STUDIES ON A NEW IMMUNOACTIVE PEPTIDE, FK-156

II. FERMENTATION, EXTRACTION AND CHEMICAL AND BIOLOGICAL CHARACTERIZATION

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An interesting immunoactive peptide, FK-156, has been isolated from the fermentation broth of *Streptomyces olivaceogriseus* sp. nov. and *Streptomyces violaceus*. The compound was purified by column chromatography with activated carbon, ion exchange Sephadex and cellulose powder. FK-156, obtained as white powder, exhibits a wide variety of immunostimulatory activity *in vivo* and *in vitro* with experimental animals. Pretreatment of mice or rats with this peptide protected the animals against lethal infection with *Escherichia coli* and resulted in prolongation of life span of tumor bearing animals.

During the course of a screening program directed toward the isolation and evaluation of new immunostimulating agents, we isolated two strains of *Streptomyces*, strain No. C-353 and strain No. 6724, which were found to produce a new immunoactive peptide, FK-156. Taxonomic study on these strains is presented in the preceding paper[1]. Strain No. C-353 was found to be a new species and designated as *Streptomyces olivaceogriseus* sp. nov. Strain No. 6724 was identified as *Streptomyces violaceus*.

In this paper, we describe fermentation, isolation procedures and some chemical and biological properties of FK-156. Determination of the chemical structure of FK-156 as D-lactyl-L-alanyl-γ-D-glutamyl-(L)*meso*-diaminopimelyl (L)-glycine will be described in the succeeding paper[2].

Fermentation

FK-156 is produced by submerged fermentation in stainless-steel fermentors with the media described in Table 1.

A mature slant culture of the producing organism was used to inoculate 500-ml flasks containing

Table 1. Media used for production of FK-156.

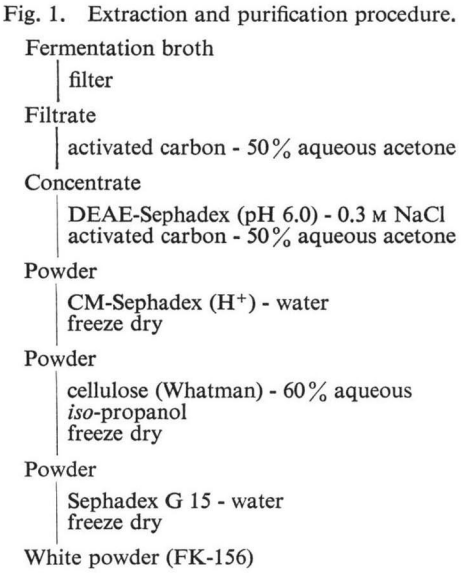
Seed medium 1		Seed medium 2		Production medium	
Potato starch	2%	Soluble starch	2%	Soluble starch	2%
Gluten meal	1%	Cotton seed meal	0.5%	Cotton seed meal	0.5%
Dried yeast	1%	Gluten meal	0.5%	Wheat germ	0.5%
C.S.L.	1%	Dried yeast	0.5%	Dried yeast	0.25%
pH	7.0	C.S.L.	0.5%	C.S.L.	0.25%
				KH_2PO_4	0.5%
				$Na_2HPO_4 \cdot 12H_2O$	0.5%
				$CoCl_2 \cdot 6H_2O$	1.25×10^{-4}%

100 ml each of sterile seed medium No. 1, which were cultured for 48 hours at 30°C with shaking (220 rpm, 5.1 cm throw). The whole volume of the seed culture thus prepared was inoculated into 30-liter jar fermentors containing 20 liters of the seed medium No. 2 and cultured for 24 hours, aerated at 20 liters per minute and agitated at 300 rpm. The whole volume of the seed culture was used to inoculate a 2,000-liter fermentor containing 1,600 liters of production medium. Fermentation was carried out for 72 hours at 30°C with agitation at 170 rpm and with air flow of 1,600 liters per minute. Production of the peptide in the fermentation broth was monitored by *in vivo* antibacterial tests.

Isolation Procedure

The diagram of the extraction and purification procedures is outlined in Fig. 1. Most of the activity was found in the broth filtrate (1,600 liters), which was passed through a column of activated carbon (800 liters) and the active principle adsorbed to the column was eluted with 50% aqueous acetone. After concentration of the solution to 600 liters, the concentrate was applied to a column of DEAE-Sephadex previously buffered with phosphate buffer of pH 6.0 (200 liters). After washing with 0.1 M NaCl, the active principle was eluted with 400 liters of 0.3 M NaCl solution. The eluate was desalted over activated carbon and lyophilized to give 800 g of crude powder. The powder was dissolved in water and the solution was passed through a column of CM-Sephadex (H^+ cycle, 25 liters). The active principle was then eluted with 25 liters of water and the active fractions were concentrated and lyophilized to give 33 g of yellowish white powder. The powder was placed on the top of a column of cellulose (1.2 liters). After washing with 1 liter of 70% aqueous propanol, the active principle was eluted with 1 liter of 60% aqueous propanol and lyophilized to yield 4 g of white powder. The powder was dissolved in 300 ml of water and passed through a column of DEAE-Sephadex (buffered at pH 6.0, 1.4 liters). The column was washed with 0.1 M NaCl solution and then eluted with 3 liters of 0.2 M NaCl solution. The active fractions were combined and desalted by the use of activated carbon. The solution was lyophilized to give 700 mg of white powder. The powder was dissolved in 20 ml of water and passed through a column of CM-Sephadex (H^+ cycle, 0.5 liter). The elution was carried out with water. The active fractions were collected and concentrated to a volume of 10 ml. The concentrate was subjected to a column chromatography of Sephadex G-15 (500 ml) and developed with water. The active fractions were combined and lyophilized to give 70 mg of FK-156 in the form of white powder.

Fig. 1. Extraction and purification procedure.



Physicochemical Properties of FK-156

FK-156 is a white powder which decomposes at 143~148°C. It is soluble in water, slightly soluble in methanol, and almost insoluble in other organic solvents. Its Rf values on TLC are listed in Table 2. Color reactions are as follows: Positive in ninhydrin, potassium permanganate, and iodine tests, negative in DRAGENDORFF and EHRLICH tests.

Measurement of optical rotation gave $[\alpha]_D^{25}$ $-30.0°$ (c 0.5, H_2O). Potentiometric titration gave pKa' values of 4.3, 5.8, 6.3 and 10.9 (67% DMSO). Elemental analysis indicated the following composition:

Calcd. for $C_{20}H_{33}N_5O_{11}$: C 46.24, H 6.36, N 13.49
Found C 46.10, H 6.43, N 13.43

Table 2. Chromatographic behavior of FK-156.

TLC	Solvent system	Rf
Cellulose	*n*-Butanol - acetic acid - water (4: 1: 2)	0.35
Silicagel	*iso*-Propanol - water (6: 4)	0.65

Field desorption mass spectroscopy gave M+1 peak at 520, thus establishing the molecular formula. The IR and NMR spectra of this compound are shown in the succeeding paper[2]. Amino acid analysis indicated the presence of the following amino acids in equimolar ratio; glycine, glutamic acid, alanine, and α,ε-diaminopimelic acid.

Biological Properties

Protective Efficacy in Experimental Infections in Mice

Table 3 shows the protective efficacy of FK-156 in experimental infection in mice with *Escherichia coli* strain No. 22. Cells of *Escherichia coli* No. 22 were cultured overnight at 30°C in Difco nutrient broth. The fully grown culture was diluted in fresh nutrient broth ($\times 1/10$) and incubated at 30°C for further 2 or 3 hours. When the cell density of 1×10^7/ml was obtained the culture was used to infect experimental animals. One-fifth ml of the culture was injected intraperitoneally to mice. This challenge dose killed the untreated mice within 48 hours of infection. FK-156 was administered subcutaneously to mice 1, 4, 5 and 6 days before the infection. The numbers of the dead and survived mice were recorded for 2 days after the infection. As can be seen from Table 3, treatment of mice with FK-156 afforded marked protection against the rapidly lethal infection. All mice survived when FK-156 was administered at a dose as low as 1 μg/mouse/day ($\times 4$).

Enhancing Activity on Delayed Type Hypersensitivity (DTH) and Humoral Antibody Production in Guinea Pigs

Delayed type hypersensitivity experiments were performed as described by AUDIBERT *et al.*[3].

Table 3. Protective efficacy of FK-156 and MDP in experimental infection in mice with *E. coli* No. 22.

Exp. No.	Compound	Dose (mg/kg/day)*1	Survivor/infected*2
I	FK-156	0.01	10/10
		0.003	10/10
		0.001	10/10
		0.0003	8/10
		0.0001	4/10
	Vehicle	—	0/10
II	FK-156	0.001	8/8
		0.0001	1/8
	MDP*3	0.01	1/8
		0.001	0/8
	Vehicle	—	0/8

*1 Compound was adminstered subcutaneously 6, 5, 4 and 1 day before infection.
*2 One fifth ml of the culture of *E. coli* No. 22 (2×10^7/ml) was injected intraperitoneally to mice. Fourty eight hours after the infection, survival was counted.
*3 *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (Muramyl dipeptide)

Briefly, a half mg of ovalbumin was emulsified with FK-156 in Freund's incomplete adjuvant (0.1 ml). Guinea pigs given 0.1 ml of this emulsion in both posterior footpads were skin tested on day 14 and bled on day 16. The skin test was performed by intradermal injection of 5 μg of antigen on the back. Skin reaction of the test sites was measured 48 hours after the antigen challenge.

The titration of antibody was carried out with sheep blood cells which were coated with ovalbumin by the use of chromium chloride. The antibody titer was expressed as the reciprocal of the highest dilution of serum evoking the threshold hemagglutination or hemolysis. As shown in Table 4, FK-156 strongly enhanced both humoral antibody production and DTH reaction. The optimal dose of the drug in enhancing the responses is $1 \sim 10$ μg/site. The higher dose, 100 μg/site, showed only a weak stimulatory activity.

Table 4. Enhancing activities on cellular immunity and humoral antibody production in guinea pigs.

Dose (μg/site)	Cellular immunity*2 (mm diameter)	Humoral immunity	
		Hemagglutinin*3 titer ($\log_2$)	Hemolysin titer*3 ($\log_2$)
0	0	9.1±0.19	4.5±0.45
0.1	8.2±2.8*	9.9±0.10*	6.4±0.76
1	14.5±2.1*	11.5±0.61*	7.7±0.64*
10	11.0±1.3*	12.2±0.56*	7.1±0.58*
100	4.2±1.7*	10.0±1.01	5.9±0.89

* $P<0.01$

*2 Ovalbumin (500 μg/50 μl saline) was emulsified in Freund's incomplete adjuvant (50 μl) with the drug and injected to posterior foot pads of Guinea pigs. Fourteen days later, 5 μg of antigen was injected intradermally and diameter of erythema appearing in 48 hours was measured.

*3 Guinea pigs were bled 16 days after antigen challenge. Hemagglutinin and hemolysin titers are expressed as logarithm of the number of highest dilutions causing hemagglutination and hemolysis of antigen-coated SRBC, respectively.

Mitogenic Activities on Murine Splenic Lymphocytes

Spleen cells prepared from Balb/c mice (female, 9 week old) were cultured in microtiter plates, each well containing 5×10^5 cells in a volume of 0.2 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum. Graded concentration of FK-156 was added to the culture. After 48 hours of incubation at 37°C, the cultures were pulse-labeled with 1 μCi/well of [^{3}H]thymidine. Cells were harvested 24 hours later by the use of cell harvester and the incorporation of [^{3}H]thymidine into cells was determined with a liquid scintillation counter.

As shown in Table 5, FK-156 significantly increased the incorporation of labeled thymidine in mouse spleen cell culture. The maximal response was observed at a concentration of 100 μg/ml of FK-156.

Table 5. Mitogenic activity on mouse spleen cells.

Concentration (μg/ml)	[^{3}H] Thymidine uptake* net cpm	Stimulation index
100	2,028	4.9
10	1,486	3.6
1	835	2.0
0	407	1.0

* Mouse spleen cells (5×10^5/0.2 ml/well) were cultured in the presence of graded concentration of FK-156. After 48 hours, the cultures were pulse-labeled with 1 μCi/well of [^{3}H]thymidine for 24 hours.

Blood Stream Clearance of Carbon

Carbon clearance test was carried out according to the method of STOSSEL and COHN[4].

Table 6. Effect of FK-156 and MDP on blood clearance of carbon in mice.

Drug	Dose (mg/kg)	K value[1)]	$K_{treated}/K_{control}$
Vehicle	—	0.0206[2)]±0.0026	
FK-156	100	0.0402±0.0033***	1.95
	10	0.0367±0.0044**	1.78
	1	0.0303±0.0037*	1.47
	0.1	0.0253±0.0026	1.23
MDP	100	0.0251±0.0021	1.22
	10	0.0224±0.0028	1.09
	1	0.0221±0.0031	1.07
	0.1	0.0204±0.0022	0.99

* $P<0.05$ ** $P<0.01$ *** $P<0.001$

1) Ten μl of the carbon particle suspension (34 mg/ml) was injected intravenously to mice. Three and 6 minutes after the injection, blood samples were collected and concentration of carbon was measured. The phagocytic coefficient, K, was calculated as mentioned in the text.

2) Mean±S.E. of ten mice/group.

Zeichentushe drawing ink (Rotring Werke, West Germany) was diluted in saline containing 1% of gelatine to make a final concentration of 34 mg/ml. Ten microliter/g of body weight of the suspension was injected into mice (*dd*Y, male, 5~6 week old) *via* tail vein. Three and 6 minutes after the injection, 50 μl of blood samples were removed from retro-orbital venous plexus by the use of capillary pipettes. The blood samples were immediately discharged into 3 ml of sodium carbonate solution to disrupt red blood cells and the amounts of carbon were measured in a spectrophotometer at 660 nm. The density readings were converted to a logarithmic scale and plotted against time. The phagocytic coefficient, K, was calculated according to the following relationship:

$$K=\frac{(\log C_1-\log C_2)}{T_2-T_1}$$

in which T_1 and T_2 are the time in minute when the samples were withdrawn and C_1 and C_2 represent the concentration of carbon. FK-156 was administered subcutaneously 24 hours before carbon clearance test. As shown in Table 6, the phagocytic coefficients were elevated in mice treated with the drug compared with that of control animals.

Anticancer Activity on Transplantable Tumor

Cells of ascites hepatoma AH 66 were suspended in HANK's solution at a density of 1×10^5 cells/ml. 0.5 ml of the suspension was implanted intraperitoneally in Donryu rats (female, 6 week old). FK-156 was administered 5 successive days starting 9 days before the tumor cell inoculation. Life span of control animals was less than 13 days. Intraperitoneal administration of FK-156 at dosages of 0.1, 0.3 and 1.0 mg/kg/day resulted in a prolongation of the survival time of tumor-transplanted animals. At day 30 after tumor transplantation, 70% of the rats treated with these doses survived, whereas none of the animals treated with 0.03 mg/kg/day or saline survived.

Acute Toxicity of FK-156 in Mice

FK-156 exhibits low toxicity in mice. LD_{50} value is greater than 1 g/kg when administered intravenously to ICR mice (male and female, 6 week old).

Discussion

A screening program has been elaborated in our research laboratories for the discovery and isolation of microbial products which enhance the resistance of experimental animals against bacterial infection. FK-156 was discovered in this screening program. It enhances the resistance of experimental animals against microbial infection and tumor transplants and also stimulates humoral antibody production and delayed type hypersensitivity reaction when administered with antigens. It also enhances phagocytic function of reticuloendothelial systems of animals. The acute toxicity of this compound is low. As shown in the succeeding paper[2], the structure of this compound is D-lactyl-L-alanyl-γ-D-glutamyl-(L)*meso*-α,ε-diaminopimelyl(L)-glycine.

Recently, tetrapeptide composed of D-alanine, D-glutamic acid, LL-diaminopimelic acid and glycine has been found in the mycelial extract of a *Streptomyces* strain[5]. Conjugation of the peptide with lauric acid yielded lauroyl tetrapeptide (LTP) which possesses immunostimulatory activity. The structure of LTP resembles that of FK-156. However, considerable differences are also observed; in case of LTP, diaminopimelic acid is of LL configuration and glycine is bound to amino terminal of diaminopimelic acid, whereas FK-156 has *meso* diaminopimelic acid and glycine is bound to carboxyl terminal.

Small molecular microbial products with immunostimulatory activity have also been reported and examined for their efficacy in the treatment of immune deficiency, cancer and infection[6].

Cell walls or cell wall skeletons of some microorganisms, such as *Mycobacterium*[7], *Corynebacterium*,[8] and *Nocardia*[9] have been known to possess immunostimulatory activity in experimental animals and man. Some of them are now on clinical trials for their activity as cancer immunotherapeutic agents. Peptidoglycans of the cell walls have strong adjuvant effect on humoral antibody production and delayed type hypersensitivity reaction against various antigens. The minimal effective structure responsible for the immunoadjuvant activity of the peptidoglycans has been revealed to be *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP)[10].

The structure of FK-156 is related to the peptide moiety of MDP. It is interesting that FK-156, an acyl peptide without aminosugar moiety, has as strong activities as those of MDP. Elucidation of the structure-activity relationship of the derivatives of FK-156 showed that glutamyl-diaminopimelic acid is the minimal and essential structure for the activities in this series of compounds.

FK-156 and its derivatives are now under preclinical evaluation in experimental infections and transplantable tumor systems. The results will be published elsewhere.

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