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ENZYMATIC ACTIVATION OF *CLOSTRIDIUM PERFRINGENS* EPSILON PROTOTOXIN AND SOME BIOLOGICAL PROPERTIES OF ACTIVATED TOXIN

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ABSTRACT

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Maximal activation of *Clostridium perfringens* epsilon toxin was only achieved by the combined action of trypsin and chymotrypsin. Impure preparations of trypsin, presumably containing small amounts of chymotrypsin were more efficient in activating prototoxin than pure trypsin. Activated toxin was readily absorbed by brain tissue and smaller amounts were possibly absorbed by kidney tissue. Other tissues absorbed only very small amounts of toxin. Injection of mice with toxoid 3 h prior to challenge with toxin increased their resistance 32 times.

INTRODUCTION

The purification and some chemical, immunological and biological properties of a preparation of *Clostridium perfringens* epsilon prototoxin, termed purified prototoxin (Worthington, Mülders & Van Rensburg, 1973) were described in a previous paper. This prototoxin has been used in further investigations on some of the biological characteristics of epsilon toxin.

Highly purified epsilon toxins prepared by other workers varied considerably in toxicity. Toxins prepared by Thomson (1963) (2,2 to 2,4 $\times$ 10^6 LD/mgN), Verwoerd (1960) (2,2 to 5 $\times$ 10^6 MLD/mgN) and Stuart (according to Hauschild, 1971) (3 $\times$ 10^6 MLD/mgN), showed similar toxicity. Habeeb (1969) prepared a toxin with a toxicity of 3,0 to 4,2 $\times$ 10^6 MLD/mg protein. Assuming a nitrogen content of 16%, this gives a toxicity in the region of 1,8 to 2,5 $\times$ 10^7 MLD/mgN. Our purified prototoxin contained 1 to 2 $\times$ 10^7 MLD/mgN when activated by the method used by Habeeb (1969). When lower concentrations of trypsin were used for activation the toxicity was, however, considerably lower. It was therefore decided to investigate more fully the reasons for these discrepancies.

The precise mode of action of epsilon toxin is not known. Bullen & Batty (1956) showed that it causes increased intestinal permeability, allowing the absorption of undigested proteins and presumably toxin itself. After absorption the toxin appears to affect mainly the central nervous system and kidneys. The clinical disease causes severe nervous symptoms and chronic or subacute intoxication may be associated with gross brain lesions (Hartley, 1956; Griner, 1961). Griner & Carlson (1961) showed that there was an increased vascular permeability in the brain. This could be important in causing oedema and necrosis of nervous tissue (Griner, 1961). A variety of effects on kidney function have been described, as well as hyperglycaemia and alterations in the activity of certain blood enzymes (Hauschild, 1971).

We wished to formulate a working hypothesis on the mode of action of epsilon toxin and some preliminary work on the absorption of epsilon toxin by mouse tissues and the blocking of toxicity by injection of toxoid was therefore undertaken.

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MATERIALS AND METHODS

Epsilon prototoxin preparation

Purified epsilon prototoxin was prepared as described previously (Worthington *et al.*, 1973). The extinction coefficient $E^{1\%}_{280\ nm} = 10$ was used to estimate the concentration of prototoxin in solution (Worthington *et al.*, 1973).

Prototoxin activation and mouse toxicity tests

The method used for toxin activation was basically that described by Habeeb (1969). To 1 ml of a solution containing 1 mg/ml of prototoxin 8,5 ml of 0,1M phosphate buffer pH 8,0 and 0,5 ml of 5% (m/v) trypsin were added. The solution was incubated in a waterbath at 37°C for 30 min and doubling dilutions were made in peptone saline [1% (m/v) Oxoid peptone in 0,25% (m/v) NaCl]. Three mice were injected intravenously with 0,5 ml of each toxin dilution. A MLD was taken as the least amount of toxin which killed two out of three mice within 24 hours of injection.

In routine work a low grade trypsin* containing 2 000 units/g was used. This trypsin will be referred to as low grade trypsin. In other experiments a high grade trypsin* containing 2,0 U/mg which had been treated with a chymotrypsin inhibitor TPCK was used; this will be referred to as TPCK trypsin. Three times crystallized chymotrypsin** with an activity of 42 units/mg was also used (See Results for details).

Absorption of toxin by mouse tissues

Two mice were killed by decapitation. The brains, livers, spleens, kidneys, lungs and muscles of the hindlegs were immediately removed and macerated with a pair of scissors. Five ml of trypsin activated toxin containing between 2 048 and 4 096 MLD/ml was added to each tissue homogenate and stirred gently for 30 min with a magnetic stirrer at room temperature. The suspensions were then centrifuged at 27 000 *g* in a refrigerated centrifuge, doubling dilutions of the clear supernatant made in peptone saline and 0,5 ml of each dilution injected intravenously into each of three mice. The experiment was repeated as above, except that the toxin solution, used for absorption with tissues, was diluted to contain $\pm$512 MLD/ml.

* Merck

** Sigma Chemical Co

TABLE 1 Activation of purified prototoxin with TPCK trypsin, chymotrypsin and combinations of the two enzymes

Exp. No.	Enzyme	Mg enzyme/ mg prototoxin	Incubation time (min)	Toxicity (MLD/ mg prototoxin)
1.....	TPCK trypsin.....	2	30	2×10^5
	TPCK trypsin + chymotrypsin.....	2 + 2	30	$1,6 \times 10^6$
2.....	TPCK trypsin + chymotrypsin.....	2 + 2	30	$3,2 \times 10^6$
	Chymotrypsin.....	2	30	$1,6 \times 10^6$
3.....	TPCK trypsin.....	2	30	4×10^5
	TPCK trypsin + chymotrypsin.....	2 + 2	30	$1,6 \times 10^6$
	Chymotrypsin.....	2	30	8×10^5

Blocking of toxicity by formalinized toxoid

Cl. perfringens strain ET 468 was grown in Wrights' broth containing 3% insoluble dextrin. After incubation at 37°C for 24 h the supernatant was clarified by high speed centrifugation (the supernatant had a flocculation titre of 200 Lf/ml), 0,003% low grade trypsin added and reincubated for 2 h at 37°C. Formalin (0,9%) was then added and the solution incubated at 37°C for 72 h. The product was non-toxic to mice. Mice were injected intraperitoneally with 2 ml of toxoid. After 3 h had elapsed these mice and controls which had not received toxoid, were used in toxicity tests as described above.

RESULTS

Prototoxin activation

Preliminary experiments showed that activation of purified prototoxin by low grade trypsin caused a fourfold increase in toxicity as compared to TPCK trypsin. It was therefore suspected that chymotrypsin present in the low grade trypsin might be playing an important role in the activation process.

The results of three experiments, in which TPCK trypsin and chymotrypsin were used on their own and in combination, are summarised in Table 1. It can be clearly seen that TPCK trypsin was less efficient as a prototoxin activator than chymotrypsin. The combination of both enzymes was more efficient than either enzyme alone.

Further experiments were undertaken to determine the effect of enzyme concentration on the activation of prototoxin. The results are given in Table 2. In high concentration chymotrypsin was an efficient prototoxin activator but the activity declined rapidly as the amount of enzyme used was reduced. TPCK trypsin, on the other hand, was less effective as an activator but it was equally active at all the concentrations tested.

TABLE 2 Effect of enzyme concentration on the activation of purified prototoxin

Enzyme	Mg enzyme/ mg prototoxin	Incubation time (min)	Toxicity (MLD/mg prototoxin)
Chymotrypsin....	5,0	30	$3,2 \times 10^6$
	0,5	30	4×10^5
	0,05	30	2×10^5
	0,005	30	2×10^5
TPCK trypsin....	5,0	30	4×10^5
	0,5	30	4×10^5
	0,05	30	4×10^5
	0,005	30	4×10^5

In another experiment (Table 3) it was shown that a low concentration of TPCK trypsin together with a sub-optimal concentration of the chymotrypsin preparation formed an efficient system for prototoxin activation.

TABLE 3 Activation of purified prototoxin with suboptimal concentrations of chymotrypsin and low concentration of TPCK trypsin

Enzyme	Mg enzyme/ mg prototoxin	Incubation time (min)	Toxicity (MLD/mg prototoxin)
TPCK trypsin....	0,005	30	2×10^5
Chymotrypsin....	0,5	30	8×10^5
TPCK trypsin + chymotrypsin...	0,005+ 0,5	30	$3,2 \times 10^6$

An attempt was also made to determine the optimal conditions for activation with the low grade trypsin. Varying concentrations of enzyme and activation times were investigated (Tables 4 and 5). Reduction of the enzyme concentration from 2,5 mg per mg of prototoxin to 0,25 mg per mg of prototoxin resulted in a fourfold reduction in activation (Table 4). The optimal incubation time was 45 min (Table 5). At this time activation was so efficient that the resultant toxin had a toxicity of at least $6,4 \times 10^6$ MLD/mg. As titration of toxin was not taken beyond this point the toxicity might even have been higher.

TABLE 4 Activation of purified prototoxin by varying doses of low grade trypsin

Enzyme	Mg enzyme/ mg prototoxin	Incubation time (min)	Toxicity (MLD/mg prototoxin)
Low grade trypsin	2,5	30	$1,6 \times 10^6$
	0,25	30	4×10^5
	0,025	30	2×10^5
	0,0025	30	1×10^5

TABLE 5 Activation of purified prototoxin by low grade trypsin. Effect of varying incubation time

Enzyme	Mg enzyme/ mg prototoxin	Incubation time (min)	Toxicity (MLD/mg prototoxin)
Low grade trypsin	2,5	15	8×10^5
	2,5	30	$3,2 \times 10^6$
	2,5	45	$6,4 \times 10^6$
	2,5	60	$3,2 \times 10^6$
	2,5	90	$1,6 \times 10^6$

Absorption of toxin by mouse tissues

The absorption of high concentrations of toxin by the mouse tissues is shown in Table 6 and the absorption of low concentrations in Table 7. In view of the fact that doubling dilutions of toxic supernatant were used in the mouse toxicity tests, absorption of half the toxin in the original solution onto the mouse tissues represents a single step difference in the titration. Toxin absorption by tissues which absorb large amounts of toxin, e.g. brain, can best be gauged from the results given in Table 6 and absorption by tissues absorbing small amounts from the results in Table 7. Tables 6 and 7 represent results from single experiments but each experiment was repeated three times with essentially similar results.

It is clear (Table 6) that brain tissue absorbs comparatively large amounts of toxin (>9 600 and <19 840 MLD absorbed by two mouse brains) and that other tissues absorb little toxin. Kidney tissue seems to absorb slightly more than the other tissues (Table 7).

TABLE 6 Absorption of epsilon toxin (high concentration) by macerated mouse tissues

Tissue used for for absorption	Highest dilution of supernatant which killed mice	Number of mice killed*	Estimated amount of toxin absorbed (MLD)
—	1/2048	1/3	Nil
Brain.....	1/64	3/3	>9 600 <19 840
Kidney.....	1/1024	3/3	? a small amount
Liver.....	1/2048	2/3	Nil
Spleen.....	1/2048	2/3	Nil
Lung.....	1/2048	1/3	Nil
Muscle.....	1/2048	1/3	Nil

* 1/3 means one out of three mice died at the highest dilution of toxin which killed mice

TABLE 7 Absorption of epsilon toxin (low concentration) by macerated mouse tissues

Tissue used absorption	Highest dilution of supernatant which killed mice	Number of mice killed*	Estimated amount of toxin absorbed (MLD)
—	1/256	3/3	Nil
Brain.....	Not toxic		2 560
Kidney.....	1/64	2/3	1 920
Liver.....	1/128	3/3	1 280
Spleen.....	1/128	3/3	1 280
Lung.....	1/128	3/3	1 280
Muscle.....	1/128	2/3	1 280

* 3/3 means that three out of three mice died at the highest dilution of toxin which killed mice

Blocking of toxicity by formalinised toxoid

In two separate experiments it was found that 32 times the amount of toxin that was required to kill control mice was needed to kill mice which had been injected 3 h previously with toxoid.

DISCUSSION

Standardisation of toxicity tests on epsilon toxin can only be achieved if the activation conditions are carefully standardised. Of particular importance is the use of a single preparation of enzyme which must not vary from one test to another. Many low grade preparations of trypsin are contaminated with small amounts of chymotrypsin (Kosta & Carpenter, 1964). It was shown in this investigation that the two enzymes have a synergistic effect with regard to epsilon prototoxin activation (Tables 1 and 3). Activation by TPCK trypsin produced a product of lower toxicity than the toxin obtained when high concentrations of low grade trypsin were used. Maximal activation with low grade trypsin was, however, only achieved with high concentrations of enzyme (Table 4). Although TPCK trypsin activated toxin was four to 16 times less toxic than optimally activated toxin (Tables 1 and 3), increasing the concentration of enzyme used did not improve the activation. The maximal activation which can be achieved by TPCK trypsin was obtained at the comparatively low concentration of 0,005 mg/mg prototoxin (Table 2). On the other hand, chymotrypsin alone was capable of achieving near to maximal activation when used in very high concentrations but its activity fell rapidly at lower concentrations (Table 2). These findings suggest that the reason low grade trypsin had to be used in high concentrations was to obtain a sufficiently high concentration of the contaminating chymotrypsin for maximal synergistic effect. The high concentrations of chymotrypsin necessary to achieve maximal chymotrypsin activity might indicate that the preparation used was not pure and that maximal activation depended on achieving a sufficiently high concentration of a small trypsin contamination. Further work to clarify this point is indicated. Table 5 shows clearly that the time taken to achieve maximal activation is critical and it should be determined experimentally for the particular enzyme preparation and activation system used.

In the experiments on toxin absorption by tissues it could be clearly seen that brain tissue absorbed toxin. The amount of toxin absorbed by brain in terms of MLD was considerable but it should be remembered that in terms of mass of protein it is still a very small amount. Assuming a toxicity of $3{,}2 \times 10^6$ MLD/mg, 10 000 MLD is about 30 μg of protein toxin. It is not known what part of the brain tissue absorbs the toxin and whether this absorption of toxin to brain is responsible for the toxic effects. The finding is, however, highly suggestive because the symptomatology of enterotoxaemia is typical of a central nervous system derangement. The fact that other tissues absorb comparatively little toxin suggests that toxin may be interacting with some specific site in the brain. A further interesting observation is that kidneys appear to absorb slightly more toxin than other tissues. A number of effects of epsilon toxin on kidneys have been described (Hauschild, 1971).

If the hypothesis that the effects of epsilon toxin are caused by its interaction with a particular binding site or sites in the central nervous system is true, then the toxicity should be reduced by the prior injection of a non-toxic structural analogue of epsilon toxin, which can compete for the available sites. Pre-injection of formalinised toxoid resulted in a 32-fold increase in resistance to epsilon toxin. The suggested hypothesis is therefore considered worthy of further more detailed investigation.

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