AN INHIBITOR IN BACILLUS SUBTILIS OF ITS EXTRACELLULAR RIBONUCLEASE

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Suspensions of stationary phase B. subtilis cells grown in a maltose - casein hydrolysate medium synthesize ribonuclease which appears in the extracellular medium. The enzyme is secreted rather than liberated by cell lysis (Coleman and Elliott, 1964). It has now been found that the soluble fraction of a disrupted cell preparation of the same strain of B. subtilis contains a powerful inhibitor of this extracellular ribonuclease.

EXPERIMENTAL

Washed B. subtilis (Takamine**) stationary phase cells were disrupted in 0.05 M tris - HCl buffer, pH 8.0, containing 0.01 M β-mercaptoethanol, using either a French pressure cell or a Raytheon sonic oscillator. This preparation was centrifuged for 60 min. at 105,000 x g to give a supernatant fraction containing approximately 10 mg. of protein per ml.; this is referred to as 'crude inhibitor' and was

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** An unclassified strain kindly supplied by the Takamine Laboratories Inc., Clifton, N.J., U.S.A.

used in early inhibition studies and as the starting material from which the inhibitory principle has been purified.

Preparation of a 'purified inhibitor' fraction involved ammonium sulphate fractionation, precipitation at pH 4.7 followed by ion exchange column chromatography on TEAE - cellulose and gel filtration using Sephadex G-100. The purification procedure resulted in a preparation with 60% of the initial inhibitor activity but a barely detectable amount of protein. In view of the fact that the protein content was so low and since the inhibitor has not yet been conclusively established to be a protein the amount of inhibitor added to experimental systems is given in terms of volume of inhibitor solution.

Extracellular ribonuclease was purified from the medium of B. subtilis cultures by acid treatment and ammonium sulphate precipitation (Rushizky et al., 1963). Enzyme activity was measured by the method described by Josefsson and Lagerstedt (1962).

In all experiments the enzyme (0.2 ml. unless otherwise stated) and varying amounts of inhibitor as indicated were mixed in buffer and the reaction started by the addition of RNA; the latter was prepared from yeast by the method of Crestfield et al., (1955).

RESULTS AND DISCUSSION

Crude inhibitor (0.06 ml.) from a disrupted B. subtilis cell preparation inhibited the activity of extracellular ribonuclease from the same organism by 88%. This inhibitory action of the extract was completely destroyed by boiling it for 10 min. The inhibitor is greatly stabilized towards heating by β-mercaptoethanol which also prevents loss of

inhibitory activity during prolonged dialysis.

The effect of the purified inhibitor preparation was studied on the time course of ribonuclease action as shown in Fig. 1; it was found to reduce the rate of the enzymic reaction which however remained linear with time.

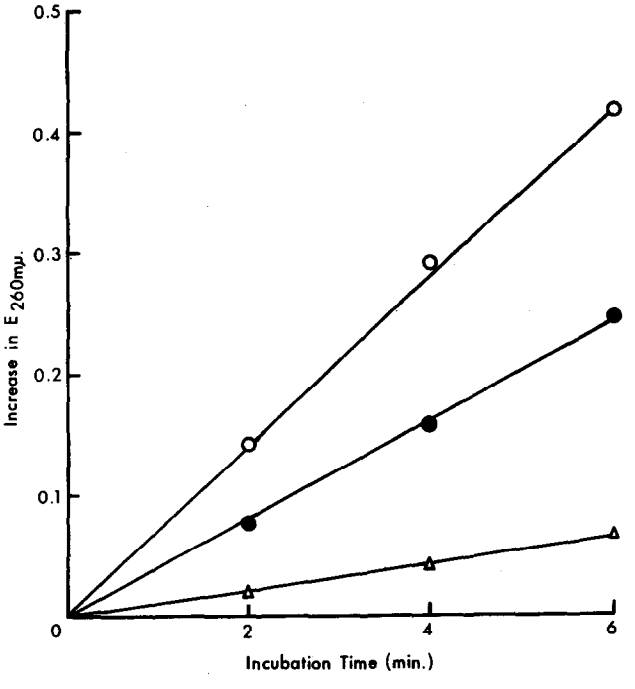

Fig. 1. Effect of 'purified inhibitor' on the time course of ribonuclease action. Assays were carried out in the absence of inhibitor (o) and in the presence of 0.01 ml. (•) and 0.02 ml. (Δ) of inhibitor.

On 'titrating' a fixed amount of ribonuclease with purified inhibitor the enzyme activity decreased linearly with increasing inhibitor concentration until only 5% activity remained. This residual activity was neutralized by doubling the amount of inhibitor (Fig. 2). This result suggests a stoichiometric combination of inhibitor and enzyme to form

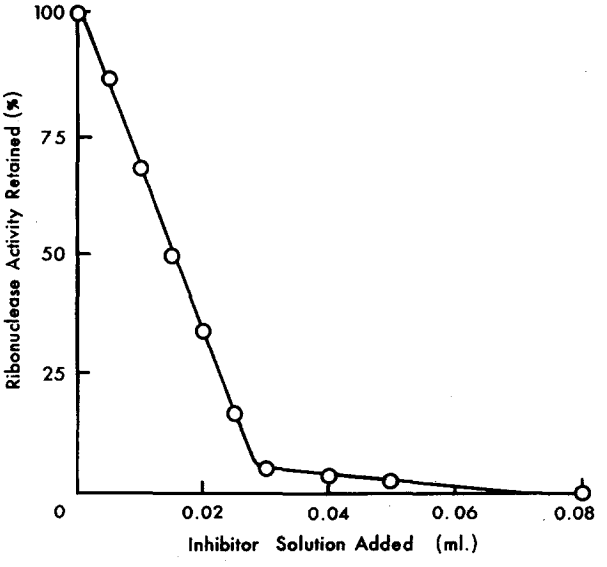

Fig. 2. Effect of increasing amounts of 'purified inhibitor' on ribonuclease activity.

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a complex with a very low degree of dissociation. Further evidence in support of this idea is obtained by operating in the opposite direction i.e. fixed amounts of the inhibitor are treated with increasing amounts of ribonuclease. It can be seen in Fig. 3 that as soon as an amount of ribonuclease sufficient to neutralize the inhibitor is exceeded the observed ribonuclease activity increases parallel to that in a control experiment containing no inhibitor. This experiment also eliminates the possibility of the inhibitor being a proteinase which causes the progressive destruction of the ribonuclease.

A study of the specificity and distribution of the inhibitor has been made. It is shown in Table 1 that the inhibitor has no effect on bovine pancreatic ribonuclease and moreover soluble extracts of a number of different micro-organisms were completely without inhibitory activity

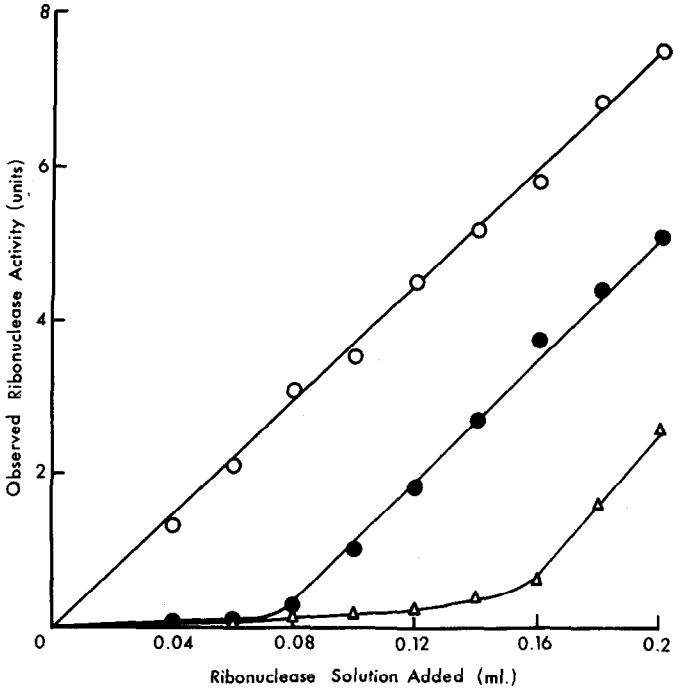

Fig. 3. Effect of 'purified inhibitor' on increasing amounts of ribonuclease. Assays were carried out in the absence of inhibitor (o) and in the presence of 0.01 ml. (•) and 0.02 ml. (Δ) of inhibitor.

towards B. subtilis extracellular ribonuclease. Thus it would appear that the inhibitor from B. subtilis is specific for its own extracellular ribonuclease being incapable of attacking other ribonucleases and absent from the other micro-organisms tested.

A bacterial inhibitor of ribonuclease has not been previously reported. Roth (1958) has described a compound which is present in rat liver and which has a similar action on pancreatic ribonuclease. Shortman (1962) has shown that this mammalian ribonuclease inhibitor is probably a protein. The nature of the B. subtilis inhibitor has not yet been determined but such data as are available suggests that it also may be a protein. Thus it is heat labile, non-dialysable and is destroyed by 'Pronase' a wide specificity proteinase

Table 1

Specificity and distribution of B. subtilis extracellular ribonuclease inhibitor.

	Ribonuclease	Additions to Assay System	RNase Activity (percent of control)
1.	B. subtilis (exo)	None (control)	(100)
2.	"	Purified inhibitor (0.1 ml.)	0
3.	Bovine pancreatic	None (control)	(100)
4.	" "	Purified inhibitor (0.1 ml.)	100
5.	B. subtilis (exo)	E. coli extract	100
6.	"	Strep. faecalis extract	100
7.	"	Staph. aureus extract	100
8.	"	Lb. leichmanii extract	100

In experiments 5 to 8 inclusive 0.1 ml. samples of sonic extracts of various micro-organisms each containing approx. 10 mg. protein per ml. were used.

(4 mg./ml., 30° for 20 min.). However, the inhibitor activity of the preparation is unaffected by incubation at 25° for periods of up to 60 min. in the presence of either crystalline trypsin or chymotrypsin, each at a concentration of 0.15 mg./ml. It is also unaffected by incubation with pancreatic deoxyribonuclease.

The observation that the inhibitor is apparently specific for the extracellular ribonuclease produced by the organism in which it occurs prompts speculation relating to the function of the inhibitor. However, there is no evidence at present to indicate its role in the economy of the bacterial cell.

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