PROTEOLYTIC-INDEPENDENT COBRA NEUROTOXIN INHIBITING ACTIVITY OF *CURCUMA SP. (ZINGIBERACEAE)*

C. Cherdchu and E. Karlsson

Department of Pharmacology, Pramongkutklao College of Medicine, Bangkok, Thailand, and Institute of Biochemistry, University of Uppsala, S—751 23 Uppsala, Sweden.

INTRODUCTION

Curcuma sp. (Zingiberaceae), a plant known in Thai as Wan Ngu, is reputed in Thailand as an antidote against cobra envenomization. It is claimed by the local people to be effective when given orally to the victim of poisonous snakebite, particularly from cobra snakes. The aqueous extract of *Curcuma sp.* rhizomes, when given intravenously, has been shown to protect mice and anesthesized dogs against *Naja naja siamensis* venom (Tejasen *et al.*, 1969 a, b; Tejasen and Sunyapridakul, 1970; Tejasen and Thongtharb, 1978). The plant extract has been found to antagonize the inhibitory action of purified *N. n. siamensis* neurotoxin in isolated rat phrenic nerve hemidiaphragm preparations. Moreover, it has also been shown to prolong the survival times of mice receiving lethal doses of *N. n. siamensis* venom, but only when administering the plant extract and venom in combination via the same routes (Cherdchu *et al.*, 1978). The mechanism of antagonization of *N. n. siamensis* neurotoxin by the plant extract is believed to be due to direct inactivation (Chantaratham and Tejasen, 1970; Cherdchu *et al.*, 1978).

The *N. n. siamensis* neurotoxin is a polypeptide, and the proteolytic activity of the *Curcuma sp.* extract, if it exists, might be responsible for the inactivation of the neurotoxin. Therefore, the purpose of this study was to investigate the proteolytic activity of the *Curcuma sp.* extract and its role on the mechanism of antagonism of *N. n. siamensis* neurotoxin.

MATERIALS AND METHODS

Lyophilized cobra *(Naja naja siamensis)* venom was purchased from Miami Serpentarium Laboratories (Miami, Florida,U.S.A.). Bio-Rex 70, -400 mesh (sodium form) was from Bio-Rad Laboratories, California. Sephadex G-50 was from Pharmacia, Uppsala, Sweden. Ammonium acetate, trichloroacetic acid and sodium dihydrogenphosphate were from Merck, Darmstadt. Azocasein was from Sigma Chemical Co., St. Louis, Missouri. The *siamensis* neurotoxin (STX) was purified as described earlier (Karlsson *et al.*, 1971). The STX (100 μg/ml) was stored at -10° until use. The tritiated STX (^{3}H-STX) was prepared by reductive methylation with formaldehyde and radioactive sodium borohydride $^3H\text{-}NaBH_4$ according to Means and Feeney (1968). The toxin was then completely methylated with non-radioactive borohydride thereby converting all amino groups (one α-and five ε-amino) to dimethylamino groups. The modified toxin retained a strong binding to the acetylcholine receptor isolated from the electric organs of *Torpedo marmorata* (Karlsson, unpublished).

Aqueous extract of *Curcuma sp.* rhizomes was prepared as described previously (Cherdchu *et al.*, 1978) with only some minor modifications. A volume of aqueous extract of *Curcuma sp.* was centrifuged at 17,000 g for 30 min at room temperature. The supernatant was carefully removed with a Pasteur pipet, and to the remaining residue was added distilled water to the original volume before centrifugation. These two fractions, the

supernatant and the residue, were subsequently assayed for proteolytic activity.

The proteolytic activity was assayed with azocasein as substrate (Barrett and Kirschke, 1981). The assay mixture contained 0.1 M phosphate buffer (pH 8.0), 1% azocasein and aliquots of plant extract or its fractions from centrifugation, in a final volume of 0.6 ml. After 20 min incubation at 37°C, 0.6 ml of 10% trichloroacetic acid was added and precipitated protein was removed by centrifugation. Aliquots of the supernatant were taken for determination of absorbance at 366 nm. The proteolytic activity was expressed as the change in absorbance at 366 nm per hour, ΔA_{366}/hr.

The *in vivo* test of neurotoxin inhibiting activity was as follows: Female albino mice of Swiss strain, weighing 20-25 gm were used. The aqueous extract of *Curcuma sp.* or its fractions from centrifugation at 17,000 g were first incubated with STX and then injected peritoneally in the same syringe. The concentrations of plant extract and STX were 16 ml/kg and 400 μg/kg, respectively. After injection, time to death was recorded. The survival times obtained from mice receiving STX alone and with the plant extract or its fractions from centrifugation at 17,000 g were then compared.

Dialysis of incubation mixture between ^{3}H-STX and aqueous extract of *Curcuma sp.* was as follows: One milliliter of *Curcuma sp.* was incubated with 10 μg, 6 μCi/ml ^{3}H-STX at room temperature for 2 hours in a tightly stoppered test tube. The incubation mixture was transferred into a dialysis bag and dialysed against 15 ml distilled water. The same amount of ^{3}H-STX was also treated as above, but 1.0 ml distilled water was replaced for the plant extract. At zero time, before submerging the bag into dialysing water, dialysates of 250 μl were taken for determination of activity in order to serve as the activity at zero time. The measurement of radioactivity of dialysates against time were further continued in Beckmann LC-200, Liquid Scintillation Counter, using 5.0 ml scintillation cocktail consisting of 25% Triton X-100 and 75% xylene with 2 gm PPO per liter (Anderson and McClure, 1972).

Gel filtration on Sephadex G-50 of the incubation mixture between ^{3}H-STX and aqueous extract of *Curcuma sp.* was prepared as follows: Sephadex G-50 was packed and equilibrated in a 1.5 × 70 cm column having 0.2 M unbuffered ammonium acetate as equilibrating solution. The incubation mixture of 1.0 ml *Curcuma sp.* extract and 1 μg, 0.6 μCi/ml ^{3}H-STX (at 25°C for 2 hours) was applied to the column and eluted with 0.2 M ammonium acetate. The same amount of ^{3}H-STX incubated with 1.0 ml distilled water was also filtered through Sephadex G-50 column. Aliquots of 250 μl were taken for scintillation counting.

RESULTS

It is well established that the aqueous extract of *Curcuma sp.* possesses neurotoxin inhibiting activity which probably exerts its antagonistic effect via direct inactivation of the neurotoxin molecules (Tejasen *et al.*, 1978; Cherdchu *et al.*, 1978). Neurotoxins are low molecular weight proteins cross-linked by four (short neurotoxins) or five (long neurotoxins) disulfide bridges. STX has five disulfides. This polypeptide neurotoxin is probably vulnerable to the enzymic hydrolysis. Based on this reasoning as well as the information obtained by Srisukawat (1977) indicating that the plant extract was particulate in nature; the aqueous extract of *Curcuma sp.* and its fractions from 17,000 g centrifugation were tested for proteolytic activity using azocasein as substrate. The aqueous extract had a proteolytic activity of about 0.342 ΔA_{366}/hr while the supernatant

from centrifugation possessed 0.366 ΔA_{366}/hr and the residue had no activity. These two fractions were also given peritoneally into mice in combination with four lethal doses of STX in the same syringe. The neurotoxin inhibiting activity was found to be present only in the residue fraction. Whilst the supernatant fraction which apparently contained proteolytic activity produced no neurotoxin inhibiting activity as seen in the similarity of the survival time of 45.5 ± 1 (n = 3) min in control mice to 46 ± 2 (n = 4) min in mice receiving supernatant fraction together with STX (Table 1).

Table 1

Proteolytic effect and neurotoxin inhibiting activity of fractions from centrifugation of *Curcuma sp.* extract.

Fractions	Proteolytic Activity	Survival Time
	ΔA_{366}/hr	min ± S.E.M.
Supernatant	0.366	46 ± 2 (4)
Residue	None	All survived (4)

Values in parenthesis indicate numbers of mice used.

When the mixture of ^{3}H-STX and the *Curcuma sp.* extract was dialysed against distilled water only trace amounts of the radioactivity were detected in the dialysates. This low activity was also observed when the same amount of ^{3}H-STX alone was dialysed. As shown in Fig. 1 the detectable activity increased as the dialysing time increased, indicating that the neurotoxin slowly passes through the dialysis tubing. The increase in activity of ^{3}H-STX alone was slightly higher than that of the neurotoxin and the plant extract. However, the amounts of detectable radioactivity in dialysates accounted for only 0.06% of the applied radioactivity for the ^{3}H-STX alone and 0.03% when combined with *Curcuma sp.* extract. ^{3}H-STX has radioactive methyl groups at six different positions in the molecule. A proteolytic degradation should therefore give rise to radioactive peptides which should pass out into the dialysate more rapidly than ^{3}H-STX alone. Thus, this experiment suggested that the polypeptide neurotoxin was perhaps not hydrolysed by the proteolytic activity present in the *Curcuma sp.* extract.

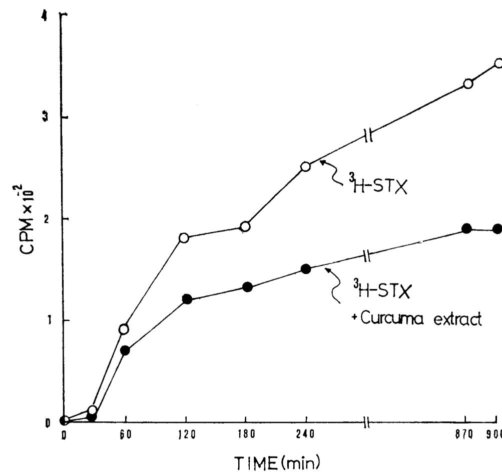

Fig. 1—Radioactivity of dialysates at different times of dialysing. One milliliter of aqueous extract of *Curcuma sp.* was incubated with 10 μg, 6 μCi/ml ^{3}H-STX at 25°C for 2 hours and was dialysed against distilled water. The same amount of ^{3}H-STX was also incubated with distilled water and was dialysed as above serving as control.

When the ^{3}H-STX was gel filtered on Sephadex G-50 column it gave the major peak at about 72 ml of elution volume which almost coincided with the major peak obtained from gel filtration of the neurotoxin treated with the plant extract (Fig. 2). The small peak that appeared at about the void volume in front of the major peak of the *Curcuma sp.* extract-treated neurotoxin chromatogram might attribute to the complex formation which resulted from the combination of the neurotoxin with the substance in the extract. Consequently, it gave rise to a higher molecular weight substance than that

of the native neurotoxin. However, this small peak accounted for only about 4% of the total activity obtained from the summation of all the peaks appearing in chromatogram. The small peaks that appeared after each major peak might probably be associated with the contaminated substances or resulted from unwanted water-exchangeable ^{3}H-hydrogen atom of the labelled neurotoxins. This experiment also indicated that the neurotoxin was not hydrolysed to small peptides by the proteolytic activity of the plant extract.

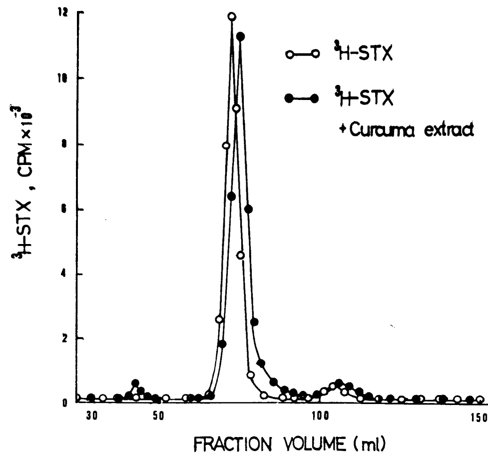

Fig. 2—Gel filtration of ^{3}H-STX alone and of the incubation mixture between ^{3}H-STX and *Curcuma sp.* extract on Sephadex G-50. One milliliter of plant extract was used for incubation with 1μg, 0.6 μCi/ml ^{3}H-STX at 25°C for 2 hours. Distilled water was substituted for plant extract in control. Flow rates were 7.4 and 7.8 ml/hr and fraction sizes 1.85 and 1.95 ml for control and treated chromatograms, respectively.

DISCUSSION

It is generally known that the primary cause of death from cobra envenomization is peripheral respiratory failure due to skeletal neuromuscular blockage by postsynaptic neurotoxins, the very toxic component of cobra venom (Lee, 1970). Therefore, any plant extract claimed to be effective as snakebite antidote must first and foremost be able to antagonize the toxic actions of this neurotoxin. There are at least two possible mechanisms by which the *Curcuma sp.* extract could inactivate the neurotoxins. Firstly, the *Curcuma sp.* extract may alter the binding of the neurotoxins on cholinergic receptors at neuromuscular junctions. Secondly, the *Curcuma sp.* extract may directly inactivate the neurotoxins. The studies made by Cherdchu *et al.* (1978) on the effect of *Curcuma sp.* extract on neurotoxins suggested that chemical inactivation can be responsible for the observed antagonism and not competition between neurotoxins and *Curcuma sp.* extract for acetylcholine receptors.

As mentioned earlier when considering the structure of neurotoxin molecules, one possible mechanism of chemical inactivation is proteolysis. The work on using trypsin for treatment of snakebite envenomation (Hsiung *et al.*, 1975; Huang and Lee, 1980) has led us to search for the existence of proteolytic activity in the extract. Although the actual chemical nature of neurotoxin-inhibitor found in *Curcuma sp.* extract is still unknown results from the present investigation confirmed that *Curcuma sp.* extract directly inactivates the neurotoxins. Since the proteolytic activity found in the plant extract was separable from the neurotoxin inhibiting activity, it suggested that no proteolysis is involved in destruction of the polypeptide neurotoxins. This was supported by the results obtained from dialysing and gel filtration of the incubation mixture between *Curcuma sp.* extract and the labelled *N. n. siamensis* neurotoxins. It should be noted, however, that though the proteolytic activity of the plant extract may not be responsible for inactivation of neurotoxins, the complex formation is perhaps attributable to a certain extent for the antagonism. Therefore, we concluded that the neurotoxin inhibiting activity of *Curcuma sp.* extract is not associated with the proteolytic activity present

in the plant extract. However, it seems premature to conclude from the present results that *Curcuma sp.* extract exerts its antagonistic effect by complex formation with the *N.n. siamensis* neurotoxins.

SUMMARY

The 1: 1 (w/v) aqueous extract of *Curcuma sp. (Zingiberaceae)* was shown to antagonize the toxic action of *Naja naja siamensis* neurotoxin possibly via direct inactivation of the toxin. The plant extract possessed proteolytic activity which could be separated from the neurotoxin inhibiting activity. The mechanism of antagonism between the plant extract and the neurotoxin was shown not to be involved with the existence of proteolytic activity in the plant extract.

ACKNOWLEDGEMENTS

This work was supported by Guest Scholarship, Swedish Institute, Sweden. The authors would like to thank Drs. A. Apisariyakul and P. Tejasen for the supply of *Curcuma sp.* used in this study.

REFERENCES

ANDERSON, L.E. and MCCLURE, W.O., (1972). An improved scintillation cocktail of high-solubilizing power. *Anal. Biochem., 51* : 173.

BARRETT, A.J. and KIRSCHKE, H., (1981). Cathepsin B, Cathepsin H, and Cathepsin L. *Methods Enzymol., 80* : 535.

CHANTARATHAM, A. and TEJASEN, P., (1970). Studies of the antagonistic effect of Wan Ngu *(Curcuma sp., Zingiberaceae)* on the action of Thailand cobra venom *(Naja naja siamensis)* at the neuromuscular junction. *Chiang Mai Med. Bull., 9* : 73.

CHERDCHU, C., SRISUKAWAT, K. and RATANABANANGKOON, K., (1978). Cobra neurotoxin inhibiting activity found in the extract of *Curcuma sp.* (Zingiberaceae). *J. Med. Ass. Thailand., 61* : 544.

HSIUNG, Y.L., TSOU, T.C., HOU, Y.T., LIU, T.C., CHOU, H.L. and LI, C.Y., (1975). Experimental studies on curing elapid bite with trypsin. *Scientia Sin., 18* : 396.

HUANG, H.C. and LEE, C.Y., (1980). Evaluation of trypsin treatment for snakebite envenomation. *Toxicon, 18* : 475.

KARLSSON, E., ARNBERG, H. and EAKER, D., (1971). Isolation of the principal neurotoxins of two *Naja naja* subspecies. *Eur. J. Biochem., 21* : 1.

LEE, C.Y., (1970). Elapid neurotoxins and their mode of action. *Clin. Toxicol., 3* : 457.

MEANS, G.E. and FEENEY, R.E., (1968). Reductive alkylation of amino groups in proteins. *Biochemistry, 7* : 2192.

SRISUKAWAT, K., (1977). Studies on the nature of the *Naja naja siamensis* toxin 3 inhibiting activity in *Curcuma sp. (Zingiberaceae)*. M.S. Thesis, Graduate School, Mahidol University.

TEJASEN, P., CHANTARATHAM, A. and KANJANAPOTHI, D., (1969a). The antagonistic effect of a medicinal plant *(Curcuma sp., Zingiberaceae)* to Thailand cobra venom *(Naja naja siamensis)*. Part I. *Chiang Mai Med. Bull., 8* : 165.

TEJASEN, P., CHANTARATHAM, A. and KANJANAPOTHI, D., (1969b). The antagonistic effect of a medicinal plant *(Curcuma sp., Zingiberaceae)* to Thailand cobra venom *(Naja naja siamensis)*. Part II. *Chiang Mai Med. Bull., 8* : 229.

TEJASEN, P. and SUNYAPRIDAKUL, L., (1970). Experimental studies of the effect of an aqueous extract of Wan Ngu *(Curcuma sp., Zingiberaceae)* on cobra envenomization. *Chiang Mai Med. Bull., 9* : 56.

TEJASEN, P. and THONGTHARB, C., (1978). The antagonistic effect against cobra envenomization: comparision of medicinal plants, corticosteroid and antivenine. *Chiang Mai Med. Bull., 17* : 159.