ENZYMATIC CONVERSION OF ACLACINOMYCIN A TO Y BY A SPECIFIC OXIDOREDUCTASE IN *STREPTOMYCES*

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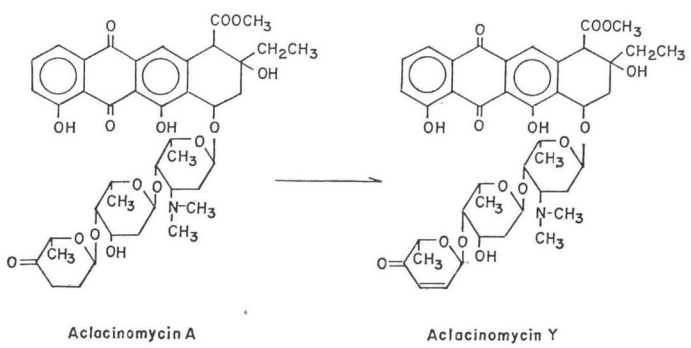
A specific oxidoreductase converting aclacinomycin A to a new analog, aclacinomycin Y, was purified to apparent homogeneity from the culture filtrate of aclacinomycin-producing microorganisms. The isolated enzyme was a weakly acidic protein (isoelectric point, 5.9) with a molecular weight of about 72,000. The enzymatic reaction requires molecular oxygen and has a pH optimum at 5.5.

The enzyme catalyzed an oxidation of the terminal sugar, L-cinerulose, of the trisaccharide moiety of aclacinomycin A to L-aculose (2,3,6-trideoxyhex-2-enopyranos-4-ulose) with removal of two electrons. Studies of substrate specificity revealed that the enzyme is an oxidoreductase capable of modifying anthracyclic triglycosides by oxidizing their terminal sugars.

Aclacinomycin A (ACM-A) and nineteen related anthracyclic compounds have been found in a culture broth of *Streptomyces galilaeus* strain No. MA144-M1 (ATCC 31133)[1,2]. More recently we found in a cultural filtrate of the strain MA144-M1 an additional analog, named aclacinomycin Y (ACM-Y)[3], which possesses an unique terminal sugar residue, 2,3,6-trideoxyhex-2-enopyranos-4-ulose (L-aculose), and which has approximately a 10-fold greater antimicrobial activity than ACM-A. The aclacinomycin analogs (*e.g.*, aclacinomycin B (ACM-B), MA144 N1, M1 and L1), including ACM-A and ACM-Y, differ structurally in the sugar residues of the trisaccharide linked to aklavinone at the C-7 position, and appear to be formed by the modification of their terminal sugar residues by oxidizing or dehydrogenating enzymes. To clarify the biosynthetic relationship among these analogs, we attempted to isolate an enzyme capable of converting ACM-A to ACM-Y (Fig. 1) from the cultural filtrates of aclacinomycin-producing microorganisms and other anthracycline producers.

Fig. 1. Enzymatic conversion of aclacinomycin A to Y.



In this paper we describe the isolation and properties of an oxidizing enzyme responsible for the formation of

ACM-Y, and the characterization of newly isolated antibiotic aclacinomycin Y.

Results

Properties of Oxidoreductase

The converting enzyme, an oxidoreductase, was purified with an overall yield of 1.7% from the cultural filtrate of *S. galilaeus* MA144-M1 strain 6U-21 as described in Experimental (Table 1).

The molecular weight and isoelectric point of the enzyme were estimated to be 72,000 (Fig. 2) and 5.9 (Fig. 3), respectively. The enzyme was stable at the range of pH 5.0~8.0 when incubated at 37°C for 3 hours. The enzyme was stable to heating at 50°C for 30 minutes in neutral pH, but lost 20% and 50% of activity at 60°C and 70°C, respectively. No loss of activity was observed on freezing at −20°C in 10% glycerol - 0.05 M Tris-HCl (pH 7.2) for at least 2 months.

Table 1. Purification of oxidoreductase from a culture filtrate of *Streptomyces galilaeus* MA144-M1, mutant No. 6U-21.

Purification step	Vol (ml)	Protein (mg)	Total act. (units)	Yield (%)	Specific act. (units/mg)	Purification (fold)
Culture filtrate	3,300	22,100	119,800	100	5.0	1.0
$(NH_4)_2SO_4$ ppt. (50%)	260	2,314	111,700	97.7	50.6	9.7
DEAE-Cellulose	38	76	38,000	31.7	500.0	100.0
DEAE-Sephadex A-50	87	7.5	6,600	5.5	800.0	176.0
Sephadex G-75	125	2.1	2,000	1.7	952.4	190.5

Fig. 2. Molecular weight determination of the enzyme by Sephadex G-75 column chromatography

(a) egg lysozyme (14,300), (b) trypsin inhibitor (21,500), (c) ovalbumin (46,000), (d) bovine serum albumin (68,000), (e) human γ-globulin (150,000). Open circle shows the oxidoreductase.

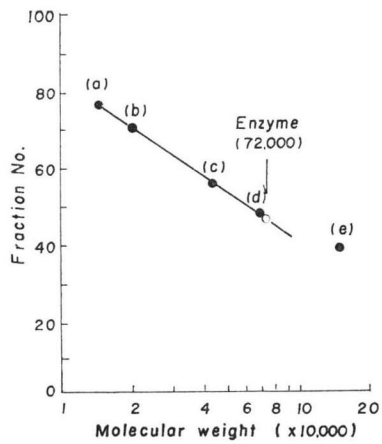
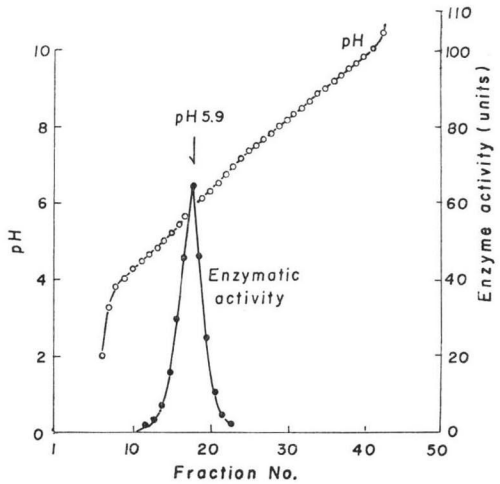


Fig. 3. Isoelectric focusing of the enzyme on pH 3~10 carrier ampholine.



The enzyme reaction proceeded optimally at pH 5.0~6.0 (Fig. 4) and required molecular oxygen as shown in Table 2. NAD^+, $NADP^+$, phenazine methosulfate and 2,6-dichlorophenolindophenol could not serve as electron acceptors in this enzyme reaction. Effects of divalent cations and some enzyme inhibitors on the reaction are shown in Table 3. NaN_3 and ascorbic acid (1 mM) inhibited

Fig. 4. pH Optimum of enzyme activity.

Buffers (50 mM) were; citric acid-NaOH buffer, acetic acid-NaOH buffer, K_2HPO_4-KH_2PO_4 buffer, and Tris-HCl buffer.

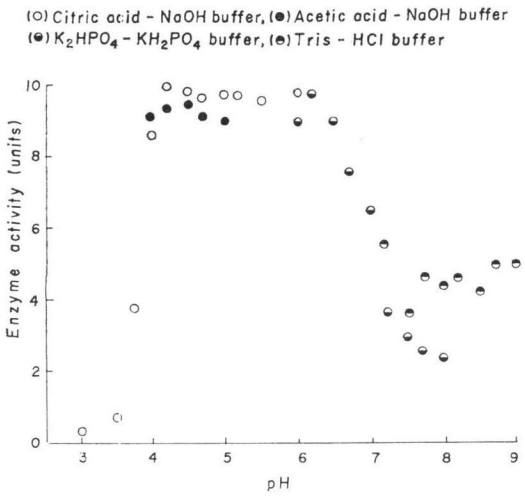


Table 2. Oxygen requirement for enzyme reaction

Electron acceptor	Concentration (mM)	ACM-Y formed (n moles)	Relative rate (%)
None		5.2	6.5
$NADP^+$	0.1	5.3	6.6
NAD^+	0.1	5.0	6.2
FAD	0.05	5.4	6.7
FMN	0.05	5.9	7.5
Phenazine methosulfate	0.1	5.0	6.2
2,6-Dichlorophenolindophenol	0.1	5.6	7.0
O_2		80.0	100.0

The reaction mixture (2 ml) contained 100 nmoles of ACM-A, 100 μmoles of citric acid - NaOH buffer (pH 5.5), 4 units of enzyme, and indicated amount of electron acceptor in a Thunberg tube changed with N_2 gas was incubated at 37°C for 30 minutes by shaking and extracted with toluene. ACM-Y was determined by thin-layer chromatography as described in Experimental. The result with O_2 as acceptor was obtained in an open Thunberg tube.

Table 3. Effect of various additions on enzyme activity.

Addition	Concn. (mM)	% Control
Mg^{++}	1	100
Mn^{++}	1	100
Fe^{++}	0.5	74.4
	1	26.8
Ni^{++}	1	100
Co^{++}	1	100
Cu^{++}	1	100
Ca^{++}	1	100
Zn^{++}	1	100
EDTA	1	100
p-Chloromercurybenzoate	1	100
Monoiodoacetate	1	100
NaN_3	1	85.3
	5	47.4
Ascorbic acid	1	85.5
	5	55.5
Sodium borate	1	100
Mercaptoethanol	1	100
Potassium ferricyanate	1	100
Brilliant blue R	1	100
Triphenol tetrazolium chloride	1	100

significantly, but metal ions, except for ferric ion, EDTA and SH reagents such as *p*-chloromercuric benzoate and monoiodoacetate did not.

Enzymatic Conversion of ACM-A and MA144 N1 to ACM-Y

Enzymatic oxidation of ACM-A to ACM-Y proceeded as shown in Fig. 5a. With the incubation time, ACM-A disappeared with nearly proportional formation of ACM-Y, and ACM-B (Fig. 7) was also formed slowly. Recently, we found that ACM-Y in 50 mM citric acid - NaOH buffer (pH 5.5) can be non-enzymatically converted to ACM-B by shaking in the absence of enzyme (unpublished data). MA144 N1 was also converted by this enzyme to ACM-Y through intermediate formation of ACM-A, low levels of which were detected in the kinetic experiments (Fig. 5b).

Substrate Specificity

Aklavinone glycosides and ϵ-pyrromycinone glycosides such as aclacinomycins[1,2,4], cinerubins[2,5]

Fig. 5. Enzymatic oxidation of aclacinomycin A (a) and MA144 N1 (b).

Reaction mixture containing 100 nmoles of ACM-A or MA144 N1 and 2 units of the enzyme in 1 ml of 50 mM citric acid-NaOH buffer (pH 5.5) was incubated at 37°C for 4.5 hours with shaking. At appropriate time intervals, a reaction mixture was extracted with 0.2 ml of toluene. Twenty μl of the extract were chromatographed on silica gel plate with a solvent of benzene - ethylacetate - methanol - formic acid - H_2O (5: 5: 1.5: 1: 0.3).

Enzymatic products were determined by a TLC scanner as described in Experimental.

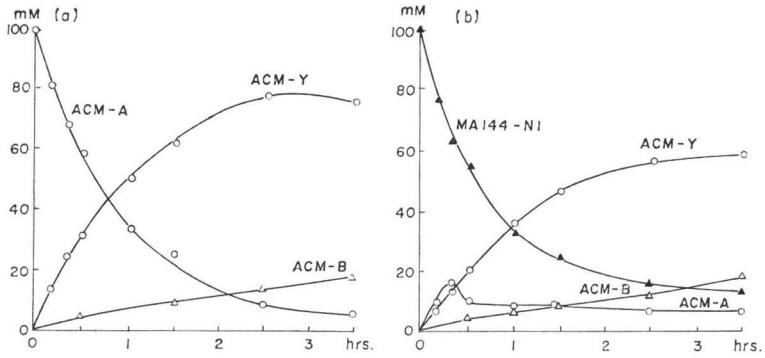


Fig. 6. Time course of production of the enzyme and antibiotic by aclacinomycin-producing strain 6U-21.

Strain No. 6U-21 was cultivated as described in Experimental. At appropriate time intervals, 2 ml of the culture were sampled and centrifuged at 10,000×g for 10 minutes. The supernatant was assayed for enzyme activity. Aclacinomycins were extracted from the mycelial pellet with 5 ml of acetone and determined by O.D at 430 nm of the extract. The residual pellet was extracted for nucleic acids (NA) with 5 ml of 5% trichloroacetic acid. Cell growth is determined by O.D at 260 nm of the extract.

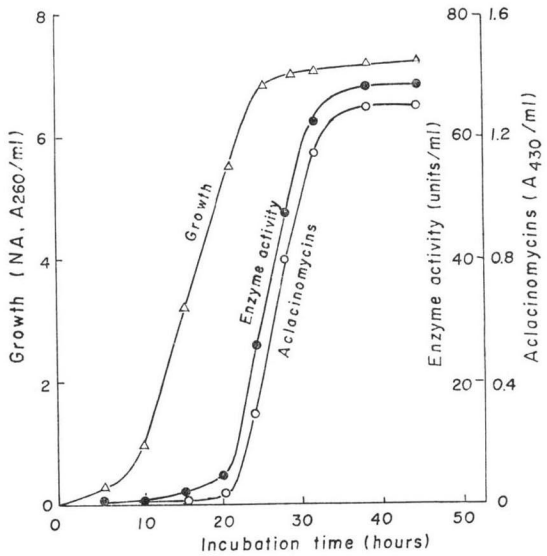
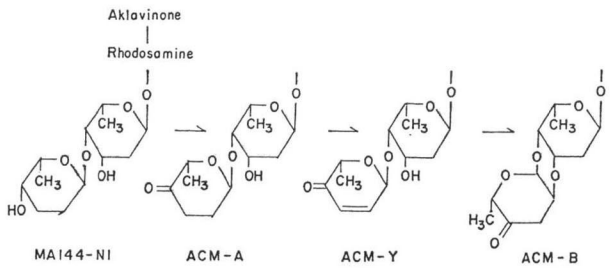


Fig. 7. Biopathway in the formation of aclacinomycin analogs from MA144 N1.



and rhodirubins[6], methyl-L-cinerulosyl-2-deoxy-L-fucoside[5] (disaccharide moiety of ACM-A) and the deoxysugar mixture obtained from ACM-A and MA144 N1 by acid hydrolysis were tested. Enzymatically oxidized products were examined by thin-layer chromatography using two solvent systems of benzene - ethylacetate - methanol - formic acid - water (5: 5: 1.5: 1: 0.3) and chloroform - methanol (20: 1) and quantitative values were determined by a TLC scanner. Rf values of products and relative rate of oxidation of substrate are shown in Table 4. Besides ACM-A and MA144 N1 and their analogs MA144 L1 and U1, the ε-pyrromycinone glycosides, rhodirubins A, B, C (cinerubin A) and E, all triglycosides containing L-cinerulose, L-rhodinose or 2-deoxy-L-fucose as a terminal sugar, were oxidized with removal of 2 or 4 electrons of the terminal sugar with various extents. However, MA144 M1, which contained L-amicetose (isomer of L-rhodinose) as terminal sugar, MA144 G1 having D-cinerulose A, MA144 U5[4] in which rhodosaminyl residue of the trisaccharide moiety of ACM-A is replaced by 2-deoxy-L-fucose, rhodirubin D (ε-pyrromycinone diglycoside with terminal 2-deoxy-L-fucose), and MA144 S1 (aklavinone diglycoside with terminal 2-deoxy-L-fucose) were not oxidized. Methyl-L-cinerulosyl-2-deoxy-L-fucoside as well as deoxysugars such as L-cinerulose, L-rhodinose and 2-deoxy-L-fucose were not oxidized. Granaticin B[7] having L-rhodinose was also resistant to this enzyme.

Table 4. Substrate specificity of the enzyme.

Substrate	Rf*	Product Rf*	Relative** rate of oxidation (%)
Aklavinone glycosides			
Aclacinomycin A (RA-DF-CinA)***	0.29	0.34	95.6
Aclacinomycin B (RA-DF-CinB)	0.43	no change	0
MA144 G1 (RA-DF-D-CinA)	0.27	no change	0
MA144 L1 (meDA-DF-CinA)	0.36	0.41	17.3
MA144 M1 (RA-DF-Ami)	0.22	no change	0
MA144 N1 (RA-DF-Rho)	0.17	0.34	87.0
MA 144 S1 (RA-DF)	0.11	no change	0
MA144 U1 (RA-DF-DF)	0.07	0.14	10.0
MA144 U5 (DF-DF-CinA)	0.53	no change	0
ε-Pyrromycinone glycosides			
Rhodirubin A (RA-DF-Rho)	0.17	0.35	90.5
Rhodirubin B (RA-Rho-Rho)	0.25	0.40	18.9
Rhodirubin C (RA-DF-CinA)	0.29	0.35	100.0
Rhodirubin D (RA-DF)	0.12	no change	0
Rhodirubin E (RA-DF-DF)	0.07	0.14	8.5

Reaction mixture contained 8 units of the enzyme and 50 nmoles of substrate in 1 ml of 50 mM citrate buffer (pH 5.5) and was incubated at 37°C for 30 minutes.

* Rf values on silica gel TLC. Solvent system: benzene - ethylacetate - methanol - formic acid - water (5: 5: 1.5: 1: 0.3).

** % of product to substrate

*** ( ) sugar moiety: RA; rhodosamine, DF; 2-deoxyfucose, Cin; cinerulose, Rho; rhodinose, Ami; amicetose, meDA; N-monomethyldaunosamine.

Occurrence of Oxidoreductase Activity in Anthracycline-producing Microorganisms

Aclacinomycin-producing strain, *S. galilaeus* MA144-M1 and its blocked mutants accumulating various aclacinomycin analogs, and producers of other anthracyclic compounds were tested for the activity capable of converting ACM-A to ACM-Y. As shown in Table 5, aklavinone glycoside- and ε-pyrromycinone glycoside-producing strains had a high level of enzyme activity which ranged from 20 to 80 units/ml. Among the mutants derived from aclacinomycin-producing strain MA144-M1, MA144 N1-accumulating mutant (strain No. KE-197) and all of non-pigmented mutants lacked the

Table 5. Oxidoreductase activity in anthracycline-producing organisms.

Organisms	Strain characteristics (major fermentation products)	Enzyme activity (units/ml)
S. galilaeus MA144-M1 (original strain)	aclacinomycins	79.3
" " MA144-M1 mutant No. 6U-21	aclacinomycin A and B	69.0
" " MA144-M1 mutant No. 7U-357	aclacinomycin B	36.3
" " MA144-M1 mutant No. KE-197	MA144 N1	0
" " MA144-M1 mutant No. 7U-491	MA144 S1 and U1	34.2
" " MA144-M1 mutant No. 3AR-33	aklavinone	16.0
" " MA144-M1 mutant No. 7U-147	aglycone (unidentified)	22.7
" " MA144-M1 mutant No. 7U-300	" "	38.3
" " MA144-M1 mutant No. KE-303	less productive	5.4
" " MA144-M1 mutant No. ANR-396	non-productive	0
" " MA144-M1 mutant No. 5AF-305	"	0
" " MA144-M1 mutant No. 7AF-100	"	0
S. ME505-HE1	rhodirubins	25.0
S. cinereoruber IFO 12756	cinerubins	45.6
S. capoamus A-593	"	24.5
S. purpurascens ISP 5310	rhodomycins	2.3
S. violarus A-530	"	1.9
S. roseoviolaceus ISP 5277	roseorubicins	0
S. steffisburgensis A-775	steffimycins	0
S. nogalater NRRL 3035	nogalamycins	0
S. rubirireticuli ISP 5464	ruticulomycins	0
S. coeruleorubidus ME130-A4	baumycins	0
S. coeruleorubidus NRRL B-3045	daunomycins	0
S. peuceticus subsp. *caesius* ATCC 27952	adriamycins	0

S. galilaeus, *S.* ME505-HE1, *S. cinereoruber* and *S. capoamus* were cultivated at 28°C for 2~3 days in the medium described in Experimental. Other organisms were cultivated at 28°C for 5 days in a medium consisted of 3% glycerol, 1% soybean meal, 2% CSL, 0.1% yeast ext., 0.3% NaCl, 0.25% $CaCO_3$, pH 7.2. The cultural filtrates were assayed for the enzyme activity.

Table 6. Antimicrobial activities of aclacinomycins A and Y.

Microorganisms	MIC (μg/ml)		Microorganisms	MIC (μg/ml)	
	ACM-A	ACM-Y		ACM-A	ACM-Y
Staphylococcus aureus FAD 209P	6.2	0.4	*Pseudomonas fluorescens* NIHJ B-254	100	100
Staphylococcus aureus Smith	0.8	0.2	*Proteus morganii*	100	100
Bacillus subtilis ATCC 6633	3.1	0.2	*Mycobacterium smegmatis* ATCC 607	100	100
Bacillus cereus ATCC 9634	0.8	0.1	*Candida albicans* IAM 4905	25	100
Sarcina lutea ATCC 9341	0.8	0.2	*Candida tropicalis* IAM 4942	50	100
Micrococcus flavus	0.4	0.1			
Corynebacterium bovis 1810	0.8	0.2			

Antimicrobial activity was determined by broth dilution method.

enzyme activity. Rhodomycin-producers showed only a low enzyme activity, but other anthracycline producers did not. Fig. 6 shows the time-course production of the enzyme and antibiotic activity by aclacinomycin-producing strain No. 6U-21 during cultivation.

Biological Activity of ACM-Y

Antimicrobial activity of ACM-Y was strikingly strong (4~10-fold stronger than ACM-A)

against Gram-positive bacteria in comparison with other aclacinomycin analogs[4], as shown in Table 6. ACM-Y inhibited markedly the growth and nucleic acid biosynthesis of cultured L1210 cells; IC_{50}, 0.03 μg/ml for the growth, 0.29 μg/ml for DNA synthesis, and 0.02 μg/ml for RNA synthesis, while it was less active against L1210 leukemia in BDF_1 mice (ILS: 130%) as compared with ACM-A[4].

Discussion

Several studies on microbial metabolism of anthracyclic compounds have been reported[8~13]. Reductive cleavage of glycosidic linkage at C-7 position of an aglycone[8,9,12,13] and reduction of 13-keto group of adriamycin, daunomycin and their aglycones[10,11,13,14] take place by NADPH-dependent cytochrome P450 reductase[12] and NADH-dependent aldo-ketoreductase[14], respectively. In the present work an enzyme capable of converting ACM-A to ACM-Y was found in *S. galilaeus* MA144-M1. This enzymatic modification was characterized by the oxidation of the terminal sugar of anthracyclic glycosides. The reaction required molecular oxygen since it did not proceed under anaerobic condition with or without electron acceptor (Table 2). This oxidizing enzyme is specifically active on anthracyclic triglycosides. It is most active on anthracyclic triglycosides with sugar sequence of RA(rhodosamine)-DF(2-deoxy-L-fucose)-Cin(cinerulose), moderately active on those with RA-DF-Rho(rhodinose), less active on those with RA-Rho-Rho, RA-DF-DF or meDA(N-monomethyl daunosamine)-DF-Cin and inactive on those with DF-DF-Cin. MA144 G1 and M1 having sugar sequence of RA-DF-D-Cin and RA-DF-Ami(amicetose) respectively, and anthracyclic mono- and diglycosides were not oxidized. These results indicate that strict structural requirement for the enzymatic oxidation is present in the sugar sequence and sugar component of trisaccharide moiety. It is obvious that aclacinomycin analogs, ACM-A, ACM-Y and ACM-B, are successively formed from MA144 N1 by enzymatic oxidation and a non-enzymatic addition of the hydroxyl group to the generated enone system as shown in Fig. 7. This biopathway was supported by the fact that a mutant strain No. KE-197, which lacks in the enzyme activity capable of converting ACM-A to ACM-Y, accumulated only MA144 N1 in the culture broth.

We have further examined the distribution of this enzymatic activity in *Streptomyces*, including various antibiotic-producers; aminoglycosides (*e.g.*, sisomicin, streptomycin and validamycin) and macrolides (*e.g.*, carbomycin, cirramycin, josamycin, lankamycin, spiramycin and tylosin) and anthracyclines (*e.g.*, adriamycin, daunomycin, nogalamycin, rhodomycin, cinerubin, rhodirubin, steffimycin and aclacinomycin), and found that the enzyme existed only in the strains capable of producing anthracyclic triglycosides (aclacinomycin, cinerubin, rhodirubin and rhodomycin).

Experimental

Microorganisms and cultivation

Streptomyces galilaeus MA144-M1 (ATCC 31133), strain No. 6U-21 was used for the isolation of the enzyme. Mutants used in this experiment were isolated by treatment with ultraviolet light, N-methyl-N′-nitro-N-nitrosoguanidine or acriflavine. Details of the mutants used will be described elsewhere. The cultivation was carried out in a 500-ml Erlenmeyer flask containing 50 ml of the medium, consisted of 1.5% soluble starch, 1% glucose, 3% soybean meal, 0.1% yeast extract, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot H_2O$, 0.3% NaCl, 0.007% $CuSO_4 \cdot 5H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0008% $MnCl_2 \cdot 4H_2O$ and 0.0002% $ZnSO_4 \cdot 7H_2O$ (pH 7.4), on a rotary shaker (210 rpm) for 2~5 days at 28°C.

Enzyme assay

Reaction mixture containing 0.1 μmole of ACM-A, 50 μmoles of citric acid - NaOH buffer (pH 5.5) and enzyme source in 1 ml in a glass tube (14 × 100 mm) was incubated at 37°C for 30 minutes with shaking. The reaction products were rapidly extracted with 0.25 ml of toluene after addition of 1 ml of 0.2 M Tris - HCl buffer (pH 7.5). Twenty μl of the extract were spotted on a silica gel plate which

was developed in a solvent of benzene - ethylacetate - methanol - formic acid - water (5: 5: 1.5: 1: 0.3). The enzyme activity was determined from the amount of residual ACM-A on silica gel plate by densitometric scanning at 430 nm (Shimazu-TLC scanner model CS-910). Linear relationship between the conversion rate (%) and the enzyme concentration was obtained in a range of 0.7 to 4 units of enzyme. One unit of the enzyme activity corresponded to the amount capable of converting 1 nmole of ACM-A per minute.

Properties of enzyme

The molecular weight of the enzyme was determined on a Sephadex G-75 column according to the method of ANDREW[15] using egg lysozyme (14,300), trypsin inhibitor (21,500), ovalbumin (46,000) and bovine serum albumin (68,000) as standard.

pH Optimum for the enzyme activity was determined by incubating the reaction mixture containing 0.1 μmole of ACM-A, 50 μmoles of buffer at various pH values and 3 units of enzyme in 1 ml at 37°C for 30 minutes. Citric acid - NaOH (pH 3~6), acetic acid - NaOH (pH 4~5), K_2HPO_4 - KH_2PO_4 (pH 6~8) and Tris-HCl (pH 7.2~9) were used as buffers. For determining the temperature effect on enzyme activity, the reaction mixture containing 3 units of enzyme were incubated at various temperatures from zero to 60°C for 30 minutes.

Isoelectric focusing

The isoelectric focusing was performed at 700 V for 48 hours in a LKB 8100 electron focusing column using pH 3~10 carrier ampholine (LKB Instrument, Inc.) by the method of VESTERBERG and SEVENSSON[16]. Fractions of 2 ml were collected, dialyzed against 20-fold volume of 0.05 M Tris-HCl buffer (pH 7.2) and assayed for the enzyme activity.

Purification of enzyme

S. galilaeus MA144-M1, strain 6U-21 was shake-cultured at 28°C for 60 hours. The enzyme was extracted and purified from 3.3 liters of the cultural filtrate as follows.

All operations were carried out at 4°C.

Step 1. Ammonium sulfate precipitation.

The crude enzyme was obtained from the cultural filtrate by ammonium sulfate precipitation (50% saturation, pH 7.2) and dissolved in 200 ml of 0.01 M Tris-HCl buffer (pH 7.5) (buffer A) followed by dialysis against a 20-fold volume of buffer A overnight.

Step 2. DEAE-Cellulose column chromatography.

The dialyzed solution was applied on a column (26×4 cm) of DEAE-Cellulose equilibrated with buffer A. After washing with buffer A, the enzyme protein was eluted with a convex gradient of NaCl between 0.02 M (500 ml) and 0.25 M (1,000 ml). The active fractions were pooled and concentrated by ammonium sulfate precipitation (80% saturation). The precipitate was collected by centrifugation and dissolved in 100 ml of buffer A followed by dialysis against 20-fold volume of buffer A.

Step 3. DEAE-Sephadex A-50 column chromatography.

The enzyme solution from step 2 was loaded on to a DEAE-Sephadex A-50 column (20×3.5 cm) equilibrated with buffer A containing 0.02 M NaCl. After the column was washed with 100 ml of the same buffer, a linear gradient of 0.02 to 0.3 M NaCl in buffer A (1,000 ml) was used for the elution of the enzyme. The active fractions were pooled and concentrated to about 40 ml by evaporation at 35°C under reduced pressure.

Step 4. Sephadex G-75 column chromatography.

The above enzyme concentrate was chromatographed on a column (40×5 cm) of Sephadex G-75 by the elution with buffer A.

Purification data are shown in Table 1. Throughout the chromatographic treatment the enzyme was purified to homogeneity as judged by electrophoresis on polyacrylamide gel performed at pH 9.4 with a current of 2 mA per tube at 4°C according to the method of DAVIS[17].

Isolation and identification of ACM-Y

ACM-A (200 μg/ml) was incubated in 50 ml of 50 mM citric acid - NaOH buffer (pH 5.5) con-

taining 5,000 units of enzyme and 100 μg of catalase by rotary-shaking at 28°C for 4 hours. The reaction mixture was extracted with toluene after adjusting to pH 7 and evaporated under reduced pressure. A yellow residue (about 700 mg) was chromatographed on a silica gel column (200 g) using toluene - methanol (100: 1.5), and the eluate was concentrated to dryness (250 mg). Further purification was carried out by countercurrent distribution with a solvent system composed of toluene and 0.1 M acetate buffer, pH 3.15 (1 : 1, 20 transfers). Fractions 9~12 gave 130 mg of pure ACM-Y, mp. 153~155°C, $[\alpha]_D^{22}$ +66 (*c* 1.0, $CHCl_3$), ν_{max}^{KBr} cm^{-1}: 1695, 1670, 1615, $\lambda_{max}^{90\%\ MeOH}$ nm (ε): 229.5 (46,970), 259 (25,920), 290 (10,200), 432 (12,800), NMR ($CDCl_3$) δ in ppm: 1.09 (3H, t, J=7.0), 1.19 (3H, d, J=6.5), 1.29 (3H, d, J=6.5), 1.41 (3H, d, J=6.5), 1.63 (2H, q, J=7.0), 1.8 ~2.1 (4H, m), 2.18 (3H, s), 2.42 (2H, m), 3.7 (3H, s), 4.01 (1H, q, J=6.5), 4.1 (1H, s), 4.55 (1H, m), 4.56 (1H, q, J=6.5), 4.76 (1H, q, J=6.5), 5.02 (1H, m), 5.27 (1H, dd, J=2. 4), 5.51 (1H, m), 6.1 (1H, d, J=10), 6.88 (1H, dd, J=3. 1), 7.26 (1H, dd, J=2. 8), 7.63 (1H, s), 7.63 (1H, t, J=8), 7.78 (1H, dd, J=2. 8). Anal. Calcd. for $C_{42}H_{51}NO_{15}$: C, 62.29; H, 6.35; N, 1.73. Found: C, 61.98; H, 6.30; N, 1.70.

These analytical data agree with those of ACM-Y described in a subsequent paper[3].

General

Thin-layer chromatography was performed on silica gel plate F_{254} (E. Merck & Co.) for the separation of anthracyclic glycosides and their oxidized products using the following solvent systems: chloroform - methanol (20 : 1 or 10 : 1); benzene - ethylacetate - methanol - formic acid - water (5: 5: 1.5: 1: 0.3). Thin-layer chromatographic analysis of sugars was done as previously described[1,2].

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