protein extracts. Furthermore, an enzymatic assay shows that this protocol can obtain active PPOs in pigment-free soluble proteins.

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Author contributions

Seyit Yuzuak (Conceptualization [supporting], Data curation [lead], Formal analysis [equal], Investigation [equal], Methodology [equal], Validation [supporting], Visualization [lead], Writing—original draft [lead]) and De-Yu Xie (Conceptualization [equal], Formal analysis [supporting], Funding acquisition [lead], Investigation [equal], Methodology [equal], Project administration [lead], Resources [lead], Supervision [lead], Validation [supporting], Writing—review & editing [lead])

Supplementary data

Supplementary data is available at *Biology Methods and Protocols* online.

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Data availability

The data underlying this article are available in the article and its online supplementary material.

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