Production of Anticapsin by *Streptomyces griseoplanus*¹

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Anticapsin is a new fermentation product which inhibits formation of the hyaluronic acid capsule of *Streptococcus pyogenes*. Production of this metabolite in a complex medium by *S. griseoplanus* is enhanced by high levels of carbohydrate. A number of carbon sources support biosynthesis but sucrose is most effective, the optimum level being 150 g/liter. Neither glucose nor fructose, alone or in combination, serves as an equivalent substitute for sucrose. The addition of dibasic potassium phosphate to the medium further increases anticapsin production. Dissolved oxygen levels are important for synthesis and stability of the metabolite. Anticapsular activity diminishes rapidly in previously aerated broths which are held under static conditions. This decrease does not occur in pasteurized broths or unpasteurized filtrates.

Beta-hemolytic streptococci of Lancefield group A form mucopolysaccharide capsules of hyaluronic acid (4). The capsule, in addition to being implicated in the virulence of these bacteria (15), imparts resistance to infection by bacteriophage (6). The latter characteristic permitted development of the *Streptococcus pyogenes* plate test for capsule-inhibiting compounds described by Whitney et al. (Bacteriol. Proc., p. 7, 1970). Subsequent screening by this method resulted in the detection of activity in a culture filtrate and the isolation of a compound which was named anticapsin. Anticapsin is produced by a strain of *Streptomyces griseoplanus*, NRRL-3507, isolated from a soil sample. A proposed mode of action for anticapsin was reported by Whitney and Funderburk (Abstr. 10th Int. Cong. Microbiol., p. 101, 1970). They found that it competitively inhibits L-glutamine-d-fructose-6-phosphate amidotransferase, the initial enzyme involved in the biosynthesis of uridine diphosphate-N-acetylg glucosamine, a precursor of hyaluronic acid. Isolation and characterization by Shah et al. (12) determined anticapsin to be a water-soluble, ninhydrin-positive compound having the empirical formula C₉H₁₃O₄N and yielding tyrosine upon acid hydrolysis. The structure has been reported by Neuss et al. (9) as an epoxy-keto-amino acid (Fig. 1) identical to the C-terminal residue of the dipeptide antibiotic bacilysin (10, 14) which is produced by *Bacillus subtilis*.

Initial efforts to isolate anticapsin were hampered by the low potency (23 µg/ml) of fermentation broths. This study was therefore undertaken for the purpose of enhancing production of the antibiotic.

MATERIALS AND METHODS

Culture preservation. *S. griseoplanus* was preserved by lyophilization of spores suspended in beef serum. Sporulated cultures were produced on agar slants by incubating for 6 days at 34°C on a medium containing 1% dextrin 700 (A. E. Staley Mfg. Co.), 1.5% nutri-soy flour (Archer-Daniels-Midland Co.), 0.2% nadorisol (National Distiller's Products Co.), 0.2% blackstrap molasses, 0.1% CaCO₃, and 2.5% agar in distilled water (pH 6.5).

Preparation of fermentor inoculum. Suspensions of sporulated slant cultures were added aseptically to wide-mouth 250-ml Erlenmeyer flasks which contained 50 ml of a medium composed of 1.5% glucose, 1.5% nutri soy grits (Archer-Daniels-Midland Co.), 1% corn steep liquor (Corn Products Co.), 0.5% NaCl, and 0.2% CaCO₃ in tap water (pH 6.5). Incubation was performed at 30°C for 48 hr on a model G35 shaker (New Brunswick Scientific Co., New Brunswick, N.J.), orbiting in a 2-inch diameter (5.08-cm) circle at 250 rev/min. Fermentors were inoculated with a volume of the mycelial suspension equal to 1% (v/v) of the fermentation medium.

Fermentors. Primary studies were conducted in wide-mouthed 250-ml Erlenmeyer flasks containing 50 ml of medium, which were incubated on a rotary shaker as previously described. Larger volume fermentations were conducted in stirred vessels of conventional design with a total capacity of 40 liters and a 1:1 height-diameter ratio for the 25-liter medium volume. The dissolved oxygen content of the broth,

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as measured by a modification of the galvanic membrane electrode described by Johnson et al. (5), was maintained above 40% saturation by controlling impeller speed at 600 rev/min and sparging with 0.5 volumes of air per volume of medium per minute.

Assay procedure. Biological activity was determined by a disc-plate agar diffusion test employing S. pyogenes strain C203 and a homologous bacteriophage capable of infecting only in the absence of host hyaluronic acid capsules. The simultaneous incorporation of host and phage into an agar layer produced normal growth of the encapsulated bacteria. Application of anticapsin-containing paper discs to the surface of test plates prior to incubation of the plates permitted growth of S. pyogenes but inhibited capsule synthesis, resulting in bacteriophage infection and lysis of nonencapsulated cells, as signified by clear zones surrounding the disc. The use of control plates lacking bacteriophage permitted differentiation between antibiotic and anticapsular activity. This quantitative assay method was capable of detecting anticapsin at levels of 4 µg/ml (Whitney et al., Bacteriol. Proc., p. 7, 1970).

Chromatographic identification of anticapsin. Fermentations were monitored by descending adsorption chromatography on Whatman no. 1 paper in water-saturated 1-butanol; water-saturated 1-butanol and methanol (80:20); and in water-acetic acid-1-butanol (4:1:4). Anticapsin was detected on developed chromatograms by a bioautographic technique employing the same S. pyogenes-bacteriophage system used for assay.

Carbohydrate determinations. Sucrose was measured by the anthrone method (7), reducing sugar by the Nelson method (8), and glucose by the glucose oxidase method of Comer and Brickley (2).

RESULTS

Preliminary testing. Individual components of the original screening medium (no. 1, Table 1), plus several additional substrates, were evaluated on the basis of their effects on growth of the microorganism, pH of the medium, and biosynthesis of anticapsin. Integration of these data resulted in medium 2, which supported a 65% increase of anticapsin production in shaken flasks and 240% in stirred vessels. The omission of glucose and dextrin from this formulation gave medium 3, which was used as the basal medium for subsequent supplementation.

Effect of glucose and dextrin. A linear relationship existed between anticapsin biosynthesis and the quantity of glucose or dextrin, or both, incorporated in basal medium 3. At concentrations up to 105 g/liter, both carbohydrates afforded similar anticapsin titers. A sharp reduction occurred with glucose at concentrations above 105 g/liter. Dextrin gave maximum titers at 120 g/liter without apparent inhibition at levels as high as 160 g/liter.

| Medium component | 1 | 2 | 3 | 4 | 5 | 6 |
|------------------|---|---|---|---|---|---|
| Glucose          | 15| 15|   |   |   | 70|
| Dextrin 700      | 10| 10| 150| 150| 137|
| Sucrose          | 15| 20| 20 | 20 | 20 |
| Nutrisoy grits   | 1 |   | 3  | 3  | 3  |
| Casein           | 1 | 1 | 1  | 1  | 1  |
| Amber EHC        | 3 | 3 | 3  | 3  | 3  |
| 2019 Yeast       | 1 | 1 | 1  | 1  | 1  |
| NaCl             | 5 |   | 2  | 2  | 2  | 2  |
| CaCO₃            |   | 2 | 2  | 2  | 2  | 2  |
| K₂HPO₄           |   | 1 |     |     |     |     |

| Terminal pH values for media 1 through 6 were 6.8, 7.6, 8.6, 6.8, 6.8, and 5.8, respectively. Per cent of growth (solids) in media 1 through 6 was 30, 40, 20, 42, 44, and 53, respectively. Anticapsin titers (micrograms per milliliter) in shaken flasks for media 1 through 6 are 49, 82, <8, 1,480, 1,910, and 2,140, respectively, and in stirred fermentors are medium 1, 23; medium 2, 79; medium 3, <8; medium 4, 1,370; medium 5, 2,060; and medium 6, 2,030. |

*Expressed as grams per liter.
With glucose as the carbon source, detectable levels of anticapsin were present at 24 hr, when growth was only 25 to 30% of the maximum (Fig. 2). Low levels of glucose caused the idiophase to be limited to the trophophase. Biosynthesis of anticapsin extended into the stationary phase only when high levels of glucose were employed. Peak titers coincided with depletion of the exogenous glucose.

**Comparative effectiveness of various carbon sources.** Of the other carbon compounds tested as substrates for the fermentation, only pentoses and glucosamine failed to support growth or anticapsin production. Optimum levels of the useful substrates were 100 g/liter or higher for all compounds except inulin, glycerol, and the oils. Sucrose gave higher titers of anticapsin than comparable levels of any other single carbon source (Table 2).

**Sucrose metabolism.** The kinetics of sucrose catabolism in relation to anticapsin biosynthesis are shown in Fig. 3. Maximal accumulation of anticapsin coincided with depletion of the exogenous carbohydrate, as previously observed with glucose and dextrin. Enzymatic hydrolysis of sucrose by invertase would be expected to yield equimolar quantities of glucose and fructose. Paper chromatograms confirmed the progressive disappearance of sucrose and the accompanying increase of free glucose and fructose in the medium. Glucose was present in greater quantity than fructose, however, which was verified by quantitative determinations.

**Molar comparison of sucrose, fructose, and glucose.** At concentrations of 0.2 to 0.4 M, sucrose was superior as a substrate to an equimolar level

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**TABLE 2. Effect of various carbon sources on biosynthesis of anticapsin by Streptomyces griseoplanus in medium 3**

| Substrate tested | Anticapsin titer (μg/ml) |
|------------------|-------------------------|
| Fructose         | 750                     |
| Galactose        | 615                     |
| Glucose          | 790                     |
| Mannose          | 360                     |
| Lactose          | 740                     |
| Maltose          | 690                     |
| Sucrose          | 1,090                   |
| Raffinose        | 510                     |
| Dextrin          | 780                     |
| Starch           | 470                     |
| Inulin           | 470                     |
| Glycerol         | 540                     |
| Refined soybean oil | 690                   |
| Cottonseed oil   | 745                     |

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**TABLE 3. Comparative value of equimolar levels of fructose, glucose, and sucrose regarding ability to support the production of anticapsin in medium 3**

| Carbohydrate molarity | Fructose | Glucose | Sucrose |
|-----------------------|----------|---------|---------|
| 0.1                   | 50       | 45      | <17     |
| 0.2                   | 220      | 230     | 660     |
| 0.3                   | 380      | 390     | 1,090   |
| 0.3*                  | 790      | 1,280   |         |
| 0.4                   | 550      | 550     | 1,320   |
| 0.4*                  | 1,170    | 1,010   | 2,030   |
| 0.5                   | 705      | 690     | 970     |
| 0.6                   | 340      | 860     |         |
| 0.7                   | N*       | 480     |         |

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* See Table 1.
* Supplemented with 0.3 M sucrose.
* Supplemented with 7% dextrin.
* No growth.
TABLE 4. Concentration of minerals* in medium 4a before and after conducting the anticapsin fermentation with Streptomyces griseoplanus

| Mineral      | Mineral level* (μg/ml) |
|--------------|------------------------|
|              | Prior to fermentation  | After fermentation |
| Sodium       | 56                     | 54                    |
| Potassium    | 448                    | 260                   |
| Calcium      | 157                    | 746                   |
| Magnesium    | 68                     | 70                    |
| Cobalt       | 0.3                    | 0.3                   |
| Copper       | 0.5                    | 0.6                   |
| Iron         | 0.8                    | 0.5                   |
| Manganese    | 0.7                    | 0.8                   |
| Zinc         | 0.7                    | 0.6                   |
| Chloride     | 102                    | 97                    |

* Particulate material was removed by centrifugation prior to analysis.

a Determined by atomic absorption spectroscopy.

TABLE 5. Effect of K2HPO4 on production of anticapsin by Streptomyces griseoplanus in medium 4a

| K2HPO4 Level b (ml) | Anticapsin titer (μg/ml) |
|---------------------|--------------------------|
| 0                   | 1,370                    |
| 0.25                | 1,560                    |
| 0.5                 | 1,740                    |
| 1.0                 | 2,060                    |
| 3.0                 | 1,940                    |

b Incorporated into the medium prior to autoclaving.

d of fructose or glucose (Table 3). Combining the yields obtained from glucose and fructose, tested individually at 0.3 m, should give a theoretical yield of 770 μg of anticapsin per ml from 0.3 m sucrose; the actual yield was 1,090 μg/ml.

In terms of anticapsin production, fructose and glucose were comparable at levels of up to a 0.5 m concentration. Higher levels of fructose inhibited growth and biosynthesis, whereas glucose was tolerated at a 0.6 m concentration, with a resultant increase in anticapsin production.

When medium 3 contained 70 g of dextrin per liter, the addition of 0.05 m glucose increased anticapsin synthesis 100%; the addition of 0.05 m fructose increased synthesis 180%. However, 0.04 m sucrose provided greater stimulation than either of its monomers, increasing synthesis 280%.

Effect of phosphate and minerals. Typical mineral content of medium 4 in stirred fermentors is illustrated in Table 4. The potassium level characteristically decreased by almost 200 μg/ml during the course of the fermentation. The calcium increase of approximately 600 μg/ml may have resulted from disassociation of CaCO3.

Numerous monovalent and divalent ions were examined as additions to medium 4, but only inorganic phosphate enhanced production of anticapsin. The stimulatory nature of dibasic potassium phosphate is shown in Table 5. Although inorganic phosphate increased titers 50% in the sucrose medium, it was not stimulatory in a sucrose-dextrin medium.

Effect of oxygen tension. Low concentrations of dissolved oxygen exerted a limiting effect on anticapsin production in medium 5 (Fig. 4). The minimum level necessary for unrestricted synthesis was between 7 and 20% of saturation. Product formation occurred at the same rate whether oxygen tension was maintained at 20 or 60%. Growth rates were identical over the range of 1 to 60% saturation, indicating that the minimum level necessary for growth was lower than the minimum level critical for the accumulation of anticapsin.
TABLE 6. Stabilization of anticapsin in nonaerated, whole broths by pasteurization

| Treatment of whole broths after withdrawal from fermentor | Time of mycelial removal from broth | Anticapsin titer (μg/ml) |
|-----------------------------------------------------------|-----------------------------------|--------------------------|
| Untreated, Pasteurized                                   | Immediate                         | 2,070, 65                |
| X X                                                        | X X                               | 1,890                    |
| X X                                                        | X X                               | 1,880                    |

*a Heated for 10 min at 80 C.

Instability after terminating aeration-agitation. The anticapsin titer of 160-hr fermentation broths remained constant when aerobic incubation was continued for an additional day (Fig. 3). Titters also remained constant in broth filtrates incubated for a similar period either with or without aeration. However, static incubation of whole, unfiltered broths resulted in approximately a 95% reduction of the capsular titer within 7 hr (Table 6). This loss in activity could be averted by pasteurizing the unfiltered broth immediately upon conclusion of the aerated fermentation, although a 10% reduction in capsular activity occurred during the heating process.

DISCUSSION

Stirred-fermentor titers of anticapsin in a complex medium were increased almost 100-fold. Preliminary titration of glucose and dextrin indicated a quantitative correlation between anticapsin biosynthesis and the carbohydrate concentration of the medium. High levels of carbohydrate did not enhance the rate of anticapsin synthesis. Initiation of the idiophase was actually retarded, and higher titers were achieved only through prolongation of the biosynthetic period.

A number of sugars and oils would support anticapsin production when substituted for glucose or dextrin. The superior potencies afforded by sucrose appear to result from the tolerance of the microorganism for high levels of the disaccharide, maintenance of nontoxic levels of free hexoses through their gradual hydrolytic release from sucrose, the glucose-fructose combination, and the apparently preferential utilization of the fructose moiety early in the fermentation. Experiments employing glucose, fructose, or dextrin in combination with sucrose indicated that mixtures richer in the aldose were preferred for optimal anticapsin production.

Inorganic phosphate markedly enhanced the production of anticapsin in the sucrose medium. Stimulation of metabolite production by phosphate in other fermentations has also been observed (1, 3, 13). CaCO₃ was important in the fermentation; the frequent observance of pH values near 5.5 prior to its inclusion suggested its role as a buffer.

Shikimic acid has been reported to be a precursor of bacilysin formation by B. subtilis, although tyrosine is not (11). The biosynthetic pathway to anticapsin in S. griseopsis has not yet been elucidated.

This appears to be the first report of a biologically active, secondary metabolite which is synthesized aerobically but is destroyed by the producing microorganism under conditions of low oxygen tension. The nature of the molecular alteration resulting in inactivation of anticapsin under semianaerobic conditions is being investigated.

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