IMPROVEMENT OF THE PRODUCTIVITY OF ELASNIN, A SPECIFIC ELASTASE INHIBITOR, BY *STREPTOMYCES NOBORITOENSIS* KM-2753

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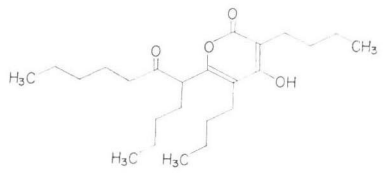
Streptomyces noboritoensis KM-2753, an elasnin-producing strain, co-produces an antimycin complex and its elasnin productivity is low (0.006 mg/ml). To obtain mutants possessing higher degrees of elasnin productivity and deficient in antimycin production, the strain was treated with N-methyl-N′-nitro-N-nitrosoguanidine (NTG), ultraviolet light, acriflavine (AF) and high temperature. Mutant N-134 obtained by treatment with NTG was 108 times higher than the original strain in elasnin productivity and produced no antimycin. Strain AF-17 obtained by AF treatment and strain H-80 obtained by incubation at high temperature showed 715 and 428 times higher productivities than that of the original strain, respectively. The productivity of elasnin was further increased 1.6~2.6 times by addition of saturated fatty acids, especially lauric acid, to the culture. Consequently, strain AF-17 produced 11.1 mg/ml of elasnin in the presence of 10 μg/ml of lauric acid.

In the course of screening for specific inhibitors of human granulocyte elastase, the authors have discovered a novel specific elastase inhibitor, elasnin (Fig. 1), which is produced by a soil isolate named *Streptomyces noboritoensis* KM-2753[1~3]. The strain co-produces an antimycin complex and its productivity of elasnin is low. Therefore, attempts were made to obtain mutants possessing higher productivity of elasnin but no productivity of antimycin.

Fig. 1. The structure of elasnin.



This paper deals with the improvement of elasnin production by monocolony isolation from the original strain KM-2753, the treatment of the monocolony isolate MC-1 by NTG, UV, AF and high temperature, and the addition of various fatty acids to the culture medium.

Materials and Methods

Monocolony isolation

Streptomyces noboritoensis KM-2753, the original strain, was grown on a modified WAKSMAN's agar slant (pH 7) consisting of 1.0% glucose, 0.5% peptone, 0.5% meat extract, 0.5% NaCl and 2% agar for 2 weeks at 27°C. Spores from a slant were suspended in saline water containing 1% (v/v) Tween 80. One-tenth ml of diluted spore suspension was spread with a glass rod on the modified WAKSMAN's agar in a Petri dish and incubated at 27°C. The colonies formed after one week were transferred onto a modified WAKSMAN's agar using sterilized tooth-picks and incubated for one week. They were transferred into 50-ml large test tubes containing 10 ml of elasnin production medium (2.0% glucose, 2.0% soybean meal and 0.1% NaCl, pH 7.0) and incubated for 4 days at 27°C. Elasnin

Abbreviations: NTG, N-methyl-N′-nitro-N-nitrosoguanidine; AF, acriflavine; UV, ultraviolet.

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produced in each culture broth was assayed using human granulocyte elastase as described in previous papers[1,2].

NTG treatment[4]

Strain MC-1 obtained from strain MK-2753 by monocolony isolation was transferred on a modified WAKSMAN's agar slant and cultured for two weeks at 27°C. The spores of fully grown slant culture were suspended in 10 ml of 1% (v/v) Tween 80 solution and centrifuged at 3,000 rpm for 5 minutes. The precipitate was resuspended in 10 ml of 0.05 M Tris-maleate buffer (pH 9), and NTG as a mutagen was added to 4 ml of the suspension to give a final concentration of 1,000 μg/ml. After incubation of the spore suspension for 2 hours at 27°C, 1 ml of L-cysteine solution (50 mg/ml) was added to decompose the remaining NTG and a sample (4 ml) was centrifuged at 3,000 rpm for 5 minutes. The precipitate was washed twice with 4 ml of sterile water and resuspended in 2 ml of sterile water. The suspension was then serially diluted and spread uniformly on the modified WAKSMAN's agar plate. The colonies formed after 3 or 4 days were transferred onto an agar medium containing glucose, soybean meal, NaCl and agar (10, 10, 0.5 and 12 g, respectively, per liter of tap water) using sterilized tooth-picks. After incubation of the agar plate for one day, the colonies were cut into agar pieces. The pieces were incubated for an additional 3 days and then transferred onto an agar plate containing *Candida albicans* as a test organism. After incubation for one day at 27°C, strains which showed no activity against *Candida albicans* were selected, and grown on a modified WAKSMAN's agar plate to obtained pure cultures. They were trasferred into 50-ml large test tubes containing 10 ml of elasnin production medium and incubated for 4 days at 27°C. Amount of elasnin in each cultured broth was assayed as described above.

UV treatment[5]

The NTG mutant, N-134, was then exposed to UV-radiation emitted from a germicidal lamp (15-W) at a distance of 30 cm for 30 seconds. The mutants obtained by UV irradiation were isolated according to the above procedure.

AF treatment[6] and incubation at high temperature[7,8]

Spores of strain MC-1 from modified WAKSMAN's agar slants were transferred to 100 ml of a seed medium containing dextrin, glucose, soybean meal, yeast extract, and $CaCO_3$ (20, 2, 15, 3 and 3 g, respectively, per liter of tap water, pH 7) in a 500-ml SAKAGUCHI flask. After cultivation for one day at 27°C, 1-ml aliquots were transferred into 10 ml of the same medium containing 3 μg/ml of acriflavine in a 50-ml test tube and incubated for 4 days at 27°C. The cultured broth was centrifuged. The precipitated mycelia and spores were washed twice with saline water, treated with a sonic oscillator for 2 minutes at 10 KHz to cut mycelia, and then transferred onto the following media to examine the production of melanin and formation of aerial mycelium. Melanin production medium contained glycerol (15.0 g), L-tyrosine (0.5 g), L-asparagine (1.0 g), KH_2PO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), NaCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), $MgCl_2 \cdot 4H_2O$ (0.001 g), $ZnSO_4 \cdot 7H_2O$ (0.001 g) and agar (20 g) in a total volume of 1 liter of distilled water; aerial mycelium formation medium contained starch (10.0 g), KH_2PO_4 (1.0 g), $MgSO_4 \cdot 7H_2O$ (1.0 g), NaCl (1.0 g), $(NH_4)_2SO_4$ (2.0 g), $CaCO_3$ (2.0 g), $MgCl_2 \cdot 4H_2O$ (0.001 g), $ZnSO_4 \cdot 7H_2O$ (0.001 g), $FeSO_4 \cdot 7H_2O$ (0.001 g) and agar (20 g) per liter of distilled water.

To examine antibiotic production, each colony was transferred onto the modified WAKSMAN's agar medium and incubated for 3 days at 27°C. The antibiotic productivity of each colony grown on this medium was assayed by transferring a cylinder of agar bearing the colony on an antibiotic assay plate which contained *Candida albicans* as a test organism for antimycin. Colonies which had lost the ability to produce antimycin were transferred into 10 ml of production medium in 50-ml large test tubes. After incubation for 2 days, elasnin produced in each cultured broth was assayed as described above.

The highest temperature permitting growth was 37°C for parent (MC-1). The procedure of preincubation at high temperature was similar to that used for the AF treatment, except for incubation which was performed at 37°C.

Effect of various long chain fatty acids

Strain N-134, which had lost ability to produce antimycin, was cultivated in the production medium

in a SAKAGUCHI flask at 27°C. After incubation for 1 day a slightly alkaline solution (KOH added) of a fatty acid in an aqueous ethanol solution (1 : 3, v/v) was added to the culture medium at a final concentration of 10 μg/ml; incubation was for 4 days. Elasnin concentration of the cultured broth was determined as described above.

Results and Discussion

The elasnin-producing strain, *Streptomyces noboritoensis* KM-2753 isolated by the authors from a soil sample, co-produces a candidicidal substance, which was identified as an antimycin complex[2]. The amount of elasnin produced by the organism was small (0.006 mg/ml). So, we attempted to improve the elasnin-producing strain.

Since elasnin has been suggested to be biosynthesized from 12 molecules of acetate *via* the polyketide or fatty acid[3] pathway, we attempted to improve the elasnin production by the addition of various fatty acids.

Monocolony Isolation

Twenty eight hundred strains were obtained by monocolony isolation from the original strain KM-2753. Among them, strain MC-1 produced 0.17 mg/ml of elasnin (Fig. 2). The cultural characteristics of strain MC-1 was similar to that of the original strain. Elasnin production of the strain was 28 times higher than that of the original strain. But, strain MC-1 co-produced the antimycin complex as the original strain did.

Treatment with NTG and UV

Spores of a fully-grown slant culture of the strain MC-1 cultured on a modified WAKSMAN's agar for 2 weeks at 27°C were treated with NTG as described in Materials and Methods. First, 1580 isolates possessing lower candidicidal activity than that of the parent strain were selected. Next, 4 strains with increased productivity of elasnin were selected from the above 1580 isolates. Among these 4 strains, strain N-134, which had different growth characteristics on inorganic salts-starch agar from those of the parent strain (Table 1), was selected as the best producer of elasnin and it produced no antimycin even in submerged culture.

The mutant strain N-134 was then treated with UV radiation. One mutant (U-134) selected from 630 colonies also had different morphological aspects and produced an amount of elasnin almost equal to that produced by the NTG mutant N-134 at 4 days.

The cultural characteristics of the mutant strains are summarized in Table 1. The cultural characteristics of these mutants were similar to those of the parent strain on various media except on inorganic salts-starch agar medium. Cottony and mossy aerial hyphae of the parent strain were moderately formed on the medium and extended straightly but mutants (N-134 and U-134) formed no aerial mycelium and were different from the parent strain in soluble pigment formation.

Typical time course of elasnin production by strains MC-1 and N-134 are shown in Fig. 2. Strains MC-1 and N-134 were cultivated in 30-liter jar-fermentors containing 20 liters of the production medium under the following condition: temperature, 27°C; aeration, 10 liters/min; agitation, 250 r.p.m.; and pressure, 0.5 Kg/cm^2. Assay of elasnin in cultured broth was carried out as described in previous papers[1,2]. The amount of elasnin produced by mutant N-134 obtained by NTG treatment was over 100 times larger at 4th day than that of the original strain, and increased gradually even after the 4th day to reach 1.05 mg/ml. Strain U-134 produced an amount of elasnin equal to that produced

Fig. 2. Time courses of elasnin production by *Streptomyces noboritoensis* KM-2753-MC-1 and mutants N-134 and AF-17.

Elasnin, ——; residual glucose, -·-·-; pH, ------.

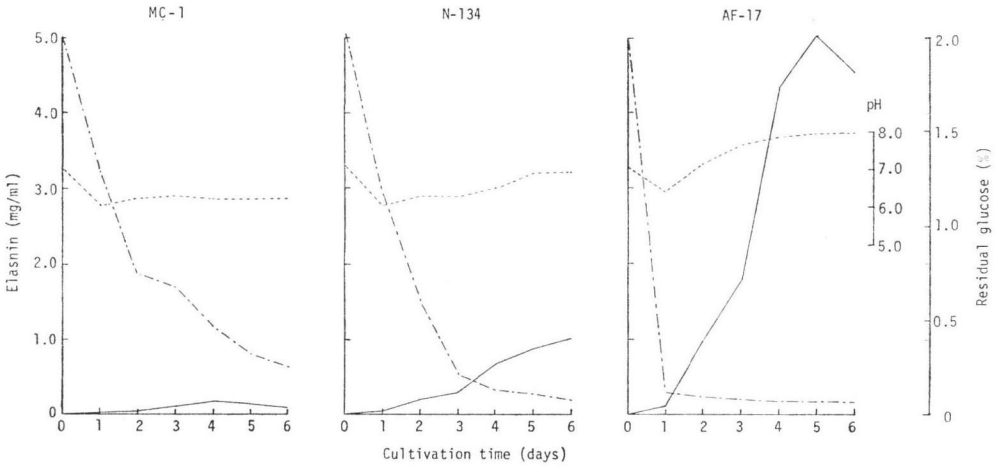

Table 1. Cultural characteristics of parent and mutants.

Medium		MC-1	N-134	AF-17	H-80
Glucose-nitrate agar	G*	Deep brown (4pl)	Light brown (4ng)	Chocolate brown (4pn)	Clove brown (3pl)
	R*	Deep brown (4pl)	Light brown (4ng)	Chocolate brown (4pn)	Clove brown (3pl)
	AM*	None	None	None	None
	SP*	Light yellow (1ea)	None	Deep brown (4pl)	None
Glycerol-calcium malate agar	G	Chestnut brown (4ni)	Light spice brown (41g)	Light olive (1½ie)	Light olive (1½ie)
	R	Chocolate (4nl)	Light spice brown (41g)	Golden olive (1½ng)	Golden olive (1½ng)
	AM	None	None	None	None
	SP	Cork tan (4ie)	None	Golden olive (1½ng)	Golden olive (1½ng)
Oatmeal agar	G	Yellow maple (3ng)	Adobe brown (3lg)	Luggage tan (4ne)	Dark brown (3pn)
	R	Yellow maple (3ng)	Adobe brown (3lg)	Luggage tan (4ne)	Dark brown (3pn)
	AM	None	None	None	None
	SP	Yellow maple (3ng)	None	Luggage tan (4ne)	Covert brown (2li)
Inorganic salts-starch agar	G	Dark brown (3pn)	Topaz (3ne)	Covert brown (2li)	Dark brown (2pn)
	R	Dark brown (3pn)	Adobe brown (3lg)	Mustard brown (2pl)	Dark brown (2pn)
	AM	Cottony (4ca)	None	Cottony (c)	Cottony (4ca)
	SP	Citron (1gc)	None	Olive (1pi)	Olive (1pi)

* Abbreviation: G, growth; R, reverse; AM, aerial mycelium; SP, soluble pigment.

Each strain was cultivated on various media described by WAKSMAN[9] and ISP[10]. The color and hue number indicated were based on those of the Color Harmony Mannual (4th edition) published by Container Cooperation of America.

by strain N-134.

Treatment with AF and Preincubation at High Temperature

After AF treatment 515 isolates were obtained which were deficient in antimycin productivity. Among them, strains AF-3, 6, 17 were selected for production of elasnin. Strain AF-17 showed

cultural characteristics similar to those of the parent strain (Table 1) and exhibited 715 times higher productivity of elasnin than the original strain, as shown in Fig. 2.

Also, 620 isolates were obtained by preincubation at high temperature (37°C) and it was found that one of them, strain H-80, showed higher productivity of elasnin (2.57 mg/ml, 428 times). Cultural characteristics of H-80 strain were similar to those of the parent strain and of strain AF-17, as shown in Table 1.

In contrast to the NTG and UV mutants, strains AF-17 and H-80 maintained aerial mycelium formation on inorganic salts-starch agar (Table 1) and showed much increased elasnin production. The fact that AF treatment and incubation at elevated temperature which are known as plasmid-eliminating methods[6~8] are more useful to obtain mutants having high elasnin productivity than treatment with other physical and chemical mutagens is noteworthy.

Effect of Various Long-chain Fatty Acids

An attempt was made to increase elasnin production by adding various long-chain fatty acids using strain N-134. As shown in Table 2, saturated fatty acids such as lauric acid (12: 0), myristic acid (14: 0) and palmitic acid (16: 0) much increased elasnin production when added to 1-day cultures, whereas unsaturated fatty acids such as myristoleic acid (14: 1), palmitoleic acid (16: 1) and vaccenic acid (18: 1), and odd-numbered fatty acids such as undecanoic acid (11: 0), tridecanoic acid (13: 0) and pentadecanoic acid (15: 0) had little effect in increasing elasnin productivity.

In contrast, the addition of capric acid (10: 0) to the culture markedly caused cell lysis.

The results of improvement of elasnin production by *Streptomyces noboritoensis* are sum-

Table 2. Effect of long-chain fatty acids on elasnin production by strain N-134.

Fatty acids (10 μg/ml) were added on the 1st day of incubation as described in the text.

Long-chain fatty acids	Carbon Nos.	Elasnin production	
		mg/ml	ratio
None		0.31	1.0
Capric acid	10: 0	0.06	0.2
Undecanoic acid	11: 0	0.35	1.1
Lauric acid	12: 0	5.12	16.5
Tridecanoic acid	13: 0	0.34	1.1
Myristic acid	14: 0	3.81	12.3
Myristoleic acid	14: 1, *cis*-9	0.68	2.2
Pentadecanoic acid	15: 0	0.98	3.2
Palmitic acid	16: 0	2.29	7.4
Palmitoleic acid	16: 1, *cis*-9	0.44	1.4
Stearic acid	18: 1	1.61	5.2
Vaccenic acid	18: 1, *cis*-11	0.31	1.0
Eicosanoic acid	20: 1	1.27	4.1

Table 3. Elasnin production and phenotype of mutants obtained by various treatments from the original strain KM-2753.

Each strain was cultivated in the production medium for 4 days at 27°C. Lauric acid was added to 1-day culture.

Strain	Parent strain	Treatment with	Elasnin production (mg/ml)		Phenotype*			
			No addition	Lauric acid (10 μg/ml)	Ela.	Ant.	Mel.	AM
KM-2753			0.006	—	+	+	+	+
MC-1	KM-2753	Monocolony	0.17	0.37	+	+	+	+
N-134	MC-1	NTG	0.65	1.05	+	−	+	−
U-134	N-134	UV	0.58	—	+	−	+	−
AF-17	MC-1	AF	4.29	11.1	+	−	+	+
H-80	MC-1	High temp.	2.57	—	+	−	+	+

* Ela., elasnin production; Ant., antimycin production; Mel., melanin formation; AM, formation of aerial mycelium.

marized in Table 3. The amount of elasnin produced by the original strain KM-2753 was 0.006 mg/ml. On the other hand, the amount produced by strain AF-17 in the presence of 10 μg/ml of lauric acid reached 11.1 mg/ml. The concentration of elasnin is 1850 times higher than that produced by the original strain in the original medium.

The increase of elasnin production by the addition of lauric acid cannot be explained only by the incorporation of lauric acid into elasnin. This effect may be due to the alteration of the membrane permeability or of the fatty acid biosynthesis by lauric acid. The relationship of elasnin and fatty acid biosynthesis and the mechanism of elasnin production enhancement by adding saturated long-chain fatty acids is under investigation.

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