Purification and properties of the chymotrypsin-like serine proteinase overproduced by *Streptomyces* sp. strain C5-A13


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Abstract. The major serine proteinase of *Streptomyces* sp. strain C5-A13, a proteinase-overproducing mutant strain, was purified to homogeneity. It has a relative molecular mass (Mr) of 19500 as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Superose-6 gel filtration chromatography, a low isoelectric point, optimal activity at pH 8.5-9.5, and an optimal temperature of approx. 55 °C. This purified enzyme has a high specific activity on azocasein of 11000 units mg⁻¹ of protein, a Kᵣ of 2.7 × 10⁻⁵ M and a Vₘₐₓ of 2.0 μmol min⁻¹ mg⁻¹ of protein when succinyl-alanyll-alanyl-prolyl-phenylalanine p-nitroanilide was used as substrate. It was inhibited by the serine proteinase inhibitors diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride but not by ethylenediaminetetraacetate (EDTA) or N-tosyl-L-phenylalanine chloromethyl ketone. Thus, this serine proteinase appears to be a chymotrypsin-like enzyme and represents nearly 90% of the total extracellular azocasein-hydrolyzing activity produced by strain C5-A13. Since CaCO₃ did not stimulate activity of the purified enzyme itself, the carbonate effect may be a transcriptional or translational rather than a post-translational one.

Introduction

*Streptomyces* sp. strain C5-A13 was isolated as a N-methyl-N'-nitro-N-nitrosoguanidine-induced, proteinase-overproducing mutant strain, and antibiotic-non-producing pleiotropic mutant of C5, an anthracycline antibiotic producer (Gibb et al. 1987). More recently, we have shown that the increased C5-A13 proteinase activity was partially due to a stimulation by carbonate anions in the medium (Gibb et al. 1989). These previous efforts, however, did not discern whether the increased azocasein hydrolysis activity was due to a single or to multiple proteinases. Furthermore, we had not determined whether the increase in proteolytic activity generated by the presence of carbonate anions was due to production of the proteinase, e.g., transcriptional or translational activation, or to the increased activity of the enzyme(s) themselves.

In this paper, we report the purification of the carbonate-stimulated formation of serine proteinase by C5-A13, show that it is the major serine proteinase of this strain, and that it is the primary proteinase, the synthesis of which is stimulated by carbonate in the medium.

Materials and methods

**Bacterial strains and cultivation.** *Streptomyces* sp. strain C5-A13 was maintained on yeast-malt agar (YMA) as described previously (Dekleva et al. 1985). For purification of the extracellular proteinase, 50 ml trypticase soy broth (TSB) in 250-ml flasks containing a coiled spring for mycelial dispersion (Dekleva et al. 1985) were inoculated from agar plates, incubated on a rotary shaker (250 rpm) at 30 °C for 48 h, and then used to inoculate 400 ml TSB in 2-1 flasks also containing coiled springs. After 24 h of incubation at 30 °C on a rotary shaker (250 rpm), these 400-ml seed cultures were used to inoculate a 14-1 New Brunswick stirred tank fermentor containing 91 glucose-yeast extract (GYE) medium. GYE medium contained (grams per liter): glucose (CPC International, Englewood Cliffs, N.J., USA), 31.25; Amberex yeast extract (Universal Foods, Milwaukee, Wis., USA), 12.5; NaCl, 3.0; and CaCO₃, 6.25 and adjusted to pH 7.25 with 5 M NaOH (Gibb et al. 1989). Fermentation was carried out at an air-flow rate of 101-min⁻¹, 400 rpm, 30 °C, and initial pH of 7.25 (not controlled). Autoclave-sterilized antifoam, DF-60P (Mazer Chemical Corporation, Gurnee, Ill., USA), was added as necessary.

**Purification procedures.** All purification steps, including the initial harvesting, were carried out at 4 °C. At the end of a 72-h fermentation, biomass was removed by tangential-flow filtration using a Pellicon system (Millipore, Bedford, Mass., USA) equipped with 0.45-μm membranes. The mycelium-free culture fluid, con-
Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Dreyfuss et al. (1984). The gel was run using a Pharmacia Flat Bed Apparatus FBE 3000 according to the methods of Dreyfuss et al. (1981). The Mr was calculated by linear regression analysis as obtained with SAAPF-pNA (0.25 mM final concentration) as the substrate and 100 mM HEPES and 10 mM CaCl₂ (pH 8.0) as the buffer. The temperature was controlled using the Haake water bath, which adjusted the cuvette holder temperature as needed.

The following proteinase inhibitors (all obtained from Sigma) were examined for their effect on SAAPF-pNA hydrolysis: DIFP, phenylmethylsulfonyl fluoride (PMSF), N-tosyl-l-phenyl-alanine chloromethyl ketone (TPCK), and ethylenediaminetetraacetic acid (EDTA). PMSF was dissolved in DMSO. The enzyme samples were preincubated at room temperature with each inhibitor, or in the presence of a DMSO control as indicated. The assays were then carried out at 37°C using SAAPF-pNA (0.25 mM final concentration) as substrate and 100 mM HEPES (pH 8.0) as buffer.

The effect of carbonate on azocasein hydrolysis by the purified proteinase was determined by incubating 70 mM (final concentration) CaCO₃, NaHCO₃, NaCl, CaCl₂, or water (i.e., no salt addition) with 20 μg of the purified enzyme for 15 min at room temperature, followed by the assay for azocasein hydrolysis activity at pH 8.0 and 37°C according to the methods described by Gibb et al. (1989).

Determination of kinetic constants. The K_m and V_max values of the purified proteinase for SAAPF-pNA were determined from an Eadie-Hofstee plot generated from the initial reaction velocities obtained with SAAPF-pNA concentrations of 10-50 μM and 1.5 μM purified enzyme per assay. Each assay was carried out in duplicate at 37°C in 100 mM HEPES (pH 8.0) buffer plus 10 mM CaCl₂. The change in absorbance at 410 nm was monitored continuously and the initial linear velocity was used for calculation of the kinetic constants.

Results and discussion

Production of serine proteinase by C5-A13

Approximately 90% of the azocasein hydrolysis activity produced by C5-A13 grown in GYE medium eluted from DEAE-Sephadex in a single broad peak prior to application of the salt gradient (Fig. 1). The azocasein hydrolysis activity in these fractions was completely inhibited by DIFP, a serine proteinase inhibitor. When the cell-free culture broth was tested with 3H-DIFP to deter-