AGELENIN, A SPIDER NEUROTOXIN: DETERMINATION OF THE C-TERMINUS AS AMIDE FORM, AND INVESTIGATION OF THE DISULFIDE BOND ARRANGEMENT

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ABSTRACT

Agelenin is a neurotoxic peptide previously isolated from the venom of a spider, *Agelena opulenta*, and consists of 35 amino acid residues. In the present study, the carboxyl terminus of agelenin was found to be amide form but not carboxylic acid form by dansyl-Edman degradation analysis as well as by peptide synthesis. Among six cysteine (Cys) residues in agelenin, formation of disulfide bond between Cys¹⁰ and Cys²⁴ was determined. As for the rest of Cys residues, two possible arrangements of disulfide bonds were Cys³-Cys¹⁸ and Cys¹⁹-Cys³⁴, or Cys³-Cys¹⁹ and Cys¹⁸-Cys³⁴.

Spiders have venoms to paralyze or kill insects. During recent years, the venoms of several spiders have been recognized as neurotoxins, and are expected to serve as useful probes in the field of neuroscience (7). To date, spider neurotoxins seem to be chemically classified into two groups. The first is a group of polyamine-like toxins which potentially block neuromuscular transmission mediated by glutamic acid, acting on postsynaptic glutamate receptor (8). The second is a group of cysteine (Cys)-rich neuroactive peptides isolated from *Atrax robustus* (12), *Atrax versutus* (2), *Agelenopsis aperta* (13), *Eurypelma californicum* (11), *Agelena opulenta* (6), *Hololena curta* (14) and *Phoneutria nigriventer* (4).

Agelenin isolated from the venom of *Agelena opulenta* (6), belongs to the second group. Agelenin is known to suppress excitatory postsynaptic potentials in the lobster neuromuscular junction, possibly by blocking the presynaptic calcium channel. The amino acid sequence of agelenin, consisting of 35 amino acid residues, was previously determined and its carboxyl terminal (C-terminal) residue was reported to be leucine (Leu) by using carboxypeptidase (CP) method (6). However, the method using

CP hardly revealed whether Leu³⁵ at the C-terminus was carboxylic acid free (Leu-OH) form or amide (Leu-NH₂) form. In the present study, we determined the C-terminus of agelenin to be Leu-NH₂ form, by analytical as well as peptide synthesis approach. In addition, we investigated the arrangements of the disulfide bonds in agelenin which has six Cys residues in its amino acid sequence.

MATERIALS AND METHODS

Materials

Spiders (*Agelena opulenta*) were collected in the field in Tokyo. Natural agelenin was purified from the spider according to the method previously described (6). Dansyl chloride (DNS-Cl) was from Kanto Chemical. DNS-L-Leu-OH and DNS-L-cysteic acid (Cys SO₃⁻) were from Sigma. DNS-L-Leu-NH₂ was prepared from DNS-Cl and L-Leu-NH₂ (Peptide Institute, Osaka, Japan). Trypsin (EC 3.4.21.4) was from Worthington and thermolysin (EC 3.4.24.4) was from Seikagaku Kogyo. Dowex 50 X-8 was obtained from Muromachi Kagaku Kogyo. Chemical synthesis of peptides was performed by the solid phase method.

Oxidation and Digestion of Agelenin

Purified agelenin (1.5 nmol) was oxidized with performic acid to cleave disulfide bonds by the method described elsewhere (16). The oxidized agelenin was digested with trypsin (about 1 μ g) in 50 μ l of 25 mM Tris-HCl buffer (pH 8.0) at 40°C for 4 h.

Dowex 50 Chromatography

Diluted HCl solution was added to the tryptic digest of the oxidized agelenin to adjust pH to about 3, and the digest was applied onto a Dowex 50 X-8 column (250 μ l of resin) equilibrated with 2.5 mM HCl/2-propanol(2-PrOH) (80/20, v/v). The column was washed with 2.5 ml of 2.5 mM HCl/2-PrOH (80/20, v/v) (fraction I), and eluted with 2.5 ml of 2 M pyridine (fraction II).

Dansyl-Edman Degradation Analysis

The fraction from the Dowex column containing the C-terminal peptide fragment; CysSO₃⁻³⁴-Leu³⁵ was dissolved in 100 μ l of 50% pyridine aqueous solution. The sample was allowed to react with 5 μ l of phenylisothiocyanate at 55°C for 30 min under nitrogen gas and the excess reagent was discarded by three extractions with 200 μ l of heptane/ethyl acetate (10/1, v/v), then three extractions with 200 μ l of heptane/ethyl acetate (2/1, v/v). After the sample was evaporated to dryness, 30 μ l of trifluoroacetic acid (TFA) was added and heated at 55°C for 5 min under nitrogen gas. To the dried sample, 30 μ l of water and benzene were added, shaken vigorously and the organic phase was discarded.

The aqueous phase containing C-terminal residue was dried and dissolved in 50 μ l of 0.5 M NaHCO₃. DNS-Cl solution (6 mg/ml of acetone) was added and dansylation was proceeded at room temperature overnight. The dansylated sample was analyzed by reversed-phase high performance liquid chromatography (HPLC) using an ODS column (ODS-80_{TM}, 4.6×250 mm, Tosoh). The eluent system used was 10 mM sodium acetate buffer (pH 6.8)/acetonitrile (95/5, v/v) (A) and 10 mM sodium acetate buffer (pH 6.8)/acetonitrile (30/70, v/v) (B). The column was eluted with a linear gradient from A to B in 70 min at a flow rate of 1.0 ml/min. The column temperature was at 50°C. DNS-derivatives were detected by fluorescence (excitation at 350 nm and emission at 530 nm).

Fig. 1 The strategy for the C-terminus analysis

Comparison of Elution Profiles between Agelenin and Synthetic Peptides

Two types of peptides having the same amino acid sequence as agelenin except the C-terminal residue, i.e. Leu-OH (Syn-agel-OH) or Leu-NH₂ (Syn-agel-NH₂), were chemically synthesized. The elution profile of agelenin, Syn-agel-OH and Syn-agel-NH₂ on an ODS column (Inertsil ODS-2, 4.6×250 mm, GL Science) were compared. The column was eluted with water/acetonitrile (83/17, v/v) containing 0.05% TFA at a flow rate of 1.0 ml/min. The column temperature was at 40°C. Detection was performed by UV absorption at 215 nm. Chromatogram was recorded by an integrator 807-IT (Jasco, Tokyo, Japan).

Fig. 2 The C-terminus analysis by dansyl-Edman degradation. The elution positions of authentic dansyl derivatives (DNS-OH, DNS-NH₂, DNS-Leu-OH, DNS-Leu-NH₂ and DNS-CysSO₃⁻) are indicated by arrows. a, the dansylated sample; b, acid hydrolyzate of the peak fraction identified as DNS-Leu-NH₂ in (a)

Analysis of Disulfide Bonds

Agelenin (2 nmol) was dissolved in 20 μ l of 8 M urea, and then 80 μ l of 25 mM Tris-HCl buffer (pH 8.0)/5 mM CaCl₂ was added to reduce urea concentration to 1.6 M. Then, agelenin was digested with trypsin (1 μ g) or thermolysin (1 μ g) at 40°C for 12 h. The digest was applied onto the Inertsil ODS-2 column to isolate the peptide fragments. The eluents used were water/acetonitrile (95/5, v/v) (C) and water/acetonitrile (40/60, v/v) (D), both of which contained 0.05% TFA. Gradient elution from C to D in 80 min was performed at a flow rate of 1.0 ml/min. The column was maintained at 40°C. Detection was by UV absorption at 215 nm. The purified peptide fragments were subjected to amino acid composition analysis by ion-exchange chromatography using *o*-phthalaldehyde method

(3), and sequence analysis by the Edman degradation method (15). For microsequencing, an automatic protein sequencer (Type 477A, Applied Biosystems) was used.

RESULTS

Fig. 1 summarizes the strategy for the C-terminus analysis. When the tryptic digest of the performic acid oxidized agelenin was applied onto the Dowex 50 column, acidic peptide fragments were expected to be recovered in fraction I and neutral fragments in fraction II. Actually, amino acid sequence analysis suggested that the fraction I was a mixture of CysSO₃⁻³⁴-Leu³⁵ (-OH or -NH₂ form; the C-terminal dipeptide fragment) and CysSO₃⁻¹⁸-Lys²³, whereas the fraction II was of Gly¹-Arg⁸, Phe⁹-Arg¹⁷, CysSO₃⁻²⁴-Lys²⁵ and Glu²⁶-Arg³³ (data not

Fig. 3 Elution profiles of agelenin, Syn-agel-OH and Syn-agel-NH₂. a, Syn-agel-NH₂ (peak 1) and Syn-agel-OH (peak 2) were eluted at 6.4 min and 6.9 min, respectively; b, agelenin was eluted at 6.4 min.

shown). Thus, the fraction I contained the C-terminal peptide and was therefore subjected to dansyl-Edman degradation analysis. The dansylated sample gave a peak corresponding to the authentic DNS-Leu-NH₂ but not DNS-Leu-OH by HPLC as shown in Fig. 2a. When this peak fraction corresponding to DNS-Leu-NH₂ in Fig. 2a was collected, hydrolyzed with 5.7N HCl for 5 h and subjected to rechromatography, a new peak corresponding to the authentic DNS-Leu-OH appeared due to amide bond hydrolysis from Leu-NH₂ to Leu-OH (Fig. 2b; sulfone-amide bond is almost stable under this hydrolysis condition). The peak in Fig. 2a was thus determined to be DNS-Leu-NH₂, and therefore the C-terminal of agelenin was decided to be Leu-NH₂ but not Leu-OH.

To support the result of the dansyl-Edman analysis, we compared elution profiles of agelenin and two synthetic peptides; Syn-agel-OH and Syn-agel-NH₂, on the reversed-phase HPLC. The retention time (*t_R*) of agelenin (*t_R*=6.4 min) completely coin-

Table 1 Amino Acid Composition of Cys-containing Peptides

	T-1	TH-1	TH-2	TH-3
Asx	1.0(1)	1.0(1)	3.0(3)	3.1(2)
Glx	—(0)	1.2(1)	1.4(1)	—(0)
Ser	1.0(1)	—(0)	2.7(3)	2.5(3)
Thr	—(0)	—(0)	—(0)	—(0)
Pro	0.9(1)	—(0)	1.8(2)	1.8(2)
Gly	1.1(1)	—(0)	5.0(4)	4.0(4)
Ala	0.9(1)	0.9(1)	1.2(1)	—(0)
Val	—(0)	—(0)	—(0)	—(0)
Met	—(0)	—(0)	—(0)	—(0)
Leu	1.0(1)	0.8(1)	2.5(3)	1.2(2)
Ile	—(0)	—(0)	0.9(1)	0.8(1)
Tyr	—(0)	—(0)	—(0)	—(0)
Phe	0.9(1)	0.9(1)	0.7(1)	—(0)
His	—(0)	—(0)	0.8(1)	0.9(1)
Lys	1.0(1)	1.9(2)	1.5(2)	—(0)
Arg	1.0(1)	—(0)	2.7(3)	3.3(3)
Cys	+(2)	+(2)	+(6)	+(4)

Assignment

The theoretical numbers of residue are shown in parentheses. T-1, TH-1, TH-2 and TH-3 correspond to the peak fractions shown in Fig. 4.

cided with that of Syn-agel-NH₂ (*t_R*=6.4 min) but not with Syn-agel-OH (*t_R*=6.9 min) as shown in Fig. 3, confirming that the C-terminus of agelenin was amide form. Fig. 4 shows the chromatogram obtained from the tryptic and the thermolytic digests of agelenin. Each peak fraction was subjected to amino acid composition analysis and the fraction containing Cys was successively subjected to amino acid sequence analysis. Data concerning disulfide bonds are summarized in Table 1 and Fig. 5. T-1 and TH-1 indicated a disulfide bridge formation between Cys¹⁰-Cys²⁴. TH-2 and TH-3 suggested two possible arrangements; i.e. Cys³-Cys¹⁸ and Cys¹⁹-Cys³⁴, or Cys³-Cys¹⁹ and Cys¹⁸-Cys³⁴.

DISCUSSION

In the present study, we determined the C-termi-

Fig. 4 Chromatograms of peptide fragments derived from digested agelenin. a, digestion by trypsin; b, digestion by thermolysin

Fig. 5 Disulfide bonds arrangements in agelenin. $\rightarrow$, Identified as phenylthiohydantoin derivatives by the sequencer. A disulfide bond was defined between Cys¹⁰ and Cys²⁴. Cys³ and Cys³⁴ form disulfide bonds with Cys¹⁸ and Cys¹⁹ (the exact arrangement is unknown).

nus of agelenin as Leu-NH₂ by dansyl-Edman degradation analysis and also by authentic peptides synthesis. The C-terminal amide which often occurred in bioactive peptides is known to be post-translational product during biosynthesis, via cleavage at-NH-CH₂-bond of glycine in the precursor peptides which yields glyoxylic acid and the matured peptides of the C-terminal amide (1, 9). We suppose the biosynthesis of agelenin follows the similar processing route.

In Fig. 2a, DNS-Leu-OH was slightly detected, but we consider this to be an artifact generated during the dansyl-Edman process of peptide fragment. The elution profile of agelenin was entirely the same as that of Syn-agel-NH₂, without any peak of Syn-agel-OH (Fig. 3). In dansyl-Edman analysis, it is noted that the dansylated sample was a mixture of DNS-Leu-NH₂³⁵ and DNS-CysSO₃⁻¹⁹-(N^e-PTC) Lys-OH²³ as indicated in Fig. 1. However, DNS-CysSO₃⁻¹⁹-(N^e-PTC)Lys-OH²³ was not coeluted within the peak fraction of DNS-Leu-NH₂³⁵ (Fig. 2a). Acid hydrolysis and rechromatography of the peak fraction corresponding to DNS-Leu-NH₂³⁵ in Fig. 2a gave a peak corresponding to the authentic DNS-Leu-OH but not DNS-CysSO₃⁻ (Fig. 2b).

In our previous paper (6), CP-P (EC 3.4.16.1) was used to determine the amino acid sequence in the C-terminal region of agelenin. Although the method using CP is widely used for the determination of the C-terminal amino acid sequence, it is not appropriate for the C-terminal amide analysis, since CP releases amino acid amide at the C-terminus (for example, X-Leu-NH₂) as either amide (H-Leu-NH₂) form or carboxylic acid (H-Leu-OH) form. Even though carboxylic acid form is exclusively detected by the CP analysis, there still remains the possibility that the C-terminus is amide form.

Agelenin is known to have six Cys residues. Our preliminary study suggested that all Cys residues formed disulfide bonds (data not published). In the present study, we determined that Cys¹⁰ was bonded to Cys²⁴, judging from the tryptic and thermolytic peptide fragments of T-1 and TH-1. Assuming that Cys³ and Cys³⁴ form a disulfide bond, Gly¹-Arg⁸ and Ile²⁹-Cys³⁴ should be bonded as one peptide by Cys³-Cys³⁴. From the results of the amino acid composition and sequence analysis (Table 1 and Fig. 5), this assumed peptide is expected to be eluted in both fractions (TH-2 and TH-3) having different retention times; however, this is inconsistent with the general principle that one peptide has

a unique retention time in HPLC. Thus, formation of a disulfide bond between Cys³ and Cys³⁴ is unlikely. On the contrary, it is possible that the disulfide bond is formed as follows; Cys³-Cys¹⁸ and Cys¹⁹-Cys³⁴, or Cys³-Cys¹⁹ and Cys¹⁸-Cys³⁴. We could not define the precise arrangement because of a limited amount of agelenin available for analysis. However, in view of a recent report discussing all possible arrangements of three disulfide bridges in amino acid sequence (17), and of the characteristic of two adjoining Cys (Cys¹⁸ and Cys¹⁹) in agelenin, agelenin seems to have a similar disulfide bond arrangement as ω -conotoxin GVIA (5). Based on the cystine arrangement in ω -conotoxin GVIA (10), the putative disulfide bond arrangement in agelenin is supposed to be Cys³-Cys¹⁹, Cys¹⁰-Cys²⁴ and Cys¹⁸-Cys³⁴. Although several Cys-rich toxins have been purified from spider venom (2, 4, 11-14), the investigation of disulfide bonds was reported only for μ -agatoxin (13). μ -Agatoxin was reported to have four disulfide bonds (i.e. eight Cys residues) in the molecule and the arrangement of two bonds have been determined. However, the other two bonds have not been defined yet, remaining two possible arrangements.

Synthesis of agelenin (Syn-agel-NH₂) used in the present study will be reported elsewhere.

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414

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