SYNTHESIS AND PROPERTIES OF A PROTOPLAST-BURSTING FACTOR FROM BACILLUS AMYLOLIQUEFACIENS

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SUMMARY

The cells of young cultures of B. amyloliquefaciens, formerly called B. subtilis,[1] which are not producing significant amounts of extracellular enzymes yield stable protoplasts; by contrast, secreting cells a few hours older fail to give protoplasts at all.[2] It has now been found that this protoplast instability is not due to a change of membrane structure associated with protein secretion, but to a heat stable protoplast-bursting factor which is secreted simultaneously with extracellular enzymes.

The bursting factor is a peptide-lipid virtually identical to "surfactin" isolated from B. subtilis supernatants by Arima et al.[3] An assay method for the factor has been devised and its production by cells studied. The factor inhibits the growth of a number of Gram positive organisms.

EXPERIMENTAL

Protoplast-bursting factor was isolated from 30 hr culture supernatants using essentially the ether-extraction procedure of Arima et al.[3] No attempt was made to crystallise the compound. The preparation gave a single band after thin layer chromatography and paper electrophoresis and all protoplast bursting activity was present in the corresponding band of a duplicate.

Thin layer chromatography was carried out on silica gel G using ethanol: chloroform (35:65, v/v) and paper electrophoresis in pyridine-acetic acid-H_2O (25:1:225, v/v), pH 6.5 for 3 hr at

340 V. Detection of the compound was by a chlorination procedure[4] or by I_2 vapour (it did not react with ninhydrin).

RESULTS AND DISCUSSION

Identification of protoplast-bursting factor. The isolated factor after hydrolysis in 6 N HCl at 110° for 24 hr yielded L-aspartate, L-glutamate, L-valine, L-isoleucine, L-leucine, and D-leucine together with a brown oil which was identified as fatty acid from its infra-red spectrum. It is concluded that the factor is a peptide-lipid. (At 3 μg/ml it causes the complete and instantaneous lysis of B. amyloliquefaciens protoplasts.) The infra-red spectrum of the isolated factor was virtually identical to that reported for "surfactin".[3]

Apparent membrane fragility of secreting cells. Previous workers[5,6] have reported on the apparent instability of membranes of older secreting B. subtilis cells as evidenced by inability to form protoplasts. This, however, is not due to a fundamental property of the cell membrane associated with secretion as suggested by earlier workers[5] nor to the extracellular enzymes themselves, but rather to the presence of remaining traces of the protoplast-bursting factor, the synthesis of which closely parallels that of extracellular enzymes.

There is a good inverse correlation between the formation of extracellular enzymes by B. amyloliquefaciens cells and their capacity to form protoplasts. The effect is sharp; unwashed 18 hr cells which have not synthesised a significant amount of extracellular enzymes or bursting factor are readily converted to protoplasts but with 19 hr cells accumulation of extracellular enzymes and bursting factor have begun and protoplast formation then does not occur, but only lysis. However, if such actively

secreting cells of any age are first thoroughly washed to remove traces of factor prior to the addition of lysozyme, stable protoplasts can be readily obtained.[2] A single wash in buffer is not adequate.

Antibiotic activity of protoplast-bursting factor. The factor at concentrations of 10-20 μg/ml completely inhibited growth of a number of Gram positive bacteria including B. cereus T and 9946, B. stearothermophilus 1503-4R, B. megaterium KM (thy^- $tryp^-$) and S. aureus N.C.T.C. 9789. However, this was not a general effect, since it did not inhibit growth of either S. aureus N.C.T.C. 2237; B. licheniformis 749; B. subtilis W168, SB133, SB19 or of B. amyloliquefaciens itself. In addition, the factor

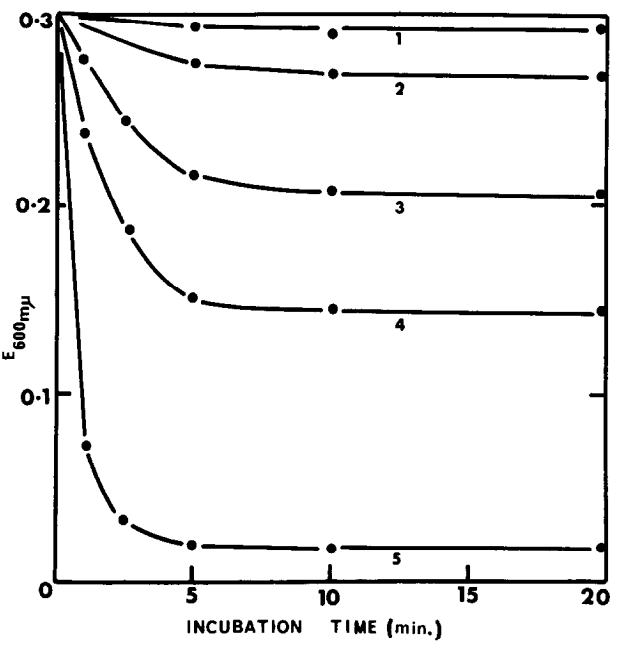

Fig. 1. Effect of bursting factor on protoplasts. Different volumes of four-fold diluted standard supernatant were added to cuvettes containing 1.2 ml of 2,4-dinitrophenol-treated protoplasts[2] prepared as described previously except that the buffer contained 10 mM-Mg^{2+}. The final volume of the mixture was 3.0 ml. The cuvettes were incubated at 30° and the $E_{600m\mu}$ measured. Curve 1, no addition; curve 2, 0.1 ml; curve 3, 0.2 ml; curve 4, 0.3 ml and curve 5, 0.5 ml of supernatant.

at concentrations of 0-400 μg/ml did not inhibit growth of any Gram-negative organism tested. These included E. coli B586, (even though protoplasts of this organism were lysed by the factor), K_{12}596, CA7 and Crooke's strain; E. freundii CA31, Shigella sonnei P9 and Salmonella typhimurium (SA_2 and Adelaide). Apparently, the factor inhibits growth by disorganising the cell membrane since addition of growth inhibitory concentrations to suspensions of B. cereus or B. stearothermophilus cells caused the instantaneous release of protein and U.V.-absorbing material without detectable cell lysis. Moreover, factor treatment of isolated B. amyloliquefaciens cytoplasmic membranes resulted in their immediate disruption to very small spherical pieces of membrane.

Assay for protoplast-bursting activity. Fig. 1 shows the rate of protoplast lysis by bursting factor upon the addition of different volumes of a four-fold diluted supernatant. (Undiluted culture supernatant was used as a reference standard and defined as containing 100 units of activity.) Protoplast lysis produced after 8 min. by a given volume of this diluted supernatant was measured as the fall in $E_{600m\mu}$. When the respective $\triangle E_{600m\mu}$ values were plotted against the volume of supernatant added, a "sigmoid-type" curve was obtained (Fig. 2, curve 3). Similar plots were made for other dilutions of the supernatant (Fig. 2, curves 1, 2 and 4). From Fig. 2 it can be seen that a linear inverse relationship exists between the amounts of lytic factor (in different dilutions of supernatant) and the volumes of the respective supernatants (arrows) needed to produce a $\triangle E_{600m\mu}$ = 0.15. (See Fig. 3.) Hence the number of units in an unknown solution can be calculated from plots as in Fig. 2.

Effect of inhibitors on factor formation by washed cells. Using

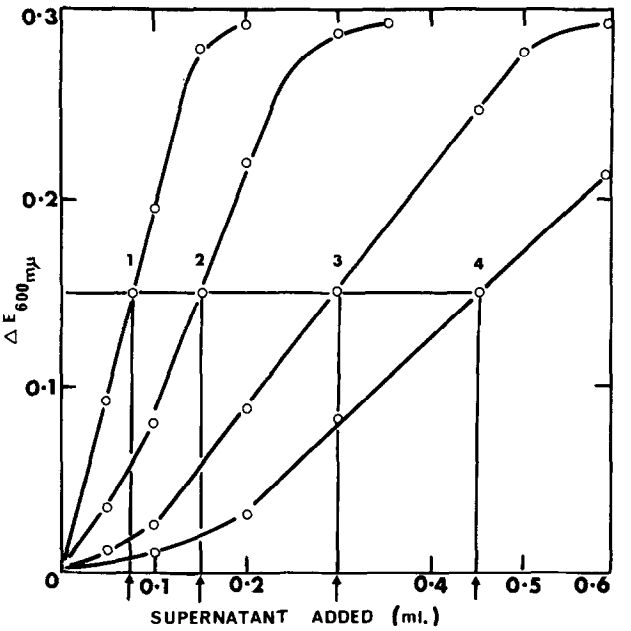

Fig. 2. Relationship between extent of protoplast lysis ($\triangle E_{600m\mu}$) and amount of lytic factor added (volume of supernatant). Curve 1, standard undiluted supernatant; curve 2, supernatant diluted two-fold; curve 3, supernatant diluted four-fold and curve 4, supernatant diluted six-fold.

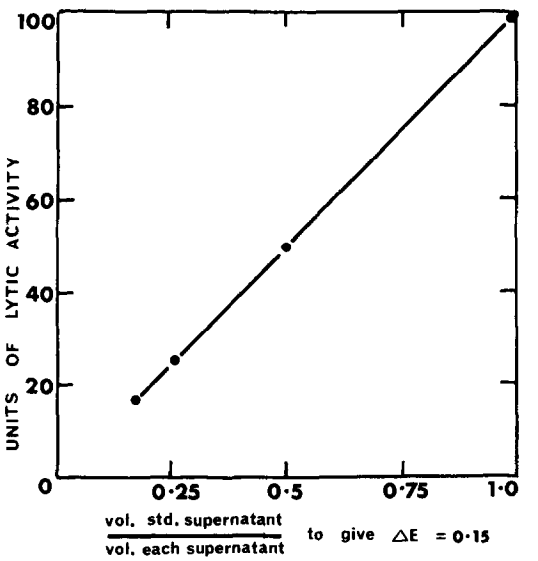

Fig. 3. Relationship between amounts of bursting activity in different supernatants and the volumes of the respective supernatants needed to produce $\triangle E_{600m\mu} = 0.15$.

the above assay procedure, it was found that washed 22 hr cells suspended in maltose-casein hydrolysate medium[7] secreted factor into the external medium in an almost linear fashion (Fig. 4). Synthesis proceeded unaffected for at least 60 min. in the presence of either chloramphenicol (10 μg/ml), actinomycin D (10 μg/ml) or puromycin (20 μg/ml) (Fig. 4). Inhibition after this time may be due to the involvement of an unstable enzyme or protein in factor production which must be continually resynthesised. At the drug concentrations used, general protein synthesis (and extracellular enzyme synthesis) are almost completely inhibited within a few minutes. Separate experiments showed that the factor is synthesised de novo in the presence of these drugs since the incorporation of L-[^{14}C]-valine into isolated factor continued

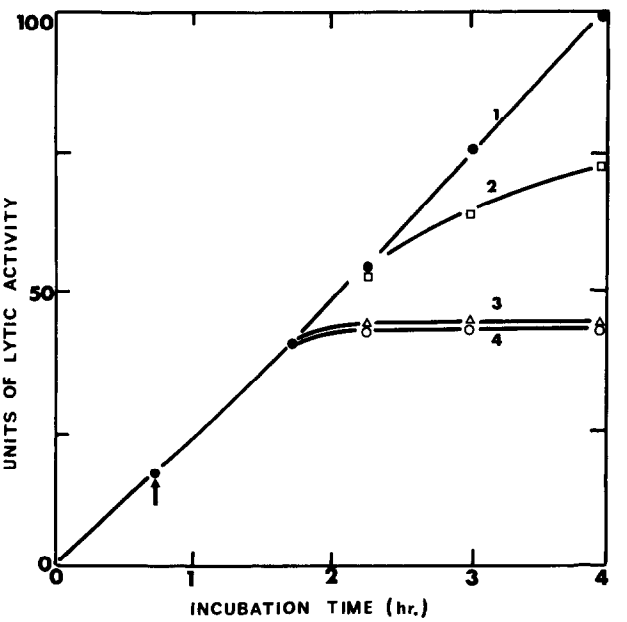

Fig. 4. Effect of protein synthesis inhibitors on bursting factor formation by washed cells. Inhibitors were added (arrow) to cells after 45 min. preincubation at 30°. Curve 1, no addition; curve 2, chloramphenicol (10 μg/ml); curve 3, actinomycin D (10 μg/ml); curve 4, puromycin (20 μg/ml).

unaffected for 60 min. agreeing with the result in Fig. 4. It appears therefore that synthesis of the protoplast-bursting factor proceeds by a mechanism differing from protein synthesis, as indeed would be expected from the presence of D-leucine and from its antibiotic nature.[8]

Since the synthesis of the protoplast-bursting factor by cell cultures closely paralleled that of extracellular enzymes, it was tempting to postulate that the bursting factor may facilitate the passage of extracellular enzyme peptides[2] through the hydrophobic membrane. Such a proposal, however, was made unlikely by the isolation of a mutant of B. amyloliquefaciens which synthesises normal levels of extracellular enzymes yet produces less than 8% of the normal level of factor. Since this mutant sporulates poorly, compared with wild-type, a possible connection between sporulation and bursting factor synthesis may exist here also, as discussed by Schaeffer[9] in connection with antibiotic production by B. subtilis.

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