ISOLATION OF A NEW ANTITUMOR SUBSTANCE FROM *BACILLUS STEAROTHERMOPHILUS*

Yasuhiro KOHAMA,* Tetsuyuki TERAMOTO, Kentaro IIDA, Norihito MURAYAMA, Taro SEMBA, Noriko TATEBATAKE, Yuzo KAYAMORI, Kazutake TSUJIKAWA and Tsutomu MIMURA

Faculty of Pharmaceutical Sciences, Osaka University, Yamadaoka 1-6, Suita, Osaka 565, Japan

A new antitumor substance, BS-1, was isolated from the autolysate and culture filtrate of *Bacillus stearothermophilus* UK563 by ethylacetate extraction and HPLC. BS-1 inhibited the proliferation of mouse macrophage-like cells, P388-D1 (IC₅₀: 4 µg/ml) and mouse mastocytoma, P-815 (IC₅₀: 0.6 µg/ml), but not that of Balb/c 3T3.

KEYWORDS *Bacillus stearothermophilus*; thermophile; antitumor substance; P388-D1; P-815

Recently, great interest has developed in the application of thermostable enzymes produced by the thermophile for bioreactor, biosensor and clinical reagents, in addition to its uses in industrial technology.¹⁾ Thermophile contains a variety of components such as lipids and polyamines which are chemically different from those of mesophile.²⁾ In connection with our studies on products of thermophile with potentially useful biological activity, we focused on *B. stearothermophilus* which has much short generation time (11min).³⁾ In this paper, we present the isolation of antitumor substance from the autolysate and culture filtrate of *B. stearothermophilus* UK563.

EXPERIMENTAL

Isolation *B. stearothermophilus* UK563 (Fermentation Research Institute, Deposit No. 7275) was cultured in a 500-liter continuous fermenter at 60°C at a flow rate of 400 l/h in a medium (adjusted to pH 7.0) of the following composition: glucose (0.35%), yeast extract (0.3%), pepton (0.1%), KH₂PO₄ (0.2%), Na₂HPO₄ (0.2%), MgSO₄·7H₂O (0.1%), FeSO₄·7H₂O (0.005%), MnSO₄·H₂O (0.0001%) and Na₂MoO₄ (0.0001%).⁴⁾ After 4 days culture, the culture broth was separated into cells (approximately 50 g) and fluid (approximately 10 l) by centrifugation. The collected cells were autolyzed by incubating in 25 mM phosphate buffer (pH 8.0)-2 mM ethylenediaminetetraacetic acid at 40°C for 2 h. Cell debris was removed by centrifugation. The supernatant was concentrated to 1/5 volume *in vacuo* and extracted with ethylacetate. After removal of the solvent, the extract was dissolved in deionized water and activated ODS resin (Waters, 55-105 µm) was added. The ODS resin recovered by filtration on a glass filter was washed with 0.5% CH₃CN and eluted with 18% CH₃CN. The 18% CH₃CN fraction was evaporated *in vacuo*, and approximately 40 mg of oily residue containing the substance that inhibits the proliferation of mouse macrophage-like cells, P388-D1, was obtained from 1 kg of the mycelium. The oily residue of the 18% CH₃CN fraction was dissolved in 0.05% HCl and subjected to preparative HPLC on a Cosmosil 10C₁₈ column. A sample of the column effluent was assayed for antitumor activity. An antitumor substance in the culture filtrate as well as the autolysate was concentrated by extraction with ethylacetate and treatment with ODS resin, and purified by HPLC on the ODS column.

Antitumor Activity Mouse mastocytoma, P-815, and P388-D1 were supplied by the Japanese Cancer Research Resources Bank. Culture medium was RPMI 1640 (Nissui) supplemented with 50 µM 2-mercaptoethanol, 100 units/ml penicillin, 100 µg/ml streptomycin and 10 % fetal calf serum. The cells (2x10⁵/ml) were incubated with samples in 24-well multiplates (Sumilon) for 48 hr at 37 °C. A viability test was carried out by a trypan blue dye exclusion method.⁵⁾ Bleomycin A₂ (BLM) and 5-fluorouracil (5-FU) were used as positive controls.

RESULTS AND DISCUSSION

An active component in the autolysate of the strain UK563 for inhibition of the proliferation of P388-D1 was purified by HPLC on Cosmosil 10C₁₈ (Fig. 1a). The activity was recovered in a peak which eluted at 26.5 min. The homogeneity was

Fig.1 Separation of Antitumor Substance on HPLC

Arrows indicate peaks with antitumor activity.

a) Column (Cosmosil 10C₁₈): 0.46x25cm, solvent: 5-30 % CH₃CN in 0.05% HCl, flow rate: 1 ml/min, sample: 18% CH₃CN fraction in 100 μ l of 0.05% HCl (equivalent to approximately 10 g of the mycelium).

b) Column (Asahipak GS-320): 0.76x50cm, solvent: 50 mM CH₃COONH₄ (pH6.7)/CH₃CN (90:10), flow rate: 1 ml/min, sample: active fraction from Cosmosil in 100 μ l of solvent.

confirmed by another HPLC of the final preparation on Cosmosil 5C₁₈-AR (0.46x25cm) under the same condition as shown in Fig. 1a and on Asahipak GS-320 as shown in Fig. 1b. The final preparation was designated as BS-1. The antitumor activity in the culture filtrate of UK-563 was also derived from BS-1: because a single peak of absorbance co-eluting with antitumor activity in a final purification step showed the same retention time as that of BS-1. The yield of BS-1 was approximately 50 μ g from each of the cells (1 kg) and the culture filtrate (200 l). Therefore, BS-1 must be a secondary metabolite of the strain

Table I. Antitumor Activity *in Vitro* of BS-1

Sample	Concentration (μ g/ml)	Inhibition percent of proliferation		
		P-815	P-388-D1	Balb/c 3T3
BS-1	0.5	45	1	0
	1	66	5	0
	2.5	99	25	0
	10	—	87	0
	25	—	—	29
BLM	1.5	94	14	77
5-FU	0.01	63	10	21

UK563. BS-1 dose-dependently inhibited the proliferation of P388-D1 at doses ranging from 0.5 to 10 $\mu\text{g/ml}$ (Table I), and its 50% inhibitory concentration (IC_{50}) was 4 $\mu\text{g/ml}$. On the other hand, BS-1 inhibited the proliferation of mouse whole embryo, Balb/c 3T3 by 29% at 25 $\mu\text{g/ml}$. The ratio of the IC_{50} against Balb/c 3T3 to the IC_{50} against P388-D1 was more than 6. BS-1 also was markedly antitumor against P-815 (IC_{50} :0.6 $\mu\text{g/ml}$) (Table I), and the ratio of IC_{50} against Balb/c 3T3 to IC_{50} against P-815 was more than 40. So far as we know, this is the first report with respect to an antitumor substance in products of thermophile. Physicochemical properties will be presented shortly. Further, the action mechanism and antitumor activity *in vivo* of BS-1 should be studied.

ACKNOWLEDGEMENT We are grateful to Nippon Kayaku Co.Ltd., for the supply of bleomycinA₂.

REFERENCES

- 1) H. Kondo and K. Nagata, *Kagaku to Kogyo* (Tokyo), 60, 428 (1986); K. Tomita, *Bio Industry*, 3, 707 (1986).
- 2) S. M. Friedman, "Biochemistry of Thermophile," Academic Press, New York, 1978.
- 3) T. D. Brock, *Science*, 158, 1012 (1967); T. Saiki, *Protein Nucleic Acid Enzyme*, 20, 188 (1975).
- 4) K. Tomita, K. Nagata and H. Okuno, "Free Radicals in Chemical Medicine," Vol 1, ed. by M. Kondou, Nipponigakukan, Tokyo, 1987, p83.
- 5) J. A. Metcalf, J. I. Gallin, W. M. Nauseef and R. K. Root, "Laboratory Manual of Neutrophil Function," Raven Press, New York, 1986.

(Received August 1, 1991)