INSENSITIVITY OF BACILLUS AMYLOLIQUEFACIENS EXTRACELLULAR PROTEASE FORMATION TO RIFAMPICIN AND ACTINOMYCIN D

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SUMMARY

Extracellular protease formation by washed cells of B. amyloliquefaciens, suspended in a medium containing high amino acid levels occurs in two distinct phases separated by a plateau region. Phase 1 in which extracellular protease formation occurs for 30-40 min. at the maximum rate of which the cells are capable is completely independent of messenger RNA (mRNA) synthesis as shown by insensitivity to rifampicin and actinomycin D. Phase 2 starts at 90 min. when the amino acid concentration has fallen and protease synthesis is released from repression; this phase is sensitive to rifampicin and actinomycin. In phase 1, despite the insensitivity to these drugs, protease production in their presence is sensitive to chloramphenicol and puromycin. Assuming that the latter drugs at the concentrations used are acting specifically as inhibitors of protein synthesis, the results suggest the presence in harvested cells of a pool of protease-specific mRNA which is translated and exhausted over a period of 30-40 min. The possible implications of this for the mechanism of extracellular enzyme production are discussed.

INTRODUCTION

It has earlier been shown[1] that extracellular protease formation by washed cell suspensions of B. amyloliquefaciens (formerly classified as B. subtilis[2]) shows a non-linear time course when the medium contains high levels of amino acids. There is an initial rapid production for 30-40 min (phase 1) followed by a period during which extracellular protease does not increase. At approx. 90 min., extracellular protease production recommences and goes on indefinitely (phase 2). It has been established[1] that extracellular protease

production is repressed by mixtures of certain amino acids; the onset of phase 2 production can be explained by utilisation of amino acids during the first 90 min. of incubation and consequent derepression of protease synthesis. However, phase 1 is more difficult to explain since a requirement of 30-40 min. to establish repression seems unlikely.

We have now shown that phases 1 and 2 of protease production are different. While both are sensitive to chloramphenicol and puromycin, phase 1 is totally insensitive to actinomycin and rifampicin but phase 2 shows the sensitivity normally expected for bacterial protein synthesis. The explanation of these results and their possible implications for the mechanism of extracellular enzyme synthesis are discussed.

EXPERIMENTAL

Washed cell experiments were carried out as follows. Cultures were grown for 25 h. at 30°C as described previously[1]. The cells were then harvested, twice washed in a salts-maltose-0.5% casamino acids medium[1] and resuspended in this medium to the original cell density. Samples (25 ml) of cell suspension were shaken at 30°C and at appropriate times, samples (1 ml) were withdrawn, centrifuged and the supernatants assayed for protease activity as previously described[1].

To determine the incorporation of L-[^{14}C]-valine into total protein, a 2 ml sample of washed cell suspension containing 1 μC of [^{14}C]-valine was incubated with shaking. At various times, 0.1 ml samples were withdrawn and treated as previously described[1] except that the Oxoid filters were counted by liquid scintillation.

[2-^{14}C]-Uracil incorporation into total cellular RNA was measured as described previously[1] except liquid scintillation

counting was again used; 3 ml of washed cells were incubated with 0.75 μC of [^{14}C]-uracil.

Actinomycin D was a generous gift from Merck, Sharp and Dohme.

RESULTS

Fig. 1 (curve 1) shows the typical biphasic time course of extracellular protease appearance when washed cells are shaken in the presence of 0.5% casamino acids. Curves 2 and 3 show that rifampicin (0.5 μg/ml) and actinomycin (2 μg/ml) respectively

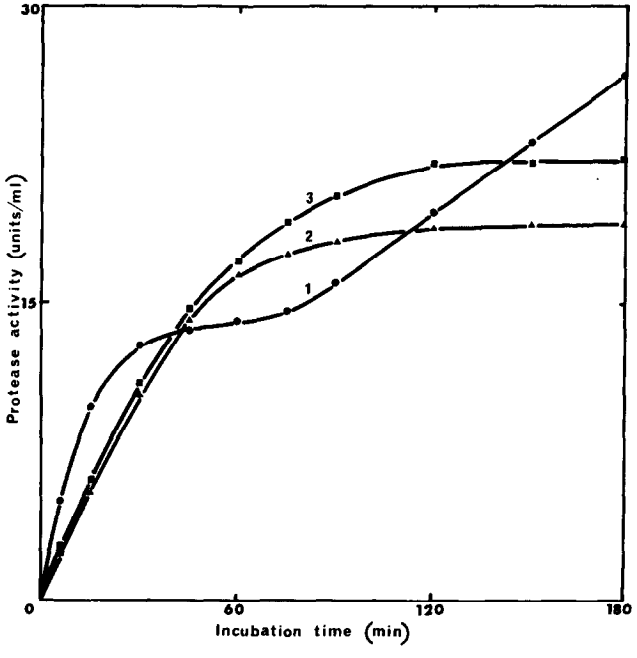

Fig. 1. Effect of actinomycin and rifampicin on protease formation by washed cells. The drugs were added at zero time. Curve 1, no addition; curve 2, 0.5 μg/ml of rifampicin; curve 3, 2 μg/ml of actinomycin.

added at zero time have little effect on the rate of protease accumulation in the external medium and indeed the total amount of extracellular enzyme at 90 min. is increased by these antibiotics.

Rifampicin and actinomycin at the concentrations used almost completely inhibit incorporation of [^{14}C]-uracil (Fig. 2) into cellular material. In addition, Fig. 3 shows that general protein synthesis measured by [^{14}C]-valine incorporation into cellular material is inhibited 97% and 90% by rifampicin and actinomycin respectively, at the concentrations that have no inhibitory effect on protease appearance in the external medium.

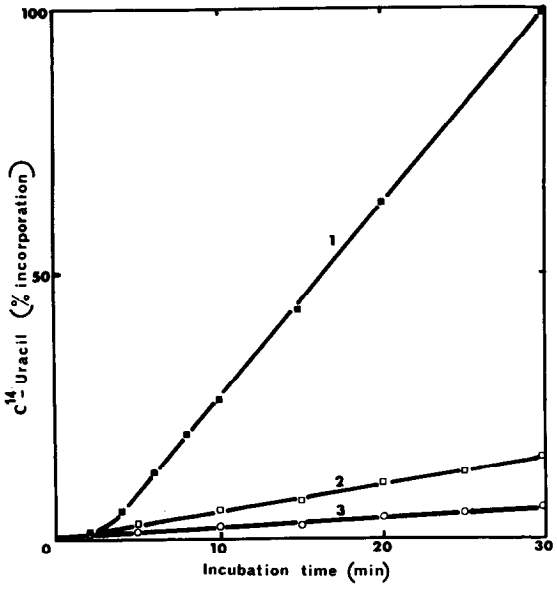

Fig. 2. Effect of actinomycin and rifampicin on [^{14}C]-uracil incorporation by washed cells. Drugs and [^{14}C]-uracil were both added at zero time.
Curve 1, no addition of drugs; curve 2, 2 μg/ml of actinomycin; curve 3, 0.5 μg/ml of rifampicin. The 100% value for [^{14}C]-uracil incorporation was 21,800 counts/min.

In marked contrast to this, when the addition of rifampicin is delayed for 90 min. it is seen from Fig. 4 that phase 2 of protease production is sensitive to this agent, inhibition being complete in a few minutes. Actinomycin when added at 90 min. gave a similar result.

These results indicate that phase 1 of protease production either does not involve protein synthesis or alternatively if it

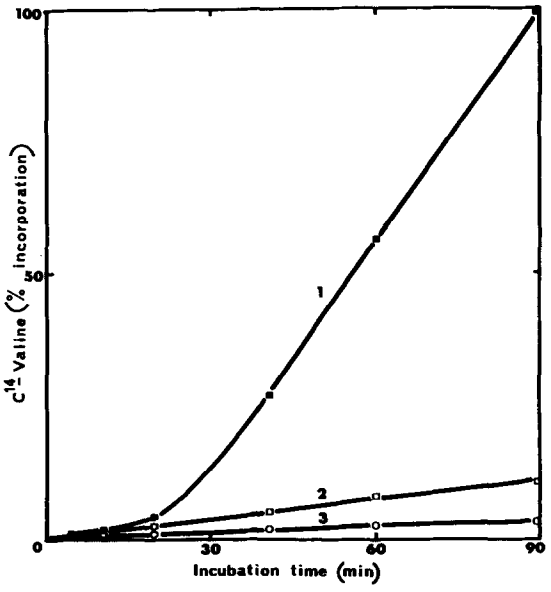

Fig. 3. Effect of actinomycin and rifampicin on [^{14}C]-valine incorporation by washed cells. Drugs and [^{14}C]-valine were both added at zero time.
Curve 1, no addition of drugs; curve 2, 2 μg/ml of actinomycin; curve 3, 0.5 μg/ml of rifampicin. The 100% value for [^{14}C]-valine incorporation was 3,470 counts/min.

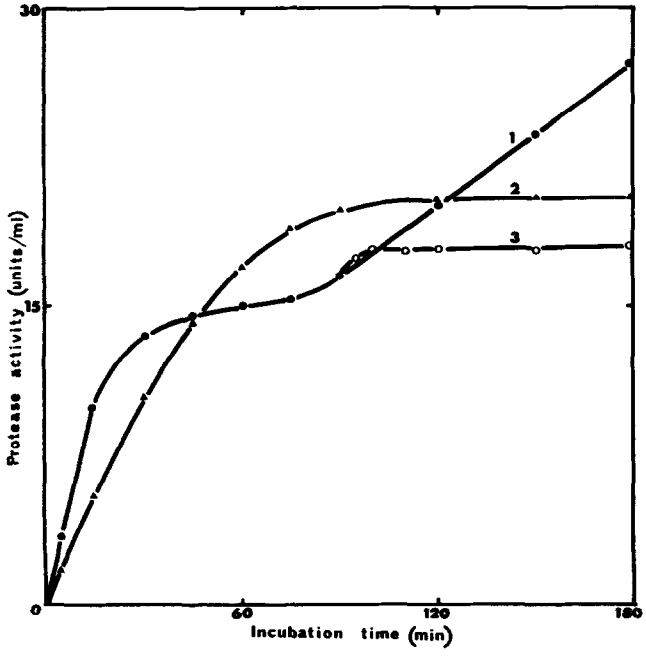

Fig. 4. Effect on protease production by washed cells of adding rifampicin at zero time compared with adding the drug after 90 min. incubation.
Curve 1, no addition; curve 2, 0.5 μg/ml of rifampicin added at zero time; curve 3, 0.5 μg/ml of rifampicin added after 90 min.

does, this protein synthesis is not dependent on mRNA synthesis, a situation virtually unique so far as bacteria are concerned. To examine this, the effect of chloramphenicol both in the presence and absence of actinomycin was tested. Fig. 5 shows that in both situations, chloramphenicol (10 μg/ml) immediately and completely inhibited the appearance of extracellular protease. Similar results were obtained with puromycin (20 μg/ml).

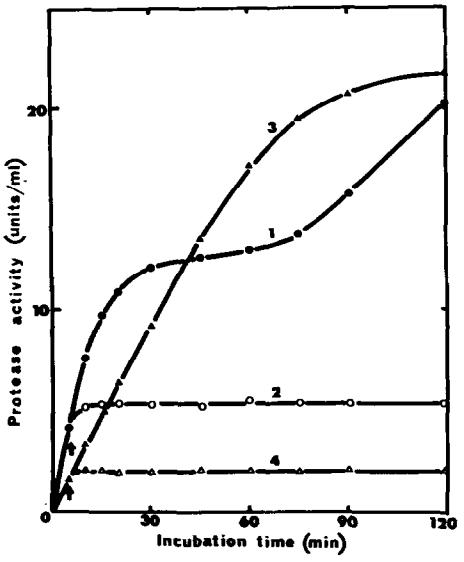

Fig. 5. Effect of chloramphenicol on protease production by washed cells in the presence and absence of actinomycin. Chloramphenicol was added (arrows) to cells after 5 minutes pre-incubation. Actinomycin was added at zero time.
Curve 1, no addition of drugs; curve 2, 10 μg/ml of chloramphenicol; curve 3, 2 μg/ml of actinomycin; curve 4, 2μg/ml of actinomycin + 10 μg/ml of chloramphenicol.

DISCUSSION

The biphasic time course of protease accumulation in the external medium can be accounted for in the following way: the high level of amino acids initially present completely represses synthesis of protease-specific mRNA for 90 min. At this time the amino acid level has been sufficiently reduced by metabolism and protein synthesis to release the repression and permit transcription of the

protease gene(s) to proceed. This results in phase 2 production, and this phase is sensitive to actinomycin and rifampicin as expect ed in the normal way.

Superimposed on this, however, is a situation which permits extracellular protease appearance to proceed rapidly for the first 30-40 min. (phase 1) which does not depend on mRNA synthesis. When this potentiality is exhausted, extracellular protease production ceases until derepression and phase 2 synthesis starts.

The most important question is the nature of phase 1 production. The simplest explanation would be that preformed enzyme or a zymogen precursor inside the cell is secreted. However, the inhibition by chloramphenicol and puromycin strongly suggest that protein synthesis is involved. This is further supported by the fact that pactamycin, an antibiotic believed to inhibit initiation of protein synthesis, also abolishes the actinomycin-insensitive phase 1 protease production.

Assuming that protein synthesis is involved the idea of a conventionally 'stable' mRNA for protease does not seem feasible since the effects of rifampicin on phase 2 synthesis clearly shows that protease mRNA is not long lived under these circumstances. However, it is possible that the apparent stability may be a repercussion of the mechanism of extracellular enzyme secretion.

It has earlier been postulated by Elliott and May[3] that extracellular enzyme synthesis by B. amyloliquefaciens occurs at special sites at the membrane, the nascent polypeptide chain being extruded through it. Thus synthesis and secretion would be one inseparable event. The model implies that mRNA must somehow migrate from the gene to the translation-extrusion sites on the membrane. If one postulates that the mRNA for protease is somehow stabilised until translated (to permit this migration) then a positive imbalance of

transcription over translation would cause 'queueing up' of mRNA molecules for a limited number of translational-extrusion sites and so cause a pool of mRNA to accumulate. The existence of this pool would show up only when transcription was suppressed by amino acids or drugs and could thus account for phase 1 synthesis and its characteristics.

While this explanation is attractive and appears to fit both the facts and the postulated model for enzyme secretion well, it is necessary to add the caution that it depends upon the assumption that in these studies chloramphenicol, puromycin and pactamycin are specifically inhibiting protein synthesis and not acting in other secondary ways. While this assumption is reasonable and is usually accepted, the matter can be finally settled only by appropriate labelling studies and isolation of the enzyme produced in the absence of mRNA synthesis. This work is in progress.

ACKNOWLEDGEMENT

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