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GUIDELINE

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GL-0009	**July 8, 2013**	**July 2008**	**001**

Subject	**SEPABEADS EC AND RELIZYME IMMOBILIZATION PROTOCOLS**

1) Enzyme immobilization on epoxy polymer: Direct covalent binding methods

2) Post-blocking procedure

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5) Enzyme immobilization on EC-Q1A: adsorption method

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1) Enzyme immobilization on epoxy polymer: Direct covalent binding methods

1.1 - PGA (Penicillin-G Acylase)

Procedure

- Put in reaction 5 g wet carrier with 20 ml of enzyme solution in a K phosphate buffer 1.25 M pH 8.0 ± 0.2, at a suggested stirring of 150 rpm (overhead stirrer) and immobilisation temperature 25°C.
- After 1 minute, stop stirring and check/adjust the pH at 8 ± 0.1.
- Restart stirring and let the immobilisation reaction proceed 48 hours.
- Check the residual surnatant activity.
- Eliminate the surnatant and wash the immobilised enzyme with 20 ml K phosphate buffer solution 0.02 M at pH 8.0 ± 0.2 stirring 1 - 2 minutes at 25°C.
- Eliminate the surnatant and wash again the immobilised enzyme with the same buffer solution 45 minutes under stirring.
  This last washing will allow desorbing any eventual amount of adsorbed, but not covalently bound, enzyme.
  Based on the activity check on this last surnatant, adjustment at the immobilisation step, like molarity increase, different loading, immobilisation time, or blocking steps will be operated in the successive experiments.
- Eliminate the surnatant and wash the immobilised enzyme with 20 ml K phosphate buffer solution 0.02 M pH 8.0 ± 0.2 over a buchner and filter 5 minutes under vacuum.
- Check the activity of the immobilised enzyme[1].

[1] *Add min 30 mg of immobilised enzyme to 20 ml PenG 2% in K phosphate buffer solution 0.02 M pH 8 and at 28°C, control pH titrating with*
NaOH 0.05 N for 10 minutes.
U/g = (ml NaOH x 1000 x 0.05)/(t_r g)
t_r = Reaction time in minutes (10)
g = Immobilised enzyme in g wet

1.2 - DAAO (D-AMINO ACID OXIDASE)

Procedure

- Put in reaction 5 g wet carrier with 20 ml of enzyme solution in a K phosphate buffer 1.25 M at pH 8.0 ± 0.2, at a suggested stirring of 150 rpm (overhead stirrer) and immobilisation temperature 20 - 25°.
- After 1 minute, stop stirring and check/adjust the pH at 8 ± 0.1.
- Restart stirring and let the immobilisation reaction proceed 18 hours.
- Stop stirring and incubate 20 - 24 h at the same temperature.
- At the end of incubation, check the residual surnatant activity and proteins.
- Eliminate the surnatant and wash the immobilised enzyme with 20 ml of K phosphate buffer solution 0.02 M at pH 8.0 ± 0.2 stirring 1 – 2 minutes minute at 20 - 25°C.
- Eliminate the surnatant and wash again the immobilised enzyme with the same buffer solution 45 minutes under stirring.
  This last washing will allow desorbing any eventual amount of adsorbed, but not covalently bound, enzyme.
  Based on the activity and protein check on this last surnatant, adjustment at the immobilisation step, like molarity increase, different loading, immobilisation time, or blocking steps will be operated in the successive experiments.
- Eliminate the surnatant and wash the immobilised enzyme with 20 ml of K phosphate buffer solution 0.02 M at pH 8.0 ± 0.2 over a buchner and filter 5 minutes (or 10 minutes in a closed reactor) under vacuum.
- Check the activity of the immobilised enzyme.

1.3 - GA (Glutaryl Acylase)

Procedure

- Put in reaction 5 g wet carrier with 20 ml of enzyme solution in a K phosphate buffer 1.25 M pH 8.0 ± 0.2, at a suggested stirring of 150 rpm (overhead stirrer) and immobilisation temperature 25°C.
- After 1 minute, stop stirring and check/adjust the pH at 8 ± 0.1.
- Restart stirring and let the immobilisation reaction proceed 48 hours.
- Check the residual surnatant activity.
- Eliminate the surnatant and wash the immobilised enzyme with 20 ml K phosphate buffer solution 0.02 M at pH 8.0 ± 0.2 stirring 1 - 2 minutes at 25°C.
- Eliminate the surnatant and wash again the immobilised enzyme with the same buffer solution 45 minutes under stirring.
  This last washing will allow desorbing any eventual amount of adsorbed, but not covalently bound, enzyme.
  Based on the activity check on this last surnatant, adjustment at the immobilisation step, like molarity increase, different loading, immobilisation time, or blocking steps will be operated in the successive experiments.
- Eliminate the surnatant and wash the immobilised enzyme with 20 ml K phosphate buffer solution 0.02 M pH 8.0 ± 0.2 over a buchner and filter 5 minutes under vacuum.
- Check the activity of the immobilised enzyme[2].

[2] *Add 30 – 50 mg of immobilised enzyme to 20 ml PenG 2% in K phosphate buffer solution 0.02 M pH 8 and at 28°C, control pH titrating with*
NaOH 0.05 N for 10 minutes.
U/g = (ml NaOH x 1000 x 0.05)/(t_r g)
t_r = Reaction time in minutes (10)
g = Immobilised enzyme in g wet

1.4 - LIPASE IMMOBILISATION

Principle

Lipase is covalently attached to Sepabeads polymeric carriers bringing an epoxy active group.

Procedure

- ❒ Enzyme is dissolved or conditioned in 0,5 - 1.0 M K phosphate buffer pH 8.0.
- ❒ Sepabeads wet carrier is conditioned in same buffer. A ratio 1 : 2 wet weight carrier(W)/buffer volume(V) is adopted under overhead stirring at 300 rpm for 20 minutes, at room temperature (not higher than 25°C).
- ❒ Decant the supernatant and filter the carrier in a Büchner (under vacuum) for 2 - 3 minutes.
- ❒ Start the immobilization procedure adding the enzyme solution to the filtered carrier. The quantity of enzyme activity to be loaded is in the range of 1000 - 3000 Units (as tributyrin hydrolysis) per g wet carrier. A ratio W/V of 1 : 2 as above is suggested.
- ❒ Stir at 300 rpm and at 25°C for 20 - 24 hours controlling periodically the activity in the supernatant.
- ❒ Filter the slurry and determine the residual protein on the filtered solution.
- ❒ Separately, re-suspend the immobilized preparation obtained in K phosphate buffer 20 mM pH 8.0 buffer. W/V ratio 1 : 3. Stir for 30 minutes then filter the slurry.
- ❒ Re-check protein content on filtered solution and suspend the immobilized in a 0,5 M NaCl in 20 mM buffer pH 8.0. Solution ratio: W/V 1 : 3.
- ❒ Let the immobilized incubate for 1 - 2 hours.
- ❒ Filter the supernatant and check the protein content.
- ❒ Proceed for the immobilized washing with acetone till complete water displacement.
- ❒ Dry the immobilized under reduced pressure till residual water < 5 -10%; then check activity and store the immobilized at 4°C.

1.5 - GLUCOAMYLASE IMMOBILIZATION

Materials:

Glucoamylase	Enzyme
Sepabeads EC-EP	Carrier
Potassium dihydrogen phosphate	Buffer
Sodium hydroxide	pH adjusting agent
Glucose	Preserving agent
Sodium benzoate	Preserving agent
Potassium sorbate	Preserving agent

Procedure

In 200 ml water, 0.28 g of potassium dihydrogen phosphate was dissolved and sodium hydroxide was added to adjust the pH to 7. The solution was stirred and heated to 75°C and 100 ml of glucoamylase was added. The temperature dropped to 55°C. Directly after, 200 g of Sepabeads EC-EP/M were added and the mixture was stirred and heated to 50°C.
The mixture was stirred for 5 hours at 50°C; the reaction content was filtered and the immobilized enzyme was washed 8 times with 500 ml water.

1.6 - PULLULANASE IMMOBILIZATION

Materials:

Pullulanase	Enzyme
Sepabeads EC-EP/M	Carrier
Potassium dihydrogen phosphate	Buffer
Citric acid	Buffer
Sodium hydroxide	pH adjusting agent
Glucose	Preserving agent
Sodium benzoate	Preserving agent
Potassium sorbate	Preserving agent

Procedure

To a mixture of 300 ml 1M phosphate buffer and 75 ml of pullulanase, 300 g of Sepabeads EC-EP/M were added. The mixture was stirred at room temperature for 20 h. The immobilized enzyme was filtered and washed 5 times with 900 ml 4 g/l citrate, pH 5.

2) Post-blocking procedure

2.1 – GLYCINE ON SEPABEADS EC-EP

Principle

This test has the aim to increase the hydrophilicity degree of SEPABEADS EC-EP.

Procedure

- ❒ After immobilization and final rinse, suspend the immobilized in (min) 2 ÷ 5 BV (max) of 1 M glycine solution at pH 9.0 and room temperature (max 28°C).
- ❒ Let the immobilized be incubated under gentle stirring for (min) 12 ÷ 24 h (max).
- ❒ Filter 5 minutes under vacuum the glycine solution (for its possible re-use), then rinse the immobilized with 2 BV DI water pH 7 ± 0,5 to eliminate residual glycine.
- ❒ Filter 5 minutes under vacuum and re-suspend the immobilized in low molarity buffer before the use (or adding glycerol for storage stabilization).

3) Enzyme immobilization on amino polymer: Indirect covalent binding methods

3.1 - PGA (Penicillin-G Acylase)

Pre-activation

- Rinse 5 g wet carrier with 20 ml K phosphate buffer 0.1 M at pH 4.2 ÷ 4.5 under stirring (overhead stirrer) at 150 rpm 15 minutes at 25°C controlled temperature.
- Check the pH, which has to be in the range of 8.0 ± 0.2 and adjust it, if necessary.
- Let the carrier settle and eliminate the surnatant.
- Rinse the carrier with 20 ml K phosphate buffer 0.02 M at pH 8.0 ± 0.2, 5 minutes under stirring at 25°C
- Eliminate the surnatant and add 20 ml Glutaraldehyde 2% solution in K phosphate buffer 0.02 M pH 8.0 ± 0.2
  Stir the slurry 60 minutes under 25°C controlled temperature.
- Let the resin settle and eliminate the surnatant.
- Rinse the resin twice with K phosphate buffer 0.02 M at pH 8.0 ± 0.2

Immobilisation

- Put in reaction 5 g wet carrier with 20 ml enzyme solution in a K phosphate buffer 0.02 M at pH 8.0 ± 0.2
- 25°C immobilisation temperature is suggested so as a stirring speed of 150 rpm (overhead stirrer). After 1 minute, stop the stirring and check/adjust the pH 8 ± 0.2.
- Restart stirring and let the immobilisation reaction proceed, as initial proposal, 20 hours
- Check the residual surnatant activity and proteins.
- Eliminate the surnatant and wash the immobilised enzyme with 20 ml of K phosphate buffer solution 0.02 M at pH 8.0 ± 0.2 stirring 1 – 2 minutes at 25°C.
- Eliminate the surnatant and wash again the immobilised enzyme with 20 ml of the same buffer solution, but containing 0.5 M NaCl, 45 minutes under stirring.

- This last washing will allow desorbing any eventual amount of adsorbed, but not covalently bound enzyme.
  Based on the activity check on this last surnatant, adjustment at the immobilisation step, like Glutaraldehyde increase, different loading, immobilisation time will be operated in the successive experiments.

- Eliminate the surnatant and wash the immobilised enzyme with K phosphate buffer solution 0.02 M at pH 8.0 $\pm$ 0.2 over a Buchner and filter 5 minutes under vacuum.

- Check the activity of the immobilised enzyme.

3.2 - DAAO (D-AMINO ACID OXIDASE)

Pre-activation

- Rinse 5 g wet carrier with 20 ml K phosphate buffer 0.1 M at pH 4.2 ÷ 4.5 under stirring (overhead stirrer) at 150 rpm 15 minutes at 20° - 25°C controlled temperature.
- Check the pH, which has to be in the range of 8.0 ± 0.2 and adjust it, if necessary.
- Let the carrier settle and eliminate the surnatant.
- Rinse the carrier with 20 ml K phosphate buffer 0.02 M at pH 8.0 ± 0.2 for 5 minutes under stirring at 20° - 25°C
- Eliminate the surnatant and add 20 ml Glutaraldehyde 2% solution in K phosphate buffer 0.02 M pH 8.0 ± 0.2 Stir the slurry 60 minutes under controlled temperature of 20° - 25°C.
- Let the resin settle and eliminate the surnatant.
- Rinse the resin twice with K phosphate buffer 0.02 M at pH 8.0 ± 0.2

Immobilisation

- Add 20 ml of enzyme solution in a K phosphate buffer 0.02 M at pH 8.0 ± 0.2
- Immobilisation temperature at 20 - 25°C is suggested so as a stirring speed of 150 rpm (overhead stirrer). After 1 minute of initial operations, stop the stirring and check/adjust the pH at 8 ± 0.2. 20 - 24 hours of immobilisation are initially proposed. Check the residual surnatant activity and proteins.
- Eliminate the surnatant and wash the immobilised enzyme with 20 ml of K phosphate buffer solution 0.02 M at pH 8.0 ± 0.2 stirring 1 - 2 minutes at 25°C.
- Eliminate the surnatant and wash again the immobilised enzyme with the same buffer solution, but containing 0.5 M NaCl , 45 minutes under stirring.
- This last washing will allow desorbing any eventual amount of adsorbed, but not covalently bound enzyme.
  Based on the activity and protein check on this last surnatant, successive adjustment at the immobilisation step, like increase of Glutaraldehyde, different loading, immobilisation time will be operated in the successive experiments.

- Eliminate the surnatant and wash the immobilised enzyme with K phosphate buffer solution 0.02 M at pH 8.0 ± 0.2 over a Buchner and filter 5 minutes (or 10 minutes in a closed reactor) under vacuum.

- Check the activity of the immobilised enzyme.

3.3 - GA (GLUTARIL ACYLASE)

Pre-activation

- Rinse 5 g wet carrier with 20 ml K phosphate buffer 0.1 M at pH 4.2 ÷ 4.5 under stirring (overhead stirrer) at 150 rpm 15 minutes at 20° - 25°C controlled temperature.
- Check the pH, which has to be in the range of 8.0 ± 0.2 and adjust it, if necessary.
- Let the carrier settle and eliminate the surnatant.
- Rinse the carrier with 20 ml K phosphate buffer 0.02 M at pH 8.0 ± 0.2 for 5 minutes under stirring at 20° - 25°C
- Eliminate the surnatant and add 20 ml Glutaraldehyde 2% solution in K phosphate buffer 0.02 M pH 8.0 ± 0.2 Stir the slurry 60 minutes under controlled temperature of 20° - 25°C.
- Let the resin settle and eliminate the surnatant.
- Rinse the resin twice with K phosphate buffer 0.02 M at pH 8.0 ± 0.2

Immobilisation

- Put in reaction 5 g wet carrier with 20 ml of enzyme solution in a K phosphate buffer 0.02 M at pH 8.0 ± 0.2.
- Immobilisation temperature at 20 - 25°C is suggested so as a stirring speed of 150 rpm (overhead stirrer). After 1 minute of initial operations, stop the stirring and check/adjust the pH at 8 ± 0.2. 18 hours of immobilisation are initially proposed. Check the residual surnatant activity and proteins.
- Eliminate the surnatant and wash the immobilised enzyme with 20 ml of K phosphate buffer solution 0.02 M at pH 8.0 ± 0.2 stirring 1 - 2 minutes at 25°C.
- Eliminate the surnatant and wash again the immobilised enzyme with the same buffer solution, but containing 0.5 M NaCl, 45 minutes under stirring.
- This last washing will allow desorbing any eventual amount of adsorbed, but not covalently bound enzyme.
  Based on the activity and protein check on this last surnatant, successive adjustment at the immobilisation step, like increase of Glutaraldehyde, different loading, immobilisation time will be operated in the successive experiments.

- Eliminate the surnatant and wash the immobilised enzyme with K phosphate buffer solution 0.02 M at pH 8.0 ± 0.2 over a Buchner and filter 5 minutes under vacuum.

- Check the activity of the immobilised enzyme.

4) Enzyme immobilization on amino polymer: Ionic binding method

4.1 - STANDARD PROTOCOL FOR AMYLOGLUCOSIDASE ENZYME IMMOBILIZATION

Activation

- ❒ Put 200 g = about 300 ml resin wet DIAION HPA25L in a 1 l beaker and add 400 ml Na Acetate buffer 0,1 M pH 8.3 (resin quantity/solution volume 1/2 w/v). Stir the slurry for 15 minutes under 25°C controlled temperature.
- ❒ Check the pH and, if necessary, adjust it by NaOH or CH_3COOH.
- ❒ Let the resin settle and eliminate the surnatant.
- ❒ Rinse the resin again with 400 ml Na Acetate 0,1 M pH 8,3, let the resin settle and eliminate the surnatant.

Immobilization

- ❒ Prepare a solution containing 3000 Units (corresponding to 15 U/ g wet resin) by dissolving the needed quantity of AMG enzyme as it is into 250 ml Na Acetate buffer 0,1 M pH 8,3.
- ❒ Mix under stirring 200 g = about 300 ml ml resin + 300 ml solution and let incubate 20 h at 25°C
- ❒ Drain the surnatant and rinse with 3 BV of the same buffer under agitation 1h.
- ❒ Pick up 10 ml of surnatant to check the activity (and proteins); enzyme binding should correspond to min 99% (=max 1% in surnatant); if not, repeat the immobilization procedure.

Bioconversion

- ❒ Transfer the so obtained immobilized enzyme in a jacketed column and percolate at 50°C the solution to be converted into Dextrose by applying a flowrate in a range of 1 – 6 BV/h , adjusted according to syrup DE at the inlet and the expected syrup DE at the outlet.

5) Enzyme immobilization on EC-Q1A: adsorption method

5.1 - GA (GLUTARYL ACYLASE)

Procedure

- Rinse 10 g wet carrier with 40 ml K phosphate buffer 0.1 M, pH 4.2 ÷ 4.5 under stirring (overhead stirrer) at 150 rpm 15 minutes at 20° - 25°C controlled temperature.
- Check the pH, which has to be in the range of 8.0 ± 0.2 and adjust it, if necessary.
- Let the carrier settle and eliminate the surnatant.
- Put in reaction 10 g wet carrier with 40 ml of enzyme solution in a K phosphate buffer 0.02 M pH 8.0 ± 0.2
- Start the immobilisation step under stirring (overhead stirrer) at 150 rpm at 20° - 25°C controlled temperature
  18 hours immobilisation are initially proposed.
- Stop the stirring and, after the resin settlement, check the residual activity and total proteins on surnatant.
- Eliminate the surnatant and wash the immobilised enzyme with 40 ml K phosphate buffer solution 0.02 M,
  pH 8.0 ± 0.2 under stirring 1 - 2 minutes.
- Stop the stirring and, after resin settlement, eliminate the surnatant.
- Add to the immobilised enzyme 40 ml 2% Glutaraldehyde solution in K phosphate buffer 0.02 M, pH 8.0 ± 0.2
  Stir the slurry 60 minutes min. under 20 - 25°C controlled temperature.
- Stop stirring and, after resin settlement, eliminate the surnatant.
- Rinse the immobilised enzyme with 40 ml K phosphate buffer solution 0.02 M pH 8.0 ± 0.2 under stirring for 1 - 2 minutes then, after resin settlement, eliminate the surnatant.
- Add to the immobilised enzyme 40 ml K phosphate buffer solution 0.02 M, pH 8.0 ± 0.2, containing 0.5 M NaCl and keep the slurry under stirring 30 - 45 minutes to desorb any eventual amount of adsorbed, but not covalently bound, enzyme.

- Stop stirring and, after resin settlement, check the surnatant activity and proteins content. In case of partial desorption repeat the immobilisation modulating the quantity of total proteins and activity in loading and/or increase the Glutaraldehyde quantity in the crosslinking step above mentioned.

- Eliminate the surnatant and wash the immobilised enzyme with 40 ml K phosphate buffer 0.02 M at pH 8.0 $\pm$ 0.2, over a Buchner and filter 5 minutes under vacuum.

- Check the immobilised enzyme activity.

6) Enzyme immobilization on carboxylic polymer

Principle

The most common method of activation for carboxyl functions is via their N-hydroxysuccinilmidylesters, by use of N-hydroxysuccinimide (NHS) and a carbodiimide. These active esters are suitable for coupling primary amines leading to stable amide (Rif.: Linqiu Cao, Rolf D. Schmid, *Carrier-bound immobilized enzymes: principles, applications and design*, (2006), ed. Wiley.).

Procedure

- Wash the polymer with DMF, DMF/MeOH and MeOH (three times, 1 gwet/4 ml carrier/solution ratio).
- Filtrate (under 860 mbar).
- Wash the polymer with potassium phosphate (Kpi) buffer 0.1M pH 8.0 (three times, 1 gwet/4 ml carrier/solution ratio).
- Filtrate (under 860 mbar).
- Second washing step with Kpi solution that will be subsequently used for enzyme immobilization (1 gwet/4 ml carrier/solution ratio).
- Filtrate (under 860 mbar).
- Prepare of enzyme solution in Kpi buffer, add the carrier keeping approximately 1 gwet/4 ml carrier/solution ratio. The immobilization time is 12-24 hours under stirring at controlled temperature.
- Filtrate (under 860 mbar) and collect the supernatant.
- Wash with Kpi low molarity pH 8.0 (twice, 1 gwet/4 ml carrier/solution ratio) at room temperature.
- Filtrate (under 860 mbar) and collect the washing solution.