

Covalent Immobilization of Enzymes Using Commercially Available CDI-Activated Agarose

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1. Introduction

Carbonyldiimidazole (CDI) activated supports for enzyme immobilization are commercially available. The urethane linkage that is formed when a protein is bound to these supports is about 20-fold more stable than the N-substituted isourea linkage formed during protein immobilization on cyanogen bromide-activated matrices (1). Complete characterization of the immobilized preparation requires the development of new methodology or the modification of existing methodology to measure the amount of protein bound to, and the enzymatic activity residing in, the immobilized preparation. In this chapter we present detailed descriptions of the immobilization of two enzymes, lipoxygenase (2,3) and lactamase, on CDI-activated agarose, and procedures for characterization of the immobilized enzymes.

2. Materials

Water was purified to a resistance of 18 m Ω -cm using a Barnstead (Dubuque, IA) NANO pure system.

2.1. Lipoxygenase Immobilization

1. Soybean lipoxygenase (LOX EC 1.13.11.12) (lipoxidase, type 1-B, Sigma, St. Louis, MO).
2. CDI-activated support. Reacti-Gel, a 6% crosslinked agarose activated with 1,1'-carbonyldiimidazole (CDI) (Pierce, Rockford, IL).
3. Coupling buffer: 0.2M borate buffer, pH 9.0 (*see* Note 1).
4. Quenching reagent: 2M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris)-HCl buffer, pH 8.0.

5. Storage buffer: 0.1M sodium phosphate buffer, pH 7.0, containing 0.9% NaCl, 0.05% bovine serum albumin (BSA), and 0.02% NaN₃.
6. LabQuake rotary shaker (Lab Industries, Berkeley, CA).
7. 10-mL polypropylene column fitted with a porous polyethylene disk.
8. Buchner flask and filter funnel for vacuum filtration of gel.

2.2. Lactamase Immobilization

1. β -Lactamase (EC 3.5.2.6) derived from *Bacillus cereus* 569/H9, lactamase I:lactamase II activity, 10:1 (Calbiochem, La Jolla, CA).
2. CDI-activated support. Reacti-Gel, a 6% crosslinked agarose activated with 1,1'-carbonyldiimidazole (Pierce, Rockford, IL).
3. Coupling buffer: 0.1M borate buffer, pH 8.5, containing 0.9% NaCl.
4. Quenching reagent: 2M Tris-HCl buffer, pH 8.0.
5. Storage buffer: 0.05M sodium phosphate buffer, pH 7.2, containing 0.9% NaCl, 1% gelatin, 0.02% NaN₃.
6. Dialysis buffer: 0.1M borate buffer, pH 8.5.
7. Dilution buffer: 0.1M phosphate buffer, pH 7.2.
8. Dialysis tubing with M_r cutoff of 1000.
9. Poly-Prep column with a two-way stopcock (Bio-Rad, Richmond, CA).

2.3. Protein Measurement and Lipoxygenase Activity Assay

2.3.1. Efficiency of Immobilization of Lipoxygenase

Protein assay reagent (Bio-Rad). Protein standard containing 50 mg/mL albumin and 30 mg/mL globulin (Sigma).

2.3.2. LOX Assay: HPOD Generation in Aqueous Media

The assay is based on the generation of hydroperoxyoctadecadienoic acid (HPOD) from linoleic acid (LA).

1. 0.2M borate buffer, pH 9.0.
2. Linoleic acid.
3. Sonicator.

2.3.3. LOX Assay: HPOD Generation in Organic Media

1. Linoleic acid.
2. Water-saturated octane.
3. 0.2M borate buffer, pH 9.0.
4. Diethyl ether.

2.3.4. Spectrophotometric Assay

1. Xylenol orange reagent: 100 μ M xylenol orange, sodium salt (Aldrich, Milwaukee, WI), 250 μ M ammonium ferrous sulfate, 25 mM H₂SO₄, and 4 mM 2,6-di-*t*-butyl-4-methylphenol in methanol/water (9:1, v/v) (4; see Notes 2,4).

2. Ethanol.
3. Cumene hydroperoxide (Sigma).

2.3.5. Analysis by Thin-Layer Chromatography (TLC)

1. TLC plates: silica gel HPTLC, with preadsorbent zone, 10 × 10 cm (Analtech, Newark, DE). Plates are developed in a twin-trough TLC tank (Camag, Wrightsville, NC).
2. 5% boric acid in methanol.
3. Solvent I: diethyl ether, benzene, ethanol, acetic acid (40:50:2:0.2, v/v/v/v). Solvent II: isooctane, diethyl ether, acetic acid (50:50:2, v/v/v).
4. 60% sulfuric acid solution.

2.3.6. Analysis by High Performance Liquid Chromatography (HPLC) (see Note 3)

1. Methylene chloride; linoleic acid; 1,3 dilinolein; monolinolein; diolein (Sigma or Nu Chek Prep, Elysian, MN).
2. Dry nitrogen gas.
3. 0.3M N-tris(hydroxymethyl)-methylglycine (Tricine), pH 8.0.
4. 100 mM deoxycholate in water.
5. 15°C water bath (Lab-Line, Orbit Shaker Bath, Melrose Park, IL).
6. 1M citric acid solution.
7. Methanol/chloroform, 2:1 (v:v) solution.

2.4. Protein Measurement and Lactamase Activity

2.4.1. Efficiency of Immobilization of Lactamase

1. BCA protein reagent: Dilute as described by supplier (Pierce).
2. Microtiter wells (Immunolon strips from Dynatech Laboratories, Chantilly, VA).
3. Biotek ELISA Reader (Burlington, VT).
4. DeltaSoft program (Biometallics, Princeton, NJ).

2.4.2. Evaluation of Lactamase Activity: Preparation of β -Lactam Standard

1. 3 mg/mL penicillin G in dilution buffer.
2. 1 mg/mL lactamase in dilution buffer.
3. Dilution buffer: 0.1M phosphate buffer, pH 7.0.
4. Microfiltration centrifugation tube with 10 kDa filter (Amicon).

2.4.3. Analysis by TLC

1. 35- μ m porous polypropylene disk, 2.5 × 1.7 μ m (Bel Art Products, Pequannock, NJ).
2. TLC plates: silica gel HPTLC-GLC, 10 × 20 cm, score at 2.5 cm (Analtech) and high performance LHP-KD, 10 × 10 cm, with preadsorbent zone (Whatman, Clifton, NJ). For developing use a wide mouth 118-mL glass jar for the 2.5 × 10 cm plates and a twin-trough TLC tank (Camag) for the 10 × 10 cm plates.

3. Methanol.
4. Penicilloic acid prepared as described in Section 3.4.2.
5. Developing solvent: chloroform, acetone, acetic acid, 10:9:1 (v:v:v).
6. Iodine crystals.
7. 1% aqueous gelatinized potato starch solution.

3. Methods

3.1. Immobilization of Lipoxygenase

1. Add a 1 mL settled volume of CDI-activated agarose to a 10 mL polypropylene column fitted with a porous polyethylene disk. Remove the acetone, in which the CDI-activated gel is suspended, from the gel with vacuum filtration without allowing the gel to dry completely. If a polypropylene disk is not available, a Büchner flask and funnel may be used.
2. Wash the gel with 3×5 mL of chilled distilled water. Remove the water with vacuum filtration as before.
3. Covalently link the activated gel to the LOX within 30 min of washing. Dissolve 2.0 mg/mL of LOX in chilled coupling buffer and transfer 3 mL to a covered polypropylene container holding the CDI-activated gel. Set aside 0.2 mL for total protein and activity measurements.
4. Incubate the gel with the LOX for 42 h at 4°C with gentle mixing using a rotary shaker. Adjust the pH of the gel/enzyme to the original pH (this may require three adjustments).
5. After the incubation, collect the effluent to determine the concentration of unbound protein and LOX activity. Rinse the gel with 5 mL of the borate coupling buffer and pool with the effluent.
6. Block the remaining active sites on the gel with 4×5 mL washes with quenching reagent (2M Tris-HCl, pH 8.0) and allow the last 5 mL to remain in the column for 30 min at room temperature.
7. Rinse the gel containing the immobilized enzyme with 3×10 mL washes of cold water and store at 4°C in storage buffer.

3.2. Immobilization of Lactamase

1. Dissolve 30 mg of β -lactamase in 3 mL of chilled 0.1M borate coupling buffer, pH 8.5, and transfer to a dialysis tubing for dialysis overnight against 1 L of coupling buffer to remove the buffer salts and to equilibrate the protein in the borate coupling buffer. Change the buffer three times during the dialysis.
2. Transfer the dialysate to a calibrated conical tube to measure the volume of the dialysate.
3. Fit a Poly-Prep column with a two-way stopcock and close the bottom outlet. Transfer the CDI-activated agarose gel to the column and allow it to settle to a 2 mL volume. Drain the acetone from the gel using vacuum filtration.
4. Wash the gel with 2×1 mL of chilled distilled water, using vacuum filtration between the washes.

5. Close the bottom of the column. Couple the enzyme to the activated gel within 30 min.
6. Set aside 0.2 mL of the lactamase dialysate from step 2 for total protein measurement. Add 4 mL of the chilled lactamase dialysate in coupling buffer to the CDI-activated column. Rinse the sample tube with 0.5 mL coupling buffer and add to the gel.
7. Cap the top of the column and incubate the gel mixture for 40–42 h at 4°C with gentle mixing on a rotary shaker. Check and maintain the pH at 8.5.
8. After the immobilization period, collect the effluent and determine the concentration of the unbound lactamase.
9. Remove excess imidazole from the unbound lactamase by dialysis or ultrafiltration in borate coupling buffer prior to quantification of the protein concentration. Rinse the gel with 2 × 1 mL washes of borate coupling buffer and pool with the effluent.
10. Rinse the column further with 2 × 1 mL washes of borate coupling buffer and quench with 4 × 5 mL washes of 2M Tris-HCl buffer, pH 8.0. Allow the last 5 mL to remain in the column for 30 min at room temperature. This step blocks the remaining active sites on the gel.
11. Rinse the gel containing the immobilized enzyme with 3 × 5 mL washes of degassed storage buffer and store at 4°C.

3.3. Protein Measurement and Lipoxygenase Activity Assay

3.3.1. Efficiency of Immobilization of Lipoxygenase

1. Determine the percent of protein load on the Reacti-Gel by measuring the total protein before and after coupling. The difference between the amount added and recovered protein is divided by the initial amounts and multiplied by 100. The amounts of protein in the LOX/borate buffer mixture are estimated using the Bio-Rad Protein Reagent and Sigma protein standards for calibration (*see Note 4*).
2. Dilute the protein standards to provide concentrations of 0.0125–1.0 mg protein/mL of 0.2M borate buffer, pH 9.0.
3. Transfer 20- μ L aliquots of standards and samples (protein solution prior to immobilization and unbound protein) to test tubes in duplicate.
4. Add 0.2 mL of Bio-Rad reagent protein sample and 0.2M borate buffer, pH 9.0 (0.8 mL), to each tube, mix gently, and incubate the mixture for 30 min at room temperature.
5. Measure the absorbances at 565 nm using a spectrophotometer.

3.3.2. LOX Assay: HPOD Generation in Aqueous Media

1. Remove the storage buffer from the immobilized LOX (IMM-LOX) by vacuum filtration.
2. Wash the IMM-LOX with two 3.0-mL aliquots of 0.2M borate buffer, pH 9.0.
3. Add 0.59 g of IMM-LOX (containing 3 mg protein) to 40 mg of linoleic acid (LA) that had previously been suspended by sonication for 30 min in 20 mL of 0.2M borate buffer, pH 9.0.

4. Allow the reaction to proceed at 15°C with agitation at 250 rpm. After 15 min, filter the IMM-LOX from the reaction mixture, wash with two 10-mL aliquots of borate buffer, and place in storage buffer at 5°C or return to a fresh suspension of LA.
5. Assay an aliquot from the reaction solution for hydroperoxide content as described in Sections 3.3.4., 3.3.5., and 3.3.6.

3.3.3. HPOD Generation in Organic Media

1. For assays in organic solvent, dissolve 40 mg of LA in 15.0 mL of water-saturated octane in a 125-mL, glass-stoppered Erlenmeyer flask.
2. Wash the IMM-LOX with 0.2M borate buffer, pH 9.0, then add 1.5 g IMM-LOX containing 1.5 mg protein and 187 μ L of 0.2M borate buffer, pH 9.0, to the LA solution.
3. Allow the reaction to proceed at 15°C with agitation at 250 rpm. After 15 min, filter the gel from the reaction mixture and wash the IMM-LOX with two 10-mL aliquots of borate buffer that has been similarly filtered.
4. Lower the pH of the water layer to 3.0 using 0.1M HCl and separate the aqueous and organic layers.
5. Extract the aqueous layer with 3 \times 5 mL diethyl ether. Combine the ether and the organic fractions and take an aliquot for hydroperoxide assay as described in Sections 3.3.4, 3.3.5., and 3.3.6.

3.3.4. Spectrophotometric Assay

HPOD concentration is determined spectrophotometrically using the xylenol orange reagent.

1. Add 2.0 mL of the xylenol orange reagent to 10–50 μ L of sample and raise the volume of the mixture to 2.1 mL by addition of ethanol.
2. Incubate the assays at room temperature for 45 min and then measure the absorbance at 560 nm vs a blank of 2.0 mL xylenol orange reagent and 100 μ L of ethanol (*see* Note 5).
3. Use a commercial preparation of cumene hydroperoxide to prepare a calibration curve of the dye reagent. Prepare the calibration curves on the same day as the reaction assay using freshly diluted (in 95% ethanol) cumene hydroperoxide.

3.3.5. Analysis by TLC

TLC analysis is performed on each reaction mixture as a check on the hydroperoxide levels given by the xylenol orange method and to determine if any anaerobic byproduct formation and/or decomposition of HPOD has occurred (5).

1. Dip 10 \times 10 cm silica gel-HL TLC plates in 5% boric acid in methanol and allow to air dry prior to spotting with samples and standards.
2. Develop the TLC plates sequentially in developing solvents I and II with air drying between developments.

3. Visualize the hydroperoxides by charring after spraying the TLC plates with 60% sulfuric acid (*see* Note 6).

3.3.6. Analysis by HPLC (*see* Note 3)

Analysis by HPLC is particularly convenient for determining the activity of IMM-LOX on several substrates simultaneously. Prepare an equal molar mixture of substrates containing an internal standard. Measure the loss of the substrate with time caused by oxidation by IMM-LOX and compute the relative oxidation rates (6).

1. Prepare in methylene chloride a substrate mixture consisting of 30 $\mu\text{mol/mL}$ linoleic acid, 15 $\mu\text{mol/mL}$ 1,3-dilinolein, and 30 $\mu\text{mol/mL}$ 1-monolinolein, with 30 $\mu\text{mol/mL}$ diolein as the internal standard.
2. Subject a 20- μL aliquot of the substrate mixture to analysis by HPLC and adjust the concentrations of the substrates accordingly (*see* Note 3).
3. Add a 0.2-mL aliquot of the substrate mixture to nine 10-mL Erlenmeyer flasks equipped with stoppers.
4. Evaporate the methylene chloride from the flasks under a stream of dry nitrogen.
5. Add 1.8 mL of 0.3M N-tris(hydroxymethyl)-methylglycine (Tricine), pH 8.0, and 0.2 mL 100 mM deoxycholate in water to each flask.
6. Stopper the flasks and place them in a 15°C water bath. Agitate the flasks at 250 rpm.
7. At time zero add IMM-LOX to each flask except for one. The IMM-LOX should contain approx 100 μg LOX.
8. The reaction mixture in the flask: receiving no IMM-LOX should be quenched immediately with 0.4 mL of 1M citric acid. Thereafter, each oxidation should be quenched at 5-min intervals.
9. Immediately after quenching, each reaction mixture is extracted with 2 \times 1.5 mL methanol/chloroform (2:1).
10. Evaporate the methanol/chloroform from the flasks under a stream of dry nitrogen.
11. Redissolve the substrates and products in methylene chloride. Filter the solutions and subject them to analysis by HPLC.

3.4. Protein Measurement and Lactamase Activity

3.4.1. Efficiency of Immobilization of Lactamase

Determine the coupling efficiency by measuring protein concentration before and after coupling. Use lactamase as a specific protein standard (*see* Note 7).

1. Prepare lactamase standards in concentrations of 0.0125–1.0 mg protein per mL of 0.1M borate buffer, pH 8.5, containing 0.9% NaCl.
2. Transfer 50- μL aliquots of standards and samples (protein solution prior to immobilization and unbound dialysate) to the test tubes in duplicate.

3. Add 1 mL of the BCA reagent to each tube, mix gently, and incubate the mixture for 30 min at 37°C.
4. Transfer 200- μ L aliquots to microtiter wells.
5. Measure the absorbances at 562 nm using a Biotek ELISA Reader equipped with the DeltaSoft program that can analyze and determine the protein concentration in the samples (*see* Notes 7,8).

3.4.2. Evaluation of Lactamase Activity:

Preparation of β -Lactam Standard

Use penicilloic acid as a standard for enzyme activity and prepare in the laboratory from penicillin G.

1. Transfer 100 μ L of 3 mg/mL penicillin G and 50 μ L of 1 mg/mL lactamase to a microfuge tube.
2. Adjust the volume to 1 mL by addition of 850 μ L of 0.1M phosphate buffer, pH 7.0, (dilution buffer) and mix in a rotary mixer for 10 min.
3. Transfer the mixture into a microfiltration centrifuge tube (10 kDa filter) and centrifuge for 20 min at 10,000g. Dilute the penicilloic acid in the filtrate 100X with dilution buffer and use as a standard (*see* Notes 9–11).

3.4.3. Analysis by TLC

1. Make a microcolumn by inserting a 35- μ m porous polypropylene disk, 2.5 \times 1.7 μ m into the bottom of a 1-mL disposable pipet tip.
2. Transfer a 100- μ L portion of the immobilized enzyme preparation to a microfuge tube and wash with 1 mL of 0.1M phosphate buffer, pH 7.0. Recover the immobilized enzyme by centrifugation at 10,000g for 10 min.
3. Add 100 μ L of 3 mg/mL penicillin G to the lactamase-agarose and place the mixture in a rotary mixer for 10 min.
4. After centrifugation at 10,000g for 10 min, analyze the supernatant by TLC using a modification of a procedure reported by Moats (7) as follows.
5. Clean the TLC plates by immersion in methanol for 10 min and subsequent drying at 85°C for 20 min.
6. Apply 1–2 μ L samples to the 2.5-cm TLC plates and air dry using a hair dryer. For the detection of low concentrations of penicillin G and penicilloic acid, apply 25- μ L samples to 10 \times 10 cm Whatman TLC plates.
7. Place the TLC plates in developing tanks that have been equilibrated with 10 mL of the developing solvent, chloroform:acetone:acetic acid, 10:9:1 (v:v:v), and develop for 7 min. The Whatman TLC plate is developed for 10 min with the same solvent mixture in a twin-trough TLC tank in which both chambers are filled with 10 mL of the developing solvent.
8. Air dry the developed plate and visualize spots for penicillin G and penicilloic acid by exposing the plate to iodine vapor for 3–5 min (*see* Note 12).
9. Allow the background iodine on the TLC plate to vaporize for 30 s and then spray the spots with a 1% aqueous gelatinized potato starch (8). The starch suspension is previously heated to >70°C and cooled prior to use as a spray.

4. Notes

1. If the influence of coupling pH is to be investigated, use a coupling buffer that contains a mixture of buffers: borate, ethylenediamine-tetraacetic acid (EDTA), and N-tris(hydroxymethyl)methylglycine (Tricine) all at 0.2M.
2. The H₂SO₄ must be added immediately after the solvent is added. The reagent should be bright yellow.
3. HPLC analysis is conducted on an Alltech (Deerfield, IL) C18 hydroxyethylmethacrylate (HEMA) column (250 × 4.6 mm) installed on a Waters (Milford, MA) LCM1 HPLC instrument (9,10). The detector is a Varex evaporative light-scattering detector MK III (Alltech) operated at a temperature of 48°C, and with N₂ as the nebulizing gas at a flow rate of 1.3 mL/min. The mobile phase has the following composition and gradient: methanol:10 mM aqueous trifluoroacetic acid (TFA) (86:14), 0–4 min; methanol:10 mM TFA (90:20), 4–8 min; acetonitrile:methanol:10 mM TFA (30:65:5), 8–10 min; acetonitrile:methanol:10 mM TFA (51:48:1), 10–25 min. The flow rate is 1 mL/min. Calibration curves are prepared that allow the conversion of peak area to micromoles for each substrate.
4. Absorbance at 280 nm should not be used to measure protein, since the hydrolysis product of the CDI-activated agarose is imidazole, which absorbs at this wavelength.
5. Old samples of LA exposed to air may be partially oxidized. If the degree of oxidation of LA is not known, the blank should be a substrate mixture that was not exposed to LOX.
6. A 6-mm thick sheet of aluminum is placed on top of a laboratory hot plate to give even heat distribution during charring. The R_f values of LA, HPOD, and hydroxyoctadecenoic (ricinoleic) acid were 0.77, 0.62, and 0.53, respectively. Since this TLC developmental system cannot differentiate between fatty acids having differing degrees of unsaturation, the R_f value of hydroxyoctadecadienoic acid, the reduced product of HPOD, is also 0.53.
7. Although the absorbance at 562 nm of an equal weight of BSA is 10 times higher than that of lactamase I, BSA may still be used as a protein standard for coupling efficiency, since this parameter is the ratio of the amount of bound lactamase I to the amount of lactamase I initially added times 100.
8. The coupling efficiency as measured by the BCA protein assay is typically 55%, with approx 3.8 μmol lactamase (M_r 31,500)/mL of agarose gel.
9. β-lactamase I catalyzes the hydrolysis of the lactone ring of penicillins and β-lactams forming penicilloic acid (11,12).
10. The immobilized enzyme retained its activity and was more stable than the enzyme in solution.
11. Penicilloic acid was used as a standard to determine the activity of the immobilized enzyme. The yield of penicilloic acid as described in Section 3.4.2. was quantitative as shown by a single band on a TLC plate.
12. A pinch of iodine crystals is placed in the bottom of the 118-mL glass jar that is capped to allow the iodine vapor to saturate the jar.

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