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The Effect of 2-Mercapto-5-Methyl-1,3,4-Thiadiazole on Enzymatic Synthesis of Cefazolin

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ABSTRACT

The effect of unreacted residual 2-mercapto-5-methyl-1,3,4-thiadiazole (MMTD), the reagent for 3-[5-methyl-1,3,4-thiadiazole-2-yl]-7-aminocephalosporanic acid (M-7-ACA) synthesis, on the enzymatic acylation of M-7-ACA by the methyl ester of 1,2,3,4-tetrazol-1-acetic acid (MeTzAA) to produce cefazolin (CEZ) was studied. In the two-step process of synthesizing CEZ from 7-aminocephalosporanic acid (7-ACA), one of the key parameters controlling the overall CEZ yield was the ratio of MMTD to 7-ACA in M-7-ACA synthesis. The increase of the ratio showed opposing effects by increasing the M-7-ACA yield in the first step, while decreasing CEZ yield in the subsequent enzymatic reaction by the inhibitory effect of the increased content of MMTD as an impurity in the M-7-ACA preparation. It was revealed that the decrease of CEZ yield in the enzymatic reaction was caused by the selective retardation of the rate of CEZ synthesis reaction by a typical competitive inhibition, while not affecting the rate of MeTzAA hydrolysis reaction. The optimum MMTD-to-7-ACA ratio rendering the highest overall CEZ yield over 7-ACA was 1.2:1.

Index Entries: Cefazolin; enzymatic synthesis; kinetics; inhibition; 2-mercapto-5-methyl-1,3,4-thiadiazole; 7-aminocephalosporanic acid.

INTRODUCTION

Since the early 1970s, numerous attempts to prepare semisynthetic cephalosporin antibiotics by enzymatic methods, instead of conventional

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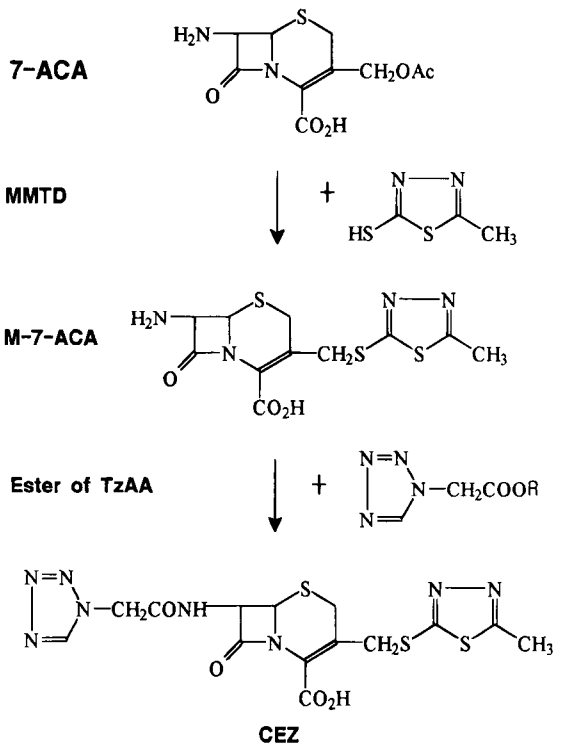

Fig. 1. General scheme of synthesis of CEZ from 7-ACA. Abbreviations: 7-ACA = 7-aminocephalosporanic acid, MMTD = 2-mercapto-5-methyl-1,3,4-thiadiazole, M-7-ACA = 3-[5-methyl-1,3,4-thiadiazole-2-yl]-7-aminocephalosporanic acid, TzAA = 1,2,3,4-tetrazol-1-acetic acid, CEZ = cefazolin.

chemical methods, have been reported *(1)*. The major advantages of an enzymatic process in cephalosporin antibiotic production are that the enzymatic process is usually less complicated than a chemical process, because of the high selectivity of biocatalysts, and that it is more environment friendly, since it does not require toxic solvents. Despite these advantages, successful commercialization of the enzymatic process so far is limited, because of lack of overall economic feasibility, to only a small number of cephalosporins, such as 7-aminodeacetoxicephalosporanic acid (7-ADCA), cephalexin, and 7-aminocephalosporanic acid (7-ACA) *(2)*. Cefazolin (CEZ) is one of the cephalosporin antibiotics that is mass-produced by a chemical process; alternative ways of producing it by enzymatic methods have been extensively studied. Various microorganisms, including *Escherichia coli (3)*, *Arthrobacter viscoseous (4)*, *Baccilus megaterium (5)*, and *Kluyvera noncitrophila (6)* are reported to produce penicillin amidase, E.C. 3.5.1.11, capable of synthesizing CEZ.

Figure 1 represents the overall scheme of the process synthesizing CEZ from 7-ACA. The process consists of two major reaction steps: The first step prepares 3-[5-methyl-1,3,4-thiadiazole-2-yl]-7-aminocephalos-

poranic acid (M-7-ACA) by replacing the acetyl group of the C-3 chain of 7-ACA with 2-mercapto-5-methyl-1,3,4-thiadiazole (MMTD); the second step synthesizes CEZ by the acylation of M-7-ACA by a derivative of 1,2,3,4-tetrazol-1-acetic acid (TzAA). The first reaction is common to both the chemical process and the enzymatic process. It is general practice to use excess amounts of MMTD to enhance the M-7-ACA yield over 7-ACA, since the price of 7-ACA is much higher than that of MMTD. When an excess amount of MMTD is used, however, a large portion of the residual MMTD coprecipitates with M-7-ACA, and is contained in M-7-ACA crystals as an impurity in the subsequent recovery step. The second reaction can be carried out either chemically or enzymatically. In conventional chemical processes, TzAA reacts with M-7-ACA at a very low temperature (–35 ~ –20°C). A large amount of toxic organic solvents, such as acetonitrile, triethylamine, pyvaloyl chloride, and methylene chloride, are required for the chemical reaction to proceed. In the enzymatic process, on the other hand, the reaction is carried out near room temperature, and a biocatalyst and aqueous buffer solution can replace all those organic chemicals potentially harmful to the environment. Because of these advantages, it is expected that the enzymatic process could compete with a chemical process if some key problems of the enzymatic process, such as the low product yield and the low solubility of M-7-ACA in the operating condition, are solved. In the process of improving the biocatalyst and the enzymatic reaction system, it was found that the enzymatic reaction was influenced by the quality of the M-7-ACA produced by the first reaction, and that MMTD was the substance that caused the problems. The present work reports these findings.

MATERIALS AND METHODS

Biocatalyst

The biocatalyst used in this study was prepared by immobilizing penicillin amidase extracted from *E. coli* CFC-04017, a mutant strain of *E. coli* (ATCC 9637). The conventional crosslinking technique with acryl amide was adopted as the immobilization method *(7)*. The fresh biocatalyst thus prepared contained CEZ-synthesizing activity of about 400 U/g on a dry basis.

Chemicals

7-ACA produced by Cheil Jedang, (Seoul, Korea) was used to synthesize M-7-ACA. MMTD and TzAA were supplied by Wako Chemical (Osaka, Japan). Methyl ester of 1,2,3,4-tetrazol-1-acetic acid (MeTzAA) were prepared by esterification of TzAA by a conventional method *(8)*.

Synthesis of M-7-ACA

M-7-ACA was synthesized by the method previously developed *(9)*. Twenty g/L of 7-ACA, and controlled amounts of MMTD, were dissolved in 0.1 *M* sodium bicarbonate buffer, pH 6.0. The reaction was carried out to equilibrium for 3–5 h at 65°C. After that, the M-7-ACA produced was recovered by precipitating it at pH 4.0.

Enzymatic Synthesis of CEZ

The enzymatic reaction was carried out in an 1 L jacketed reactor and the temperature was maintained at 30°C throughout the experiment. Thirty g/L of M-7-ACA dissolved in 0.3 *M* ammonium phosphate buffer, pH 7.5, was poured into the reactor containing biocatalyst suspension. The amount of biocatalyst was adjusted to provide 5.5 U/mL of enzyme dosage. The reaction was started by adding MeTzAA into the reactor. After 45–60 min, pH was dropped to about 6.3–6.4 by the hydrolysis of MeTzAA, and the reaction was stopped. The biocatalyst was recovered from the reaction mixture by vacuum filtration and returned to the reactor for the subsequent runs.

Assay Methods

CEZ and related compounds were assayed by HPLC equipped with a μ-Bondapack C18 column (Waters, Milford, MO) and a 214-nm UV detector. The mobile phase was 0.05 *M* solution of $NH_4H_2PO_4$ and methanol with a ratio of 75:25, and the flow rate was 1.2 mL/min.

The unit of CEZ-synthesizing activity is defined as one mol of CEZ formed for 10 min under the following conditions: initial pH 7.4, temperature 30°C, and initial concentrations of M-7-ACA and MeTzAA 75 m*M* and 300 m*M*, respectively. The amount of biocatalyst is adjusted to convert about 10–20% of M-7-ACA to CEZ.

RESULTS AND DISCUSSION

General Behavior of the Enzymatic Reaction

Besides the synthesis of CEZ, the biocatalyst simultaneously catalyzes two other enzymatic reactions; MeTzAA is hydrolyzed to its acid form and CEZ is hydrolyzed back to M-7-ACA and TzAA. The following is the scheme of the three reactions catalyzed by the biocatalyst:

Main Reaction

$$\text{M-7-ACA} + \text{MeTzAA} \rightarrow \text{CEZ} + CH_3OH \quad (1)$$

Side Reactions

$$\text{MeTzAA} + H_2O \rightarrow \text{TzAA} + CH_3OH \quad (2)$$

$$\text{CEZ} + H_2O \rightarrow \text{M-7-ACA} + \text{TzAA} \quad (3)$$

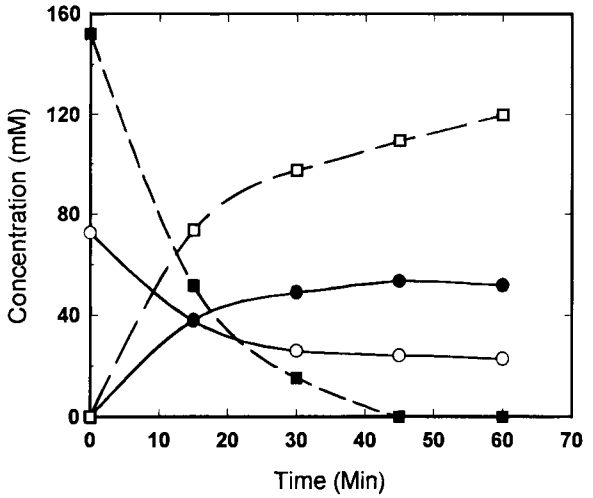

Fig. 2. Typical time pattern of enzymatic CEZ synthesis. Enzyme dosage = 5.5 U/mL, initial pH = 7.5, temperature = 30°C, [MeTzAA]/[M-7-ACA] = 2.0:(○), M-7-ACA; (●), CEZ; (■), MeTzAA; (□), TzAA.

The two most important parameters affecting the equilibrium and kinetic behaviors of these reactions are pH and the ratio of MeTzAA to M-7-ACA. The optimum initial pH, determined by taking into account its effects on the reaction kinetics, as well as on the solubility of M-7-ACA, was 7.5. It has been reported that CEZ yield can be increased by increasing the molar ratio of MeTzAA to M-7-ACA *(3,10)*. However, the practical ratio should be determined based on the overall process economics, considering not only the product yield, but also the prices of substrates and the effect on subsequent steps. The optimum ratio determined in this way was 2.0:1.

Figure 2 demonstrates a typical time-course of the enzymatic reaction under this condition. In this system, there are two acyl acceptors competing with each other to take the acyl moity from MeTzAA. Consequently, the proportion of MeTzAA consumed by the main reaction *(1)* is only 36%, and the remaining 64% is hydrolyzed to TzAA by the reaction *(2)*. The molar yield of CEZ reaches its maximum, 72%, at 45 min of incubation time, when MeTzAA is exhausted. After that the CEZ concentration decreased slowly by the reverse reaction *(3)*.

The general behavior of this enzymatic reaction system appears to be analogous to other reactions catalyzed by penicillin amidases to produce β-lactam antibiotics, such as cephalexin or benzylpenicillin *(11–13)*.

Effect of MMTD on the Enzymatic Reaction

An important finding in this study was that the enzymatic reaction was influenced by the content of MMTD in M-7-ACA. As previously men-

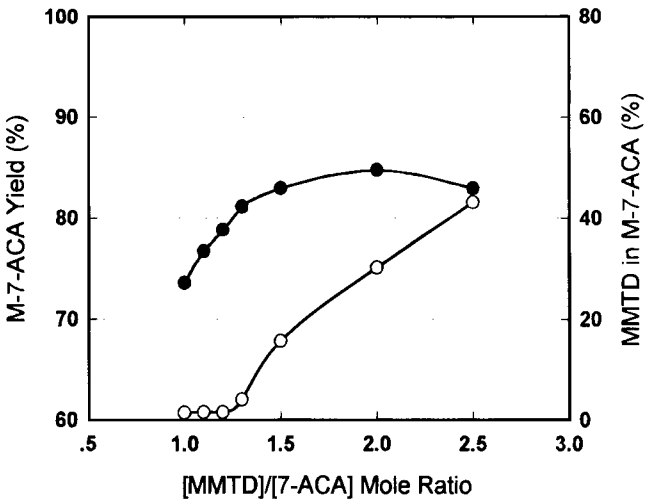

Fig. 3. The effect of the molar ratio of MMTD to 7-ACA on M-7-ACA synthesis. 20 g/L of 7-ACA and controlled amounts of MMTD was dissolved in 0.1 *M* sodium bicarbonate buffer (pH 6.0). Reaction time = 4 h, temperature = 65°C: (●), M-7-ACA yield; (○), MMTD content in M-7-ACA.

tioned, MMTD is contained in M-7-ACA as an impurity in the recovery step when excess amount of MMTD is used. Figure 3 shows the patterns of increase of both the M-7-ACA yield and the MMTD content in the M-7-ACA product as the ratio of MMTD to 7-ACA is increased. A maximum M-7-ACA yield of 85% was obtained when the molar ratio of MMTD to 7-ACA ≥ 2.0:1. It is notable that the MMTD content in M-7-ACA remains essentially zero until the ratio > 1.2:1. The MMTD content in M-7-ACA thus prepared strongly affected the enzymatic synthesis of CEZ in the next step. Figure 4 shows the gradual decrease of CEZ yield as the MMTD content in M-7-ACA increases. This phenomenon was explained by studying the basic inhibition kinetics of MMTD on enzymatic reactions. Initial reaction rate for each M-7-ACA concentration with varying MMTD concentration was measured, and the result was presented in Hanes-Woolfe plot. (Figure 5) The parallel straight lines on this plot clearly show that MMTD is a competitive inhibitor of the CEZ synthesis reaction (reaction [1]). The inhibition constant, K_i, calculated from this plot is 40 m*M*. On the contrary, the rate of MeTzAA hydrolysis reaction (reaction [2]) was not affected by the presence of MMTD (Figure 6). Since the reaction (1) and the reaction (2) compete with each other for limited amount of MeTzAA, the decrease of the rate of reaction (1) in the presence of MMTD results in the decrease of the final CEZ yield.

As a whole, the increase of MMTD to 7-ACA ratio exerts opposing effects in each step by increasing M-7-ACA yield in the first reaction, while decreasing CEZ yield in the second reaction as a result of

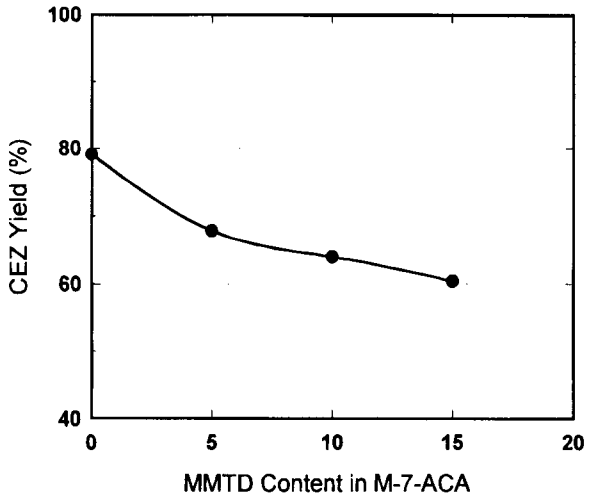

Fig. 4. The effect of MMTD content in M-7-ACA on CEZ yield in the enzymatic synthesis of CEZ. Enzyme dosage = 5.5 U/mL, reaction time = 50 min, initial pH = 7.5, temperature = 30°C, [MeTzAA]/[M-7-ACA] = 2.0.

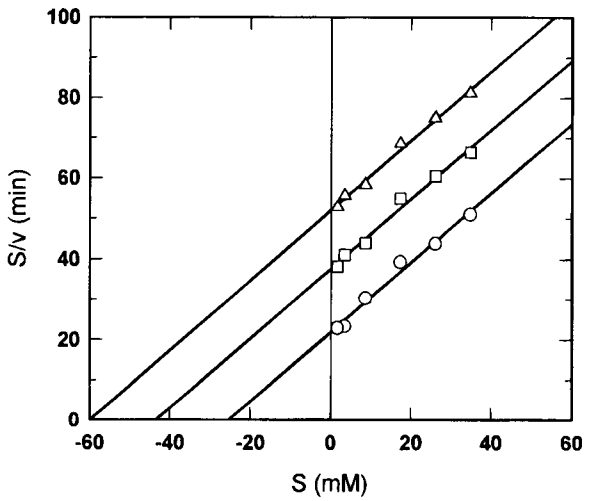

Fig. 5. Hanes-Woolfes plot of the enzymatic reaction for CEZ synthesis challenged with various concentrations of MMTD. S and v denote the substrate concentration and the reaction rate, respectively. Enzyme dosage = 5.5 U/mL, initial pH = 7.5, temperature = 30°C, [MeTzAA] = 100 m*M*, reaction time = 10 min. Concentration of MMTD: (○), 0 m*M*; (□), 30 m*M*; (△), 60 m*M*. Each data point is the average of three trials.

increased MMTD content in M-7-ACA. In Table 1, the overall yield of CEZ over 7-ACA was calculated for each value of MMTD-to-7-ACA molar ratio. The optimum MMTD-to-7-ACA ratio rendering the highest overall yield was found to be 1.2:1. In the large-scale production, however, the value should be determined taking into account not only the

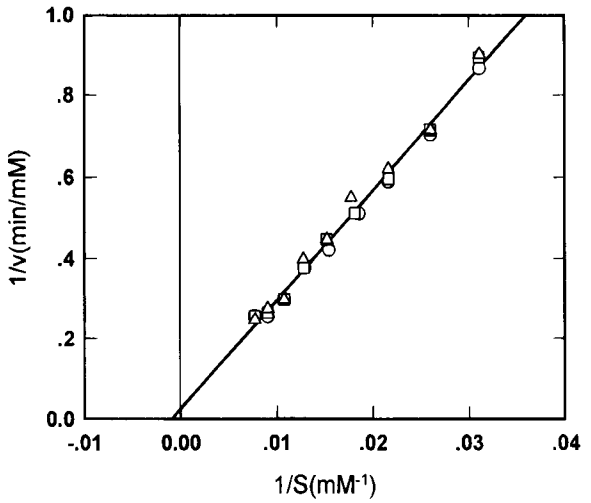

Fig. 6. Double reciprocal plot of the enzymatic reaction for MeTzAA hydrolysis challenged with various concentrations of MMTD. S and v denote the substrate concentration and the reaction rate, respectively. Enzyme dosage = 5.5 U/mL; initial pH = 7.5; temperature = 30°C, [MeTzAA] = 140 m*M*, reaction time = 5 min. Concentration of MMTD: (○), 0 m*M*; (□), 30 m*M*; (△), 60 m*M*. Each data point is the average of three trials.

Table 1
Optimization of Molar Ratio of MMTD to 7-ACA in Chemical Synthesis of M-7-ACA Used as Substrate for Enzymatic Synthesis of CEZ

Molar ratio of MMTD to 7-ACA	1.0	1.1	1.2	1.3	1.5
M-7-ACA yield (%)[a] A	73.6	76.7	78.8	81.1	82.9
MMTD content (%)	1.4	1.5	1.5	4.0	15.7
CEZ yield (%)[b] B	75.9	75.7	75.7	70.1	59.9
Total yield (%) A × B	55.9	58.1	59.7	56.9	49.7

[a] These values were taken from Fig. 3.
[b] These values were taken from Fig. 4 by linear interpolation between data points.

overall yield, but also all other economic factors, such as raw material prices, utilities, and labor.

REFERENCES

1. Matsumoto, K. (1980), *Kakko to Kogyo* **38,** 216–237.
2. Nikkei Biotechnology Annual Report (1994), Nikkei BP, Tokyo.
3. Kostardinov, M., Nikolov, N., Tsoneva, N., and Petkov, N. (1992), *Appl. Biochem. Biotechnol.* **33,** 177–182.
4. Kuboda, I. Banyu Pharmaceuticals (1979), Japan Patent 79-59397.

5. Fujii, T., Sibuya, Y., and Matsuda, T. Toyo Jozo Co. (1979), Japan Patent 79-31080.
6. Kimura, K., Mori, Y., Shimizu, M., Masuike, T., and Fujida, H. Kyowa Hakko Kogyo Co. (1979), Japan Patent 79-31078.
7. O'Driscoll, K. F. (1976), in *Methods in Enzymology*, vol. 44, Mosbach K., ed., Academic, New York, pp. 169–183.
8. Brenner, M. and Huber, W. (1953), *Helv. Chim. Acta.* **36,** 1109–1114.
9. Jackson, B. Eli Lilly (1978), US Patent 4,115,645.
10. Satarova, D., Kurochkina, V., Libinson, G., and Nys, P. (1993), *Proceedings of 6th European Congress on Biotechnology*, vol. 1, TU255.
11. Nam, D. H., Kim, C., and Ryu, D. D. Y. (1985), *Biotechnol. Bioeng.* **27,** 953–960.
12. Blinkovsky, A. M. and Markaryan, A. N. (1993), *Enzyme Microbiol. Technol.* **15,** 965–973.
13. Svedas, A. N., Margolin, I. L., Borisov, I. L., and Berezin, I. V. (1980), *Enzyme Microbiol. Technol.* **2,** 313–317.