WA8242A$_1$, A$_2$ and B, Novel Secretory Phospholipase A$_2$ Inhibitors Produced by *Streptomyces violaceusniger*

I. Taxonomy, Production and Purification

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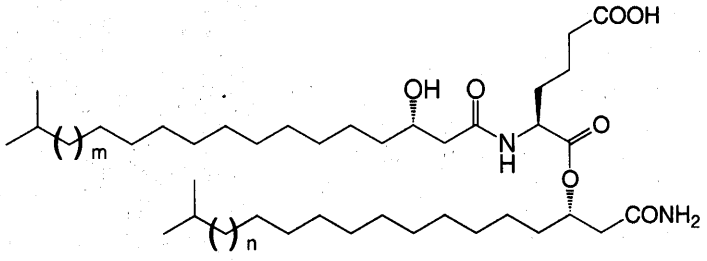
WA8242A$_1$, A$_2$ and B, novel inhibitors of secretory phospholipase A$_2$, were obtained from the cultured mycelium of *Streptomyces violaceusniger* No. 8242. WA8242A$_1$, A$_2$ and B are structurally related compounds with α-aminoadipic acid and with various length of alkyl chains.

Phospholipase A$_2$ (PLA$_2$; EC 3.1.1.4) is a lipolytic enzyme that catalyzes the hydrolysis of the acyl ester bond at the *sn*-2-position of phosphoglycerides, *e.g.*, phosphatidylcholine and phosphatidylethanolamine. Mammalian PLA$_2$ exists in both extracellular and intracellular forms. As the extracellular PLA$_2$s are secreted and act extracellularly, these enzymes are called secretory PLA$_2$s. Mammalian secretory PLA$_2$s are divided into two groups on the basis of the amino acid sequence, though they have the similar molecular weight of 14 kD and need high level of Ca^{2+} for their activity[1]. Group I PLA$_2$ is secreted from pancreas. While the group I PLA$_2$ normally digests phospholipids in foods, its increase in serum has been reported in acute pancreatitis or pancreatic cancer[2]. Group II PLA$_2$ has been regarded as an acute phase enzyme, because its increase in serum and/or tissue has been found in several inflammatory diseases, such as shock[3], tuberculosis[4], arthritis[5] and heart disease[6]. Furthermore, it is reported that group II PLA$_2$ stimulates the degranulation of mast cells *in vitro*[7]. Accordingly, the inhibition of secretory group II PLA$_2$ may be useful for the treatment of various inflammatory and allergic diseases.

In the course of searching for secretory PLA$_2$ inhibitors from microorganisms by using a group I PLA$_2$, we found that *Streptomyces violaceusniger* No. 8242 produced novel type PLA$_2$ inhibitors, WA8242A$_1$, A$_2$ and B (Fig. 1). Interestingly, it was revealed that these compounds had a stronger inhibitory activity against group II PLA$_2$ than against group I PLA$_2$.

Fig. 1. Chemical structures of WA8242A$_1$, A$_2$ and B.



WA8242A$_1$: m=1, n=0
WA8242A$_2$: m=0, n=1
WA8242B: m=1, n=1

This paper describes the taxonomy of the producing strain, production and purification of these inhibitors. Biological properties are described in an accompanying paper[8]. Structure elucidation and total synthesis will be published in this journal[9].

Materials and Methods

Materials

Porcine pancreatic PLA_2 was purchased (Boehringer Mannheim). The cDNA of human synovial PLA_2 was cloned from human placenta cDNA library and expressed in CHO cells in our laboratories. The recombinant human synovial PLA_2 was partially purified by using Heparin-Toyopearl affinity column chromatography. 1-Palmitoyl-2-[ω-(1-pyreno)decanoyl]-*sn*-glycero-phosphocholine, a substrate for group I PLA_2, was purchased (Sigma). 1-Triacontanoyl-2-[ω-(1-pyreno)decanoyl]-*sn*-glycero-phosphoethanolamine, a substrate for group II PLA_2, was prepared in our laboratories. All other chemicals were of the analytical grade.

Taxonomy

The methods and media described by SHIRLING and GOTTLIEB[10] and WAKSMAN[11] were employed for taxonomic study. The observations were made after 14 to 21 days cultivation at 30°C. The morphological observations were made on the cultures grown on yeast-malt extract agar, inorganic salts-starch agar, and oatmeal agar using an optical microscope and a scanning electron microscope. The color names used in this study were taken from the Methuen Handbook of Colour[12]. The temperature range for growth was determined on yeast extract-malt extract agar. The preparation of cells and detection of the isomer of diaminopimelic acid were performed by procedure of BECKER *et al.*[13].

PLA_2 Assay

Porcine pancreatic PLA_2 was used as group I PLA_2 at 100 ng/ml. The recombinant human synovial PLA_2 was used as group II PLA_2 at 10 ng/ml.

Substrates described in materials were hydrolyzed by PLA_2 to liberate (1-pyreno)decanoic acid, and the reactions were monitored by measuring the fluorescence of pyrene (excitation at 345 nm and emission at 380 nm) as described by HENDRICSON *et al.*[14]. The standard reaction buffer in a total volume of 1200 μl contained Tris-HCl buffer (50 mM, pH 7.4), NaCl (100 mM), $CaCl_2$ (5 mM), EDTA (1 mM) and PLA_2. Substrates were dried under a gentle stream of nitrogen gas and suspended in the reaction buffer without $CaCl_2$ by sonication at 70°C for 30 minutes. The reaction was started by addition of substrate (1 μM) and incubated at room temperature for 30 minutes. To terminate the reaction, 10 μl of EDTA (1 M) was added. BSA coated polystylene tube (coated with 0.1% BSA for over night) was used to avoid the adhesion of free (1-pyreno)decanoic acid to the reaction tube.

In the PLA_2 assays, inhibitor and vehicle was preincubated with the enzyme for 30 minutes at room temperature. Each sample was dissolved in methanol (final 1.25%). All data are the average of at least duplicate determinations.

Results

Identification of the Producing Strain

The strain No. 8242 was isolated from a soil sample obtained from Atami City, Shizuoka Prefecture, Japan.

The vegetative mycelium developed well without fragmentation. The aerial mycelium branched monopodially and formed spiral chains of spores with more than 10 spores per chain. The spores had a rougose surface and were spherical to cylindrical shaped with a size of $0.6 \sim 0.8 \times 0.6 \sim 1.0$ μm. No sclerotic granules, sporangia or zoospores were observed (Fig. 2). The results of cultural characteristics are shown in Table 1.

The aerial mycelium was white to gray. Part of the colony became black and moist, and showed hygroscopic appearance on the most media used. On the reverse side, the growth was yellowish brown, brown and dark brown. Reverse mycelial pigment was not pH sensitive. Melanoid

Fig. 2. Scanning electron micrograph of spore chains of strain No. 8242 grown on yeast-malt extract agar for 14 days at 37°C.

Bar represents 5 μm.

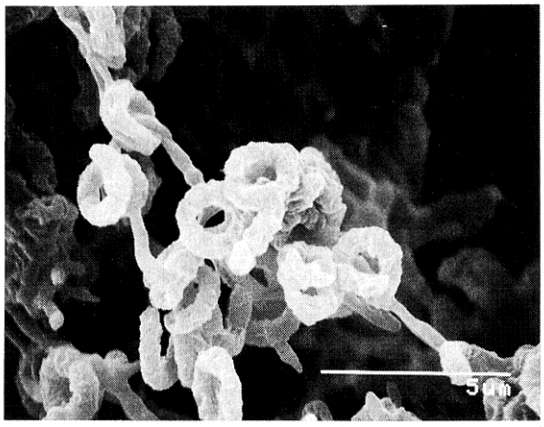


Table 1. Cultural characteristics of strain No. 8242.

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Yeast extract-malt extract agar	Good	Abundant, white, gray (1D1) and black	Brown (6D6)	None
Oatmeal agar	Good	Abundant, gray (1E1), white and black	Yellowish brown (5D4)	None
Inorganic salts-starch agar	Good	Abundant, gray (1D1), white and black	Light brown (6D5)	None
Glycerol-asparagine agar	Good	Moderate, white	Brownish orange (6D5)	None
Peptone-yeast extract-iron agar	Moderate	None	Pale yellow (4A3)	None
Tyrosine agar	Good	Moderate, yellowish white (4A2)	Grayish brown (8F4)	None
Sucrose-nitrate agar	Good	None	Light brown (6D6)	None
Sucrose-asparagine agar	Good	Moderate, white and gray (1D1)	Brown (7E5)	None

Table 2. Physiological characteristics of strain No. 8242.

Condition	Characteristic
Temperature range for growth (°C)	13~39
Optimum temperature for growth (°C)	32
Gelatin liquefaction	+
Milk coagulation	+
Milk peptonization	±
Starch hydrolysis	+
Production of melanoid pigments	−
Decomposition of cellulose	−
Carbohydrate utilization:	
D-Glucose	+
L-Arabinose	+
D-Xylose	+
Inositol	+
Sucrose	+
D-Fructose	+
D-Mannitol	+
L-Rhamnose	+
Raffinose	±

+: Positive, ±: weakly positive, −: negative

pigments and other soluble pigments were not produced in tryptone-yeast extract broth, peptone-yeast extract-iron agar and tyrosine agar.

Analysis of whole cell hydrolysates of strain No. 8242 showed the presence of L,L-diaminopimelic acid. Physiological properties and utilization of carbon sources are shown in Table 2.

The morphological and chemical characteristics of strain No. 8242 permitted a clear assignment to the genus *Streptomyces*. Strain No. 8242 was compared with *Streptomyces* species described in the literature[15,16]. As strain No. 8242 proved to resemble *Streptomyces violaceusniger*, it was compared with the type strains *S. violaceusniger* IFO 13459^T and *S. hygroscopicus* IFO 13472^T in detail[17]. Strain No. 8242 proved to closely resemble *S. violaceusniger* IFO 13459^T with a few differences (Table 3). It is considered that these differences were too small to separate the two strains into different species. Therefore, strain No. 8242 was identified as a strain of *Streptomyces violaceusniger*.

The strain No. 8242 was deposited as FERM BP-4241 at the National Institute of Bioscience and Technology, Japan.

Production

A loopful of a mature slant culture of *Streptomyces violaceusniger* No. 8242, was inoculated into a seed medium (200 ml) containing soluble starch 5% glucose 1%, corn steep liquor 1%, polypeptone 0.5%, yeast extract 0.2% and $CaCO_3$ 0.3% (pH 7.0) in a 500 ml Erlenmeyer flask and cultured at 30°C for 4 days on a rotary shaker with 5.1 cm throw at 220 rpm.

The resultant seed culture (2% inoculum) was used to inoculate 220 liters of a sterile production medium containing soluble starch 2%, glycerin 1%, glucose 1%, soybean powder 1%, polypeptone 0.5%, yeast extract 0.5% and $CaCO_3$ 0.5% (pH 7.0) in a 300 liter jar fermenter. The fermentation was carried out at 30°C for 4 days under aeration at 300 liters/minute and agitation

Table 3. Comparisons among strain No. 8242, IFO 13459[T] and IFO 13472[T]

Condition		No. 8242	*S. violaceusniger* IFO 13459[T]	*S. hygroscopicus* IFO 13472[T]
Spore mass		Gray, hygroscopic	Gray, hygroscopic	Gray, hygroscopic
Spore chain		Spiral	Spiral	Spiral
Spore surface		Rugose	Rugose	Rugose
Production of melanoid pigments		None	None	None
Formation of aerial mycelium on peptone-yeast extract iron agar		None	None	Moderate
Production of soluble pigments on tyrosine agar		None	Trace of reddish brown	None
Tempereture range for growth (°C)		13~39	14~44	12~45
Carbon utilization	Sucrose	+	+	−
	Raffinose	±	+	+
NaCl tolerance	4%	+	+	+
	6%	−	−	+
	8%	−	−	+

+: Positive, ±: weakly positive, −: negative, [T]: type strain.

at 200 rpm.

The amount of the product in the fermentation broth was quantified by the PLA_2 inhibition assay. The sample for the assay was prepared as follows; ten ml of broth was centrifuged at 2000 rpm for 10 minutes, and acetone was added to the mycelial pellet to a volume of 8 ml with mixing. The mixture was allowed to stand for an hour, and 2 ml of methanol was then added, followed by mixing and centrifugation at 2000 rpm for 5 minutes. The supernatant thus obtained was employed for the assay. The PLA_2 inhibition assay method was described in the materials and methods section.

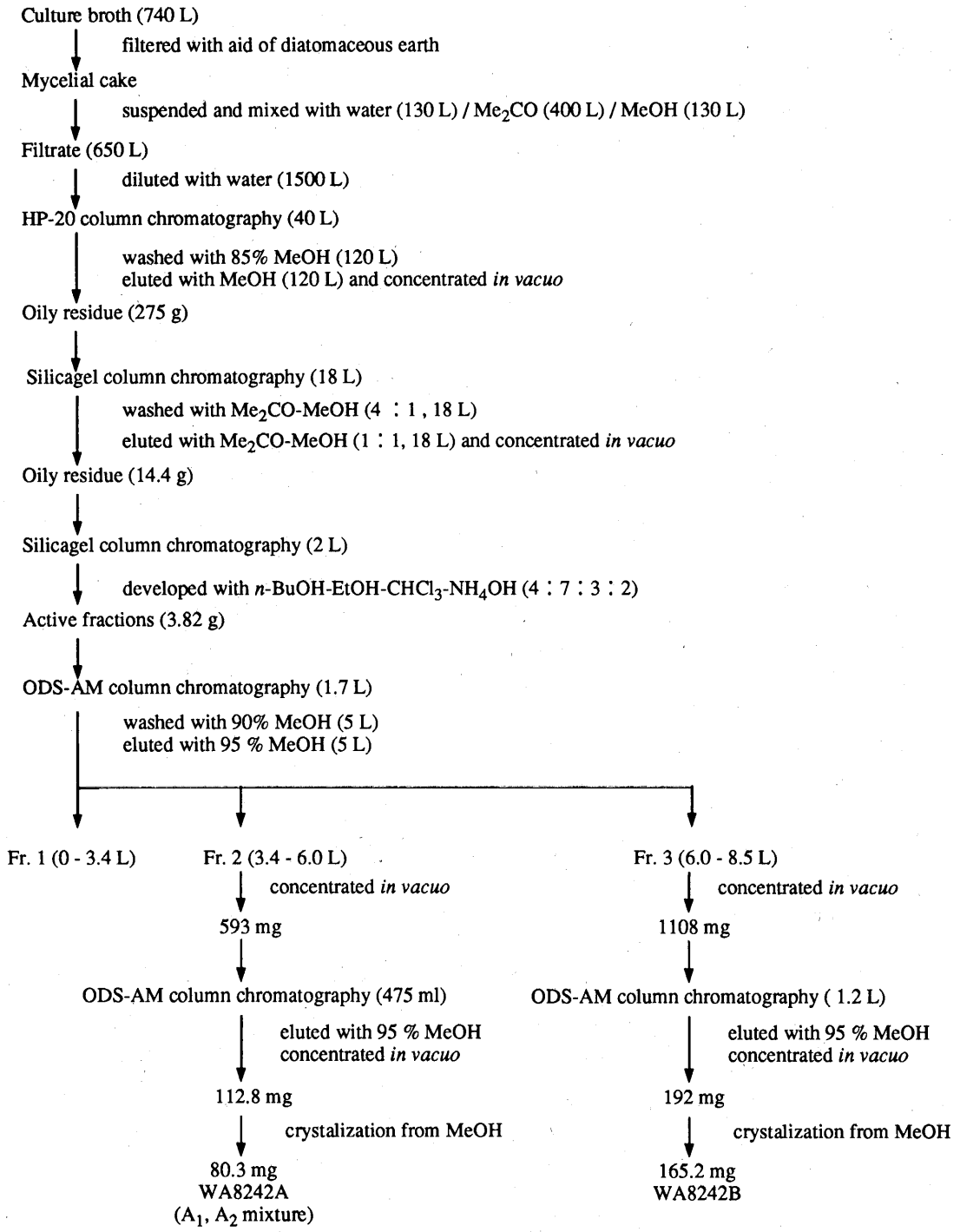
Purification

The purification procedure is shown schematically in Fig. 3. The pooled 740 liters of culture broth was filtered with diatomaceous earth. The mycelial cake was suspended in 130 liters of water and 400 liters of acetone was added to the suspension with stirring. The mixture was allowed to stand overnight and 130 liters of methanol was added to the mixture with vigorous mixing. After the filtration of the mixture, 1500 liters of water was added to the filtrate and passed through a column of polymeric adsorbent, Diaion HP-20 (40 liters). The column was washed with 120 liters of 85% methanol and eluted with 120 liters of methanol. The eluate was concentrated under reduced pressure to give a 275 g of oily residue. The residue was applied to an 18 liter silica gel column for chromatography. After washing with 18 liters of acetone-methanol (4:1), active substances were eluted by 18 liters of acetone-methanol (1:1). The eluate was concentrated *in vacuo* to dryness and dissolved in a mixture of *n*-butanol-ethanol-chloroform-ammonium hydroxide (4:7:3:2). The mixture was rechromatographed on a 2 liter silica gel column and developed with the same solvent. The active fractions were combined and concentrated to give an oily residue (3.82 g). The residue was then applied to a 1.7 liter of ODS column packed with 80% methanol. The column was washed with 5 liters of 90% methanol and eluted with 95% methanol. The first fraction of eluate (0~3.4 liters) was discarded, and the second fraction (3.4~6 liters) containing the minor component (WA8242A) and the third fraction (6~8.5 liters) containing the major component (WA8242B) were collected.

The fraction containing WA8242A was concentrated to give an oily residue (593 mg). The residue was rechromatographed on 475 ml of ODS column and the active constituent was eluted with 95% methanol. The active fraction was concentrated to dryness to give 112.8 mg of powder. The powder was dissolved in a small amount of hot methanol and was allowed to stand at room temperature to yield 80.3 mg of colorless crystals of WA8242A.

The fraction containing WA8242B was concentrated to give a 1108 mg of oily residue. The residue was chromatographed on 1.2 liter of ODS column and developed with 95% methanol. The active fraction was concentrated to dryness to give 192 mg of powder. The powder was dissolved in a small amount of hot methanol and was allowed to stand at room temperature to yield 165.2 mg of colorless crystals of WA8242B.

Fig. 3. Purification scheme for WA8242A (A_1, A_2 mixture) and B.



The chemical structures of WA8242A$_1$, A$_2$ and B were shown in Fig. 1. In the course of structural elucidation, it was found that WA8242A was composed of two related compounds (WA8242A$_1$ and WA8242A$_2$) with same molecular weight.

As listed in Table 4, both compounds inhibited PLA_2s at nealy same IC_{50} against each type of PLA_2. In addition, the effect of both compounds were more

Table 4. IC_{50} values of WA8242A and B against PLA_2s (10^{-9} M).

Type of PLA_2	WA8242A[a]	WA8242B
Group I	1.47	1.10
Group II	0.25	0.14

[a] Mixture of WA8242A$_1$ and A$_2$.

selective against group II PLA_2 than group I PLA_2.

Discussion

WA8242A$_1$, A$_2$ and B were obtained from the cultured mycelium of *Streptomyces* sp. which was identified as a strain of *Streptomyces violaceusniger* No. 8242. As a result of isolation and purification procedure, two active constituents were obtained and named WA8242A and B. During the structural elucidation, however, it was revealed that WA8242A was a mixture of two related compound with same molecular weight of 682. The difference of these compounds, designated as WA8242A$_1$ and A$_2$, was only the length of alkyl-chains. WA8242B had one longer alkyl-chain than WA8242A$_1$ or A$_2$ (Fig. 1).

Many types of natural products with PLA_2-inhibitory activities have been reported. Manoalide, Luffariellolide and Scalaradial were obtained from marine sponge[18] and YM-26567-1 was isolated from a plant[19]. Plipastatins[20~22], Duramycins[23], Thielocins[24,25], Cinatrins[26,27], Folipastatin[28] and so on were obtained from microorganisms. WA8242A$_1$, A$_2$ and B were different from these known inhibitors in the points of structure and inhibitory potency. WA8242A$_1$, A$_2$, B and/or its derivatives will be useful and informative for developing a new type of anti-inflammatory agent.

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