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Journal of Molecular Catalysis B: Enzymatic 9 (2000) 275–281

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Penicillin acylase-catalyzed synthesis of cefazolin in water–solvent mixtures: enhancement effect of ethyl acetate and carbon tetrachloride on the synthetic yield

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Received 31 July 1999; received in revised form 20 October 1999; accepted 20 October 1999

Abstract

The effects of organic solvents on the penicillin acylase-catalyzed, kinetically controlled synthesis of cefazolin have been examined in various water–solvent mixtures. In the presence of water-miscible solvents, the initial rate and maximum yield of cefazolin (CEZ) synthesis reaction were found to be reduced. The extent of inhibition was increased with increasing hydrophobicity of the solvent in the reaction mixtures. Enzymatic synthesis of cefazolin was also carried out in the water–solvent biphasic systems. Among the water-immiscible solvents tested, ethyl acetate (EtOAc) and carbon tetrachloride (CCl_4) were found to markedly improve the yield of cefazolin in the two-phase reaction system. Our study showed that the enhancement effect of EtOAc and CCl_4 on the synthetic yield was mainly caused by a reduction of the hydrolysis of acyl donor and product in the two-phase system rather than extraction of the product into the solvent phase.

Keywords: Cefazolin; Penicillin acylase; Organic solvents; Two-phase system; Ethyl acetate; Carbon tetrachloride

1. Introduction

Cefazolin (CEZ), which is a semisynthetic cephalosporanic β-lactam antibiotic, has been synthesized enzymatically in an aqueous buffer solution, but only resulted in a low synthetic yield mainly due to the hydrolytic side reactions [1]. As shown in Scheme 1, penicillin acylase catalyzes not only the synthesis of CEZ but also the hydrolytic reactions of tetrazolylacetic methyl ester (TzAA-OMe) and CEZ. Therefore, prevention of the hydrolysis of acyl donor substrate (TzAA-OMe) and product (CEZ) is desirable for the improvement of synthetic yield of CEZ.

Organic solvents added to the aqueous medium often suppress the enzymatic hydrolysis reactions by lowering the water activity of reaction medium and/or altering the pK values of reactants [2–9]. Owing to recent development, many synthetic reactions have been successfully carried out using hydrolytic enzymes in nonaqueous organic media [10]. On the other hand, organic solvents can reduce the produc-

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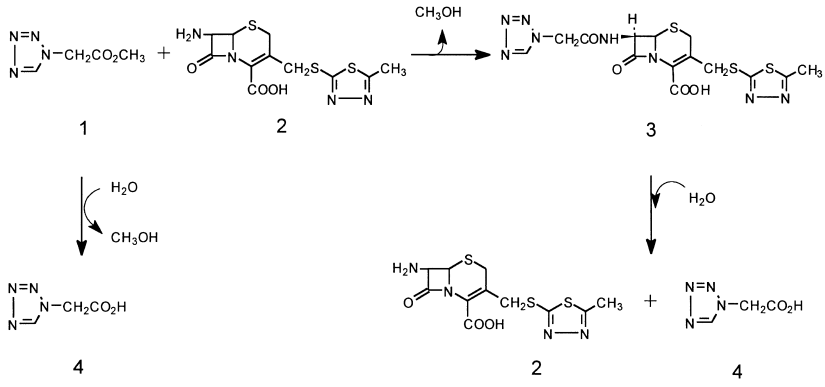
1381-1177/00/$ - see front matter
PII: S1381-1177(99)00106-X



1 : tetrazolylacetic methyl ester

2 : 3-[5-methyl-1,3,4-thiodiazol-2-yl]-7-ACA

3 : cefazolin

4 : tetrazolylacetic acid

Scheme 1. Reaction mechanism for penicillin acylase-catalyzed synthesis of cefazolin.

tion yield by inhibiting the catalytic activity of the enzyme or by lowering the stability of the three-dimensional structure of the enzyme [11–13]. Considering these two opposing effects associated with organic solvents, careful design of the reaction medium is required to obtain a higher synthetic yield of the product in water–solvent mixtures.

Enzymatic synthesis of β-lactam antibiotics in reaction media other than pure aqueous solutions has been previously attempted [14–20]. In the presence of suitable organic solvents or additives, penicillin acylase-catalyzed synthesis of β-lactam antibiotics such as cephalothin, cephalexin, ampicillin, and penicillin G have been reported to attain a higher yield than that in an aqueous buffer solution. For example, in case of a kinetically controlled synthesis of ampicillin, which follows a reaction route similar to that shown in Scheme 1, the maximum yield of ampicillin can be improved to over two-fold with the addition of 40% (v/v) methanol to the aqueous medium [20].

In this work we investigated the effects of organic solvents on the synthesis of CEZ from 3-[5-methyl-1,3,4-thiodiazol-2-yl]-7-amino-cephalosporanic acid (M-7-ACA) and TzAA-OMe using an *Esherichia coli* penicillin acylase (penicillin amidohydrolase; EC 3.5.1.11). This study shows that the synthetic yield of CEZ can be significantly improved in the two-phase system composed of an aqueous buffer and a water-immiscible organic solvent.

2. Materials and methods

2.1. Materials

Eupergit PcA, an *E. coli* penicillin acylase immobilized on Eupergit C, was purchased from Fluka (Switzerland) and used as a biocatalyst. The activity of Eupergit PcA was 106 IU/g (wet). M-7-ACA, TzAA-OMe, and CEZ were kindly provided by Dr. Y.J. Jeon at Cheiljedang (Korea). Acetonitrile used for HPLC eluent were from Malinckrodt (USA). All other reagents were of analytical grade and obtained from Sigma or Aldrich.

2.2. Enzyme reactions

All experiments were carried out at 30°C in vials agitated at 100 rpm in a temperature-con-

trolled incubator shaker (New Brunswick Scientific, USA). For enzymatic reactions in an aqueous solution, 0.1 M sodium phosphate buffer (pH 6.2) was used as a standard reaction medium. Synthesis of CEZ was carried out using 20 mM TzAA-OMe and 5 mM M-7-ACA and hydrolysis of TzAA-OMe and CEZ was carried out at 20 mM TzAA-OMe and 5 mM CEZ, respectively. The reaction was started by adding 0.12 g of enzyme suspensions (Eupergit PcA) to the reaction mixture (10 ml), and 0.1 ml sample was withdrawn during the reaction for the HPLC analysis. Enzymatic reactions in water–solvent mixtures were carried out under the same conditions that were employed in an aqueous solution except that organic solvent was added to the standard aqueous medium composed of phosphate buffer and the reactant(s). The yield of CEZ was determined based on the initial concentration of M-7-ACA and expressed as a percentage.

2.3. Analysis

Substrates and products were identified and analyzed by HPLC (Dionex Bio LC) and a UV detector (250 nm) with a μ-Bondapak C18 column (3.9 × 300 mm, Waters). Eluent was composed of 30% (v/v) acetonitrile and 70% (v/v) deionized water (pH 3.0). The pH of the eluent solution was adjusted with phosphoric acid and the flow rate of the eluent was 1.0 ml/min in all cases.

3. Results and discussion

3.1. Synthesis of CEZ in the single-phase system

We investigated the effect of water-miscible organic solvents on penicillin acylase-catalyzed synthesis of CEZ to examine the possibility of enhancing the CEZ synthesis yield with the addition of polar organic solvents (30% v/v). As shown in Table 1, completely water-miscible solvents such as 1,2-ethanediol, dimethylsulfoxide, dimethylformamide, methanol, and acetonitrile were found to reduce both the initial reaction rate and the maximum yield of CEZ as compared to those obtained in an aqueous buffer solution. The extent of inhibition increased gradually with the solvent hydrophobicity, which is represented by the log P value [21] in Table 1. Considering our previous results on the penicillin acylase-catalyzed synthesis of pivampicillin (PVM) [18,19], where the degree of enzyme inhibition increased in the presence of a less polar organic solvent, the decrease of synthetic rate of CEZ is likely due to interaction of the solvent with penicillin acylase as in the case of PVM synthesis.

Although the initial reaction rates are generally decreased with the addition of water-miscible organic solvents in many penicillin acylase-catalyzed reactions [18–20], dependence of a synthetic yield on the solvent volume varies with chemical natures of the reactants [20]. In

Table 1
Effect of organic solvents on the rate and yield of cefazolin synthesis reaction

Solvents	v/v°[a] (%)	Y/Y°[b] (%)	log P[c] (−)	$S^{d}_{o/w}$ (wt.%)
A. Water-miscible solvents				
1,2-ethanediol	22	64	−1.90	∞
dimethylsulfoxide	16	54	−1.35	∞
dimethylformamide	6	38	−1.00	∞
methanol	8	44	−0.77	∞
acetonitrile	1	11	−0.34	∞
B. Water-immiscible solvents				
methyl acetate	18	109	0.15	24.5
ethyl acetate	96	165	0.67	8.1
butyl acetate	101	105	1.70	0.68
pentyl acetate	104	106	2.13	0.17
cyclohexyl acetate	65	80	2.31	0.29
carbon tetrachloride	114	156	2.98	0.077
hexane	100	103	3.52	0.0012

[a] Relative initial reaction rate: Initial velocity in water–solvent mixtures (v) normalized to that obtained in an aqueous buffer solution (v°).

[b] Relative maximum yield: Maximum yield in water–solvent mixtures (Y) normalized to that obtained in an aqueous buffer solution (Y°).

[c] Hydrophobicity of organic solvent [21].

[d] Solubility of organic solvent in water at 25°C [22].

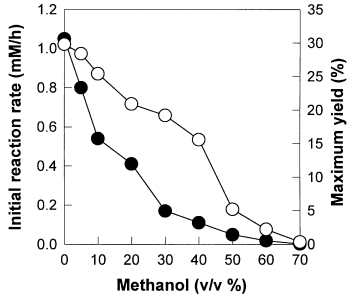


Fig. 1. Effect of solvent content on the enzymatic synthesis of CEZ in water–methanol mixtures. (●) Initial reaction rate, (○) maximum yield. Reaction conditions: 5 mM M-7-ACA, 20 mM TzAA-OMe, 0.1 M phosphate buffer, 10 ml reaction volume, 0.12 g Eupergit PcA, 30°C, and pH 6.2.

the case of ampicillin synthesis, for example, over a two-fold increase of the yield was obtained in 40% (v/v) methanol mixture, but the maximum yield of PVM, which is an ester compound of ampicillin, gradually decreased with increasing methanol fraction in the reaction medium [20]. In this study we tested the effect of methanol content on the synthesis of CEZ. As shown in Fig. 1, both maximum yield and initial reaction rate were gradually decreased with increasing methanol fraction in the reaction mixture, which indicates that the effect of methanol on synthetic yield of CEZ is similar to that of PVM synthesis.

3.2. Synthesis of CEZ in the two-phase system

Although there are several reports concerning the enzymatic synthesis of β-lactam antibiotics in the medium composed of water and water-miscible organic solvents, the synthesis of β-lactam antibiotics in a two-phase reaction medium has not been studied rigorously. In this study, we have also investigated the effect of water-immiscible solvents (30% v/v) on the synthesis of CEZ in the two-phase system (Table 1).

Contrary to the case of water-miscible solvents, water-immiscible solvents used in this work showed lesser suppression of the synthesis rate (except methyl acetate). This may be due to lower solubility of water-immiscible solvents in water (see $S_{o/w}$ in Table 1). When compared among alkyl acetate compounds, the reaction rate increased with increasing solvent hydrophobicity or decreasing the aqueous solubility of the solvent. Although the volume of organic solvent is the same in all cases (30% v/v), solvent concentration in the aqueous phase varies with the solvent solubility. Thus, lower concentration of organic solvents in the aqueous phase may result in a lower degree of enzyme inhibition.

In the presence of cyclohexyl acetate, which is almost as hydrophobic as pentyl acetate, the enzyme was subjected to a relatively higher degree of inhibition. Such greater inhibition of penicillin acylase by cyclic or aromatic solvents has previously been observed: the synthesis rates of pivampicillin and ampicillin were significantly reduced by the presence of ring-structured solvents such as cyclohexanol, cyclohrxanone, dioxane, tetrahydrofuran, pyridine, and phenol [19]. The results presented in this work also support the view that there might be strong interaction of ring-structured compounds with the active site of penicillin acylase.

Among seven water-immiscible solvents tested in this work, EtOAc and CCl_4 showed a significant enhancement of the synthetic yield of CEZ; the relative maximum yields in the corresponding two-phase systems were 165% and 156%, respectively (see Table 1). In Fig. 2,

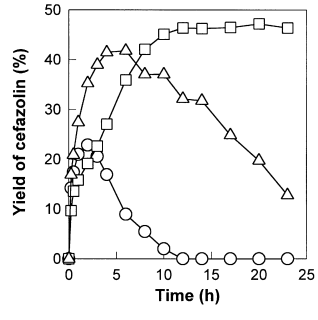


Fig. 2. Effect of reaction medium on the synthesis of CEZ. (○) Aqueous buffer solution, (△) CCl_4/buffer two-phase medium, (□) EtOAc/buffer two-phase medium. Reaction conditions: 20 mM TzAA-OMe, 5 mM M-7-ACA, 10 ml phosphate buffer (0.1 M), 4.3 ml solvent, 0.12 g Eupergit PcA, 30°C, and pH 6.2.

the time profiles of the CEZ synthesis reaction in three different reaction media (buffer, CCl_4/buffer, EtOAc/buffer) are compared. Synthetic profiles in aqueous buffer solution and CCl_4/buffer follow typical kinetically controlled patterns, where hydrolysis of product occurs after the synthetic yield reaches a maximum. In contrast, hydrolytic dissipation of produced CEZ was quite suppressed in the case of the EtOAc/buffer two-phase system. We also investigated the solvent volume effect on the maximum yield of CEZ. It was found that, contrary to the results observed in water-methanol mixtures (see Fig. 1), the maximum yields were not significantly different depending on the volume of EtOAc or CCl_4 (up to 40% solvent) in the reaction medium (Table 2).

3.3. Suppression of hydrolysis reactions in the two-phase system

To examine the role of EtOAc and CCl_4 in enhancing the yield of CEZ production, we investigated the dependence of the hydrolysis of TzAA-OMe and CEZ on reaction media. Hydrolysis reactions were carried out under the same conditions as those used in the CEZ synthesis reaction and the initial concentrations of TzAA-OMe and CEZ were 20 and 5 mM, respectively. The results of the hydrolysis reaction of TzAA-OMe and CEZ in three different reaction media (buffer, CCl_4/buffer, EtOAc/buffer) are shown in Figs. 3 and 4, respectively.

Table 2
Effect of solvent volume fraction on the maximum yield of cefazolin

Solvent fraction (v/v)	$Y/Y°$ (%)[a]	
	EtOAc/buffer (%)	CCl_4/buffer (%)
Saturated buffer[b]	112	100
9.1%	137	155
16.7%	152	153
28.6%	158	149
33.3%	152	148

[a] Relative maximum yield.
[b] Solvent volume fraction in saturated buffer solutions: 8.1% (EtOAc), 0.08% (CCl_4).

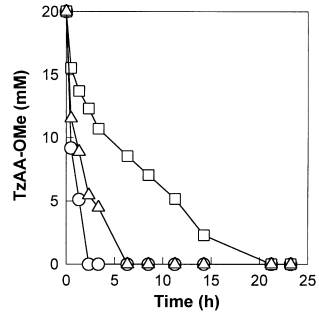


Fig. 3. Effect of reaction medium on the hydrolysis of TzAA-OMe. (○) Aqueous buffer solution, (△) CCl_4/buffer two-phase medium, (□) EtOAc/buffer two-phase medium. Reaction conditions: 20 mM TzAA-OMe, 10 ml phosphate buffer (0.1 M), 4.3 ml solvent, 0.12 g Eupergit PcA, 30°C, and pH 6.2.

In an aqueous buffer system, 20 mM of TzAA-OMe was completely hydrolyzed to the corresponding acid, tetrazolylacetic acid (TzAA), in about 3 h (Fig. 3). When the same reaction was carried out in CCl_4/buffer, TzAA-OMe was not completely hydrolyzed until 6 h, and in EtOAc/buffer, TzAA-OMe took about 22 h for complete hydrolysis. It is interesting to note that the reaction time at a maximum conversion of CEZ approximately corresponds to the time at which an acyl donor (TzAA-OMe) is completely hydrolyzed.

When 5 mM of CEZ was hydrolyzed with Eupergit PcA in an aqueous buffer solution, more than 90% of CEZ was hydrolyzed within

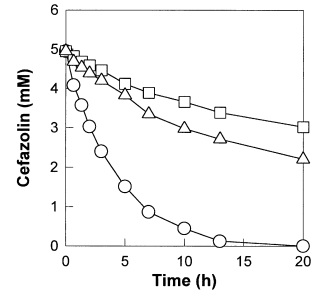


Fig. 4. Effect of reaction medium on the hydrolysis of CEZ. (○) Aqueous buffer solution, (△) CCl_4/buffer two-phase medium, (□) EtOAc/buffer two-phase medium. Reaction conditions: 5 mM CEZ, 10 ml phosphate buffer (0.1 M), 4.3 ml solvent, 0.12 g Eupergit PcA, 30°C, and pH 6.2.

10 h, whereas in the two-phase system containing either EtOAc or CCl_4, the hydrolysis rates of CEZ were remarkably reduced and more than half of initial CEZ remained in the reaction mixture after 20 h of reaction (Fig. 4). From these results, we can deduce that the enhancement effects of EtOAc and CCl_4 on the CEZ synthesis are closely related to the suppression effect of these solvents on the hydrolysis of TzAA-OMe and CEZ.

3.4. Partitioning of substrates and product in the two-phase system

When the product is subjected to hydrolysis in an aqueous medium, synthetic yield of the product can be improved if the product is extracted into the solvent phase in the two-phase system. It was however found that CEZ was hardly solubilized in either EtOAc or CCl_4, and that most of CEZ was partitioned into the aqueous phase in the corresponding two-phase system. The distribution coefficient (D), defined as the ratio of the concentration in the solvent phase to that in the aqueous phase at pH 6.2, was lower than 0.01. This indicates that an increase of maximum yield in the two-phase system was not caused by extraction of the synthesized CEZ to the solvent phase.

We also examined the distribution of the substrates in EtOAc/water and CCl_4/water two-phase systems. Similar to the result of CEZ, most of M-7-ACA was partitioned to the aqueous phase in both solvent systems ($D < 0.01$). On the other hand, TzAA-OMe was partitioned into the solvent phase in the EtOAc/water system ($D = 2.1$), whereas no appreciable partitioning of TzAA-OMe was observed in the CCl_4/water system ($D < 0.01$). Lower hydrolysis rate of TzAA-OMe in EtOAc/water than in CCl_4/water can be explained, in part, by the partitioning of TzAA-OMe into the solvent (EtOAc) phase. However, it is unlikely that the partitioning of substrates and/or products plays a major role in enhancing the yield of CEZ in the two-phase system.

3.5. Concluding remarks

In this study, we have investigated the synthesis of CEZ in water–solvent mixtures and found that maximum yield of CEZ can be improved in the two-phase system composed of EtOAc/buffer or CCl_4/buffer. Further experiments showed that hydrolysis of TzAA-OMe and CEZ could be suppressed to a greater extent with the addition of EtOAc or CCl_4 to the reaction medium. On the other hand, there was no appreciable partitioning of CEZ to the solvent phase tested. It appears that the main reason for the increase in the CEZ synthesis yield is a reduction of side reactions (hydrolysis of TzAA-OMe and CEZ; see Scheme 1) in the two-phase system rather than partition of the product into the organic phase. This reaction system is therefore different from water–solvent two-phase systems, where extraction of the product plays an important role in increasing the production yield [23–25].

To the author's knowledge, this is the first report that shows an improvement of the yield of kinetically controlled β-lactam antibiotics synthesis reaction in the two-phase system. The use of water-immiscible solvents in enzyme-catalyzed synthesis reaction appears to be very attractive because enzymes are generally more stable in water-immiscible solvents than in water-miscible solvents. Before this approach could become practical, however, the general principle related to the positive effect of water-immiscible solvents should be further studied with several other solvents for penicillin acylase-catalyzed reactions. Further studies on the detailed mechanism of the enzyme reaction and the effects of solvent–enzyme and solvent–substrate, and solvent–product interactions remain to be performed in the future.

Acknowledgements

This work was supported by the Korea Science and Engineering Foundation and the Ministry of Education.

References

[1] M. Kostadinov, A. Nikolov, N. Tsoneva, N. Petkov, Appl. Biochem. Biotechnol. 33 (1992) 177.
[2] R.G. Ingalls, R.C. Squires, L.G. Butler, Biotechnol. Bioeng. 17 (1975) 1627.
[3] G.A. Hommandberg, A. Mattis, M. Laskowski Jr., Biochemistry 17 (1978) 5220.
[4] K. Martinek, A.N. Semenov, J. Appl. Biochem. 3 (1981) 93.
[5] K. Nilsson, K. Mosbach, Biotechnol. Bioeng. 26 (1984) 1146.
[6] H.D. Jakubke, P. Kuhl, A. Konnecke, Angew. Chem., Int. Ed. Engl. 24 (1984) 85.
[7] A.M. Klibanov, CHEMTECH 16 (1986) 354.
[8] C. Laane, J. Tramper, CHEMTECH 20 (1990) 502.
[9] K.-A. Hwang, S.B. Lee, K.H. Lee, Biotechnol. Lett. 17 (1995) 71.
[10] A.M.P. Koskinen, A.M. Klibanov (Eds.), Enzymatic Reactions in Organic Media, Chapman & Hall, London, 1996.
[11] V.V. Mozhaev, Y.L. Khmelnitsky, M.V. Sergeeva, A.B. Belova, N.L. Klyachko, A.V. Levashov, K. Martinek, Eur. J. Biochem. 184 (1989) 597.
[12] G. Carrea, G. Ottolina, S. Riva, Trends Biotechnol. 13 (1995) 63.
[13] G. Bell, P.J. Halling, B.D. Moore, J. Patridge, D.G. Rees, Trends Biotechnol. 13 (1995) 468.
[14] V. Kasche, U. Haufler, L. Riechmann, Methods Enzymol. 136 (1987) 280.
[15] F.R. Lafuente, C.M. Rosell, J.M. Guisan, Enzyme Microb. Technol. 13 (1991) 898.
[16] F.R. Lafuente, G. Alvaro, R.M. Blanco, J.M. Guisan, Appl. Biochem. Biotechnol. 27 (1991) 277.
[17] C.K. Hyun, J.H. Choi, J.H. Kim, D.D.Y. Ryu, Biotechnol. Bioeng. 41 (1993) 654.
[18] M.G. Kim, S.B. Lee, J. Mol. Catal. B: Enzym. 1 (1996) 71.
[19] M.G. Kim, S.B. Lee, J. Mol. Catal. B: Enzym. 1 (1996) 181.
[20] M.G. Kim, S.B. Lee, J. Mol. Catal. B: Enzym. 1 (1996) 201.
[21] C. Hansch, A. Leo, D. Hoekman, Exploring QSAR: Hydrophobic, Electronic, and Steric Constant, American Chemical Society, Washington, DC, 1995.
[22] I.M. Smallwood, Handbook of Organic Solvent Properties, Halsted, New York, 1996.
[23] G. Carrea, Trends Biotechnol. 2 (1984) 102.
[24] K. Martinek, A.N. Semenov, Biochim. Biophys. Acta 658 (1981) 76, 90.
[25] K. Morihara, Trends Biotechnol. 5 (1987) 164.