A Study of a New Antitumor Substance, Cellostatin

I. On the Isolation and Some Properties of Cellostatin

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In recent several years, attempts to search antitumor substance in actinomycetes antibiotics have been tried, and it has been confirmed that the culture filtrates of some streptomyces strains produce the active agents on the growth of a variety of mouse and rat tumors. The study on the screening of these substances necessitates the rapid, sensitive, efficient and quantitative methods by which the antitumor activity is estimated.¹⁾ Also, the methods are required strongly from stand points of extraction of the active agents in the metabolic filtrates of streptomyces.

Although the correlation between antibacterial and antitumor effects are not able to be prematurely concluded²⁾, some substances³⁾, for example Azaserine⁴⁾ and Puromycin⁵⁾ may possess both, antimicrobial and antitumor properties. Consequently, it is worthy to utilize its antimicrobial activity as an indicator for the isolation of the tumor-inhibiting agents.

The author has isolated a new antitumor substance "Cellostatin" produced by a strain of Streptomyces against the Ehrlich ascites carcinoma, which is active also to some yeast and fungi. Therefore, the isolation of antitumor agents was plotted with the antifungal activity as an indicator.

In this paper, isolation and some properties of this substance are reported.

EXPERIMENTAL

Taxonomic Studies

A strain (our collection No. E-150) of Streptomyces was isolated from soil samples collected at Omiyamachi, Ibaragi prefecture. The strain was a species of streptomyces and seemed to be related to *Str. flocculus* at first. However, its soluble pigment in organic media was brown to dark brown, and in this respect it has no similarities to any of the streptomyces

species hitherto described. Some similarity was able to be found in the description of *Str. lavendulae*. But in the respects, such as it does not produce a lavender color and characteristic spirals, this strain differed from *Str. lavendulae* too. The results of the mycological studies are shown in Table I. From the reason mentioned above cellostatin-producing strain, E-150, is deemed to be a new species and is named *Str. cellostaticus*, n. sp.

Production of the Substance

The antibiotic was produced in the medium containing 2.5% starch,

TABLE I
Mycological Properties of No. E-150 Strain

Cultural characteristic	Media	Growth	Aerial mycelium	Soluble pigment	Remarks
	Glucose-asparagine agar	Colorless to pale yellow (7-19-2)	Thick, abundant, cottony like, white to reddish grey (1-16-1)	None	
	Nutrient agar	Dark brown (4-12-2)	White to greyish white	Blackish brown	
	Yeast extract agar	Some elevated, pale yellowish brown	Abundant, thick, cottony, light purplish gray (24-17-1)	Faint brown	
	Ca-malate agar	Colorless to pale yellow (7-19-2)	abundant, velvety, light purplish grey (24-18-1)	None	
	Czapek's agar	Glistening, pale yellowish brown (6-18-3); center of colony elevated	White	None or dull yellow orange (6-18-4)	
	Starch agar	Thin, colorless	Abundant, white	None	
	Potato	Spreading, thick, much wrinkled, blackish	Abundant, cottony, white	Color of plug: Black	
	Carrot	Spreading, thick, wavy, faint brown	Abundant, white	Color of plug: Dark brownish	
	Egg media	Very much wrinkled in margin, dark yellowish brown (7-13-2)	White in center	Dark brown	

	Gelatin	Thin, colorless	Thin, white	Brown colored liquefied portion	Liquefaction: about 10.1 mm of depth of gelatin in tube is liquefied in 30 days
	Milk 37°C				Coagulation: Rapid (7 days), clot formation. Peptonization: Rapid (8–15 days), clearing up all the milk. Change of reacto: Alkaline (pH 8.0)
	Tyrosine medium Czapek's solution	Ring formation on the dry portion of tube, thick colorless	abundant, some powdery, white to grayish white	Pale orange (4–19–3)	Tyrosinase: not formed Nitrites formation: Excellent
Morphology	Spirals Conidia	Glucose-asparagine agar: None, a few spirals may be formed at times Spherical or oval.			
Utilization test of various carbon sources	Utilization	Rhamnose, lactose, raffinose, inulin, inositol, sodium citrate, L-xylose, maltose, glycerol, glucose, starch, sucrose, D-fructose, D-mannitol, L-arabinose, sodium acetate			
	No utilization	Phenol, D-sorbitol, dulcitol, sodium succinate, D-galactose, salicin			

3.5% soy bean meal, 0.5% dried yeast, 0.25% NaCl, 0.005% $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.0005% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.005% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.35% CaCO_3 , (pH 7.2) under the shaking condition.

The potency was determined by the pulp disc method and broth dilution method with *Candida albicans* (M-9) as test organism. But the border line of the inhibition circle produced by cellostatin on candida plate was turbid and indistinct. So the diffusion method was not accurate for assay.

The maximal production of the antibiotic was obtained on the 4th to 5th day of incubation with pH 7.4; its potency was 1:8–16 dilution unit against *Candida albicans*, and about 200mcg/ml of final substance was obtained from this filtrate.

Purification

The mycelium was removed from the fermentation broth by filtration at pH 6.0. The clear filtrate was passed through IRC-50 cation exchange resin column (hydrogen form) corresponding to 1/30 volume of

Fig. 1

the filtrate at pH 7.5 by s.v. 5–6 in order to adsorb almost all active agents. The column was washed with water to remove impurities, and the substance was eluted with 80% aqueous acetone, and further with 80% acetone containing 0.2 *N* HCl at s.v. 1–2. At that time, 2nd substance (active against *S. lutea*) was eluted mainly in 80% aqueous acetone fraction, while 1st substance (active against *C. albicans*) in 80% acetone containing hydrochloric acid.

As shown in the summarized and electrophoretic papergram (Fig. 1), these two substances are different from one another. By the further experiments of antitumor activity, it was confirmed that the 1st substance exhibited the destructive effect against Ehrlich ascites carcinoma, while the 2nd substance no activity. Consequently, the extraction of antitumor agents was followed using the antifungal activity as an indicator; the 1st substance was designated Cellostatin. The potent fractions eluted by 80% acetone containing hydrochloric acid were combined, adjusted to pH 6.0 with NaOH, concentrated in vacuo, and dried. The brownish residue was extracted with anhydrous methanol, and the insoluble impurities were filtered off. The methanolic extract was dried in vacuo and again taken up in an hydrous methanol. These processes were repeated two or three times. The resulting active residue was dissolved in acidic methanol and precipitated with acetone. The crude antibiotic was further purified by Al_2O_3 and carbon (Darco G-60) chromatography after pretreatment with H_2SO_4 according to Cartor *et al.*⁶⁾ In this way, sulphate of Cellostatin was obtained as a colorless platelet crystals. Crystalline reineckate (d. p. 156–166°C) was also isolated from the pure H_2SO_4 salt.

Chemical and Physical Properties

Cellostatin was a basic substance, very soluble in water, slightly soluble in methanol, but insoluble in ethanol, butanol, acetone, ether, and other organic solvents. It is precipitated from its aqueous solution by picric acid, Reinecke acid, and phosphotungstic acid. The antibiotic is very stable at pH 7.0, 5.0 and 2.0 and relatively unstable at pH 9.0.

The sulfate crystal is colorless platelets and the melting point of its reineckate is 156–166°C (decomp.). It indicated positive reaction in ninhydrin, Sakaguchi and Molisch, but the negative in biuret, Millon, Fehling, Maltol, FeCl_3 , and Elson-Morgan test. The elemental analysis indicated: C 22.10%, H 4.52%, N 13.15%, O 27.77%.

As shown in Fig. 2, the ultraviolet absorption spectrum represented that max $E_{1\text{cm}}^{1\%}$ value is 115 at 265 $m\mu$ and min $E_{1\text{cm}}^{1\%}$ value 98 at 245 $m\mu$ in distilled water. The infrared absorption spectrum of the antibiotic is shown in Fig. 3.

Fig. 2

Fig. 3

Biological Properties

Antimicrobial spectrum of Cellostatin by agar streak dilution method is shown in Table II and III. The antibiotic was found slightly active against some yeast and fungi. It also indicated static action against *Trichomonas vaginalis* at the concentration of 100 mcg/ml.

The LD₅₀ for mice by single intraperitoneal injection is about 15 mg/kg. The daily injection of 2 mg/kg for one week into mice did not exhibit any toxic signs. The histological findings of toxicity were examined in the autopsied mice after lethal dosis injection. It was characterized by the advanced parenchymatous degeneration and haemorrhage in liver and kidney.

SUMMARY

Streptomyces Sp. No. 150 produced two antibiotics in the culture broth, which were separated from one another with a cation exchange resin. The first substance was isolated as sulfate in a crystalline form, and characterized as a basic, water-soluble, antifungal substance with anti-

TABLE II
Antibacterial Spectrum

Test organism	MIC $\mu\text{g/ml}$
<i>M. pyog. v. aureus</i> (Terashima)	100<
<i>M. pyog. v. albus</i>	100<
<i>M. citreus</i>	50
<i>B. subtilis</i> (NRRL-558)	100<
<i>B. anthracis</i>	100
<i>S. lutea</i>	100
<i>E. coli</i> (Umezawa)	100<
„ SMR	100<
<i>S. typhosa</i>	100
<i>S. paratyphi</i> A	100
<i>S. paratyphi</i> B	100
<i>S. enteritidis</i>	100<
<i>Sh. dysenteriae</i>	100
<i>Sh. sonnei</i>	100<
<i>P. aeruginosa</i>	100
<i>P. vulgaris</i> (OX-19)	100
<i>Cl. welchii</i> *	100<
<i>Cl. tetani</i> *	100<
<i>Cl. botulinum</i> *	50

* Tested in Tarozzi's broth.

TABLE III
Antifungal Spectrum

Test organism*	MIC mcg/ml
<i>Sacch. cerevisiae</i>	100<
<i>Zygosaccharomyces</i>	100<
<i>C. albicans</i> (M-2)	25
„ (M-8)	100
„ (M-9)	25
„ (M-10)	100
„ (Takeda)	25
<i>C. krusei</i>	100<
<i>C. parakrusei</i>	100<
<i>C. tropicalis</i>	100<
<i>C. pseudotropicalis</i>	25
<i>Torula</i>	100
<i>Mycotorula</i>	100<
<i>Rhodotorula</i>	100<
<i>Willia</i>	100<
<i>Asp. niger</i>	100
<i>Asp. oryzae</i>	100
<i>Trich. interdigitalis</i>	12.5
„ <i>gypseum</i>	100
„ <i>purpurealis</i>	6.3
<i>Mucor pusils</i>	100

* Tested in 1% glucose nutrient agar.

tumor activity. The antibiotic seems to differ from any other formerly reported antifungal substances.⁷⁾ It was recognized as a new antibiotic and named “Cellostatin”. The second substance is active to *S. lutea*, soluble in organic solvent, and inactive for the growth of tumors.

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