CHEMICAL CHARACTERIZATION OF SPIDER TOXIN, NSTX

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ABSTRACT

Toxic principles in the venom of the spider, *Nephila maculata*, were purified with the reverse phase high performance liquid chromatography, and three kinds of the major toxins (NSTX-1, -2 and -3) were isolated. These principles commonly possessed 2,4-dihydroxyphenylacetyl-asparaginy-l-cadaveryl moiety in the molecule. The structure of NSTX-3 was concluded to be *N*-(2,4-dihydroxyphenylacetyl-L-asparaginy-l)-*N'*-(L-arginyl-putreanyl)-cadaverine.

A spider toxin from the venom of *Nephila clavata* (Joro spider) has been recognized as a neurotoxin (JSTX) which suppresses irreversibly the excitatory postsynaptic potential (EPSP) and the glutamate potential in the lobster neuromuscular junction with high degree of specificity (7, 8). JSTX is also pharmacologically active in other systems where glutamate is a putative neurotransmitter (10).

We purified the toxic principles from the venom of *Nephila maculata*, a Papua New Guinean species, and found that the similar neurotoxins (NSTX) were present in the venom. The structures of both toxins were examined recently and the following chemical properties were proposed. The toxic principles (NSTX and JSTX) contained 2,4-dihydroxyphenylacetyl-asparaginy-l-cadaverine as a common component in the molecule, and in addition, various kinds of polycationic basic moieties such as polyaminergic substituent and/or basic amino acid residues were attached to this common moiety. One of the major toxin, NSTX-3, in *N. maculata* was investigated in details by NMR spectroscopic analysis, and also taking into consideration of other analytical data obtained by amino acid analysis, Edman procedure, and Fab-Mass spectroscopy. The structure of NSTX-3 was determined as *N*-(2,4-dihydroxyphenylacetyl-

L-asparaginy-l)-*N'*-(L-arginyl-putreanyl)-cadaverine.

MATERIALS AND METHODS

Spiders (*Nephila maculata*) were collected at Wau, Papua New Guinea. After the venom glands were taken out, they were immediately lyophilized, and stored in a deep freezer at -80°C until use.

The toxins were usually purified as follows. The dried venom glands (10 mg) from about five spiders were homogenized with 1 ml of 60% acetonitrile, and the homogenate was centrifuged at 3,000 rpm for 5 min. The supernatant was subjected to high performance liquid chromatography with a reverse phase column (TSKgel ODS 120-T, Toyo Soda; 4.6 mm i.d. $\times$ 250 mm; particle size 5 μm). Column temperature was maintained at 40°C . The elution was carried out with a linear concentration gradient from 0.02% hydrochloric acid to 60% acetonitrile containing 0.02% hydrochloric acid in 60 min, at a flow rate of 0.5 ml/min. The eluate was monitored by absorbance at 210 nm, and respective peak fractions were collected and concentrated *in vacuo*.

Each fraction was assayed for irreversible inhibition of EPSP using the neuromuscular

preparation of the lobster's walking leg as described previously (8).

The active substance in each peak was hydrolyzed with 6 N hydrochloric acid, and amino acids and polyamines in the hydrolysate were determined with an amino acid analyser equipped with an ion-exchange column (TSKgel IEX-215, Toyo Soda; 4 mm i.d. $\times$ 50 mm; particle size 5 μ m). To analyse both amino acids and polyamines in one column, the fourth buffer, 0.35 N sodium citrate (pH 5.28) containing 2 M NaCl and 20% methanol, was used following the conventional three buffer systems for the usual amino acid analysis. The eluate from the column was mixed with an NaOCl buffer, and then mixed with *o*-phthalaldehyde (OPA) reagent. Both column temperature and reaction temperature were maintained at 65°C. The OPA derivatives of amino acids and polyamines were determined fluorometrically with excitation at 235 nm, and emission at >410 nm.

D,L-Configuration of amino acids in the hydrolysate was determined by chiral separation of amino acids derivatized with OPA and *N*-acetyl-L-cysteine, according to the method of Nimura and Kinoshita (12).

The subtractive Edman procedure was performed by the modified method for picomolar analysis (14). After coupled with phenylisothiocyanate (PITC), PITC-NSTX-3 was reacted with trifluoroacetic acid, and evaporated. The residue was dissolved in water, and extracted with benzene. A portion of the water layer was hydrolyzed with 6 N hydrochloric acid, and subjected to the amino acid analysis, and the residue was subjected to the next cycle of degradation.

Fast atom bombardment mass spectrometry (Fab-Mass) was performed with glycerol as the supporting matrix.

Proton nuclear magnetic resonance (^1H -NMR) spectra, two dimensional NMR spectra in the mode of double quantum filter correlational spectroscopy (DQF-COSY) and relay COSY in DMSO- d_6 and/or D_2O were measured at 400 MHz. The spectra were corrected with signals of H_2O and DMSO (4.8 ppm and 2.5 ppm, respectively).

Apparatus

The isolation of the spider toxin was performed with a JASCO TRIOTAR-VI HPLC system, equipped with TSKgel ODS-120T

columns (4.6 mm i.d. $\times$ 250 mm for analytical separation, and 7.8 mm i.d. $\times$ 300 mm for preparative separation). UV spectra were measured with a JASCO MULTI-320 or a UVIDECE-505 UV/VIS digital spectrophotometer, and fluorescence spectra with a Hitachi 650-10(S) fluorescence spectrophotometer. Hydrolysis was done with a PICO-TAGTM work station, (Waters Assoc.). Amino acids and polyamines were analysed with a Toyo Soda HLC-803D pump using a postcolumn-OPA system. Fab-Mass spectra were measured with a JEOL DX-303 (Nippon Denshi). NMR spectra were measured with a Bruker AM-400 equipped with ASPECT 3000.

RESULTS AND DISCUSSION

Chromatogram of the extract from spider venom glands is shown in Fig. 1. Biological activity was detected in peak fractions indicated by arrows. Major active substances were eluted at around 18 min. All these peaks possessing the biological activity (EPSP inhibition) commonly showed similar UV spectra with maxima at 210 nm and 280 nm, and exhibited natural fluorescence at 310 nm when excited at 280 nm.

To obtain enough amounts of toxins for structural analysis, preparative isolation of active principles starting from about 200 mg of the dried venom glands was performed using an enlarged semipreparative TSKgel ODS-120T column (7.6 mm i.d. $\times$ 300 mm) with the same elution system and at a flow rate of 1.5 ml/min. The respective active principles eluted at around 18 min were rechromatographed with isocratic elution of 4% acetonitrile containing 0.02% HCl. Three kinds of principles (NSTX-1, -2, -3) were obtained as a chromatographically single peak.

The amount roughly calculated by the UV absorbance was approximately 1 μ mol for NSTX-1, 1.6 μ mol for NSTX-2 and 3 μ mol for NSTX-3.

Fab-Mass spectra of NSTX-1, -2 and -3 were measured. Molecular ion of NSTX-1 was not observed. Those of NSTX-2 and NSTX-3 were detected at $m/z=567[\text{M}+\text{H}]^+$ and at $m/z=665[\text{M}+\text{H}]^+$, respectively.

^1H -NMR spectra of NSTX-1, -2 and -3 commonly showed two characteristic aromatic signals in D_2O (6.4 ppm and 7.1 ppm). The integrations of these signals were almost 1 H:1 H.

Amino acids and polyamines were analysed

Fig. 1 Chromatogram of spider toxin (*Nephila maculata*)

in these principles. One mole each of asparatic acid, cadaverine and unknown substances were determined in the hydrolysate of NSTX-1 and NSTX-2. One mole each of asparatic acid, cadaverine, putreanine and arginine were determined in the NSTX-3 hydrolysate. Asparatic acid and arginine in these hydrolysates were both detected as L-configuration according to the method of Nimura and Kinoshita (12).

These observations suggest that the spider toxins were composed of the similar substances of low molecular weights. These principles were unlikely to be of a simple peptidic nature.

Structural Characterization of NSTX-3

Structural analysis was carried out on NSTX-3. Subtractive Edman degradation against NSTX-3 caused a decrease of about a half amount of arginine, but the degradation did not proceed to the next cycle, suggesting that arginine was located at a terminal end of NSTX-3 and that α -amino group of arginine was not protected.

Further structural analysis was performed by NMR spectroscopy. ^1H -NMR data in D_2O and DMSO-d_6 of NSTX-3 are shown in Fig. 2. In DMSO-d_6 , the aromatic protons were observed at 6.2 ppm (doublet), 6.3 ppm (singlet), and 6.8 ppm (doublet), indicating that NSTX-3 possesses three substituted aromatic ring or benzene in its molecule. Rapidly D-H exchangeable two singlet signals at 9.1 ppm and 9.6 ppm seemed to represent two kinds of phenolic OH protons.

Two aromatic protons at 6.2 ppm and 6.3 ppm observed in DMSO-d_6 converted into single proton signal with the same chemical shift to give 1H integration when the spectrum of NSTX-3 was measured in D_2O . These aromatic protons seem to be slowly exchanged by deuterium under the effect of the respective adjacent hydroxyl groups in D_2O .

The doublet proton at 6.8 ppm with no shift when the signal was measured in D_2O or DMSO-d_6 , was considered to be at *m*-position against two respective hydroxyl groups, since the protons located at *o*- or *p*-position of phenolic hydroxyl group are recognized to shift about 0.4 ppm to a high magnetic field (3)

Fig. 2 ^1H -NMR spectrum of NSTX-3 in D_2O (upper part) and in DMSO-d_6 (lower part)

when the spectrum was measured in DMSO-d_6 . Two doublet protons at 6.2 ppm and 6.8 ppm in DMSO-d_6 , showing spin-spin coupling (see DQF-COSY spectrum in DMSO-d_6 in Fig. 3), suggested that these protons were in vicinal position in a benzene ring. A long range coupling between protons at 6.2 ppm and 6.3 ppm indicated that they were at *m*-position. These data suggested the presence of 2,4-dihydroxyphenyl group in the molecule. In addition, the value of the chemical shift of these aromatic protons strongly suggested that the dihydroxyphenyl group is connected to an alkyl substituent.

Two signals at 3.8 ppm and 4.5 ppm were supposed to represent α -protons of amino acids, namely aspartic acid and arginine in the molecule. Two protons at 6.9 ppm and 7.4 ppm in DMSO-d_6 (both singlet) were easily exchanged by adding D_2O , and exhibited cross peaks in DQF-COSY and nuclear overhauser effect in NOESY spectrum (data not

shown). Thus, these protons were supposed to be amido protons of asparagine.

Although the signals at around 3.5 ppm were complicated by the presence of impurities, it eventually exhibited A-B pattern (quartet, $J=15$ Hz), and was assumed to be methylene protons of 2,4-dihydroxyphenylacetyl moiety. Geminal coupling of these protons suggests the existence of asymmetric carbon which may be due to α -proton of L-asparagine at the adjacent position.

In DQF-COSY spectra in DMSO-d_6 (Fig. 3) and in D_2O (Fig. 4), respective diagonal peaks and cross peaks were connected with lines, which correspond to 2,4-dihydroxyphenylacetic acid (line a), asparagine (line b), cadaverine (line c), putrescine (line d), and arginine (line e). The above characterization of the components was also supported by measurement of relay COSY spectrum (data not shown).

As mentioned above, aspartic acid, argi-

Fig. 3 DQF-COSY spectrum of NSTX-3 in DMSO- d_6

nine, cadaverine, and putrescine were detected in the hydrolysate of NSTX-3. Aspartic acid and cadaverine were commonly detected in the hydrolysate of NSTX-1 and NSTX-2. Preliminary study indicates that one minor fraction with weak biological activity contains aspartic acid and cadaverine. This observation also suggests that cadaverine is connected to the next part to the asparaginyl residue, and 2,4-dihydroxyphenylacetyl-asparaginyl-cadaverine may be a minimum requirement for revealing activity. Together with the results from Edman degradation, it is assumed that arginine is located at the end of NSTX-3, and putrescine is positioned between arginine and cadaverine.

Therefore, the presumptive structure of NSTX-3 was *N*-(2,4-dihydroxyphenylacetyl-L-asparaginyl)-*N'*-(L-arginyl-putrescanyl) cadaverine (Fig. 5). Its calculated molecular weight, 664, agrees with the data by Fab-Mass.

JSTX has been recognized as a potent and

irreversible blocker of the glutamate receptor in the lobster neuromuscular junction (8) and in mammalian brain (7). The similar activity reported in the venom of *Araneus ventriconus* (9), spider venom in the genera, *Nephila*, *Araneous* or *Argiope*, belonging to the same family of the spider, *Argiopidae*, has been effectively used in the study of the glutaminergic system (2, 6, 11, 15).

We previously reported that the toxic principles isolated from the venom of *N. maculata* and *N. clavata* commonly possessed 2,4-dihydroxyphenylacetyl-asparaginyl-cadaverine in the molecule (1). The 2,4-dihydroxyphenylacetyl-moiety was quite unique, and may be important for revealing activity, since the removal of this moiety by treatment with dilute acid causes inactivation of the toxicity. Most of the toxic principles were linked with aminoethylcarbonyl group in another amino side of cadaverine. In the case of NSTX-3, this part was putrescanyl moiety. Putrescine was

Fig. 4 DQF-COSY spectrum of NSTX-3 in D₂O (partially enlarged)

Fig. 5 Proposed structure of NSTX-3

first reported by Kakimoto *et al.* (5) in mammalian brain, and was synthesized by Shiba and Kaneko (13). Although the biological activity of putrescine is not yet clear, it is of interest that this compound is incorporated into spider toxin.

The spider toxins are usually similar in their structures, and the difference is found in a polybasic part in the molecule.

Recently the toxic principle, argiopine, was also characterized in the venom of the spider, *Argiope*, by Grishin *et al.* (4). In this toxin, arginine (free α -NH₂) is linked with polyamine (NHCH₂CH₂CH₂NHCH₂CH₂CH₂NHCH₂CH₂CH₂CH₂CH₂NH) through amido bond, and this polyamine is connected to α -carboxyl of 2,4-dihydroxyphenylacetylaspargine.

Thus the structures of NSTX, JSTX and

argiopine are similar to each other except that argiopine lacks β -carboxyethylamino moiety like putrescine in NSTX-3.

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