STUDIES ON THE COBRA NEUROTOXIN INHIBITING ACTIVITY IN AN EXTRACT OF *CURCUMA SP.* (ZINGIBERACEAE) RHIZOME

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Abstract. A study was carried on the mode of action and some properties of a cobra neurotoxin inhibitor found in the extract of *Curcuma* sp. (Zingiberaceae). When the principal postsynaptic neurotoxin (STX) of the Thai cobra *(Naja naja siamensis)* was mixed with an aqueous extract of *Curcuma* sp. rhizome, the STX was inactivated as tested in mice or *in vitro* using a rat hemidiaphragm preparation. The 'neurotoxin inhibitor' ('NTxI') was found only in the water insoluble fraction of the rhizome extract. Using radioactively labeled neurotoxins, ^{125}I-STX and ^{3}H-STX, it was demonstrated that the neurotoxin did not form a stable complex with the 'NTxI'; the inactivated neurotoxin remained in the supernatant of the reaction mixture. After inactivation by 'NTxI', the STX exhibited an unchanged molecular weight as judged by SDS-polyacrylamide gel electrophoresis and an unchanged isoelectric point in isoelectric focusing. Extraction of the *Curcuma* sp. rhizome with at least 0.2% Triton X-100 resulted in solubilization of a component capable of forming a soluble and stable complex with ^{3}H-STX. By column chromatography on Sephadex G-200 in the presence of 0.1% Triton X-100, the toxin-binding compound was shown to have a molecular weight of about 150 kDa. This 150 kDa component was obtained by Triton extraction of the water-insoluble fraction, and much less from the water soluble fraction, of *Curcuma* sp. rhizome. It did not possess any carbohydrate side-chain capable of binding the lectin Concanavalin A. The time course of the 150 kDa-^{3}H-STX complex formation was extremely slow (approx 22 hours). The bond between the toxin and the 150 kDa component was of the non-covalent type. Quantitative calculation showed that the 150 kDa component was present in only 2.3 nmole/ml of the original aqueous extract. Incubation of ^{3}H-STX with thin slices of *Curcuma* sp. rhizome followed by autoradiography did not result in specific binding of the toxin with any particular cell type or subcellular structure of the rhizome. The nature of the 'NTxI' and the possibility that the 150 kDa component is the soluble form of 'NTxI' are discussed.

INTRODUCTION

A great number of drugs used in modern medicine are obtained from medicinal plants, for example, cardiac glycosides, vinca alkaloids, reserpine, etc. Many modern drugs are semisynthetic derivatives of natural products. It is therefore possible that medicinal plant(s) containing antidotes to some poisonous snake bites may exist. Nakagawa *et al* (1982) reported two compounds found in a South American medicinal plant (Cabeca de Negra) to be potent antisnake venom agents against *Bothrops atrox.*

A few medicinal plants are claimed by the Thais and their neighbors to be antidotes against poisonous snake envenomation. The use of these medicinal plants can be extremely dangerous if they are not efficacious. Cherdchu *et al* (1977) reported the ineffectiveness of *Clinacantus nutans* Burm in antagonizing crude cobra venom and its purified principal postsynaptic neurotoxin, STX (Karlsson *et al,* 1971). Tejasen *et al* (1969a, b) were the first to demonstrate that an aqueous extract of the rhizome of *Curcuma* sp. (Zingiberaceae) could antagonize the lethal effect of Thai cobra venom. A preliminary study of this plant, its mechanism of action and the chemical nature of its 'neurotoxin inhibitor' ('NTxI') was attempted by Cherdchu *et al* (1978). They showed, *in vitro,* that aqueous extracts of the plant rhizome could abolish the effect of STX. In addition, the results from *in vivo* studies indicated that the antagonistic effect could be observed only when the extract

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Abbreviations: BSA, bovine serum albumin; NTxI, neurotoxin inhibitor; STM, sieve tube member and STX, *N.n. siamensis* postsynaptic toxin 3.

was precombined with STX or the crude cobra venom before injection into mice. They also found that the 'NTxI' could be activated by acidification and inactivated by alkalinization. Furthermore, the 'NTxI' was particulate in nature, heat stable and was resistant to hydrolysis by pepsin, alpha-amylase, and beta-amylase (Cherdchu *et al*, 1978). They concluded that 'NTxI' exerted its effect directly on the STX molecule, since it did not inhibit acetylcholinesterase nor act on the nicotinic acetylcholine receptor. Cherdchu and Karlsson (1983) reported that the inactivation of STX was not by the action of proteases present in the aqueous extract of *Curcuma* sp. rhizomes. The present study deals with further characterization of the mode of action and the nature of 'NTxI' found in the *Curcuma* sp. (Zingiberaceae).

MATERIALS AND METHODS

Chemicals and biochemicals

Lyophilized Thai cobra *(Naja naja siamensis)* venom was purchased from Queen Suavabha Memorial Institute, Bangkok, Thailand. The *Curcuma* sp. rhizomes were kind gifts from Dr P Tejasen and Dr A Apisariyakul of the Department of Pharmacology, Faculty of Medicine, Chiang Mai University. Chemicals of reagent grade were obtained from Sigma Chemical Co, unless otherwise indicated.

Purification and radioactive labeling of *N.n. siamensis* neurotoxin 3

The *N.n. siamensis* neurotoxin 3 (STX) was purified on Bio-Rex 70 (Bio-Rad Laboratories, California), according to the method described by Karlsson *et al* (1971). The tritiated STX (^{3}H-STX) was prepared by reductive methylation with formaldehyde and ^{3}H-NaBH$_4$ according to Means and Feeney (1968). Iodination of STX was carried out according to the method of Greenwood *et al* (1963).

Preparation of the aqueous extract of *Curcuma* sp. rhizomes

Aqueous extract of *Curcuma* sp. rhizomes was prepared as described by Cherdchu *et al* (1978). The young and old rhizomes were arbitarily separated and were extracted separately. The fresh rhizome was weighed and chopped into small pieces then ground in double distilled water in a mortar. The ground rhizome was transferred into a Omni-Mixer 17220 (Ivan Sorval Inc), along a small volume of double distilled rinsing water. The total volume of water used (in ml) was equal to the weight (in g) of the rhizome. The material was then blended at a speed setting of 6 for 15 minutes. The resulting paste was forced through a tissue press (Harvard Apparatus Company, Inc, MA) to remove large fibers, homogenized and then centrifuged in a clinical centrifuge at 3,000 rpm for 20 minutes. Aliquots of 2-3 ml of the slightly cloudy extracts were bubbled with nitrogen gas and tightly stoppered prior to storage at -20°C. Each tube of the extract was used only once in an individual experiment; the remaining extract from the experiment was discarded. Storage of the extract at -70°C over several years did not diminish the antagonistic activity of the extract (Cherdchu *et al*, 1978).

Reaction between *Curcuma* sp. extract and radioactive STXs

Curcuma sp. extract (0.25 ml) was incubated with 3×10^5 cpm/30 µg of ^{125}I-STX in 1.05 mM reaction mixture containing 20 mM Tris HCl pH 7.4, 0.047% of BSA, and 52.5 mM sodium chloride. After incubation for 30 minutes at 25°C, the reaction was terminated by the addition of 1.95 ml of 40 mM Tris HCl pH 7.4 containing 0.1% BSA, and the mixture was then centrifuged at 100,000*g* for 45 minutes. The supernatant and the pellet were separated and counted for radioactivity in a gamma counter. In some experiments, the reaction mixture was filtered through a Millipore HA membrane filter (pore size 0.45 µm). The Millipore filtrate and the retentate were counted for radioactivity. When ^{3}H-STX was used, the membrane was dissolved in 5 ml Bray's scintillation fluid.

Solubilization of the *Curcuma* sp. aqueous extract

Solubilization of the *Curcuma* sp. extract was performed by the addition of 25% Triton X-100 into 2 ml of the aqueous extract to obtain 0.1%, 0.2%, 0.4% or 1.0% final concentrations of the detergent. The mixtures were incubated at 25°C for 60 minutes. ^{3}H-STX (3×10^6 cpm with specific activity of 3×10^5 cpm/µg) was added followed by incubation at 25°C for 2 hours before centrifugation of the reaction medium at 100,000*g*, for

45 minutes. The supernatant and pellet were separated and the radioactivity of each fraction counted. In some experiments the supernatant was further fractionated on Sephadex G-200 or Sephadex G-100 columns equilibrated with buffer containing 0.1% Triton X-100. Fractions were collected and aliquots taken for counting in a liquid scintillation counter.

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (1971). SDS-agarose gel electrophoresis was carried out using agarose gel prepared with 0.3% agarose, 0.375 M. Tris HCl pH 8.8 and 0.1% SDS. The electrode buffer was 0.025 Tris base, 0.192 M glycine and 0.1% SDS at a final pH of 8.3. When the electrophoresis was complete, the gel was cut into 3 mm slices and heated at 60°C with 60 µl 35% hydrogen peroxide for 3 hours. Subsequently, 0.4 ml of scintillation fluid was added and the radioactivity was counted.

Isoelectric focusing

Isoelectric focusing of the ^{3}H-STX before and after reaction with the extract was carried out according to O'Farrell *et al* (1977). The gel contained pH 3.5-10 ampholine (LKB Co, Uppsala). After the electrophoresis, the gel was cut into 2 mm slices and processed for radioactive counting as described above.

Assays of the 'NTxI' activity of the *Curcuma* sp. extract

The *in vivo* assay of the 'NTxI' activity in the plant extract was carried out in female albino mice weighing 20 ± 2 g. STX (2 µg/g body weight) was mixed with the extract (16 µl/g body weight) prior to peritoneal injection into the mice. The numbers of mice that survived were recorded 24 hours after injection.

The *in vitro* test of the 'NTxI' activity of the plant extract was carried out using isolated rat phrenic nerve-hemidiaphragm preparations (Cherdchu *et al*, 1978). The entire nerve-muscle preparation was submerged in 60 ml Kreb's solution.

Autoradiographic study of *Curcuma* sp. rhizome incubated with ^{3}H-STX

Thin slices of *Curcuma* sp. rhizome (approx 0.5 mm thickness) were incubated with 10^6 cpm of ^{3}H-STX (specific activity 3 × 10^5 cpm/µg) in normal saline solution for 30 minutes at 25°C. The slices were extensively washed with saline, fixed with glutaraldehyde and processed for autoradiography. Control slices were also prepared using 10 cpm ^{3}H-STX plus an excess (1 mg) of non-radioactive STX.

Protein determination

Protein concentration was determined by the method of Lowry *et al* (1951) using BSA as the standard.

RESULTS

The *in vivo* neurotoxin-inhibiting activity of young and old *Curcuma* sp. rhizomes

Mice injected with STX (2 µg/g body weight) all died (Table 1). When *Curcuma* sp. rhizome extract (16 µl/g body weight) was combined with STX, the number of dead mice decreased (Table 1). Extracts made from young or old rhizomes showed comparable activity against STX.

The *in vitro* reaction between the *Curcuma* sp. extract and STX

When isolated rat phrenic nerve hemidiaphragm was incubated with 0.58 µg/ml of STX, indirectly stimulated muscle contraction was slowly but completely inhibited (Fig 1). When *Curcuma* sp. extract (8.3 µl/ml), was added to the organ bath with STX, the neuromuscular blockade produced by STX was drastically reduced.

When these amounts of STX and the *Curcuma* sp. extract were mixed and the reaction mixture was filtered through a Millipore HA membrane filter (pore size 0.45 micron), the filtrate (containing approximately 95% of the neurotoxin, see below), did not inhibit neuromuscular transmission (Fig 1). The resuspended Millipore retentate gave similar results.

Reactions of *Curcuma* sp. extract and radioactive STX

After the *Curcuma* sp. extract was incubated with ^{125}I-STX and the reaction mixture was centri-

Table 1

Antagonistic effect of extract of *Curcuma* sp. and STX in mice.

Experiment*	Number of deaths/ total tested
1. *Curcuma* sp. extract (young rhizome)	0/6
2. STX	6/6
3. *Curcuma* sp. extract (young rhizome) + STX	3/7
4. *Curcuma* sp. extract (old rhizome) + STX	3/7

* see Materials and Methods for details.

Table 2

Interaction of ^{125}I-STX with *Curcuma* sp. extract.

Experiment*	Radioactivity (cpm)	
	Pellet (%)	Supernatant (%)
1. *Curcuma* sp. extract + ^{125}I-STX	15,525 (4.90)	299,598 (95.00)
2. *Curcuma* sp. extract + ^{125}I-STX + 2 mg STX	13,029 (3.86)	324,381 (96.14)

* see Materials and Methods for details.

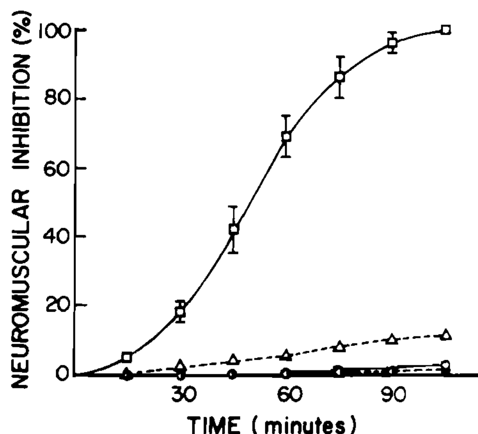

Fig 1—Cobra neurotoxin inhibiting activity of *Curcuma* sp. aqueous extract on isolated rat hemidiaphragm preparations. The neuromuscula inhibitions were produced by : 0.58 μg/ml STX (--□--□--); 0.58 μg/ml STX + 8.3 μl/ml *Curcuma* sp. extract (--△--△--); Millipore filtrate of the reaction mixture of STX and *Curcuma* sp. extract (--○--○--) and Millipore retentate of the reaction mixture of STX and *Curcuma* sp. extract (--●--●--). Each point represents mean ± SE of six determinations.

fuged (see Materials and Methods), 95% of the radioactive toxin remained in the supernatant while the rest was found in the pellet (Table 2). The radioactivity in the pellet was slightly decreased when an excess of non-radioactive STX (2 mg) was included in the incubation mixture. If, instead of centrifugation, the reaction mixture was filtered through Millipore HA membrane filter; the filtrate contained between 90-95% of the radioactive toxin.

Similar results were obtained when 3 × 10^5 cpm of ^{3}H-STX was used in place of ^{125}I-STX (data not shown).

Some physicochemical properties of ^{3}H-STX after being incubated with *Curcuma* sp. extract

The molecular weights of ^{3}H-STX before and after incubation with the *Curcuma* sp. extract were studied by gel filtration chromatography on a Sephadex G-50 column. After incubation of ^{3}H-STX with *Curcuma* sp. extract and centifugation, the ^{3}H-STX in the clear supernatant (containing

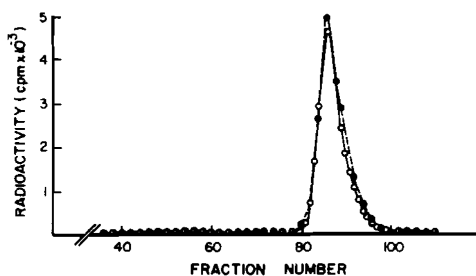

Fig 2—Gel filtration chromatography of ^{3}H-STX before (--○--○--) and after (--●--●--) incubation with the aqueous extract of *Curcuma* sp. Sephadex G-50 column (size 2.6 × 97 cm) was equilibrated and eluted with 25 mM ammonium acetate. The flow rate was 20 ml/hour and the fraction volume was 3 ml.

about 95% of the radioactivity) showed the same molecular weight as that of untreated ^{3}H-STX (Fig 2).

A similar study was carried out on the isoelectric point of the ^{3}H-STX before and after incubation with the *Curcuma* sp. extract. It was found that the treated and untreated ^{3}H-STX showed identical isoelectric focusing patterns (data not shown).

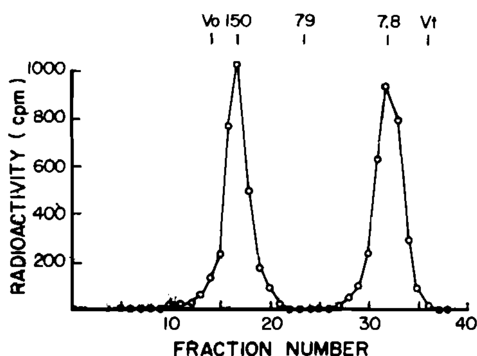

Fig 3—Chromatography of *Curcuma* sp. aqueous extract after solubilization in 0.2% Triton X-100 and incubation with ^{3}H-STX. A Sephadex G-200 column (1 × 40 cm) was equilibrated and eluted with 50 mM sodium phosphate, pH 7.4, containing 0.1% Triton X-100 and 0.01 mg/ml BSA. The flow rate was 2 ml/hour and the fraction size was 1 ml. The elution volumes of standard proteins with MW's in kDa are shown on the top of the profile.

Solubilization of the *Curcuma* sp. extract

Aqueous extracts of *Curcuma* sp. rhizome were incubated with various concentrations of Triton X-100 at 25°C for 60 minutes followed by centrifugation at 100,000*g* for 45 minutes. It was found that the supernatants contained a component capable of binding ^{3}H-STX as shown by Sephadex G-200 column chromatography (Fig 3). This component, when bound to ^{3}H-STX, eluted in the same fraction as standard rabbit IgG chromatographed under the same conditions. Thus, the molecular weight of this component was approximately 150 kDa.

The sizes of these 150 kDa peaks were comparable when the *Curcuma* sp. extracts were treated with 0.2%, 0.4% or 1.0% Triton X-100. This peak was considerably smaller when solubilization was done with 0.1% detergent.

The radioactive peak at 150 kDa was not the result of ^{3}H-STX being trapped in or being part of the micellar structure of Triton X-100. When ^{3}H-STX was treated with detergent under conditions similar to those described above but in the absence of *Curcuma* sp. extract, no radioactive peak at 150 kDa was observed.

The source and concentration of the 150 kDa component

The aqueous extract of *Curcuma* sp. rhizome was centrifuged at 100,000*g* for 45 minutes, the supernatant and pellet were separated. The supernatant and the pellet were then separately treated with 0.2% Triton X-100 as described previously. It was found that most, and in some experiments, all of the 150 kDa component was found in the detergent extract of the pellet (Fig 4). In order to determine the time course and extent of 150 kDa-^{3}H-STX complex formation, ^{3}H-STX was added to the Triton X-100 extract and then incubated for 2, 4, 10 or 22 hours. Reaction mixtures were chromatographed on Sephadex G-100 columns (0.5 × 10 cm) to separate the 150 kDa-^{3}H-STX complex from the free ^{3}H-STX. It was found that the rate of complex formation was very slow (Fig 5).

From the specific activity of the ^{3}H-STX, it was calculated that the amount of 150 kDa at maximum complex formation (Fig 5) was 2.3 nmole/ml of the *Curcuma* sp. original aqueous extract.

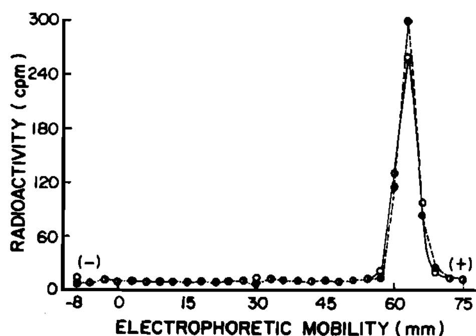

Fig 4—SDS-agarose gel electrophoresis of ^{3}H-STX (--○--○--) and the 150 kDa-^{3}H-STX complex (--●--●--).

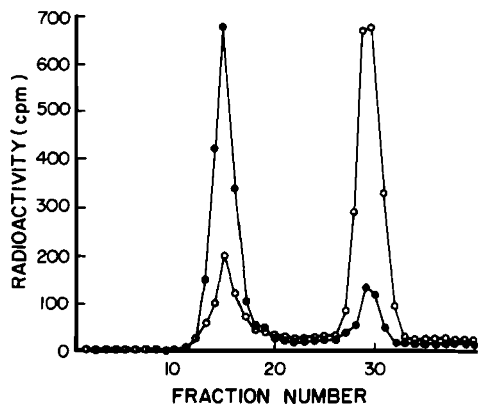

Fig 5—Sephadex G-200 chromatography of the Triton X-100 treated pellet or Triton X-100 treated supernatant fraction of the *Curcuma* sp. aqueous extract. Each fraction was incubated with ^{3}H-STX (see Materials and Methods). The reaction mixtures were chromatographed on identical Sephadex G-200 columns under the same conditions as shown in Fig 3. The figure shows the profiles of Triton X-100 treated pellet plus ^{3}H-STX (--●--●--) and Triton X-100 treated supernatant plus ^{3}H-STX (--○--○--).

Nature of the 150 kDa-^{3}H-STX complex

After the 150 kDa-^{3}H-STX complex was formed, further incubation of the complex with Concanavalin A (final concentration 10 μg/ml) at 25°C for 30 minutes did not result in a complex with higher molecular weight than 150 kDa. This was shown by chromatography of the incubation mixture on a Sephadex G-200 column (data not shown). This result suggests that the 150 kDa component did not contain D-mannopyranosyl residue in an (α 1→3)-linked manno-oligosaccharide moiety capable of binding to the lectin Concanavalin A (Van Landschoot *et al*, 1980).

When the 150 kDa-^{3}H-STX complex was subjected to SDS-polyacrylamide gel electrophoresis (under non-reducing conditions), all of the radioactivity was found at the free ^{3}H-STX band (Fig 6). Thus the bonding between the 150 kDa component and the ^{3}H-STX was of the non-covalent type.

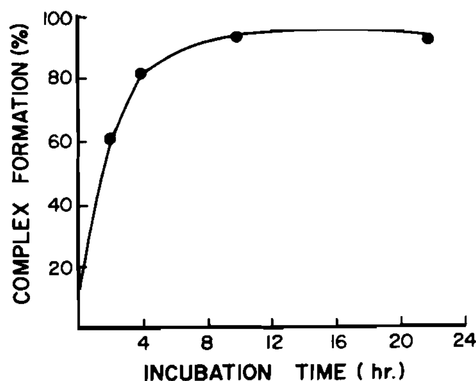

Fig 6—Time coures of 150 kDa-^{3}H-STX complex formation. The Triton X-100 solubilized *Curcuma* sp. extract was incubated with ^{3}H-STX for varying periods of time and the amounts of 150 kDa-^{3}H-STX complex formed were quantitated by chromatography on Sephadex G-100 columns.

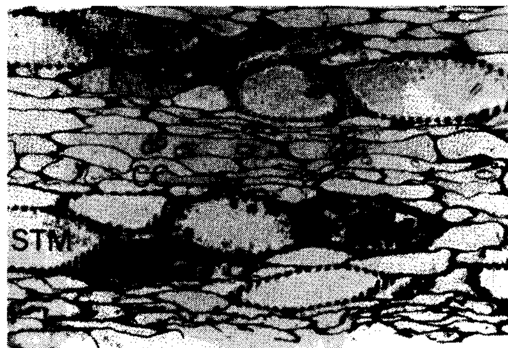

Fig 7—Autoradiography of *Curcuma* sp. rhizome incubated with ^{3}H-STX. STM, sieve tube member; CC, companion cell. The magnification was 720x.

After incubation of ^{3}H-STX with thin slices of *Curcuma* sp. rhizome, the radioactive toxin was mainly located in the sieve tube member (STM) of the phloem (Fig 7). Similar autoradiographs were obtained when an excess of non-radioactive STX was added to the incubation medium. This result suggests that there was no cell or subcellular organelle which specifically bound ^{3}H-STX.

DISCUSSION

The aqueous extracts of *Curcuma* sp. rhizome, young or old, showed comparable activity against STX. The rhizomes used in the present experiments showed slightly lower activity than those used previously (Cherdchu *et al*, 1978). In previous experiments, the same volume of extract (16 μl/g mouse) completely protected all mice injected with the same dose of STX. However, in the present experiments, this dose of the *Curcuma* sp. extract allowed about half of the mice to survive; if there had been a difference in the anti-neurotoxin activity between young and old rhizomes, this would have been observed.

In fact, the aqueous extract of *Curcuma* sp. rhizome as first described by Tejasen *et al* (1969a) was not an extract in the strict sense. It would more accurately be described as a 'blend' of rhizomes in water. This blend was centrifuged at 3,000*g* and so the slightly cloudy supernatant still contained a large amount of fine insoluble particles. Centrifugation of the cloudy extract at 100,000*g* for 45 minutes resulted in a pellet which contained most of the 'NTxI' activity. Filtration of the aqueous extract on Millipore HA membrane also resulted in an insoluble retentate with most of the 'NTxI' activity.

Two kinds of radioactive STXs, one labeled at tyrosine residues and one labeled at lysine residues, were used in the present study. This was done to avoid confusion that could have resulted should 'NTxI' inactivation of STX have been dependent upon interactions with STX tyrosine and been blocked by iodination. As it turned out, ^{3}H-STX was found to give similar results to those of ^{125}I-STX. Because of its longer half-life and less hazardous nature, ^{3}H-STX was used in succeeding experiments.

It was conclusively shown in this study that 'NTxI' did not inhibit the action of STX by forming a stable complex with the latter. The radioactive STX bound to the pellet was only about 5% of the total added; this component was formed nonspecifically since it was not reduced by the addition of excess non-radioactive neurotoxin. The toxin which was pharmacologically inactive remained in the supernatant. The molecular weight and the pI of the inactivated STX remained unchanged indicating that certain uncharged amino acid residue(s) were modified by 'NT × I. In addition, the observation that the two radioactive STX's gave similar binding results suggested that tyrosine(s) was probably not involved. A possible candidate target is the invariant tryptophan at position 25 of STX (Karlsson, 1979). This modification by 'NTxI' must be extremely effective since the biological activity of STX was completely abolished. It is most likely that 'NTxI' is an enzyme but not a protease (Cherdchu and Karlsson, 1983).

Solubilization of the *Curcuma* sp. extract with non-ionic detergent, Triton X-100, resulted in a 150 kDa component capable of forming a non-covalent, stable complex with STX. Moreover, the 150 kDa component was found predominantly in the pellet of the aqueous extract of the rhizome. These observations suggest that the 150 kDa may be the soluble form of 'NTxI'. This possibility is not weakened by the finding that the concentration of the 150 kDa molecule (2.3 nmole/ml) was quite low. This concentration is only 15% of that needed to interact with STX if 1 mole of 150 kDa inactivated 1 mole of STX in the *in vivo* experiment. However, since evidence points to the enzymatic nature of 'NTxI', the 150 kDa component need be present in only catalytic amounts, and need not be present in a 1:1 stoichiometry with the neurotoxin. Another observation was the extremely slow rate at which the 150 kDa and ^{3}H-STX reacted to form the complex. It is conceivable that 'NTxI' is structurally altered by the detergent, particularly the enzyme active site, in such a way that an enzyme-substrate complex can be formed (albeit slowly) but that further catalytic activity is lost.

Because of the presence of detergent, characterization of the 150 kDa component was very difficult. Various hydrolytic enzymes could be tested with the 150 kDa component and might shed light on its chemical nature, but they were inactive in the detergent solution. Therefore, the excess

detergent must be removed before further study on the nature of the 150 kDa component can be carried out.

Because of these difficulties, an attempt was made to see whether ^{3}H-STX would bind to specific cells or subcellular structures of the *Curcuma* sp. rhizome. However, no specific binding was observed; this is not unexpected in the light of the result from Table 2 where no stable 'NTxI'-^{3}H-STX complex was found.

Further investigations are needed to gain information on the chemical nature of 'NTxI' and/or the 150 kDa component.

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