Enzymatic Synthesis of Amoxicillin by the Cell-Bound α-Amino Acid Ester Hydrolase of Xanthomonas citri

Koichi KATO, Kenji KAWAHARA, Takeshi TAKAHASHI* and Seizi IGARASHI**

Microbiological Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532, Japan

Received October 4, 1979

Whole cells of Xanthomonas citri K24, a penicillinase-deficient α-amino acid ester hydrolase producer, were effectively used for synthesis of amoxicillin from D-α-(p-hydroxyphenyl)-glycine methyl ester (HPGME) and 6-aminopenicillanic acid (6-APA). The yield of amoxicillin from 6-APA was increased by reducing the ionic strength of the reaction mixture and by adding 2-butanol to the reaction mixture. Increasing the HPGME/6-APA ratio above 1:1 raised the yield of amoxicillin. The optimum pH for the synthetic reaction was between 6 and 7, and the optimum temperature, 20°C. The addition of 2-butanol repressed the enzymatic hydrolysis of HPGME, and enhanced the acylation of 6-APA. More than 90% of the added 6-APA was converted into amoxicillin under the optimum conditions.

Attempts to synthesize some penicillins by means of penicillin amidase [EC. 3.5.1.11] have been made by several workers. The enzymatic synthesis of benzylpenicillin from 6-aminopenicillanic acid (6-APA) and phenylacetic acid by bacterial penicillin amidase was first reported in 1960 by Rolinson et al. and Claridge et al. Kaufmann et al. found that Escherichia coli transferred the acyl groups from various phenylacetic acid derivatives to 6-APA. Later, Cole demonstrated enzymatic syntheses of p-hydroxybenzylpenicillin and ampicillin by means of E. coli penicillin amidase. Nara et al. and Okachi et al. reported the synthesis of ampicillin by Kluyvera citrophila and Pseudomonas melanogenum, respectively.

We described in 1972 that some bacteria belonging to the family Pseudomonadaceae catalyzed N-acylation of 7-aminocephem compounds with α-amino acid esters. By using these bacterial strains we synthesized various semi-synthetic cephalosporins having α-amino acid side chains, including cephalexin and cephaloglycin. The bacteria also acylated 6-APA with α-amino acid esters to give various semi-synthetic penicillins. The substrate specificity of the enzyme catalyzing these synthetic reactions was different from those of penicillin amidases and other known enzymes. The enzyme, which we named α-amino acid ester hydrolase, catalyzed the hydrolysis of α-amino acid esters and the transfer of acyl group from the amino acid esters to such acyl acceptors as 7-aminocephem compounds and 6-APA.

Using these bacterial strains producing α-amino acid ester hydrolase, we tried to synthesize amoxicillin from D-α-(p-hydroxyphenyl) glycine methyl ester (HPGME) and 6-APA. In this paper we describe the synthesis of amoxicillin by means of a penicillinase-deficient mutant strain, X. citri K24.

MATERIALS AND METHODS

Microorganism and culture conditions. X. citri K24,
a penicillinase-deficient mutant strain derived from *X. citri* IFO 3835, was used. The microorganism was cultured and harvested as described previously. The washed cells were suspended in water at a concentration of 70 g in wet weight/liter and stored at -20°C until use. One milligram in wet weight of the washed cells showed 0.2 unit of α-amino acid ester hydrolase activity. One unit of the enzyme activity was defined as the activity that hydrolyzed 1 μmol of D-α-phenylglycine methyl ester at pH 6.0 and 30°C.

**Chemicals.** HPGME hydrochloride, [α]_D+126° (c=1.0, H2O), was prepared from D-α-(p-hydroxyphenyl) glycine (HPG) by the method of von Brenner and Huber. 6-APA was a product of Gist-Brocades N. V., Delft. Aminex A-27 was purchased from Bio-Rad Laboratories Inc., Calif. Other chemicals were of reagent grade and were used without further purification.

**Determination of amoxicillin, HPG and HPGME.** A high-performance liquid chromatograph (Waters Associates, model 202) was used to determine the concentrations of amoxicillin, HPG and HPGME in the reaction mixture. Ten microliters of the reaction mixture appropriately diluted with 0.05 M citrate buffer (pH 5.0) was applied to a column (2.2 x 450 mm) of Aminex A-27 resin, and eluted with 0.35 M sodium acetate buffer (pH 4.0) at a flow rate of 0.4 ml/min. The column temperature was kept at 45°C and the effluent was monitored by the absorbance at 254 nm. Under these conditions the retention times for HPGME, HPG and amoxicillin were 2, 4 and 7.5 min, respectively.

**Determination of 6-APA.** The colorimetric method of Marrelli was used for the determination of 6-APA in the reaction mixture.

**RESULTS**

**Enhancement of amoxicillin synthesis by reduction of ionic strength**

The reaction mixture (pH 7.0) containing HPGME hydrochloride (20 mg/ml), 6-APA (10 mg/ml), potassium phosphate buffer (100 mM) and washed cells of *X. citri* K24 (35 mg/ml) was incubated with stirring at 20°C. The concentration of the synthesized amoxicillin reached a plateau after about 6 hr and the ratio of conversion of 6-APA into amoxicillin was only 23% on a molar basis (Fig. 1). Both the reaction rate and the yield of amoxicillin were increased by omitting phosphate buffer and controlling the reaction pH with an NaOH solution (Fig. 1). Figure 2 shows that the synthetic reaction was strongly inhibited by high concentrations of phosphate buffer or NaCl. Plots of the degree of inhibition versus log ionic strength of phosphate or NaCl gave straight lines (Fig. 2).

**Effect of alcohols on amoxicillin synthesis**

Some alcohols increased the ratio of conver-
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TABLE I. STIMULATIVE EFFECT OF ALCOHOLS ON AMOXICILLIN SYNTHESIS

The reaction mixture (100 ml) containing HPGME hydrochloride (20 mg/ml), 6-APA (10 mg/ml) and washed cells of X. citri K24 (30 mg wet weight/ml) was incubated at 15°C and pH 6.8 for 6 hr with stirring. Each alcohol was added to 5% (v/v).

<table>
<thead>
<tr>
<th>Alcohol added</th>
<th>Conversion of 6-APA into amoxicillin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>50</td>
</tr>
<tr>
<td>Methanol</td>
<td>48</td>
</tr>
<tr>
<td>Ethanol</td>
<td>65</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>71</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>74</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>79</td>
</tr>
<tr>
<td>2-Butanol</td>
<td>86</td>
</tr>
<tr>
<td>2-Methyl-2-propanol</td>
<td>75</td>
</tr>
<tr>
<td>1-Pentanola</td>
<td>73</td>
</tr>
<tr>
<td>2-Methyl-2-butanol</td>
<td>72</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>65</td>
</tr>
<tr>
<td>1-Octanol</td>
<td>60</td>
</tr>
</tbody>
</table>

* Partially insoluble in the reaction mixture.

TABLE II. EFFECT OF CONCENTRATION OF 2-BUTANOL ON AMOXICILLIN SYNTHESIS

Reaction conditions were the same as those described in Table I except that the temperature was raised to 20°C and the reaction time was 12 hr.

<table>
<thead>
<tr>
<th>2-Butanol (v/v)</th>
<th>Conversion of 6-APA into amoxicillin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>2.5</td>
<td>82</td>
</tr>
<tr>
<td>5.0</td>
<td>93</td>
</tr>
<tr>
<td>7.5</td>
<td>93</td>
</tr>
<tr>
<td>10.0</td>
<td>91</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of pH (A) and Temperature (B) on Amoxicillin Synthesis by X. citri K24 in the Presence of 5% (v/v) 2-Butanol.

(A): the reaction mixture (100 ml) containing per ml, 20 mg of HPGME hydrochloride, 10 mg of 6-APA and 35 mg of washed cells of X. citri K24 was incubated with stirring at 20°C for 8 hr in the presence of 5% (v/v) 2-butanol. The pH of the reaction mixture was kept at the values indicated in the Figure by the addition of an NaOH solution. (B): the reaction conditions were the same as those for (A), except that the pH was kept at 6.8 and that the temperature was varied from 15°C to 35°C.

**Optimum pH and temperature**

As shown in Fig. 3 (A), the optimum pH for...
amoxicillin synthesis was 6–7. In our previous paper, we described that the optimum temperature of the *Xanthomonas* α-amino acid ester hydrolase was 35°C for the transfer reaction. However, in the presence of 5% (v/v) 2-butanol, the ratio of conversion of 6-APA into amoxicillin diminished above 25°C (Fig. 3 (B)). This may be due to inactivation of the enzyme by the added 2-butanol.

**Effect of substrate concentration on amoxicillin synthesis**

The ratio of conversion of 6-APA into amoxicillin was increased by increasing the molar ratio of HPGME to 6-APA (Table IV, Expt. 1). This is because, as shown in Table III, the acyl group of the added HPGME is not only transferred to 6-APA to form amoxicillin, but also to water during the course of the synthetic reaction. On the other hand, the addition of more than 20 mg/ml of HPGME hydrochloride and of more than 10 mg/ml of 6-APA markedly suppressed the ratio of conversion of 6-APA into amoxicillin (Table IV, Expt. 2).

**Time-course of amoxicillin synthesis**

The time-course of the synthesis of amoxicillin from HPGME and 6-APA is shown in Fig. 4. The concentration of amoxicillin and that of HPG reached plateaus after about 15 hr, and about 96% of the added 6-APA was converted into amoxicillin with almost complete consumption of the added HPGME. The concentrations of amoxicillin, HPG, HPGME and 6-APA in the reaction mixture at 15 hr were 44.1 mm, 48.1 mm, 3.4 mm and 1.5 mm, respectively.

**DISCUSSION**

In the present paper we have shown that a penicillinase-deficient α-amino acid ester hydrolase producer, *X. citri* K24, synthesized amoxicillin from HPGME and 6-APA. The accumulation of amoxicillin reached a plateau when 23% of the added 6-APA was converted into amoxicillin (Fig. 1). Whereas, reduction of the ionic strength of the reaction mixture accelerated the rate of amoxicillin synthesis and consequently increased the yield of amoxicillin to about 60% on a molar basis. This may be explained by the finding that high concentra-

![Fig. 4. Time-course of Amoxicillin Synthesis by X. citri K24.](image-url)
tions of inorganic salts induced dissociation of the enzyme molecule resulting in a drastic decrease of the enzyme activity.\(^{13}\)

The addition of an alcohol to the reaction mixture further increased the yield of amoxicillin. Of the tested alcohols, 2-butanol was the most effective (Table I). It repressed the hydrolysis of HPGME by the α-amino acid ester hydrolase and enhanced the transfer of the acyl group from HPGME to 6-APA (synthesis of amoxicillin). But the addition of 2-butanol did not significantly affect the sum of the reaction rates of hydrolysis and transfer (Table III). The mechanism of the repression of ester hydrolysis and the enhancement of the acyl transfer by the added alcohol remains to be elucidated.

Acknowledgments. We wish to thank to Dr. S. Tatsuoka, Dr. R. Takeda, Dr. E. Ohmura and Dr. M. Isono for their interest and encouragement during this work. We are also indebted to Mr. T. Takahashi and Mrs. C. Kawahara for their skillful assistance.

REFERENCES