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Cephalosporin C acylase in the autolysis of filamentous fungi

F. REYES, M. J. MARTINEZ, C. ALFONSO, J. L. COPA-PATIÑO*, J. SOLIVERI*, *Departmento de Microbiología Aplicada, Centro de Investigaciones Biológicas (CSIC), Calle Velázquez 144, Madrid 28006 and * Departmento de Microbiología y Parasitología, Universidad de Alcalá de Henares, Spain*

Abstract—Cephalosporin C acylase activity was studied using fluorescamine determination of free $-NH_2$ groups produced in the deacylation of cephalosporin C by the enzyme. Fourteen fungi from different genera were studied and low extracellular cephalosporin C acylase activity was found in the genera *Aspergillus, Fusarium* and *Penicillium*. Forty one fungi of these genera were checked but not all presented acylase activity. The enzyme was generally found to be an extracellular enzyme and during the process of autolysis its activity increased with incubation time and with increasing pH of the medium. In no case was β-lactamase activity detected. *Penicillium rugulosum* and *Penicillium griseofulvum* were identified as good cephalosporin C acylase producers. Deacetyl esterase activity was also detected in these fungi.

The starting compound for the industrial synthesis of semisynthetic cephalosporins is 7-amino-cephalosporanic acid (7-ACA). This compound is generally prepared by removing the side chain of the naturally occurring cephalosporin C (CPC) (Huber et al 1972). The complicated chemical techniques used to produce 7-ACA have led to attempts to establish an alternative enzymatic method.

The intermediate of semisynthetic penicillin antibiotics, 6-amino-penicillanic acid (6-APA) has been produced from penicillin G or homologous penicillins by deacylation using penicillin acylase (Huber et al 1972; Vandamme & Voets 1974; Cole et al 1975). It is well known that semisynthetic cephalosporins such as benzylcephalosporin and cephalothin are also deacylated to 7-ACA by this enzyme (Huang et al 1963; Sjöberg et al 1967: Shimizu et al 1975). However, an enzyme which deacylated CPC to 7-ACA was found only in some *Schizomycetes* (Walton 1964, 1966) and recently in species of *Paecilomyces* (Kawate et al 1987a, b). Also, CPC might be cleaved to 7-ACA by two-step enzymic reactions, oxidation and deacylation (Shibuya et al 1981; Matsuda & Komatsu 1985).

The presence of penicillin acylases in the autolysis of filamentous fungi (Alfonso et al 1989), was the reason for our study on the production of CPC acylases during the autolysis of filamentous fungi.

Materials and methods

Microorganisms and culture conditions. Fourteen fungi from different sources were used (Alfonso et al 1989). In addition, nineteen *Aspergillus*, six *Fusarium* and sixteen *Penicillium* strains were checked. These fungi were isolated from peach trees by Dr Paloma Melgarejo (Instituto de Investigaciones Agronómicas, Madrid) and the selected fungi classified by her.

Stock cultures were maintained on potato-dextrose agar and transferred to malt-agar for an inoculum. The basal medium was (g L^{-1}); glucose 10; ammonium tartrate 2, KH_2PO_4 1, $MgSO_4$ $7H_2O$ 0·5, KCl 0·5, yeast extract 1, and trace elements solution 1 mL (containing mg L^{-1}: $Na_2B_4O_7$ $10H_2O$ 100, $ZnSO_4$ $7H_2O$ 70,

Table 1. Mycelium dry weight (MDW), proteins (Pr) and cephalosporin C acylase activity (CPC-A) in the metabolic liquid during autolysis (AD) of *Penicillium oxalicum*.

Incubation time (days)	pH	MDW ± s.d. mg/flask	AD* %	Pr ± s.d. mg/flask	CPC-A U/flask
1	5·0	420 ± 1·8	—	70 ± 2·0	—
2	6·1	900 ± 3·8	0	35 ± 4·2	—
3	7·2	652 ± 5·7	28	40 ± 3·5	6
7	8·4	150 ± 4·5	83	50 ± 2·8	24
14	8·5	171 ± 1·9	87	50 ± 4·9	50

* degree of autolysis.

Correspondence to: F. Reyes, Departmento de Microbiología Aplicada, Centro de Investigaciones Biológicas, Calle Velázquez 144, Madrid 28006, Spain.

Table 2. pH and cephalosporin C acylase activity in the metabolic liquid and mycelial extract at 3, 4, 7 and 14 days of incubation (autolytic period) of different strains of *Aspergillus, Fusarium* and *Penicillium*.

	pH at days				Metabolic liquid units/flask at days			
Fungi	3	4	7	14	3	4	7	14
Aspergillus								
6	6·0	6·5	7·1	6·9	—	4	24	70
9	5·2	5·8	7·4	8·0	—	4	30	90
29	4·5	5·0	7·7	8·3	—	6	70	94
35	6·5	7·0	8·3	8·3	50	60	128	72
47	7·2	7·7	8·4	8·3	80	80	78	76
68	6·8	7·3	8·4	8·2	50	56	66	66
88	7·0	7·5	8·4	8·2	36	42	50	60
120	6·9	7·4	7·4	7·5	20	24	42	38
148A	7·2	7·7	8·4	8·2	30	34	40	56
258	5·1	5·6	5·7	6·9	—	—	—	20
349	5·0	5·0	8·1	8·3	—	4	70	60
414	3·6	3·7	5·1	6·0	—	—	—	—
452	5·5	5·9	6·4	8·7	—	—	—	70
464B	3·5	3·6	4·2	5·4	—	2	4	—
470	3·5	3·9	4·7	5·6	—	—	—	—
494	3·4	3·8	4·8	6·1	—	—	—	—
496	6·5	6·8	7·5	7·5	—	8	70	44
501	4·0	3·1	3·4	4·1	—	—	—	—
505	3·5	3·6	4·6	5·5	—	—	—	—
Fusarium								
197	6·1	6·6	6·9	7·7	—	4	20	64
240	3·5	3·6	4·0	5·0	—	—	—	—
249C	6·9	7·4	7·9	8·4	56	60	80	90
368D	6·5	7·0	7·0	8·1	10	12	16	60
611	6·5	6·7	7·5	8·1	—	4	28	68
614	6·0	6·3	7·2	8·1	—	—	—	60
Penicillium								
21	4·5	4·5	3·8	7·4	—	—	—	20
45	3·6	3·7	7·1	8·2	—	10	44	60
49E	3·5	3·3	8·0	8·6	—	6	24	68
54	4·0	4·5	8·2	8·3	—	8	50	36
76	5·2	5·7	7·5	8·2	—	8	56	80
121	4·5	3·4	8·2	8·7	—	6	34	32
141F	3·6	3·2	8·3	8·6	—	10	96	58
184	5·6	6·2	8·4	8·1	—	6	40	110
194G	4·0	3·5	7·1	7·7	—	6	22	36
195	3·5	3·3	7·7	8·3	—	—	—	70
204	4·5	4·9	6·2	8·2	—	—	—	36
207H	4·2	3·8	7·8	8·5	—	4	46	60
221	4·0	4·0	7·3	7·5	—	4	24	56
222I	6·2	6·2	8·4	8·1	12	24	48	100
230J	4·5	3·5	7·5	8·0	2	6	10	20

Mycelial extract-units per flask/day:
A 3/14. B 2/3, 1/4. C 3/3, 2/4. D 1/14. E 4/14. F 1/4, 1/7, 4/14. G 1/4, 2/7. H 3/14. I 3/3, 4/4, 5/7, 3/14. J 5/3, 4/4, 3/7, 2/14.

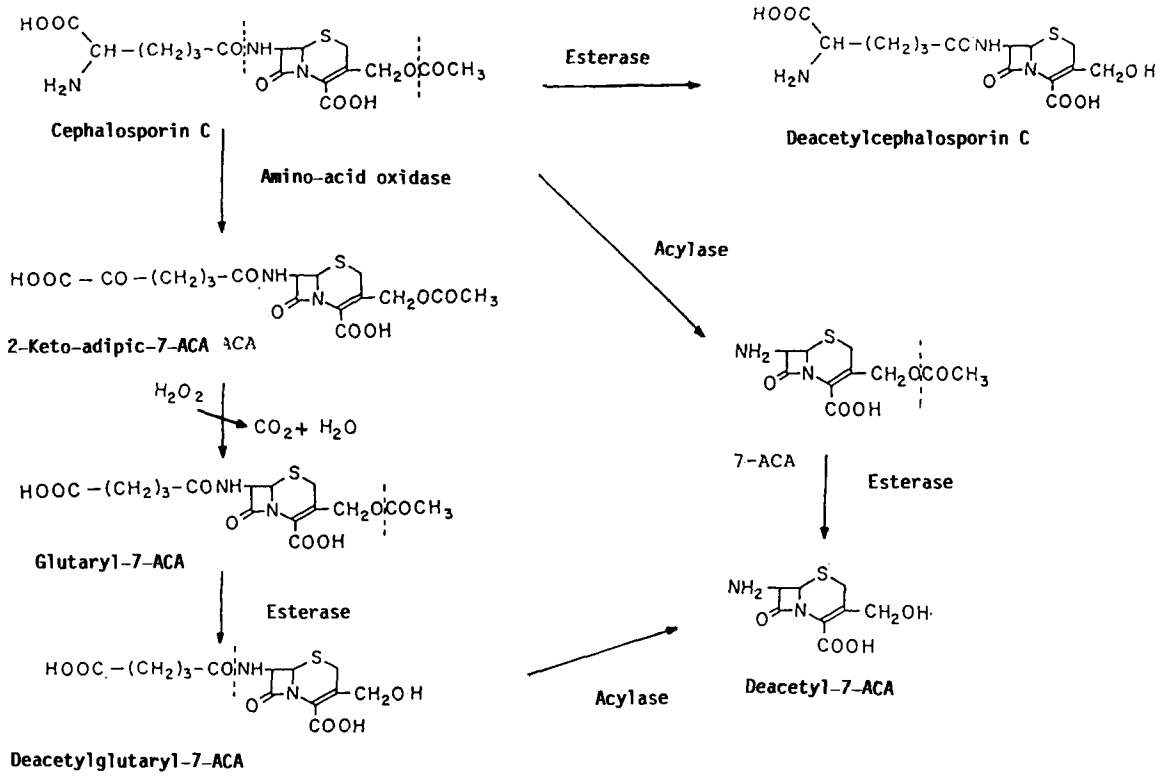

FIG. 1. Possible scheme of degradation of cephalosporin C by lytic enzymes from autolysed cultures of filamentous fungi.

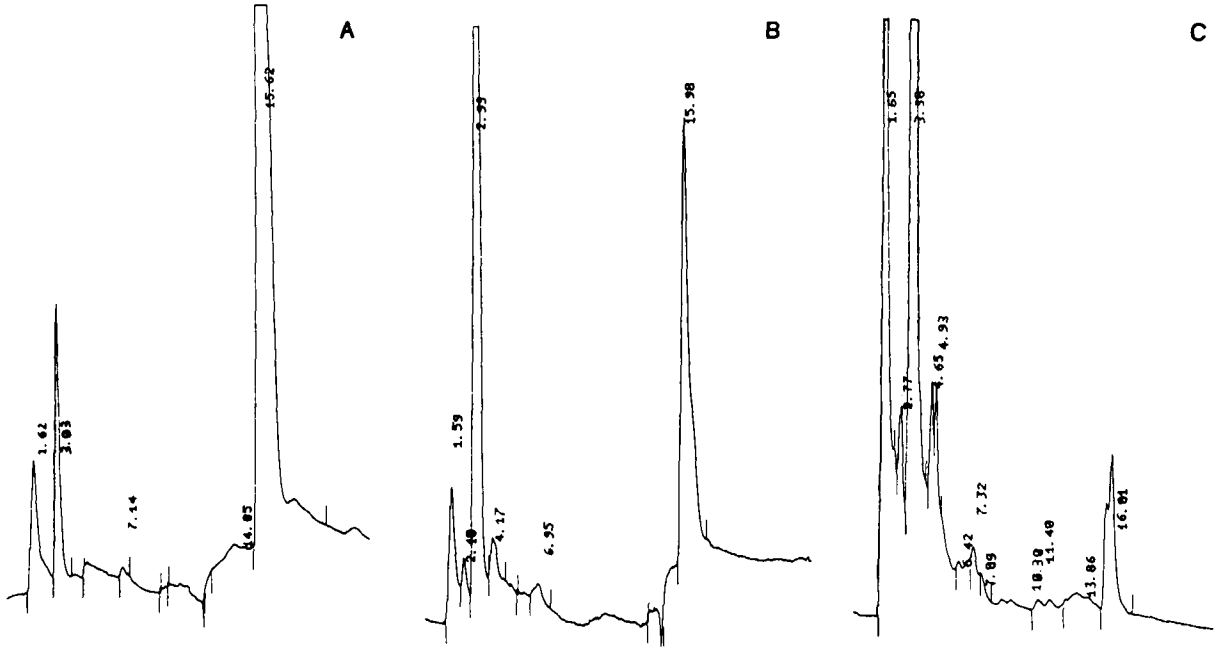

FIG. 2. Chromatographic analysis of degradation products of CPC by enzymic complex of *P. oxalicum* autolysed cultures at different incubation times (A: 0 h; B: 1 h and C: 8 h). The retention times were: (1) CPC 15·8–16 min; deacetyl-CPC, 3·0–3·3 min; deacetyl-7-ACA, 2·4–2·7 min; deacetyl-glutaryl-7-ACA, 1·5–1·7 min.

$FeSO_4$ 50, $CuSO_4$ $5H_2O$ 10, $MnSO_4$ $4H_2O$ 10, and $(NH_4)_6Mo_7O_{24}$ $4H_2O$ 10). Each medium was distributed in 200 mL portions in 1 L sterile flasks. For each fungus and each medium batches of 5 flasks were prepared and steam sterilized on 3 successive days for 30 min each day. Each flask was inoculated with 1 mL of a spore suspension containing 10^6 spores mL^{-1}. The inoculated flasks were incubated at 25°C with shaking (110 rev min^{-1}) At 3, 4, 7 and 14 days of incubation a sample was taken. CPC acylase activity was determined in both the culture liquid and the mycelial extract of each fungal culture. The culture liquid was separated from the mycelium by centrifugation (5000 *g*). The mycelium was washed with distilled water

and frozen at −20°C. The frozen mycelium was ground with a pestle and mortar for 15 min at 4°C to obtain the mycelial extract.

Assay procedures. The CPC acylase activity was determined by the fluorimetric method of Reyes et al (1989) using fluorescamine to detect free $-NH_2$ groups produced after incubation of 0·5 mL of culture liquid or mycelial extract with 0·5 mL of CPC (2 mg mL^{-1}) in 0·05 M borate-citrate-phosphate buffer, pH 7·5 during 2 h at 37°C. Controls at zero time were carried out. One unit was defined as the amount of enzyme that catalyses the production of one μmol of 7-ACA, or equivalent products, in 120 min. The β-lactamase activity was determined by the method of O'Callaghan et al (1972) with a chromogenic cephalosporin substrate (Nitrocefin Oxoid SR 112) and esterase activity was determined with nitrophenyl acetate as substrate in 50 mM borate-citrate-phosphate buffer, pH 5·5.

Production of hydrolytic enzymes. Lytic enzymes were precipitated from the supernatant of 14 day-old autolysed cultures using tannic acid as described by Shibata & Nisizawa (1965). The dry precipitate was stored at 4°C and the CPC acylase activity was stable for at least one year. Enzyme activity was determined as a function of pH over a range from 2 to 10 in 0·05 M borate-citrate-phosphate buffer. The variation of activity with the incubation time was also assayed. The kinetics of the hydrolysis of CPC was followed by HPLC using a Polygosil 60-5 C 18, 5 μm (25 × 0·4), column and 50 mM KH_2PO_4-water in a gradient from 95% to 5% of phosphate as mobile phase; flow rate was 1 mL min^{-1} and detection at 262 nm.

Results and discussion

Generally the autolysis of filamentous fungi takes place in a similar manner under the same shaking conditions. Growth and autolysis of *Penicillium oxalicum* (Alfonso et al 1989) were studied using the stated conditions (Table 1) to determine the start and the end of autolysis (maximum and constant mycelium dry weight, respectively) as a model to study other fungi. The degree of autolysis was defined as the percentage loss in mycelium dry weight between the day of maximal growth and the day on which the sample was taken.

Fungi from different genera which previously had been studied to determine the presence and variation during autolysis of penicillin G and penicillin V acylases (Alfonso et al 1989) were also tested to determine the production of CPC acylases throughout the incubation time. Low activity of CPC acylase which increased with the incubation time was only detected in *Aspergillus nidulans*, *Fusarium culmorum* and *Penicillium oxalicum*; the activity was always higher in the metabolic liquids than in the mycelial extracts.

Based on these results, 19 *Aspergillus*, 6 *Fusarium* and 14 *Penicillium* strains were checked (Table 2). In the mycelial extract, most fungi did not show CPC acylase activity. In the metabolic liquid CPC acylase activity was found in 34 of the fungi tested. This extracellular acylase activity increased with the incubation time and a close relationship with the pH of the metabolic liquid was found. In most fungi in which the pH of the metabolic liquid was below 6 during autolysis, no CPC acylase activity was found, but when pH was higher than 6, activity was detected.

This acylase activity was first detected in bacteria by Walton (1964, 1966). Later, Shibuya et al (1981), Matsuda & Komatsu (1985) and Matsuda et al (1987) studied this activity in a strain of *Pseudomonas* and Kawate et al (1987a, b) have recently found an intracellular CPC acylase in the fungus *Paecilomyces* C-2106 sp. The CPC acylase activity found by us in strains of *Aspergillus*, *Fusarium* and *Penicillium* are extracellular enzymes.

It has not yet been established whether deacylation of CPC is due to the activity of only one enzyme or by an enzyme system.

β-Lactamase activity was not detected in any of the fungi studied.

Penicillium strains 184 and 222 were chosen as good producers of CPC acylase and classified as *P. rugulosum* and *P. griseofulvum*, respectively.

The enzymic complex from autolysed cultures of *P. oxalicum* was precipitated with tannic acid. The CPC acylase specific activity was 1 unit mg^{-1} of protein. The CPC acylase activity as a function of pH showed a maximum of activity at pH 7·5. The enzyme activity increased with the incubation time up to 1 h, after which a decrease in activity was observed. This was interpreted as a possible deamination of CPC by an amino-acid oxidase. High levels of esterase activity were detected in this enzymic complex. Walton (1964, 1966), has also found esterase activity in bacteria. The hydrolysis of cephalosporin C by enzymes obtained from the fungi *P. oxalicum*, *P. rugulosum*, *P. griseofulvum*, *A. niger* and *A. 35* was followed by HPLC (Fig. 1). The chromatogram of the hydrolysis products of cephalosporin C by *P. oxalicum* enzymes shows that deacetylation takes place simultaneously and deacetyl CPC, deacetyl glutaryl-7-ACA and deacetyl 7-ACA were produced, and confirmed by the retention time and spectrum of each substance (Fig. 2). It is important to consider that extracellular acylase activity is produced by different genera of filamentous fungi during their autolysis and a higher activity with increasing pH of the medium during this degradative process is observed. It has not yet been determined for glutaryl-7-ACA, or for CPC, or for the two substrates simultaneously.

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Inhibitory effects of catecholamines on cholinergically and non-cholinergically mediated contractions of guinea-pig isolated bronchial muscle

YUICHIRO KAMIKAWA, YASUO SHIMO, *Department of Pharmacology, Dokkyo University School of Medicine, Mibu, Tochigi 321-02, Japan*

Abstract—The actions of catecholamines on the responses evoked by electrical field stimulation or by acetylcholine and substance P in guinea-pig bronchial strip chain have been examined. Electrical field stimulation evoked a biphasic contraction, consisting of a cholinergically-mediated fast contraction followed by a non-cholinergically-mediated slow contraction. All catecholamines tested caused a concentration-dependent reduction in the height of the biphasic contraction, where non-cholinergic contractions were more potently inhibited. The inhibitory effect of isoprenaline was largely prevented by propranolol (2 μM) alone, whereas those of noradrenaline and adrenaline were prevented by treatment with both propranolol (2 μM) and yohimbine (2 μM). The inhibitory effect of dopamine was unaffected either by propranolol (2 μM), yohimbine (2 μM) or haloperidol (10 μM). Submaximal contractions of bronchial muscle evoked by exogenous acetylcholine (2 μM) or substance P (0·2 μM) were also inhibited by catecholamines, except dopamine, but the effects were antagonized by propranolol (2 μM) alone. The results suggest that in guinea-pig isolated bronchial muscle, catecholamines can inhibit both cholinergic and non-cholinergic excitatory neurotransmissions not only by postjunctional β-adrenoceptors but also by prejunctional α_2-adrenoceptors.

Previously, we have reported that catecholamines and related symphatomimetic amines can inhibit cholinergic neurotransmission of the guinea-pig trachea not only via postjunctional β_2-adrenoceptors but also by prejunctional α_2-adrenoceptors (Kamikawa & Shimo 1986). Similar observations were made by Vermeire & Vanhoutte (1979) and Grundström et al (1981). Recent evidence indicates that guinea-pig peripheral airways are predominantly innervated by excitatory cholinergic and non-cholinergic nerves and that substance P (SP) or related tachykinins might function as the transmitter substance of the non-cholinergic nerves (Håkanson et al 1983; Lundberg et al 1983; Leander et al 1984; Andersson & Grundström 1987; Kamikawa & Shimo 1989). Hence, in the present study we have investigated the modulating effects of catecholamines on neurogenic contractions of the guinea-pig bronchi. A preliminary report of some of these results has been made (Kamikawa & Shimo 1987).

Correspondence to: Y. Kamikawa, Department of Pharmacology, Dokkyo University School of Medicine, Mibu, Tochigi 321-02, Japan.

Materials and methods

Male guinea-pigs (300 to 700 g) were stunned, the tracheobronchial tree excised and the bronchial strip chain prepared (Kamikawa & Shimo 1989). Briefly, two pieces of right and left bronchial transverse strips, 2–3 mm wide, were connected in alignment with threads and immersed in a 10 mL organ bath filled with modified Krebs bicarbonate solution of the following composition (mM); NaCl 120, KCl 4·7, $CaCl_2$ 2·5, $MgCl_2$ 1·2, $NaHCO_3$ 25, KH_2PO_4 1·2, disodium edetate 0·03, ascorbic acid 0·12 and glucose 11 (pH 7·4). The Krebs solution always contained 20 μM choline chloride and was bubbled with 5% carbon dioxide in oxygen, and maintained at 37°C.

The preparation was suspended under an initial tension of 0·5 g and 60 min was allowed to elapse before experiments were started. The bronchial response was isometrically recorded by means of a force-displacement transducer (Nihon Kohden SB-IT-H) and a Nihon Kohden polygraph recorder (RJG-4004). Electrical field stimulation was with rectangular pulses of 8 Hz frequency, 0·5 ms duration and supramaximal voltage, through bipolar platinum electrodes which were 10 mm apart and connected to a Nihon Kohden stimulator (SEN-1011). The total number of stimulating pulses was kept constant at 40. For the elimination of endogenous prostaglandin biosynthesis in response to field stimulation, the Krebs solution contained 2 μM indomethacin. When the strip was electrically stimulated, a biphasic contraction was obtained composed of an initial fast contraction mediated by cholinergic- followed by a sustained contraction mediated by non-cholinergic-nerve stimulation (Kamikawa & Shimo 1989). The heights of these contractions were comparable to those of submaximal contractions induced by exogenous acetylcholine (ACh, 2 μM) and SP (0·2 μM), respectively. The effects of catecholamines on the electrically induced contractions were measured as the percentage changes of the original contraction height obtained just before the drug was applied to the bath.

Drugs used were noradrenaline bitartrate, adrenaline bitartrate, isoprenaline bitartrate, yohimbine hydrochloride, propranolol hydrochloride, carbachol chloride (Sigma), indomethacin (Sankyo), dopamine hydrochloride (Wako), acetylcholine chloride (Dai-ichi), substance P (Peptide Institute) and haloperidol (Searle). To prepare the drug solutions, catecholamines were