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Studies on Screening and Isolation of Esterase Inhibitors from Soil Microorganisms(I). Identification of strain DMC-498 producing esterase inhibitors

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Abstract □ To find microorganisms producing esterase inhibitors, microbes were isolated from soil samples that were collected at different locations in Korea and screened for inhibitory activities. One of the inhibitor-producing strains was named strain DMC-498. This strain was found to be a new species of the genus *Streptomyces* by comparison with the characteristics of morphology and metabolisms of the other species of the genus.

Keywords □ Esterase inhibitors, *Streptomyces*, Morphology, Carbon source utilization.

Enzyme inhibitors are useful tools in analysis of homeostasis of living organisms and disease processes. Inhibitors of enzymes involved in growth mechanisms must have cytotoxic action and some of them may exhibit therapeutic action on certain type of tumors. It was though that if enzymatic mechanisms of metastasis or contact inhibitions are clarified, then it is possible to find inhibitors of these enzymes to prevent metastasis or abnormal growth of tumors. If enzymatic process of tumor immunity is understood, it is possible to find enzyme inhibitors which enhance tumor immunity and which are useful in cancer therapy. Many biologists have studied inhibitors of various enzymes in recent years. Inhibitors of aminopeptidases, alkaline phosphatases and esterases were found and their functions have been studied. Forphenicin, an inhibitor of alkaline phosphatase, enhanced delayed type hypersensitivity and increased the number of antibody forming cells[1]. Bestatin, an inhibitor of aminopeptidase B, was found by Umezawa *et al.*[2] It hydrolyzes and N-terminal peptide bond containing N-terminal arginine or lysine. Bestatin also inhibits leucine aminopeptidase and enhances delayed-type hypersensitivity[3]. Muller *et al.* described that bestatin increased the incorporation rate of ^{3}H-thymidine into DNA only in T Cells[4]. Ishizuka *et al.* reported that bestatin was mitogenic on spleen cells and caused the proliferation of T cells probably through the activation of macrophage[5]. Esterastin, an inhibitor of esterase, suppressed both delayed-type hypersensitivity and antibody-forming cells[1]. Ebelactone, an inhibitor of the same enzyme, inhibited lipase and N-formyl methionine aminopeptidase[6]. This inhibitor enhanced immune response.

As described above, inhibitors of cell surface enzymes play an important role in studying functions of enzymes and immunity. In our screening test of enzyme inhibitors, we found several strains producing esterase inhibitors from Korean soil microorganisms. One of them was identified as a new species of the genus *Streptomyces* by International Streptomyces Project (ISP) and Bergey's Manual of Determinative Bacteriology. This strain was named strain DMC-498 and its characteristics are here reported.

EXPERIMENTAL METHODS

Screening Method

1) Soil Samples

one gram of each soil samples which were collected in various locations in Korea was used for screening procedure.

2) Media for culture

Oatmeal yeast medium (oatmeal 20 g, yeast extract 1 g, agar 20 g and distilled water 1000 m*l*) was used for isolation of colonies. And shaking culture medium (oatmeal 20 g, yeast extract 1 g, and distilled water 1000 m*l*) was used for production of esterase inhibitory substances.

3) Isolation of Soil Microorganisms

One gram of soil sample was diluted with 10 m*l* of sterilized water in sterilized capped tubes. After the suspension was agitated, one m*l* of this suspension was diluted in the same way to final dilution of 10,000 fold. One m*l* of this suspension was smeared on the oatmeal agar plate with a sterile glass rod and incubated for three days at 27 ± 1 °C. The colonies which were considered to be *Actinomycetes* on the oatmeal agar plate were transferred aseptically into a new oatmeal agar plate and further incubated for three days at 27 ± 1 °C. Then the colonies of *Actinomycetes* were selected and finally transferred into oatmeal agar slants and screened for esterase inhibitory activity.

4) Culture Method

The colonies maintained in oatmeal agar slants were inoculated into 10 m*l* of the shaking culture media in 50-m*l* flask and incubated for three days in a rotary shaker at 27° ± 1 °C and 180 rpm.

5) Activity Test

Enzyme; Esterase EC 3.1.1.1 Type 1 (a commercial product from porcine liver by Sigma Chem. Co.), Substrate; p-nitrophenyl acetate N-8130 (Sigma Chem. Co.), Buffer; 0.1 M phosphate buffer containing 0.06% Triton X-100, pH 7.0. The reaction mixture was composed of 2.5 m*l* phosphate buffer, 0.03 m*l* of esterase solution (dissolved at the concentration of 1.6 unit/m*l* in phosphate buffer) and 0.3 m*l* of each culture filtrate. This reaction mixture was preincubated at 28 °C for filtrate. This reaction mixture was preincubated at 28 °C for five minutes and 0.03 m*l* of substrate solution (0.05 M p-nitrophenyl acetate) was added. After incubation for 15 minutes, the optical density was measured at 400 nm. As a control, 0.3 m*l* of water was added instead of culture filtrate and the mixture was incubated in parallel with the test sample. As a blank, 0.03 m*l* of water was added instead of enzyme solution and the mixture was also incubated in parallel with the test sample. The percent inhibition of esterase inhibitory activity was calculated from the following equation:

$$\text{P.I.} = \frac{C-T}{C-B} \times 100$$

where T, C and B are the optical density of the test, control and blank, respectively. One inhibition unit (I.U.) in this systems was defined as the amount of inhibitor providing 50% inhibition compared to the original activity.

Identification of Strain DMC-498

During the screening procedure, a strain which had inhibitory activity against esterase was isolated from soil sample. This strain was named as strain DMC-498 and used in this study.

1) Biochemical Studies

Staneck's method was taken in this studies.[7]

a) Identification of isomers of diaminopimelic acid (DAP)

Approximately three mg (dried weight) of cells was placed into a small ampoule with one m*l* of 6 N HCl. The sealed ampoule was kept at 100 °C in an oven for 18 hours. After cooling, the hydrolysate was filtered through Whatman No. 1 paper. The filtrate was evaporated to dryness on a boiling water bath, redissolved in one m*l* of distilled water, and made to dryness again. This residue was dissolved in 0.3 m*l* of distilled water, and 0.002 m*l* was applied at the base line of the TLC sheet (microcrystalline cellulose plate). Ascending TLC was performed with the solvent system MeOH-distilled water-6 N HCl-pyridine (80:26:4:10, v/v) for approximately four hours. After the chromatogram was air dried, spots were visualized by spraying with 0.2% ninhydrin in acetone and heating at 100 °C for three min. As a DAP standard, 1 μl of 0.01 M DL-DAP (Sigma Chem. Co.) which contains both meso-and L-DAP isomers was used.

b) Identification of monosaccharides in whole cell hydrolysates

Approximately 25 mg (dry weight) of cells was placed into an ampoule with 1.5 m*l* of 1 N sulfuric acid. The sealed ampoule was heated for two hours on a boiling water bath. After cooling, the hydrolysate was transferred to 25-m*l* centrifugation tube and saturated barium hydroxide was added dropwise until the pH was between 5.2 and 5.5. The precipitate was removed by centrifugation and discarded. The supernatant fluid was evaporated in a 50-m*l* beaker under stream of air, and the residue was redissolved in 0.3 m*l* distilled water (any insoluble material remaining at this step was removed by centrifugation); 1.0 μl of this hydrolysate was applied to the base line of the TLC sheet (microcrystalline cellulose plate). One percent solution of galactose, xylose, glucose and rhamnose were used as a standard mixture. Ascending TLC was performed with the solvent system, n-butanol-distilled water-pyridine-toluene (10:6:6:1, v/v), for approximately six hours. Spots were visualized by spraying on the chromatogram with acid anilin

phthalate (3.25 g phthalic acid dissolved in 100 m*l* water saturated butanol plus two m*l* of aniline) and heating at 100 °C for four minutes.

2) Morphological Studies

The color of aerial mycelium, substrate mycelium, soluble pigment, the form of the spore chain, spore-bearing hyphae and the growth of strain DMC-498 were observed after incubation at 27 ± 1 °C on the ISP media and some glucose containing media. All processes of morphological studies were performed according to the ISP methods.[8] The composition of media used were as follows.

a) Yeast extract-malt extract agar (ISP No. 2 medium)

yeast extract (4.0 g), malt extract (10.0 g), glucose (4.0 g), distilled water (1.0 liter), agar (10.0 g).

b) Oatmeal agar (ISP No. 3 medium)

oatmeal (20.0 g), agar (18.0 g), distilled water (1.0 liter), trace salt solution (1.0 m*l*). Composition of trace salt solution: $FeSO_4{\cdot}7H_2O$ (0.1 g), $MnCl_2{\cdot}4H_2O$ (0.1 g), $ZnSO_4{\cdot}7H_2O$ (0.1 g), distilled water (1.0 liter).

c) Inorganic salt-starch agar (ISP No. 4 medium)

soluble starch (10.0 g), K_2HPO_4 (anhydrous basis) (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), trace salt solution (1.0 m*l*), agar (20.0 g).

d) Glycerol-asparagin agar (ISP No. 5 medium)

glycerol (10.0 g), L-asparagine (anhydrous basis) (1.0 g), K_2HPO_4 (anhydrous basis) (1.0 g), distilled water (1.0 liter), agar (20.0 g).

e) Glucose-asparagine agar (Krainsky's medium)

glucose (10.0 g), asparagine (0.5 g), K_2HPO_4 0.5 g), distilled water (1.0 liter), agar (15.0 g).

f) Glucose-nitrate agar

glucose (5.4 g), $NaNO_3$ (1.5 g), KH_2PO_4 (1.0 g), $MgSO_4{\cdot}7H_2O$ (0.5g), thiamine HCl (1000 ppm) (2.0 m*l*), distilled water (1.0 liter).

g) Glucose-peptone agar

glucose (10.0 g), peptone (2.0 g), $MgSO_4.7H_2O$ (0.5 g), K_2HPO_4 (0.5 g),agar (15.0 g), distilled water (1.0 liter).

) Microscopic Features

Strain DMC-498 was inoculated on an oatmeal agar medium (oatmeal 20.0 g, agar 20 g, yeast extract 1 g, and distilled water 1 liter) and sterilized cover glasses were set on the oatmeal agar layer at an angle of 45 degrees. After three-day incubation at 27 ± 1 °C, the cover glasses were picked up to observe the microscopic features. Substrate and spore chain were observed at 650 magnification, aerial mycelium and spore chain were observed at 650 and 1500 magnification with scanning electron microscopy (SEM).

4) Physiological Tests

a) Formation of melanoid pigment

The formation of melanoid pigment was observed two days after the inoculation of strain DMC-498 on the following medium: glycerol 15.0 g, L-tyrosine 0.5 g, L-asparagine 1.0 g, K_2HPO_4 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, NaCl 0.5 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, trace salt solution 1.0 m*l*, agar 20.0 g and distilled water one liter.[8]

b) Starch hydrolysis

Strain DMC-498 was inoculated on the NB agar medium containing 0.2% soluble starch[9] and incubated at 27 ± 1 °C for four days. Then the color changes were observed around the colonies after adding iodine solution.

c) Gelatin hydrolysis

Strain DMC-498 was inoculated to the nutrient broth agar medium containing 0.4% gelatin, and was incubated for three days at 27 ± 1 °C. Fifteen percent solution of mercuric chloride in 20% HCl (v/v) was spread on the growth medium, and presence of clear zone around the colony was examined.[10]

d) Utilization of carbon sources

This test was performed according to the procedure recomended by Shirling and Gottlieb.[8] After autoclaving the basal medium, it was cooled to 60 °C and carbon sources were added aseptically to give a concentration of approximately one percent. Inoculum of strain DMC-498 was inoculated on the complete medium. After two-week incubation at 27 ± 1 °C, each plate was observed for the degree of the growth compared with the glucose containing medium (positive control) and carbon source free medium (negative control). Composition of basal agar medium: $(NH_4)_2SO_4$ 2.64 g, $K_2HPO_4{\cdot}3H_2O$ 5.65 g, KH_2PO_4 anhydrous 2.38 g, $MgSO_4{\cdot}7H_2O$ 1.00 g, Pridham and Gottlieb trace salt 1.00 m*l*, distilled water 1.00 liter, agar 15.00 g.

Pridham and Gottlieb trace salts: $CuSO_4{\cdot}5H_2O$ 0.64 g, $FeSO_4{\cdot}7H_2O$ 0.11 g, $MnCl_2{\cdot}4H_2O$ 0.79 g, $ZnSO_4{\cdot}7H_2O$ 0.15 g, distilled water 100.0 m*l*. Used carbon sources: no carbon source (negative con-

Table I. Esterase inhibitor producing strains that were collected in Korean soil samples

Strain	Optical density*			P.I.(%)**
	Test	Control	Blank	
DMC-26	0.76	0.91	0.30	25
DMC-45	0.84	0.96	0.28	18
DMC-174	0.57	0.95	0.21	51
DMC-354	0.70	0.95	0.25	36
DMC-498	0.39	0.94	0.34	92

* Optical density was measured at 400 nm.
** P.I. = Percent inhibition.

trol), D-glucose (positive control), L-arabinose, sucrose, D-fructose, D-xylose, rhamnose, raffinose, D-mannitol and salicin.

RESULTS AND DISCUSSION

Screening of Strains Producing Esterase Inhibitors

About 400 strains isolated from soil samples collected in various locations in Korea were tested for esterase inhibitory activity. Several strains which produced esterase inhibitors were isolated from the soil samples and one of them named strain DMC-498 (Table I).

Identification of Strain DMC-498

1) Biochemical Studies

As shown in Figs. 3 and 4, whole cell hydrolysates of strain DMC-498 contained LL-DAP and glucose, but galactose, arabinose and xylose were

Table II. Morphological studies of strain DMC-498

Medium	Growth	Reverse phase	Aerial mycelium		Soluble pigment
			Color	No. of spore	
Yeast extract malt extract agar (ISP No. 2)	very good	brown (517)	pink (467)	1-10 straight	brown (339) exopigment
Oatmeal agar (ISP No. 3)	very good	white	pink (469)	2-10 straight	dark brown (508) dark yellow (240)***
Inorganic salt-starch agar (ISP No. 4)	good	pink (468)	pink (468)	1-10	none
Glycerol-asparagine agar (ISP No. 5)	good	white	white	single	none
Glucose-asparagine agar	poor	white	white	none	none
Glucose-nitrate agar	poor	white	white	none	none
Glucose-peptone agar	good	brown (334)	pink (468)	1-10	dark green (379)

*** Dark yellow at pH 3.0
Color numbers were due to Color Guide(Dainippon Ink and Chemicals Incorporation in Japan).

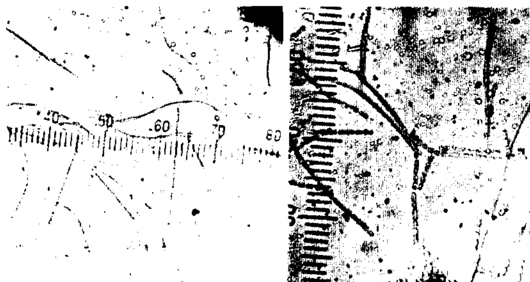

Fig. 1. Morphology of aerial spore chain of strain DMC-498.
One graduations means 1/1000 mm.

not present. These facts indicated that this strain belonged to cell type 1, one of the genus *Streptomyces,* as described in Bergey's Munual of Determinative Bacteriology[11] and International Streptomyces Project (ISP) methods.

2) Morphological Studies

As shown in Table II, this strain grew well on the ISP medium. Reverse phase colors of this strain were brown and pink on ISP No. 2 and 3 media, respectively. Aerial mass color was pink on ISP No. 2, 3 and 4 media. Number of the spore was 1-10. Soluble pigments were produced, and their colors were brown or green as shown on Table II. Exopigment was produced on ISP No. 2 and glucose peptone agar media. Brown pigment produced on the oatmeal agar medium changed to yellow by dropping 0.05 N HCl.

3) Microscopic Features

Shape of spore-bearing hyphae was rectus-flexibilus as shown in Fig. 1. Type of spore surface was observed as smooth type as shown in Fig. 2.

Fig. 2. Scanning electron micrograph of strain DMC-498.
Each bar indicates 2 μm.

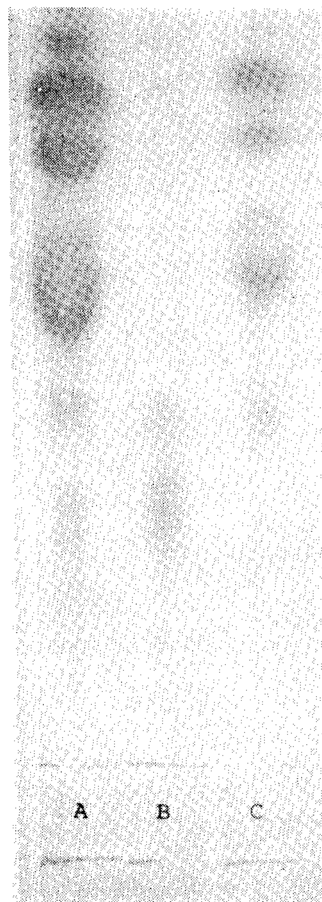

Fig. 3. Identification of DAP isomers by TLC.
A) Hydrolysate of *E. coli*
B) Standard mixture of DAP isomers
Upper: LL-DAP
Lower: meso-DAP
C) Hydrolysate of strain DMC-498.

4) Physiological Test

The results of starch hydrolysis and gelatin hydrolysis were positive. Melanoid pigment was not produced. This strain grew well on the medium containing glucose or salicin, but did not utilize any other carbon sources. The pattern of carbon source utilization was very similar to that of *Streptomyces lavendulae.* But there were differences between the two strains in morphological and physiological characteristics. Therefore, it was suggested that this strain would be a new species in the genus *Streptomyces* by ISP and Bergey's Manual of Determinative Bacteriology (Tables III and IV).

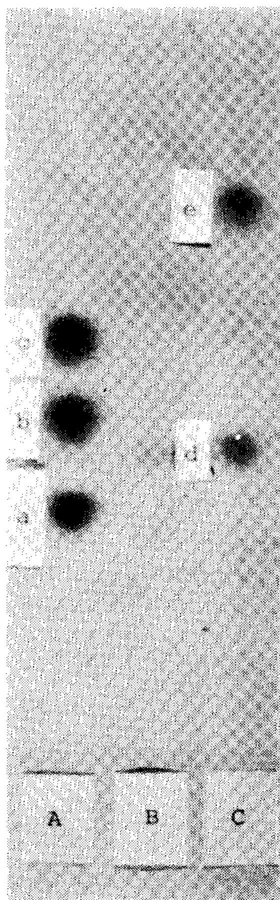

Fig. 4. Identification of monosacchrides in whole cell hydrolysate.
A) Standard mixture
a) galactose
b) arabinose
c) xylose
B) Hydrolysate of strain DMC-498
C) Standard mixture
d) glucose
e) rhamnose

CONCLUSION

A strain that inhibited esterases was found from the soil samples and named strain DMC-498. This strain belonged to cell wall type 1, one of the genus *Streptomyces*. Its aerial mycelium was rectus-flexibilus and its spore surface was smooth. The color of the aerial mycelia was pink. This strain was found to be a new species of the genus *Streptomyces*.

Table III. Utilization of carbon sources in comparison with *Streptomyces lavendulae*.

	Strain DMC-498	*S. lavendulae*
No carbon sources	−	−
D-Glucose	+	+
D-Xylose	−	−
L-Arabinose	−	−
L-Rhamnose	−	−
Raffinose	±	+
D-Mannitol	−	−
Inositol	−	−
Salicin	+	+
Sucrose	−	−

(− : Not utilized, + : Utilized, ± : Utilization doubtful)

Table IV. Morphological characteristics of strain DMC-498 in comparison with those of *Streptomyces lavendulae*

	Strain DMC-498	*S. lavendulae*
Aerial mass color	Red	Red
Spore surface	Smooth	Smooth
Spore chain	Rectus Flexibilus	Spira
Melanoid pigment	Not produced	Produced
Reverse phase color	Present	none
Antibiotic	Not produced	produced

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