Screening of Lipase Inhibitors from Marine Algae

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ABSTRACT: The possible presence of an inhibitor of pancreatic lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) was screened in 54 marine algae. An active inhibitor, caulerpenyne, was purified from an extract of *Caulerpa taxifolia,* using ethyl acetate extraction, followed by successive chromatographies on ODS and silica gel columns. The purified inhibitor was identified by thin-layer chromatography, infrared and nuclear magnetic resonance spectroscopy. Caulerpenyne competitively inhibited lipase activities using emulsified triolein and dispersed 4-methylumbelliferyl oleate (4-MU oleate) as substrates. The concentrations producing 50% inhibition against triolein and 4-MU oleate hydrolysis were 2 mM and 13 μM, respectively. *In vivo,* oral administration of corn oil with or without caulerpenyne to rats demonstrated a reduced and delayed peak plasma triacylglycerol concentration with caulerpenyne.

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Pancreatic lipase is a key enzyme of dietary triacylglycerol absorption, hydrolyzing triacylglycerols to 2-monoacylglycerols and fatty acids. Typical substrates for these enzymes are long-chain triacylglycerols which are separated from the aqueous medium by the surface phase. Thus, lipase must be adsorbed on the lipid surface, and the nature of the surface of the substrate is an important factor for lipase activity. Therefore, amphoteric substances would be expected to influence the lipase reaction rate. There have been many reports of lipase inhibitors derived from natural materials, for example, proteins (1,2), phytic acid (3), and tannin (4). Most of these inhibitors are amphoteric substances. It is well known that bile salts and synthetic detergents (5) behave as inhibitors of lipolysis. Hydrophobic proteins, such as bovine serum albumin and β-lactoglobulin (6), inhibit lipase activity toward triacylglycerol substrates by competing for the substrate surface.

Only a few substances directly interact with lipases themselves, one example being lipstatin (7), from *Streptomyces toxytricini,* which strongly inhibits lipases. In this study, we report the isolation from marine algae of caulerpenyne, an inhibitor which interacts directly with pancreatic lipase.

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Abbreviations : 4-MU oleate, 4-methylumbelliferyl oleate.

MATERIALS AND METHODS

Materials. The enzyme substrates and reagents used were obtained as follows. Triolein, cholesterol oleate, taurocholic acid, pancreatic lipase (Type VI-S, from porcine pancreas), and 4-methylumbelliferyl oleate (4-MU oleate) were purchased from Sigma Co. (St. Louis, MO). Silica gel (Kiesel gel 60, Merck) was purchased from Kanto Chemicals (Tokyo, Japan). Dimethyl sulfoxide and octadecyl silica gel columns (Cosmosil 75C18-PREP) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). A normal-phase high-performance liquid chromatographic column (YMC-pack SIL-06) was obtained from YMC Co. (Kyoto, Japan).

Measurement of lipase activity and inhibitory activity. Lipase activity was determined by measuring the rate of release of oleic acid from triolein (8). A suspension of 90 μmol triolein, 45 mg gum arabic, and 9.45 μmol taurocholic acid in 9 mL 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.0, containing 0.1 M NaCl) was sonicated for 5 min. The assay system comprised the following components in a total volume of 0.2 mL: 0.05 mL enzyme solution, 0.05 mL inhibitor solution, 1 μmol triolein, 0.106 μmol taurocholic acid, 0.5 mg gum arabic, 20 μmol *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, and 20 μmol NaCl. Incubation was carried out at pH 7.0 and 37°C for 30 min, and the amount of oleic acid produced was determined by the method of Zapf *et al*. (9) with a slight modification (10). The incubation mixtures were added to 3 mL of a 1:1 (vol/vol) mixture of chloroform/heptane containing 2% (vol/vol) methanol and extracted by shaking the tubes horizontally for 10 min in a shaker. The mixture was centrifuged ($2000 \times g$, 10 min), the upper aqueous phase was removed, and 1 mL of copper reagent (10) was added to the lower organic phase. The tubes were then shaken for 10 min, the mixture was centrifuged ($2000 \times g$, 10 min), and 1 mL of the upper organic phase, which contained the copper salts of the extracted oleic acid, was treated with 1 mL 0.1% (wt/vol) bathocuproine containing 0.05% (wt/vol) 3-*tert*-butyl-4-hydroxyanisole and the absorbance determined at 480 nm.

Lipase activity was also measured using 4-MU oleate as a substrate (11). The reaction mixture was prepared with 0.1 mL 0.1 mM 4-MU oleate and 0.05 mL inhibitor solution. The reaction was started by adding 0.05 mL lipase, all in a final volume of 0.2 mL. After incubation at 37°C for 15 min, 0.5

mL of 0.1 M HCl and 1 mL of 0.1 M sodium citrate were added. The amount of 4-methylumbelliferone released by the lipase was measured fluorometrically at an excitation wavelength of 320 nm and an emission wavelength of 450 nm.

The concentration of the tested compounds giving 50% inhibition of the enzyme activity was estimated from the least-squares regression line of the plots of the logarithm of the concentration vs. the inhibitory activity.

Extraction from marine algae. Fresh marine algae were collected around Shikoku Island, Japan, in 1993–1994, freeze-dried, powdered, and kept at −20°C. Ten grams of the marine algal powder was extracted with 100 mL methanol (or ethyl acetate) with shaking at room temperature for 24 h. After filtration, the extract was evaporated *in vacuo*, dissolved in 2 mL dimethyl sulfoxide, and used to measure the lipase inhibitory activity.

Purification of inhibitor. Caulerpa taxifolia, obtained from a tropical fish store, was cultured in a tank. The cultured alga was freeze-dried and ground to give 100 g of powder and then extracted with 1000 mL of ethyl acetate. After filtration, the extract was evaporated *in vacuo*. The evaporated sample was dissolved in a small amount of acetonitrile, passed through an octadecyl silica gel column (Cosmosil 75C18-PREP, 40 g) to remove pigments such as chlorophyll, and eluted with acetonitrile. The eluate was concentrated *in vacuo*, applied to a silica gel column (Kiesel gel 60, 200 g), and eluted with a stepwise solvent system of *n*-hexane/ethyl acetate. The eluate from hexane/ethyl acetate (4:1, vol/vol) that exhibited lipase inhibitory activity was applied to a normal-phase high-performance liquid chromatographic column (YMC-pack SIL-06) and eluted with hexane/ethyl acetate (2:1, vol/vol). The fractions containing lipase inhibitory activity were pooled and concentrated to dryness *in vacuo*.

Oral administration of caulerpenyne. A suspension of 6 mL corn oil, 80 mg cholic acid, and 2 mg cholesteryl oleate in 6 mL water was sonicated for 5 min. Male Wistar King rats, weighing 150–190 g, were starved overnight, then divided into two groups, and 1 mL corn oil suspension was administered to each rat *via* a stomach tube (8). One group received this suspension containing 1 mL caulerpenyne (15 mg) solution and the control group received the suspension containing 1 mL water. After caulerpenyne administration, blood samples were collected from the tail vein or artery into heparinized microcapillary tubes, at regular intervals, and centrifuged immediately at 10,000 rpm for 5 min. Plasma triacylglycerol concentrations were determined using the Triglyceride E-Test (Wako Pure Chemical Industries, Osaka, Japan).

Statistics. The data were analyzed for statistical significance using Student's *t*-test.

RESULTS

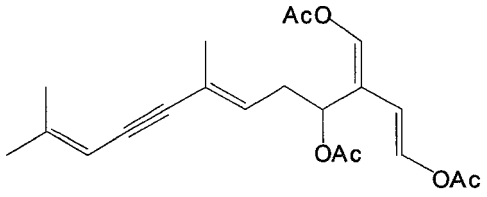
Screening of lipase inhibitors. Marine algae (54 species of natural materials) were extracted using methanol or ethyl acetate, and the inhibitory activities of these extracts were screened against triolein-hydrolyzing activity of porcine pancreatic lipase. The results from 27 species are shown in Table 1. Inhibitory activity was not observed in the extracts from *Gracilaria bursa-pastoris*, *G. chorda*, *G. textorii*, *Gymnogongrus flabelliformis*, *Eckloniopsis radicosa*, *Chaetomorpha crassa*, and algae of the genus *Laurencia* (data not shown). Higher activity was found in methanol extracts from *C. taxifolia* and *Asparagopsis tociformis.*

Purification of inhibitor. Since both the methanol and ethyl acetate extracts from *C. taxifolia* had relatively high lipase inhibitory activity, isolation of the lipase inhibitor from its ethyl acetate extract was attempted. Although the methanol extract had a higher lipase inhibitory activity than that of the ethyl acetate extract, it also contained a high amount of polyphenols or their oxidized compounds such as tannins. Since tannin-like compounds bind strongly to enzyme proteins and may inhibit their enzyme activity (12), we isolated the lipase inhibitor from the ethyl acetate extract. Purification was achieved by sequential chromatography on octadecyl silica gel, silica gel and normal-phase high-performance liquid chromatographic columns. The purified inhibitor (0.2 g, slightly yellowish oil) was obtained from 100 g of dried *C. taxifolia*. The purified inhibitor was identified as caulerpenyne by comparing its thin-

TABLE 1
Inhibition of Lipase Activity by Algal Extracts

Species	Inhibition activity (%)	
	Methanol extract	Ethyl acetate extract
Phaeophyta		
Cutleria cylindrica	88	14
Dictyopteris latiuscula	90	—[a]
D. prolifera	97	—
Hizikia fusiformis	71	21
Ishige okamurai	77	41
I. sinicola	76	22
Myelophycus simplex	62	49
Padina arborescens	52	—
P. crassa	—	98
Sargassum muticum	94	—
S. ringgoldianum	85	—
S. thunbergii	63	—
Chlorophyta		
Caulerpa okamurae	94	—
C. toxifolia	100	52
Codium latum	95	3
C. pugniformis	94	5
Rhodophyta		
Asparagopsis taxiformis	100	10
Chondrus giganteus	51	—
Eucheuma amakusaensis	70	39
E. serra	44	31
Gloiopeltis tenax	94	—
Gracilaria verrucosa	—	41
Gracilariopsis chorda	58	5
Hypnea charoides	100	1
H. japonica	88	—
Lomentaria catenata	43	13
Porphyra tenera	63	—

[a]Not detected.



SCHEME 1

layer chromatographic, infrared and nuclear magnetic resonance spectroscopic, and electron impact-mass spectrometric data with those reported previously (13) (Scheme 1). ^{1}H and ^{13}C nuclear magnetic resonance spectra were measured using EX-270 (270 MHz) (JEOL Ltd., Tokyo, Japan), and electron impact-mass spectrometric data were obtained using JMS-SX102A (JEOL Ltd.).

Inhibitory action of caulerpenyne. The hydrolytic activity of porcine pancreatic lipase toward triolein emulsified with gum arabic and 4-MU oleate was determined in the presence of increasing concentrations of caulerpenyne (Figs. 1 and 2). In a concentration dependent manner caulerpenyne inhibited the triolein- and 4-MU oleate-hydrolyzing activities of pancreatic lipase, showing 50% inhibition at 2 mM and 13μM, respectively. Caulerpenyne also inhibited the tributyrin-hydrolyzing activity in a concentration-dependent manner (data not shown). The inhibitory action of caulerpenyne was examined using different concentrations of pancreatic lipase and substrates. In using triolein as a substrate, the inhibitory activity of caulerpenyne was not affected by an increase in the triolein concentration (Fig. 3A), but it decreased with increasing enzyme concentration (Fig. 3B). Similar results were observed using 4-MU oleate as a substrate; the inhibition was not affected by an increase of 4-MU oleate, but it decreased with an increase in enzyme concentration (data not shown). To clarify the enzyme-inhibiting mechanism, the kinetics of the inhibitory effect of caulerpenyne on lipase activity were studied. The mode of inhibition was analyzed by changing the concentration of substrate in the presence of two concentrations of caulerpenyne. A double-reciprocal plot of the reaction rate vs. triolein concentration was linear in the presence and absence of caulerpenyne, suggesting that the observed inhibition of lipase activity by caulerpenyne was due to competitive inhibition of the enzyme (data not shown). A similar result was obtained using 4-MU oleate as a substrate (data not shown).

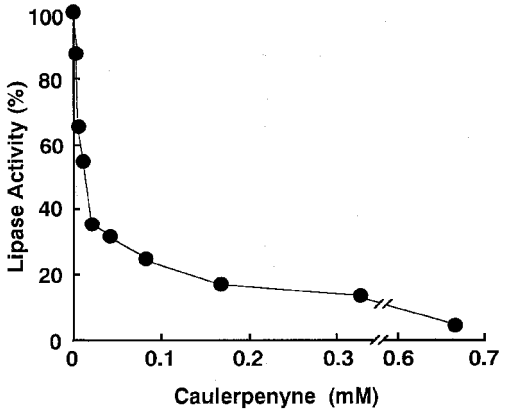


FIG. 2. Effect of increasing concentrations of caulerpenyne on the rate of hydrolysis of 4-methylumbelliferyl (4-MU) oleate by pancreatic lipase (18.75 μg/mL). The specific activity of 4-MU oleate-hydrolyzing activity was 17.8 μmol 4-methylumbelliferone released/mg protein of pancreatic lipase/min.

Oral administration of caulerpenyne. Figure 4 shows the time course of the plasma triacylglycerol concentration when corn oil suspension with or without caulerpenyne was administered orally to rats. Two, 3 and 6 h after caulerpenyne administration, the plasma triacylglycerol concentration decreased significantly in comparison with the controls. The peak plasma triacylglycerol concentration was reduced and delayed by caulerpenyne administration.

DISCUSSION

Caulerpenyne competitively inhibited the lipase activity. The inhibitory activity of caulerpenyne was dependent on the li-

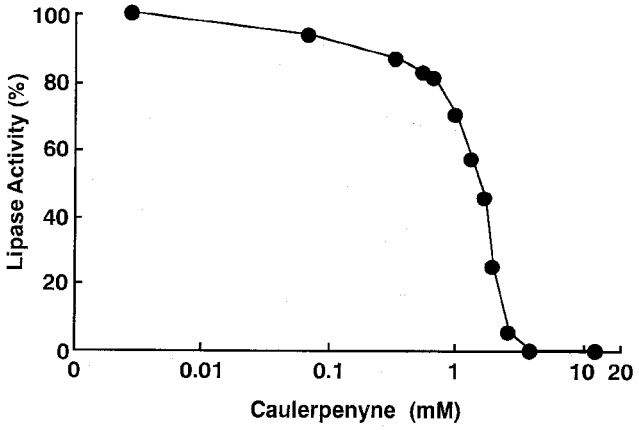


FIG. 1. Effect of increasing concentrations of caulerpenyne on the activity in hydrolysis of triolein emulsified with gum arabic by pancreatic lipase (1.0 μg/mL).

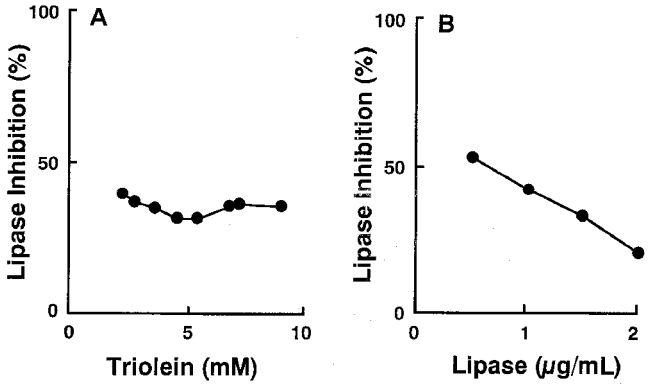


FIG. 3. Effect of substrate and enzyme concentrations on the inhibition by caulerpenyne. Lipase activity was determined using triolein as substrate with 330 μM caulerpenyne. (A) Effect of substrate concentrations (lipase, 1.0 μg/mL); (B) effect of enzyme concentrations (triolein, 5 mM).

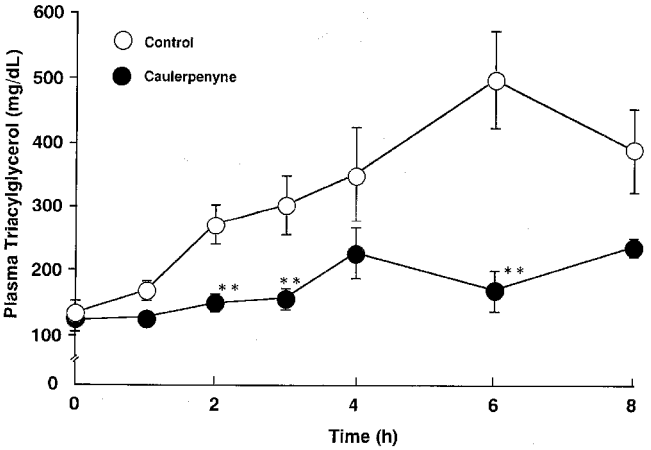


FIG. 4. Effect of caulerpenyne on rat plasma triacylglycerol levels after oral administration of lipid emulsion (○) or lipid emulsion containing caulerpenyne (●). The results are expressed as means ± SE of 10 experiments. $^{**}P < 0.01$ (compared with the corresponding control, Newman-Keuls' range test).

pase concentration but independent of substrate concentration (Fig. 3). Caulerpenyne also inhibited lipase activity for monomeric substrate such as 4-MU oleate or tributyrin, as well as when using emulsified triolein. These results suggest that caulerpenyne interacts directly with the lipase protein, rather than interacting with the substrate.

Caulerpenyne was the major metabolite in *C. taxifolia*, which is widely distributed in the tropics and is presently invading part of the Mediterranean (14). Fischel *et al.* (15) reported that caulerpenyne inhibited cell growth. However, as a result of evaluations conducted using human hematopoietic progenitors, melanocytes, and keratinocytes in culture, the toxicological risk of caulerpenyne to humans has been reported as low (16). In the Philippines, *C. taxifolia* is actually available as a food for medicinal purposes. It acts as an antifungal agent and reportedly decreases blood pressure (17). These results suggest that caulerpenyne (or *C. taxifolia*) may have potential as an agent that inhibits lipid absorption.

For lipase catalysis, the surface characteristics of substrate lipid micelles are the most important factors. Lipases are sometimes activated or denaturated by adsorption (or penetration) on to the substrate surface (18). Most lipase inhibitors reported thus far are adsorbed onto (or penetrate) the lipid surface, thus blocking the surface and affecting lipase activity. Several proteins, including mellitin, β-lactoglobulin A, serum albumin, ovalbumin and myoglobin, inhibit pancreatic lipase activity, and their inhibitory effects might be the result of lipase desorption from the substrate due to a change in interfacial quality (19,20). Hydrocarbons, fatty alcohols, and fatty acids also adsorb on the substrate interface and inhibit lipase activity (21–23). In this study, we purified caulerpenyne, a substance which may interact directly with lipase. A few substances acting as direct inhibitors of lipase have been reported. Lipstatin, isolated from *S. toxytricini,* is a stable covalent inhibitor of lipase (7). It interacts with lipase, probably with 1:1 stoichiometry, and inactivates the enzyme through the formation of a stable covalent intermediate. Fat absorption from the intestine of rats is also inhibited by lipstatin: the absorption of emulsified triolein was significantly decreased by 10^{-4} M lipstatin using the constant infusion method (24). We showed that caulerpenyne, administered orally in a single dose, reduced the concentration of plasma triacylglycerol in an *in vivo* experiment using rats (Fig. 4).

Marine algae (or seaweeds) have long been a food source for maritime people, as sea vegetables. In Japan, they have been eaten as a healthy food since antiquity. They contain proteins, fats, carbohydrates, cellulose, ash, minerals, and nucleic acids and are especially rich in iodine and certain vitamins. In this study, we demonstrated that many marine algae inhibited the activity of pancreatic lipase (Table 1), the extracts from Phaeophyta, Chlorophyta, and Rhodophyta having particularly high inhibitory activities. Phaeophyta generally contain large amounts of polyphenols such as tannin, which has lipase-inhibiting activity. It has been reported that most compounds with a porphyrin structure inhibit lipase activity (11). The weak inhibitory activities recognized in many algae might be caused by chlorophyll in the algal extracts. Therefore, algae are one of the most useful sources of lipase inhibitors.

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