Supplementary material

Crosslinked enzyme aggregates: a novel and effective method for enzyme immobilization

Linqiu Cao, Fred van Rantwijk and Roger A. Sheldon*

Laboratory of Organic Chemistry and Catalysis, Julianalaan 136, 2628 BL Delft, The Netherlands

Tel: +31-15-2782607, Fax: +31-15-278-1415

E-mail: r.a.sheldon.tnw.tudelft.nl

Experimental details

Materials. 6- Aminopenicillanic acid (APA), D-phenyl glycine amide and penicillin G potassium salt were kindly donated by DSM Anti-Infectives (Delft, The Netherlands). Acetonitrile, *tert*-butyl alcohol, ammonium sulfate, SDS, glutaraldehyde, Na_2HPO_4, NaH_2PO_4 of analytical grade were purchased from Acros Organics (Geel, Belgium). Semi-purified liquid *E.coli* penicillin G acylase (activity 810 U/ml) was generously donated by DSM Anti-infectives (Delft, The

Netherlands). CLEC-EC® (crosslinked enzyme crystals of *E.coli* penicillin G acylase) was purchased from Altus Biologics (Cambridge, USA).

Hydrolytic activity. The enzymic activity of the samples was measured by adding a definite volume or weight to 25 ml 100 mM phosphate buffer pH 8.0, containing 2% (w/v) fresh penicillin G at 25 C. The solution was automatically titrated with 0.1 M NaOH to the desired pH 8.0 using a Metrohm Dosimat, a Metrohm Impulsomat 614 and a Metrohm pH-meter 654 (Titrino, Metrohm, Schwitzerland). One unit (U) of penicillin G acylase activity will liberate 1 μmol phenylacetic acid per min.

The dry weight fraction of the crosslinked preparations – which consist of pure protein – was determined by exhaustively washing a sample with acetone followed by drying in vacuo. The protein dry weight fraction of the native enzyme was measured by lyophilizing a previously dialyzed sample.

HPLC analysis. All synthetic reaction mixtures were analysed on an HPLC system equipped with with a Waters M6000A pump, a custom-packed 10 μm Nucleosil C18 column (Waters Radial-Pak, 8×100mm), a Shimadzu SPD-6A UV-detector at 215 nm and and a Spectra Physics SP 4400 integrator; mobile phase acetonitrile-water (30:70, v/v), containing 5 mM phosphate buffer pH 3.0, and 0.68g/l SDS at 0.7 ml/min.

General procedure for the preparation of crosslinked enzyme aggregates (CLEAs). An aqueous solution of semi-purified penicillin G acylase was diluted seven times by addition of 0.1 M potassium phosphate buffer pH 7.0 under gentle stirring at 0°C. Aggregation of the enzyme molecules in the solution was induced by slow addition of the precipitant (ammonium sulfate, PEG 8000 or *tert*-butyl alcohol) to the clear enzyme solution under gentle stirring at 0°C. Aliquots were taken from the mixture at time intervals of 30 minutes and centrifuged. The activity of supernatant was measured using the method described above. When no more activity was detected in the supernatant, this was taken as an indication that the enzyme was completely aggregated and precipitated.

The enzyme aggregates were crosslinked by slow addition of glutaraldehyde (25% aqueous solution) to the mixture under stirring at 0°C. The final concentration of glutaraldehyde was 0.5% (w/v). After the addition of the glutaraldehyde solution the mixture was incubated in an ice-water bath under gentle stirring with a magnetic bar (300 rpm) for 1-2 hours. Aliquots (100 μl) were taken from the mixture at time intervals of 30 minutes. The samples were dissolved in 2 ml phosphate buffer (0.1 M, pH 7.0) and centrifuged. The activities of the supernatant and the residue (solid immobilized enzyme) were separately measured as described above. The crosslinking reaction was stopped when no more activity was determined in the supernatant. The crosslinked enzyme

aggregates were then collected by filtration and washed thoroughly in turn with water and buffer (50 mM phosphate buffer pH 7.0). The solid crosslinked enzyme aggregates were further dispersed in 50 mM phosphate buffer pH 7.0 under stirring and stored at 4 °C before use. No leakage of enzyme – due to hydrolysis of the Schiff's base – was observed after storage at 4 °C for 100 days.

Crosslinking of spray-dried penicillin G acylase.[1] An aqueous solution of penicillin G acylase was dialyzed to remove all adjuvants. The resulting solution (145 ml, 1.01 kU/ml) was spray-dried using a Büchi 190 Mini Spray Dryer and collected (50.6 kU, 35 % recovery). An amount of enzyme powder corresponding with 3.9 kU was dipersed in 7 ml of an ice-cooled solution of sodium sulfate and stirred vigorously. Glutaraldehyde was added to a final concentration of 1 % (v/v). The crosslinked enzyme preparation was filtered and washed extensively with demineralized water, yield 230 U (5.9 % on crosslinking, corresponding with 2 % overall recovery of activity).

Measurement of S/H in aqueous medium. The S/H ratio of various penicillin G acylases was determined according to the procedure described by Bruggink *et al*.[6] with a slight modification. The synthesis of ampicillin was performed in 20 ml of an aqueous solution containing 300 mM 6-APA, 500 mM D-phenylglycine

[1] See: Amotz, S (Novo Industri A/S) **1987** US 4,665,028.

amide and 20 U penicillin G acylase at 20 °C. The pH was maintained at 7.0 by titration with 6 N H_2SO_4 using a Metrohm Dosimat, a Metrohm Impulsomat 614 and a Metrohm pH-meter 654 (Titrino, Metrohm, Schwitzerland). Aliquots were taken from the reaction mixture at time intervals of 15 minutes. The sample was dissolved in HPLC eluent (acetonitrile-water (30:70, v/v), containing 5 mM phosphate buffer pH 3.0, and 0.68 g/l SDS) and analysed by HPLC.

Enzyme leakage test. A synthesis of ampicillin was performed in 20 ml of an aqueous solution containing 300 mM 6-APA, 500 mM D-phenylglycine amide and 960 U T-CLEA at 20 °C. The pH was maintained at 7.0 as described above. The solids (catalyst, reactants and products) were filtered off after 10 min, when the product started to precipitate. The composition of the filtrate was monitored by HPLC for over 1 h; no changes were observed.

Enzyme recovery test. A synthesis of ampicillin was performed in 50 ml of an aqueous solution containing 300 mM 6-APA, 500 mM D-phenylglycine amide and 285 U T-CLEA at 20 °C. The pH was maintained at 7.0 as described above. Samples were taken at intervals using a cut-off pipette. T-CLEA was recovered from the samples by repeated washing and centrifugation and its hydrolytic activity assayed. The activity corresponded with full recovery up to the point were

ampicillin started to precipitate, were the recovery decreased to 70 %. No further loss of activity was observed.

Ampicillin synthesis in organic solvent. To 10 ml organic solvent containing 4% water (v/v), 3 mmol 6-APA and 5 mmol D-phenylglycine amide thermostated at 0°C in a water-ice bath, was added 100 U crosslinked penicillin G acylase. The reaction mixture was gently stirred at 0°C. Samples (20 µl suspension) were taken at intervals (30 minutes), dissolved in 1ml HPLC eluent (acetonitrile-water (30:70, v/v), containing 5 mM phosphate buffer pH 3.0, and 0.68g/l SDS) and kept in a water-ice bath for analysis.