PLIPASTATINS: NEW INHIBITORS OF PHOSPHOLIPASE A_2, PRODUCED BY *BACILLUS CEREUS* BMG302-fF67

I. TAXONOMY, PRODUCTION, ISOLATION AND PRELIMINARY CHARACTERIZATION

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Plipastatins have been isolated as part of a program designed to find inhibitors of porcine pancreatic phospholipase A_2. They were purified from fermentation broth of *Bacillus cereus* BMG302-fF67 and finally separated into four fractions by reverse phase HPLC. The respective fractions were designated as plipastatins A1, A2, B1 and B2. Plipastatins also inhibited phospholipases C and D.

Phospholipase A_2 [EC 3.1.1.4] (PL-A_2) catalyses the hydrolysis of the fatty acid ester bonds at the 2-position of phospholipids liberating the corresponding free fatty acid and lysophosphatide[1]. This enzyme has recently received much attention because arachidonic acid released by the reaction is the rate-limiting precursor of inflammatory mediators such as prostaglandins and leukotrienes[2]. It is also reported to play an important role in the pathogenesis of acute pancreatitis[3].

As reported in this paper, PL-A_2, depending on sources, differ in specificity to inhibitors. Thus, we searched for new PL-A_2 inhibitors in culture filtrates of microorganisms. In this communication we report the taxonomy, production, isolation, and characterization of the inhibitors, plipastatins A1, A2, B1 and B2.

Materials and Methods

Chemicals

Chemicals employed were as follows: Silica gel 60, Silanised silica gel 60 and TLC-plate Silica gel F_{254} (0.25 mm thickness) from E. Merck, Darmstadt, FRG; packed column of Nucleosil $5C_{18}$ from Senshu Scientific Co., Tokyo, Japan; dipalmitoyl phosphatidylcholine (DPC) from Sigma Chemical Co., St. Louis, U.S.A.; [*dipalmitoyl*-1-^{14}C]DPC, [*choline-methyl*-^{14}C]DPC and a scintillator Aquasol-2 from New England Nuclear, Boston, U.S.A. All other chemicals were of analytical grade.

Enzymes

Phospholipases A_2 (hog pancreas, 700 U/mg; bee venom, 2,400 U/mg; *Crotalus durissus*, 200 U/mg) and phospholipase D (cabbage, 0.3 U/mg) were purchased from Boehringer Mannheim, Mannheim, FRG. Phospholipase C (*Clostridium perfringens*, $1 \sim 2$ U/mg) was from Calbiochem-Behring Co., La Jolla, U.S.A.

Microorganism

Strain BMG302-fF67 was isolated from a soil sample collected in the premises of Institute of Microbial Chemistry, Shinagawa-ku, Tokyo and has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan under the accession number FERM-P7843.

Taxonomic Characterization

The strain was cultured at 30°C for 7 days on media which were commonly used for taxonomic studies of bacteria. The identification was made from descriptions in BERGEY's Manual of Determinative Bacteriology 8th Ed.[4], Manual for Identification of Medical Bacteria 2nd Ed.[5] and Taxonomy and Identification of Microorganisms[6].

Production of Plipastatins

Spores of the strain BMG302-fF67 were inoculated into 110 ml of a production medium consisted of glycerol 2.5%, meat extract 0.5%, Polypeptone 0.5%, yeast extract 1.0%, NaCl 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.05% and $CaCO_3$ 0.32% (pH 7.4) in a 500-ml Erlenmeyer flask, and cultured at 27°C for one day on a rotary shaker (180 rpm). One milliliter of the above seed culture was transferred to 120 ml of the same medium in a 500-ml Erlenmeyer flask and cultured for approximately 20 hours under the same conditions.

Isolation of Plipastatins

The inhibitors produced in the culture filtrate were adsorbed on an Amberlite XAD-7 column (10% filtrate), after washing the column with H_2O ($\times 5$ column), the inhibitors were eluted with 80% MeOH ($\times 3$ column). The active eluate was concd under reduced pressure to dryness and the residue was extracted with MeOH. The extract was concd to give a brown crude powder. A solution of this crude powder in propanol ($\times 4$ v/w powder) was passed through a propanol-filled column of silica gel ($\times 20$ w/w powder). This was followed by successive elution with propanol ($\times 1$ column), 90% propanol ($\times 2$ column) and 80% propanol ($\times 2.5$ column) to collect the fractions containing the active substances, which were then concd to dryness. A brown powder containing plipastatins B1 and B2 (fraction B) was obtained from the 90% propanol eluate, and a second brown powdered fraction containing plipastatins A1 and A2 (fraction A) was obtained from the 80% propanol eluate.

The powder of fraction A was chromatographed on a column of Silanised silica gel ($\times 100$ w/w powder) with a 6 : 4 mixture of MeOH and acetate buffer (1% potassium acetate plus 3% acetic acid, pH 5), and on a Sephadex LH-20 column (2.5×120 cm) with 80% MeOH, successively. The active eluate was concd under reduced pressure to give a pale yellow powder (plipastatin A group). This preparation appeared a single spot on silica gel TLC plate as shown in Table 3. The preparation was further separated into two fractions by a reverse phase HPLC using a Nucleosil $5C_{18}$ column (2×30 cm, flow rate 8 ml/minute, detection at 254 nm, using Waters ALC/GPC 200 system) with a 1:1 mixture of MeCN and acetate buffer (2% potassium acetate plus 6% acetic acid, pH 4). The respective fractions designated as plipastatins A1 and A2 in the order eluted were concd to dryness. The resulting residues were passed through Sephadex LH-20 columns with 80% MeOH to remove potassium acetate, thereby giving plipastatins A1 and A2 as colorless amorphous powders of their potassium salts.

The powder of fraction B obtained from the 90% propanol eluate by the silica gel column chromatography as described above was rechromatographed on a column of Silanised silica gel ($\times 100$ w/w powder) with a stepwise elution using 40% MeOH ($\times 7$ column) and 60% MeOH ($\times 7$ column), and a Sephadex LH-20 column (2.5×120 cm) with 80% MeOH, successively. The active eluate was concd to give a pale yellow powder (plipastatin B group). This preparation also showed a single spot by silica gel TLC (Table 3), but it was likewise separated into two fractions by a reverse phase HPLC (Nucleosil $5C_{18}$, a 65: 35 mixture of the same ingredients presented above). The respective fractions designated as plipastatins B1 and B2 in the order eluted were then concd and applied on Sephadex LH-20 columns in the same manner outlined above to give plipastatins B1 and B2 as colorless amorphous powders of their potassium salts.

Assay for Phospholipase (PL)[7] and Inhibitory Activity

The reaction mixture (total 0.5 ml) for PL-A_2 assay consisted of 50 mM Tris-HCl (pH 7.0), 1 mM $CaCl_2$, 0.2 mM DPC which was sonicated at 70~80°C for 5 minutes with [*dipalmitoyl*-1-^{14}C]DPC (5×10^4 dpm), PL-A_2 (0.01~0.04 μg-protein) and an inhibitor. The enzyme reaction was started by the addition of the enzyme, following incubation at 37°C for 20 minutes, and stopped by the addition of 1.5 ml of $CHCl_3$ - MeOH (2: 1) and 1.0 ml of 50% MeOH containing 0.1 M potassium chloride and

Table 1. Physiological characteristics of strain BMG302-fF67.

	Strain BMG302-fF67
Attitude toward oxygen	Facultative anaerobic
OF-test (by HUGE-LEIFSON's method)	Fermentative
Growth temperature (nutrient agar)	10~37°C (optimum, 27~37°C)
pH for growth (nutrient agar)	5~9 (optimum, 6~8)
Citrate utilization (KOSER's synthetic medium and CHRISTENSEN's nutrient medium)	Positive
Inorganic nitrogen sources	Positive (KNO_3 and $(NH_4)_2SO_4$)
Starch hydrolysis	Positive
Liquefaction of gelatin	Positive
Peptonization of BCP milk	Positive without coagulation
Nitrate reduction to nitrite	Positive
MR-test	Negative
Acetylmethylcarbinol formation	Positive
Indole production	Negative
H_2S formation (ISI agar)	Negative
Production of dye (Kings A and B medium)	Weak production of yellowish pigment
Urease activity	Negative
Oxidase activity	Negative
Catalase activity	Positive
Growth in 7% NaCl (nutrient broth)	Positive

The cultivation was performed at 30°C for 7 days unless otherwise specified.

0.1 M sodium carbonate (pH 11). After mixing for 10 seconds with a Vortex-Genie mixer and centrifugation at 3,000 rpm for 5 minutes, the radioactivity of upper phase (1.5 ml) was counted with a scintillator (Aquasol-2, 5 ml).

The reaction mixture (total 0.5 ml) for PL-C assay consisted of 50 mM Tris-HCl (pH 7.0), 1 mM $CaCl_2$, 0.2 mM DPC sonicated with [*choline-methyl*-^{14}C]DPC (4.4×10^4 dpm), PL-C (0.2~0.5 μg-protein) and an inhibitor. For PL-D assay, 50 mM Tris-HCl (pH 5.6), 1 mM $CaCl_2$, 0.2 mM DPC sonicated with [*coline-methyl*-^{14}C]DPC (4.4×10^4 dpm) and PL-D (10~20 μg-protein) were used. After incubation at 37°C for 20 minutes, the enzyme reaction was stopped by the addition of 1.5 ml of $CHCl_3$ - MeOH - conc HCl (2: 1: 0.02) and 1.0 ml of 50% MeOH containing with 0.1 M potassium chloride. The radioactivity of upper phase (1.5 ml) was counted by the same procedure mentioned above.

The percent inhibition was calculated by the formula $(A-B)/A\times100$, where A is dpm of liberated radioactive product by the enzyme in the system without an inhibitor and B is that with an inhibitor. IC_{50} value shows the concentration of inhibitor at 50% inhibition of enzyme activity.

Physico-chemical Properties

Melting points were taken using a Yanaco MP-S3 apparatus and are uncorrected. UV spectra were recorded on a Beckman DU-8 spectrophotometer, and IR spectra on a Hitachi 260-10 spectrophotometer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Mass spectra were obtained on a Hitachi M-80H mass spectrometer.

Hydrolysis of Plipastatin and Analysis of Amino Acids

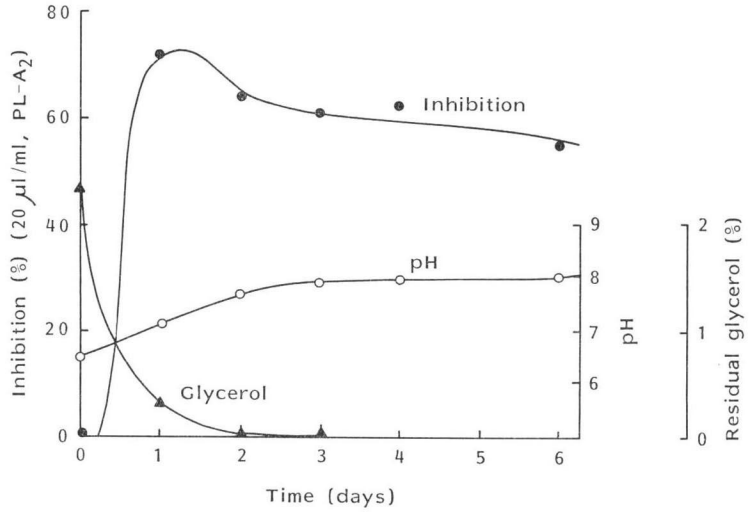
Each plipastatin was hydrolyzed at 105°C for 24 hours with constant boiling hydrochloric acid in a sealed tube. The hydrolysate was obtained by evaporation with H_2O to remove hydrochloric acid and analyzed on a Hitachi 835 automatic amino-acid analyzer.

Table 2. Comparison of characteristics of strain BMG302-fF67 with *Bacillus cereus* and *B. coagulans*.

Category	BMG302-fF67	*B. cereus*	*B. coagulans*
Form of spore	Ellipsoidal	Ellipsoidal or cylindrical	Ellipsoidal or cylindrical
Swelling of cell at spore formation	−	−	+ or −
Position of spore	Center	Center	Center or end or near end
Acid formation from glucose	+ or −	+	+
Gas formation from glucose	−	−	−
Acetoin formation from glucose	+	+	+ or −
Cell size (μm)	1.0×2.6	1.0~1.2×3~5	0.6~1×2.5~5
Motility	+	d	+
Growth temperature (°C)	10~37	(10~20)~(35~45)	(12~25)~(55~60)
Acid formation from L-arabinose, D-xylose and D-mannitol	−	−	d
Anaerobic growth	+	+	+
Reduction of nitrate	+	+	d
Gram-stain	+	+	+
Catalase	+	+	+
Hydrolysis of gelatin	+	+	−
Growth on the medium containing 7% NaCl	+	+	−

+; Positive in 90 to 100% of samples, −; negative in 90 to 100% of samples, d; positive in 11 to 89% of samples.

Fig. 1. Time course of plipastatins production by *Bacillus cereus* BMG302-fF67.



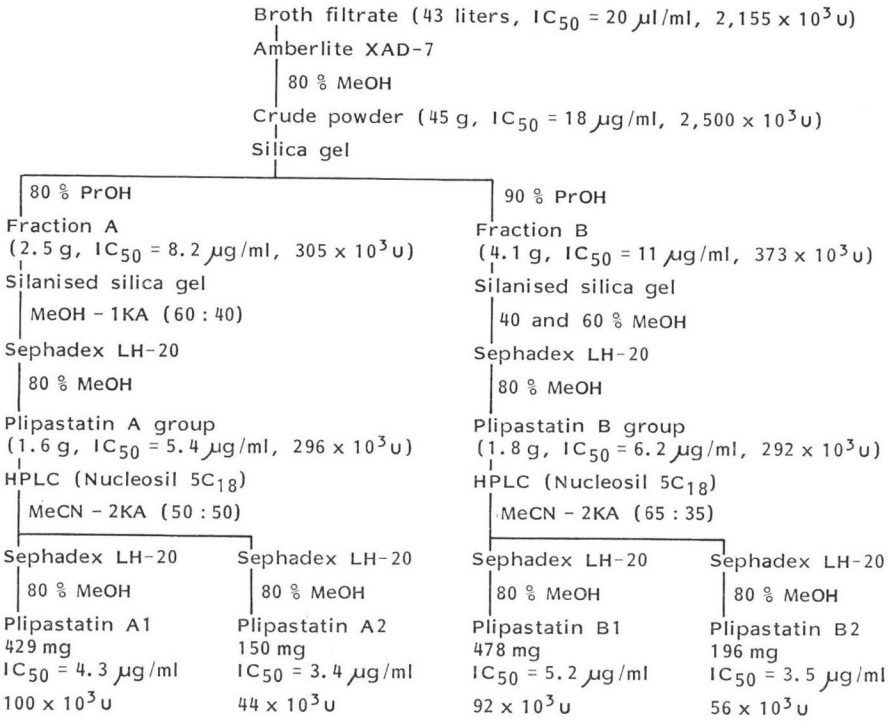
Results and Discussion

Taxonomic Characterization of Producing Strain

The taxonomic characteristics of the strain BMG302-fF67, from which the strain was identified as *Bacillus cereus*, are described below.

Morphology: Vegetative cells (30°C, 2~3 days) on nutrient agar were Gram-positive rods, approximately 1.0 by 2.6 μm with round ends. They existed singly or in mass and were motile with peritrichous flagella. Irregular forms were not observed. Spores (30°C, 3~4 days) on nutrient agar

Fig. 2. Extraction and purification of plipastatins.



1KA; 1% KOAc+3% AcOH, 2KA; 2% KOAc+6% AcOH.

were mostly 0.6 by 2.0 μm, easily stainable, elliptical at central position and heat resistant. Swelling of cells was not observed. Non-acid-fast.

Culture Characteristics: Nutrient agar colonies (30°C, 1~7 days) were dull, opaque, substantially circular and had a irregular, root-like periphery. Wrinkles appeared on the surface of the colonies with age. No diffusible pigment was observed. On nutrient agar slant (30°C, 1~7 days), the growth was moderate, filiform, opaque, dull, pale yellowish brown; the surface was smooth (1 day), changing to dull with wrinkles after 3 days. In nutrient broth (30°C, 1~7 days), the growth was moderate, and pellicles were observed on the surface of the tube (1 day). Some sediment was present (5 days).

Physiological characteristics of the producing strain BMG302-fF67 are listed in Table 1. In the acid formation test, the acid formation was negligible from D-glucose, D-fructose and sucrose, and no gas evolved. No acid or gas was formed from L-arabinose, D-xylose, D-mannose, D-galactose, maltose, lactose, trehalose, D-sorbitol, inositol, D-mannitol, glycerol or starch.

The above characters indicated that this strain should belong to *Bacillus cereus* or *Bacillus coagulans*. The comparison of the characteristics of the strain BMG302-fF67 with those of *B. cereus*[4] and *B. coagulans*[4] is shown in Table 2. As shown in the table, the strain BMG302-fF67 did not grow at 50°C. It is more closed to *B. cereus* than *B. coagulans* in its positive hydrolysis of gelatin and growth on the medium containing 7% NaCl. Therefore, we concluded that the strain BMG302-fF67 should be classified as a strain of *B. cereus*.

Production and Isolation of Plipastatins

The strain of *B. cereus* BMG302-fF67 was cultured in Erlenmeyer flasks at 27°C for 6 days on a

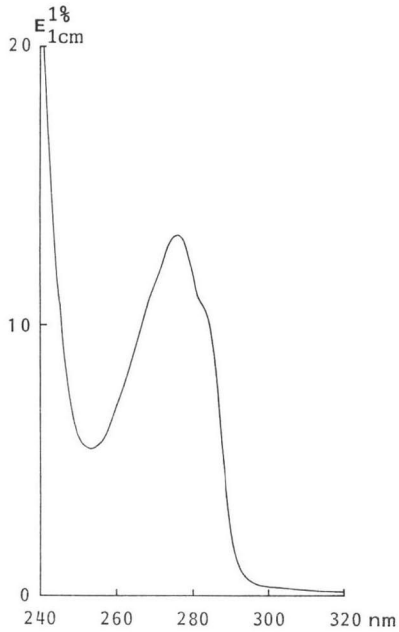
Table 3. Physico-chemical properties of plipastatins potassium salts.

	A1	A2	B1	B2
MP (°C)	193~195	188~190	186~187	184~186
MW	1,462	1,476	1,490	1,504
SI-MS m/z (M^++1)	1,463	1,477	1,491	1,505
Molecular formula	$C_{72}H_{110}N_{12}O_{20}$	$C_{73}H_{112}N_{12}O_{20}$	$C_{74}H_{114}N_{12}O_{20}$	$C_{75}H_{116}N_{12}O_{20}$
Elemental analysis C	54.44	54.67	58.07	58.50
H	7.46	7.86	7.54	7.84
N	9.78	10.84	11.07	10.63
K	6.86	6.64	3.24	3.13
UV λ_{max}^{MeOH} (ε)	276 (1,923)	276 (1,781)	276 (1,707)	276 (1,820)
$[\alpha]_D^{20}$ (c 1, MeOH)	+48.7	+51.2	+49.2	+46.2
Rf* value (a)	0.24	0.24	0.33	0.33
(b)	0.21	0.21	0.32	0.32

Rf*: Silica gel TLC (Kieselgel 60F 0.25 mm, Merck) was used with developing solvent (a) BuOH - AcOH - H_2O (4: 1: 1) or (b) 80% PrOH.

rotary shaker. The time course of the production is shown in Fig. 1. The maximum peak of plipastatin production in the flasks was obtained at one day, thereafter the production slowly decreased with a pH change to alkaline. From the culture filtrate (43 liters) plipastatins were isolated as shown in Fig. 2. The total yield of plipastatins potassium salts was 1.253 g. The purity of each preparation was examined by TLC and HPLC.

Fig. 3. UV spectrum of plipastatin A1 (in MeOH).



Physico-chemical Properties

The physico-chemical properties of plipastatins potassium salts are summarized in Table 3. The molecular weight and formula of plipastatins were determined by secondary ion mass spectrometry (SI-MS) and the calculation from the contents of the fatty acid and amino acid residues. The UV spectra of all plipastatins were similar and showed an absorption maximum at 276 nm. A UV spectrum of plipastatin A1 in Fig. 3 represents that of all the plipastatins studied. The IR spectra of plipastatins also were similar and indicated the presence of peptide bonds (1660 and 1520 cm^{-1}) and an ester or lactone linkage (1760 cm^{-1}) as shown in Fig. 4. Plipastatins are soluble in water, methanol, ethanol, propanol, butanol and dimethyl sulfoxide, but insoluble in acetone, ethyl acetate, chloroform and benzene. The spots on silica gel TLC plates were visible by ninhydrin, Rydon-Smith reagent or sulfuric acid.

Each plipastatin was hydrolyzed with hydrochloric acid and the hydrolysate analyzed with an amino-acid analyzer. The results of amino acid analyses of plipastatins indicated two kinds of peptide parts: A plipastatin A group (plipastatins A1 and A2) comprised of Ala (1), Thr (1), Glu (3), Pro (1),

Fig. 4. IR spectrum of plipastatin A1 (KBr).

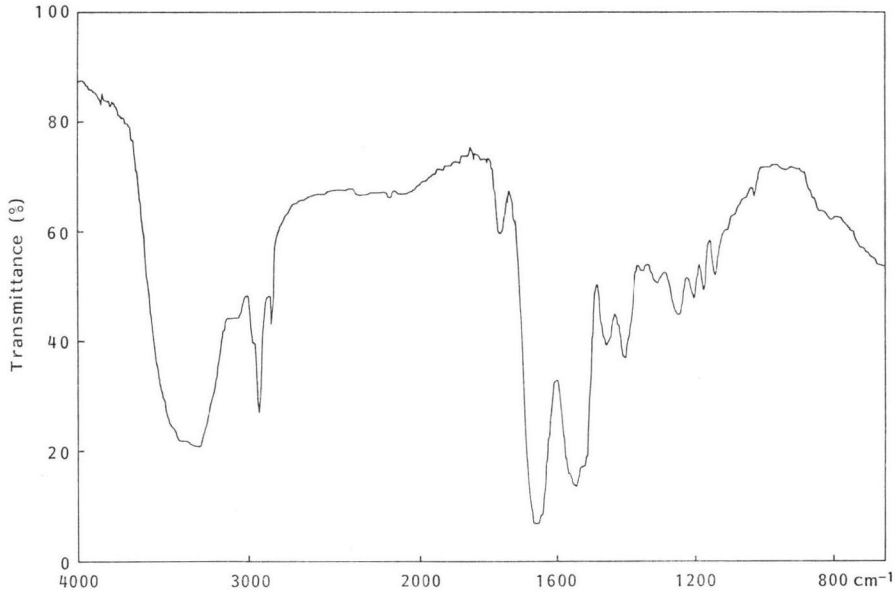


Table 4. Inhibitory effects of plipastatins on phospholipases.

Plipastatin	MW	IC_{50} ($\times 10^{-6}$ M)		
		PL-A_2	PL-C	PL-D
A1	1,462	2.9	1.3	1.4
A2	1,476	2.3	2.7	2.6
B1	1,490	3.5	2.4	1.9
B2	1,504	2.3	3.0	2.2

Ile (1), Tyr (2) and Orn (1) residues; and a plipastatin B group (plipastatins B1 and B2) comprised of the same amino acid residues except Val (1) instead of Ala. One mol of ammonia from each peptide suggests that each contains one mol of glutamine residue. The analysis of the ethereal extracts obtained from the methanolysis (30% HCl - MeOH 50°C 48 hours) product indicated the presence of two different fatty acids. Thus, plipastatins were suggested to be acylpeptides. The structural study will be published in the next reports.

By comparison with the amino acid composition of known acylpeptide antibiotics[8~10] or inhibitors[11], it can be concluded that plipastatins are a new acylpeptide family.

Biological Properties

The inhibitory activities of plipastatins on phospholipases are shown in Table 4. All plipastatins inhibited not only phospholipase A_2 but also phospholipases C and D at the range of 1.3 to 3.5×10^{-6} M. No significant inhibition was observed with bee and snake venom PL-A_2 preparations.

Plipastatins at 100 μg per ml had no antimicrobial activity except against *Corynebacterium bovis* 1810 (MIC: 6.25 μg/ml).

Intraperitoneal injection of 0.1 mg per mouse of plipastatin A1 or B1 slightly suppressed delayed-type hypersensitivity; T/C was 49% and 58%, respectively. *In vitro*, plipastatin A1 at 100 μg per ml

suppressed the ConA-stimulated T-lymphocyte blastogenesis and the LPS-stimulated B-lymphocyte blastogenesis; T/C was 52% and 15%, respectively. The acute toxicity (LD_{50} values) of plipastatin A1 in mice is as follows: 250~500 mg/kg (ip) and 200~400 mg/kg (iv).

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