In Vitro Antitumor Activity of Polymyxin Acylase from *Pseudomonas* sp. M-6-3

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Polymyxin acylase from *Pseudomonas* sp. M-6-3 can deacylate not only polymyxin group antibiotics, but also the long-chain fatty acyl group of proteins and peptides. We found the *in vitro* antitumor activity of polymyxin acylase against murine and human tumor cells, especially KB cells. The mechanism of the antitumor activity remains equivocal, but we speculate that it may result from the affinity of polymyxin acylase for long-chain fatty acyl proteins in human carcinoma cells.

Key words polymyxin acylase; antitumor activity; KB cell

Since Broome *et al.*¹⁾ proved the antitumor property of guinea-pig serum in 1961, several L-asparaginases have been purified from microorganisms and used clinically in the treatment of leukemia and lymphosarcoma. However, use of these microbial enzymes is limited because the preparations show immunological side effects ranging from mild allergic reactions to anaphylactic shock. In addition to asparaginase,²⁾ several other enzymes have been observed to inhibit tumor growth, such as glutaminase,³⁾ arginase,⁴⁾ leucine dehydrogenase,⁵⁾ and phenylalanine ammonia-lyase,⁶⁾ all of which have an effect on amino acid metabolism. Carboxypeptidase G₁,⁷⁾ a folate-cleaving bacterial enzyme, and ascorbic acid oxidase,⁸⁾ which inhibits the growth and DNA synthesis of tumor cells, also have been reported.

In an earlier study,⁹⁾ we found polymyxin acylase from *Pseudomonas* sp. M-6-3 to be useful for preparing semi-synthetic polymyxins, and we purified it to homogeneity. This enzyme can deacylate not only polymyxin group antibiotics, but also the long-chain fatty acyl group of proteins and peptides. Recently, many proteins which possess the capacity to be acylated have been reported to be important in cell growth and cellular regulation, and the membrane binding mediated by protein acylation has been found to direct the protein phosphorylation by protein kinase C.¹⁰⁾ In the present work, we investigated the cytotoxicity to clarify the physiological function of polymyxin acylase and found the *in vitro* antitumor activity of polymyxin acylase against murine and human tumor cells. This is the first report of *N*-acyl amino acylase group enzymes inhibiting the growth of human tumor cells. This paper describes our findings on the antitumor activities of polymyxin acylase.

MATERIALS AND METHODS

Preparation of Polymyxin Acylase Previously, polymyxin acylase was purified to homogeneity from an acetone-dried cell powder of *Pseudomonas* sp. M-6-3.⁹⁾ We improved the solubilization and purification methods, using colistin treatment as a solubilized reagent and employing hydrophobic chromatography. The cell-free

enzyme solubilized by colistin was further purified by four-stage chromatography on DEAE-cellulose, hydroxyapatite, butyl-Toyopearl and Superdex 200 columns. By these procedures, three kinds of polymyxin acylase (types α , β , and γ) were obtained.

Enzyme Assay Acyl amino acid acylase activity and glutaminase activity were assayed using the ninhydrin color spot of the liberated amino acid on TLC plate. The reaction mixture (100 μ l) containing 5 μ mol of acyl amino acid or glutamine and the enzyme solution (25 μ l) in 20 mM Tris buffer (pH 8.0) was incubated at 37 °C for several hours. A 1- μ l portion of the reaction mixture was developed by TLC on a silica gel plate [solvent system: upper layer of *n*-BuOH–AcOH–H₂O (4:1:3) containing 1/20 pyridine by volume]. The amount of amino acid liberated was measured by the ninhydrin method with Chromoscan 200/201 (Joyce-Loebl, England). During enzyme purification, this assay method was used with *N*-octanoyl-DL-glutamic acid as the substrate. The amount of liberated amino acid in the mixture was measured colorimetrically by the ninhydrin method. One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μ mol of glutamic acid from *N*-octanoyl-DL-glutamic acid per min.

***In Vitro* Antitumor Activity of Polymyxin Acylase** The tetrazolium-based semiautomated colorimetric assay (MTT assay) developed by Carmichael *et al.*¹¹⁾ was modified and used for the *in vitro* assay. Briefly, 2000 cells in 180 μ l of RPMI-1640 medium (Nissui Pharmaceutical Co., Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco Lab., Grand Island, NY, U.S.A.), penicillin G (100 units/ml) and streptomycin (100 μ g/ml) were seeded in a 96-well flat bottom microtest plate (InterMed, Roskilde, Denmark), and 20 μ l each of the drug solutions of graded concentrations was simultaneously added to each well for triplicate samples. The plate was incubated for 3 d at 37 °C in a humidified atmosphere of 5% CO₂. MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] (Sigma Chemical Co., St. Louis, MO, U.S.A.) was prepared at a concentration of 2 mg/ml in Dulbecco's phosphate buffered saline without calcium and magnesium and stored

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in a refrigerator. On the third day, MTT reagent (25 μ l) was added to each well. After another 4-h incubation at 37°C, the microplate was centrifuged at 1500 rpm for 10 min and the medium was removed by aspiration. To solubilize the resulting MTT-formazan, 0.2 ml of dimethylsulfoxide was added to each well, followed by thorough mixing with a mechanical plate mixer. Absorbance at 540 nm (OD_{540}) was measured with an ImmunoReader NJ-2000 (InterMed Japan, Tokyo, Japan). The percentage of cell growth inhibition was calculated by the following formula: % of cell growth inhibition = $(1 - T/C) \times 100$, where C is the mean OD_{540} of the control group and T is that of the treated group. IC_{50} value was determined graphically from the dose-response curve with at least three drug concentration points.

RESULTS AND DISCUSSION

Polymyxin acylase from the *Pseudomonas* M-6-3 strain is a unique enzyme which can be called a long-chain fatty acyl peptide deacylase. The substrate specificity for the peptide moiety is broad, while that for the acyl moiety is rather specific. The enzyme was particularly active for caprinoyl, lauroyl, and myristoyl groups. Recently, we found that the composition of the medium which was used for the growth of the *Pseudomonas* sp. M-6-3 strain affected the properties of polymyxin acylase such as substrate specificity. In the present experiment, we used polymyxin acylase which had been produced by a medium containing peptone as the sole source of carbon. We therefore named it polymyxin acylase P to differentiate it from the formerly reported polymyxin acylase we call polymyxin acylase C, which was produced by a medium containing citrate as the sole source of carbon. The cytotoxicity of polymyxin acylase P was greater than that of polymyxin acylase C; their IC_{50} values were 2.3 μ g/ml (P) and 10.5 μ g/ml (C) against the growth of L5178Y cells. This relationship was also true for the enzyme activity; 4.5 units/mg (P) and 3.2 units/mg (C), respectively. Polymyxin acylase P was separated into three fractions (P- α , P- β , and P- γ) on a butyl-Toyopearl column. Their acylase activities were 5.2 units/mg (P- α), 3.2 units/mg (P- β), and 0.23 units/mg (P- γ), respectively. The purpose of this study was to identify the type of polymyxin acylase worthy of more detailed study.

To investigate the *in vitro* antitumor activity of polymyxin acylase, murine leukemia cells and a variety of human neoplastic cells were used. As shown in Table 1, polymyxin acylase P- α was the most effective (IC_{50} : 0.58 μ g/ml) among the three kinds of polymyxin acylase P against the growth of KB cells. The growth inhibitory effect of polymyxin acylase P- α on KB cells was compared with L-asparaginase (5000 kU/mg, Lot. 40803-00) from *Escherichia coli* which was kindly donated by Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. The IC_{50} value of L-asparaginase was 1.15 μ g/ml.

We also investigated the inhibitory effects of polymyxin acylase P- α on the growth of various murine and human tumor cell lines *in vitro*. Table 2 shows that polymyxin acylase P- α has remarkable antitumor activity.

Table 1. Inhibitory Effects of Polymyxin Acylase P on the Growth of KB Cell and L5178Y Cell *in Vitro*

Polymyxin acylase	IC_{50} (μ g/ml)	
	KB cells	L5178Y cells
P- α	0.58	2.1
P- β	2.8	1.4
P- γ	> 10	> 10

Table 2. Inhibitory Effects of Polymyxin Acylase P- α on the Growth of Various Murine and Human Tumor Cell Lines *in Vitro*

Cell line	Origin	IC_{50} (μ g/ml)
L5178Y	Murine leukemia	2.1
L1210	Murine leukemia	0.95
KB	Human epidermoid carcinoma	0.58
NPC-TY861	Human epipharynx squamous carcinoma	0.75
PC-3	Human lung adenocarcinoma	4.6
PC-8	Human lung adenocarcinoma	2.1
KATO-III	Human stomach adenocarcinoma	1.2

In 1982, Carr *et al.*¹²⁾ first discovered that myristate became covalently bound through an amide bond to the NH_2 -terminal glycine residue of a protein in the catalytic subunit of adenosine 3',5'-phosphate-dependent protein kinase type II. Subsequently, Garber and Hanafusa¹³⁾ showed that pp60^{V-SRC}, which is the transforming protein in Rous sarcoma virus (RSV²)-infected cells, has a covalently bound myristate at the NH_2 -terminal glycine residue of the protein, and its myristoyl group is required for the transformation activity and for plasma membrane association.

Chow *et al.*¹⁴⁾ reported that poliovirus and other picornavirus particles are specifically modified by having myristate covalently bound to a capsid protein. Buss *et al.*¹⁵⁾ reported that myristate associated with α subunits of G protein is linked to the polypeptide through an amide bond and that myristate may play an important role in stabilizing the interactions of G proteins with phospholipids or with membrane-bound proteins.

Shoji *et al.*¹⁶⁾ synthesized *N*-fatty acyl glycinal derivatives which strongly inhibited the myristoylation of pp60^{V-SRC} and suggested that the lengths of both the fatty acyl and glycyl moiety of the compounds are very important for the prevention of protein myristoylation, and that nonmyristoylated pp60^{V-SRC} reduces tumorigenicity.

Turuta *et al.*¹⁷⁾ also clarified that polymyxin acylase (commercial product; Wako Pure Chemical Industries, Ltd.) is capable of liberating the NH_2 -terminal myristate from *N*-myristoyl-Gly-Ser-Ser-Lys, the NH_2 -terminal tetrapeptide of pp60^{V-SRC}, at 3.59 μ mol/min/mg. Previously, we investigated the possibility of the deacylation of *N*-octanoyl-lysozyme by polymyxin acylase C and detected octanoic acid, which had been liberated.¹⁸⁾ These findings led to the speculation that polymyxin acylase can inhibit the growth of human carcinoma cells as an NH_2 -terminal myristate-releasing enzyme.

L-Asparaginase from *Escherichia coli* is presently being used in the treatment of acute lymphoblastic leukemia as

an anticancer agent. Asparaginase-sensitive tumor cells generally show a diminished capacity to synthesize L-asparagine.

In 1974, Schmid and Roberts¹⁹⁾ demonstrated that glutaminase-asparaginases were effective against asparaginase-resistant cells and in asparaginase-resistant patients. Ohshima *et al.*²⁰⁾ showed that an isozyme of glutaminase from *Pseudomonas aeruginosa*, glutaminase A, was highly inhibitory to Ehrlich ascites carcinoma *in vivo*, because glutaminase A hydrolyzes both L-glutamine and L-asparagine, but that glutaminase B has essentially no asparaginase activity.

Polymyxin acylase (P- α) acted on L-glutamine (4.9 μ mol/min/mg), but scarcely on L-asparagine. On the other hand, glutaminase did not act on polymyxin. Therefore, polymyxin acylase is clearly different from glutaminase A.

We have speculated that the antitumor activity of polymyxin acylase results from its acting as a myristate-releasing enzyme against human carcinoma cells or from hydrolyzing L-glutamine, as does glutaminase A from *Pseudomonas aeruginosa*. Furthermore, we investigated whether or not glutamine would affect the inhibitory effect of polymyxin acylase (P- α) on the growth of KB cells using RPMI-1640 medium with or without glutamine, but found no significant difference (the IC₅₀ was 0.58 with glutamine and 0.76 without it). This study showed that polymyxin acylase (P- α) would be worthy of a more detailed study. Furthermore, work is necessary to elucidate the actual mechanism of its antitumor effect and physiological function.

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