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Extraction and Purification of Laccases from Rice Stems

Chenna Swetha[1, 2] and P. V. Shivaprasad[1, *]

[1]National Centre for Biological Sciences, Tata Institute of Fundamental Research, GKVK Campus, Bangalore 560065, India; [2]SASTRA University, Thirumalaisamudram, Thanjavur, 613401, India
*For correspondence: shivaprasad@ncbs.res.in

[Abstract] Laccases are found in cell walls of plants in very low amounts. This protocol provides an efficient method to purify laccases from rice stems. The method involves three steps: 1) Isolation of total protein from rice stems using buffers with high salt concentration to extract protein from cell walls; 2) Purification of laccases using concanavalin-A beads; and, 3) In-gel staining of laccases with 4-hydroxyindole. Concanavalin-A specifically binds to internal or non-reducing terminal α-D-mannosyl and α-D-glucosyl groups found in glycoproteins and glycolipids. Laccases being glycoproteins binds to concanavalin-A during purification process and eluted with mannose.
Keywords: Laccase, Lignin, Cell wall, Phenylpropanoid pathway, Rice, Plants

[Background] Laccases are oxidases ubiquitously present in bacteria, fungi, animals, and plants. They are some of the oldest enzymes identified. Laccase is involved in diverse functions such as pigmentation of fungal spores, regeneration of plants, as fungal virulence factors, and in lignification of cell walls and delignification during wood rotting. They oxidize the biosynthesis of secondary metabolite called lignin in vascular tissues of plants. Purification of laccases is very challenging as laccases are expressed in very low amounts. Existing protocols to purify laccases are from either microorganism or softer tissues of plants such as leaves. This protocol provides an efficient approach to extract and purify laccases from harder tissues of plants such as rice stems.

Materials and Reagents

1. Pipette tips (Tarsons)
2. Spatula (Fisher Scientific, catalog number: 11533462)
3. Filter paper (Whatman filters, No. 1)
4. 15 ml and 50 ml centrifuge tubes (Tarsons)
5. Dialysis membrane (Thermo Fisher Scientific, 10 kDa molecular weight cut-off (MWCO); catalog number: 68100)
6. Econo-column glass chromatography columns (Bio-Rad, catalog number: 7371512)
7. *O. sativa* var. Pusa Basmati-1 (PB-1)
   Note: PB-1 is an aromatic variety of domesticated rice.
8. Liquid nitrogen
9. Concanavalin-A beads (Sigma-Aldrich, catalog number: C7555-5ML)

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10. Sodium chloride (NaCl) (HiMedia, catalog number: MB023-1Kg)
11. Magnesium chloride ($MgCl_2$) (Fischer Scientific, catalog number: 15535-500G)
12. Manganese (II) chloride (**M**nCl_2) (Sigma, catalog number: M8054)
13. Calcium chloride ($CaCl_2$) (Sigma, catalog number: C3306-250G)
14. Tris-base (Fisher Scientific, catalog number: 15965-500G)
15. Hydrochloric acid (HCl) (Fisher Scientific, catalog number: 29505)
16. Tris-HCl (pH 6.8 and 8.8) (Bio-Rad)
17. Sodium acetate (CH_3COONa) (HiMedia, catalog number: MB048-500G)
18. Polyvinylpyrrolidone (PVP) (Sigma, catalog number: P5288-100G)
19. Phenylmethylsulfonyl fluoride (PMSF) (Sigma, catalog number: P7626-5G)
20. Dithiothreitol (DTT) (Roche, catalog number: 11096176001)
21. Glycine (Sigma, catalog number: G7126-100G)
22. Protein cocktail inhibitor (Sigma-Aldrich, catalog number: 5056489001)
23. 4-hydroxyindole (Sigma-Aldrich, catalog number: 219878)
24. Mannose (Sigma-Aldrich, catalog number: 112585)
25. Protein concentrator (Sartorius, Vivaspin, 30,000 d MWCO, catalog number: VS15T21)
26. 30% acrylamide (Bio-Rad, catalog number: 161-0156)
27. 10% sodium dodecyl sulfate (SDS) (Invitrogen, catalog number: 15525-017)
28. 10% ammonium persulfate (Sigma, catalog number: 161-0700)
29. N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma, catalog number: T7024-25ml)
30. Coomassie brilliant blue (CBB-R-250) (Sigma, catalog number: B-7920-10G)
31. Bromophenol blue (Sigma, catalog number: B5525-5G)
32. Glycerol (Millipore, catalog number: DC4P640148)
33. Ethanol (Emsure, catalog number: 1.00983.0511)
34. Acetic acid (Fisher Scientific, catalog number: 11005)
35. Methanol (Merck, catalog number: 60600905001730)
36. Isopropanol (Fisher Scientific, catalog number: 13825)
37. Dialysis buffer (see Recipes)
38. Extraction buffer (see Recipes)
39. Equilibration buffer (see Recipes)
40. Wash buffer (see Recipes)
41. Regeneration buffer (see Recipes)
42. Storage buffer (see Recipes)
43. 50 mM Mannose (see Recipes)
44. 12% SDS-PAGE gel (see Recipes)
45. TGS buffer (see Recipes)
46. Native-PAGE gel (see Recipes)
47. Native gel sample loading dye (6x) (see Recipes)
48. TG buffer (see Recipes)

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49. CBB-R-250 staining solution (see Recipes)
50. Destaining solution (see Recipes)
51. 5 mM 4-hydroxyindole (see Recipes)

Equipment

1. Scissors
2. Spatula
3. Conical flask
4. Mortar and pestle (Sigma, catalog numbers: Z247472 and Z247510)
5. Pipettes (Gilson, models: P1000, P200, P100, P20 and P10)
6. Cold centrifuge (Thermo Scientific, Heraeus Megafuge 16R, 75003694)
7. -20 °C refrigerator (Panasonic)
8. Thermomixer (Eppendorf, Thermomixer comfort)
9. Chemical fume hood
10. Rocker
11. Magnetic stirrer
12. Magnetic beads
13. Peristaltic pump (Cole Parmer 7520-67 Masterflex Console Drive, model: 77200-52)

Note: The materials, reagents and equipment not provided with company and catalog number can be ordered from any qualified company for using in this experiment.

Procedure

A. Extraction of total protein from rice stems

1. Chill the mortar and pestle with liquid nitrogen and divide 30 g of fresh or -80 °C frozen stem samples into 4 parts of 7.5 g each for convenience and freeze in liquid nitrogen. Take each part of sample, cut the tissue into small pieces of about 1-2 cm long with pre-chilled scissors in a pre-chilled mortar.
2. Grind the tissue to a fine powder by using mortar and pestle using liquid nitrogen. Collect the powder in a 50 ml centrifuge tube with the help of an ice-chilled spatula and place it in liquid nitrogen. Repeat the same for the remaining sample.
3. Add 45 ml of extraction buffer to each centrifuge tube and invert the tubes to homogenize. Place the centrifuge tubes on a rocker at 4 °C for 1 h with a set speed of 50 rockings per min.
4. Centrifuge for 45 min at 7,177 *x g*, 4 °C.
5. Filter the supernatant through Whatman filter paper to a fresh autoclaved conical flask.
6. Transfer the filtrate into 10 cm long dialysis bags and dialyze overnight in 3 L of dialysis buffer at 4 °C on a stirrer at 120 rpm.
7. The dialyzed protein sample is collected and used for purification of laccases.

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B. Purification of laccases from crude protein extract

1. Slowly load the econo-column with concanavalin-A beads using a cut 1 ml pipette tip. Allowed it to settle at the base of the column. Length of separation column was 1.5 cm x 2.5 cm (diameter x length).
2. Wash the column with 4 column volume (CV) of wash buffer. A flow rate of 1 ml/min was maintained throughout the procedure using a peristaltic pump.
3. Pre-equilibrate the column with 4 CV of equilibration buffer.
4. Pass the total crude protein sample through the walls of column slowly without disturbing using a 1 ml pipette.
5. Wash the column with 3 CV of wash buffer to wash the unbound protein.
6. Elute laccases with 9 ml of 50 mM mannose in three elutions of 3 ml each.
7. Load the protein concentrators with purified laccase and centrifuge at 7,177 *x g*, 4 °C to concentrate the eluted protein to up to 200 µl.
8. Wash the column with 3 CV of equilibration buffer and then with Milli-Q-filtered water.
9. Regenerate the column by passing regeneration buffer A and B alternatively for 4 times.
10. Pass 1 CV of storage buffer through the column and store the beads in storage buffer at 4 °C.
11. Obtained purified laccases can be used for in-gel staining (Swetha *et al.*, 2018). Perform standard mass-spectrometry (LC-MS) using cut prominent bands around 50-65 kDa from native gel to confirm the presence of laccases and to rule out contamination from peroxidases (as plant peroxidases range from 40-50 kDa).
12. Load concentrated protein in about 30 µl onto 12% SDS gel and subject to electrophoresis in TGS buffer for 2 h at 80 V.
13. Stain the gel with CBB overnight and then destain for 4 h in destaining solution at room temperature.

C. In-gel staining of purified laccases

1. Sample preparation was done by adding 8 µl of native gel sample loading dye (6x) to the concentrated protein of about 30 µl.
2. Load the protein sample onto a 12% native gel and subject to electrophoresis in TG buffer for 2 h at 80 V and 4 °C.
3. Wash the gel with water 3 to 4 times and then stain the gel with 30 ml of 5 mM 4-hydroxyindole for 2 h.

Data analysis

Data analysis could refer to Figures 3 and 4, and also Supplementary figures S7 and S8 from Swetha *et al.*, 2018.

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Notes

1. All the buffers required for protein extraction and purification should be pre-cooled to 4 °C before use.
2. While making the extraction buffer, first add Tris and salts, cool the buffer to 4 °C and then adjust the pH. Add PMSF and protein cocktail inhibitor to the cooled buffer at the end.
3. To regenerate the concanavalin column, pass regeneration buffers A and B alternatively starting with buffer A and also ending with buffer A.
4. Mass spectrometry (LC-MS) has to be employed to confirm the presence of laccases after purification.

Recipes

1. Dialysis Buffer (3 L)
   100 mM CH_3COONa (40.8 g)
   Add Milli-Q-filtered water to a volume of 2.5 L
   Cool to 4 °C and then adjust pH to 4.5 with glacial acetic acid
   Mix and make up the volume to 3 L and store at 4 °C
2. Extraction buffer (500 ml)
   50 mM Tris (3.025 g)
   1 M NaCl (30 g)
   1.5 M $CaCl_2$ (110.25 g)
   Add Milli-Q-filtered water to a volume of 400 ml
   Cool to 4 °C and then adjust pH to 8 with 1 N HCl
   150 mg DTT
   0.1% (w/v) PVP (0.5 g)
   0.5 M PMSF (87 mg) (first dissolve in 1.5 ml of ethanol and then add to the buffer)
   1 tablet of protein cocktail inhibitor
   Mix and make up the volume to 500 ml and store at 4 °C
   Note: This buffer should be freshly prepared each time.
3. Equilibration buffer (750 ml)
   50 mM Tris (4.533g)
   Add Milli-Q-filtered water to a volume of 650 ml
   Cool to 4 °C and then adjust pH to 7.5 with 1 N HCl
   0.5 M NaCl (22.5 g)
   1 mM $MgCl_2$ (152.25 mg)
   1 mM $CaCl_2$ (110.25 mg)
   1 mM $MnCl_2$ (148.44 mg)
   Mix and make up the volume to 750 ml and store at 4 °C

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4. Wash buffer (500 ml)
   1 M NaCl (29.22 g)
   5 mM $MgCl_2$ (238 mg)
   5 mM $CaCl_2$ (367.5 mg)
   5 mM $MnCl_2$ (491.6 mg)
   Add Milli-Q-filtered water to a volume of 500 ml and store at 4 °C
5. Regeneration buffers (each 500 ml)
   a. Regeneration buffer A
      0.1 M Tris (6.057 g)
      Add Milli-Q-filtered water to a volume of 400 ml
      Cool to 4 °C and then adjust pH to 8.5 with 1 N HCl
      0.5 M NaCl (15 g)
      Mix and make up the volume to 500 ml and store at 4 °C
   b. Regeneration buffer B
      0.1 M CH_3COONa (6.8 g)
      Add Milli-Q-filtered water to a volume of 400 ml
      Cool to 4 °C and then adjust pH to 4.5 with glacial acetic acid
      1 M NaCl (29.21 g)
      Mix and make up the volume to 500 ml and store at 4 °C
6. Storage buffer (40 ml)
   0.1 M CH_3COONa (544 mg)
   0.1 M NaCl (2.33 g)
   1 mM $CaCl_2$ (5.88 mg)
   Add Milli-Q-filtered water to a volume of 30 ml
   20% ethanol (8 ml)
   Mix and make up the volume to 40 ml, store at 4 °C
7. 50 mM Mannose (20 ml)
   180 mg of Mannose in 20 ml equilibration buffer
8. 12% SDS-PAGE gel
   a. Resolving gel buffer (8 ml)
      3.2 ml 30% acrylamide
      2.64 ml Milli-Q-filtered water
      2 ml 1.5 mM Tris-HCl (Bio-Rad) (pH 8.8)
      0.08 ml 10% SDS
      0.08 ml 10% ammonium persulfate
      0.008 ml TEMED
      Note: After pouring stacking gel into the cast, add 500 µl of isopropanol on top.
   b. Stacking gel buffer (5 ml)
      0.83 ml 30% acrylamide

3.44 ml Milli-Q-filtered water

0.63 ml 1.5 mM Tris-HCl (Bio-Rad) (pH 6.8)

0.05 ml 10% SDS

0.05 ml 10% ammonium persulfate

0.005 ml TEMED

Note: Before pouring stacking gel, remove isopropanol, rinse with water and dry by inserting Whatman paper pieces.

9. TGS buffer (1.5 L, 1x working)

   25 mM Tris (4.5 g)

   192 mM Glycine (21 g)

   0.1% SDS (1.5 g)

   Add Milli-Q-filtered water, mix and make up the volume to 40 ml. Store at room temperature

10. Native-PAGE gel

    a. Resolving gel buffer (10 ml)

       4 ml 30% acrylamide

       3.3 ml Milli-Q-filtered water

       2.5 ml 1.5 mM Tris-HCl (Bio-Rad), pH 8.8

       0.1 ml 10% ammonium persulfate

       0.01 ml TEMED

       Note: After pouring stacking gel into the cast, add 500 µl of isopropanol on top.

    b. Stacking gel buffer (5 ml)

       0.83 ml 30% acrylamide

       3.44 ml Milli-Q-filtered water

       0.63 ml 1.5 mM Tris-HCl (Bio-Rad), pH 6.8

       0.05 ml 10% ammonium persulfate

       0.005 ml TEMED

       Note: Before pouring stacking gel, remove isopropanol, rinse with water and remove water thoroughly. Pre-run the gel at 4 °C for 30 min before loading the samples.

11. Native gel sample loading dye (6x)

    187.5 mM Tris-HCl, pH 6.8 with 25% glycerol and 1% bromophenol blue

12. TG buffer (pH 8.3, 1.5 L, 1x working)

    25 mM Tris (4.5 g)

    192 mM Glycine (21 g)

    Add Milli-Q-filtered water, mix and make up the volume to 1,500 ml and store at 4 °C

13. CBB-R staining solution (100 ml)

    0.25 g CBB R-250

    45 ml Methanol

    45 ml double-distilled (dd) H_2O

    10 ml acetic acid

14. Destaining solution
    Ethanol:acetic acid:ddH_2O = 1:1:2
15. 5 mM 4-hydroxyindole
    First, dissolve 133.2 mg of 4-hydroxyindole in 2 ml of ethanol (500 mM 4-hydroxyindole). Then take 300 µl of 500 mM in 30 ml of Milli-Q-filtered water to dilute it to 5 mM 4-hydroxyindole

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Competing interests

The authors declare no competing interests.

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