ISOLATION AND CHARACTERIZATION OF NEW THIOL PROTEASE INHIBITORS ESTATINS A AND B

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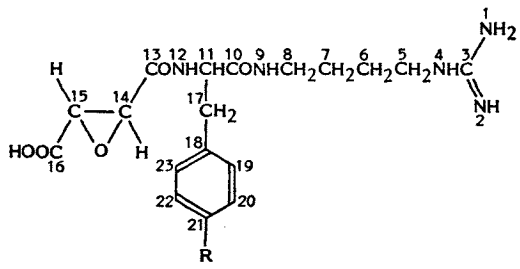
New thiol protease inhibitors, estatins A and B, were isolated from the culture filtrate of *Myceliophthora thermophila* M4323. The basic, water-soluble inhibitors were characterized as having an agmatine, *trans*-epoxysuccinic acid and L-phenylalanine or L-tyrosine moieties in the structure. The molecular formulas $C_{18}H_{25}N_5O_5$ and $C_{18}H_{25}N_5O_6$ for A and B were indicated by elemental analysis and fast atom bombardment MS. Estatins were specific inhibitors against thiol proteases such as papain, ficin and bromelain. They suppressed IgE antibody production in mice, but not IgG.

Low molecular weight protease inhibitors are useful at least as medicines or reagents for research. During our screening for thiol protease-specific inhibitors, a fungal culture produced inhibitors against papain. The inhibitors were isolated and named estatins A and B (Fig. 1).

The producing organism was isolated from a soil sample collected at Iriomote island, Okinawa Prefecture, Japan, and designated to M4323 (FERM P-8134 and FERM BP-979). It was classified as *Myceliophthora thermophila* from cultural characteristic or microscopic observation.[1,2]

In this paper, we report the isolation, physico-chemical and biological properties of estatins.

Fig. 1. Structures of estatins A and B.



Estatin A R=H
Estatin B R=OH

Materials and Methods

Materials

The following materials were purchased from the sources indicated: Papain, bromelain, ficin, β-nicotinamide adenine dinucleotide reduced form (NADH), pyruvate and L-lactate dehydrogenase, Boehringer-Mannheim; α-chymotrypsin and pepsin, P-L Biochemicals, Inc.; trypsin, Wako Pure Chemical Industries, Ltd.; pronase E, Nakarai Chem. Ltd.; milk casein (acc. to Hammerstem), Merck Co., N.B. Corp.; ovalbumin and evans blue, Sigma Chemical Company; agmatine, Tokyo Kasei Co.; *trans*-epoxysuccinic acid, Kokusan Chemical Works, Ltd.

Assays for Enzyme Inhibitory Activities

Inhibitory activities against papain, ficin and bromelain were assayed as follows: The assay mixture (total volume, 3.5 ml) consisted of casein 1.3%, cysteine 2.29 mM, EDTA 1.14 mM, phosphate buffer 50 mM (pH 6.8), and 60 μg of ficin or 100 μg of bromelain with or without inhibitors. The reaction was started by the addition of casein solution and then the assay mixture was incubated for 30 minutes at 40°C. The reaction was terminated by the addition of 2.0 ml of 1.1 M TCA solution.

After 1 hour at room temperature, the extinction of the supernatant of the centrifuged reaction mixture (3,000 rpm, 10 minutes) was read at 280 nm. Inhibitory activities against trypsin, α-chymotrypsin and pepsin were determined in the same manner except that the cysteine and EDTA were omitted from the reaction mixture. The assay mixtures (total volume, 3.5 ml) were as follows: Casein 1.3%, borate buffer 100 mM (pH 7.5) and enzyme for trypsin 1 mg; casein 1.3%, borate buffer 100 mM (pH 7.5) and enzyme for α-chymotrypsin 200 μg; casein 1.3%, HCl-KCl buffer 20 mM (pH 2.0) and enzyme for pepsin 100 μg, with or without inhibitors. The percent inhibition was calculated as follows: % inhibition=100 (A−B)/A; in this, A stands for the absorbance without inhibitor and B for the absorbance with inhibitor.

Assay of L-lactate dehydrogenase was based on the method of NEILANDS[3] by using of pyruvate as a substrate. The assay mixture (total volume, 3.0 ml) consisted of NADH 20 mM, pyruvate 0.5 mM, phosphate buffer 100 mM (pH 6.8) and L-lactate dehydrogenase (1 mU) with or without inhibitors. The reaction was started by the addition of 100 μl of pyruvate to the quartz cuvette and was followed by the determination of loss of absorbance at 340 nm for 4 minutes at 37°C with the Hitachi Model 220A spectrophotometer.

Hydrolysis of Estatins A and B by Pronase E and Detection of the Agmatine, *trans*-Epoxysuccinic Acid and L-Phenylalanine or L-Tyrosine

A water solution (4 ml) containing estatin A (10 mg) or estatin B (10 mg) and pronase E (20 mg) was incubated at 30°C for 2 days and followed by the gel filtration on Sephadex G-25 column in order to eliminate the used enzyme. The low molecular fractions were combined and concentrated to 0.5 ml. These hydrolysate containing agmatine, *trans*-epoxysuccinic acid and L-phenylalanine or L-tyrosine were analyzed using a Shimadzu Model LC-6A HPLC instrument. The separations were performed on a Ultron PS 80 H column (8 mm i.d.×30 cm) with UV detection at 210 nm for *trans*-epoxysuccinic acid, TSK gel SP-2 SW (4.6 mm i.d.×25 cm) with UV detection at 200 nm for agmatine and TSK gel Enanio L 1 (4.6 mm i.d.×25 cm) with UV detection at 254 nm for L-phenylalanine or L-tyrosine. The mobile phases were as follows: pH 2.0 aqueous perchloric acid at a flow rate of 1 ml/minute for *trans*-epoxysuccinic acid; 10% acetonitrile containing sodium phosphate 0.15 M (pH 2.4) at a flow rate of 1 ml/minute for agmatine and cupric sulfate 1 mM at a flow rate of 1 ml/minute for L-phenylalanine or L-tyrosine.

Measurement of the IgE and IgG Antibody Production in Mice

Activity on the production of IgE antibody was examined by the following method. Estatin A or B dissolved in physiological saline were administered ip to BDF_1 strain male mice weighing about 20 g (one group 5 mice) at a dose of 100 mg/kg. Control group (7 mice) received only physiological saline. After 2 hours from the administration, 4 mg of aluminum hydroxide together with 10 μg of ovalbumin was administered ip to immunize them. After 14 days from the immunization, blood was collected and the homocytotropic antibody titer of this serum (mouse anti-ovalbumin serum) was assayed by PCA reaction. The mouse anti-ovalbumin serum, diluted serially with saline, was given intradermally in a dose of 0.1 ml into 3 sites on the shaved backs of intact Wistar strain male rats weighing about 200 g. A similar dose of saline was injected into the other side. After 48 hours, the animals were given iv 1.0 ml of 1% Evans blue solution containing 2 mg of ovalbumin as an antigen. Thirty minutes later, the animals were exsanguinated and the skin of each reaction locus was removed. The amount of extravasated dye resulting from PCA was then estimated colorimetrically after extraction by the method of HARADA *et al.*[4] The last dilution giving a definite positive blueing reaction in the animals was expressed as the titer of the antiserum.

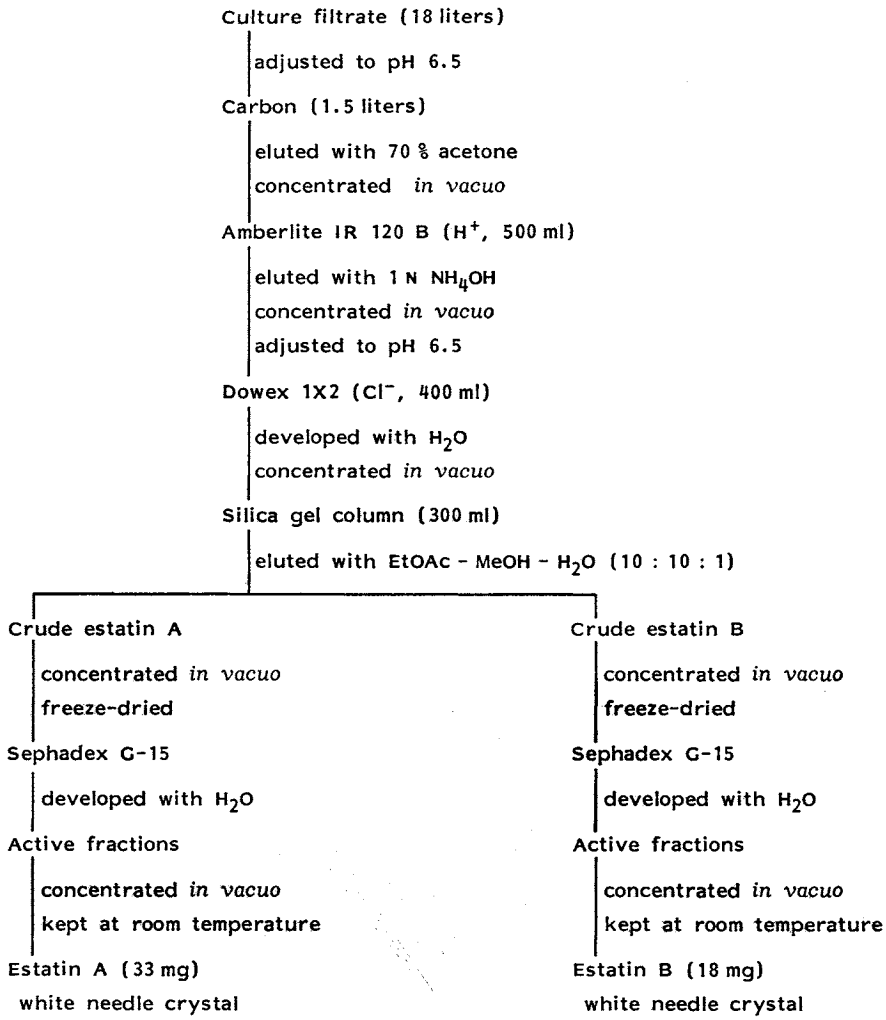
Activity on the production of IgG antibody was examined by the following method. Estatin A or B were administered ip to BDF_1 strain male mice weighing about 20 g (one group 5 mice) at dose of 100 mg/kg. Control group (7 mice) received only physiological saline. After 2 hours from the administration, each mouse was immunized by intradermal injection with 0.2 mg/0.1 ml of ovalbumin together with equal volume of Freund complete adjuvant into the 2 separate sites of the back of the mice. After 2 weeks, blood was collected and antibody titers were determined by passive hemagglutination test using glutaraldehyde as coupling reagent.

Results and Discussion

Fermentation

A 30-liter jar fermenter containing 20 liters of the fermentation medium was inoculated with 200 ml of the seed culture grown in 500-ml Erlenmeyer flasks containing 100 ml of the seed medium on a rotary shaker at 30°C for 3 days. The seed medium consisted of glucose 1.0%, dextrin 1.0%, yeast extract 0.5%, casein hydrolysate 0.5%, $CaCO_3$ 0.1% and Celite 1.0% (pH 6.5 before sterilization). The fermentation medium consisted of glucose 2.0%, peptone 1.0%, corn steep liquor 1.0%, KH_2PO_4 0.2% and $MgSO_4 \cdot 7H_2O$ 0.1% (pH 6.5 before sterilization). Fermentation was carried out for 4 days at 30°C with aeration (20 liters/minute) and agitation (200 rpm). Production of estatins began after the first 40 hours, by which time the glucose concentration was markedly decreased and just before the cessation of cell growth. Production continued throughout the stationary phase. Almost all the inhibitor was secreted into the medium. Maximum titer of estatins of about 10 μg/ml was obtained after 4 days in the production medium.

Fig. 2. Isolation and purification of estatins.



Isolation and Purification

The culture broth (20 liters) was filtered, and the filtrate was passed through a carbon column (1.5 liters). The estatins isolation procedure is shown in Fig. 2. These inhibitors were isolated by column chromatography using carbon, cation-exchange resins, anion-exchange resins and silica gel. Estatins were successively separated into two components by silica gel chromatography. In the final stage, each component was purified by Sephadex G-15 chromatography. Estatins A and B were isolated as white needle crystals. The total yield of estatins was 51 mg. The purity of each preparation was examined by TLC.

Physico-chemical Properties

Estatins behaved as weakly basic substances, and were stable at pH 2.0, 7.0 and 9.0 at 60°C for 30 minutes. They were soluble in water, acetic acid, dimethyl sulfoxide and pyridine, and insoluble in most organic solvents such as ethyl acetate, chloroform and benzene. They gave positive reactions to thiosulfate, Feigl and Sakaguchi reagents, though negative to TOLLEN's and ninhydrin reactions. The color reactions of estatins revealed that it is positive for Sakaguchi, Feigl and thiosulfate, indicating the existence of guanidyl, 1,2-dicarboxylic acid and epoxide groups in its structure.

The IR spectra of estatins suggested the existence of amide I, guanidyl, carboxyl (1650 cm^{-1}),

Fig. 3. IR spectra of estatins A and B (KBr pellet).

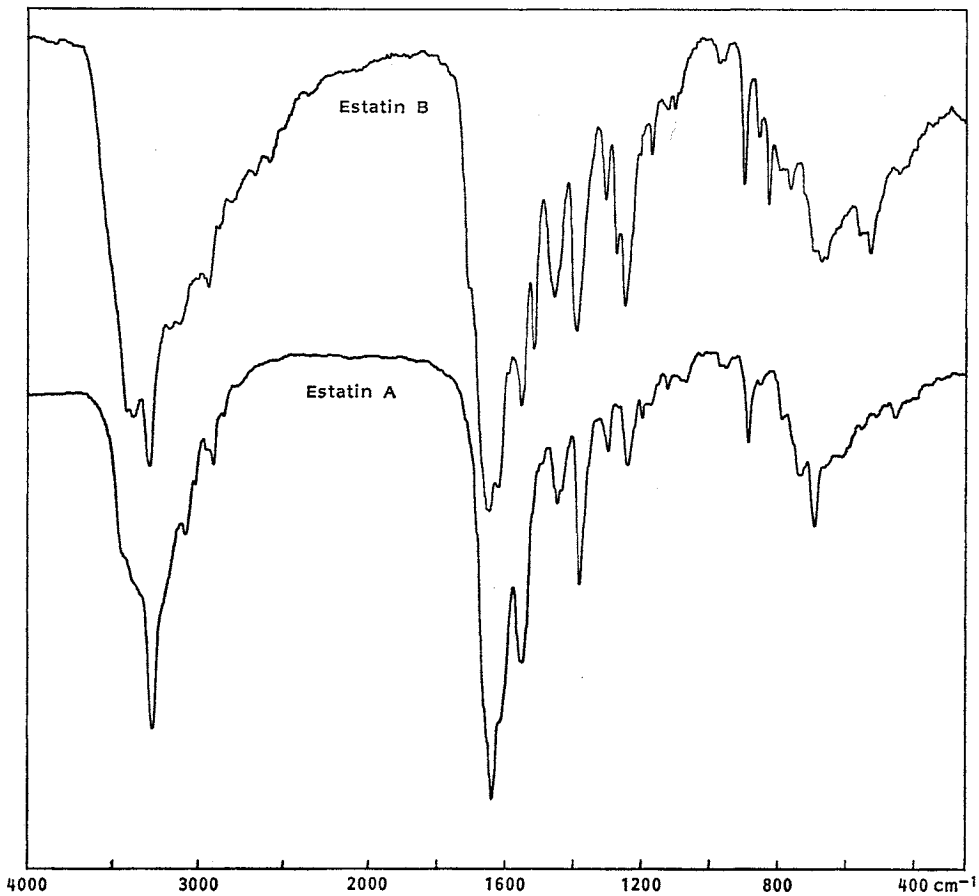


Table 1. Physico-chemical properties of estatins A and B.

	A	B
Analysis Calcd:	C 52.80, H 6.65, N 17.10	C 50.82, H 6.40, N 16.46
Found:	C 52.81, H 6.37, N 17.40	C 50.93, H 6.49, N 16.73
Formula	$C_{18}H_{25}N_5O_5 \cdot H_2O$	$C_{18}H_{25}N_5O_6 \cdot H_2O$
MW (FAB-MS)	391	407
MP (°C, dec)	223~225	217~218
$[\alpha]_D^{24}$	+41.8°±5° (*c* 0.6, H_2O)	+46.8°±5 (*c* 0.2, 0.1 N HCl)
UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$)	247(4.1), 252(4.6), 259(5.1), 264(4.1), 269(2.2)	222(274.1), 276(38.3), 283(31.8)
Hydrolysis:		
With 6 N HCl	Phenylalanine (1 mol)	Tyrosine (1 mol)
With pronase E	*trans*-Epoxysuccinic acid, agmatine, L-phenylalanine	*trans*-Epoxysuccinic acid, agmatine, L-tyrosine
Rf value[a]	0.33	0.22

[a] Plate: Silica gel f (Tokyo Kasei Co.), solvent: $CHCl_3$ - MeOH - 14% NH_4OH (20 : 30 : 3).

amide II (1550 cm^{-1}), epoxide (900 cm^{-1}), phenyl (750, 700 cm^{-1}) and amine groups (3300 cm^{-1}) (Fig. 3). Other physico-chemical properties are listed in Table 1. Elemental analysis and fast atom bombardment (FAB)-MS established the molecular formula of estatins A and B, $C_{18}H_{25}N_5O_5$ and $C_{18}H_{25}N_5O_6$, respectively.

When estatins A and B were hydrolyzed with 6 N HCl in the usual manner and the hydrolysate were analyzed by an amino acid analyzer, phenylalanine and tyrosine were detected, respectively. On treatment with pronase E, estatin A gave three components. Three components were identified as *trans*-epoxysuccinic acid, agmatine and L-phenylalanine by direct comparison in HPLC with those of authentic samples. From the data of the molecular formula and the digested products by pronase E of estatin A, the structure of estatin A revealed that each of 1 mol of *trans*-epoxysuccinic acid, agmatine and L-phenylalanine are connected with amide bond in its structure.

The ^1H NMR data (DMSO-d_6, 400 MHz) for estatins A and B are shown in Table 2. The ^1H NMR of estatin A showed the existence of four amine protons at 8.78 ppm as doublet, at 8.45 ppm as triplet, at 8.25 ppm as triplet and at 7.46 ppm as broad singlet, and one amino protons at 7.46 ppm as broad singlet, which disappears on addition of deuterium oxide. The aliphatic part of the spectrum showed the presence of methylene protons at 1.38 ppm as broad singlet and at 3.04 ppm as multiplet. In addition, this spectrum indicated the presence of aromatic protons at 7.23 ppm as multiplet.

The ^{13}C NMR spectrum (DMSO-d_6, 100 MHz) of estatin A showed eighteen carbon signals (Table 3). The distortionless enhancement by polalization transfer (DEPT) NMR spectrum assigned them to five methylenes, eight methines consisting of three aliphatic methines and five aromatic methines, and five quaternary carbon atoms consisting of three carbonyl carbons, one aromatic quaternary carbon and one guanidyl carbon.

The ^1H-^{13}C 2D correlated spectroscopy (COSY) of estatin A showed as follows: The C-6 (25.67 ppm), C-7 (26.04 ppm) carbons and the C-5 (37.83 ppm), C-8 (40.23 ppm) carbons showed the connectivity with resonance of methylene protons 6-H, 7-H at 1.38 ppm and 5-H, 8-H at 3.04 ppm, respectively. The C-17 carbon (37.45 ppm) showed the connectivity with resonance of a nonequivalent methylene protons 17-H_a at 2.82 ppm and 17-H_b at 2.98 ppm. The C-14 (52.14 ppm) and C-15 (54.35

Table 2. ^{1}H NMR data for estatins A and B.

A			B		
Position	Chemical shift	Coupling constant (J, Hz)	Position	Chemical shift	Coupling constant (J, Hz)
6-H, 7-H	1.38 (4H, br s)		6-H, 7-H	1.53 (4H, br s)	
17-H_a	2.82 (1H, dd)	9.3, 13.7	17-H_a	2.84 (1H, dd)	9.3, 13.7
15-H	2.93 (1H, d)	2.0	15-H	3.09 (1H, d)	2.0
17-H_b	2.98 (1H, dd)	5.4, 13.7	17-H_b	3.00 (1H, dd)	5.4, 13.7
5-H, 8-H	3.04 (4H, m)		5-H, 8-H	3.20 (4H, m)	
14-H	3.30 (1H, d)	2.0	14-H	3.44 (1H, d)	2.0
11-H	4.43 (1H, ddd)	5.4, 8.3, 9.3	11-H	4.48 (1H, ddd)	5.4, 8.3, 9.3
19-H, 20-H, 21-H, 22-H, 23-H	7.23 (5H, m)		20-H, 22-H	6.79 (2H, d)	8.3
			19-H, 23-H	7.15 (2H, d)	8.8
1-H, 2-H	7.46 (3H, br s)		1-H, 2-H	7.59 (3H, br s)	
9-H	8.25 (1H, t)	5.9	9-H	8.35 (1H, t)	5.9
4-H	8.45 (1H, t)		4-H	8.53 (1H, t)	
12-H	8.78 (1H, d)	8.3	12-H	8.82 (1H, t)	8.3
			21-H	9.44 (1H, s)	

^{1}H NMR spectrum recorded at 400 MHz in DMSO-d_6 at 27°C. Chemical shifts in ppm referenced to DMSO-d_6 as internal standard at 2.50 ppm.

Table 3. ^{13}C NMR data for estatins A and B.

A		B	
Position	Chemical shift (m)	Position	Chemical shift (m)
C-10	170.78 (s)	C-10	170.97 (s)
C-16	170.75 (s)	C-16	170.74 (s)
C-13	167.20 (s)	C-13	167.17 (s)
C-3	157.12 (s)	C-3	157.09 (s)
C-18	137.80 (s)	C-21	155.85 (s)
C-19	129.06 (d)	C-19	129.98 (d)
C-23	129.06 (d)	C-23	129.98 (d)
C-20	128.05 (d)	C-18	127.77 (s)
C-22	128.05 (d)	C-20	114.89 (d)
C-21	126.29 (d)	C-22	114.89 (d)
C-11	54.42 (d)	C-11	54.82 (d)
C-15	54.35 (d)	C-15	54.35 (d)
C-14	52.14 (d)	C-14	52.16 (d)
C-8	40.23 (t)	C-8	40.24 (t)
C-5	37.83 (t)	C-5	37.79 (t)
C-17	37.45 (t)	C-17	36.69 (t)
C-7	26.04 (t)	C-7	26.10 (t)
C-6	25.67 (t)	C-6	25.69 (t)

^{13}C NMR spectrum recorded at 100 MHz in DMSO-d_6 at 27°C. Chemical shifts in ppm referenced to DMSO-d_6 as internal standard at 39.50 ppm.
m: Multiplicity.

ppm) carbons were connected with 14-H at 3.30 ppm and 15-H at 2.93 ppm, respectively. The C-11 (54.42 ppm) carbon showed the connectivity with resonance of methine proton 11-H at 4.43 ppm. The C-19 and C-23 (129.06 ppm), C-20 and C-22 (128.05 ppm) and C-21 (126.29 ppm) carbons showed the connectivity with resonances of aromatic protons at 7.23 ppm.

In the ^{1}H-^{1}H COSY of estatin A, the methine proton 11-H at 4.43 ppm coupled with methylene

protons 17-H_a at 2.82 ppm, 17-H_b at 2.98 ppm and amide proton 12-H at 8.78 ppm. The amide proton 9-H at 8.25 ppm and amine proton 4-H at 8.45 ppm coupled with the methylene protons 5-H and 8-H at 3.04 ppm. The aliphatic methylene protons 6-H and 7-H at 1.38 ppm coupled with the methylene protons 5-H and 8-H at 3.04 ppm.

In the ^1H-^1H 2D nuclear Overhauser effect spectroscopy (NOESY) of estatin A, the amine proton 4-H at 8.45 ppm coupled with the amine proton 2-H and amino protons 1-H at 7.46 ppm. The amide protons 12-H at 8.78 ppm and 9-H at 8.25 ppm coupled with the methine protons 14-H at 3.30 ppm and 11-H at 4.43 ppm, respectively. The ^1H and ^{13}C NMR spectra of estatin B were closely similar to those of signals due to a phenolic hydroxy group in place of an aromatic proton in estatin A.

Based on the data presented, we propose the structures shown in Fig. 1 for estatins A and B, and all the signals were assigned as in Tables 2 and 3. The absolute structures of estatins A and B were established by the comparison with there optical isomer which were obtained synthetically.[5] The structures of estatins A and B were concluded to be *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-phenylalanyl]agmatine and *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-tyrosyl]agmatine, respectively. They were structurally similar to E-64[6] containing L-*trans*-epoxysuccinic acid and agmatine, but differ to each other in amino acid moieties.

Biological Properties

Estatin A showed strong inhibitory activities against papain, ficin and bromelain, but it showed no inhibition against α-chymotrypsin, trypsin and pepsin (Table 4). The anti-protease spectrum and the potency of estatin B were similar to that of estatin A. Estatins did not inhibit L-lactate dehydrogenase although it contains a functional thiol group. Accordingly, estatins were specific inhibitors of thiol proteases.

Various thiol protease inhibitor have been isolated from cultured broth of actinomycetes,[7,8] mold[9] and bacteria,[10] but thiol protease specific inhibitor are few.

HAMADA *et al.*[9] reported the inhibitor E-64 which can be considered as being the first thiol protease specific inhibitor isolated from *Aspergillus japonicus*. Now we have also obtained new thiol protease specific inhibitors.

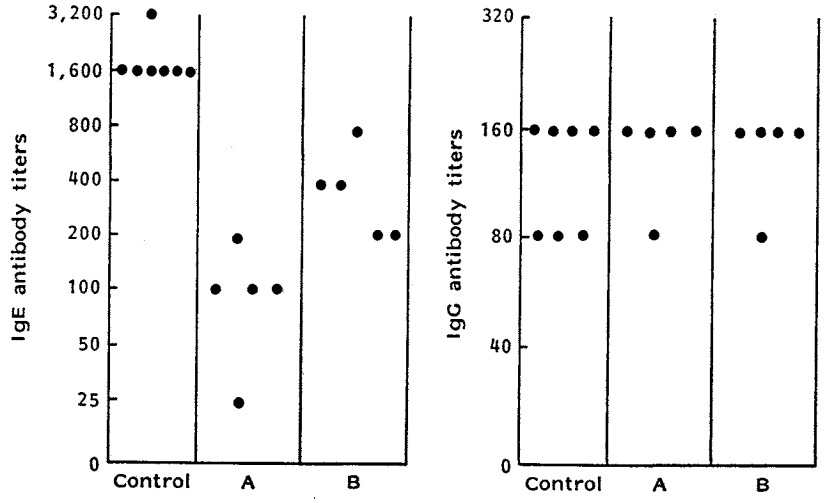
Estatins A and B showed no toxic effect when they were administered ip into mice at a dose of 400 mg/kg.

The effects of estatins on IgE and IgG antibodies production in mice are shown in Fig. 4. Estatins suppressed the production of IgE antibodies causing allergic deseases, but did not suppressed IgG antibodies production which pertains to normal immune reaction. Recently, Taisho Phar-

Table 4. Inhibitory effects of estatins on various enzymes.

Enzymes	Quantity of enzyme (μg)	IC_{50}(μg/ml)	
		Estatin A	Estatin B
Papain	60	0.026	0.027
Ficin	60	0.039	0.041
Bromelain	100	0.145	0.145
α-Chymotrypsin	200	>50	>50
Trypsin	1,000	>50	>50
Pepsin	100	>50	>50
L-Lactate dehydrogenase	0.01	>50	>50

Fig. 4. Effects of estatins A and B on IgE and IgG antibodies production in mice.



maceutical Co., Ltd. researchers[11] reported the same phenomenon with a thiol protease inhibitor E-64. It is interesting that thiol protease inhibitors such as estatins and E-64 show the property of suppressing IgE antibody production.

It is expected that thiol protease inhibitors such as estatins will provide a new aid in the clarification of the mechanisms of IgE antibody production and regulation.

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