Extracellular Serine-proteinases Isolated from *Streptomyces alboniger*: Partial Characterization and Effect of Aprotinin on Cellular Structure

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Streptomyces alboniger *ATCC 12461 grown in brain heart infusion (BHI) medium produced two extracellular serine-proteinases, denoted SP I and SP II, which were purified by ammonium sulfate precipitation and aprotinin-agarose affinity chromatography. SP I was purified 88,9-fold and SP II 66,7- fold, with 33.4% and 10.4% yield, respectively. The optimum pH for the proteinases activity, using α-N-p-*tosyl-*L-arginine-methyl ester (TAME) as substrate, was 9-10 and the optimum temperature was 37ºC. The proteolytic activity of SP I and SP II was inhibited by aprotinin and SP I was partially inhibited by leupeptin, both serine-proteinase inhibitors.* S. alboniger *growth in BHI-liquid medium decreased when 5 μg/ml, 10 μg/ml of aprotinin was used, being completely inhibited with 20 μg/ml and 40 μg/ml. At the ultrastructural level, aprotinin-treated* S. alboniger *cells showed swelling of the bacterial body and condensation of the genetic material, probably related to the inhibition of its growth.*

Key words: *Streptomyces alboniger* - serine-proteinases - growth - aprotinin

Streptomycetes are Gram-positive bacteria with an unusual morphological complexity, including the sequential production of substrate and aerial mycelia and spores (Chater & Hopwood 1984). This group of bacteria is industrially important because they produce several antibiotics, exoenzymes, such as proteinases, and other secondary metabolites of commercial value (Berdy 1984, Peczynska-Czoch & Mordask 1988, Sanglier et al. 1993).

Several proteinases have been obtained from streptomycetes and biochemically characterized, such as *Streptomyces pactum* serine-proteinase, *Streptomyces exfoliatus* metallo- and serine-proteinases and *Streptomyces rimosus* aminopeptidase (Bockle et al. 1995, Kim & Lee 1995, Vitale et al. 1996). These enzymes are involved in the assimilation of proteinaceous nitrogen sources, degradation of aerial mycelium and sporulation processes, as well as in antibiotic production (Ginther 1979, Kitadokoro et al. 1994, Kang et al. 1995, Kim & Lee 1996).

In the present study, we performed a qualitative analysis in SDS-PAGE-gelatin of proteinases produced by *S. alboniger.* Additionally, two serine-proteinases were purified and partially characterized. The effects of aprotinin, a serine-proteinase inhibitor, on *S. alboniger* growth and upon the ultrastructure of this microorganism were also analyzed.

MATERIALS AND METHODS

Microorganism and growth conditions - S. alboniger ATCC 12461 was maintained on brain heart infusion (BHI) containing 1.5% (w/v) agar. Liquid cultures were obtained by inoculating 30 ml BHI medium with a suspension of approximately 10^5 spores. This culture was used to inoculate 1l of BHI. The microorganism was grown for 14 days at 28ºC.

Obtention of cellular extract (C) and extracellular concentrate (E) - The cell-free culture supernatant (E) was obtained by centrifugation at 10,000 g for 15 min at 4ºC and concentrated against polyethyleneglycol 4,000 for 24 hr at 4ºC. E was

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mixed with SDS-PAGE sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol and 2% bromophenol blue) in a proportion of 7:3 (v/v). Cells were washed three times with isotonic buffer saline solution (0.85% NaCl) and ressuspended in 100 ml of sample buffer in order to obtain the C.

SDS polyacrylamide gels containing co-polymerized gelatin - Proteinases in C and E were assayed and characterized by electrophoresis in 7.5% SDS-PAGE (Laemmli 1970) with 0.1% copolymerized gelatin (Heussen & Dowdle 1980). After running, the gels were soaked for 1 hr at 25ºC in 2.5% Triton X-100 solution, and incubated overnight at 37ºC under two different conditions: 50 mM phosphate buffer, pH 5.5, and 100 mM glycine-NaOH buffer, pH 10.0. Proteolytic activity was detected by staining gels for 1 hr with 0.1% amido black in methanol-acetic-acid-water (30:10:60, v/v/v) and destaining in the same solvent. For enzymatic secretion studies, aliquots of 30 ml from culture medium were daily collected during 14 days, concentrated and analyzed in 7.5% SDS-PAGE gelatin using 50 mM glycine-NaOH buffer, pH 10.0.

Proteinases purification - The *S. alboniger* cell-free culture supernatant was used as the starting material for the purification of extracellular proteinases. (1) Ammonium sulfate precipitation – The culture supernatant was brought to 40% saturation with ammonium sulfate, followed by centrifugation at 10,000 g for 30 min. The precipitated fraction was dissolved and dialysed against distilled water. After dialysis the fraction was centrifuged at 10,000 g for 45 min at 4ºC. (2) Affinity chromatography – The active fraction obtained from the ammonium sulfate fractionation was applied to an aprotinin-agarose column (Sigma), pre-equilibrated with 0.01 M Tris-HCl buffer, pH 5.0, containing 5 mM $CaCl_2$. After exhaustive washing in equilibrating buffer, the adsorbed proteins were eluted with the same buffer in a linear gradient of $CaCl_2$ (5 mM-15 mM). Fractions of 1 ml were collected and the protein content detected at 280 nm. Protein-containing fractions were pooled and used for further studies.

Protein concentration determination - Protein concentration was determined using bovine serum albumin as standard (Lowry et al. 1951).

Assay of proteinase - Activity of the extracellular proteinase was performed at 37ºC by measuring the *tosyl* derivative released from α-N-p-*tosyl*-L-arginine-methyl ester (TAME). The assay mixture (1 ml) consisted: 800 µl 0.2 M glycine-NaOH buffer and 100 µl 10mM substrate (TAME). The reaction was initiated by addition of 100 µl of enzyme solution and incubated for 30 min. Enzyme activity was determined at 247 nm (Aguiar et al. 1996). Assays were run in triplicate with simultaneous controls (substrate only). One unit of proteolytic activity was defined as the amount of enzyme required to produce an increase of 0.001 absorbance at 247 nm unit under standard assay conditions.

Optima pH and temperature - The effect of pH on enzymatic activity was determined under the standard assay conditions described above, using the following buffers: 0.1 M citrate (pH 4.0-5.0), 0.2 M sodium phosphate (6.0-8.0), 0.2 M glycine-NaOH (9.0-10.0) and 0.1 M glycine-NaOH (11.0-12.8). The optimum temperature for enzyme activity (28ºC, 40ºC, 50ºC and 60ºC) was also determined, using 0.2 M glycine-NaOH, pH 10.0 as buffer.

Effect of proteolytic inhibitors - For inhibition studies, the enzymes and inhibitors *trans*-epoxysuccinyl-L-leucylamido- (4-guanidino) butane (E-64), ethyleneglycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA), ethyl-enediaminetetraacetic acid (EDTA), 1.10 phenantroline, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (STI), aprotinin, leupeptin, tosyl-L-phenylalanylchloromethylketone (TPCK) and pepstatin were pre-incubated for 30 min prior to addition of the substrate (TAME). Assays were run in triplicate with simultaneous controls.

Effect of aprotinin on Streptomyces alboniger growth - The effect of aprotinin was observed using solid and liquid media. Briefly, 37 g of BHI was dissolved in 1l of distilled water. In solid media, agar was added at the concentration of 1.5%. Twenty ml of culture medium was then poored per plate and allowed to harden. Different quantities of each amount of aprotinin (25 µg, 50 µg, 100 µg and 200 µg) were added to a 20 µl spore suspension (about 10^{-3}). This mixture was then inoculated on BHI agar plates and BHI-liquid medium. Aprotinin in BHI-liquid medium was diluted to a final concentration of 5 µg/ml, 10 µg/ml, 20 µg/ml and 40 µg/ml. The media were incubated at 28ºC during seven days.

Ultrastructural analysis of aprotinin treated cells - Effects of aprotinin on *S. alboniger* growth on liquid media were determined by transmission electron microscopy. Cells were fixed for 1 hr at 4ºC with glutaraldehyde (GA) in 0.1 M sodium cacodylate buffer, pH 7.2 and subsequentely for 1 hr at 4ºC with 1% OsO_4. The cells were then dehydrated through an ascending series of acetone and embedded in Epon. Thin sections were picked up on 300 mesh copper grids and stained with uranyl acetate and lead citrate. The material was examined in a Zeiss EM 10B transmission electron microscopy.

RESULTS

SDS-PAGE-gelatin - The proteinases in E and C extracts were most active at pH 10.0 (Fig. 1A). Proteinases migrating at 200 kDa, 45kDa and 35 kDa were detected in both extracts. The extracellular proteinase profile showed additional bands migrat-

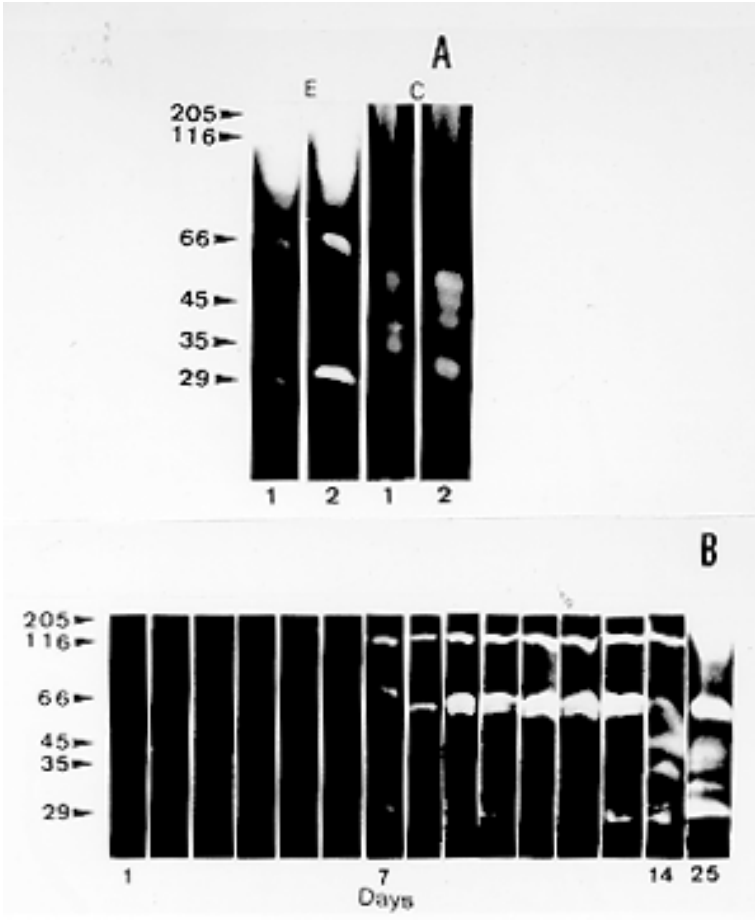

Fig. 1: SDS-PAGE-gelatin analysis of *Streptomyces alboniger* proteinases. A: cellular (C) and extracellular (E) proteinases. 1: pH 5.5 and 2: pH 10.0; B: E proteinases secretion profile until the 25th day. Molecular weight markers in kDa are indicated in the left side of the figure.

ing at 66 kDa and 29 kDa while proteinases with 55 kDa, 50 kDa and 20 kDa were observed only in cellular extracts. Secretion studies revealed that during the first seven days of culture, only the 200 kDa and 66 kDa proteinases were secreted to culture medium (Fig. 1B). After seven days, the 29 kDa enzyme was also detected. No qualitative difference was observed until the 13th day. Additional bands migrating at 35 kDa and 45 kDa were found in the 14th day. This profile was observed until the 25th day of cultive.

Proteinase purification - Fig. 2 shows the elution profile of *S. alboniger* extracellular proteinases using an aprotinin-agarose affinity column chromatography. Two peaks of serine proteinases (SP I and SP II) eluted with 8-10 mM $CaCl_2$ were obtained. Fractions 2-6 (corresponding to SP I) and 7-12 (corresponding to SP II) were collected and pooled due to protein content and proteolytic activity. The final enzyme preparations showed single protein and protease bands on SDS-PAGE and SDS-PAGE - gelatin (Fig. 3). This analysis demonstrated that SP I and SP II molecular masses are aproximately 29kDa and 66 kDa, respectively. The remaining E proteolytic activity was not retained by the affinity column. The purification procedure is summarized in Table I. The SP I was purified 88.9 fold with a yield of 33.4% and the SP II was purified 66.7 fold with a yield of 10.4%.

Optima pH and temperature - The optimum pH for SP I and SP II activities was 9-10, with a markedly reduction in activity at pH values above pH 11.0 (Fig. 4). The optimum temperature for SP I and SP II activities was 37ºC. At 28ºC weak enzymatic activity was observed with both proteinases. SP I and SP II were inactivated when incubated at 60ºC (Fig. 5).

Effects of proteolytic inhibitors - The effects of several inhibitors on SP I and SP II activities are shown in Table II. The activity of SP I and SP II were significantly inhibited by aprotinin and SP I was partially inhibited by leupeptin, both serine-proteinase inhibitors.

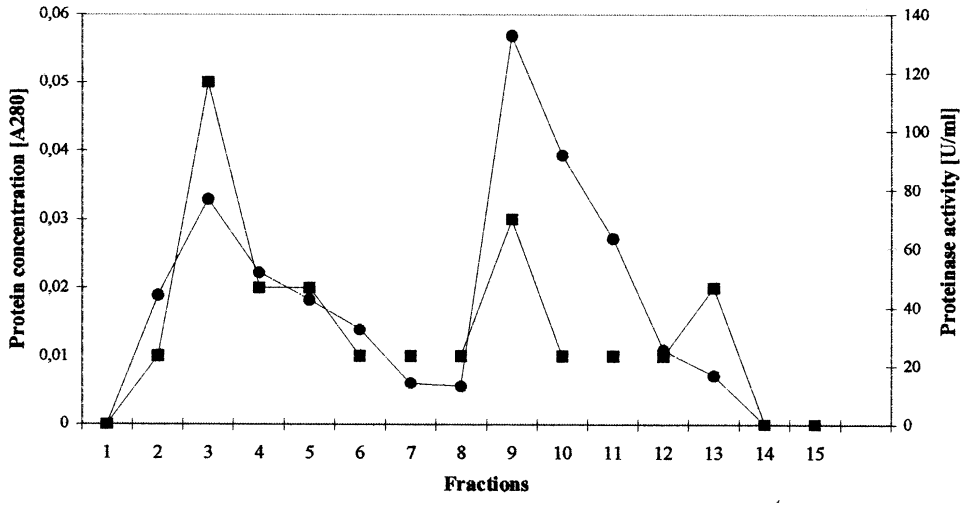

Fig. 2: elution profile of *Streptomyces alboniger* extracellular proteinases using aprotinin-agarose affinity chromatography (■) protein concentration and (•) proteinase activity. Fractions of 1 ml were collected.

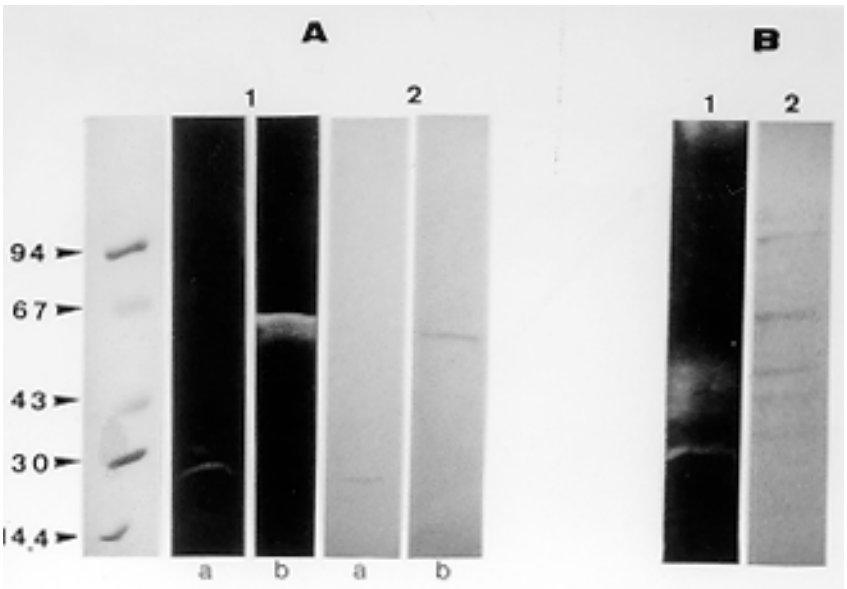

Fig. 3 - A: SDS-PAGE-gelatin (1) and SDS-PAGE (2) analyses of affinity-purified *Streptomyces alboniger* extracellular proteinases SP I (a) and SP II (b); B: extracellular proteases (1) and proteins (2) not adsorbed in the aprotinin-agarose column.

TABLE I

Purification of *Streptomyces alboniger* extracellular proteinases

Fractions	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U mg ptn^{-1})	Purification (-fold)	Yield (%)
Culture medium	300	320.8	479.6	1.5	1	100
Ammonium sulfate precipitation	15	25.74	229.3	8.9	5.9	47.8
Aprotinin-agarose						
SP I	4	1.2	160	133.3	88.9	33.4
SP II	5	0.5	50	100	66.7	10.4

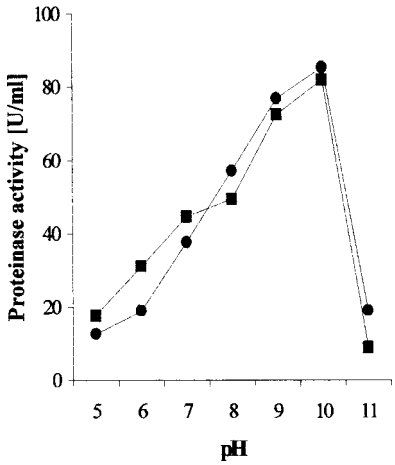

Fig. 4: effect of pH for SP I and SP II proteolytic activity (■) SP I and (•) SP II.

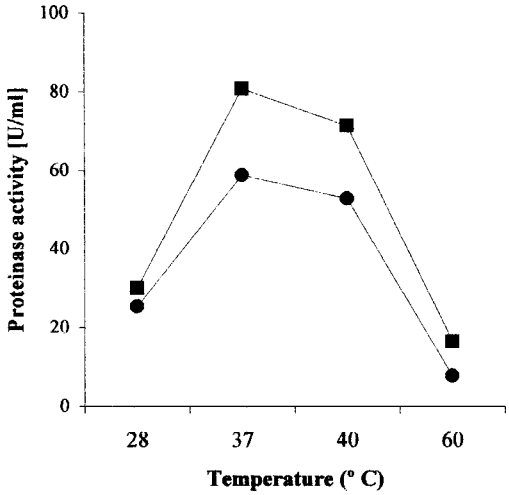

Fig 5: effect of temperature for SP I and SP II proteolytic ativity (■) SP I and (•) SP II.

Effect of aprotinin on S. alboniger growth - *S. alboniger* cells were completely inhibited by addition of the aprotinin inhibitor to BHI-agar in all concentrations used (data not shown). The effect of addition of aprotinin on *S. alboniger* growth in BHI-liquid medium was analyzed at the ultrastructural level. When 40 μg and 20 μg/ml aprotinin was added, complete inhibition was achieved during the seven days of growth. At concentrations of 5 μg/ml and 10 μg/ml, of the proteolytic inhibitor a significant decrease in growth was observed.

Ultrastructural analysis - The concentrations of 5 μg/ml and 10 μg/ml were used in ultrastructural analysis and the same effects were observed. Aprotinin treatment of *S. alboniger* caused a swelling of the bacterial body. Fig. 6B shows the septum and the cell envelope thicker than control cells (Fig. 6A). A low electron density in these structures was also observed after aprotinin treatment. However, the major alteration caused by aprotinin was a condensation of the genetic material (Fig. 6B).

TABLE II

Effect of various proteolytic inhibitors on *Streptomyces alboniger* SP I and SP II activity measured using TAME as substrate. The results are the mean of three experiments

Inhibitor	Concentration	Residual activity (%)	
		SP I	SP II
—	—	100	100
PMSF	2 mM	98	99
STI	100 μg/ml	68	81
Aprotinin	100 μg/ml	28	30
TPCK	100 μl	83	70
Leupeptin	100 μM	43	88
E-64	20 μM	73	72
EGTA	1 mM	81	72
EDTA	1 mM	97	79
1,10 phenantroline	50 μM	71	73
Pepstatin	2 μM	97	90

DISCUSSION

This work describes the purification and some biochemical properties of two extracellular proteinases isolated from *S. alboniger*. In this genus the extracellular serine-proteinases are expressed in the stationary phase and regulate the morphogenesis process closely linked to mycelial growth (Kim & Lee 1995). These enzymes were purified using aprotinin affinity chromatography. Studies with inhibitors suggest that both enzymes were serine-proteinases because they were inhibited by aprotinin. SP I was inhibited by leupeptin and partially inhibited by STI, which are used to trypsin-like proteinases. Interestingly, the proteinases SP I and SP II were insensitive to PMSF, as previously observed for an extracellular serine-proteinase isolated from *Streptomyces* sp. (Bono et al. 1996). This finding was consistent with the possibility that they could be trypsin-like proteinases since PMSF reacts very slowly with trypsins (Powers & Haper 1986). At the present moment all serine-proteinases found in *Streptomyces* spp. are chymotrypsin-, trypsin- and subtilisin-like enzymes (Sidhu et al. 1994). The optima pH and temperature values determined for these enzymes are in agreement with previous reports that described neutral to alkaline values of pH and temperatures ranging from 35°C to 75°C for proteinases purified from streptomycetes (Chandrasekaran & Dhar 1987, Kang et al. 1995, Kim & Lee 1996, Yeoman & Edwards 1997).

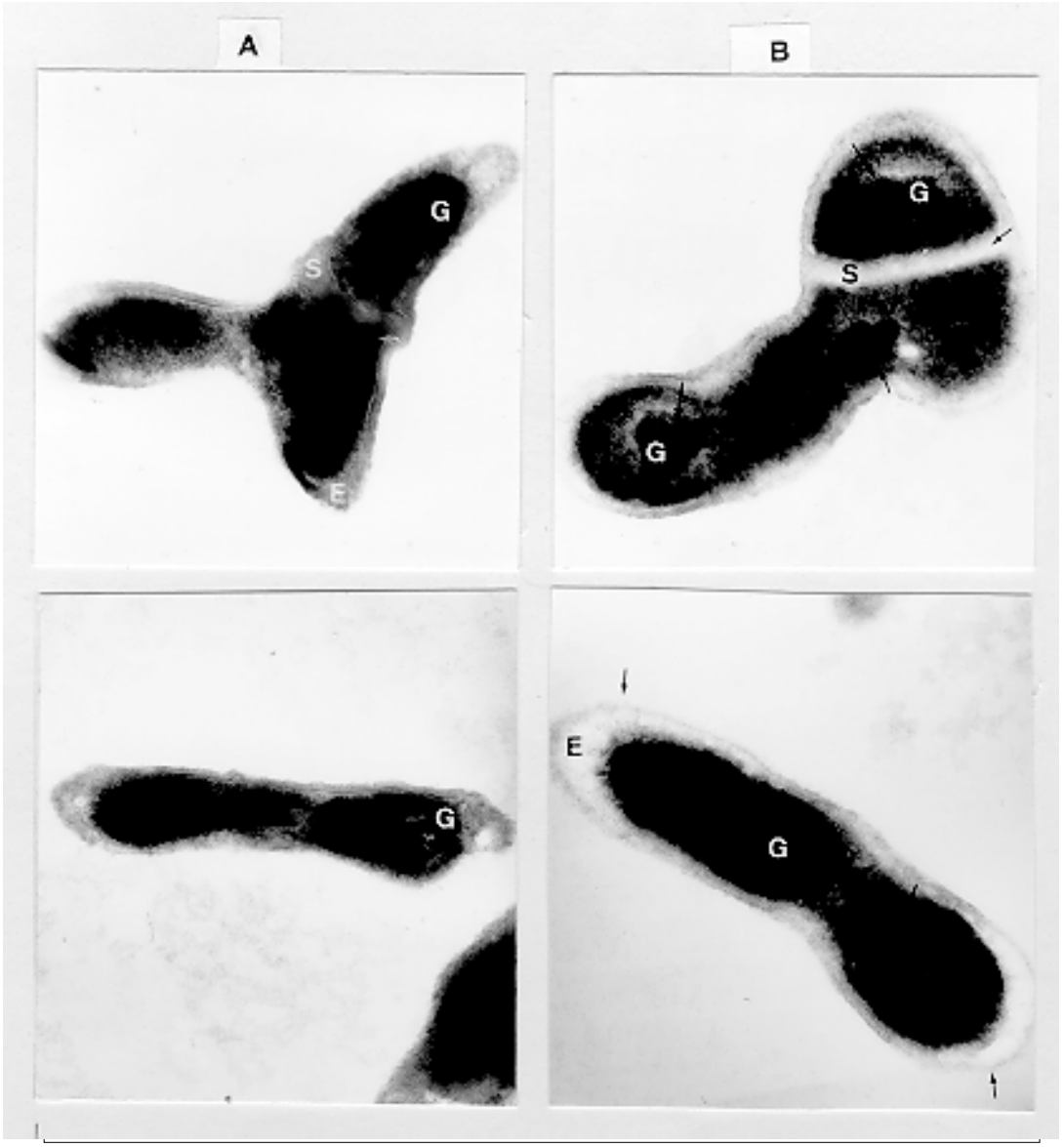

Fig 6: ultrastructural analysis of aprotinin-treated *Streptomyces alboniger* cells. A: control cells; B: aprotinin-treated cells. E: cellular envelope; S: septum; G: genetic material. Arrows indicate the major alterations detected in *S. alboniger* cells induced by aprotinin. For more details, see results.

When *S. alboniger* was grown in the presence of aprotinin its growth was partially or completely inhibited depending on the inhibitor concentration. Ultrastructural analysis showed a low electron density of the cell wall and of the septum as well as a little swelling of the bacterial body consistent with the observations of Pellegrini et al. (1992). The images exhibited centrally the genetic material, displaying a more condensed structure.

Proteolytic enzymes such as serine-proteinases may be important in the regulation of cell proliferation (Berezney 1979). Several studies with eukaryotic cells have demonstrated mitogenic effects of proteinases and suppression of cellular proliferation due to proteinases inhibitors (Ku et al. 1981, Wong et al. 1987, Jensen & O'Leary 1990). The coordination of cell cycle progression and cell differentiation with the initiation of DNA replication is fundamental, yet poorly understood in procaryotic cells. The participation of serine-proteinases in the DNA replication of *Escherichia coli, S. aureus, S. epidermidis* and *Bacillus subtilis*, has been de-

scribed (Irisawa et al. 1993, Kato et al. 1994, Jiang et al. 1998). Jenal and Fuchs (1998) suggested that the ubiquitous occurrence of the serine-proteinases in various bacteria is linked to their pivotal role in the onset of DNA synthesis by the specific degradation of proteins responsible for the negative control of chromosome replication. With regard to the role played by proteases in DNA synthesis of eukaryotic cells, a number of mechanisms have been suggested. In particular one of them indicated that serine-proteinases may be involved in the conversion of chromatin into an acceptable DNA polymerase substrate, probably acting upon nuclear proteins involved in DNA folding (Jensen & O'Leary 1990). On the other hand, the *S. alboniger* DNA alterations detected after aprotinin treatment could be due to antibacterial activity of the inhibitor as shown previously (Pellegrini et al. 1992).

Our results point to a direct correlation between the action of aprotinin and the inhibition of *S. alboniger* growth. Additional studies will be required to elucidate the precise mechanisms behind this inhibition.

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