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Alpha Sarcin, a New Antitumor Agent

I. Isolation, Purification, Chemical Composition, and the Identity of a New Amino Acid

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ABSTRACT

OLSON, B. H. (Michigan Department of Health, Lansing), AND GORDON L. GOERNER. Alpha sarcin, a new antitumor agent. I. Isolation, purification, chemical composition, and the identity of a new amino acid. Appl. Microbiol. **13**:314–321. 1965.—Isolation and purification procedures are given for the new antitumor agent, alpha sarcin. These procedures include the use of column ion exchange with a carboxylic resin (Amberlite IRC50), dialysis, decolorization with activated charcoal, gradient salt chromatography, salt removal, and drying from the frozen state. The final product has an activity of 800 sarcoma 180 mouse dilution units per mg. The amino acid composition of the purified material is reported. All of the usual amino acids found in proteins were present except methionine. In addition to the usual amino acids, an unknown amino acid was present in the acid hydrolysate. The latter was isolated, and was found to yield phenylalanine and kynurenine. This compound, which has been named "sarcinine," is extremely stable in 6 N hydrochloric acid in the absence of air, and is unstable in alkali. Sarcinine has also been found in two other antitumor peptides produced by aspergilli, and so may relate significantly to the antitumor properties of these peptides.

In 1956 the antibiotic screening program of the Michigan Department of Health, Division of Laboratories, was converted to include an anticancer agent screening program. This conversion was facilitated by a request for culture filtrates by the Cancer Chemotherapy National Service Center (CCNSC) of the National Institutes of Health. The culture filtrates were to be assayed against a series of three different induced tumor systems, namely, sarcoma 180, carcinoma 755, and leukemia 1210.

Early in the testing program, a mold, *Aspergillus giganteus* MDH 18894, was isolated from a sample of Michigan farm soil. This mold was found to produce a substance inhibitory to both sarcoma 180 and carcinoma 755. Olson (1963) named this substance alpha sarcin and presented some of its identifying characteristics. Schuurmans, Duncan, and Olson (1964) reported R_F values of alpha sarcin in four solvent systems and its migration in an electrical field.

The production of alpha sarcin by fermentation is described in the second paper of this series (Olson et al., 1965). Included in the same paper are descriptions of the antitumor spectrum of alpha sarcin and of its lack of antibacterial or antifungal activity. This lack of antimicrobial activity is surprising because of its high cytotoxic activity even at 5 μg/ml. The present paper reports the isolation and purification procedures for alpha sarcin and some associated peptides produced by the same organism. The peptides have been broken down, and their composition has been determined. Acid hydrolysates of alpha sarcin contained an amino acid not previously reported to be found in nature. This substance has been called sarcinine.

MATERIALS AND METHODS

Assay of antitumor activity of alpha sarcin against sarcoma 180 in mice. Water or physiological saline solutions of alpha sarcin were shell frozen in 100-ml serum bottles closed with rubber stoppers and aluminum overseals. Frozen samples packaged in Dry Ice, and lyophilized samples, were shipped to a CCNSC screening contractor. The screening contractor determined the antitumor activity of these samples against sarcoma 180 in mice. The test was carried out according to the CCNSC routine dose-response procedure. Four mice were used at each dilution, and three dilutions were run per sample. Assays of samples of special importance, such as those used to establish the relationship between activity measured by the tissue culture assay, by ultraviolet absorption, and by the sarcoma 180 mouse assay, were repeated several times in different control series. The potency of each alpha sarcin

preparation is given in the S180 mouse dilution units of Olson et al. (1965) unless otherwise stated.

Assay by tissue-culture activity. The sarcoma 180 plate diffusion assay was done according to the procedure developed by Schuurmans, Duncan, and Olson (1960). This method employs the inhibition of cellular dehydrogenase activity as the indication of cytotoxicity. Schuurmans et al. (1964) adapted this method for use in a bioautographic system, and used it to measure the R_F values of alpha sarcin in four solvent systems and the movement in paper strip electrophoresis.

The fluid suspension assay of Perlman et al. (1959) has been used with several available cell lines after adaptation to the agitation of a rotary shaker rather than a roller drum. This procedure with sarcoma 180 cells was used to establish stability of alpha sarcin in solution and to relate tissue-culture activity with sarcoma 180 mouse tumor inhibition. The inactivation of alpha sarcin by slow freezing and drying has also been demonstrated by use of this assay.

Assay by ultraviolet absorption. To measure alpha sarcin potency, we developed a procedure similar to the spectrophotometric assay method used by Camiener et al. (1960) to measure the activity of antimycin A and actinomycin. The ultraviolet spectrum of the solution to be tested was run on a Cary model 14 spectrophotometer, and a tangent was drawn common to the curve at the minimum near 2,500 A and the inflexion near 3,050 A. The height of the curve above the tangent at 2,800 A was taken to be proportional to the alpha sarcin content of pure alpha sarcin solutions. The culture filtrate from which alpha sarcin was isolated contained a number of compounds which interfered in this assay. However, most of these interfering substances were eliminated in the first ion-exchange isolation step, and the method became practical for following the isolation and purification of alpha sarcin.

To determine the activity in culture filtrate, the following method was developed. A 100-ml amount of culture filtrate was passed at a flow rate of 1 ml/min through a column (1-cm diameter) containing 10 ml of resin. The column was packed with Amberlite IRC 50 resin of 20 to 50 mesh and regenerated as later described for the first stage of isolation. The effluent was discarded, and the column was washed with 20 ml of distilled water. The alpha sarcin was eluted with 1 N HCl, and 2.7-ml fractions were taken and examined for ultraviolet absorption. The fractions containing alpha sarcin, as determined by ultraviolet-absorption characteristics, were combined as a pool, and the ultraviolet-absorption spectrum of the pool was determined. The activity was then calculated from the height of the line at 2,800 A between the curve and the tangent, the dilution used for the spectrum and the volume of the pool. The activity was expressed in absorption-height units per milliliter. Pure alpha sarcin gave 106 units per mg. This compares with 800 S180 mouse dilution units per mg.

Fermentation procedures. Olson et al. (1965) reported the fermentation procedures used for the production of alpha sarcin. The medium and the optimal conditions of growth suggested by them have been used for the production of culture filtrate in a 100-gal (378.5-liter) fermentor. The recommended medium contained 1.5% beef extract, 2% peptone, 2% corn starch, 0.5% sodium chloride, and an antifoam of 3% octadecanol in lard oil (Swift's Melocrust). The organism *A. giganteus* MDH 18894 was grown in this medium at 30 C with an air-flow rate of 0.36 volumes of air per volume of medium per minute and an agitation rate of 220 rev/min for 48 hr. The starch had disappeared by 32 hr, and the *p*H followed the pattern described by Olson et al. (1965). The culture filtrate contained an average of 21.2 units per ml, or a total of 4.86×10^6 units in 230 liters, and was used as the starting material for the isolation of alpha sarcin.

Isolation of crude alpha sarcin. The whole culture was filtered through a plate-and-frame press. Because the alpha sarcin was largely extracellular, the active principle was recovered in the culture filtrate and in a 0.1-volume water wash of the mycelium. The mycelium was discarded, and the alpha sarcin was adsorbed onto a column of carboxylic resin. One volume of resin adsorbed all of the active principle from more than 65 volumes of culture filtrate. Therefore, a Pyrex pipe (10.2 cm in diameter) fitted as a chromatographic column was filled to contain 4 liters of carboxylic exchange resin that was regenerated by mixing the hydrogen form of Amberlite IRC 50 (20 to 50 mesh) with a total of 10 volumes of 1 N NaOH. This regeneration was done in batches by agitation of the resin with three separate portions of NaOH. Each time, the resin was separated from the spent hydroxide solution. After the final separation, the resin was washed with distilled water and suspended in 3 volumes of distilled water; the suspension was adjusted to *p*H 6.8 with concentrated phosphoric acid. All *p*H adjustments were done with adequate stirring times to insure accurate *p*H measurement. The supernatant solution was removed; the resin was suspended in 3 volumes of water and adjusted to *p*H 6.8. After removal of the supernatant and resuspension in 3 volumes of water, a final adjustment was made to *p*H 7.0 with 0.2 M phosphoric acid. The resin was transferred to a resin column (10.2 cm in diameter) of Pyrex pipe and washed with 20 liters of distilled water.

The culture filtrate was passed through the resin column at a flow rate of 0.22 ml per ml of resin per min. The culture filtrate was followed by a 20-liter water wash at the same flow rate. Then the elution of adsorbed materials was begun at a flow rate of 0.04 ml per ml of resin per min. The first eluent used was 0.2 M NaCl. This concentration of salt eluted some of the inactive peptides, which were discarded. Then the elution of the active material was brought about with 1.5 M NaCl. All eluates were collected in 1-liter fractions beginning

at the time of addition of the 0.2 M NaCl to the column. The ultraviolet-absorption spectrum of each fraction was recorded, and the fractions containing the active component were selected.

The active fractions were divided into two pools. Pool A was composed of fractions which contained 75% or more of the total activity; pool B contained the remainder of the activity. Normally, pool A included fractions 10 through 14, and pool B contained fraction 9 and fractions 15 through 17. The two pools were concentrated separately in a circulating vacuum evaporator at a temperature not exceeding 35 C. The resulting concentrates were sterile-filtered and dialyzed against distilled water at 0 to 4 C in an agitated dialysis bath. After 10 changes of dialysis water (4 to 5 days), the solutions were removed from the cellophane dialysis tubing, combined, treated with 1% activated charcoal (Darco G60), stirred 5 min, and filtered through Whatman no. 3 paper. The filtrate was sterile-filtered through a Millipore filter (type HA47). The pools were kept separate until after dialysis to decrease the length of time the bulk of the active material was exposed to heat during concentration. The Darco G60 adsorption removed some of the inactive material not normally separated in the later stages of purification. Unfortunately, some of the active material was adsorbed as well. This caused a loss of 13% of alpha sarcin. After Darco G60 removal and sterile filtration, the solution containing the crude alpha sarcin was quick-frozen in a solvent-Dry Ice bath and dried from the frozen state. The product was a white fluffy powder of 200 units per mg.

It should be emphasized that solutions of alpha sarcin must be quick-frozen. Material placed in trays and frozen in a household freezer, or otherwise slowly frozen, was partially inactivated. A single slow freezing inactivated up to 60% of the alpha sarcin.

Purification. The crude dry alpha sarcin is known to contain four contaminating pepides. Each peptide has been separated in pure form, and the amino acid composition of these peptides has been determined. The purification procedure given here is the most practical for alpha sarcin. The same procedure also yields two of the contaminating peptides in pure form; one is an antifungal antibiotic with neither antitumor nor antibacterial action, and the other has not yet been examined for biological activity.

The purification was based on a *p*H buffered-salt gradient elution of the peptides from a carboxylic resin, Amberlite XE64. New resin, when first used, was specially treated as follows. The resin was wet-screened in the hydrogen form, and the resin fraction passing a 50-mesh sieve, but not passing a 150-mesh sieve, was air dried and washed with acetone to remove impurities. After complete removal of the acetone, the resin was converted into the sodium form by batch treatment with 10 volumes of 1 N NaOH. Resin which had previously been treated in this way and used in one or more gradient purifications did not require the foregoing treatment.

The routine procedure then began at this point. The resin was converted to the hydrogen form with 10 volumes of 1 N HCl and then back into the sodium form by the batch method with 10 volumes of 1 N NaOH. The resin was washed twice with water and suspended in 4 volumes of fresh distilled water. The suspension was adjusted to *p*H 5.95 by adding concentrated phosphoric acid. Sufficient stirring was used to reach equilibrium throughout the resin preparation.

The following procedure was developed to avoid the long period of column washing with buffer required by carboxylic resins buffered with phosphate (because of *p*H change with phosphate ion concentration). The supernatant fluid from the first *p*H 5.95 adjustment was removed and replaced with 0.4 M sodium phosphate buffer (*p*H 5.95). The suspension was adjusted to *p*H 5.95 with 0.4 M phosphoric acid. One additional replacement of the 0.4 M buffer and adjustment to *p*H 5.95 with 0.4 M phosphoric acid was usually necessary for complete equilibration of resin and buffer.

The resin was placed in a column (4 by 60 cm) and packed to a height of 52 to 54 cm. One volume of *p*H 5.95 buffer, equivalent to the resin volume, was used as wash. The column influent and effluent were of identical *p*H. A solution of 4 g of crude alpha sarcin in 10 ml of 0.4 sodium phosphate buffer (*p*H 5.95) was carefully added to the top of the column and allowed to pass into the column. A magnetically stirred gradient mixer (2 liter, three-necked, round-bottomed flask) was filled with 2,200 ml of the 0.4 M sodium phosphate buffer (*p*H 5.95) and connected to the top of the resin column. A reservoir containing 0.9 M sodium phosphate buffer (*p*H 6.0) was connected to the gradient mixer. The eluent of changing composition was then started through the column at a flow rate of 125 ml/hr, and the column effluent was collected in 20-ml fractions.

The optical density of each fraction at 2,760 A was determined to select the fractions containing the separated peptides. Peptide 3 was found in fractions 20 to 30, alpha sarcin in fractions 76 to 95, and an antifungal peptide in fractions 172 to 190. The antifungal peptide was eluted by converting to a 1.5 M NaCl eluent at the time tube 100 was collected. The pools of the individual peptides were dialyzed in an agitated distilled water bath until the disappearance of phosphate. The materials were removed from the dialysis tubing, quick frozen, and dried from the frozen state.

Chemical composition of peptides. Molecular weight was determined by the gel-filtration method of Andrews (1964). Sephadex G100 was used with a buffer of 0.1 M sodium phosphate (*p*H 6.5) and 0.4 M sodium chloride. Human serum globulin, ribonuclease, and mannitol were used to calibrate the column.

Amino acid analysis was done as follows. The

procedure of Crestfield, Moore, and Stein (1963) was used for acid hydrolysis and preparation of the sample. The samples of purified rechromatographed alpha sarcin were hydrolyzed in a toluene vapor bath at 110 C for 24, 48, and 72 hr. The 6 N HCl was removed on a rotary evaporator under reduced pressure. Tryptophan was determined by the spectrophotometric method of Bencze and Schmid (1957). The hydrolysates were analyzed for amino acid composition by the usual procedure on a Beckman Spinco 120B automatic amino acid analyzer. It is important to note, however, that the short column of our analyzer had a resin height of 17 cm. This extra length of resin separated the prelysine peaks more completely than does the usual 15-cm column. Lysine appeared at 59.5 ml, histidine at 71.5 ml, ammonia at 84.6 ml, and arginine at 137.5 ml.

The unknown amino acid was isolated from alpha sarcin as follows. The unknown substance appeared as a discrete peak at 40.5 ml on the 17-cm column of the Spinco model 120B amino acid analyzer. It could also be separated on the 50-cm column as a discrete peak at 133 ml with the same buffer, that is, 0.35 M sodium citrate (*p*H 5.28). A quantity of the purified unknown was prepared by the following procedure. A 500-mg amount of alpha sarcin was placed in a 100-ml round-bottomed flask and dissolved in 50 ml of 6 N HCl. The flask was freed from air and sealed under vacuum. The flask was heated in an autoclave at 110 C for 24 hr, and the 6 N HCl was removed at reduced pressure on a rotary evaporator. The residue was dissolved in *p*H 2.2 Beckman sample diluter buffer, and the solution was placed on the 50-cm preparative column (1.8-cm diameter) of the analyzer. This column had been equilibrated with the 0.35 M citrate buffer (*p*H 5.28), and was connected to a fraction collecter which provided 8-ml fractions. Tyrosine appeared in fractions 26 to 34. The unknown was found in fractions 54 to 60, and lysine first appeared in fraction 71. The fractions containing the unknown were pooled, and a portion of the pool was run on the analytical column of the analyzer to prove the absence of contaminating material. The remainder of the pool of the unknown was desalted and separated from the buffer additives, thio-diglycol and BRIJ 35, by the procedure of Drèze, Moore, and Bigwood (1954). The 4 N HCl in which the unknown was eluted was removed under reduced pressure in a rotary evaporator. The crystalline residue of the unknown was immediately placed in a nitrogen atmosphere.

RESULTS

Isolation and purification. Figure 1 provides an outline of the procedure used to isolate and purify alpha sarcin, together with the per cent recovery obtained at various stages in the purification. It should be noted that the crude alpha sarcin of 200 units per mg mentioned in Fig. 1 is different from that described in the paper by Olson et al. (1965). The earlier isolation procedure did not have the advantage of the ultraviolet assay for selection of fractions for pooling the active fractions; hence, a number of inactive substances were included in the alpha sarcin crude. The earlier crude contained only 50 units per mg. The 200 units per mg crude indicated in Fig. 1, representing 61% of the starting activity, contained four peptides other than alpha sarcin.

Figure 2 indicates the separation of alpha sarcin when purified according to the purification procedure of Fig. 1. When subjected to 0.4 to 0.9 M sodium phosphate (*p*H 5.95) gradient chromatography, two of the four contaminating peptides flushed right through a column of XE64 and, therefore, do not appear in Fig. 2. These two peptides can be separated, and appear as the first two peaks when a 0.1 to 0.9 M sodium phosphate (*p*H 6.0) buffered column of XE64 is used (Fig. 3). This column, however, did not completely separate the alpha sarcin from the antifungal peptide, as shown by antifungal assays. Furthermore, the alpha sarcin peak was delayed until peak fraction 188. The 0.4 to 0.9 M (*p*H 5.95) buffered column of Fig. 2 was much more practical for use in large-scale recovery, because the alpha sarcin peak appeared at fraction 84 and was completely freed from the antifungal fraction.

The per cent recovery of pure alpha sarcin by this procedure was 60.6% from the crude, or, overall, 36.8%. By ultraviolet assay, 84 to 88% of all peptide absorption units added to the gradient column were recovered in the eluates. So, in all probability, the recovery of alpha sarcin from the crude to the purified state would be above 80%, or an overall recovery of greater than 48%. Rechromatography of the pure alpha sarcin by the gradient method yielded a single symmetrical peak. All fractions of the rechromatographed material had identical ultraviolet-absorption spectra. This rechromatographed material was quick-frozen and dried from the frozen state for use in determination of the amino acid composition. This material had 800 S180 mouse dilution units per mg, as did the material before the rechromatography. Both preparations were pure white, fluffy materials.

As indicated earlier, the concentration of the buffer used in the gradient influenced the fraction in which alpha sarcin appeared: the more concentrated the buffer, the earlier the fraction in which alpha sarcin appeared. The position of alpha sarcin was also influenced by *p*H: the higher the *p*H of the column and buffer, the more rapidly the material moved down the column. It was found possible to use buffers of one-tenth the

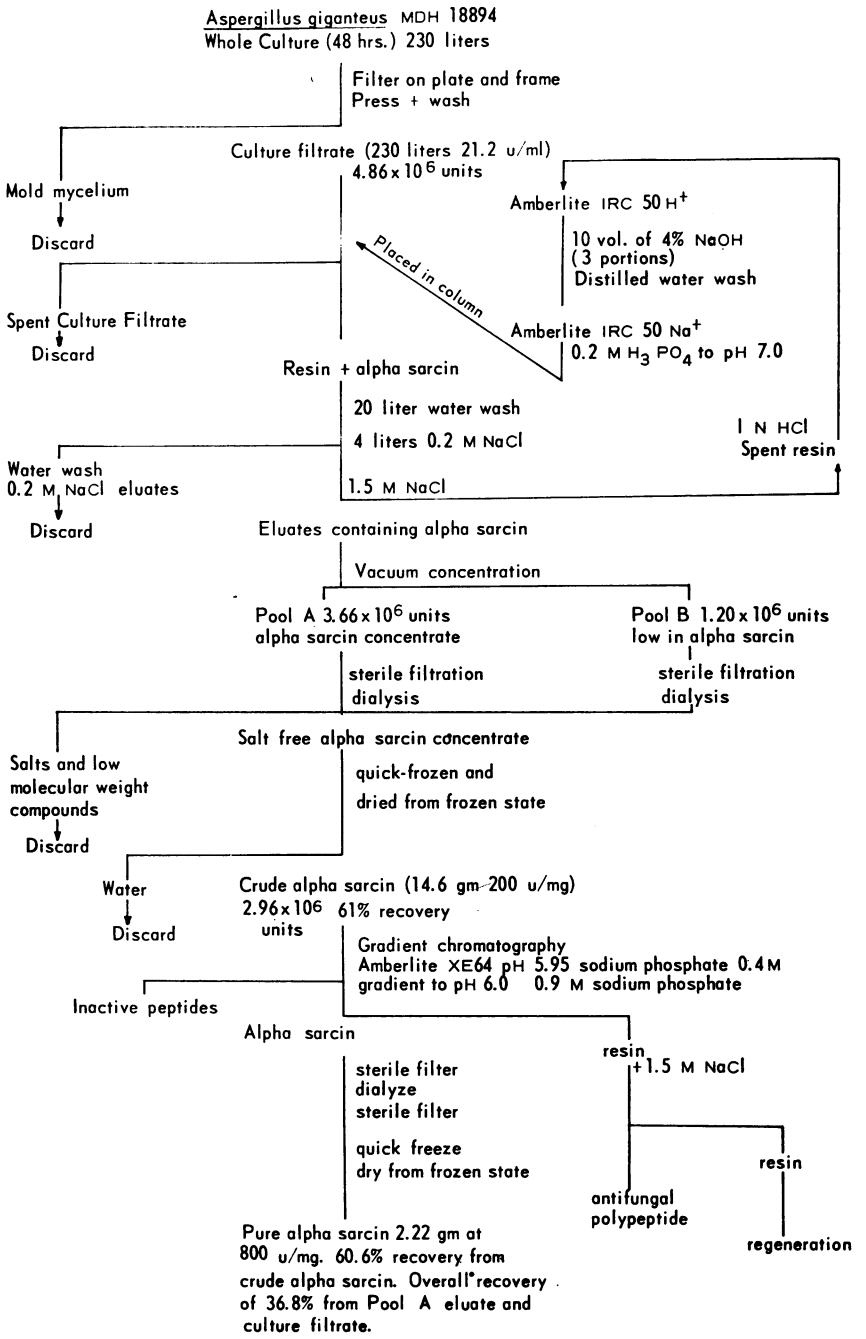

FIG. 1. *Isolation and purification procedures for alpha sarcin. Yields from 230 liters of whole culture.*

usual concentration, if the *p*H values of the column and buffers were raised to 7.0. The antifungal peptide appeared to be the peptide least influenced by changes in the chromatographic conditions. It was not possible to use a *p*H higher than 7.0 for gradient elution, because the alpha sarcin was unstable in alkaline solution.

Chemical and physical properties of alpha sarcin. The material was found to be a polypeptide with a molecular weight of approximately 16,000,

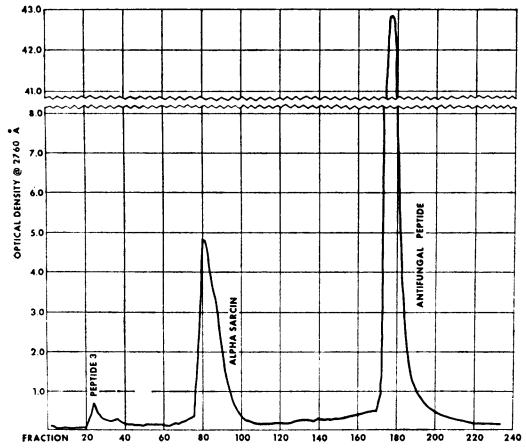

FIG. 2. *Separation of peptides produced by Aspergillus giganteus MDH 18894 by gradient elution. Sample used was 4.0 g of crude (200 units per mg) alpha sarcin. The column was 50 cm by 4 cm (diameter), packed with Amberlite XE64 resin of 50 to 150 mesh size. Gradient used was 0.4 M, pH 5.95, to 0.9 M, pH 6.0, sodium phosphate. Elution was changed to linear elution with 1.5 M sodium chloride at fraction 100. Fractions taken were 20 ml each.*

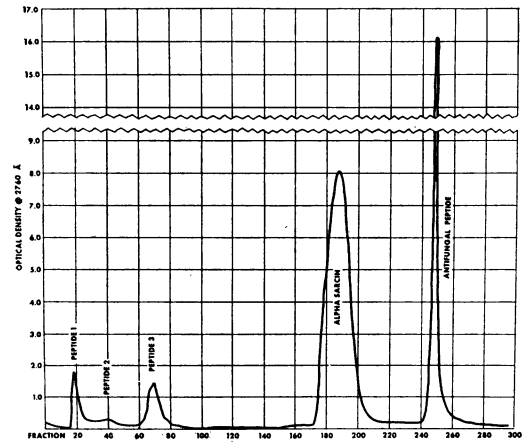

FIG. 3. *Separation of peptides produced by Aspergillus giganteus MDH 18894 by gradient elution. Sample used was 4.0 g of crude (200 units per mg) alpha sarcin. The column was 50 cm by 4 cm (diameter), packed with Amberlite XE64 resin of 50 to 150 mesh size. Gradient used was 0.1 M, pH 6.0, to 0.9 M, pH 6.0, sodium phosphate. Elution was changed to linear elution with 1.5 M sodium chloride at fraction 214. Fractions taken were 20 ml each.*

stable at *p*H 2.0 to 7.0, and much less stable above *p*H 7.0. Physiological saline solutions of alpha sarcin have been stored for long periods at 0 to 4 C and at 37 C. There was no loss of activity within the limits of accuracy of the tissue-culture assay in a 4-month storage at 0 to 4 C. The same material stored for 4 months at 37 C lost 56% of its activity. Alpha sarcin gave positive ninhydrin and biuret tests, but negative starch, Molisch, ferric chloride, pentose, and ketose tests. It could be precipitated from water solutions by addition of 2 volumes of methanol, ethyl alcohol, or acetone. It did not dialyze through a cellophane membrane, but was easily adsorbed on activated carbon from which it was not readily eluted.

Water solutions of alpha sarcin at *p*H 6.8 were inactivated by slow freezing and drying. This inactivation was measured by the fluid suspension assay of Perlman et al. (1959; Table 1). The potency of lot 4 is significantly lower than the other two lots and the control ($P = <0.01$). The inactivation occurring in lot 4 was also shown by a decrease in ultraviolet-absorbance height units. The loss in potency was accompanied by the formation of a water-insoluble precipitate.

Alpha sarcin is further characterized by the ultraviolet-absorption spectra given in Fig. 4 and by infrared-absorption peaks at 3.02, 3.41, 6.07, 6.62, 7.0, and 7.2 μ, as measured in a KBr pellet on a Perkin and Elmer Infracord 137.

Biological properties of alpha sarcin. The results of two mouse assays, as performed by a contractor of CCNSC, are shown in Table 2. Sarcoma 180 tumors in mice were inhibited in one test by 62.5 μg of alpha sarcin per kg per day. Alpha sarcin, at 100 times an S180 mouse tumor daily dose per

TABLE 1. *Comparison of alpha sarcin tissue culture sarcoma 180 activity of vacuum-dried preparations made from slow- and quick-frozen solutions*

Alpha sarcin sample	Type of freezing used before vacuum drying	ID_{50} by fluid suspension tissue culture assay (μg/ml)				Loss of activity
		Vial 1	Vial 2	Vial 3	Avg ID_{50}	
						%
Lot 3	Slow*	2.2	0.8	3.4	2.1	23
Lot 4	Slow*	4.3	4.4	5.8	4.8	66.7
Lot 5	Quick†	0.8	2.3	1.4	1.5	0
C2-64 control	Quick‡	1.1	1.9	1.8	1.6	0

* Plug frozen as 1-ml amounts in 5-ml vials in paper-wrapped trays placed in shelf freezer. Freezing time varied between vials, but average freezing time required several hours.

† Lot 5 was plug frozen as 1-ml amounts in 5-ml vials in a solvent-Dry Ice mixture. Average freezing time was less than 3 min.

‡ Control was shell frozen with a freezing time of 5 min.

ml, does not inhibit the growth of any one of 45 species of bacteria, as reported by Olson et al. (1965). The same report indicates cytotoxic action against six tissue-culture cell lines. One of these, S180, was inhibited by 5 μg/ml. Some of the other cell lines were even more sensitive.

Amino acid composition. The amino acid composition of alpha sarcin and the antifungal peptide are given in Table 3. The antifungal peptide was totally lacking in histidine, methionine, and leucine; but, of the amino acids normally found in proteins, alpha sarcin lacked only methionine. Alpha sarcin was unique in that an unknown amino acid was present in the acid hydrolysate. This amino acid appeared on the amino acid analyzer chart from the 17-cm column at 40.5 ml, a position which was between kynurenine and tryptophan. The antifungal peptide did not contain this unknown substance. A small quantity (10 mg) of this unknown, prepared according to the procedure described above, was used to determine its identity. The substance does not appear to have been described previously.

This substance was stable in the absence of air and alkali; however, when air was present, the material broke down rapidly in the cold to phenylalanine and kynurenine. The kynurenine formed has been compared with, and found identical to, both a preparation of kynurenine made by us according to the procedure of Hayaishi and

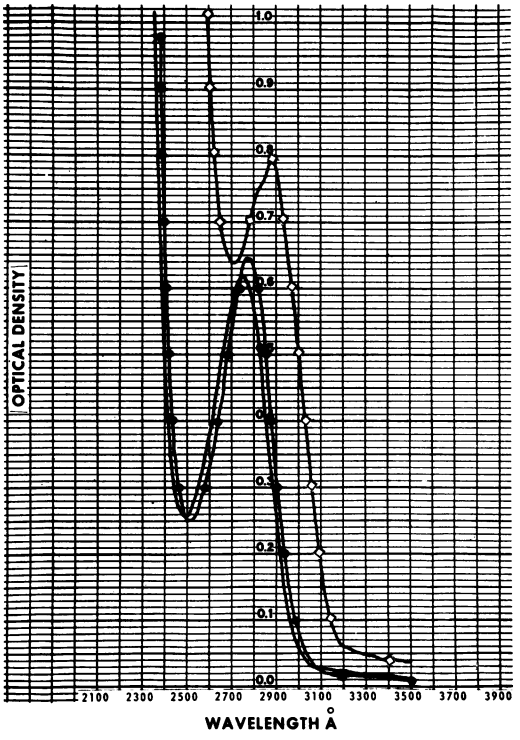

FIG. 4. *Ultraviolet-absorption spectra of alpha sarcin, 0.5 mg/ml in 0.1 N HCl (solid line), in water (●), and in 0.1 N NaOH (□).*

TABLE 2. *Inhibitory action of alpha sarcin against sarcoma 180 tumors in mice as measured by a CCNSC contractor in an 8-day test with seven injections of drug**

Material tested	Dose	Wt difference (test − control)	Tumor wt	
			Test	Test/control
	mg/kg	*g*	*mg*	%
Alpha sarcin	0.5	+0.8	165	9
Lot 5	0.25	+0.7	596	35
Sample 1	0.125	+1.3	735	44
	0.063	+0.2	1,507	90
Alpha sarcin†	0.5	−2.6	62	3
Control	0.25	−0.4	161	9
Sample 2	0.125	+1.8	362	21
	0.063	+1.3	698	42

* Four mice were tested with each material; all mice survived. The untreated tumor used as a control weighed 1,657 mg.

† Standard preparation which has been shown to have 800 mouse dilution units per mg (average value).

TABLE 3. *Amino acid composition of alpha sarcin and an antifungal peptide*

Amino acid	Amino acid in 1 mole of alpha sarcin	Proportion of amino acid in antifungal peptide
	moles	*moles*
Sarcinine	1	0
Tryptophan*	2	—
Lysine	17	9.8
Histidine	6	0
Ammonia	17	3.7
Arginine	2	1.0
Aspartic acid	16	5.1
Threonine	8	1.9
Serine	7	1.8
Glutamic acid	10	2.1
Proline	10	1.0
Glycine	13	4.2
Alanine	6	4.0
½ Cystine	2	8.0
Valine	3	1.0
Methionine	0	0
Isoleucine	3	1.9
Leucine	7	0
Tyrosine	6	5.8
Phenylalanine	5	1.0

* Determined by the spectrophotometric method of Bencze and Schmid (1957).

Stanier (1951) and a preparation of DL-kynurenine obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Kynurenine appeared at 36.8 ml on our 17-cm column and at 554 ml on the 150-cm column; both runs were at 50 C. The kynurenine position was at 66.5 ml after the phenylalanine on the 150-cm column. Each of the kynurenines and the original unknown amino acid, when dissolved in 0.1 N NaOH, were decomposed on heating in a boiling-water bath for 15 min. Under these conditions, the unknown did not degrade to kynurenine, but to *O*-aminoacetophenone and some other compounds. This unknown amino acid, which we refer to as sarcinine, has been found by us to be present in two other antitumor substances produced by aspergilli.

DISCUSSION

Alpha sarcin is a compound of very high biological activity. Only 1.25 μg per mouse per day are required to significantly inhibit the growth of sarcoma 180 tumors in mice, and 5 μg/ml inhibits sarcoma 180 tissue-culture cells when added to the medium. The toxicity of the compound to normal animals has been determined and will be reported at a later date. Alpha sarcin is now in phase II of the CCNSC human clinical trials.

The unique character of the amino acid composition of alpha sarcin is exemplified by the absence of methionine and by the presence of an unknown amino acid which we have named "sarcinine." There is little chance that the substance contains a peptide bond, since heating for 72 hr at 110 C in 6 N HCl in the absence of air does not cause hydrolysis.

Tests run on the small quantity of sarcinine isolated thus far have shown a compound stable in strong acid in the absence of air, unstable when air is present, and unstable in alkali even in the absence of air. Neutral or acidic oxidative decomposition results in the formation of phenylalanine and kynurenine.

This unusual compound, sarcinine, may well be associated with the antitumor activity, because we have found it to be present in hydrolysates of two other peptide antitumor agents which we have obtained from aspergilli.

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