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IN VIVO AND IN VITRO INHIBITION OF PROTEIN SYNTHESIS IN BACILLUS STEAROTHERMOPHILUS BY OCHRATOXIN A

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SUMMARY

Ochratoxin A, a mycotoxin produced by Aspergillus ochraceus and other molds, inhibits preferencially protein synthesis of Bacillus stearothermophilus in vivo. The peptide synthesis of a polyuridylic acid dependent cell free system is inhibited competitively. The inhibition is dependent on the Mg^{2+}-concentration. When phenylalanyl-tRNA is applied no inhibition occurs. This confirms earlier findings in Bacillus subtilis which show that ochratoxin inhibits the amino acylation reaction.

INTRODUCTION

Recently it has been shown that ochratoxin A (OTA), a mycotoxin which is produced by some strains of Aspergillus ochraceus, Penicillium viridicatum and some other aspergilli (1), inhibits growth in Streptococcus faecalis (2) and Bacillus subtilis. This growth inhibition involves protein and RNA synthesis. In B.subtilis the growth inhibition implicates an accumulation of the MS-nucleotides ppGpp and pppGpp (3), and thus suggested a binding to the ribosomes of an uncharged tRNA (4). In vitro it was demonstrated that OTA acts as a competitive inhibitor for phenylalanine in the aminoacylation of $tRNA^{Phe}$ (5).

If this inhibition was the principal reason for growth inhibition, one could expect an in vitro inhibition of protein synthesis, and especially of a polyuridylic acid (poly-U) dependent peptide synthesis system.

In this paper we show that OTA also inhibits growth and

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protein synthesis in B.stearothermophilus cells and peptide synthesis in a cell-free, poly-U dependent polyphenylalanine synthesizing system from this organism.

MATERIALS AND METHODS

Ochratoxin A was isolated and purified from wheat kernels infected by Aspergillus ochraceus as previously described (2).

B.stearothermophilus cultures were grown in penassay medium (Difco) at pH 6.0, 60°C and strong aeration. For labelling of RNA and protein 5 µCi (^{3}H)-uridine/ml of growth medium or 2 µCi (^{14}C)-phenylalanine/ml were added in the exponential phase. At different time intervals samples were taken, placed in 5% trichloracetic acid (TCA), filtered on membrane filters (Sartorius, 0.45 µm pore size), and the dried filters were counted in liquid scintillator (Omnifluor, NEN-Chemicals) using a Packard scintillation spectrometer (Modell 3320). Before each sample was taken the optical density at 546 nm (O.D. 546) was measured by means of an Eppendorf photometer.

For the preparation of the cell-free, poly-U dependent peptide synthesis system (called iS 30), the cells were grown in a medium containing Bacto-trypton, 2% and yeast extract, 0.4% using a 10 liter fermentor (Microferm, New Brunswick) at maximal aeration and agitation, and at 60°C. The cells were harvested by centrifugation at an O.D.546 of 1.5 and were washed twice with a buffer containing 0.01 M Tris/HCl pH 7.5, 0.01 M Mg-acetate, and 0.06 M KCl (TM-buffer + KCl).

The cells were ground with alumina (Alcoa A 305) using twice the weight of the bacterial cell mass. To the paste a small amount of pancreatic DNase and sufficient TM-buffer + KCl was added to be able to pour the suspension into centrifuge tubes. The suspension was centrifuged at 15 000 x g, 4°C, for 30 min. The supernatant was dialyzed for 5 hrs against TM-buffer containing 0.7 mM ß-mercaptoethanol. After this procedure the extract was found to be poly-U dependent.

The incubation mixture for peptide synthesis contained: 0.1 M cacodylate buffer, pH 7.4; 0.2 M NH_4Cl; 0.06 M KCl; 0.014 M Mg-acetate; 0.005 M GTP; 0.0025 M ATP; 0.01 M PEP; 5 µg/ml pyruvate kinase (Boehringer); 0.03 M ß-mercaptoethanol; 2 µCi (^{3}H)-phenylalanine together with unlabelled phenylalanine to give a concentration of 5.5 nM; and 2 µl of iS 30 in a volume of 100 µl. This volume contained additionally 2 µg of polyuridylic acid and 100 µg stripped B.stearothermophilus tRNA. The tubes with the mixture were incubated at 60°C (a temperature destroying the pancreatic DNase) for 15 min. The reaction was stopped by addition of 2 ml of 5% TCA, then the tubes were heated at 85°C for 15 min. The precipitates were collected on membrane filters and the radioactivity counted as described above.

The aminoacylation of B.stearothermophilus tRNA was carried out with E.coli aminoacyl-tRNA synthetase as previously described (5). The (^{3}H)-phenylalanyl-tRNA was isolated from the aminoacylation mixture by phenolyzation and precipitation with ethanol.

RESULTS

OTA is a potent inhibitor of growth of B.stearothermophilus (data not shown). The inhibition involves protein and RNA synthe-

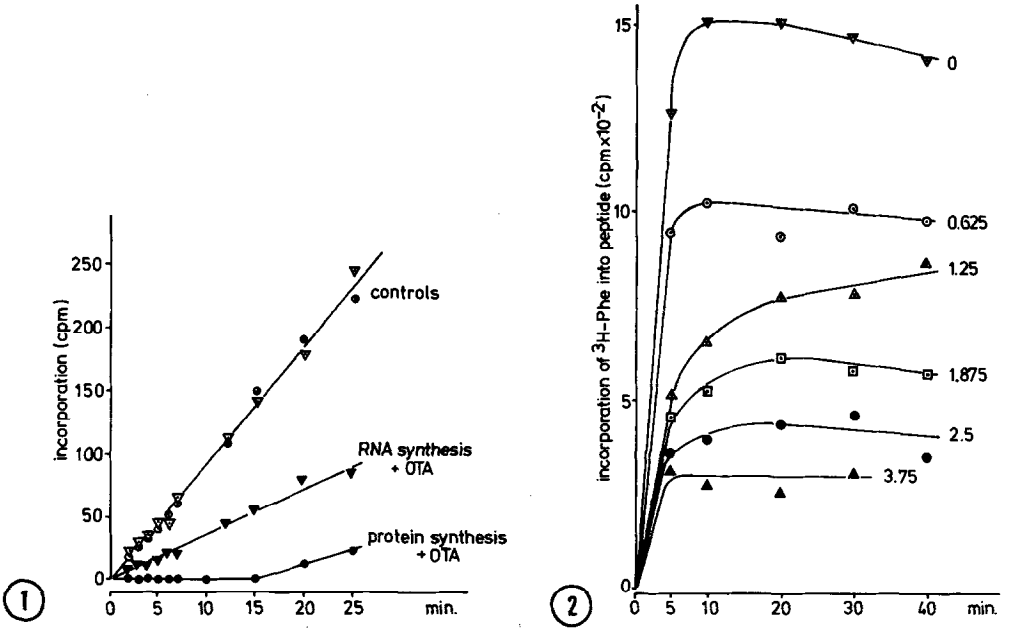


Fig.1 : Incorporation of (^{14}C)-phenylalanine and (^{3}H)-uridine into protein and RNA of an exponentially growing B.stearothermophilus culture.
Open circles:control (^{14}C)-phenylalanine incorporation into protein; open triangles: incorporation of (^{3}H)-uridine into RNA in the absence of OTA. Closed symbols: in the presence of 0.313 mM OTA (12.5 µg/ml).

Fig.2 : Inhibition of poly-U dependent incorporation of phenylalanine in the presence of different concentrations of OTA.
The numbers on the curve designate concentration (mM) of OTA.

sis (Fig.1). The RNA synthesis is less inhibited than protein synthesis in this organism. At the concentration of 0.3 mM OTA (12.5 µg/ml) the protein synthesis is almost completely blocked for 15 min. after addition of OTA, and then begins to increase but still with a lower rate than RNA synthesis. These results are similar to that obtained with B.subtilis.

Fig.2 shows peptide synthesis in a cell-free, poly-U dependent polyphenylalanine synthezising system derived from B.stearothermophilus. The incorporation of (^{3}H)-phenylalanine in the absence of poly-U was about 150 cpm under equal con-

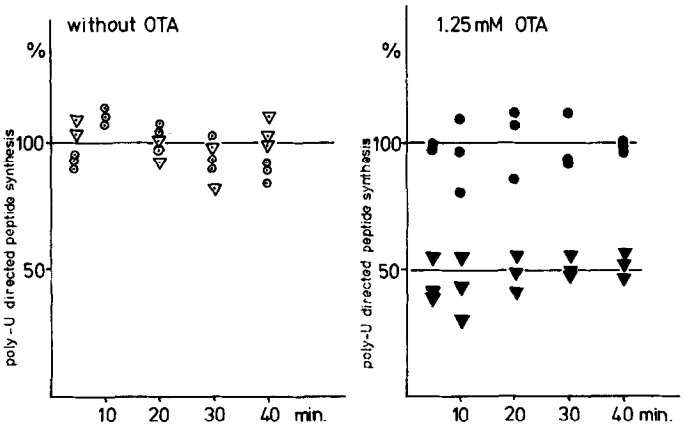


Fig.3 : The influence of Mg^{2+} concentration on the inhibition of poly-U directed peptide synthesis by OTA. Open symbols: incorporation of (^{3}H)-phenylalanine in the absence of OTA; triangles: with 0.014 M Mg^{2+}, circles: with 0.03 M Mg^{2+}. Closed symbols: same conditions but in presence of 1.25 mM OTA.

ditions. A strong dependence of the inhibition of the peptide synthesis on the concentration of OTA is observed, although the concentration necessary to inhibit the incorporation by approximately 50% is almost 10 times as high as necessary to inhibit the aminoacylation of tRNA of B.subtilis to the same extent. However this result may be explained by a 70 times higher concentration of phenylalanine in the peptide synthesis mixture.

The extent of inhibition by a given concentration of OTA is strongly dependent on the concentration of Mg^{2+}present in the incorporation mix (Fig.3). A decrease from 30 mM to 14 mM Mg^{2+} effects an increase in inhibition from 0% to 50%. Thus only in a certain range of Mg^{2+} the inhibition of the peptide synthesis by OTA is observed (Table 1). Also, the inhibition due to a higher concentration of OTA can be overcome by a higher concentration of Mg^{2+}.

The type of inhibition which OTA exerts on the peptide

Table I

Influence of Mg^{2+} concentration on the inhibition of poly-U directed peptide synthesis.

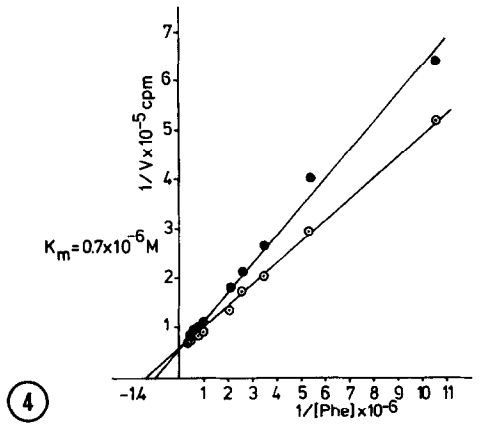
Concentration of Mg^{2+} in the incubation mix. (M)	Incorporation without OTA cpm	Incorporation in the presence of 1.25 mM OTA cpm	Inhibition %
0.0	10	10	-
0.010	200	125	34
0.012	255	150	39
0.014	265	170	39
0.016	285	185	37
0.018	345	205	41
0.020	420	290	28
0.024	585	460	21
0.030	750	725	3

synthesizing system is illustrated in Fig.4. The incorporation of phenylalanine into polyphenylalanine is inhibited competitively in a concentration range of phenylalanine as indicated. The K_m of the system was found to be 0.7×10^{-6} M.

In order to find out whether the inhibition of peptide synthesis was due to inhibition by OTA of the aminoacylation, (^{3}H)-phenylalanine in the mixture was replaced by (^{3}H)-phenylalanyl-tRNA. Fig.5 indicates that under these conditions no inhibition of peptide synthesis by OTA occurred. The slow rate of the reaction during the first 5 min. can not be explained but it could be due to competition with endogenous Phe-tRNA in the iS30. Nevertheless, this result shows that the peptide synthesizing system remains intact, and that it is obviously the aminoacylation reaction which is inhibited by the mycotoxin.

DISCUSSION

In a previous paper we have shown that Phe-tRNA synthetase of B.subtilis is inhibited by OTA (5). The in vivo measurements of the aminoacylated Phe-tRNA by the periodate oxidation method always showed significantly lower levels after OTA treatment of



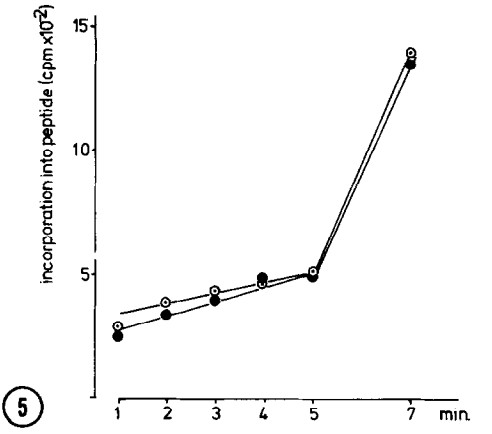


Fig.4 : Lineweaver-Burk plot of the inhibition of (^{3}H)-phenylalanine incorporation into polyphenylalanine by OTA. Open circles: control without OTA; closed circles: in the presence of 1.25 mM OTA.

Fig.5 : Poly-U directed peptide synthesis with phenylalanyl-tRNA as substrate. Open circles: control without OTA; closed circles: in the presence of 1.25 mM OTA.

the cells. However, as this method has been criticized recently in the case of B.subtilis (6), we tried to find another way to show that the inhibition of protein synthesis is the reason for growth inhibition in bacteria.

From the data on the inhibition of Phe-tRNA synthetase it could be expected that poly-U directed peptide synthesis should be inhibited by OTA. In fact, this inhibition could be observed (Fig.2). As it was known that the inhibition of the Phe-tRNA synthetase was of the competitive type (5), it was investigated whether this type of inhibition is also observed in the poly-U dependent peptide synthesis system. As both, the inhibition of aminoacylation and the inhibition of peptide synthesis (Fig.4) are of the competitive type it can be suggested that the reason for the inhibition of protein synthesis in vivo is due to inhibition of the Phe-tRNA synthetase.

A strong dependence of the inhibition of peptide synthesis on the Mg^{2+} concentration was found (Fig.3). A similar dependence has been described for the growth inhibition by OTA in S.faecalis (7) and has also been found in B.subtilis (not published). Therefore it could be expected that Mg^{2+} may also play a role in the inhibition by OTA of the aminoacylation reaction. Preliminary results (not shown) show that this is probably the case. The growth inhibition is also strongly dependent on the pH of the growth medium (2). No such dependence of inhibition of aminoacylation has been found in vitro (not shown). Therefore, this effect is probably related to the uptake of the mycotoxin by bacteria.

From the results discussed above it could be predicted that no inhibition would be observed when phenylalanyl-tRNA was used as substrate for the polyphenylalanine synthesis. The fact that in this case no inhibition occurs (Fig.5) shows that OTA is probably not involved in reactions of the protein synthesis other than aminoacylation. This finding is in agreement with results showing an increase of the MS-nucleotides ppGpp and pppGpp in B.subtilis cells after addition of OTA (3). For the synthesis of these nucleotides an intact ribosomal system is assumed to be necessary (4).

The in vivo inhibition of protein and RNA synthesis in B.stearothermophilus is very similar to B.subtilis but not to S.faecalis. In S.faecalis both syntheses are inhibited to the same extent (2). As we have demonstrated that in B.subtilis the MS-nucleotides are involved in the growth inhibition, it can be assumed that inhibition of RNA synthesis is secondary. The regulation of this synthesis, therefore, seems to be more stringent controlled in S.faecalis in comparison to the bacilli.

The mode of action of OTA in bacteria, therefore, seems to be quite peculiar for a mycotoxin. Similar modes of action for naturally occurring metabolic products have been described so far only for two antibiotics, borrelidin (8) and granaticin (9).

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