ORGANIC LETTERS

2000
Vol. 2, No. 10
1361−1364

Cross-Linked Enzyme Aggregates: A Simple and Effective Method for the Immobilization of Penicillin Acylase

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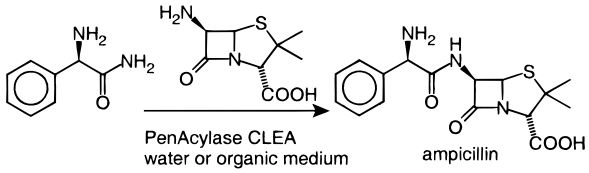
Received January 28, 2000

ABSTRACT



Penicillin G acylase (penicillin amidohydrolase, E.C. 3.5.1.11) was immobilized in a simple and effective way by physical aggregation of the enzyme, using a precipitant, followed by chemical cross-linking to form insoluble cross-linked enzyme aggregates (CLEAs). These had the same activity in the synthesis of ampicillin as cross-linked crystals of the same enzyme, but the accompanying hydrolysis of the side-chain donor was much less. Penicillin G acylase CLEAs also catalyzed the synthesis of ampicillin in a broad range of organic solvents.

The development of robust immobilized biocatalysts that are stable over a broad range of pH and temperature and are tolerant to organic solvents is a major challenge in industrial biocatalysis.[1] A major breakthrough in this field was the development of cross-linked enzyme crystals (CLECs), biocatalysts which combine the features of essentially pure protein with high specific activity and high tolerance to organic solvents.[2,3] However, the preparation of CLECs requires the crystallization of the enzyme prior to cross-linking.

It is well-known that the physical aggregation of protein molecules into supermolecular structures can be induced by the addition of salts, organic solvents, or nonionic polymers to protein solutions[4] without perturbation of the original three-dimensional structures of the protein. Indeed, aggregation induced by ammonium sulfate, poly(ethylene glycol), and some organic solvents such as alcohols, is a commonly used method of protein purification.[5] These solid aggregates are held together by noncovalent bonding and readily collapse and redissolve when dispersed in an aqueous medium.

We surmised that chemical cross-linking of these physical aggregates would produce cross-linked enzyme aggregates in which the preorganized superstructure of the aggregates, and, hence, their activity, would be maintained. Consequently, cross-linking of preformed physical aggregates of enzymes could constitute a simple method for the preparation of what we call cross-linked enzyme aggregates (CLEAs), with activities comparable to CLECs.

To test our hypothesis we chose penicillin G acylase (penicillin amidohydrolase, E.C. 3.5.1.11) as our model enzyme. Penicillin G acylase is widely used in the industrial synthesis of 6-aminopenicillanic acid (6-APA) by enzymatic deacylation of penicillin G.[6,7] The free enzyme is known to have a limited thermal stability and a very low tolerance toward organic solvents.[8] Currently much attention is being devoted to the synthesis of semisynthetic penicillins and

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10.1021/ol005593x CCC: $19.00
Published on Web 04/20/2000

cephalosporins by the enzymatic acylation of 6-APA and 7-aminodesacetoxycephalosporanic acid (7-ADCA) with the appropriate amino acid side chain (see Figure 1).[9] However,

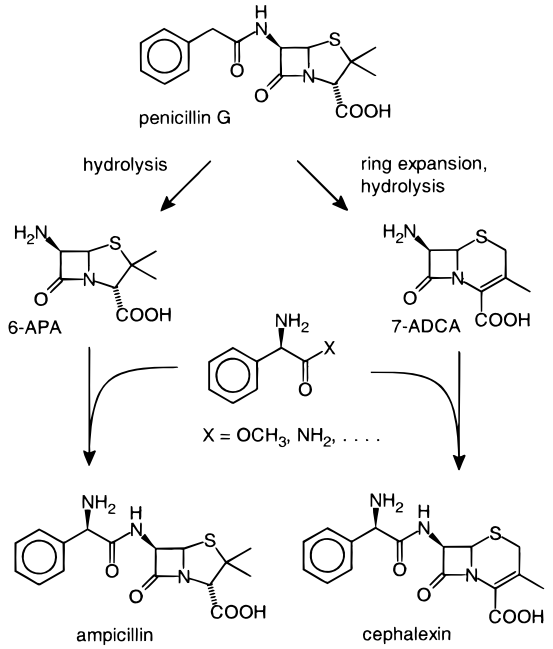

Figure 1. Enzymatic synthesis of β-lactam antibiotics.

an inherent drawback is that, owing to the competing hydrolysis of the side chain donor and the secondary hydrolysis of the products, a large excess of the side chain is usually required to complete the reaction in aqueous media.[10] For this reason, an important parameter—the so-called synthesis/hydrolysis ratio (S/H, the molar ratio of product and hydrolyzed side-chain donor formed)—is often used to evaluate the economic viability of the process.[6]

Recently, it was found that the S/H ratio depends not only on the nature of the side chain and the reaction conditions (pH, temperature, cosolvents)[10,11] but also on the enzyme immobilization method.[6] The latter effect was ascribed to diffusion limitations. The generally accepted catalytic mechanism involves reaction of an acyl−enzyme intermediate with 6-APA (coupling) or water (hydrolysis).[12] The 6-APA nucleus is a much larger molecule than water, and the diffusional limitation created in an immobilized enzyme is expected to lead to a lower S/H ratio.

Preparation of CLEAs. To test our CLEA concept we selected three precipitants that are representative for the three types of precipitants mentioned above, ammonium sulfate, an organic solvent (*tert*-butyl alcohol), and a nonionic polymer (poly(ethylene glycol), PEG). The activities of the recovered insoluble cross-linked enzyme increased with the precipitant concentration, reaching a maximum which corresponded to almost 100% retention of the activity compared with that of the enzyme in solution. The maximum activity was observed at the concentration of the precipitant required to precipitate all of the enzyme from the solution (ammonium sulfate, 30%; *tert*-butyl alcohol, 20%; PEG, 20%). Below this critical concentration, the enzyme is present mainly in solution; upon cross-linking, a cross-linked enzyme preparation (CLE) resulted in which only a small fraction of the original activity is retained (Figure 2).[13]

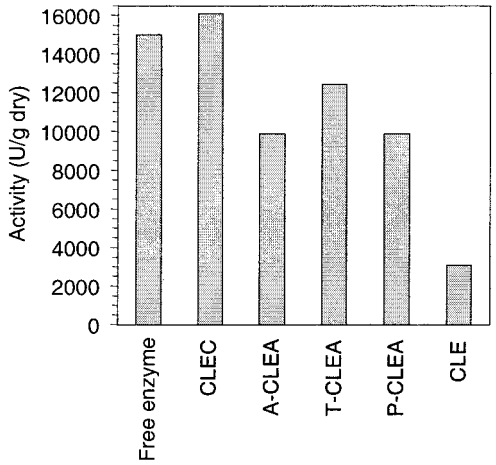

Figure 2. Hydrolytic activity of free (FE) and cross-linked penicillin G acylases. A-CLEA, T-CLEA, and P-CLEA represent the CLEAs prepared respectively by using ammonium sulfate, *tert*-butyl alcohol, and PEG 8000 as the precipitant. Synthesis of CLEAs: penicillin G acylase was dissolved (6 mg/mL) in 0.1 M Mops buffer pH 7 at 0 °C. The precipitant was added slowly to the enzyme solution under stirring. After strirring for 2 h at 0 °C, glutaraldehyde was added to cross-link the aggregate for 2 h at 0 °C. The CLEAs were collected by filtration and washed. CLE represents the preparation obtained by cross-linking penicillin G acylase in solution. Assay: 1 unit (U) of penicillin G acylase will liberate 1 μmol of phenylactic acid per minute.

Despite a partial loss of activity upon isolation of the CLEAs, their specific activity in the hydrolysis of penicillin G (Figure 2) is still on the same order as that of the penicillin G acylase CLEC and substantially higher than that of the CLE. Presumably, in solid enzyme aggregates, as in enzyme crystals, the enzyme molecules can adopt an orderly arrangement in which their conformation is fixed in a rigid superstructure that is essential for their stability and activity and which is maintained upon cross-linking.

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(11) Fernandez-Lafuente, R.; Rosell, C. M.; Guisan, J.-M. *Biotechnol. Appl. Biochem.* **1996,** *24*, 139−143.
(12) Nam, D. H.; Kim, C.; Ryu, D. D. Y. *Biotechnol. Bioeng*. **1985**, *27*, 953−960.
(13) A somewhat related system, involving the cross-linking of spray-dried enzymes, has been claimed in a patent application (Amotz, S. (Novo Industri A/S) 1987, US 4,665,028). However, text, discussion, and examples do not cover the system invented by our group, which has greater robustness and can also be used in organic solvents. When we applied this method to penicillin acylase, only a minute fraction of the native activity was recovered.

Synthesis of Ampicillin. The specific activity of the penicillin acylase CLEAs in the synthesis of ampicillin from D-phenylglycine amide (PGA) and 6-aminopenicillanic acid (6-APA) was comparable to that of the free enzyme (data not shown). We found that the CLEAs mediated the reaction at an S/H ratio that was close to that of the dissolved enzyme, whereas CLEC-EC operated at approximately half that value (Figure 3). Remarkably, the S/H ratio of the T-CLEA

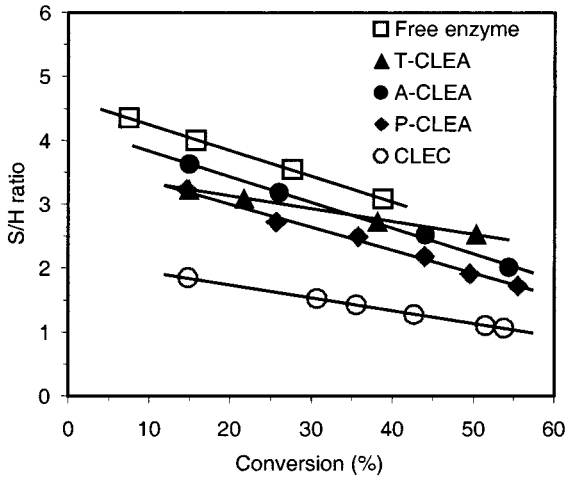

Figure 3. Relationship of S/H ratio with the conversion of 6-APA in the synthesis of ampicillin catalyzed by free and cross-linked penicillin G acylases. S/H was measured as described in ref 6. Reaction conditions: 300 mM 6-APA, 500 mM D-phenylglycine amide, and 20 U penicillin G acylase in 20 mL of water were stirred at pH 7 and 20 °C.

declined less as the reaction proceeded, compared with the free enzyme, A-CLEA, and P-CLEA. Apparently the structure of the enzyme is subtly changed by precipitation with *tert*-butyl alcohol.[14]

The chemical stability of the T-CLEA was checked by removing all solids from the reaction mixture at 60% conversion of 6-APA. The composition of the filtrate remained unchanged over time, demonstrating that no "leakage" of enzyme into the solution had taken place. We also monitored the activity of the T-CLEA in the course of the reaction. Full activity was recovered to the point were ampicillin started to precipitate; at this point approximately 30% of the original activity was lost, presumably because the crystallization caused some breakup of the aggregates. We note that in similar experiments with commercially available immobilized penicillin G acylase preparations no active enzyme was recoved at all due to mechanical disintegration of the catalyst, presumably induced by product crystallization in the matrix.

Reaction in Organic Solvent. With the aim of achieving a high S/H ratio, the enzymatic coupling reaction can be alternatively performed in a hydrophilic organic solvent at low water content. Unfortunately, as noted above, free penicillin G acylase is readily and irreversibly deactivated by organic solvents. On the basis of the high activities and tolerance to organic solvents exhibited by CLECs, we reasoned that CLEAs should also possess these desirable features. Accordingly, we found that CLEAs are active in the synthesis of ampicillin in a broad range of organic solvents (Table 1). log *P*, which is often used as an indicator

Table 1. Synthesis of Ampicillin Catalyzed by T-CLEA in Organic Solvents[a]

solvent	log *P*	convn (%)[b]	S/H
triglyme	−1.8	5	2.0
2-methoxylethyl ether	−1.3	11	2.9
1,2-dimethoxyethane	−0.8	10	3.4
acetonitrile	−0.4	17	4.8
2-ethoxyethyl ether	−0.3	25	1.8
2,2-dimethoxypropane	−0.2	56	1.3
tert-butyl alcohol	0.8	18	1.3
tert-amyl alcohol	1.4	33	1.5

[a] Reaction conditions: 300 mM 6-APA, 500 mM D-phenylglycine amide in 10 mL of solvent−water (95:5, v/v), 100 U ofenzyme at 0 °C. [b] Conversion was measured after 1 h.

of solvent behavior, does not show an obvious correlation with either the reaction rate or S/H.

Because the highest S/H ratio was achieved in acetonitrile (Table 1), this solvent was selected as the reaction medium for further study of the ampicillin synthesis, using CLEAs as well as CLECs. On the basis of dry (i.e., protein) weight, the T-CLEA preparation was more active than the CLEC (Table 2). We also took the hydrolytic activity (amount of

Table 2. Synthesis of Ampicillin in Acetonitrile Catalyzed by Crosslinked Penicillin Acylase[a]

	specific synthetic activity[b]	
biocatalyst	(μmol g^{-1} min^{-1})	(nmol U^{-1} min^{-1})
CLEC	206	1.3
A-CLEA	132	1.2
T-CLEA	214	1.9
P-CLEA	168	1.9
CLE	46	1.2

[a] Reaction conditions as in Table 1. [b] The specific synthetic activity was calculated from the conversion over the first 30 min. It is expressed in μmol of ampicillin per g (dry weight) per minute as well as in nmol of ampicillin per hydrolytic unit per minute.

U), which closely reflects the number of catalytically competent active sites,[15] into account by expressing the synthetic acitivity in nmol U^{-1} min^{-1} (Table 2). It then became apparent that the turnover frequency of the T- and

(14) Similar to the change in enantioselectivity of *C. rugosa* lipase upon treatment with isopropyl alcohol (Colton, I. J.; Ahmed, S. N.; Kazlauskas, R. J. *J. Org. Chem.* **1995**, *60*, 212−217).

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P-CLEA catalysts is 50% higher than that of the CLEC, which was only marginally better than the CLE.

In conclusion, cross-linked enzyme aggregates (CLEAs), formed by physical aggregation and subsequent chemical cross-linking, are highly active and stable biocatalysts. In the synthesis of ampicillin, CLEAs of penicillin G acylase were more efficient catalysts than cross-linked crystals (CLECs) of the same enzyme. Similar to CLECs, CLEAs of penicillin G acylase maintained their activity in organic solvents.

Acknowledgment. The authors thank Dr. E. de Vroom, Dr. E. C. Roos, Dr. J. M. van der Laan, Mr. H. Kierkels, and Prof. Dr. A. Bruggink for their interesting suggestions and helpful discussions. Thanks are due to DSM Anti-Infectives for kind gifts of penicillin G acylase, D-phenylglycine amide, and 6-APA. The spray drying and cross-linking of penicillin G acylase was performed by Messrs. M. H. A. Janssen and L. M. van Langen, to whom the authors express their thanks. Financial support by DSM Life Science Products and the Netherlands Ministry of Economic Affairs is gratefully acknowledged.

Supporting Information Available: Detailed descriptions of experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

OL005593X