Studies on Screening and Isolation of Esterase Inhibitors from Soil Microorganisms (II). Isolation of Inhibitors and Associated Lipids from *Streptomyces* Strain DMC-498

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한국 토양균중 Esterase 저해제 검색 및 분리에 관한 연구(제 2 보) *Streptomyces* Strain DMC-498 균주의 저해성분 및 관련 지질의 분리

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ABSTRACT — To find esterase inhibitors and in the metabolites of *Streptomyces* strain DMC-498, two active compounds were isolated from the methanol extract of the mycelia of the strain by Silica gel column chromatography and preparatory argentation TLC. These compounds were proved to show competitive inhibition. Compound B was found to consist of linoleic and oleic acids. Fifty percent inhibition concentration (IC_{50}) of linoleic acid was 0.045μg/ml, whereas oleic acid exhibited no inhibitory activity. Associated lipids: isostearic acid, isostearic acid methyl ester, oleic acid methyl ester and linoleic acid methyl ester, were isolated from the same extract, showing no inhibition of the esterase. Compound A was found to be a liquid inhibitor with an alicyclic ring and two or more oxygens, its molecular weight being more than 500.

Keywords □ Esterase inhibitor, *Streptomyces* strain DMC-498, Linoleic acid, Oleic acid, Linoleic acid methyl ester, Oleic acid methyl ester, Isostearic acid, Isostearic acid methyl ester.

For the last fifteen years, several enzyme inhibitors have been found and have contributed to the research for biological and biochemical mechanisms of enzymes and immunity. Protease inhibitors have been used for identification of enzymes in biological functions or disease processes.

Received for publication 15 October, 1987
Reprint request; Dr. B.K. Kim at the above address

Leupeptin was originally isolated as an inhibitor against serine or thiol protease such as trypsin, plasmin, papain and cathepsin B[1-3]. And soon it was demonstrated that leupeptin suppressed chemical carcinogenesis in rats. Other protease inhibitors such as antipain, chymostatin, elastinal and elastin were found[4-10] whose biological activities were summarized by Hamill[11]. Umezawa's group found that diketocoriolin B (DKCB), a ses-

quiterpene antibiotic, prolonged the survival of the mouse inoculated with L-1210 mouse leukemia cells and increased the number of antibody-forming cells at the doses much below the antitumor effective dose.[12] The cytocidal action was found to be due to the inhibition of Na^+-K^+-ATPase, a membrane associated enzyme[13]. It was thought that the increase in the number of antibody-forming cells could be due to the binding of DKC-B to the surface of cells involved in immunity. Glycopeptides (lectins) are known to bind to the surface of immune cells, cause mitogenesis and enhance immune response. Umezawa reasoned that inhibitors of other cell surface enzymes might, therefore, be involved in immunity, and investigated the presence of enzymes on mammalian cell surfaces.[14] Elevation of glutamyl transpeptidase and alkaline phosphatase levels in the bone marrow of rats carrying tumors was observed by Greengard.[15] He postulated that the increase in glutamyl transpeptidase and alkaline phosphatase was necessary prelude to stimulation of granulocyte multiplication by appropriate growth factors and that the measurement of these enzymes in the short-term liquid culture could offer a biochemical test for such factors elaborated by cancers or non-neoplastic conditions. Also, inhibitors of alkaline phosphatase, aminopeptidase, and esterase enhanced immune responses.

Studies on various enzyme inhibitors have been already reported by the authors.[18-21] In the present report, we attempted to isolate esterase inhibitors from strain DMC-498 that was found to be a new species of the genus *Streptomyces*[22] by using Silica gel column chromatography and argentation TLC.

MATERIALS AND METHODS

Strain—Strain DMC-498, one of the genus *Streptomyces*, was used for the production of esterase inhibitors.

Fermentation—A) Time Course of Inhibitor Production: A stock culture of strain DMC-498 was inoculated into 100 m*l* of the oatmeal yeast medium (oatmeal 20 g, yeast extract 1 g, distilled water 1 liter) in a 500-m*l* Erlenmeyer flask and was incubated at $27 \pm 1°C$ for 24 hours. Fifty miliiliters of the seed culture broth were transferred into 500 m*l* of the oatmeal yeast medium in a two-liter flask. Activity was measured for every 10-12 hours. B) Culture for Inhibitor Production: A stock culture of strain DMC-498 was inoculated into 100 m*l* of the oatmeal yeast medium in a 500-m*l* flask. Incubation was carried out at $27 \pm 1°C$ on a rotary shaker for one day. Fifty miliiliters of the seed culture broth were transferred into 500 m*l* of the same medium in a two-liter Erlenmeyer flask, and was incubated on a rotary shaker for three days.

Isolation of Esterase Inhibitors—The mycelia of three-day shaking culture were extracted with methanol and concentrated under the reduced pressure. Water was added into the methanol extract and the water suspension was extracted with chloroform by agitation. The chloroform layer was concentrated under the reduced pressure and the chloroform extract was chromatographed with Silica gel G (70-230 mesh) using chloroform. Yellow sludge of the active fraction was rechromatographed with Silica gel G (fine) using chloroform-hexan-methanol (2:2:0.2, v/v). Inhibitors were isolated from the former and latter active fractions. The inhibitor of the latter active fraction was isolated by preparatory argentation TLC. Isolated inhibitors were purified with Silica gel column using chloroform-ethyl acetate (1:1, v/v). Plate for argentation TLC was made by impregnating 10% silver nitrate on the precoated layer.

Kinetic Studies of Esterase Inhibitor—A) Calibration Curve for Enzyme Kinetics: *p*-Nitrophenol was used for making the calibration curve. Working solution of *p*-nitrophenol (1.25×10^{-2} M) was prepared. Working solution was diluted to 1.74×10^{-2} mg/m*l*, 1.24×10^{-2} mg/m*l*, 8.7×10^{-3} mg/m*l*, 5.8×10^{-3} mg/m*l* and 4.35×10^{-3} mg/m*l* in 50m*l* flask. Optical density of each solution was measured at 400 nm. Calibration curve was made by linear regression methods. B) Kinetics of Esterase Inhibitors: The concentration of the substrate (*p*-nitrophenyl acetate) was changed

from 2×10^{-4} M to 6×10^{-4} M, and its effects on initial velocity were determined at a fixed concentration of the inhibitor. Then a Lineweaver-Burk plot of 1/V, 1/[S] was prepared.

Spectral Analysis—Physico-chemical properties of the esterase inhibitors and lipids of strain DMC-498 were examined by spectroscopic methods. Conditions used for spectroscopy were as follows: A) UV spectrum: Hitachi Model ESP-35. Reading spectrometer (solvent: methanol). B) IR spectrum: Beckman IR-20A for lipids analysis (neat and KBr disk), Bruker FT-IR (ISF-85) for inhibitor analysis (neat). C) NMR spectrum: Bruker FT-NMR (200 MHz), solvent ($CDCl_3$). D) Mass spectrum: Hewlett Packard Model 5985-B.

RESULTS

Fermentation—Maximum percent inhibition appeared after three days in the liquid culture as shown in Fig. 1. The inhibitors were contained in both culture filtrate and mycelia, being contained more in the mycelia than in the culture filtrate. Therefore the mycelia of the three-day liquid culture were used for isolation of inhibitors.

Isolation of Esterase Inhibitors and Associated Lipids—The isolation procedure of the esterase inhibitors and lipids was illustrated in Scheme 1.

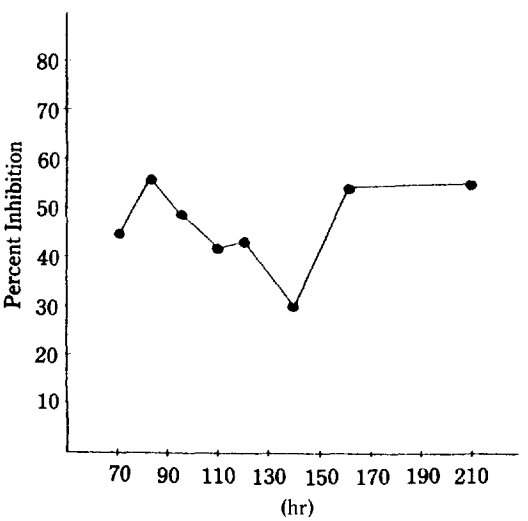
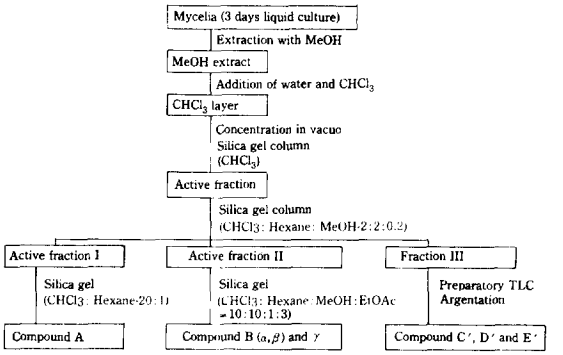


Fig. 1. Time course of inhibitor production.

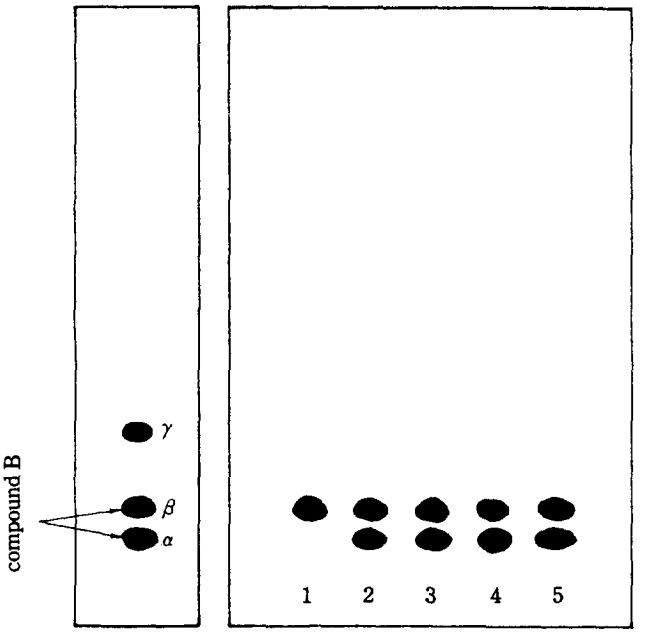


Scheme 1. Isolation procedure of esterase inhibitors from the mycelia of *Streptomyces* strain DMC-498.

Compound A was isolated from the former active fraction, and compound B was isolated from the latter active fraction. Compound B was not a single one, and it was a mixture of compound α and β. Isolated compound B was very unstable and rapidly changed into a mixture of compound α and β. The TLC pattern of compound B was illustrated in Fig. 2 in comparison with standard oleic acid and linoleic acid (Kanto Chem. Co.). It was thought that at first compound B was isolated in linoleic acid. But it was very unstable, and changed into mixed compounds of oleic and linoleic acids in a week. Because of its unstability, compound B was analyzed in a mixed state of compounds α and β by using IR, NMR and mass spectra. Several lipids were isolated from the former active fraction by preparatory argentation TLC. Total lipid fraction was separated into compounds C', D' and E'. Their TLC patterns were illustrated in Fig. 3. Also compound γ was isolated from the latter active fraction by preparatory argentation TLC[16)]

Kinetic Studies of Esterase Inhibitors—Esterase inhibitors produced by strain DMC-498 showed competitive inhibition was shown in Fig. 4.

Spectral Analysis—UV, IR and mass spectra of compound A (liquid state esterase inhibitor) were shown in Figs. 5, 6, 7 and 8. The UV spectrum showed aliphatic absorption at 211 nm and other absorption was not observed. In IR spectrum, olefinic C-H streching vibration was observed at



Solvent: chloroform-hexane-methanol
= 2:2:0.2

Silical gel plate was impregnated with 10% $AgNO_3$ solution.

Rf value: α(0.14), β(0.19), γ(0.31)
1) standard oleic acid (Kanto Chem. Co.)
2) compound B + standard oleic acid
3) compound B
4) compound B + standard linoleic acid
5) standard linoleic acid (Kanto Chem. Co.)

Fig. 2. Separation of compound B by argentation TLC.

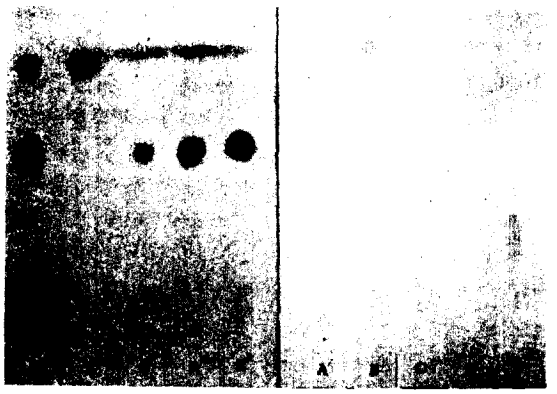


Fig. 3. Isolation of lipids from strain DMC-498 by preparatory argentation TLC.
left : normal Silica gel plate
solvent: benzene
right: Silica gel plate was impregnated with 10% $AgNO_3$

spray reagent: phosphomolybdic acid or 10% H_2SO_4
A') Total lipids
B') Straight chain fatty acid
C') iso-Stearic acid methyl ester
D') Oleic acid methyl ester
E') Linoleic acid methyl ester

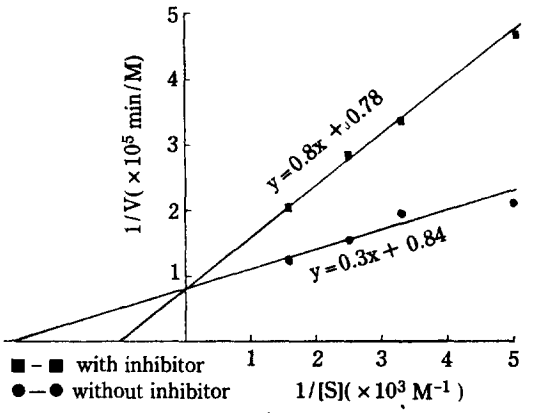


Fig. 4. Kinetic studies of the esterase inhibitor of strain DMC-498.

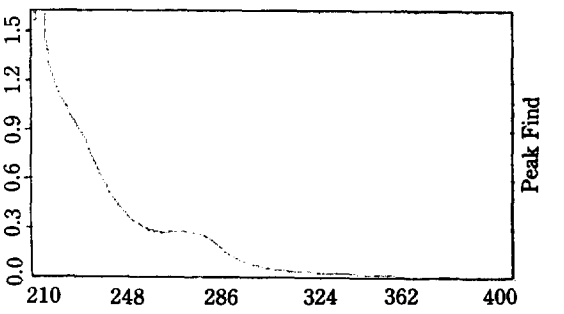


Fig. 5. UV spectrum of compound A (esterase inhibitor). solvent: methanol

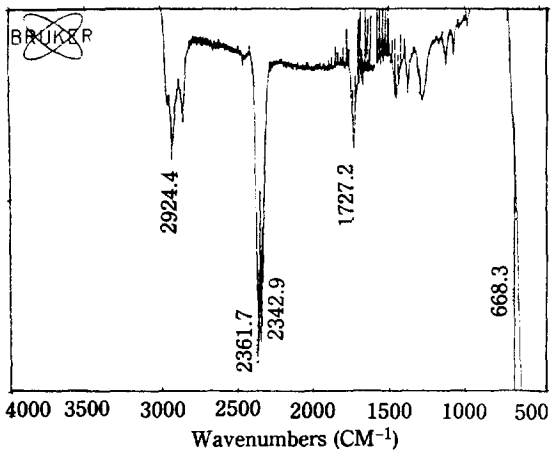


Fig. 6. IR spectrum of compound A (esterase inhibitor).

3040 cm^{-1} and C=O streching at 1727 cm^{-1}. The peak near 2361 cm^{-1} was due to CO in atmosphere. The NMR spectrum showed olefinic proton at 5.1 ppm, R-C(=O)-O-C$\underline{H}_2$ signal at 4.01, CH-HC=CH-C$\underline{H}_2$ at 2.0 ppm, C$\underline{H}_2$-C-C-(=O)-O-R at 1.7, C$\underline{H}_2$-C at 1.25 and CH_3 at 0.9 ppm. Molecular weight of compound A was not determined exactly (Fig. 8). Exact structure of compound A could not be clarified, but it was assumed that compound A would have alicyclic ring structure in respect to the presence of many protons at 1.6-1.7 ppm. Compound A gave single spot at Rf 0.38 with chloroform-hexane-ethyl acetate (5:5:1, v/v) and 0.33 with chloroform-hexane (20:1, v/v).

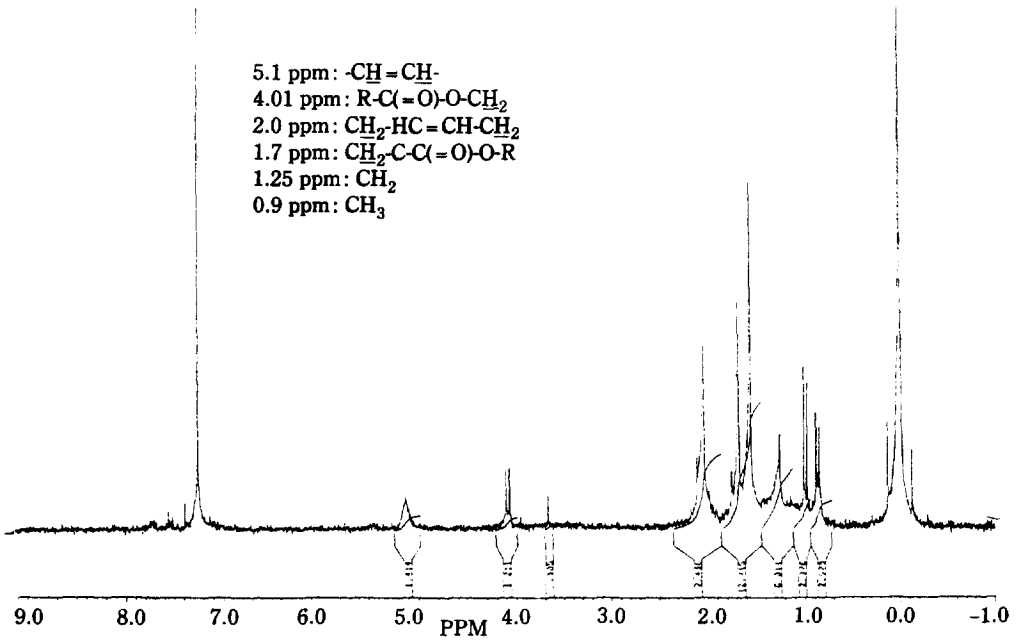


Fig. 7. NMR spectrum of compound A (esterase inhibitor). solvent: $CDCl_3$

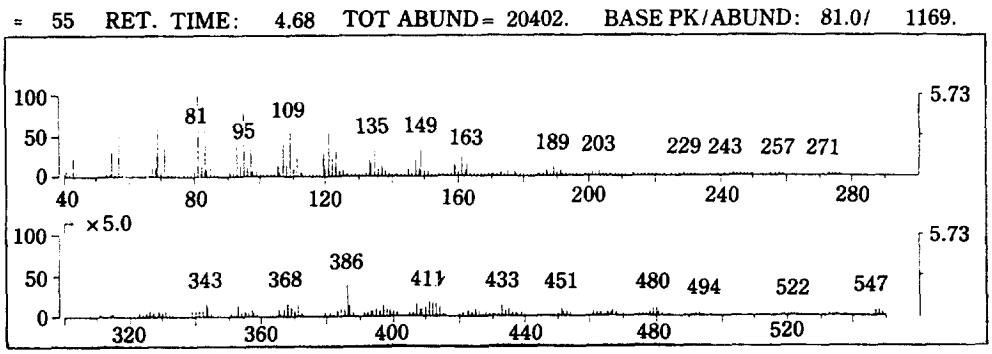


Fig. 8. Mass spectrum of compound A (esterase inhibitor).

IR and NMR spectral data of compound B, esterase inhibitors, were shown in Table I. No specific absorption was observed in the UV spectrum. The IR spectrum showed olefinic C-H streching vibration of 3040 cm^{-1}, C=O at 1711, C-O strech at 1285, C=C at 1653 and C-H strech at 2926 cm^{-1}. The IR and mass spectra of compound B were identified as those of linoleic acid ($M^+=280$). In NMR spectrum, olefinic protons were observed at 5.3ppm, -CH=CH-$C\underline{H}_2$-CH=CH- at 2.7, HO-C(=O)-$C\underline{H}_2$- at 2.3, C=C-C-$C\underline{H}_2$ at 2.0, -C(=C)-C-CH_2 at 1.6, CH_2-C- at 1.3, and CH_3- at 0.89 ppm.

The NMR spectrum of compound B was very similar to that of linoleic acid. But its integral was not exactly the same as that of linoleic acid. Isolated in the first place, compound B was a single one having lower Rf value (compound α). But approximately in a week, it became a mixture of compounds α and β. As shown in the TLC pattern (Fig. 3), compounds α and β are linoleic and oleic acids, respectively. And the difference between the integral of compound B and standard linoleic acid was interpreted in respect to the mixture. Mass spectrum also showed two molecular ion peaks: 280 of linoleic acid, CH_3-$(CH_2)_4$-CH=CH-CH_2-CH=CH-$(CH_2)_7$-COOH and 282 of oleic acid, CH_3-$(CH_2)_7$CH=CH$(CH_2)_7$COOH. Standard linoleic acid (Kanto Chem. Co.) had an esterase inhibitory activity. And its IC_{50} (50% inhibition concentration) was 0.045 μg/ml.

Molecular weight of compound C' was 298. The IR spectrum showed ketone at 1720 cm^{-1}, methyl ester signal at 1100-1300cm.$^{-1}$ The NMR spectrum showed -OCH_3 at 3.6ppm and -$C\underline{H}_2$-C(=O)-O-R at 2.3 ppm. The doublet at 0.87 ppm showed isopropyl group. Compound C' was identified as isostearic acid methyl ester, $(CH_3)_2CH(CH_2)_{13}CH_2$-C(=O)-$OCH_3$.

Molecular weight of compound D' was 296. The IR spectrum of compound D' showed olefinic C-H strech at 3040 cm^{-1}, ketone at 1710 cm^{-1}. In the NMR spectrum methoxyl proton peak appeared at 3.66ppm and olefinic proton at 5.36, -$C\underline{H}_2$-C(O)-OR at 2.3ppm. And the triplet at 0.9ppm showed proton of methyl group (-CH_2CH_3). Compound D' was identified as oleic acid methyl

Table I. Spectral data of compounds A, B, C', D', E' and γ that were isolated from the metabolite of *Streptomyces* strain DMC-498.

Compound	IR(cm^{-1})	NMR(δ ppm)
A*	1720 (C=D)	5.1 (olefinic proton)
	3040 (olefinic CH)	4.0 (R-CO-O-CH_2)
		2.0 (CH-CH = CH-CH_2)
		1.7 (CH_2-C-CO-O-R)
B*	3040 (olefinic CH)	5.3 (m, 4H, 2 CH=CH)
	1711 (C=O)	2.7 (t, 2H, CH=CH-CH_2-CH=CH)
	3300-2500 (COOH)	2.3 (t, 2H, HO-CO-CH_2)
	1285 (C-O)	2.0 (t, 4H, C=C-CH_2)
	1653 (C=C)	1.6 (t, 2H, CO-C-CH_2)
		1.3 (d, 14H, 7 CH_2)
		0.9 (t, 3H, CH_3)
C'	1720 (C=O)	3.7 (s, 3H, OCH_3)
	1100-1300	2.3 (t, 2H, CH_2)
	(methyl ester)	1.6 (m, 3H, CH, CH_2)
		1.3 (s, 24H, 12 CH_2)
		0.9 (d, 6H, CH_3)
D'	3040 (olefinic CH)	5.4 (m, 2H, CH=CH)
	1710 (C=O)	3.7 (s, 3H, OCH_3)
	1100-1300	2.3 (t, 2H, CH_2-CO)
	(methyl ester)	2.0 (d, 4H, C=C-CH_2)
		1.6 (s, 2H, CO-C-CH_2)
		1.3 (s, 20H, 10 CH_2)
		0.9 (t, 3H, CH_3)
E'	3040 (olefinic CH)	5.4 (m, 4H, CH=CH)
	1710 (C=O)	3.6 (s, 3H, OCH_3)
	1100-1300	2.7 (t, 2H, CH=CH-CH_2-CH=CH)
	(methyl ester)	2.3 (t, 2H, CO-CH_2)
		2.0 (t, 4H, C=C-CH_2)
		1.6 (t, 2H, CO-C-CH_2)
		1.3 (s, 14H, 7 CH_2)
		0.9 (t, 3H, CH_3)
γ	1100-1330 (14 CH_2)	2.3 (t, 2H, CO-CH_2)
	2600-3300 (COOH)	1.6 (m, 3H, CH, CH_2)
	1700 (C=O)	1.3 (s, 24H, 12 CH_2)
		0.9 (d, 6H, CH_3)

*Esterase inhibitors

ester, $CH_3(CH_2)_7CH=CH(CH_2)_7COOCH_3$.

Molecular weight of compound E' was 294. And the IR spectrum showed olefinic C-H strech at 3040 cm^{-1}, ketone at 1710 and characteristic methyl ester signal at 1100-1300 cm^{-1}. The NMR spectrum showed olefinic proton at 5.4ppm, $-CH=CH-CH_2-CH=CH-$ at 2.77, $-OCH_3$ at 3.66 and $-C\underline{H}_2-C(=O)-OR$ at 2.3ppm. Compound E' was identified as linoleic acid methyl ester, $CH_3(CH_2)_4-CH=CH-CH_2-CH-(CH_2)_7-C(=O)-OCH_3$.

The IR spectrum of compound γ showed characteristic signal of fourteen methylene group at 1100-1330 cm^{-1} and broad O-H signal[17] of carboxyl group at 2600-3300cm^{-1}. Molecular weight of compound γ was 284. Compound γ was identified as isostearic acid, $(CH_3)_2\ CH(CH_2)_{14}-COOH$.

DISCUSSION

Maximum percent inhibition appeared in a three-day liquid culture of the strain. Esterase inhibitors were contained in both culture filtrate and mycelia. The inhibitors were more abundant in the mycelia than in the culture filtrate. Type of inhibition was proved to be competitive by Lineweaver-Burk plot method.

The inhibitors were isolated by Silica gel column chromatography and preparatory argentation TLC. It was suggested that compound A (liquid state esterase inhibitor) had an alicyclic ring structure, two or more oxygens, and its molecular weight was more than 500. Compound B was proved to be a mixture of oleic and linoleic acids. IC_{50} (50% inhibition concentration) of standard linoleic acid (Kanto Chem. Co.) was 0.045 $\mu g/ml$. It was reported that linoleic acid inhibited the activities of various enzymes, such as trypsin, chymotrypsin, pepsin, succinic oxidase, α-ketoglutarate oxidase, isocitrate oxidase and butyrylcholine esterase[23-24] No report, however, was found that linoleic acid was an inhibitor of esterase. Thus, this shall be the first report on linoleic acid as esterase inhibitor. The main components of lipids which were isolated from the methanol extracts of the mycelia were composed of oleic acid methyl ester, linoleic acid methyl ester, isostearic acid methyl ester and isostearic acid. Further studies are in progress to determine a complete structure of compound A (liquid state esterase inhibitor).

국문 요약

한국 토양균 중에서 esterase 저해제를 생성하는 균주인 *Streptomyces* strain DMC-498을 oatmeal yeast extract 배지에서 3일간 액내 배양하였을 때 최고의 저해작용을 나타내었으며 균사체의 메탄올 추출물로부터 저해제를 분리하였다. linoleic acid가 저해작용이 있음을 확인하였으며 50% 저해작용을 나타내는 linoleic acid의 양은 0.045 $\mu g/ml$이었다. 활성이 보다 큰 compound A는 분자량이 500 이상이며 산소를 2개이상 함유하고 있는 환상구조의 지방족 화합물로 생각된다. 이것은 상경적 저해작용을 나타내었다. DMC-498 균주가 함유하는 지질의 주성분은 isostearic acid, isostearic acid methyl ester, linoleic acid methyl ester 및 oleic acid methyl ester 이었다.

감사의 말씀

이 연구에 소요되는 경비의 일부는 한국과학재단의 연구비로 충당되었음.

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