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Inhibition of the synthesis of the alkaline phosphatase of *Bacillus megaterium* KM by a novel protoplast-bursting factor obtained from *Bacillus subtilis* 202-7

We have already reported the isolation of protoplast-bursting factor[1,2] from a commercial preparation of pig pancreatic lipase, which is a heat-stable protein (mol. wt. about 13000) assumed to split the ester linkage between the carboxyl group of 12-methyltetradecanoic acid and the hydroxyl group of tyrosine in the protoplast membrane.

Whilst screening various similar factors of microbial origin, we found a strong surface-active compound containing oligopeptide and lipid from *Bacillus subtilis* 202-7, which caused instantaneous destruction of protoplasts of *Bacillus megaterium* KM in a hypertonic medium.

On examining the physiological effect of this factor on the intact cells of *B. megaterium* KM, we found it to have a selective inhibitory effect on the synthesis of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) without any lethal effect. Since no such effect was observed with other known surface-active agents (*e.g.* sodium laurylsulfate and Tween-20) we assumed that a specific interaction between this novel factor and the bacterial cell membrane must exist.

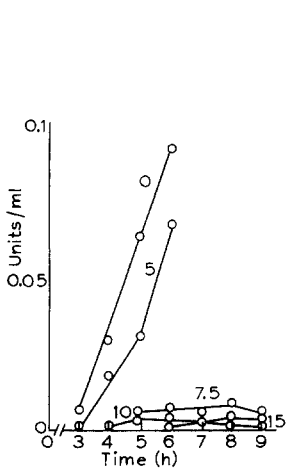
We find that many Gram-positive bacteria produce such active factors that burst protoplasts of *B. megaterium* KM. These factors fall into two groups; one group is heat stable and the other is heat labile. *B. subtilis* 202-7 produces the most active heat-stable factor (Factor I). On the other hand, the most active heat-labile factor (Factor II) is produced by *Bacillus brevis*. These factors have no antibiotic activities. Factor I can burst protoplasts at a rate of more than 90% at the concentration 6 μg/ml.

Factor I was isolated and crystallized. Work on its chemical structure is now in progress, but we have already found that it contains four kinds of amino acids (Leu (Ile):Val:Asp:Glu = 4:1:1:1) and unknown lipids. Judging from these data, Factor I differs from 'bacteriolytic principle'[3], 'autolysine'[4], 'protoplast-dissolving factor'[5] which have been described earlier.

To investigate the effect of Factor I on the synthesis of the alkaline phosphatase which is now believed to be localized on the bacterial cell's envelope[6], we cultured *B. megaterium* KM in a low-P_i medium[7]. The method of assay for alkaline phosphatase was as follows: 0.5 ml of culture which was previously treated with toluene was incubated with 2.0 ml of 0.02% disodium *p*-nitrophenylphosphate in 1 M Tris–HCl buffer (pH 8.0) containing 5 mM Mg^{2+} at 30°. After 20 min, 0.5 ml of 13% K_2HPO_4 was added to stop the reaction. The increase of absorbance at 410 mμ resulting from the liberation of *p*-nitrophenol was determined after removal of the cell debris by the centrifugation. One unit of enzyme activity was defined as that giving an increase in absorbance of 1.0 per min. The protein contents of cells were determined by the method of Lowry *et al.* after fractionating cells according to the method of Schmidt and Thannhauser.

As shown in Fig. 1, Factor I completely inhibits the synthesis of the alkaline phosphatase at a concentration (*e.g.* 10 μg/ml) which does not affect the growth of intact cells and bursts protoplasts of *B. megaterium* KM at the same concentration.

This fact is confirmed by the results in Fig. 2. The activity of the alkaline phosphatase in the absence of Factor I increased with the incubation time, but, when Factor I was added, no further increase in the alkaline phosphatase activity was observed. When the amount of the cellular protein synthesis was plotted against the incubation time, Factor I appeared not to affect the rate of protein synthesis of cells.



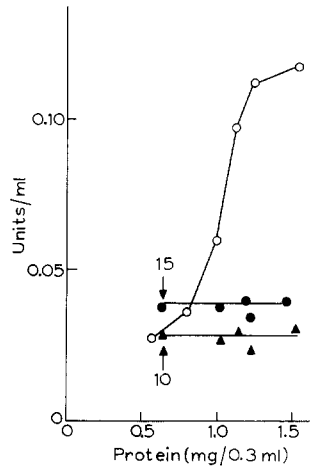


Fig. 1. The time course of the synthesis of alkaline phosphatase in the presence of various concentrations of Factor I. The assay method was as described in the text. The figures show the concentrations (μg/ml) of Factor I added at zero time.

Fig. 2. Differential synthesis of the alkaline phosphatase plotted in the presence (●, ▲) or absence (○—○) of Factor I. The assay method was as described in the text. Factor I was added (see arrow) at the concentrations shown (μg/ml).

This fact is not incompatible with the result of Fig. 1. Factor I thus inhibits the synthesis of the alkaline phosphatase of *B. megaterium* KM. On the other hand, Factor I does not inhibit the synthesis, in this organism of lactate dehydrogenase, which is also believed to be localized in the surface area.

The effect of Factor I on the synthesis of the alkaline phosphatases of other microorganisms, *e.g. B. subtilis, B. megaterium, Escherichia coli*, is not as conspicuous as in the case of *B. megaterium* KM. Factor I delays commencement of the alkaline phosphatase synthesis by 30 min.

Summarizing, we have found that *B. subtilis* 202-7 produces a most active novel heat-stable factor that bursts protoplasts of *B. megaterium* KM; and that the synthesis of the alkaline phosphatase of *B. megaterium* KM is completely inhibited by Factor I as the result of interaction between Factor I and the cell membrane, without any inhibition of the activity of alkaline phosphatase nor of the synthesis of lactate dehydrogenase.

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