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Degradation of Clavulanic Acid During the Cultivation of *Streptomyces clavuligerus*; Instability of Clavulanic Acid by Metabolites and Proteins from the Strain

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Abstract Clavulanic acid (CA) produced by *Streptomyces clavuligerus* is degraded during the bacterial cultivation. The degradation was examined in three different aspects, including physical, chemical, and enzymatic effects, in order to understand the degradation during the cultivation. The result showed that CA was unstable in the production medium containing ammonium salts and amino acids, owing to ammonium ions and amine groups. In addition, the degradation was not only due to instability of CA by metabolites and proteins, but also enzymes from *S. clavuligerus* such as β-lactamase and penicillin-binding proteins. However, the degradation caused by these enzymes was not highly significant compared with the degradation during the cultivation, owing to irreversible reactions between CA and enzymes.

Key words: Clavulanic acid degradation, metabolites, β-lactamases, penicillin-binding proteins (PBPs)

Clavulanic acid (CA) is an important antibiotic simultaneously produced by *S. clavuligerus* with other β-lactams such as cephamycin C, penicillin N, deacetoxycephalosporin C, holomycin, and various metabolites. Since CA exhibits a weak antibacterial activity by itself, it alone is not suitable for use. Therefore, it has been utilized in combination with other β-lactam antibiotics, especially amoxicillin, in the clinical field. In addition, CA has been used as an inhibitor of β-lactamase in pathogenic bacteria, because of the irreversible formation of acyl-*O*-Ser enzyme intermediates, resulting in an inactive state. Therefore, when β-lactam antibiotics such as penicillins or cephalosporins are administered together with CA, they can be protected from the hydrolysis of β-lactamase by the inhibitory action of CA. Thus, CA has been globally used in the medical field, and it has been produced in the pharmaceutical industry for a long time. However, this antibiotic is an unstable compound, which is degraded during the bacterial cultivation. To date, only a few studies have been performed related to CA degradation. The previous studies included the instability of CA in aqueous solutions [4], the production of CA and its degradation [9], CA degradation in the culture broth and the culture medium [16], reconsumption of degraded CA [8], and so on. Two decades ago, Haginaka *et al.* [4] suggested that CA degradation follows pseudo first-order kinetics under the condition of various buffers, pH, and temperature. In addition, the degradation is led by the catalysis of buffer ions including citrate, acetate, phosphate, borate, and carbonate, and the degradation rate constant k_d significantly depends on pH. The pH dependence of degradation at a logarithmic plot of k_d vs pH showed a parabolic form with a minimum at pH 6.4. It means that CA is an extremely unstable compound in both acidic and alkaline media. Furthermore, temperature also has a considerable influence on the degradation. In view of enzymatic effects, Mayer and Deckwer [9] mentioned that CA is simultaneously produced and decomposed during the cultivation in a complex medium containing soy extract, compared to soy meal itself. They estimated that β-lactamase as a growth-protecting substance might have degraded CA. Roubos *et al.* [16] studied the degradation of CA in each component of the culture medium, and calculated the degradation rate constants from the degradation experiments. By the comparison of CA degradation in nonsterilized and heat-sterilized samples of cultured broth, they also suggested the possibility that the degradation might occur by the action of enzyme or heat-sensitive metabolites from culture broth of *S. clavuligerus*.

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In this study, we attempted to clarify the factors for the degradation, and also studied the relationship between CA degradation and β-lactam resistance proteins, including β-lactamase and penicillin-binding proteins (PBPs) [11].

MATERIALS AND METHODS

Bacterial Strains and Culture Condition

Streptomyces clavuligerus NRRL3585 was cultured for the CA degradation experiments. The strain was stored with 40% glycerol at −70°C, and precultured in 250-ml baffled flasks containing 25 ml of seed medium (15 g/l glycerol, 7 g/l peptone, and 14 g/l trypticase soy broth, pH 7.0) for 36 h at 28°C with shaking at 250 rpm. The precultured strain was inoculated into 500-ml baffled flasks with 50 ml of production medium (10 g/l glycerol, 15 g/l peptone, 27 g/l trypticase soy broth, and 13 g/l MOPS) at 28°C and 250 rpm of shaking speed, and pH 6.5 was maintained with phosphoric acid.

Preparation of Ammonium and Amino Acid Solutions

Various kinds of amino acids and ammonium salts were studied for the degradation of CA. The solutions of each material were prepared as 0.05 M concentration, mixed with 10 ml of 0.05 M sodium phosphate buffer, and the pH was adjusted to 6.5.

Preparation of Supernatant Sample of the Culture Broth

The culture broth samples were taken seven times at 24 h, 53 h, 71 h, 105 h, 153 h, 202 h, and 250 h. The samples were centrifuged twice at 3,200 rpm (3,000 ×*g*) for 5 min to remove the mycelium. The supernatant obtained was divided into two parts; one for the preparation of sterilized supernatant and the other for nonsterilized supernatant. The sterilized sample was prepared by heat-sterilization at 121°C for 20 min to completely denature all proteins. The pH of the final solution was adjusted to 6.5.

Preparation of Exocellular Protein by Precipitating from the Culture Supernatant

In order to prepare the precipitated proteins, ammonium sulfate (22.5 g) was added gradually to 50 ml of the supernatant at 4°C with stirring until 80% saturation [3]. The precipitated proteins were centrifuged at 3,200 rpm for 10 min, dissolved in 5 ml of 0.05 M sodium phosphate buffer (pH 6.5), and dialyzed overnight at 4°C to remove the salt and other substances. Samples of sterilized and the nonsterilized proteins were prepared by the same manner as of culture broth. The pH of the final protein solution was adjusted to 6.5 at 28°C.

Preparation of CA Stock Solution

For the preparation of CA stock solution, 0.35 g of CA (provided by CKD Pharmaceutical Co., Korea) was dissolved in 10 ml of sodium phosphate buffer of pH 6.5 at 4°C, and sterilized through a 0.02-mm membrane filter. This stock solution was used for all experiments, and the concentration of CA in each sample was maintained at 2.5 g/l.

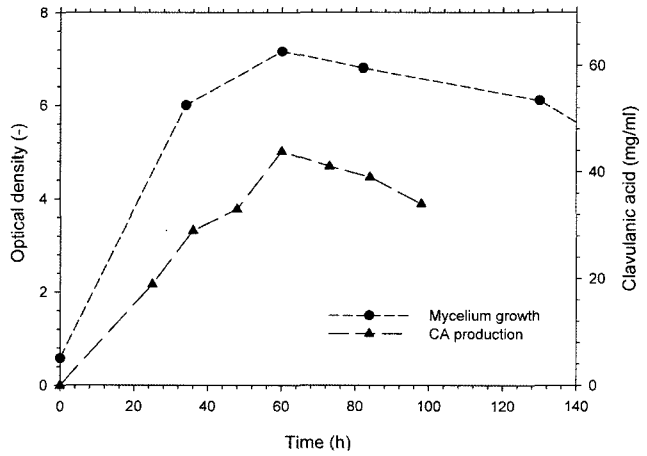

Fig. 1. CA production and mycelium growth during the cultivation of *S. clavuligerus* in a batch system containing a defined medium.

Analysis of CA in the Samples

The remaining CA concentration was measured by using HPLC equipped with a C-18 reverse-phase column (4.6 mm× 250 mm, 5 mm Kanto Chemicals, Japan). The mobile phase consisted of 0.065 M sodium phosphate monobasic (Yakuri Chemicals, Japan) buffer (pH 4.4). The flow rate was set at 1 ml/min, and the peak of CA was detected by UV at 220 nm.

Analysis of CA Degradation Rate

CA degradation was measured by taking aliquots 7–8 times during 70 h. Analysis of the degradation pattern revealed

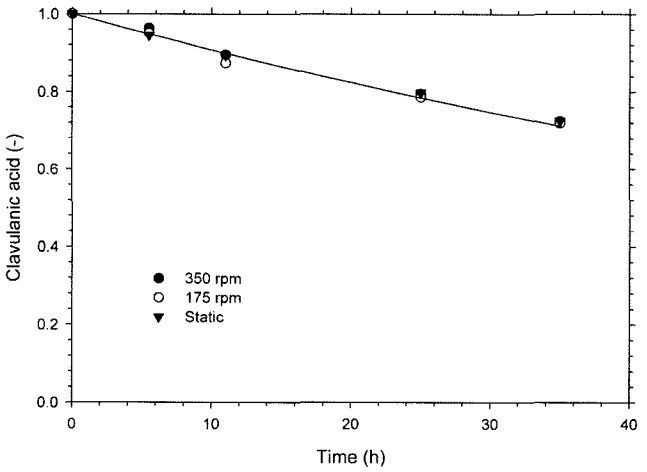

Fig. 2. CA degradation by physical effects; the effect of rotation speeds on degradation was monitored at 350, 175, and 0 rpm.

Table 1. Physical conditions; rotation speeds.

Rotation speed (rpm)	350	175	0
k_d [1/h]	0.0093	0.0093	0.0093

the first-order decay fitted to the curve, $Y=\exp(-k_d \times t)$, with Sigma plot (Aspire Software, U.S.A.). The measured data were normalized by initial CA concentration, and the relationship between the normalized concentration of CA and experiment time could be obtained.

CA Degration by β-Lactamase (Amp)

β-Lactamase (Amp) was expressed in *E. coli* BL21 (DE3)/ pET-32a(+) containing the *bla* gene [5, 13, 18]. The activity of β-lactamase (Amp) was confirmed with 50 mM penicillin G, and CA degradation was monitored after mixing the enzyme suspension with 50 mM CA solution. The concentration of CA was measured with a Multiscan Spectrum spectrophotometer (Thermo Labsystems, U.S.A.) at 220 nm and 30°C.

Results and Discussion

Mycelium Growth and CA Degradation in a Batch System

The mycelium growth and CA production curves for the strain in a batch system that contained the production medium are shown in Fig. 1. The degradation was found to start approximately 70 h later in the batch system, and it was gradually decreased during the growth of the mycelium, reaching stationary phase. Under this state, the production of metabolites or enzymes by the strain seemed to be partly responsible for the degradation of CA.

Effect of Physical Condition on CA Degradation

The effect of rotation speeds on CA degradation was monitored at 350, 175, and 0 rpm, as shown in Fig. 2 and Table 1. The result indicates that the turbulent flow caused by the rotation and the shock of the collision to the glass wall did not have any severe influence on the CA degradation, even though the significant influence of temperature had already been demonstrated.

CA Degradation in the Production Medium

Roubos *et al.* [16] suggested that compounds in the culture medium might have an influence on CA degradation, and they used synthetic medium instead of complex medium containing soy meal. However, the complex production medium containing soy broth was used in this study for the culture of *S. clavuligerus*, in order to confirm whether CA degradation was caused by amino acids in the production medium. Amino acid content was increased by heat-treatment three times, as confirmed by the ninhydrin assay (data not shown). The degradation of CA in the normal production medium and the heat-sterilized medium is shown in Fig. 3, and the degradation rate constants are summarized in Table 2. From this result, it could be possible that CA is very sensitive to chemicals such as amino acids and other inorganic materials. Although various kinds of amino acids, ammonium salts, carbon sources, and mineral salts are essential for the growth of the strain, some of them might have a strong influence on CA degradation, as shown by the increased amount of amino acids.

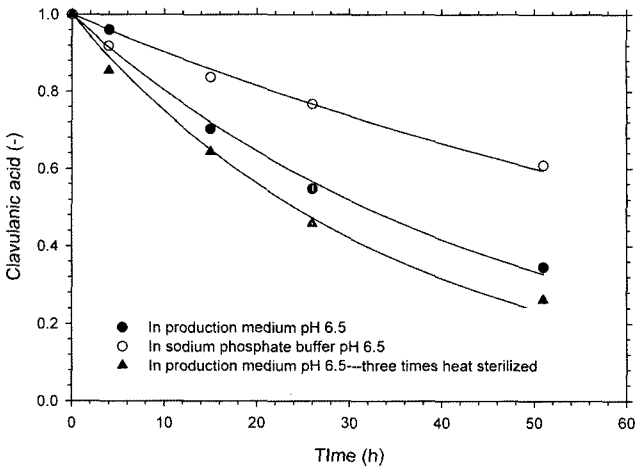

Fig. 3. CA degradation in the production medium was monitored at 4 h, 15 h, 26 h, and 50 h.

CA Degradation by Ammonium Salts and Amino Acids

The effects of ammonium salts and amino acids in the production medium on CA degradation are shown in Fig. 4 and Fig. 5, and the degradation rate constants are summarized in Table 3 and Table 4, respectively. All ammonium salts contained in the production medium had impacts on the degradation of CA, except ammonium hydroxide, in concordance with previous reports [7, 17]. Potassium sulfate as one of the mineral salts for cell growth also affected the degradation, but the degree of the degradation was not so significant, compared with ammonium salts.

Table 2. CA degradation rate constants in the production medium.

Samples	In production medium pH 6.5	In production medium three times heat sterilized (pH 6.5)	In sodium phosphate buffer pH 6.5
k_d (1/h)	0.0219	0.0288	0.0102

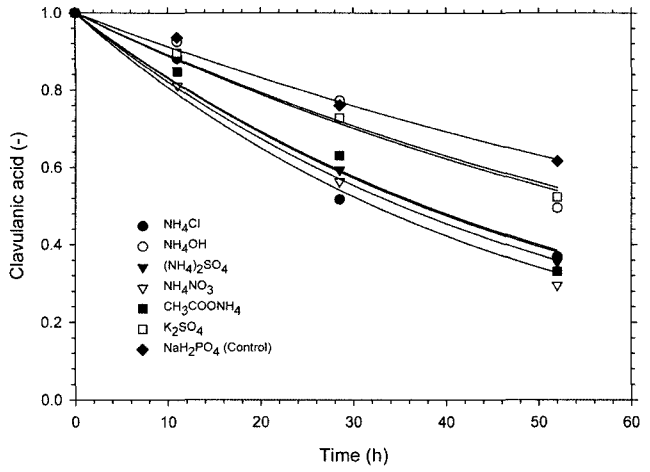

Fig. 4. CA degradation by ammonium solutions was examined at 10 h, 28 h, and 52 h.

Furthermore, CA degradation by amino acids was also identified. Previously, Andrietta *et al.* [2] reported that soy meal used for CA production includes aspartic acid, serine, arginine, and other materials. In this study, the effects of nine amino acids on CA degradation were investigated, and ornithine was found to give the strongest impact, which was based on the analysis of the degradation rate constant. Although ornithine is not required for the growth of this strain and not involved in the protein synthesis, it has been known to be utilized in arginine biosynthesis followed by CA biosynthesis. The degradation rate constant of CA was also high in the presence of arginine, but not affected by leucine and alanine. The increase of the degradation rate constant could be attributed to the side chain of amino acids, based on the fact that ornithine and arginine have polar side chains, but leucine and alanine have nonpolar and hydrophobic side chains. Probably, the amine groups contained in these polar amino acids might attack the four-ring lactam carboxyl group of CA to open the β-lactam ring. In the present study, the degradation of CA was found to be clearly affected by ammonium salts and amino acids in the production medium.

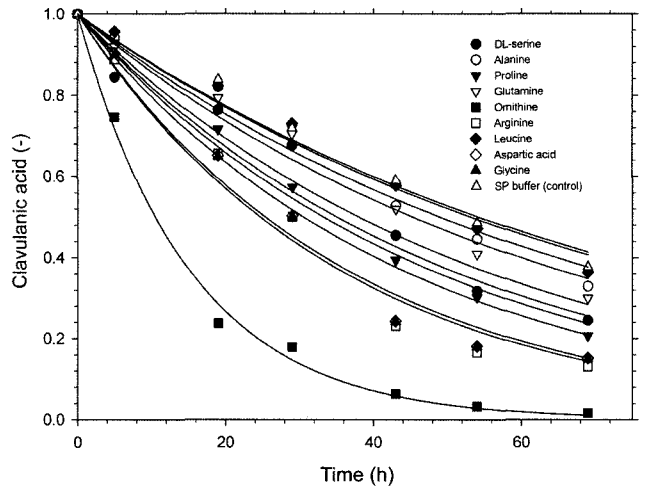

Fig. 5. CA degradation in amino acid solutions was checked at 5 h, 20 h, 28 h, 44 h, 54 h, and 65 h.

Table 3. CA degradation rate constants in ammonium solutions.

Chemicals	k_d (1/h)	Chemicals	k_d (1/h)
Ammonium chloride	0.0197	Ammonium sulfate	0.0183
Ammonium hydroxide	0.0115	Ammonium acetate	0.0186
Ammonium nitrate	0.0215	Potassium sulfate	0.0118

*SP buffer (control), 0.0092.

CA Degradation by Exocellular Products Produced by *S. clavuligerus*

CA was fed to the supernatant samples of the culture broth to clarify the cause of the degradation, as shown in Fig. 6. Previously, two research groups [9, 16] suggested that CA might be decomposed by β-lactam-resistant proteins such as β-lactamase. Mayer and Deckwer [9] described that the resistant enzyme would be simultaneously produced as a growth-protecting substance to avoid the suicide. In addition, bacterial PBPs, which promote the cell wall biosynthesis, have been known to bind to the β-lactam ring of penicillins, thus making CA bind to these proteins. In *S. clavuligerus*, two β-lactamases (*bla* in the cephamycin gene cluster [15] and *orf12* in the clavulanic acid gene cluster [6, 10]), and four PBPs (*pbp74* [1, 14] and *pcbR* [12] in the cephamycin gene cluster, and *orf18* and *orf19* in the clavulanic acid gene cluster [6, 10]) were already identified by the gene annotation.

In the present, differing from the result of Roubos *et al.* [16], the degradation of CA by the β-lactam-resistant proteins of *S. clavuligerus* could not be identified. The degradation of CA in heat-sterilized supernatant was somewhat more serious than in nonsterilized one; however, both degradation rate constants were decreased after reaching a maximum at 153 h of incubation. Consequently, it is highly likely that there are still other impact factors for the degradation of CA, as unidentified metabolites in culture broth. Since an increase of amino acid content in the heat-sterilized samples was also confirmed by the ninhydrin

Table 4. CA degradation rate constants in amino acid solutions.

Amino acid	k_d (1/h)	Amino acid	k_d (1/h)
Alanine	0.0142	Arginine	0.0281
Aspartic acid	0.0274	Glutamine	0.0153
Glycine	0.0227	Leucine	0.0130
Ornithine	0.0661	Proline	0.0209
Serine	0.0182	SP buffer	0.0128

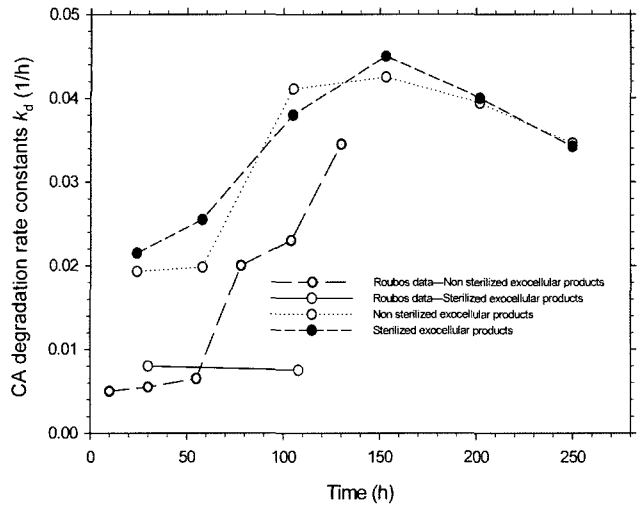

Fig. 6. CA degradation rate constant by nonsterilized and sterilized exocellular products; comparison with our data and those of Roubos *et al.* [16] (control; CA degradation rate constant in production medium).

reaction, amino acids decomposed by heat-sterilization might have an influence on the degradation of CA. Thus, this experiment indicates that the factors responsible for CA degradation might be the metabolites, but not the resistance proteins produced by the strain.

CA Degradation by Exocellular Proteins Produced by *S. clavuligerus*

Exocellular proteins were recovered by ammonium sulfate precipitation and dialysis, and nonsterilized and heat-sterilized protein samples were prepared to examine the effects of exocellular unknown proteins on the degradation of CA (Fig. 7). The degradation reactions by both non-

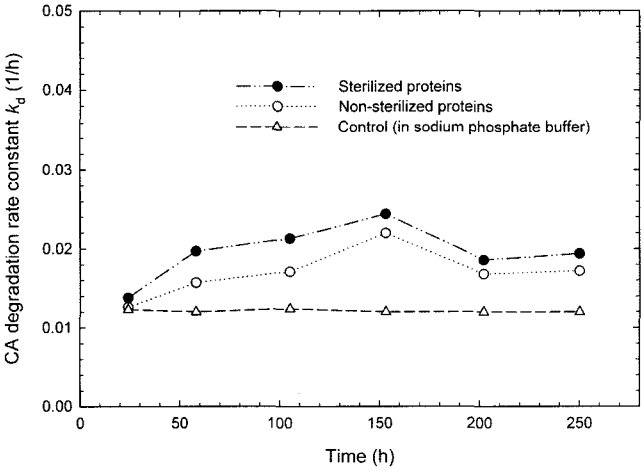

Fig. 7. CA degradation rate constant in nonsterilized and sterilized precipitated exocellular proteins (control; CA degradation rate constant in sodium phosphate buffer).

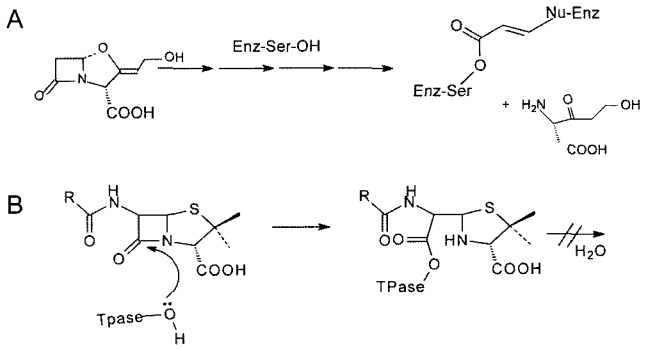

Fig. 8. A. Hydrolysis of the β-lactam ring of penicillin by class A, C, and D β-lactamases. **B.** Reaction of penicillin G as a suicide substance for transpeptidase of PBPs.

sterilized and in heat-sterilized protein samples increased gradually from the starting point, compared with the control. The degradation rate constants decreased after the maximum values of k_d, 0.024 and 0.022 1/h, at 153 h of incubation, respectively. Finally, both samples showed similar rate constants to the control, since only a small amount of proteins remained because of degradation of proteins. The degradation of CA due to the intracellular proteins might be recognized, but the influence was negligible (data not shown).

In *S. clavuligerus*, if the degradation was caused by an enzymatic factor, the degradation of CA in the precipitated exocellular protains could be identified, since the β-lactam resistance proteins are pumped out to the culture broth. However, the heat-sterilized protein sample also showed a strong influence on CA degradation, indicating that an enzymatic factor would be insignificant for the degradation. Therefore, the decomposed amino acids during sterilization were also responsible for the degradation. The difference between the k_d of the nonsterilized supernatant sample and k_d of the control, $\Delta k_{d\ \text{N.H.E. Products}}$, was 0.0241/h, and the difference between k_d of the nonsterilized exocellular protein sample and k_d of the control, $\Delta k_{d\ \text{N.H.P.E. Proteins}}$, was 0.0101/h. The difference between these two parameters, $\Delta k_{d\ \text{N.H.E Products}} - \Delta k_{d\ \text{N.H.P.E. Proteins}}$, is 0.0141/h, which might reflect the effect of the metabolites. The analysis of the degradation rate constants suggested that CA degradation might be due to proteins, enzymes, and metabolites; however, enzymes and proteins did not show such a strong influence. Since the reaction of CA with β-lactamase or PBPs is irreversible (*i.e.*, one β-lactamase must be bound to one molecule of CA, as represented in Fig. 8), the degradation of CA by the enzyme or protein cannot be completely explained.

CA Degradation by β-Lactamase (Amp)

A highly active β-lactamase (Amp) expressed by the pET-32a(+) plasmid was employed to confirm the influence of β-lactamase on CA degradation. In *S. clavuligerus*, a

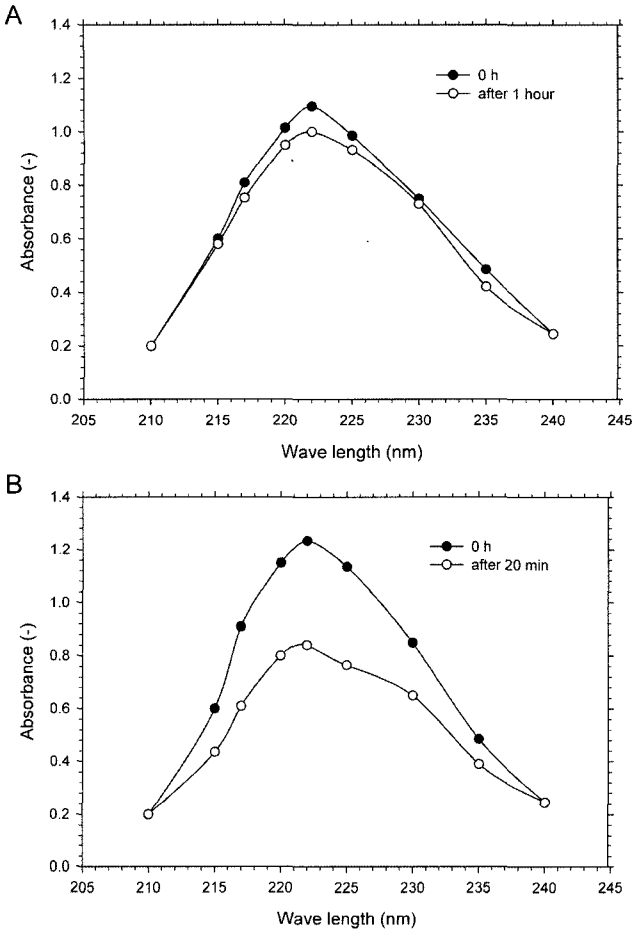

Fig. 9. CA (**A**) and penicillin G (**B**) degradation by β-lactamase (Amp) at 30°C.

highly active β-lactamase such as Amp has not yet been found, but only a weakly active β-lactamase in the cephamycin C gene cluster has been identified [15]. In addition, *orf12* in the CA gene cluster was annotated as a β-lactamase, but the activity was not identified in the reaction with penicillin G, cephalosporin C, and CA. As shown in Fig. 9, the penicillin G was decomposed immediately in the reaction with a highly active β-lactamase (Amp), but the CA was not degraded. Consequently, it could be concluded that the effect of Amp on the degradation of CA was negligible.

In the present study, the physical, chemical, and enzymatic effects on the degradation of CA were examined in detail. Neither the physical treatments nor β-lactam-resistant proteins, including β-lactamase, had any significant effect on CA degradation. However, the metabolites and non-functional proteins present in the culture medium showed more significant effects on CA degradation. Therefore, the degradation of CA during cultivation could be due to the instability caused by inorganic ions, like ammonium salts, and by polar amino acids. In spite of possible CA degradation by some other enzymes, β-lactam-resistant proteins were not responsible, because a highly active β-lactamase, such as Amp expressed by pET-32a(+), did not exhibit much significant effect on the degradation of CA.

Acknowledgments

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