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BELACTINS A AND B, NEW SERINE CARBOXYPEPTIDASE INHIBITORS PRODUCED BY ACTINOMYCETE. I. TAXONOMY, PRODUCTION, ISOLATION AND BIOLOGICAL ACTIVITIES

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Belactins A and B, new inhibitors of serine carboxypeptidase were discovered in the fermentation broth of *Saccharopolyspora* sp. MK19–42F6. They were purified by ethyl acetate extraction, silica gel chromatography, Sephadex LH20 chromatography, Capcellpak C18 SG120 reversed phase HPLC and centrifugal partition chromatography (CPC) following their inhibitory activity against carboxypeptidase Y (CP-Y). The inhibition constants (K_i) of belactins A and B against CP-Y are 0.14 and 0.27 μM respectively. Belactins A and B have highly specific inhibitory activities for CP-Y among various peptidases, have no antimicrobial activities at 100 μg/ml and have low toxicities.

KEY WORDS: Serine carboxypeptidase, carboxypeptidase Y, enzyme inhibitors, β-lactone, Actinomycete, natural compounds

INTRODUCTION

For many years, we have screened for inhibitors against various proteases or other enzymes and found various kinds of low molecular weight inhibitors,[1] which provide much information about the enzymatic kinetics or the physiological roles of enzymes and contribute greatly to the biological sciences. Among many kinds of enzymes we focus on carboxypeptidase Y (CP-Y, EC 3.4.16.1),[2] a serine carboxypeptidase from yeast.

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ABBREVIATIONS: Bz, benzoyl, CBZ, carbobenzoxy, βNA, β-naphthylamide; Z, carbobenzoxy; CPC, centrifugal partition chromatography.

CP-Y is well known among serine carboxypeptidases, which are widely distributed in higher organisms[3] and have different substrate specificities[2] from metallo carboxypeptidases, such as CP-A, CP-B[4] or CP-N.[5] Serine carboxypeptidases have an active site serine residues that forms a covalent adduct with diisopropylfluorophosphate (DFP),[6] causing irreversible loss of activity. The gene product of KEX1 of yeast,[7] CP-W of wheat[8] and platelet deamidase of human[9–11] belong in this category, and it was recently reported that a CP-Y like kinase, which is characterized as a serine carboxypeptidase, mainly contributes to the degradation of bradykinin in rat urine.[12] Irrespective of its practical usages, such as sequencing of carboxy-terminal amino acids[13] or chemical reactions,[14–16] serine carboxypeptidase is one of the physiologically unknown peptidase families and detailed studies on serine carboxypeptidases with specific inhibitors remain to be described.

In this communication we report the taxonomy, production, isolation, and enzymatic inhibitory and biological activities of new inhibitors of serine carboxypeptidase, belactins A and B from Actinomycete.

MATERIALS AND METHODS

Microorganism and Taxonomic Characterization

Strain MK19–42F6 was isolated from a soil sample collected in Katsushika-ku, Tokyo, Japan and has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba-shi, Japan, under the accession No. FERM P-14414.

Morphological, cultural and physiological characterizations[17,18] of strain MK19–42F6 were performed using the cultures at 32°C for 2 to 4 weeks (see Tables 1 and 2). Spore morphology was studied with a Hitachi model S-570 scanning electron microscope. The temperature range of growth was determined on yeast-starch agar (soluble starch 1.0% (W/V), yeast extract 0.2%, agar 3.5%, pH 7.0).

Chemotaxonomic analyses of this strain were performed according to the partially modified methods of the previous report.[19] Briefly the type of diaminopimelic acid isomers from the whole cell hydrolysate were determined by silica gel TLC, and cell wall sugars and whole cell sugars[20] were analyzed using reversed phase HPLC. The N-acyl type of muramic acid in the cell wall was determined by the glycolate test.[21] The determination of phospholipids and mycolic acids were carried out by silica gel TLC. Menaquinones were determined using HPLC and mass spectrometry.[22] Fatty acid methyl esters of whole-cell methanolysates were analyzed by a Hewlett-Packard model 5890 gas chromatograph equipped with a flame ionization detecter and J&W Scientific Megabore DB-1 column (15 m by 0.53 mm, film 1 μm).

Chemicals

Chemicals employed were as follows: acetonitrile (HPLC grade) and trifluoro acetic acid (TFA) from Wako Pure Chemical Industries, Ltd., Osaka, Japan; TLC-plate Silica gel F254 (0.25 mm thickness), Silica gel 60, Casein Hammarstein's

TABLE 1
Cultural characteristics of strain MK19–42F6.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Lt ivory (2 ca)~ Lt melon yellow (3 ea)	Scant, thin, white	None
Glycerol-asparagine agar (ISP No.5)	Lt ivory (2 ca)~ Pearl pink (3 ca)	White~ Flesh pink (4 ca)	Faint, brownish
Inorganic salts-starch agar (ISP No.4)	Colorless~ Pearl (3 ba)	Sand (3 cb)	Reddish purple~ Purplish gray
Yeast extract-malt extract agar (ISP No.2)	Colorless~ Pearl pink (3 ca)	Thin, white~ Lt gray (c)	Dark brownish
Oatmeal agar (ISP No.3)	Colorless	Scant, Oyster white (b)	None

TABLE 2
Physiological characteristics of strain MK19–42F6.

Temperature range for growth (°C)	24~37
Optimum temperature (°C)	37
Formation of melanoid pigment	negative
Hydrolysis of starch	positive
Utilization of	
L-Arabinose	+
D-Xylose	(–)
D-Glucose	+
D-Fructose	(+)
Rhamnose	+
Sucrose	(+)
Raffinose	+
Inositol	+
D-Mannitol	+

+, Positive utilization; (+), probably positive utilization; (–), probably no utilization.

(bovine milk) from E. Merck, Darmstadt, FRG; bovine serum albumin (BSA) fraction V, *p*-nitrophenyl acetate (PNP acetate), L-leucine-β-naphthylamide (Leu-βNA) from Sigma Chem. Ltd., Saint Louis, USA; DEAE-Sephadex A-50 from Pharmacia Fine Chemicals AB, Uppsala, Sweden; reversed phase column of Capcellpak C18 SG120 ϕ20×250 mm from Shiseido Co., Tokyo, Japan; benzoyl-glycyl-L-phenylalanine (Bz-Gly-Phe), benzoyl-glycyl-L-lysine (Bz-Gly-Lys), from Peptide Institute Inc., Minoh-shi, Japan; glycyl-L-arginine-β-naphthylamide (Gly-Arg-βNA), L-lysyl-L-alanine-β-naphthylamide (Lys-Ala-βNA), glycyl-L-proline-β-naphthylamide (Gly-Pro-βNA), benzoyl-L-phenylalanyl-L-valyl-L-arginine-4-methoxy-β-naphthylamide (Bz-Phe-Val-Arg-4-methoxy-βNA), carbo-benzoxy-glycyl-L-proline-β-naphthylamide (Z-Gly-Pro-βNA) from Bachem Freinchemikalien AG, Budendorf, Switzerland. All other chemicals were of analytical grade.

Enzymes

Carboxypeptidase Y (CP-Y) from yeast was obtained from Oriental Yeast Co. Ltd., Tokyo, Japan. Carboxypeptidase A (CP-A) Type-I from bovine pancreas, thrombin from human plasma and α-chymotrypsin type-II from bovine pancreas were purchased from Sigma Chem. Ltd., Saint Louis, USA. Carboxypeptidase B (CP-B) from porcine pancreas, aminopeptidase N (AP-N) from hog kidney and leucine aminopeptidase (Leu-AP) from hog kidney were purchased from Boehlinger Mannheim GmbH, F.R.G., Germany. Carboxypeptidase N (CP-N) was prepared from human plasma by ammonium sulfate precipitation (40~80%) and DEAE Sephadex A-50.[5] Dipeptidylaminopeptidase I (DPP-I)[23] and DPP-II[24,25] were prepared from rat spleen homogenate by ammonium sulfate precipitation (40~80%). DPP-IV[26,27] was prepared from rat kidney homogenate by ammonium sulfate precipitation (30~80%). Prolylendopeptidase (PEP)[28] was prepared from hog kidney by ammonium sulfate precipitation (50~65%) and DEAE Sephadex A-50.

Production of Belactins

Strain MK19–42F6 was inoculated into a 500 ml Erlenmeyer flask containing 110 ml of a medium consisting of glycerin 2.0% (W/V), soybean meal (Ajinomoto Co., Inc.) 1.5%, K_2HPO_4 0.1% and $CoCl_2 \cdot 6H_2O$ 0.0005% (pH 6.2, adjusted with 1 M KH_2PO_4) and cultured at 27°C for 2 or 3 days on a rotary shaker (180 rpm). Two ml of the above seed culture was transferred to 110 ml of the same medium in a 500 ml Erlenmeyer flask and cultured at 27°C for 2 days. The process of production of belactins was followed by the CP-Y inhibitory activity of 2 μl of broth filtrate and 4 μl of mycelial methanol extract which was obtained from the same volume of culture broth (see Figure 1).

Isolation of Belactins

The purification procedure of belactins A and B is shown in Figure 2. The fermentation broth was filtered, and the mycelia were extracted with methanol (2 liters).

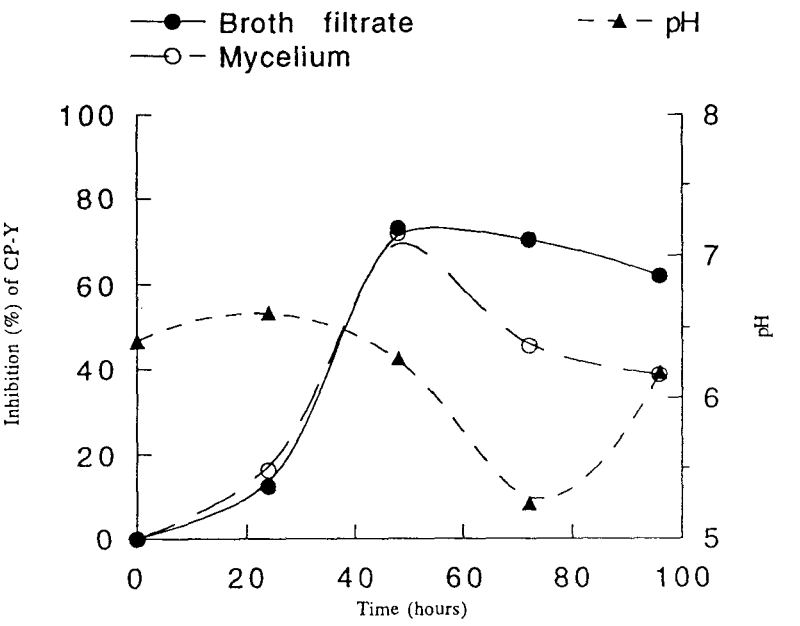

FIGURE 1 Time course of belactins production by *Saccharopolyspora* sp. MK19–42F6. (○) Inhibition (%) of broth filtrate (20 μl/ml), (●) Inhibition (%) of mycelium extract (40 μl/ml), (▲) pH.

After removal of the methanol, the extract was combined with the filtrate (5 liters). The mixtures was extracted with equal volumes of ethyl acetate and the extracts were concentrated to dryness under reduced pressure and the residue (total amount; 442.5 mg), chromatographed on a first step column of silica gel using chloroform-methanol (100/1, 50/1, 30/1 and then 10/1).

Belactin A was isolated as follows. The fractions containing belactin A (Fraction A), eluted with chloroform-methanol (100/1), were concentrated *in vacuo* to yield 39.9 mg of oily material. The oily material was further applied to a column of silica gel. The column was washed with toluene-ethyl acetate (20/1 and then 10/1) and the active fractions were eluted with toluene-ethyl acetate (6/1). After evaporation to dryness, the active fractions were chromatographed on a column of Sephadex LH20 with methanol, and pure belactin A (19.4 mg) was successively obtained as a slightly yellowish solid after lyophilization in benzene.

Belactin B was isolated as follows. The fractions containing belactin B (Fraction B), eluted with chloroform-methanol (10/1) from the first step column of silica gel, were concentrated *in vacuo* to yield 95.8 mg of oily material. It was purified further by reversed phase HPLC, performed by a GILSON's system equipped with a Waters 991J photodiode array detecter. The oily material was applied to a reversed phase HPLC column; Shiseido Capcellpak C18 SG120 ϕ20×250 mm which was equilibrated with a solvent mixture of CH_3CN-H_2O-TFA (25/74/1) and a flow rate of 6.0 ml/min

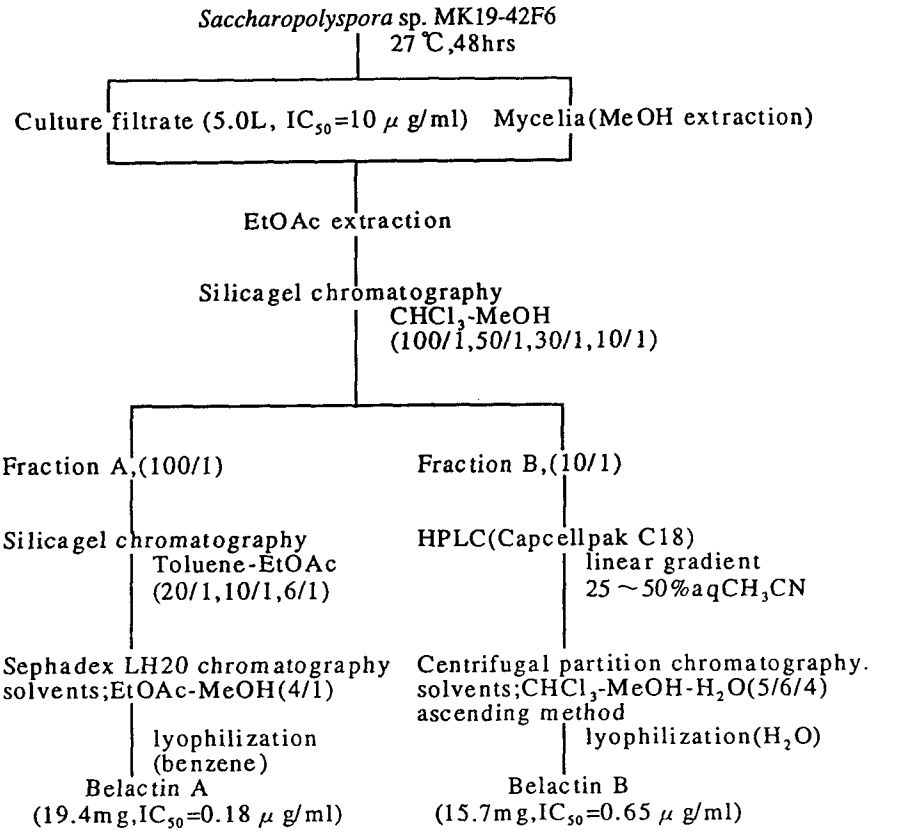

FIGURE 2 Isolation and purification of belactins.

and was eluted with gradient from CH_3CN-H_2O-TFA (25/74/1) to CH_3CN-H_2O-TFA (50/49/1) for 100 min. The active fractions was evaporated to dryness and purified with a centrifugal partition chromatography (CPC). The chromatography was performed using a CPC apparatus model NMF (Sanki Engineering Ltd.) under following conditions: solvent system, $CHCl_3$-MeOH-H_2O (5/6/4); seperation mode, ascending mode; flow rate, 5 ml/min; centrifugation, 700 rpm; temperature, 20°C. The active fractions were concentrated to a small volume and were lyophilized to give a colourless powder of belactin B (15.7 mg).

Assays of Enzymes and Inhibitory Activities

The conditions for the various enzyme assays used are summarized in Table 3.

TABLE 3
Conditions of enzyme assays.

Enzyme	Substrate	Other reaction mixture
CP-Y	1 mM Bz-Gly-Phe	25 mM Na phosphate buffer (pH 6.5)
CP-A	1 mM Bz-Gly-Phe	25 mM Na phosphate buffer (pH 7.5) containing 0.9 M NaCl
CP-B	1 mM Bz-Gly-Lys	25 mM Tris-HCl buffer (pH 8.0)
CP-N	1 mM Bz-Gly-Lys	25 mM Tris-HCl buffer (pH 7.2)
Lue-AP	0.5 mM Leu-βNA	50 mM Tris-HCl buffer (pH 7.5)
AP-N	0.5 mM Leu-βNA	50 mM Tris-HCl buffer (pH 17.0)
DPP-I	0.4 mM Gly-Arg-βNA	50 mM Citrate buffer (pH 4.0) containing 5 mM NaCl, 7 mM 2-mercaptoethanol
DPP-II	0.4 mM Lys-Ala-βNA	50 mM 3,3-dimethylglutalic acid-NaOH buffer (pH 5.5)
DPP-IV	0.5 mM Gly-Pro-βNA	50 mM Tris-Malate-NaOH buffer (pH 7.0)
Chymotrypsin	1% (W/V) Casein	17.5 mM Borate buffer (pH 7.4) containing 2.5 mM $CaCl_2$
Thrombin	0.1 mM Bz-F-V-R-4Me βNA	50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl, 25 μg/ml BSA
PEP	0.1 mM Z-Gly-Pro-βNA	25 mM Tris-HCl buffer (pH 7.8)

The principle of the assays for carboxypeptidase activity, is based on the spectrometric determination of N-benzoylglycine with cyanuric chloride using microwell plates (nunclon, F96) according to the modified method of Hayakari *et al.*[29] The reaction mixture (total 0.1 ml) for CP-Y consisted of 25 mM sodium phosphate buffer (pH 6.5), 1 mM Bz-Gly-Phe, 2.0 μg/ml enzyme, and water or aqueous solution containing the test compound. The enzyme reaction was started by the addition of the enzyme, followed by incubation at 37°C for 40 min, after then 6 μl of 1 N sodium hydroxide was added to terminate the reaction. Ten minutes later, 50 μl of 0.36 M sodium phosphate buffer (pH 7.2) and 0.15 ml of 2% (W/V) cyanuric chloride, freshly dissolved in 2-methoxyethanol, were added to the mixed solution. After the mixture was allowed to stand for 15 min at room temperature, the absorbance at 405 nm was measured with a microplate reader model 3550 (BIO-RAD).

Other carboxypeptidases inhibitory activities were measured using the same procedure as that for CP-Y except for the following conditions. For CP-A assay, 25 mM sodium phosphate buffer containing 0.9 M NaCl (pH 7.5), for CP-B assay

1 mM Bz-Gly-Lys as the substrate and 25 mM Tris-HCl buffer (pH 8.0) and for CP-N assay 1 mM Bz-Gly-Lys as the substrate and 25 mM Tris-HCl buffer (pH 7.2) were used in these reaction mixtures respectively.

For aminopeptidases,[30] dipeptidylaminopeptidases,[27,31,32] thrombin[33] and PEP,[34] β-naphthylamide or 4-methoxy-β-naphthylamide derivatives were used as the substrate and the activities of the enzymes were determined from the absorbance at 525 nm after the colour reactions of β-naphthylamine or 4-methoxy-β-naphthylamine in these assay systems. In the assay for Leu-AP, the reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.5). 0.5 mM Leu-βNA, 4.0 μg/ml enzyme and water or aqueous solution containing the test compound. The enzyme reaction was started by the addition of the enzyme, followed by incubation at 37°C for 60 min. The reaction was stopped by adding 0.1 ml of 0.5 M citrate buffer (pH 3.8) containing 0.1% (W/V) Fast Garnet GBC salt and 10% Tween 20 and the absorbance at 525 nm was determined after 15 min at room temperature.

The principle of the assay for chymotrypsin[35] is based on the spectrometric measurement of acid-soluble hydrolysate released from casein using a Hitachi model 100–41 spectrophotometer. The reaction mixture consisted of 17.5 mM borate buffer (pH 7.4) containing 2.5 mM $CaCl_2$, 1% (W/V) casein, 4.0 μg/ml enzyme and water or aqueous solution containing the test compound. The enzyme reaction was started by the addition of the enzyme, followed by incubation at 37°C for 30 min. The reaction was stopped by adding 1.5 ml of 1.13 M perchloric acid. After centrifugation (1200×g) of the mixture, the enzymatic activity was determined by measuring the absorbance at 280 nm of the supernatant.

The incubation time of various enzyme assays was determined according to the enzyme activities which were used at that time. The percentage inhibitions were calculated by the formula (A-B)/A×100, where A is the value obtained in the enzymatic assay without an inhibitor and B is that with an inhibitor. The IC_{50} value is the concentration of inhibitor giving 50% inhibition of enzyme activity.

For dialysis of the enzyme-inhibitor complex, the mixtures (1 ml) containing 25 mM sodium phosphate buffer (pH 6.5), 2 μg/ml CP-Y, 30 μg/ml BSA, and water or aqueous solution containing the test compound was incubated at 37°C for 40 min, followed by dialysis against 3 liters of 25 mM sodium phosphate buffer (pH 6.5) at 4°C. Then 0.09 ml of dialysate was taken to a 96 well plate and its carboxypeptidase activity determined and compared with that of control mixtures which were left for the same periods at 4°C without dialysis. After ten minutes preincubation at 37°C, CP-Y enzyme assays were started by adding the substrate (10 μl). All data about enzyme assays are expressed as means of triplicate experiments.

RESULTS AND DISCUSSION

Taxonomy of the Producing Organism

The cultural and physiological characteristics of strain MK19–42F6 are summarized in Tables 1 and 2 respectively and the morphological characterizations were as follows.

The strain MK19–42F6 have fragmented aerial hyphae which form hooks and loops and present a distinctive bead like appearance. The spores are cylindrical to ellipsoidal in shape, 0.5~0.6 by 0.9~1.2 μm in size and have a spiny surface. The substrate hyphae is branched and fragmented. No sporangia, motile spores or synnemata are observed.

Cell wall hydrolysates contained *meso*-diaminopimeric acid, arabinose and galactose, and whole-cell hydrolysates showed the presence of rhamnose ribose, mannose, arabinose, galactose and glucose. These data indicated that strain MK19–42F6 has a type IV cell wall and a type A whole-cell sugar pattern. The N-acyl type of muramic acid in the cell walls was determined to be the acetyl type. A type P III phospholipid pattern (phosphatidylcholine, phosphatidylethanolamine and phosphatidylmethylethanolamine present, and glucosamine containing phospholipid absent) was found. Mycolic acids were absent. The predominant menaquinones were MK-9 (H_4) and MK-10 (H_4). This strain contained major amount of *anteiso*-branched 14-methylhexadecanoic acid (*ai*-17), *iso*-branched 13-methyltetradecanoic acid (*iso*-15), *iso*-branched 14-methylpentadecanoic acid (*iso*-16) and *iso*-branched 15-methylhexadecanoic acid (*iso*-17).

Based on these characteristics, strain MK19–42F6 was placed in the genus *Saccharopolyspora*.[36]

Production and Isolation

The time course for inhibitory activity against CP-Y and the pH value of the broth were studied for 4 days. The maximum peaks, for the production of belactins in the culture filtrate and the mycelial extract were obtained at 2 days and thereafter the production slowly decreased with a pH change to acid (Figure 1).

The flow diagram for isolation is shown in Figure 2. The yields of belactins A and B were 19.4 mg and 15.7 mg respectively.

The physico-chemical properties and the structure determinations of belactins A and B are reported in the following paper.[37]

Enzymatic Inhibitory Activities or Other Biological Activities of Belactins

The inhibitory activities (IC_{50}) of belactins A and B against CP-Y were determined to be 0.18 and 0.65 μg/ml respectively. During inhibitory kinetic studies with belactins A and B, the steady-states for CP-Y inhibition by belactins were attained after 40 min preincubation of both inhibitors with the enzyme at 37°C (data not shown). To clarify whether belactins inhibit CP-Y reversibly or irreversibly, dialysis experiments with belactin A were performed, compared with a competitive inhibitor, piperastatin A.[38] More than 90% of the CP-Y activity was recovered after dialysis of the inhibitor-enzyme complex for 56 hours which showed that belactin A was a reversible inhibitor as well as piperastatin A, as shown in Table 5. The slow recovery in activity of the enzyme with belactin A might be caused by the high hydrophobicity or slow-dissociation of belactin A. Under the assay conditions with preincubation of belactin A with the enzyme for 40 min, the Lineweaver-Burk plots for the belactins exhibited mixed non-competitive inhibitions as shown in Figures 3 and 4. K_i values for belactins A (M_v, 352) and B (M_v, 514) against CP-Y were 0.14 μM and 0.27 μM respectively which

TABLE 4
Inhibitory activities of belactins against various peptidases.

Enzymes	IC_{50}(μg/ml)	
	Belactin A	Belactin B
CP-Y	0.18	0.65
CP-A	>100	>100
CP-B	>100	>100
CP-N	>100	>100
Leu-AP	>100	>100
AP-N	>100	>100
DPP-I	100	100
DPP-II	>100	>100
DPP-IV	>100	>100
Chymotrypsin	>100	>100
Thrombin	>100	>100
PEP	50	60

TABLE 5
Effect of dialysis of enzyme-inhibitor complex on CP-Y inhibition by belactin A.

(a) Dialysis 31 h

Mixtures	Dialysis		Control	
	A_{405}	Act(%)	A_{405}	Act(%)
No inhibitor	0.2	100	0.26	100
Bel A 0.25 μg/ml	0.17	85	0.03	12
Bel A 0.5 μg/ml	0.16	80	0.02	7.7
Pip A 0.15 μg/ml	0.2	100	0.08	31
Pip A 0.3 μg/ml	0.18	90	0.03	12

(b) Dialysis 56 h

Mixtures	Dialysis		Control	
	A_{405}	Act(%)	A_{405}	Act(%)
No inhibitor	0.33	100	0.39	100
Bel A 0.25 μg/ml	0.32	97	0.05	13
Bel A 0.5 μg/ml	0.3	91	0.03	7.7
Pip A 0.15 μg/ml	0.34	103	0.21	54
Pip A 0.3 μg/ml	0.34	103	0.07	18

Bel A = belactin A. Pip A = piperastatin A.

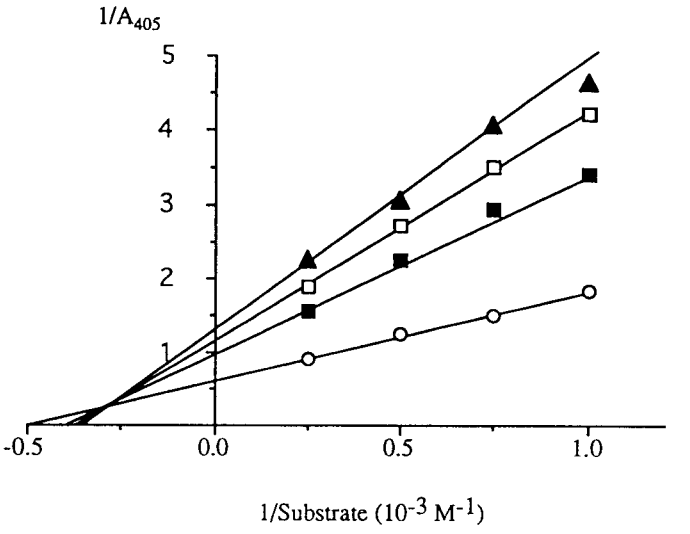

FIGURE 3 Lineweaver-Burk plot of inhibition of CP-Y by belactin A. (○) I=0 μg/ml, (■) I=0.05 μg/ml, (□) I=0.075 μg/ml, (▲) I=0.1 μg/ml. I=Inhibitor.

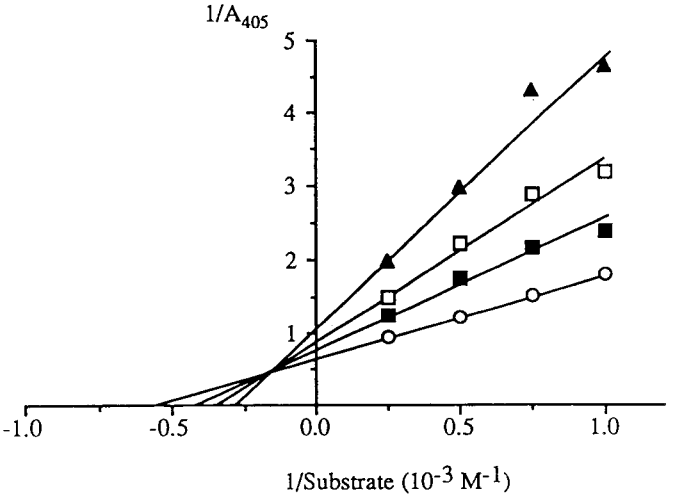

FIGURE 4 Lineweaver-Burk plot of inhibition of CP-Y by belactin B. (○) I=0 μg/ml, (■) I=0.1 μg/ml, (□) I=0.15 μg/ml, (▲) I=0.25 μg/ml. I=Inhibitor.

were calculated from the corresponding Dixon plots. More detailed kinetic studies concerning slow-binding inhibition of CP-Y by the belactins will be reported later.

In the previous studies, DFP and acylaminocids, such as CBZ-L-Phe were reported to be inhibitors of CP-Y,[6,39] but these inhibitors did not have sufficient selectivity or potency against CP-Y. In the course of the inhibitory characterization of the belactins using many kinds of peptidases, metallo carboxypeptidases (CP-A, B and N), aminopeptidases (AP-M and Leu-AP), dipeptidylaminopeptidases (DPP-I, II and IV) and serine endopeptidases (chymotrypsin, thrombin and PEP), the belactins were found to have high selectivities towards CP-Y except that they also inhibited PEP and DPP-I weakly (see Table 4). Belactins A and B have no antimicrobial activities at 100 μg/ml, and have low toxicities, since there were no deaths after intraperitoneal injection of mice with 100 mg/kg of belactins A and B (data not shown). In order to understand the cell biological or physiological roles of serine carboxypeptidase including CP-Y, potent and specific inhibitors of serine carboxypeptidase, are needed. To this end we have discovered new serine carboxypeptidase inhibitors, belactins A and B, which are characterized as potent and useful inhibitors of CP-Y.

The structures of belactins A and B, were determined to be 4-[3-[(2-amino-5-chlorobenzoyl)amino]-1,1-dimethyl-2-oxobutyl]-3-methyl-2-oxetanone and 4-[3-[[2-(β-glucopyranosylamino)-5-chlorobenzoyl] amino]-1,1-dimethyl-2-oxobutyl]-3-methyl-2-oxetanone respectively as reported in the following paper,[37] and possess a β-lactone moiety. Characterization of the inhibitory activities of belactins, compared with other β-lactone containing inhibitors such as esterasin[40,41] and ebelactones,[42,43] are reported in the following paper.[37]

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