ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 11 (2001) 665–670

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Cross-linked aggregates of penicillin acylase: robust catalysts for the synthesis of β-lactam antibiotics

L. Cao, L.M. van Langen, F. van Rantwijk, R.A. Sheldon *

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

Abstract

A novel method for the immobilization of penicillin G acylase (penicillin amidohydrolase, E.C. 3.5.1.11) is reported. It involves the physical aggregation of the enzyme, followed by chemical cross-linking to form insoluble cross-linked enzyme aggregates (CLEAs). Compared with conventionally immobilized penicillin G acylases, these CLEAs possess a high specific activity as well as a high productivity and synthesis/hydrolysis (S/H) ratio in the synthesis of semi-synthetic antibiotics in aqueous media. Moreover, they are active in a broad range of polar and apolar organic solvents.

Keywords: Penicillin acylase; β-lactam antibiotics; Enzymatic synthesis; Enzyme immobilization; Aggregates; Stabilization and cross-linking

1. Introduction

The enzymatic synthesis of β-lactam antibiotics such as ampicillin (Scheme1) offers several benefits compared to conventional chemical methods [1]. The enzymatic procedure is carried out under mild conditions in water, without any need for protection/deprotection schemes, and is environmentally benign. However, an inherent drawback is that, owing to the competing hydrolysis of the side chain donor and the secondary hydrolysis of the product, a large excess of the side chain is usually required to complete the reaction [2].

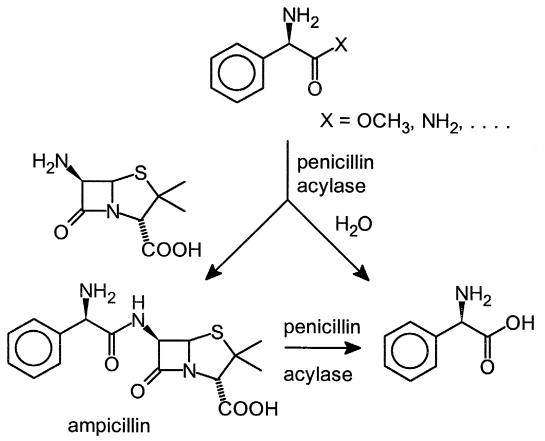
Furthermore, in order to minimize the degradation of the labile nucleus, enzymatic coupling is generally performed under conditions, such as lower pH and temperature, which are far from the optimum of the enzyme [3]. Hence, they generally suffer from a low reaction rate as well as a low space–time yield and productivity [4–9].

With the aim of achieving a high synthesis/hydrolysis ratio (S/H, molar ratio of product and hydrolyzed side chain donor formed) at high conversion (> 90%) and a high productivity and space–time yield, we have developed a novel method for the immobilization of penicillin G acylase, which involves the physical aggregation of the enzyme under non-denaturing conditions, followed by cross-linking to cross-linked enzyme aggregates (CLEAs). The performance of CLEAs is compared with those of commercially available immobilized penicillin G

* Corresponding author. Tel.: +31-15-278-2607; fax: +31-15-278-1415.

E-mail address: r.a.sheldon@tnw.tudelft.nl (R.A. Sheldon).

1381-1177/01/$ - see front matter
PII: S1381-1177(00)00078-3



Scheme 1. Enzymatic synthesis of ampicillin via kinetically controlled coupling.

acylase preparations such as cross-linked enzyme crystals and PGA-450 in the synthesis of ampicillin in aqueous media (Scheme 1) on the basis of S/H ratio, specific activity and stability in organic media.

2. Materials and methods

2.1. Materials

6-Aminopenicillanic acid (6-APA), D-phenylglycine amide (D-PGA) and penicillin G potassium salt were kindly donated by DSM Anti-Infectives (Delft, The Netherlands). Acetonitrile, poly(ethylene glycol) (PEG 8000), *tert*-butyl alcohol, ammonium sulfate, SDS, glutaraldehyde, Na_2HPO_4 and NaH_2PO_4 of analytical grade were purchased from Acros Organics (Geel, Belgium). Semi-purified liquid *Escherichia coli* penicillin G acylase and Assemblase® — immobilized penicillin G acylase — were generously donated by DSM Anti-Infectives. PGA-300 and PGA-450 — immobilised *E. coli* penicillin G acylase preparations on polyacrylic amide — were received from Roche Diagnostics (Penzberg, Germany) as a gift. Cross-linked enzyme crystals of *E. coli* penicillin G acylase (CLEC-EC) were kindly donated by Altus Biologics (Cambridge, MS, USA).

2.2. 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. During the assay, the solution was thermostated at 25°C. The solution was automatically titrated with 0.1 M NaOH using a Metrohm Dosimat, a Metrohm Impulsomat 614 and a Metrohm pH-meter 654 (Titrino, Metrohm, Switzerland). One unit (U) of penicillin acylase will liberate 1 μmol of phenylacetic acid per min.

2.3. HPLC analysis

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

2.4. Preparation of CLEAs

CLEAs were prepared by slowly adding a precipitant for protein aggregation, ammonium sulfate, PEG 8000 or *tert*-butanol, to the penicillin G acylase solution under gentle stirring at 0°C. When no more activity was detected in the supernatant, the physically aggregated penicillin G acylase was subjected to chemical cross-linking using glutaraldehyde (25% aqueous solution) at 0°C. The cross-linking reaction was allowed to continue until no more activity was determined in the supernatant. The CLEAs were then collected by filtration and washed thoroughly in turn with water and buffer (50 mM phosphate buffer (pH 7.0). The solid CLEAs were further dispersed in 50 mM phosphate buffer (pH 7.0) under stirring and stored at 4°C before use.

2.5. Synthetic activity in aqueous environment

The synthetic activity of various penicillin G acylase preparations was determined in a batch reactor

(50-ml flask, thermostated at 20°C). The reaction was started by adding approximately 200 U penicillin G acylase preparation (immobilized or free enzyme) to 50 ml of an aqueous solution containing 300 mM 6-APA and 500 mM D-PGA. The pH was kept constant 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). The ampicillin concentration was determined by HPLC as described above.

2.6. Enzyme synthetic activity in organic solvent

To determine the enzyme activity in organic solvents, a batch reactor thermostated at 0°C in a water–ice bath was used. To 10-ml organic solvent containing 5% water (v/v), 3 mmol 6-APA and 5 mmol D-PGA, immobilized enzyme (normally 100 U) was added to start the reaction. The reaction mixture was gently stirred at 0°C. Samples (20 μl suspension) were taken at intervals (30 min) and dissolved in 1-ml HPLC eluent (acetonitrile–water (30:70, v/v) containing 5 mM phosphate buffer pH 3.0 and 0.68 g/l SDS) and kept in a water–ice bath for analysis.

2.7. Determination of S/H ratio

The S/H ratio of various penicillin G acylases was determined according to the procedure described by Bruggink et al. [1] with slight modification.

3. Results and discussions

3.1. Activity of the CLEAs

CLEAs of penicillin-G acylase were prepared by slowly adding a precipitant — ammonium sulfate, PEG 8000 or *tert*-butyl alcohol — to a solution of the enzyme at 0°C and pH 8. In the next step, the aggregates were cross-linked using glutaraldehyde; the cross-linked aggregates were subsequently collected, washed and redispersed in 50 mM phosphate buffer pH 7.

The activities of CLEAs prepared in the presence of the different precipitants are listed in Table 1.

Table 1
Comparison of the hydrolytic activity of different penicillin G acylase preparations[a]

Biocatalyst	Carrier	Activity (U/g_{wet})	Activity retention (%)
Free enzyme	no	15000	na[b]
CLEC-EC	no	16085	na
A-CLEA	no	9875	41
T-CLEA	no	12440	55
P-CLEA	no	9876	61
PcA	Eupergit C	100	50–60
PGA-450	PAA	410	na

[a]A-CLEA, T-CLEA and P-CLEA represent the CLEAs prepared in the presence of ammonium sulfate, *tert*-butanol and polyethylene glycol as precipitants, respectively.
[b]na: not available.

Compared with the commercial immobilized penicillin G acylases such as PGA-450 and Eupergit C-penicillin G acylase (PcA), the specific activities of the CLEAs are generally at least one to two orders of magnitude higher. Moreover, the specific activity of the CLEAs is very close to that of CLCs of penicillin G acylase (CLEC-EC).

Compared with the free enzyme, more or less 40% of the activity of the CLEAs was lost after filtration and redispersion in buffer. Because no activity loss took place during aggregation and cross-linking, the activity loss on filtration could be attributed to the fusion of some CLEAs, leading to diffusion effects.

3.2. Synthesis of ampicillin catalyzed by CLEAs

In order to investigate the potential of penicillin acylase CLEAs in the synthesis of β-lactam antibiotics in aqueous media, we studied the T-CLEA catalyzed synthesis of ampicillin. Surprisingly, we found that the CLEA gave a two times higher S/H ratio and an almost four times higher productivity than CLEC-EC. Compared with a conventionally immobilized penicillin G acylase, e.g. PGA-450, the productivity of CLEAs was approximately 200-fold higher (Table 2). Hence, we conclude that CLEAs of penicillin G acylase combines a high productivity with a high S/H ratio.

The two types of cross-linked enzymes — CLECs and CLEAs — had comparable activities and syn-

Table 2
Synthesis of ampicillin catalyzed by different immobilized penicillin G acylase

Biocatalyst	Conversion[a] (%)	S/H ratio[b]	V_{syn} (max)[c] (μmol/U h)	Relative productivity[d] (%)
Free enzyme	88	2.0	25.5	100
CLEC	72	0.71	39.6	39
T-CLEA	85	1.58	38.2	151
PGA-450	86	1.56	15.6	0.8

[a] Maximum conversion.
[b] S/H was determined at the conversion listed in this table.
[c] V_{syn} (max) is the initial reaction rate for the ampicillin synthesis in the first half hour.
[d] Relative productivity was calculated at the point of the maximal conversion with free enzyme as 100.

thesized ampicillin at very similar rates, but the CLEA had a much higher S/H ratio. The low S/H of CLECs possibly means that CLECs have a high propensity for hydrolysis of the side chain donor. PGA-450 had the lowest synthetic reaction rate for ampicillin synthesis, resulting in the lowest productivity of all the biocatalyst used.

3.3. Synthesis of ampicillin in organic solvents using CLEA

Although considerable progress in the field of biocatalysis in non-conventional media has been made in the last decade, the enzymatic synthesis of β-lactam antibiotics in organic solvents still remains a considerable challenge owing to the extreme instability of penicillin G acylase in organic solvents. Moreover, the activity of most enzymes is generally two or three orders of magnitude lower in organic solvents than that in aqueous media. In practice, this would mean that unacceptably high amounts of immobilized penicillin G acylase are needed to obtain a viable reaction rate.

This drawback can, in principle, be overcome by using a CLEA as the catalyst, owing to its higher specific activity. To this end, we investigated the synthetic activity of T-CLEAs in mainly organic solvents of different polarities. It was found that T-CLEAs are active in a broad range of organic solvents (see Table 3). The organic solvents can be classified into three groups: (i) hydrophilic ones (log $P < 0$) in which the CLEAs are less stable; (ii) slightly polar ones($1 > \log P > 0$) in which the CLEAs are relatively stable; (iii) hydrophobic ones (log $P > 1$) in which the CLEAs are very stable. It was found, however, that hydrophilic organic solvents give higher S/H ratio, because they can efficiently reduce the water activity.

Table 3
Stability and activity T-CLEAs in organic solvents for the ampicillin synthesis[a]

Organic solvent	Log P	Conversion (%)	Reaction rate (μmol U^{-1} h^{-1})	S/H
Triglyme	−1.7	5	1.4	2.0
2-Methoxyethyl ether	−1.3	11	2.8	2.9
1, 2-Dimethoxyethane	−0.8	10	2.5	3.4
tert-Butyl alkohol	−0.8	18	4.5	1.3
Acetonitrile	−0.4	17	4.4	4.8
2-Ethoxyethyl ether	−0.3	25	6.2	1.8
2, 2-Dimethoxypropane	−0.2	56	14.0	1.3
tert-Amyl alcohol	1.4	33	8.3	1.5
tert-Butyl methyl ether	1.6	47	11.7	1.3

[a] Reaction conditions: 500 mM 6-APA, 750 mM D-PGA, 20 U/ml CLEA 0°C, 300 rpm stirring.

Table 4
Comparison of the hydrolytic and synthetic activities of different immobilized penicillin G acylases

Enzyme	Hydrolytic activity (U g^{-1} wet)	Synthetic activity (μmol g^{-1} min^{-1})	Synthetic activity (nmol U^{-1} min^{-1})
CLEC-EC	5657	72.6	1.3
T-CLEA	2247	42.8	1.9
CLE	921	10.8	1.2
PGA-450	209	1.9	0.9
PGA-300	36	0	0
Assemblase	138	1.4	1

3.4. Enzymatic synthesis of ampicillin in acetonitrile

The activity of CLEA in the synthesis of ampicillin in acetonitrile–water (95:5. v/v) were compared with conventionally immobilized penicillin G acylases. It was found that CLEAs gave a similar activity to the CLEC-EC. However, the synthetic activities of the immobilized penicillin G acylases in organic solvent were not linearly related to their respective hydrolytic activities. The ratios between these two activities are listed in Table 4. Interestingly, the activity shown by CLEAs was about 50% higher than CLECs. Compared with the conventional immobilized penicillin G acylase, the synthetic activity of CLEAs is generally at least one magnitude higher. Not unexpectedly, the synthetic activity of these enzymes in organic solvents is generally several magnitudes lower than that in aqueous media. As shown in Table 4, the activity of CLEAs is twice that of the conventionally cross-linked enzymes (CLE) prepared in solution. We suggested that this is due to pre-organized three dimensional structure and hence, the activity of the aggregates being maintained on cross-linking while the enzyme molecules in solution are more flexible and can undergo a conformational change on cross-linking, leading to loss of activity [10].

4. Conclusions

CLEAs of penicillin G acylase show considerable promise as immobilized biocatalysts for the synthesis of β-lactam antibiotics. They combine the attractive features of a high volumetric activity and a high S/H ratio in β-lactam synthesis with a higher stability and activity in organic solvents compared to conventional immobilized penicillin G acylases as well as cross-linked crystals. Moreover, the technique is readily amenable to scale-up and is universally applicable, because it does not require the time and labour-intensive process of enzyme crystallization and any enzyme can be readily induced to form preorganized physical aggregates under non-denaturing conditions.

Acknowledgements

The authors wish to thank Dr. E. de Vroom, Dr. E.C. Roos, Dr. J.M. van de Laan, Mr. H. Kierkels and Prof. Dr. A. Bruggink of DSM (Delft and Geleen, The Netherlands) for their interesting suggestions and helpful discussion. Generous gifts of penicillin G acylase by DSM Anti-Infectives are gratefully acknowledged. The authors express their thanks to Roche Diagnostics (Penzberg, Germany) for a kind donation of immobilized penicillin acylase. Financial support by DSM Life Science Products and the Netherlands Ministry of Economic Affairs is gratefully acknowledged.

References

[1] A. Bruggink, E.C. Roos, E. de Vroom, Org. Process Res. Dev. 2 (1998) 128.
[2] V. Kasche, U. Haufler, L. Riechman, Methods Enzymol. 136 (1987) 280.
[3] L. van Langen, E. de Vroom, F. van Rantwijk, R. Sheldon, FEBS Lett. 456 (1999) 89.

[4] S. Ospina, E. Barzana, O.T. Ramirez, A. Lopez-Munguia, Enzyme Microb. Technol. 19 (1996) 462.
[5] E. Boccu, C. Ebert, L. Gardossi, T. Gianferrara, M. Zacchigna, P. Linda, Farmaco 46 (1991) 565.
[6] F.-M. Cui, L.-Z. Zhu, W.-Z. Han, M. Wu, Z.-X. Wang, Acta Microbiol. Sin. 36 (1996) 151.
[7] N.K. Maladkar, Enzyme Microb. Technol. 16 (1994) 715.
[8] T. Takahashi, Y. Yamazaki, K. Kato, M. Isono, J. Am. Chem. Soc. 94 (1972) 4035.
[9] O. Hernandez-Justiz, R. Fernandez-Lafuente, M. Terreni, J.M. Guisan, Biotechnol. Bioeng. 59 (1998) 73.
[10] O. Zaborsky, Immobilised Enzymes, CRC Press, Cleveland, 1973, p. 61.