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ORIGINAL RESEARCH PAPER

Expandase-like activity mediated cell-free conversion of ampicillin to cephalexin by *Streptomyces* sp. DRS I

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Abstract Cell-free extracts of *Streptomyces* sp. DRS I converted ampicillin to cephalexin, presumably due to the activity of the enzyme, expandase. The extract was fractionated and characterized by colorimetric and chromatographic measurements coupled with disc-agar diffusion bioassay against an ampicillin-resistant, cephalexin-sensitive *E. coli* strain. Though expandase could not be identified, the presence of a hitherto unreported expandase in *Streptomyces* sp. DRS I is suggested.

Keywords Ampicillin · Biotransformation · Deacetoxycephalosporin C synthetase · *Streptomyces* sp.

Introduction

Penicillin N is converted to deacetoxycephalosporin C (DAOC) by DAOC synthetase (DAOCS), the so-called ring expanding enzyme or expandase (Kupka et al. 1983; Martin and Liras 1989). DAOCS is an Fe(II)- and α-ketoglutarate-dependent dioxygenase that catalyzes the oxidative conversion of the five-membered thiazolidine ring of the intermediate penicillin N into the six-membered dihydrothiazine ring of deacetoxycephalosporin C (Kohsaka and Demain 1979). The enzyme was studied initially in *Cephalosporium acremonium* (Kohsaka and Demain 1979) and later in *Streptomyces clavuligerus* (Jensen et al. 1982) and *S. lactamdurans* (Cortes et al. 1987). Extensive studies have been carried out with purified enzymes. Characteristics of the enzyme with regard to cofactor requirement, optimum pH, and temperature for optimum activity have been reviewed (Martin and Liras 1989).

Despite the obvious importance of the ring expansion reaction, earlier studies on DAOCS have often been impeded by poor enzyme stability and the difficulty in obtaining a large amounts of purified enzyme (Lübbe et al. 1985). Enzymes involved in the biosyntheses of secondary metabolites usually exhibit broad substrate specificities. However, expandase enzymes isolated from various sources have a very narrow substrate specificity (Cortes et al. 1987; Dotzlaf and Yeh 1987; Maeda et al. 1995). The enzyme in cell-free extracts, as well as after purification, has a very narrow substrate specificity and no detectable activity on readily available and inexpensive penicillins such as penicillin G and V produced by *Penicillium chrysogenum* (Dotzlaf and Yeh 1987; Maeda et al. 1995). However, by increasing the iron and α-ketoglutarate concentrations in the standard reaction mixture used for conversion of penicillin N, conversion of penicillin G and 14 other penicillin N

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analogues by the resting cells and cell-free extracts of *Streptomyces clavuligerus* NP1 could be detected (Cho et al. 1998). The enzymatic conversion of penicillin G is an important cost-saving step applicable for manufacturing oral cephalosporins. Hence, ensuing investigations to understand and characterize the catalysis of penicillin G has been carried out, but all these studies were carried out with *S. clavuligerus* NP1 (Adrio et al. 1999; Fernández et al. 1999). For instance, pre-incubation of crude extracts of *S. clavuligerus* NP1 in the presence of ferrous ions plus either ascorbate or α-ketoglutarate led to inactivation of the expandase enzyme (Adrio et al. 1999). Another study showed that the growth of *S. clavuligerus* NP1 in the presence of alcohol resulted in marked conversion of penicillin G by the resting cells (Fernández et al. 1999). In vitro conversion of penicillin G and ampicillin have also been carried out by recombinant *Streptomyces clavuligerus* NRRL 3585 deacetoxycephalosporin C synthase (Sim and Sim 2001).

The primary objective of this study was therefore to detect ampicillin to cephalosporin conversion in crude cell-free fractions of the soil isolate *Streptomyces* sp. DRS I.

Materials and methods

Microorganisms

Streptomyces sp. DRS I, an indigenous soil isolate, was used in this work. Its capacity to convert ampicillin to cephalexin was reported earlier (Roy et al. 1997). The absence of extracellular antibiotic in this strain facilitated detection of cephalosporin produced from added ampicillin by ring expansion.

Media and culture conditions

Streptomyces sp. DRS I was grown in medium consisting of (g/l) yeast extract, 3; malt extract, 3; peptone, 5 and glycerol 20, pH 7, in 250 ml conical flasks at 30°C with shaking. Two ml of this culture was inoculated into 400 ml Czapek-Dox plus broth (pH 7) held in 1 l flasks and shaken at 200 rpm for 72 h at 30°C. The composition of Czapek-Dox plus medium was same as that of Czapek-Dox medium but supplemented with 5 g peptone/l and 3 g yeast extract/l and sucrose was substituted for glycerol.

Preparation of cell-free extracts and its fractionation

Fermentation broths were centrifuged at 9,000×*g* for 15 min at 4°C. Pellets were washed twice by using buffer A (25 mM Tris/HCl, pH 7, 10 mM glucose, 10 mM $MgSO_4$, 10 mM KCl and 2 mM L-ascorbic acid). Cells (38.2 g wet wt) were resuspended in 45 ml buffer A and were disrupted by 3 × 5 min treatments of a Branson sonifier in an ice water bath. Cell debris was removed by centrifugation (18,000×*g*, 30 min, 4°C). The resulting extract (sup 1) was stored at 0°C. The pellet was resuspended in 45 ml buffer A, sonicated as above and the supernatant obtained after centrifugation was mixed with sup 1 (E_0 fraction). The resulting extracts, containing 9.6 mg protein/ml, were placed on ice and stored.

For further fractionation, 60 ml 40% (w/v) PEG-6000 (in buffer A) was added to 60 ml E_0 fraction and held overnight at 4°C. The precipitate was separated by centrifugation and suspended in 20 ml buffer B (25 mM Tris/HCl, pH 7, 10 mM glucose and 2 mM β-mercaptoethanol). The insoluble fraction was discarded after centrifugation (18,000×*g*, 30 min, 4°C). To 16 ml soluble fraction, 4 ml 1 M NaOH was added drop wise and held overnight at 4°C. The precipitate obtained after centrifugation (18,000×*g*, 30 min, 4°C) was dissolved in buffer B (E_s fraction, 1.6 mg protein/ml). For further fractionation of E_s fraction, 100 µl acetone was added to 400 µl E_s fraction. The precipitate was dissolved in 400 µl buffer C [(25 mM Tris/HCl, pH 7, 10 mM glucose, 2 mM BME and 2 mM $CaCl_2$), (E_S, acetone ppt. fraction, 1.1 mg protein/ml)]. An equal volume of streptomycin sulphate solution 2% (w/v) in 25 mM Tris/HCl, pH 7 was added drop wise to E_s fraction and held at 4°C. The precipitate obtained after centrifugation (18,000×*g*, 30 min, 4°C) was dissolved in buffer C and dialysed overnight against 200 volumes of buffer B at 4°C to remove streptomycin sulphate (E_{ST} fraction, 0.78 mg protein/ml). 40 µl E_{ST} fraction was applied to HPLC on a Hypersil C4 column (250 × 4.6 mm) using a mobile phase of 25 mM Tris/HCl (pH 6.5) containing 19% (v/v) acetonitrile at 1 ml/min.

Protein concentration was estimated by using the Lowry method with BSA as standard. The respective protein fractions were subjected to 12% (v/v) SDS-PAGE following the method of Maniatis et al. (1982).

Each track was loaded with 15–50 μg proteins. A set of protein molecular marker was used as control.

Ring expansion activity

To assess whether the cell-free extracts and different protein fractions can transform ampicillin, the reaction condition used in this study described by Maeda et al. (1995), with ampicillin instead of penicillin N. The standard reaction condition contained 25 mM Tris/HCl (pH 7), 2 mM β-mercaptoethanol, 5 mM each of $MgSO_4$, $FeSO_4$, ascorbic acid, KCl, α-ketoglutarate and 0.7 mM ATP. All reactions carried out in this study contained 4 mg ampicillin/ml and 0.1–2 mg protein/ml. Reaction mixtures were incubated at 30°C for 30 min. The order of addition of reaction components was carried out following the method of Shen et al. (1984). Reactions were stopped by adding equal volume of methanol to reaction mixture (Cho et al. 1998).

Detection of cephalexin in reaction mixture

Bioassay

Expandase activity was detected by paper disc-agar diffusion bioassay. Paper discs were saturated with 200 μl biosynthesis reaction mixture (cell-free extracts) as follows. Two discs were superimposed, and 50 μl samples were applied four times. After each application, the discs were dried for 30 min in air. Finally, discs were placed on nutrient agar medium and 0.8% agar medium seeded with *Escherichia coli* MTCC 48 (an ampicillin-resistant and cephalexin-sensitive strain). The plates were incubated overnight at 37°C. The diameters of zones of growth inhibition were measured and the amount of ampicillin conversion was quantified with calibration curves using pure cephalexin as standard.

Colour development

To assess the effect of varying the concentrations of cell-free protein (1–1.5 mg/ml), ampicillin (0.5–1.5 mg/ml) and incubation time (0–60 min) on cephalexin formation by the E_0 fraction, reactions carried out at 30°C as described above. Addition of NaOH to the reaction mixture containing cephalexin turned it yellow (Fujii et al. 1976), which was read at 470 nm and quantified with a calibration curve using pure cephalexin as standard. All values were subtracted by the background reading of the blank.

Thin layer chromatography

After termination of enzyme reaction, the reaction mixture was mixed with an equal volume of acetonitrile and held at 4°C overnight. The precipitate was dissolved in a minimum volume of water and 100 μl was subjected to TLC analysis (silica gel G) using butanol/acetic acid/water (2:1:1, by vol.) as solvent. The product was visualized after the exposure of oven-dried silica gel plates to iodine vapour. To detect antimicrobial activity in situ, the TLC plate was overlaid with soft NA (0.8%), containing *E. coli* and incubated overnight at 37°C.

Results and discussion

Fractionation of cell-free extracts and SDS-PAGE

A crude cell-free extract of *Streptomyces* sp. DRS I was fractionated and used for enzyme assay using ampicillin as the substrate. Figure 1 shows the protein profiles of the $E_{0,}$ E_S, E_S and E_{ST} fractions. Four bands were revealed in the E_S fraction but only a single one was present in the final E_{ST} fraction. HPLC of this fraction also showed only one peak (data not shown).

At this stage it is not possible to pinpoint which protein band shown on the acrylamide gel is expandase, the protein bands obtained from E_s fractionation by acetone precipitation or the protein band obtained by streptomycin sulfate precipitation (Fig. 1) since both the fractions showed the capacity to convert ampicillin to cephalexin as shown from bioassay against *E. coli* MTCC 48 (Table 1). It can be presumed only that the protein band obtained from the streptomycin sulfate precipitation might be a fragment of the band shown by acetone precipitation, which has not lost its active site (Fig. 1). Further work would be needed to answer this question. There is some disagreement in the molecular weight of the expandase of *C. acremonium* in the data of different authors. Kupka et al. (1983) and Scheidegger et al. (1984) reported a molecular weight for this enzyme of 31 and 33 kDa, respectively, while a molecular

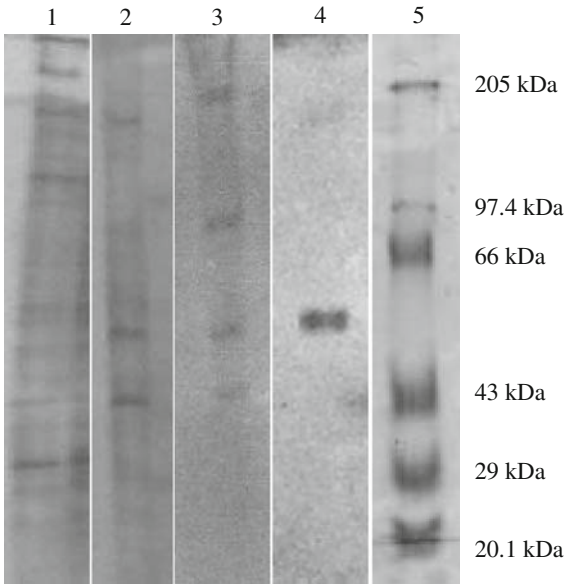

Fig. 1 SDS-PAGE of fractions of cell-free extracts with 12% (v/v) gradient gel. After electrophoresis, the gels were stained with Coomasie Brilliant Blue R-250. *Lane 1*, cell-free extracts (E_0 fraction); *lane 2*, 40% (w/v) PEG-6000 precipitate (E_S); *lane 3*, active fractions obtained after acetone precipitate (E_S, acetone ppt.); *lane 4*, active fractions obtained after streptomycin sulphate precipitate (E_{ST}); *lane 5*, molecular mass markers and sizes are shown in kilodaltons. About 50 μg proteins were loaded in lanes 2–5. *Lane 1* was loaded with 15 μg protein

weight of 41 kDa (Table 2) has been described for the same enzyme (Dotzlaf and Yeh 1987). The origin of these variations is not known; it might be due to strain differences or in vitro proteolytic degradation. The expandases of *S. clavuligerus* and *S. lactamdurans* have molecular weight values of 29.5 (Jensen et al. 1985) and 27 kDa, respectively, which are lower than the molecular weight of the fungal enzyme (Table 2).

Ring expansion activity of cell-free extracts

Using different protein fractions established for cell-free transformation of ampicillin to cephalexin, conversion was observed in all reaction mixtures against *E. coli* MTCC 48 suggesting the presence of expandase in cell-free extracts of *Streptomyces* sp. DRS I (Table 1). Table 3 shows results obtained when different concentrations of cell-free protein and ampicillin were tested with cell-free extracts. The conversion rates of ampicillin by cell-free extracts were compared over a period of 1 h. It is clear that an equal mixture of enzyme and substrate in the reaction was found to be optimum for product formation after 30 min incubation (Table 2). Using the conditions reported by Maeda et al. (1995), in which 1–2 mg protein/ml was in the cell-free extracts, we observed activity in reaction mixture. Substrate (ampicillin) or protein (in cell-free extracts) higher than 1 mg/ml did not improve the ring expansion activity (Table 3).

Increase in O.D. at 470 nm absorbance of the reaction mixture containing different concentrations of cell-free extracts (E_0 fraction) and substrate

Table 1 Transformation of ampicillin by cell-free extracts of *Streptomyces* sp. DRS I with different concentrations of protein fractions

Protein fractions	Total protein (mg/ml)	Inhibition zones (mm) against *E. coli*	Cephalexin formation in reaction mixture (μg/ml)
Cell-free extract (E_0 fraction)	0.5	12	7.1
	1	14	8.2
	2	16	9.4
40% (w/v) PEG-6000 precipitate (E_S fraction)	0.1	20	11.8
Acetone precipitate (E_S acetone ppt. fraction)	0.1	20	11.8
Streptomycin sulphate precipitate (E_{ST} fraction)	0.1	17	10
40 μg ampicillin (without protein)[a]	–	0	0
Cell-free extract (without ampicillin)[b]	1	0	0

[a] Reaction was carried out without protein

[b] Reaction was carried out without substrate (ampicillin)

All reactions contained 25 mM Tris/HCl (pH 7), 2 mM β-mercaptoethanol, 5 mM each of $MgSO_4$, $FeSO_4$, ascorbic acid, KCl, α-ketoglutarate, 0.7 mM ATP, 4 mg ampicillin/ml and 0.1–2 protein mg/ml. Reaction mixtures were incubated at 30°C. Reactions were terminated after 30 min by the addition of an equal volume of methanol and were centrifuged (14,000×*g*, 5 min); 200 μl of the reaction mixture was used in the bioassay. The amount of product detected was measured using cephalexin as the standard

Table 2 Characteristics of previously reported deacetoxycephalosporin C synthases (expandase)

Source	Enzyme activity	Requirements of co-factors for ring expansion	Stimulators for ring expansion	Optimum pH	Optimum Temp. (°C)	Mol. wt (kDa)	References
C. acremonium	Expandase and hydroxylase	α-KG	DTT (1.00 mM)	7.3–7.8	26–34	41	Dotzlaf and Yeh (1987)
		Fe^{2+}	ASC (0.25 mM)				
		O_2	ATP (0.05 mM)	7.3	36–38		
S. lactamdurans	Expandase	α-KG	DTT (0.1 mM)	5–11	25–30	27	Cortes et al. (1987)
		Fe^{2+}	ASC (2.8 mM)				
		O_2					
S. clavuligerus	Expandase	α-KG	ND[b]	ND	ND	29.5	Jensen et al. (1985)
		Fe^{2+}					
		K^+					

Abbreviations: [a] *α-KG* α-ketoglutarate, *ASC* ascorbic acid, *ND* not determined

Earlier studies have reported that DTT and ascorbate stimulate the ring expansion of penicillin N and different concentrations of DTT and ascorbate was reported to be optimal. For example, DTT added at 5 mM stimulated the expandase activity detected in the cell-free extracts of *S. clavuligerus* (Lübbe et al. 1985) whereas Cho et al. (1998) used 16 mM DTT in reaction mixture

Table 3 Effect of protein concentration (E_0 fraction), substrate concentration and incubation time on ring expansion of ampicillin and quantification of product formation

Incubation (min)	Protein (mg/ml)					
	1			1.5		
	Substrate (mg/ml)					
	0.5	1	1.5	0.5	1	1.5
	A_{470} nm [Mean ($n = 5$) ± SD][a]					
0	0.016 ± 0.002	0.074 ± 0.003	0.090 ± 0.002	0.018 ± 0.002	0.052 ± 0.002	0.097 ± 0.002
30	0.019 ± 0.002 (ND)[b]	0.108 ± 0.002 (0.056)	0.094 ± 0.003 (ND)	0.020 ± 0.003 (ND)	0.061 ± 0.002 (0.014)	0.114 ± 0.003 (0.028)
60	0.032 ± 0.003 (0.02)	0.103 ± 0.003 (0.048)	0.103 ± 0.002 (0.021)	0.023 ± 0.002 (0.01)	0.082 ± 0.002 (0.049)	0.107 ± 0.002 (0.016)

[a] A_{470} values represent the average of five readings and SD was calculated for each experiment

[b] Figures in parentheses indicate product formation in reaction mixture (cephalexin mg/4 ml)

Abbreviation: *N* number of readings (470 nm), *SD* standard deviation, *ND* no detectable activity

Different concentrations of cell-free extracts (E_0 fraction) and substrate (ampicillin) were added per ml of reaction mixture (see Table 1). Reaction mixtures were incubated at 30°C. Reactions were stopped at different times by adding equal volume of methanol. 4 ml sample from each reaction was taken and adding 1 ml NaOH and held for 30 min. All reaction mixtures were read at 470 nm and quantified with a calibration curve using pure cephalexin as standard. All 470 nm values were subtracted by the background reading of the blank

(ampicillin) was very small (Table 2). The enzyme reaction was carried out using arbitrarily selected concentrations of ingredients in the reaction mixture. As the concentration of the ingredients (and even the order of addition of ingredients) effects expandase activity that converts penicillin N to deacetoxycephalosporin C (Shen et al. 1984), biotransformation of ampicillin to cephalexin may be improved by carrying out the enzyme reaction under optimum conditions. Cho et al. (1998) found that by increasing the

concentration of the substrate, co-substrate (α-ketoglutarate) and $FeSO_4$, and also by using a high concentration of cells, conversion of penicillin G to DAOG was markedly improved. Using such conditions, they showed activity on penicillin G and 14 other penicillins including ampicillin with cell-free extract of *Streptomyces clavuligerus* NP1.

TLC analysis of the reaction mixture showed presence of two compounds in addition to ampicillin. R_f of one of the compounds was the same as that of cephalexin. Bioautography of TLC plate revealed that the compound with same R_f value with cephalexin was active against *E. coli* and the others were inactive (figure not shown). Therefore, it was experimentally substantiated that cell-free extracts and partially purified enzyme preparations of *Streptomyces* sp. DRS I, have been able to convert ampicillin to cephalexin.

Taking together from our findings, it is evident that crude cell-free extracts (E_0 fraction), fractions E_s (acetone precipitate) and E_{ST} of *Streptomyces* sp. DRS I contain an expandase responsible for transformation of ampicillin to cephalexin.

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