LIPSTATIN, AN INHIBITOR OF PANCREATIC LIPASE, PRODUCED BY *STREPTOMYCES TOXYTRICINI*

I. PRODUCING ORGANISM, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

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Lipstatin, a new and very potent inhibitor of pancreatic lipase (the key enzyme of intestinal fat digestion) was isolated from *Streptomyces toxytricini*. Lipstatin contains a β-lactone structure that probably accounts for the irreversible lipase inhibition. The IC_{50} of lipstatin for pancreatic lipase is 0.14 μM. In mice triolein absorption was dose-dependently inhibited by lipstatin, whereas oleic acid was absorbed normally. Other pancreatic enzymes, such as phospholipase A2 and trypsin, were not inhibited even at an inhibitor concentration of 200 μM.

Obesity and hypercholesterolemia are to a relevant degree related to high nutritional fat intake. The key enzyme of dietary triglyceride absorption is pancreatic lipase[1], exerting its activity at the water-lipid interphase, in conjunction with bile salts and co-lipase. A target directed screening of microbial broths from soil organisms resulted in the discovery of a very potent, selective and irreversible inhibitor of pancreatic lipase, which was named lipstatin[2]. The lipstatin molecule has an unusual β-lactone structure incorporated into a hydrocarbon backbone, comparable to esterastin[3]. Several lipase inhibitors and several compounds inhibiting fat absorption have been described[4], but a clear demonstration, that inhibition of fat absorption resulted from inhibition of the lipase activity has been lacking so far.

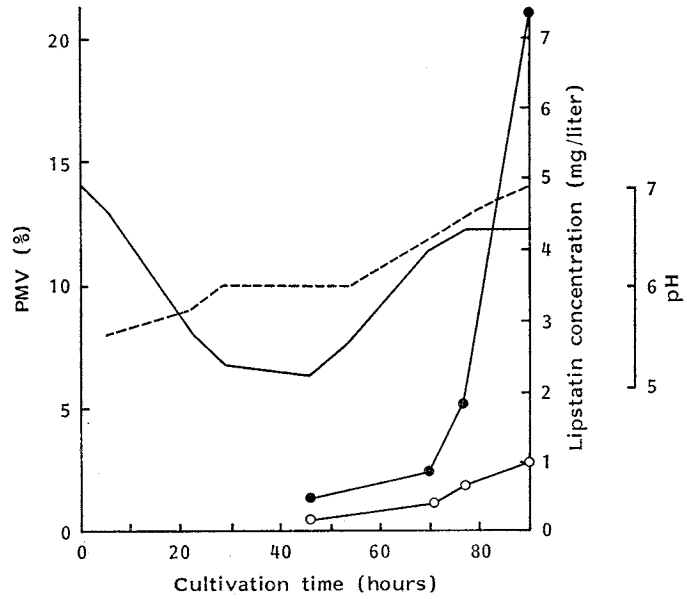
Producing Organism

Fermentation broths from actinomycetes and fungi, isolated from soil samples, were screened for inhibition of lipase activity. The most active broths were from the fermentation of two streptomyces strains, No. 85~13 found in a soil sample from Mallorca (Spain) and No. 72~21 in a soil sample from Gstaad (Switzerland). Although the appearance of the colonies on all surface cultures was different, a complete identification carried out by CBS (Centraalbureau voor Schimmelcultures, Baarn, Netherlands) showed that the strains were grey and white variants of *Streptomyces toxytricini* (Preobrazhenskaya and Sveshnikova)[5]. Both variants are equally productive and can be grown and maintained on agar medium containing soluble starch 10 g, Casamino acids 0.3 g, KNO_3 2 g, NaCl 2 g, K_2HPO_4 2 g, $MgSO_4 \cdot 7H_2O$ 50 mg, $CaCO_3$ 20 mg, $FeSO_4 \cdot 7H_2O$ 10 mg and agar 20 g in 1 liter tap water, pH 7.4. Strain 85~13 (white variant) was assigned NRRL No. 15443 and used for the fermentations and isolations presented in this paper.

Fermentation Process

Lipstatin was produced in shake flasks as well as in fermentor cultures of *S. toxytricini*, grown at

Fig. 1. 200-liter tank fermentation at 28°C; 0.4 vvm aeration and 300 rpm agitation.
—— pH, ------ packed mycelium volume (PMV), ● intracellular lipstatin, ○ extracellular lipstatin.



28 to 30°C for 4~5 days. The inoculum medium contained soya-flour 30 g, corn starch 30 g, dextrin 40 g, $(NH_4)_2SO_4$ 2 g, $CaCO_3$ 6 g and soya-oil 8 ml in 1 liter tap water, pH 7. The production medium for shake flasks and fermentors, called carbohydrate cascade medium[6] contained potato starch 5 g, glucose 5 g, ribose 5 g, glycerol 5 g, soya-flour 20 g, $(NH_4)_2SO_4$ 2 g, yeast extract 2 g and Bacto-peptone 2 g in 1 liter tap water, pH 7.

A typical tank fermentation is shown in Fig. 1. The course of the extracellular and intracellular lipstatin production is characteristic. At a certain stage of the fermentation the intracellular concentration increases rapidly up to 10 mg/liter, whereas the extracellular concentration increases only slightly. The extracellular lipstatin at the point of maximum concentration varies between 10 and 40% of the total. Prolonged incubation results in decreasing yields both intra- and extra-cellularly. The exact location of the intracellular lipstatin is not yet known, neither are the factors for its release. The pH of the fermentation broth should not be alkaline, otherwise the β-lactone of the lipstatin molecule is irreversibly opened[7].

Isolation Procedure and Structure

Upon termination of a 1,000-liter fermentation at the point of maximum lipstatin concentration, the broth was cooled to 4°C and centrifuged. The supernatant was discarded and the mycelium was stored frozen until extraction of lipstatin. Lipstatin was isolated as a pale yellow oil (Fig. 2). Samples were chromatographed on Silica gel F_{254} plates with chloroform and the compounds were visualized with chlorine - tolidine or with anisaldehyde - sulfuric acid. The purified lipstatin moved as a single spot with an Rf 0.15.

Experimental

41 kg of frozen biomass were thawed and homogenized with 40 liters of water. The suspension was extracted with 140 liters of methanol under stirring for 20 minutes. Then the mixture was filtered

Fig. 2. Isolation of lipstatin.

Mycelium (41 kg)
| 1) extraction with methanol
| 2) concentration of the extract and diluting it in water
| 3) extraction with hexane - ethyl acetate
Crude extract (428 g)
| flash chromatography on silica gel with chloroform
Enriched extract (70 g)
| flash chromatography on silica gel with hexane - ethyl acetate
Crude lipstatin (4.2 g)
| chromatography on Lobar Lichroprep RP-8 with methanol
Pure lipstatin (1.77 g) (structure see Table 1)

and the filter cake extracted a second time with 140 liters of methanol. The organic extracts were pooled and concentrated to 22 liters under reduced pressure. The concentrate was diluted with water to 50 liters and extracted three times with 50 liters of hexane - ethyl acetate (1 : 1). 1.4 kg and 0.5 kg, respectively, of sodium chloride were added during the second and third extraction. A fourth extraction was made with 60 liters of ethyl acetate. The pooled organic extracts were concentrated, dried over sodium sulfate and concentrated under reduced pressure to give 428 g of a brownish oily residue.

The crude extract (428 g) was subjected to flash chromatography on silica gel. In four cycles on a 1 kg column 70 g of enriched extract were eluted with chloroform. This material was further purified by flash chromatography on silica gel. In two cycles on a 1 kg-column 4.2 g of crude lipstatin were eluted using a gradient of hexane - ethyl acetate (9 : 1 to 4 : 1). The final purification was made with reverse phase chromatography using a Lobar Lichroprep RP-8 size C column (Merck) with methanol. A yield of 1.77 g of pure lipstatin was obtained.

Biological Activities

Experimental

For the determination of lipase activity, the hydrolysis of fatty acids from trioleate was followed at pH 8 for 10 minutes at room temperature, using a recording pH-stat. The substrate emulsion was prepared by ultrasonication of trioleate (30 mg/ml) in a solution containing taurodeoxycholate 1 mM, taurocholate 9 mM, cholesterol 0.1 mM, lecithin 1 mM, bovine serum albumin 15 mg/ml, Tris-HCl 2 mM, NaCl 100 mM and $CaCl_2$ 1 mM. This composition of the test emulsion was chosen to mimic as closely as possible the *in vivo* conditions. After addition of the test compound, or vehicle alone, the pH was set to 8.0 and the reaction was started by the addition of either porcine pancreatic lipase (Sigma, type VI-S) or intestinal fluid. The amount of lipolytic activity was adjusted to result in the generation of 0.2 to 0.3 μmol fatty acid per ml and per minute.

The activity of other hydrolases was measured according to standard methods[8]. In all enzymatic measurements 2.5% bovine serum albumin was included in the assay systems.

Absorption of dietary fat was measured in female Füllinsdorf albino mice. After fasting overnight they received a liquid test meal (0.25 ml/animal) by oral intubation, immediately followed by lipstatin in a solution of gum arabic 5% and defatted milk powder 5% (0.5 ml/animal, n=6). Controls (n=12) were given the carrier without lipstatin. Thereafter food was given *ad libitum*.

The test meal contained glyceroltri[9,10(n)-^{3}H]oleate 10%, [1-^{14}C]oleic acid 10%, glucose 28%,

starch 32.8% and defatted milk powder 14% in saline. Feces from each mouse were collected separately for 3 days, this suffices for a complete intestinal passage. [^{3}H]- and [^{14}C]-Radioactivities were separated by combustion of the dry feces in a Packard sample oxidizer and measured by scintillation counting. Percent absorption of [^{3}H]triolein and of [^{14}C]oleic acid was calculated as the difference between orally administered (100%) and fecally recovered radioactivities.

Biological Activity of Lipstatin and its Derivatives

Lipstatin inhibited the hydrolysis of trioleate by porcine pancreatic lipase dose-dependently with an IC_{50} of 0.14 μM (Fig. 3). Inhibition increased with prolonged preincubation time and was not reduced upon subsequent dilution. After preincubating lipstatin for 20 minutes with a 20-fold excess of pancreatic lipase, only 15% of the inhibitory activity could be extracted with chloroform - methanol (2:1), whereas 90% of the inhibitory activity were recovered after preincubation with bovine serum albumin. Thus an irreversible type of inhibition is very likely.

The β-lactone moiety of the molecule is essential for the activity of lipstatin and its derivatives. Opening of the β-lactone results in almost complete loss of the lipase inhibitory activity (Table 1).

Other pancreatic enzymes such as phospholipase A2, amylase, trypsin, chymotrypsin and liver esterase were not inhibited by lipstatin at concentrations up to 200 μM.

Fig. 3. Inhibition of pancreatic lipase by lipstatin.

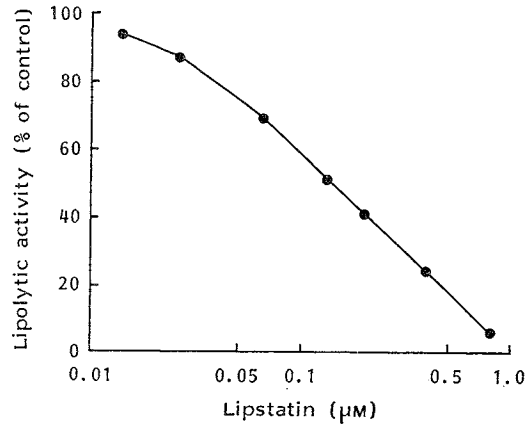
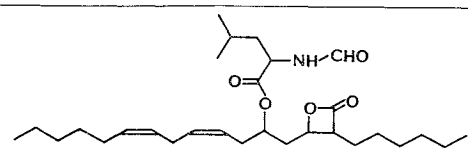
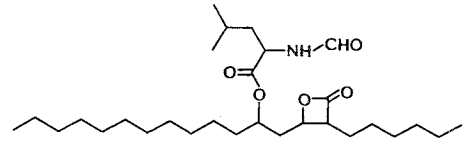
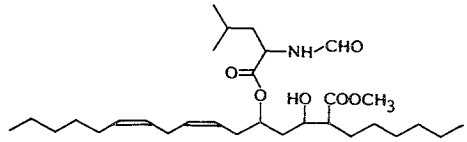
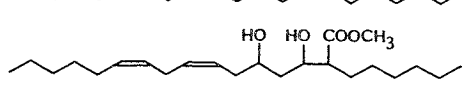
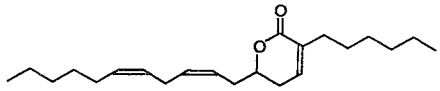


Table 1. Inhibition of pancreatic lipase *in vitro* by lipstatin and its derivatives.

Name	Structure[7]	IC_{50} (μM)
Lipstatin		0.14
Tetrahydrolipstatin		0.36
		>2,000
		630
		>3,000

The absorption of dietary triolein in mice was dose-dependently inhibited by lipstatin (Table 2). In contrast, the absorption of oleic acid, which was given simultaneously, was not impaired. Since triglycerides are absorbed only after they have been cleaved by pancreatic lipase, these data show that the hydrolytic step of lipid digestion was specifically inhibited by lipstatin. In a similar experiment, the bile acid sequestrant cholestyramine, which interferes with a post-hydrolytic step of fat absorption, inhibited triolein and oleic acid absorption to a similar degree. Inhibition of pancreatic lipase by lipstatin was demonstrated in a separate *in vivo* experiment. Lipase activity measured *ex vivo* in the intestinal fluid of mice 2 hours after an oral dose of 50 mg/kg was inhibited by 80% as compared to controls.

Table 2. Inhibition of dietary fat absorption by lipstatin in mice.

Lipstatin (mg/kg)	Lipid absorption (%, mean±S.E.M.)	
	[^{3}H]Triolein	[^{14}C]Oleic acid
0	97.2±0.4	94.8±0.4
20	31.2±1.4	90.4±0.8
60	21.1±3.8	91.2±0.5

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