

# Alpha Sarcin, a New Antitumor Agent

## II. Fermentation and Antitumor Spectrum

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### ABSTRACT

OLSON, B. H. (Michigan Department of Health, Lansing), J. C. JENNINGS, V. ROGA, A. J. JUNEK, AND D. M. SCHUURMANS. Alpha sarcin, a new antitumor agent. II. Fermentation and antitumor spectrum. *Appl. Microbiol.* 13:322-326. 1965.—*Aspergillus giganteus* MDH 18894 was found to produce alpha sarcin, a new antitumor agent inhibitory to a number of different animal tumors. This culture produced culture filtrates that inhibited mouse sarcoma 180 at dilutions up to 1:32. Optimal fermentation conditions were established for shake flasks and 30-liter and 100-gal (378.5-liter) fermentors. Results from a variety of media were reported; however, the medium which yielded the most reproducible filtrates consisted of 2% corn starch, 1.5% beef extract (Difco), 2% peptone (Difco), and 0.5% sodium chloride. Purified preparations of 800 sarcoma 180 dilution units per mg were inactive against bacteria, yeasts, molds, actinomycetes, and protozoa, but were cytotoxic to several strains of mammalian cells. Alpha sarcin inhibited sarcoma 180 in mice at a dose of 62.5  $\mu$ g per kg per day. A variety of other animal tumors were also inhibited by this compound.

In 1956 a mold, MDH 18894, was isolated from a sample of Michigan farm soil. This mold was found to produce a substance inhibitory to a number of animal tumors. This antitumor substance, along with some of its identifying characteristics, was described by Olson (1963). In 1960, the mold MDH 18894 was classified by E. S. Beneke of Michigan State University as a distinct strain of *Aspergillus giganteus*.

Both culture and antitumor agent have several unique characteristics. The culture is extremely specific in its nutritional requirements; slight changes in the medium prevent the formation of alpha sarcin and, instead, cause the production of a wide-spectrum antibiotic. Still, alpha sarcin production is reproducible when the proper medium and uniform conditions are employed. Alpha sarcin inhibits tumor growth over a wide range of concentrations without undue toxicity. Tests with mice bearing sarcoma 180 tumors have shown a sixfold difference between the minimal inhibitory level and the toxic level. It is hoped that this attribute will apply also in the human clinical trials now in progress under the supervision of the Cancer Chemotherapy National Service Center (CCNSC) of the National Institutes of Health.

Olson and Goerner (1965) described procedures of isolation and purification for obtaining alpha

sarcin in pure form. They reported also the amino acid composition of alpha sarcin and noted the presence of a previously undescribed amino acid.

### MATERIALS AND METHODS

*Fermentation procedures.* All shake-flask fermentations were carried out in wide-mouth, 500-ml Erlenmeyer flasks. Uniform aeration and agitation for these fermentations were provided by a Gump rotary shaker, operated at 250 rev/min. The rotary motion of the shaker was 2.25 inches (5.7 cm) in diameter.

Larger-scale fermentation studies were done in 30-liter and 100-gal (378.5-liter) fermentors. These fermentors were described in detail by Olson et al. (1954). The agitation was provided by turbine-type agitators of controlled speed. The aeration was by sterile air, sparged through a ring sparger located directly below the turbine.

*A. giganteus* MDH 18894 was maintained in soil stock and in lyophil since its original isolation. Agar slant cultures to be used in fermentation studies were routinely started from a soil stock at 3-week intervals. The slants were used to inoculate seed flasks only after 1 to 3 weeks of incubation. The seed-flask inoculum was grown in 500-ml Erlenmeyer flasks on a Gump rotary shaker at 30 C. The medium used was 2% soybean meal, 1% corn meal (yellow), 0.5% calcium carbonate, and 0.1% Swift Mellocrust Lard Oil, supplemented with 3% octadecanol. The flasks containing the medium were autoclaved for 1 hr, and each flask

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was inoculated with a loopful of spores from an agar slant culture. After 48 hr on the shaker, a 1% inoculum transfer was made from the initial flask to another flask containing the same medium. This second stage of inoculum was shaken for 20 hr, and was used to seed the fermentation flasks. The composition of the specific medium used in each flask is presented along with its results.

*Analytical procedures.* Free reducing sugar was determined by the method of Shaffer and Somogyi (1933), and the sugar concentration is expressed as glucose in milligrams per milliliter.

The dry weights of mycelium were determined by filtration of a 10-ml quantity of whole culture through a tared 7-cm Whatman no. 1 filter paper on a Büchner funnel. The mycelial pad was rinsed with water and dried. Each mycelial pad and filter paper was weighed after drying for 16 hr at 105 C. During the first 20 hr of fermentation, the unused starch contributed to the apparent dry weight of mycelium, but the values obtained after 20 hr were more truly representative of the mycelial weight of the mold.

*Assay of antitumor activity against sarcoma 180 in mice.* Samples were submitted to several tumor assay contractors of CCNSC for determination of antitumor activity against sarcoma 180 and adenocarcinoma 755 in mice. The antitumor activity against other tumors was determined by the Sloan Kettering Institute for Cancer Research at the request of the CCNSC.

One unit of alpha sarcin activity is defined as the amount of alpha sarcin that is needed per day per 20-g mouse to inhibit sarcoma 180 tumor growth by a minimum of 58% in the CCNSC mouse tumor test (Cancer Chemotherapy National Service Center, 1959).

*Assay of cytotoxicity by tissue-culture assays.* Tissue-culture activity was measured by the agar plate assay of Schuurmans, Duncan, and Olson (1960), and by the fluid suspension assay of Perlman et al. (1959).

## RESULTS

*Alpha sarcin fermentation.* A medium composed of 2% cornstarch, 1.5% beef extract (Difco), 1% peptone (Difco), and 0.5% sodium chloride was selected as comparative control medium for shake-flask fermentation studies.

This medium (medium A in Table 1) reproducibly supported the production of 32 units of alpha sarcin per ml. It served as control for a study of nutrients and their effect on alpha sarcin production in shake flasks (Table 1). Media O, P, and Q were the same as medium A, except for the concentration of beef extract. The lack of sensitivity of the sarcoma 180 mouse assay did not allow differentiation of yields between media A, P, and Q. Medium A had the lowest concen-

TABLE 1. Effect of medium composition on alpha sarcin production in shake flasks

Medium	Medium components (g/100 ml)						Culture filtrate dilution†				Approx units/ml	
	Beef extract	Pep-tone	Corn starch	NaCl	Nz Amine Type B*	Tryptose	Component added to only one medium	1:4	1:8	1:16		1:32
A	1.5	1.0	2.0	0.5					0.14	0.39	0.32	32
B		1.0	2.0	0.5	1.5				0.29	0.47	0.78	8
C			2.0	1.0	1.5		CaCO <sub>3</sub> , 0.5; Casamino Acids, 1.0		0.12	0.23	0.28	32
D		1.0	2.0	0.5		1.0			0.14	0.49	0.62	8
E		1.0	2.0	0.5			Soy peptone,* 1.0		0.28	0.10	0.25	32
F		1.0	2.0	0.5			Tankage, 2.0		0.92	0.96	0.77	0
G		1.0	2.0	0.5			Dried blood, 4.0		0.48	0.79	0.78	0
H		1.0	2.0	0.5			Meat-bone scraps, 2.0		0.64	0.86	1.65	0
I			2.0	1.0		1.0	Brewer's yeast, 2.0		0.22	0.50	0.59	8
J			2.0	1.0		1.0	Powdered milk,* 1.0		Toxic	0.15	0.49	16
K			2.0	1.0		1.0	Corn steep liquor, 2.0		0.96	0.80	0.73	0
L	1.5	1.0		0.5			Glucose, 2.0	0.55				0
M	1.5	1.0		0.5			Lactose, 2.0	0.16				4
N	1.5	1.0		0.5			Maltose, 2.0	0.13		0.55		8
O	0.5	1.0	2.0	0.5					0.35	0.48	0.46	8
P	1.0	1.0	2.0	0.5					0.10	0.17	0.29	32
Q	2.0	1.0	2.0	0.5					Toxic	No tumor	0.32	32
R	1.5	2.0	2.0	0.5					0.22	0.20	0.34	32

\* Product of Sheffield Farms Co., Inc.

† Results at each dilution show the inhibition of sarcoma 180 tumors in mice as the tumor weight of the test divided by that of the control.

tration of beef extract that consistently gave yields of 32 units per ml and was, therefore, selected for use as control.

An increase in peptone content of medium A, from 1 to 2%, as in medium R of Table 1, did not affect the amount of alpha sarcin produced in shake flasks. This was not true for fermentations carried out in larger vessels. It will be seen later that the yield tripled with a doubling of the peptone concentration.

The nutritive components listed in Table 1 are representative of many that have been tested. Numerous other materials were unable to stimulate production of alpha sarcin. Although some components listed in Table 1 were also ineffective, they have been given to show the nutritional specificity of the culture. For example, neither dried blood nor meat-bone scraps were able to replace beef extract. Medium C was the only medium not containing beef extract or enzymatically altered meat protein that supported good production of alpha sarcin. The beef extract and the peptone of medium A were replaced in medium C by an acid-hydrolyzed casein and an enzymatically altered milk protein. The activity obtained with medium C was comparable to that from medium A; however, more variable results were obtained with medium C than with A. Medium C required the addition of calcium carbonate to avoid a low pH which prevented the production of alpha sarcin.

Corn starch served effectively as a source of carbon for both mycelium and alpha sarcin production. Although several of the sugars were utilized more readily than starch for mycelium production, they did not effectively replace it for the production of alpha sarcin. The rate of starch hydrolysis was rather slow. Glucose was not released from the corn starch at a rate much

above that at which the glucose was utilized by the mold (Fig. 1). Figure 1 illustrates some of the changes that occurred during fermentation. Each point was obtained from the average of two flasks removed from the shaker at the time indicated. The mycelial weight reached a maximal value at 42 hr. No values were plotted prior to the 24-hr sample because of inaccuracy due to the presence of a large amount of starch in the culture in the early stages of the fermentation. It may be further noted from Fig. 1 that no starch remained after 32 hr. This disappearance of starch coincides with the initial appearance of alpha sarcin, which continued to be produced until a maximum of 32 units per ml was reached at 72 hr. Other fermentations have shown that no additional alpha sarcin was produced after 72 hr in this medium but that a curve of the activity plateaued at this point, and later decreased.

The sarcoma 180 mouse test data of Table 1 show an important characteristic of alpha sarcin. Culture filtrates from media A, C, E, P, and R show antitumor activity at all three dilutions (1:8, 1:16, and 1:32) without reaching the toxic level of the test. This, of course, shows a large safety factor for the use of alpha sarcin in the treatment of animal tumors.

Increasing interest in alpha sarcin, because of favorable characteristics, made it plain that shake-flask studies must be translated into large-scale fermentations. Therefore, a comparison of its production was made in fermentation vessels of different sizes. The conditions first used in the 30-liter and 100-gal fermentors were selected, based on the requirements found in shake-flask fermentations and the aeration efficiency of the larger fermentors to be used. The data obtained in the vessels of various sizes are listed in Table 2, along with the conditions of operation for each vessel. In 178 shake flasks, run at 30 C, between 16 and 32 units of alpha sarcin per ml were produced, in 56 to 64 hr. The reproducibility was excellent. The optimal fermentation time in the 30-liter and 100-gal fermentors was found to be shorter than in the shake flasks, but the yield was somewhat lower. The maximal activity was produced in 40 to 48 hr, and averaged between 8 and 16 units per ml. A similar fermentation time was required in the 100-gal fermentor to give an average of 12 units of alpha sarcin per ml. Only once in 22 runs were 32 units per ml produced in the 100-gallon fermentor with medium R of Table 1.

The data in Table 2 show that an increase in peptone from 1%, as used in medium A, to 2%, as in medium R, nearly tripled the production of alpha sarcin. This increased yield with increased

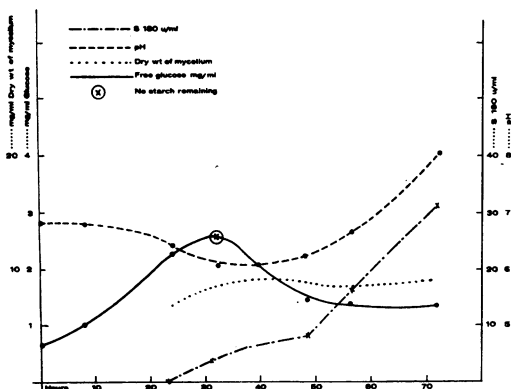


FIG. 1. Alpha sarcin production and associated chemical changes.

TABLE 2. *Alpha sarcin production in various sizes of fermentation vessels with media A and R of Table 1*

Type of vessel*	Medium	Inoculum size†	Age at peak activity	pH		Agitation		Aeration (vol of air per vol of medium per min)	Medium vol in vessel	Alpha sarcin (units/ml) in culture filtrate
				Initial	Final	Type	Rev/min			
Shake flasks (500-ml wide-mouth Erlenmeyer)	A	1.0	56-64	6.8-7.0	6.3	Gump rotary shaker	250	150 ml of medium in flasks	150 ml	16-32 (178)‡
Fermentor, 30 liter	A	1.3	40-48	6.8-7.0	5.4-5.7	8-inch turbine	577	2.0	15 liter	8-16 (15)
Fermentor, 100 gal	A	0.2	40-48	6.8-7.0	5.8	16.5-inch turbine	220	0.25	60 gal	4 (2)
Fermentor, 100 gal	A	4.0	40-48	6.8-7.0	5.8	16.5-inch turbine	220	0.25	60 gal	7 (7)
Fermentor, 100 gal	R	0.2	40-48	6.8-7.0	5.8-6.3	16.5-inch turbine	220	0.36	60 gal	12 (22)

\* Optimal temperature for all fermentations was 30 C.

† Volume of seed per volume of medium. All inocula were from 24-hr cultures.

‡ Values in parentheses show number of runs used to establish activity range.

TABLE 3. *Animal antitumor spectrum of crude alpha sarcin\**

Tumors inhibited	Dose level†	Tumors not inhibited	Dose level
Sarcoma 180‡	1	Sarcoma 387	2
Adenocarcinoma 755‡	2.25	Mecca lymphosarcoma	2 (toxic)
Sarcoma 180	4	Bashford carcinoma 63	2
Sarcoma 180 fluid form	2	Carcinoma 1025	2
Sarcoma T241	4	Ehrlich carcinoma	2
Mammary adenocarcinoma E0771	2	Bladder carcinoma	2 (toxic)
Miyono adenocarcinoma	2	Harding Passey melanoma	4
Ridgeway osteogenic sarcoma	2	Leukemia	4 (toxic)
Wagner osteogenic sarcoma	2	Jensen rat sarcoma	4
Ehrlich carcinoma fluid form	2	Murphy Sturm lymphosarcoma	2
Lung carcinoma	2	Walker carcinosarcoma 256	2
Glioma 26	2	Flexner-Jobling carcinoma	2
Friend virus leukemia solid form	4	Iglesias functioning ovarian tumor	2
Fortner adenocarcinoma of small bowel	2	Crabb hamster sarcoma	2
		Fortner adenocarcinoma of pancreas	4

\* As determined by Sloan-Kettering Institute for Cancer Research.

† Milligrams per kilogram per day.

‡ Tested by a CCNSC contractor according to CCNSC protocol, the assay used to establish units per milligram.

concentration of peptone did not occur in shake flasks. Chemical change data of the larger vessels indicated an accelerated fermentation over that of the shake flask. The initial pH of the alpha sarcin fermentation in the 100-gal-fermentor was 6.8 to 7.0 and remained at this level for the first 12 hr; the medium then became more acid and the pH went down to a low point of 5.3. This low pH normally occurred at 24 hr after inoculation. The pH rose slowly until the fermentation was harvested at 48 hr, at which time the pH was about 6.3. As stated in Table 2, 30 C was the

optimal temperature for this fermentation. This determination was made in shake flasks run at 27, 30, and 34 C. The activity at 27 and 34 C was less than half of that at 30 C.

*Alpha sarcin antitumor spectrum.* Early in the development of alpha sarcin, at the request of officers of CCNSC, a crude dry product was submitted to the Sloan-Kettering Institute for Cancer Research for the determination of an antitumor spectrum. This material had 50 units per mg of sarcoma 180 activity. Presently, our preparations of alpha sarcin contain 800 units

per mg. The methods used to isolate alpha sarcin were described by Olson and Goerner (1965). Table 3 shows that 13 of the 28 tumors tested were inhibited to some degree by the crude preparation of alpha sarcin at a dosage level of 50 to 200 units per kg per day. The same crude preparation of alpha sarcin showed no antimicrobial activity at a concentration of 100 units per ml. The microorganisms tested included 45 species of bacteria, some yeasts, molds, actinomycetes, and protozoa.

Both the crude and purified preparations of alpha sarcin showed tissue-culture activity against several cell strains when tested by the tissue-culture agar plate assay of Schuurmans et al. (1960), or in the fluid suspension assay of Perlman et al. (1959). Alpha sarcin was cytotoxic to the following mammalian cell strains: human liver (Chang), KB (Eagle), Detroit 6 (Berman), human heart (Girardi), Maben (Frisch), and sarcoma 180 (Foley). Sarcoma 180 was used for agar plate assay.

The foregoing features of alpha sarcin, together with its chemical and pharmacological properties, resulted in its selection by a clinical panel of CCNSC for human clinical evaluation.

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