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# THERMODYNAMIC EVALUATION OF ANTIBACTERIAL ACTIVITY FOR INCLUSION COMPLEXES OF AMOXICILLIN WITH CYCLODEXTRINS

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The antibacterial action of amoxicillin (AMPC) and the inclusion complexes of AMPC with α-, β- and γ-cyclodextrins (α-CD, β-CD and γ-CD, respectively) to *Escherichia coli* B (*E. coli*) was evaluated by isothermal titration microcalorimetry and by petri-dish bioassay method. The effects of the compounds on produced heat during the exponential phase of the *E. coli* growing were measured and the growing rate constants of the cells was calculated from the power-time (*p-t*) curve before and after the treatment with AMPC. Results from the both methods showed that the antibacterial activity became stronger in the following order: AMPC-βCD > AMPC-γCD ≈ AMPC-αCD > AMPC only.

***Keywords:*** *amoxicillin, antibacterial activity, cyclodextrins, inclusion complex, microcalorimetry*

## Introduction

Amoxicillin (AMPC) is a penicillin derivative belonging to a group of β-lactam antibiotics used in *Helicobacter pylori* eradication [1, 2]. However, the clinical application to *H. pylori* has been unsuccessful because of the instability in the strong acidic environment such as the stomach and the appearance of antibiotic-resistant *H. pylori*. AMPC is rapidly degraded under acidic conditions because the β-lactam ring is more susceptible to hydrolytic degradation when the pH deviated significantly from the isoelectric point (pH 4.8) [3, 4]. It is as yet unclear whether AMPC acts against *H. pylori* directly after oral administration or via the systemic circulation. In any case, the stability of AMPC in the stomach is likely to be a key feature for the delivery system. We found that the formation of inclusion complexes between AMPC and cyclodextrins (CD) could inhibit the degradation in the strong acidic solution [5]. The degree of stabilization was dependent on the concentration of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) at pH 1.2, and a maximum increase in stability of AMPC, of 4.0-fold, was observed at an AMPC to HP-β-CD molar ratio of 1:5. There was little difference in the stability at a molar ratio more than 1:5. Thus, cationic AMPC formed two types of inclusion complexes with CD at 1:1 and 1:2 molar ratios (Fig. 1), where the penam ring of AMPC was included in the hydrophobic cavity of CD and protected from the acidic degradation. In this study, the effect of CD on the antibacterial action of AMPC was evaluated by isothermal titration microcalorimetry (ITM) and compared with the petridish bioassay method.

## Experimental

### *Materials*

Amoxicillin anhydride (AMPC) and α-, β-, γ-cyclodextrins (α-CD, β-CD, γ-CD, respectively) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other materials used were obtained from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). *Escherichia coli* B (ATCC27166) (*E. coli*) was our collection in the peptone culture medium purchased

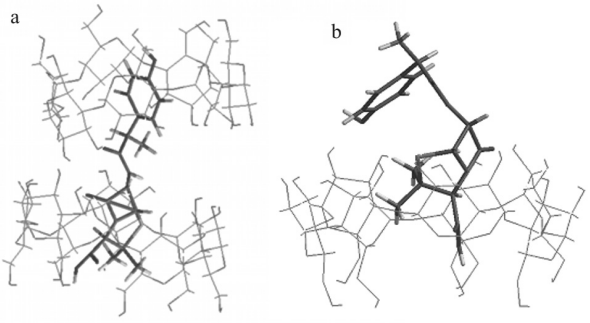


**Fig. 1** Stable structures of inclusion complexes between AMPC and βCD with a – 1:2 and b – 1:1 stoichiometries

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from Wako Pure Chemical Ind. Ltd. LB liquid culture medium was prepared according to the following prescription (g/L): peptone 10.0; east extract 5.0; NaCl 10.0; agar 15.0; and pH = 6.5, and it was sterilized in high pressure steam at 121°C and 1 kg $cm^{-2}$ for 15 min.

*Calorimetry*

An isothermal titration calorimeter (ITM; Thermal Activity Monitor 2270, ThermoMetric AB Sweden) was employed to measure heat product of *E. coli* growth. *E. coli* suspended in LB liquid medium was enclosed in the reaction cell and the thermogram was recorded under static and anaerobic conditions at 37°C. AMPC or a mixture of AMPC-CD (1:5) was prepared in the sterilized buffer solution at pH 1.2 immediately before use and injected to the reaction cell to achieve the AMPC concentrations of 1.5, 4.5 and 6.0 μg $mL^{-1}$ during the exponential growth phase of *E. coli*. The growth rate constants of *E. coli* (*k*) were calculated from the power-time (*p-t*) curves before and after treatment with AMPC and AMPC-CD complexes. Turbidity of *E. coli* culture in the reaction cell was measured by the absorbance at 660 nm at 30 min intervals.

*Microbiological analysis*

The antibacterial activity of AMPC and AMPC-CD complexes was measured by a petri-dish bioassay method (diffusion technique). The strain of *E. coli* was seeded from standardized suspension to a concentration of about $10^5$ viable cells per mL in agar medium. The seeded agar medium was poured into petri-dishes (9 cm) to a depth of about 4 mm, four disks (6 mm of diameter) containing the 10 μL samples with various concentrations of AMPC were placed in each dish, and the petri-dishes were left for 1 h then incubated at 37°C for 24 h. The inhibition ring diameter of growth of *E. coli* was measured.

## Results and discussion

A complete thermogram (power-time curve: *p-t* curve) of *E. coli* growing in LB medium under anaerobic condition at 37°C in ITC was shown in Fig. 2. The *p-t* curve represented an initial lag phase following an exponential growth phase, where the total quantity of heat of culture (*Q*) had a linear correlation with the turbidity of *E. coli* culture ($OD_{660}$). It was indicated that the increment of *Q* corresponded to amplification of the cells in the medium and the heat output rate of *p-t* curve to the growth rate of *E. coli*. So, the effect of AMPC and AMPC-CD complexes on *E. coli* cells was examined in the exponential growth phase.

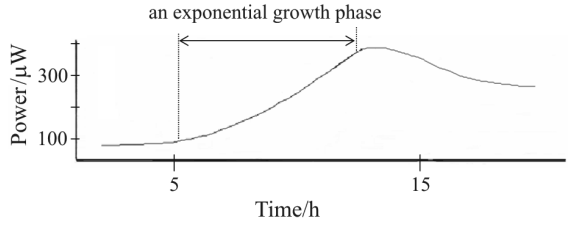


**Fig. 2** Power-time curve of *E. coli* growth in LB medium at 37°C

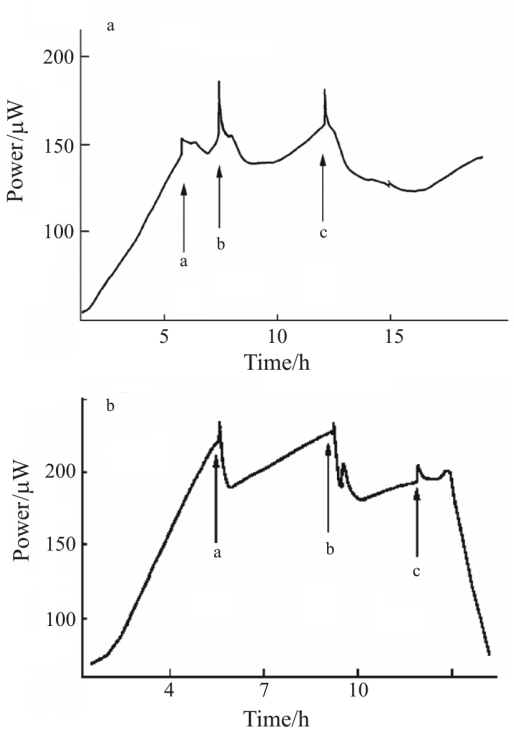


**Fig. 3** Effects of a – AMPC and b – AMPC-βCD complex on heat production of *E. coli* growth in LB medium at 37°C. Arrows indicate the injection time of the solution at various concentrations of AMPC, a – 1.5, b – 3.0, and c – 6.0 μg $mL^{-1}$

Figures 3 shows the *p-t* curves with treatment of AMPC and AMPC-βCD (1:5) solutions at pH 1.2, where each arrow represents the injection of the solution containing various dose of AMPC (a: 1.5 μg $mL^{-1}$, b: 3.0 μg $mL^{-1}$ and c: 6.0 μg $mL^{-1}$). Treatment of AMPC and all AMPC-CD complexes decreased the power output immediately when they were added to cells, but the extent and duration of the effect varied with the biological activity of the compounds. Treatment with AMPC resulted in a drop of the *p-t* curve to a lower level. The curves then either stayed at that level for a certain period of time and ascended to a second peak (after treatment with AMPC, AMPC-αCD and AMPC-γCD) or immediately decreased to the baseline and no recovery occurred to

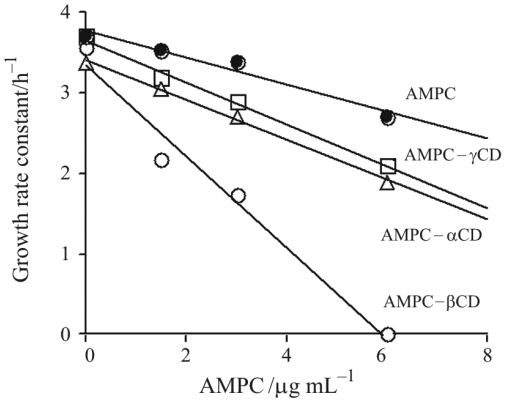


**Fig 4.** Growth rate constants calculated from *p-t* curves for treatment of AMPC only and AMPC-CD complexes

death of cells (after treatment with 6.0 μg mL$^{-1}$ AMPC-βCD). The lower slope of the second ascend *p-t* curve after treatment indicates a partial inhibition of the cell growing. Thus, the treatment with AMPC and AMPC-CD complexes to *E. coli* culture resulted in a decrease of the heat output rate.

In the exponential phase of growth, the *p-t* curve obeys [6]

$$\ln Q = kt + \ln Q_0$$

Using this equation, the heat output rate constants relating to the growth rate constant ($k$) were calculated before and after the treatment. The depressing effect on the rate constant is dependent on the concentration of AMPC. The relationship between the concentration and $k$ is nearly linear for all compounds (Fig. 4). The results show that the inhibiting action of AMPC-CD complexes on *E. coli* growth is stronger than that of AMPC only.

The antibacterial activities of AMPC and AMPC-CD complexes were examined on the basic

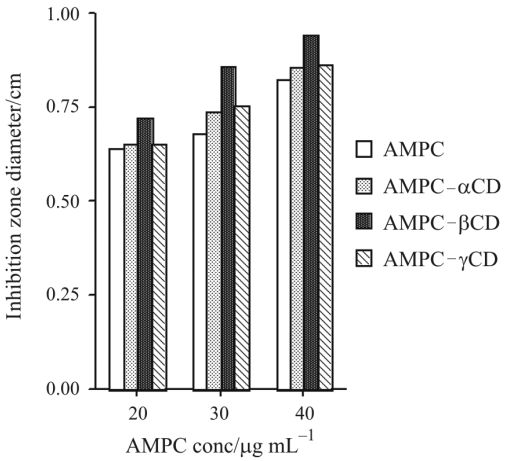


**Fig. 5** Effect of various doses of AMPC for AMPC only and AMPC-CD complexes on inhibition zone size *vs. E. coli*

inhibition zone size of *E. coli* using disk agar-diffusion technique. The disks containing various doses of AMPC in only AMPC and AMPC-CD (1:5) solutions at pH 1.2 were prepared and put on the agar medium. Figure 5 shows the inhibition zone sizes for AMPC only and AMPC-CD complexes *vs. E. coli* at various concentrations of AMPC. The minimal inhibitory concentration of AMPC to observe inhibition zone was 20.0 μg mL$^{-1}$. In the any concentrations of AMPC, AMPC-βCD complex showed the largest inhibition zone size.

The minimal inhibitory concentration of AMPC in the calorimetry was ten times lower than in the petri-dish bioassay. Because, the calorimetric method was an online recording of *E. coli* growth and hence even the slightest and short lasting effects could be detected. But the results from the petri-dish bioassay were cumulative effects of incubation for 24–48 h and influenced by inoculum size, agar consistency, solubility and diffusion potential of the test substances that didn't influence the produced heat. The treatment with AMPC or AMPC-CD complexes to *E. coli* cells in the calorimetric exponential phase resulted in a decrease of the heat output rate to a lower level, depending on the concentration of AMPC. If *E. coli* cells are not completely killed by AMPC, the heat output rate is maintained at a certain level and the *p-t* curve revives and ascends again after treatment as shown. If a proportion of *E. coli* cells are killed, others are inhibited, and some others remain unaffected, the produced heat after the treatment decrease corresponding to unaffected and growing cells. If the number of the latter is very small and their production heat below the detection limit of the calorimeter, no growth is observed as shown in the case of the treatment with 6.0 μg mL$^{-1}$ AMPC-βCD. Thus, the values of $k$ obtained from *p-t* curves after the treatment indicated the degree of the inhibition activity of AMPC. The results show that the inhibiting effect of AMPC-CD complexes on *E. coli* growth was stronger than of AMPC only. Considering both results from the calorimetry and the petri-dish bioassay, the antibacterial activity of AMPC and AMPC-CD complexes could be arranged in the following order: AMPC-βCD > AMPC-γCD ≈ AMPC-αCD > AMPC. β-CD easily formed the inclusion complex with cationic AMPC to protect the penam ring from the degradation [5]. Thus, the antibacterial effect of AMPC was mainly related to the chemical stability in the strong acid solution such as gastric juice, indicating that the complexation with cyclodextrins would be useful for *H. Pylori* infection and as a drug delivery system in an oral dosage form.

## Conclusions

Microcalorimetry is a useful mean for the estimation of the relative antibacterial activity of antibiotics to provide the kinetic and thermodynamic information, and more sensitive than the petri-dish bioassay due to an online recording of bacterial cell growing. The experimental results pointed out that the sequence of antibacterial activity to *E. coli* is AMPC-βCD > AMPC-γCD ≈ AMPC-αCD > AMPC only.

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