ISOLATION, PURIFICATION, AND CHARACTERIZATION OF ANTICAPSIN[1]

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Anticapsin is a new biologically active metabolite isolated from the culture filtrate of a strain of *Streptomyces griseoplanus*. The metabolite inhibits the formation of the hyaluronic acid capsules of *Streptococcus pyogenes* at a minimal concentration of 3 mcg/ml. Anticapsin ($C_9H_{13}O_4N$), an amino acid (pKa=4.3; 10.1 in 66 % DMF), is chemically related to bacilysin.

The hyaluronic acid capsules of microorganisms of the *Streptococcus* Group A are thought to contribute to the virulence of the organism[2]. Capsules also render the organisms resistant to attack by bacteriophage, thus permitting the use of phage to reveal, through zones of bacterial lysis, the absence of capsules. During the course of screening new cultures for metabolites which inhibit hyaluronic acid capsule formation, activity was detected in the broth of a strain of *Streptomyces griseoplanus*[3]. The active metabolite was isolated and named anticapsin. This paper describes the isolation, purification, and characterization of anticapsin.

Production

Fermentation inoculum was prepared by aseptically adding suspensions of sporulated slant cultures from *Streptomyces griseoplanus*, NRRL 3507, to a medium composed of 15 g/liter glucose, 15 g/liter Nutrisoy grits (Archer-Daniel-Midland Co.), 10 g/liter corn steep liquor (Corn Products Co.), 5 g/liter sodium chloride, and 2 g/liter calcium carbonate in tap water. Flasks contained 50 ml of medium and were incubated 48 hours at 30°C on a G53 New Brunswick Shaker rotating at 250 rpm. A 1 percent (v/v) level of the mycelial cell suspension from these flasks was transferred to a fermentation medium composed of 15 g/liter glucose, 30 g/liter dextrin 700 (A. E. Staley Mfg. Co.), 20 g/liter Nutrisoy grits, 3 g/liter Amber EHC (Amber Laboratories), 1 g/liter yeast 2019 (Standard Brands), 0.2 g/liter Antifoam "A" (Dow Chemical Co.), and tap water. The fermentation was conducted at 30°C in aerated and stirred 40-liter fermentors of conventional design. Maximum accumulation of anticapsin was observed after 70~76 hours, when the pH was 6.8 and the potency had reached a level of 300 mcg/ml.

Further modification of the fermentation medium showed that when glucose and dextrin 700 were replaced with 150 g/liter of sucrose and the fermentation time was extended to 144 hours, the yield of anticapsin increased to 1,300~1,500 mcg/ml.

Isolation and Purification

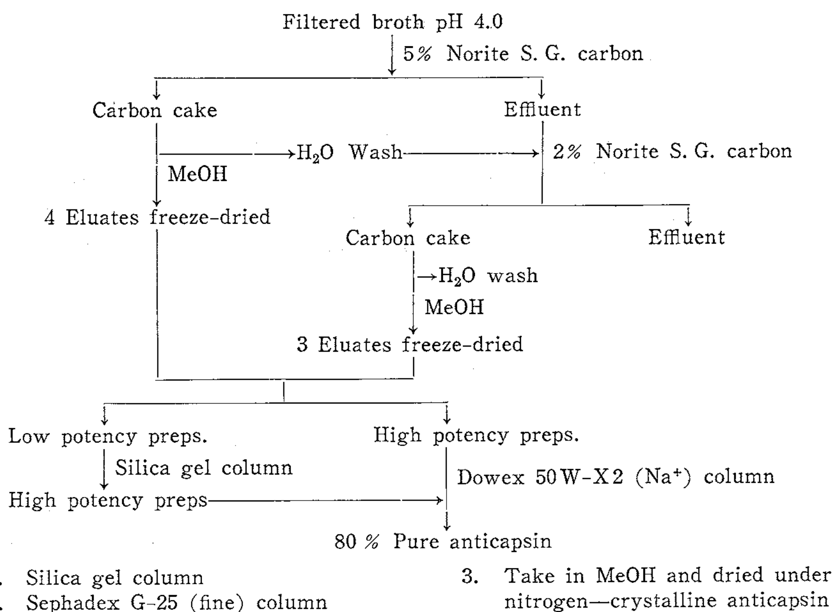
The whole broth was filtered with 2 percent Hyflo Supercel (John Mansville Co.), and the pH of the filtrate adjusted to 4.0. Norite S. G. carbon (American Norite Co.) was added to the filtrate to a concentration of 5 percent and the mixture was stirred for one hour. After filtering, the carbon was washed two times with distilled water and eluted four times with one-third volume of 30 percent methanol. The water washes and effluent from carbon, which still contained 20 % of the biological activity of the original broth, were combined and retreated with 2 % (w/v) carbon and stirred for one hour. The carbon was filtered, washed two times with distilled water, and eluted three times with one-third volume of 30 % methanol. The active eluates from both the carbon treatments were concentrated and freeze dried. At this stage, the dried preparations contained 165 mcg/mg of the activity. The microbiological activity at each step was measured using the method described by WHITNEY *et al.*[3] for inhibition of capsule formation and phage lysis of *Streptococcus pyogenes*, ATCC 12384.

Freeze-dried preparations dissolved in water were chromatographed on a silica gel column (W. R. Grace Co., Type 950, U. S. Mesh 60~200, 1 g of preparation per 50 g of silica). The column was eluted with deionized water. The elution of activity at this stage was followed using thin-layer chromatography on silica plates (Analtech, Inc.), developed in water and sprayed with ninhydrin reagent. The fractions richest in the ninhydrin-positive spot corresponding to anticapsin activity were combined and freeze-dried. The dried preparations contained 310 mcg/mg activity.

Further purification was accomplished on a Dowex 50WX2 (Na^+) column (Dow Chemical Co., U. S. mesh 100~200). The column was washed with deionized water affording a three-fold purification of the material from the silica column. The elution of anticapsin was again monitored by thin-layer chromatography on silica plates (Brinkman Instruments, Inc.), developed this time in a 70 % aqueous acetonitrile system and sprayed with ninhydrin or benzidine hypochlorite reagent. After spraying with benzidine hypochlorite reagent, active fractions showed a colorless spot, against a light-blue background, which turned yellow after standing. Fractions with highest color intensity after ninhydrin spray were combined and freeze-dried. Dry preparations were white, fluffy, and contained 830 mcg/mg activity. The recovery was 30 % at this stage from the filtered broth.

Final purification was achieved by chromatography on silica gel column (W. R. Grace Co., Type 950, U. S. mesh 60~200) in 70 % aqueous acetonitrile and then on a Sephadex G-25 (fine) column (Pharmacia Fine Chemicals, Inc., Upsala, Sweden) in deionized water. The fractions were again evaluated by thin-layer chromatography as described above. Crystallization occurred when a methanol solution of amorphous anticapsin was evaporated under nitrogen. The crystalline compound appeared as a single substance when chromatographed as described above; however, using cellulose plates (Brinkman Instruments, Inc.) and developing in 70 % aqueous propanol, the material appeared to contain traces of other ninhydrin-positive spots[5]. The biological activity was defined as 1,000 mcg/mg.

Fig. 1. Isolation and purification scheme of anticapsin.



Modification in the fermentation media[4] gave preparations having 280~350 mcg/mg activity after carbon treatment. With these preparations, the early silica gel column chromatography step could be eliminated and purification was done directly on Dowex 50WX2 (Na^+) columns, yielding material with activity of 830 mcg/mg, identical to that described above. The isolation procedure is summarized in Fig. 1.

Physical and Chemical Properties of Anticapsin

Anticapsin, a white crystalline material, is quite stable at room temperature between pH 4.0 and 7.5 and melts at 240°C with decomposition. It is readily soluble in water and dimethylformamide, slightly soluble in methanol, and insoluble in most other organic solvents.

Anticapsin shows a positive color development with nynhydrin and potassium permanganate reagents, but it does not react with BENEDICT, biuret, FEHLING, ferric chloride, MOLISCH or SAKAGUCHI reagents.

Paper Chromatograms of anticapsin were bioautographed against either *Streptococcus pyogenes* or *Salmonella gallinarum* and yielded the Rf values as indicated:

(i) *n*-BuOH saturated with water-0.12, (ii) *n*-BuOH saturated with water+2% *p*-toluenesulphonic acid - 0.58, (iii) MeOH - 0.1 N HCl (3 : 1) - 0.65, (iv) Propanol - pyridine - acetic acid - water (15 : 13 : 3 : 12) - 0.60, (v) MeOH - 0.05 M sodium citrate at pH 5.7 (70 : 30) - 0.66.

Electrometric titration showed the presence of two titrateable groups with pKa=4.3 and pKa=10.1 in 66% DMF, typical of an α-amino acid, affording an apparent molecular weight of 210. Elemental analysis of anticapsin revealed the following composition:

Calculated for $C_9H_{13}O_4N$:	C 54.26,	H 6.58,	O 32.13,	N 7.03.
Found:	C 53.91,	H 6.71,	O 32.01,	N 6.91.

Anticapsin does not give a satisfactory mass spectrum. The empirical formula ($C_9H_{13}\cdot O_4N$=199 M.W.) was substantiated by a high resolution mass spectrum of N-acetyl-anticapsin methyl ester[5].

Ultraviolet absorption spectroscopy of anticapsin shows a very weak absorption with λ_{max} 310 nm, ε=32.4 (water), typical of certain isolated ketones[5]. Reaction with 6 N hydrochloric acid at 110°C for 19 hours changes the ultraviolet spectrum to that typical of a simple phenol. The hydrolysis product in acid shows an absorption maximum with λ_{max} 223 nm, ε=5,300, and λ_{max} 276 nm, ε=1,300, and in alkali with λ_{max} 243 nm, ε=6,700, and λ_{max} 295 nm, ε=1,900.

Infrared absorption spectrum of anticapsin measured in a KBr disc is shown in Fig. 2. The carbonyl band at 1,720 cm^{-1} is not attributable to the carboxyl group of the amino acid, but again appears to support a ketonic function. Anticapsin is optically active[5], $[\alpha]_D^{25}$ +125° (*c* 1, H_2O).

Amino acid analysis[6] of unhydrolyzed anticapsin showed a major peak appearing after valine which did not correspond to any known amino acid. A small peak appeared between alanine and valine. This was due to a compound chemically related to anticapsin[5], but it could not be further purified by the methods discussed above. When anticapsin was subjected to 6 N hydrochloric acid at 110°C for 19 hours,

Fig. 2. Infrared absorption spectrum of anticapsin (KBr pellet)

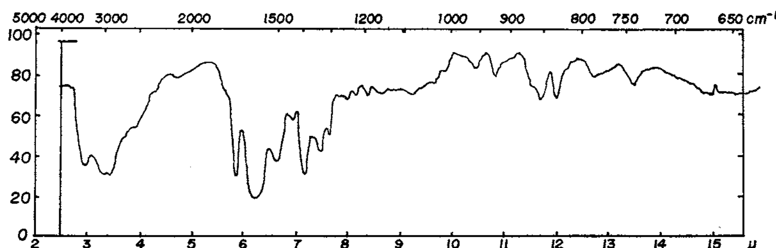
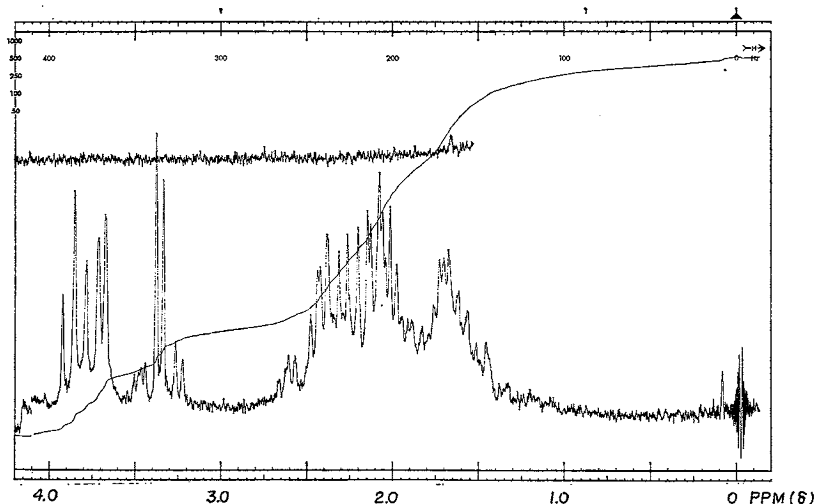


Fig. 3. Nuclear magnetic resonance spectrum of anticapsin in D_2O (TMS used as external standard)



a major peak appeared in the position of tyrosine, consistent with the ultraviolet spectrum of the hydrolysate as described above. The amino acid was isolated from the acid hydrolysate. The physical properties of the isolated compounds were identical to those of L-tyrosine[5].

Fig. 4. Bacilysin.

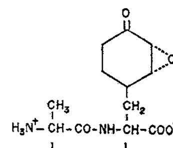
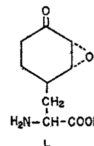


Fig. 5. Anticapsin.



The nmr spectrum of anticapsin in D_2O is shown in Fig. 3. The chemical shifts of the protons correspond to values characteristic for hydrogens of an α,β-epoxyketone[5].

From all available imformation, it appeared that anticapsin was an α-amino acid containing an epoxy-ketone group which was converted by acid to tyrosine. Bacilysin, an antibiotic from *B. subtilis*[7,8] was shown to have the structure shown in Fig. 4. Anticapsin, Fig. 5, has been shown to be identical to the epoxy-keto-amino acid obtained by cleavage of bacilysin[5,8]

Anticapsin inhibits the formation of hyaluronic acid capsules in *Streptococcus pyogenes* at 3 mcg/ml as measured in a phage-containing system[9]. With the exception of activity against *Salmonella gallinarum* (MIC 6.25 mcg/ml), anticapsin had no significant *in vitro* antimicrobial activity. While bacilysin has antibacterial activity against staphylococci, it is not active as an inhibitor of capsule formation in *Streptococcus pyogenes*[6,7]

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