ELSEVIER

PEPTIDES

Peptides 20 (1999) 1027–1034

Assignment of the three disulfide bonds of *Selenocosmia huwena* lectin-I from the venom of spider *Selenocosmia huwena*☆

Feng Li, Songping Liang*

Department of Biology, Hunan Normal University, Changsha, Hunan 410006, People's Republic of China

Received 16 March 1999; accepted 17 April 1999

Abstract

The positions of the disulfide bonds of *Selenocosmia huwena* lectin-I (SHL-I) from the venom of the Chinese bird spider *S. huwena* have been determined. The existence of three disulfide bonds in the native SHL-I was proved by matrix-assisted laser desorption ionization time-of-flight mass spectroscopic analysis. To map the disulfide bonds, native SHL-I was proteolytically digested. The resulting peptides were separated by reverse phase high-performance liquid chromatography. Matrix-assisted laser desorption ionization time-of-flight mass spectroscopic analysis indicated the presence of one disulfide bond Cys7–Cys19. The partially reduced peptides by using Tris-(2-carboxyethyl)-phosphine at pH 3.0 were purified by reverse phase high-performance liquid chromatography. Four M Guanidine-HCl was found to increase the yields of partially reduced peptides prominently. The free thiols were carboxamidomethlate by iodoacetamide. The specific location of another disulfide bond Cys2–Cys14 was proved by comparing N-terminal sequencing analysis of the partially reduced and alkylated SHL-I with that of the intact peptide. Finally, the three disulfide linkage of SHL-I could be assigned as Cys2–Cys14, Cys7–Cys19, Cys13–Cys26.

Keywords: Disulfide bonds; SHL-I; Spider; MALDI-TOF mass spectroscopic analysis; Carboxamidomethlation; TCEP

1. Introduction

SHL-I is a lectin isolated from the venom of the Chinese bird spider *Selenocosmia huwena*. It can agglutinate human and mice erythrocytes at minimum concentration of 125 μg/ml and 31 μg/ml, respectively [10]. The hemagglutinating activity of SHL-I can be inhibited by mannosamine [9]. The amino acid sequence of SHL-I has been determined as following: NH2-Gly-Cys-Leu-Gly-Asp-Lys-Cys-Asp-Try-Asn-Asn-Gly-Cys-Cys-Ser-Gly-Try-Val-Cys-Ser-Arg-Thr-Trp-Lys-Trp-Cys-Val-Leu-Ala-Gly-Pro-Trp-COOH. There are 6 Cys residues and two of them are adjacent. It is one of the smallest lectins so far found with the molecular weight less than 5000Da. SHL-I has high sequence similarity with a fragment of great nettle lectin [10]. It could be used as a model molecule to study the relationship between the structure and functions of lectins. Disulfide bonds play a very important role in establishing and maintaining the three-dimensional structure and disulfide linkage pattern is critical for biologic activity. The disulfide location is, therefor, essential for further understanding of the relationship of its structure to function. Because SHL-I contains a pair of adjacent cysteine residues and is resistant to enzymatic digestion with common conditions, the traditional method can not resolve all the problems of the disulfide bond assignment. In this paper, we report the determination of the disulfide bridge positions of SHL-I by using the modified method described by Gray [7], that involves partial reduction by Tris-(2-carboxyethyl)-phosphine (TCEP) at acid pH and rapid alkylation and identification of labeled thiols by Edman degradation.

2. Materials and methods

2.1. Materials

SHL-I was isolated from the venom of the spider (*Selenocosmia huwena*) and purified by means of ion exchange and reverse phase high-performance liquid chromatography

☆ This work was supported by Project 863 of the Science Committee of China and by National Science Foundation of China.

* Corresponding author. Tel.: +86-731-8872556; fax: +86-731-8871340.

E-mail address: liangsp@public.cs.hn.cn (S. Liang)

0196-9781/99/$ – see front matter
PII: S0196-9781(99)00096-0

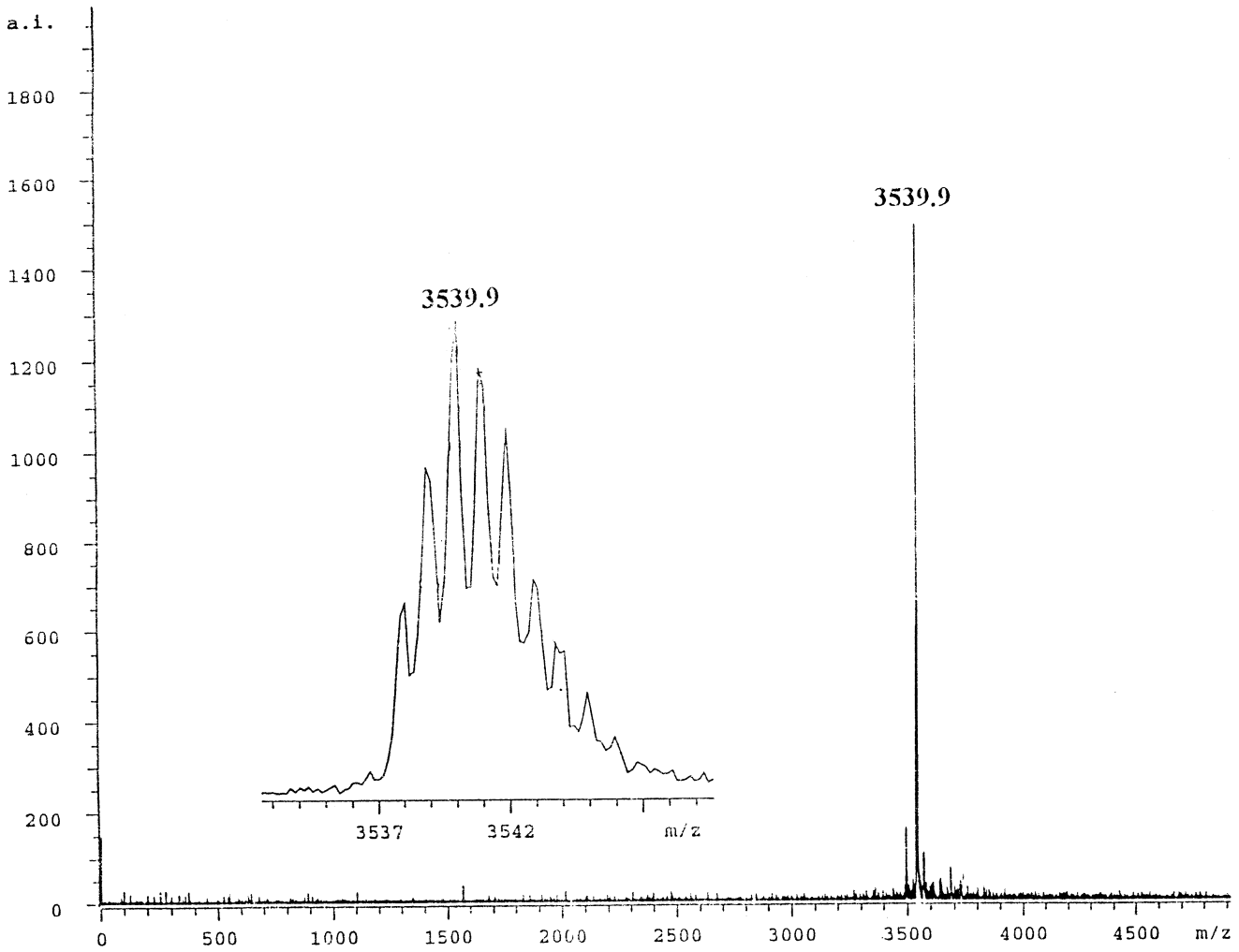

Fig. 1. MALDI-TOF mass spectra of native SHL-I. Analysis was carried out with a Bruker Proflex III mass spectrometer, α-cyano-4-hydroxycinnamic acid was used as matrix. The sub-figure is close-up view of the isotope peaks.

(RP-HPLC) as described in our previous paper [10]. Trypsin, chymotrpsin, and endopeoteinase Glu-C, iodoacetamide, α-cyano-4-hydoxycinn acid, 2-mercaptoethanol were purchased from Sigma (St. Louis, MO, USA). TCEP was from Pierce Chemical (Rockford, Illinois, USA). Acetonitrile and trifluoroacetic acid was HPLC grade. All other reagents were analytical grade. The 0.1 M TCEP solution in 0.1 M citrate buffer at pH 3.0 was prepared as stock solution and stored under N_2 at -20°C. The 2 M iodoacetamide solution in 0.5 M Tris-HCl, pH 8.0, containing 2 mM ethylenediaminetetraacetic acid, was freshly prepared. All the buffers used in the experiments were degassed and the reduction and alkylation were carried out under N_2.

2.2. Multi-enzymatic digestion of the native SHL-I and disulfide-linked peptide identification

Incubated for 16 h at 37°C with a mixture of trypsin (4 μg), chymotrypsin (4 μg), and endopeoteinase Glu-c (4 μg), 100 μg of native SHL-I was dissolved in 200 μl of 0.2 M Tris-HCl buffer (pH 7.5). A digested mixture of 2 μl was directly analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The remained was injected to HPLC for purification. The fractions were collected and lyophilized for MALDI-TOF MS analysis. The peptide containing one disulfide bond then was dissolved in 5 μl of 50 mM ammonium bicarbonate solution (pH 8.0) and a solution of 1 μl of 100 mM 2-mercaptoethanol in water was added to reduce the disulfide bond. The mixture was incubated at 37°C for 1 h and finally analyzed by MALDI-TOF MS.

2.3. Partial reduction of SHL-I and rapid carboxamidomethlation of reduced isomers

Containing 4 M guanidine-HCl, 100 μg of native SHL-I was dissolved in 30 μl of 0.1 M citrate buffer (pH 3.0).

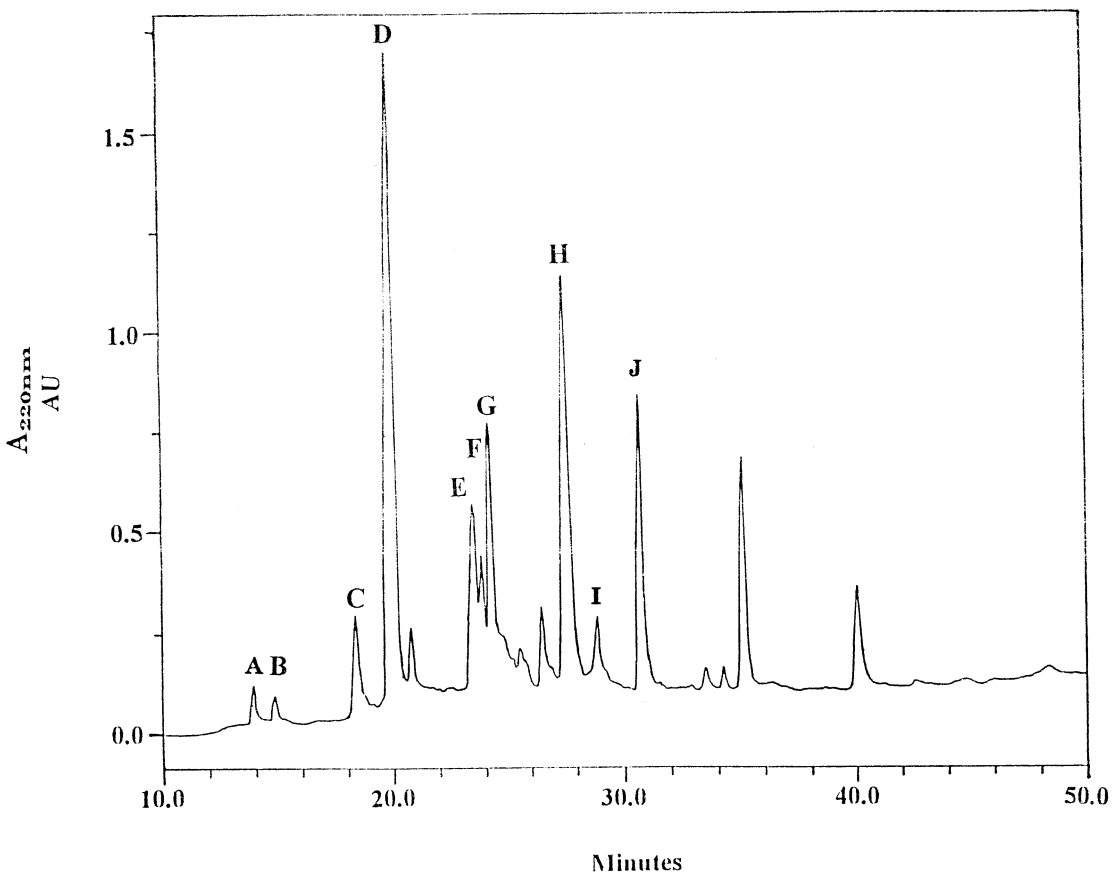

Fig. 2. RP-HPLC separation of multi-enzymatic digested SHL-I. Peptides were separated on a Vydac C18 column at a flow rate of 0.2 ml/min. The peaks labeled with A, B, C, D, E, F, G, H, I, and J were subjected to MALDI-TOF mass analysis and the results are listed on Table 1.

Added to the mixed solution was 15 μl of 50 mM TCEP in 0.1 M citrate buffer (pH 3.0), and placed at room temperature for 15–20 min. Then, the reaction mixture was injected directly to HPLC. The partially reduced peptide fraction, about 200 μl, was mixed with 300 μl of 2 M iodoacetamide solution. After reaction of about 30 s in darkness, 500 μl of 0.5 M citric acid was added to acidify the mixture to terminate the reaction, and the mixture was applied to HPLC. The predominant fractions were collected and identified by MALDI-TOF MS and amino acid sequence analysis.

2.4. HPLC separation

All chromatographic experiments were performed on a Waters Alliance HPLC separation system with a Waters 996 photodiode array detector and a Millenium[32] chromatography workstation to record the data. The mobile phases were A, 0.1% trifluoroacetic acid (TFA) in water; B, 0.08% TFA in acetonitrile. A Vydac C_{18} column (300-Å pore, 2.1 × 250 mm) and a YWG-C18 pak column (300-Å pore, 3.9 × 300 mm) were used. The gradients were described in the legends of the HPLC chromatogram figures.

Table 1
MALDI-TOF MS analysis of the peptides by multi-enzymatic digestion of native SHL-I

Partial sequence[a]	Retention time	$[M + H]^+_{(calc.)}$	$[M + H]^+_{(obed.)}$
29–32	19.9	429.5	430.4
(7–9)-S-S-(18–21)	13.9	860.9	861.2
(4–9)-S-S-(18–21)	14.8	1161.2	1160.7
(6–10)-S-S-(18–21)	14.8	1103.2	1103.6
(1–5)-S-S-(12–17)-S-S-(24–28)	18.4	1695.8	1696.2
(4–11)-S-S-(18–25)	[c]	1993.1	1993.9
(1–6)-S-S-(9–17)-S-S-(24–28)	[c]	2215.4	2214.9
(1–6)-S-S-(10–17)-S-S-(22–28)	[c]	2339.5	2339.7
(1–5)-S-S-(9–17)-S-S-(25–32)	[c]	2370.5	2369.9
(1–21)-S-S-(26–28)[b]	24.3	2544.7	2545.4
(1–17)-S-S-(18–21)[b]	23.9	2562.7	2563.4
(1–6)-S-S-(7–17)-S-S-(1–3) └S-S-(26–28)	23.6	2580.7	2579.9
(1–6)-S-S-(10–17)-S-S-(22–32)	28.9	2751.0	2750.6
(1–21)-S-S-(24–28)[b]	27.5	2859.1	2858.4
(18–21)-S-S-(1–17)-S-S-(24–28)	27.5	2877.1	2875.6
(1–21)-S-S-(24–32)	30.7	3270.6	3269.8

[a] Numbers denote amino acid positions as found in the sequence of the native SHL-I.
[b] Two intra-chain disulfide bonds present in the one chain of the fragment.
[c] Date was obtained from MS analysis of the digested mixture.

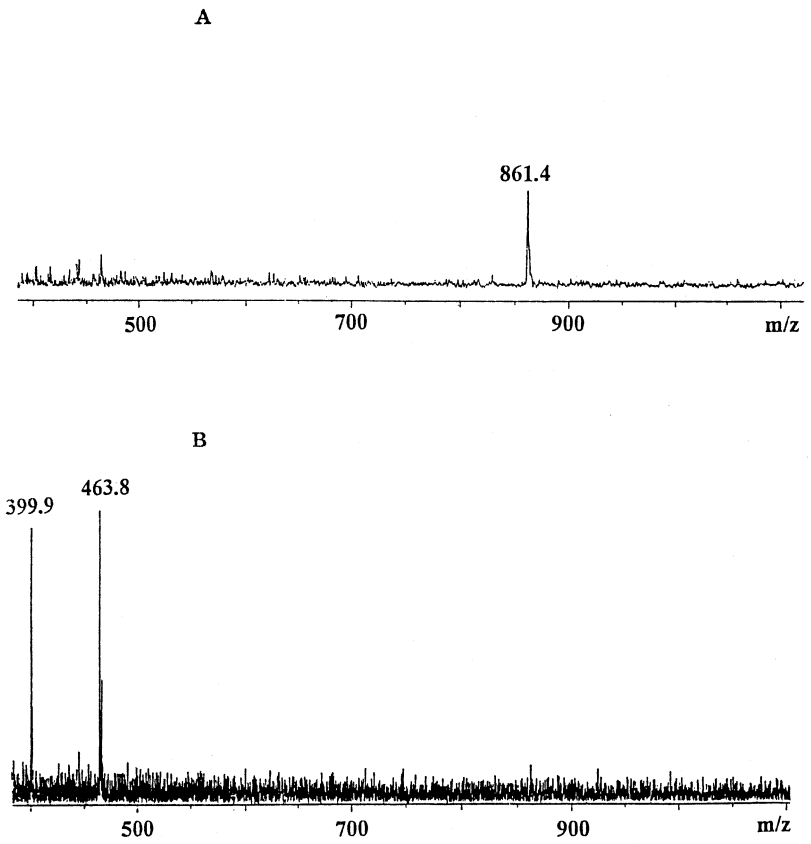

Fig. 3. MALDI-TOF mass spectra of the fraction A of Fig. 2. A, before reduction of the peptide with 2-mercaptoethanol in solution; B, after reduction of peptide with 2-mercaptoethanol in solution, indicating the disulfide bond Cys7–Cys19.

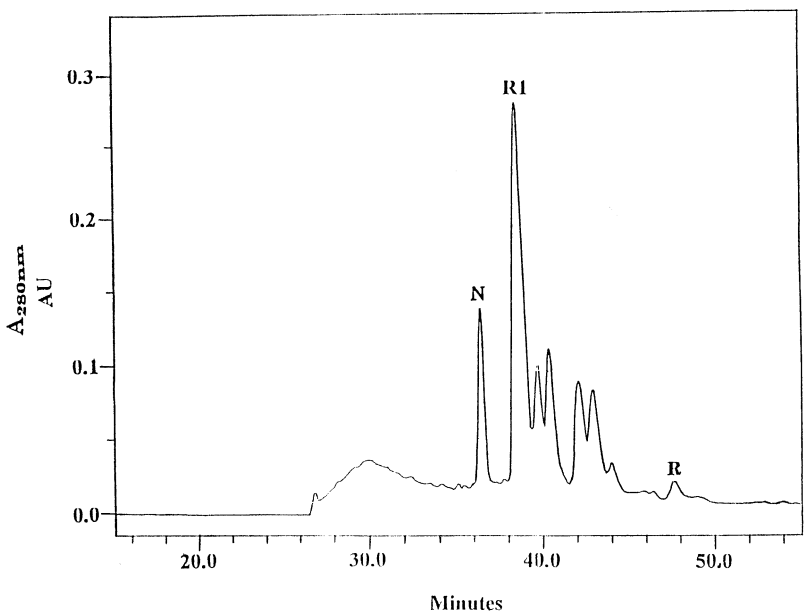

Fig. 4. RP-HPLC chromatogram of the partially reduced products of SHL-I. Partial reducing conditions were described in the text. Resulting peptides were separated on a Vydac C18 column at a flow rate of 0.2 ml/min with a linear gradient 27–31% B in 40 min. The peaks labeled with N and R are native and entirely reduced SHL-I, respectively. R1 and the following small peaks are products of partial reduction.

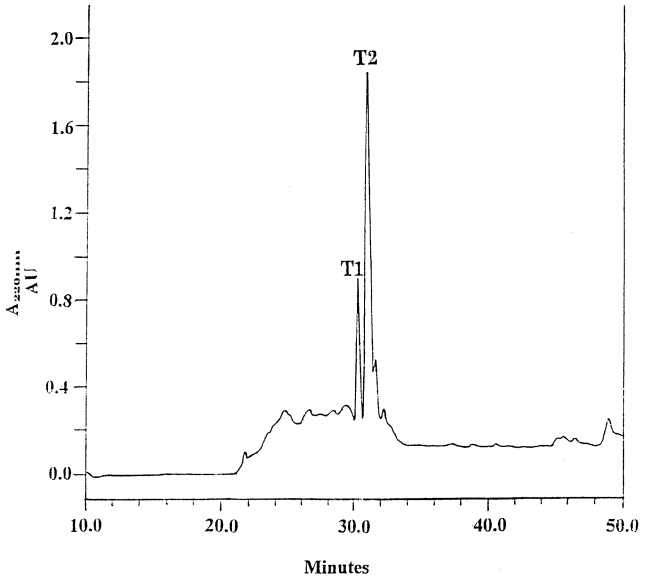

Fig. 5. RP-HPLC chromatogram of carboxamidomethylation of fraction R1 in Fig. 4. A YWG-C18 pak column (Dalian Scientific Instrument Corp. China) was used and a linear gradient 10–55% B in 30 min was adopted. Peak T1 and T2 were discussed in the text.

2.5. MS analysis

MALDI-TOF MS analysis was carried out with a Bruker Proflex III mass spectrometer that was equipped with a nitrogen laser of 337 nm. The acceleration voltage was set to 20 KV. The spectra data were calibrated with a fragment of adrenocorticotropic hormone (2466.7 Da) and HWTX-I (3750.3 Da) externally or internally. The matrix—α-cyano-4-hydroxycinnamic acid—was dissolved in a 0.1% TFA acetonitrile/water (1:2) to a saturated solution. Peptide samples were dissolved in a 0.1% TFA acetonitrile/water (1:2) solution and mixed in equal volumes with matrix solution, then applied to a stainless steel sample plate and air dried before being introduced into the mass spectrometer.

2.6. Amino acid sequencing

The intact and carboxamidomethylation partial reduced SHL-I were analyzed by the ABI Model 475A protein sequencer.

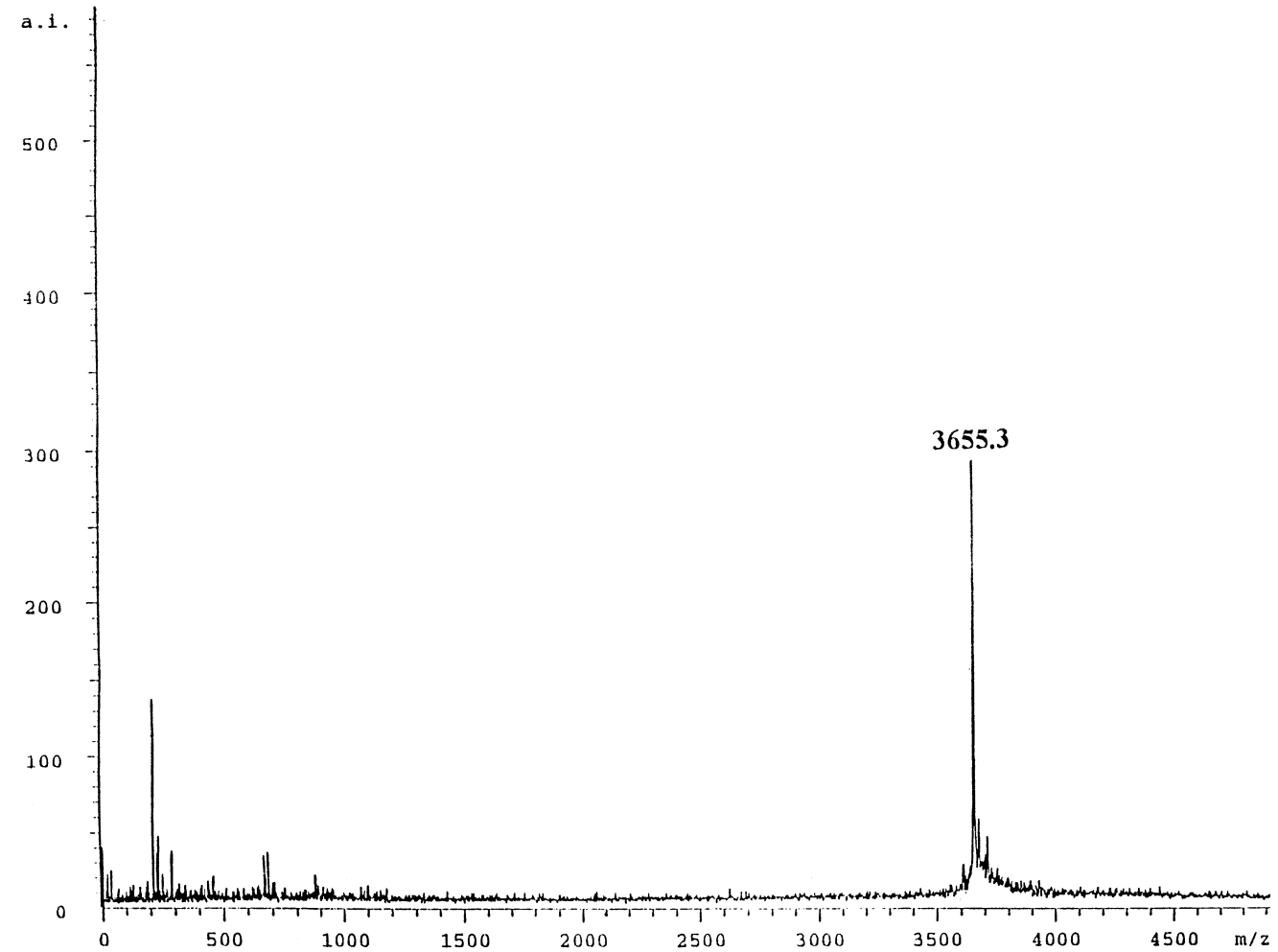

Fig. 6. MALDI-TOF MS analysis of fraction T2 in Fig. 5. α-Cyano-4-hydroxycinnamic acid was used as matrix. It indicates two thiols were carboxamidomethylated.

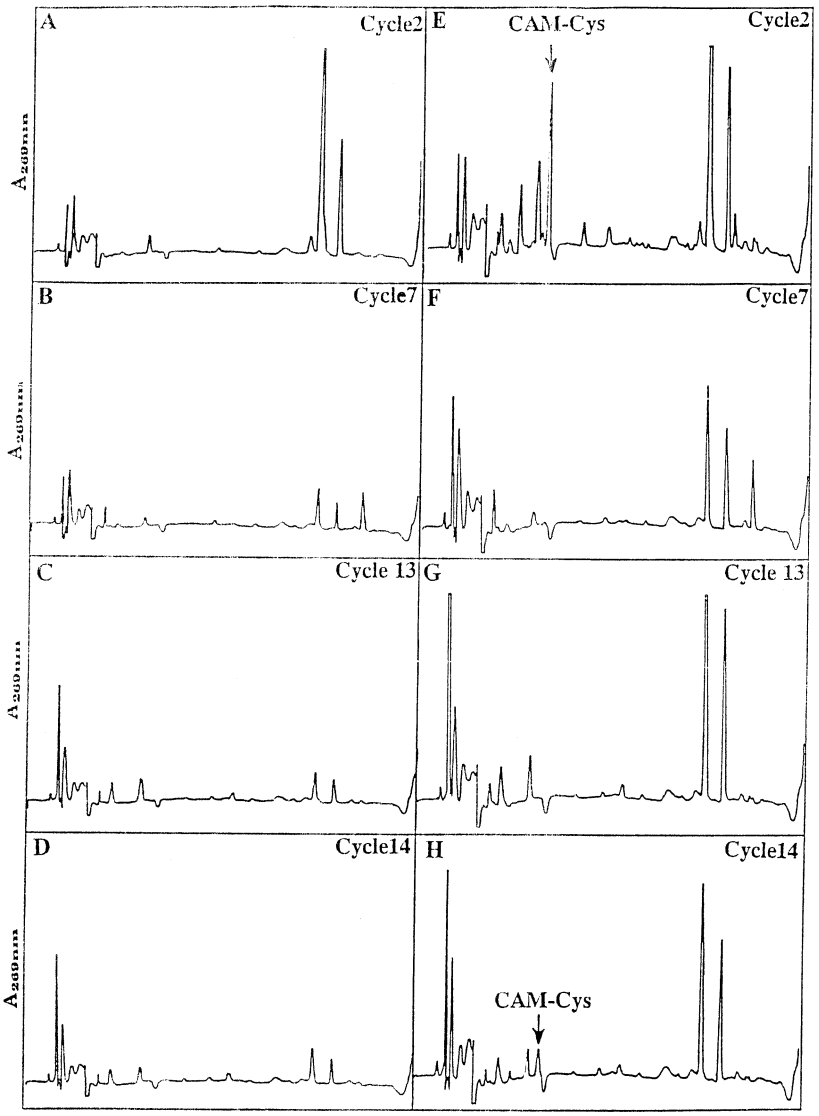

Fig. 7. The chromatograms of phenylthiohydantoins of Edman degradation observed at cycles 2, 7, 13, 14 of the native SHL-I (A, B, C, D) and the partially reduced and carboxamidomethylated SHL-I, the peak T2 in Fig. 5 (E, F, G, H).

3. Results

Fig. 1 shows the result of MALDI-TOF MS analysis of the native SHL-I. The exact mass was determined to be 3539.9 ± 1. There were about 6 mass units different from the value, 3546.0, that was calculated based on the amino acid sequence. It indicates that the six Cys residues are all involved in three disulfide bonds.

The RP-HPLC chromatogram for the multi-enzymatic digestion was given in Fig. 2. All fractions were collected and analyzed by MALDI-TOF MS. Table 1 shows the mass mapping of the peptides produced from digestion. The peptide of HPLC peak A in the Fig. 2, with a mass of 861.4 Da (Fig. 3A), was assigned as dipeptide containing one disulfide bond. Then the dipeptide was reduced and analyzed by mass spectrum. In addition to disappearance of the signal at *m/z* 861.4, two new ion signals were observed on reduction at *m/z* 399.9 corresponding to the fragment: CDY and *m/z* 463.9 that corresponds to fragment VCSR (Fig. 3B). That establishes a disulfide linkage between Cys7 and Cys19.

An approach for analyzing disulfide linkage patterns in highly bridged small peptide was developed by Gray [7]. TCEP, used at acid pH, was proved an excellent reagent that can partially reduce the disulfide bonds [4]. After separation of these intermediates containing both disulfides and thiols by HPLC, alkylation was carried out, and the labeled thiols can be identified by sequence analysis. Fig. 4 is the RP-HPLC chromatogram of residual intact SHL-I and partially reduced intermediates. The R1 peak in Fig. 4 was carboxamidomethlated by a rapid reaction procedure, and the profile of purification by RP-HPLC was shown in Fig. 5. Other minor peaks and shoulders were not investigated further. The predominant peak T2 with a mass ($M + H^+$) of 3655.3 Da (Fig. 6), that has two camboxamidmethnal groups (+58 Da), was

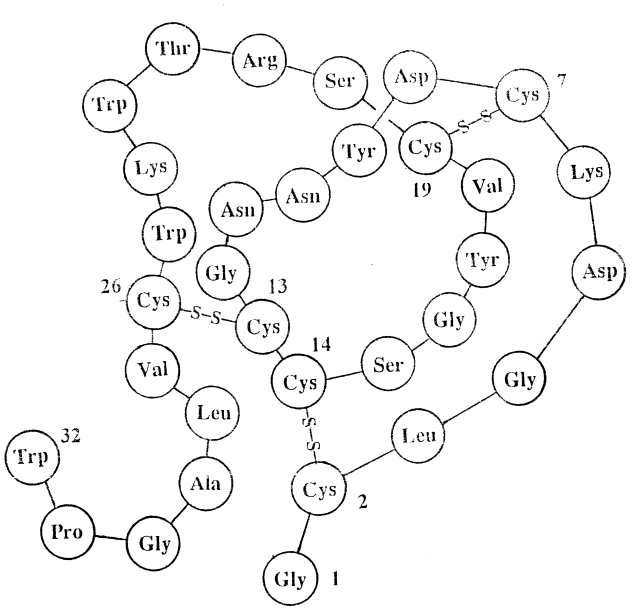

Fig. 8. Schematic representation of disulfide bond arrangement in SHL-I.

analyzed by sequencing. In the case of partially reduced/carboxamidomethylated peptide, the phenylthiohydantoin signals of *S*-carboxamidomethylcysteine are legible in both cycle 2 and cycle 14, but these signals don't appear in cycle 7 and cycle 13 (Fig. 7). By contrast, there are no phenylthiohydantoin signals in these four cycles of the native SHL-I (Fig. 7). Therefore, we can conclude that Cys2 and Cys14 are linked by a disulfide bond. By process of elimination, the third disulfide linkage is between Cys13 and Cys26.

4. Discussion

The disulfide bond is one of the weakest types of covalent bonding in proteins and polypeptides. The intra-chain disulfide bonds serve to confer conformational stability on the folded peptide chain of protein. So the assignment of disulfide linkage pattern is necessary for further studies relating structure to function. The mass spectral results demonstrated that all six Cys residues of SHL-I are involved in three intra-chain disulfide bonds. To obtain the fragment with a single disulfide bond, we used multi-enzymatic digestion to cleavage the peptide chain between Cys residues. Initially, we employ chymotrypsin only, but the process of digestion is very slow. We didn't get the target fragment even the digestion lasted for 2 days. The Asp8 residue adjacent to Try9, which is a cleavage site of chymotrypsin, decreases the rate of digestion [1]. Furthermore, the longer time in this condition will promote disulfide bond scrambling [13]. We used three kinds of enzyme to assure the cleavage between Cys residues and accelerating the digestion speed. Because of a tightly folded structure, the yields of target peptides were low even in the condition of multi-enzymatic digestion. No scrambled isomers were found in this condition. Due to the high sensitivity and accuracy, MS analysis has become the frequently used method for the disulfide bond assignment of proteins [2,3,5,6,14,18]. The key step of the assignment of disulfide bond is isolating and then reducing the single disulfide bond containing peptide and combining with MS analysis. Just through this approach we solved the linkage of Cys7–Cys19 of the SHL-I.

Partial reduction to generate a series of intermediates containing both disulfides and thiols was widely used in assignment of disulfide bonds [7,8,15,16]. Water-soluble TCEP has proved to be more excellent reducing reagent than dithiothreitol and 2-mercaptoethanol for doing this. The advantage is that TCEP can selective reduce the disulfide bonds depend on their accessibility and the more important, the process can be performed at acid pH to suppress the scrambling among the disulfide bonds [4,7,8]. In the beginning we used the condition of partial reduction described by Gray and got relatively low yields of the partially reduced peptide. Finally we found that the reaction buffer containing 4 M guanidine-HCl can increase the yields evidently. In our procedure, the target partial reduced isomer is rapidly carboxamidomethylated and followed by amino acid sequence analysis. The result is definitely indicated the disulfide between Cys2 and Cys14. The partial reduced isomers can refold easily. About 20% fraction eluate of R1 (Fig. 4) can be reoxidized and refold to native structure at room temperature for 2 h (data are not shown). In the basic condition the refold is more distinct. The peak T1 in Fig. 5 is refolded from fraction R1 (Fig. 4) to native SHL-I, that has the same behavior on chromatogram and same molecular weight with native SHL-I (data are not shown). Fig. 8 shows the finally assigned disulfide arrangement in the SHL-I.

SHL-I has the same disulfide location (I–IV; II–V; III–VI) with another peptide, huwentoxin-I [17], which is a neurotoxin from the same spider and its three-dimensional structure has been established by two-dimensional nuclear magnetic resonance [12]. Searching the protein database, We found many peptides have the same disulfide linkage pattern with that of SHL-I. Two adjacent Cys at III and IV were also found in their primary structure. Their three-dimensional structures have been established. Despite the quite different resources and functions, most of these peptides have the inhibitor cystine knot motif [11]. Because SHL-I has exactly the same disulfide bond pattern as that of these peptides, it would be interesting to see whether SHL-I does in fact contain this motif.

References

[1] Allen G. Sequencing of proteins and peptides. Amsterdam: Elsevier, 1989. pp. 90–1.
[2] Bauer M, Sun Y, Degenhardt C, Kozikowski B. Assignment of all four disulfide bridges in echistatin. J Protein Chem 1993;12:759–64.
[3] Burman S, Wellner D, Chait B, Chaudhary T, Breslow E. Complete assignment of neurophysin disulfide indicates pairing in two separate domains. Proc Natl Acad Sci USA 1989;86:429–33.

[4] Burns JA, Butler JC, Moran J, Whitesides GM. Selective reduction of disulfides by tris-(2-carboxyethyl)-phosphine. J Org Chem 1991;56: 2648–50.
[5] Chang JY, Schindler P, Chatrenet B. The disulfide structures of scrambled hirudins. J Biol Chem 1995;270:11992–7.
[6] Cosenza L, Sweeney E, Murphy JR. Disulfide bond assignment in human interleukin-7 (IL-7) by Matrix-assisted Laser Desorption/Ionization mass spectroscopy and site-directed Cysteine to Serine mutational analysis. J Biol Chem 1997;272:2995–3000.
[7] Gray WR. Disulfide structures of highly bridged peptides: a new strategy for analysis. Protein Sci 1993;2:1732–48.
[8] Gray WR. Echistain disulfide bridges: selective reduction and linkage assignment. Protein Sci 1993;2:1749–55.
[9] Liang SP, Lin L. Haemagglutination activity analysis of *Selenocosmia huwena* lectin-I from the venom of the spider *Selenocosmia huwena*. Chinese J Biochem Mol Biol (in press) 1999.
[10] Liang SP, Pan X. A lectin-like peptide isolated from the venom of the Chinese bird spider *Selenocosmia Huwena*. Toxicon 1995;33:875–82.
[11] Pallaghy PK, Nielsen KJ, Craik DJ, Norton RS. A common structural motif incorporating a cystine knot and a triple-stranded β-sheet in toxic and inhibitory polypeptides. Protein Sci 1994;3:1833–9.
[12] Qu YX, Liang SP, Ding JZ, Liu XC, Zhang RJ, Gu XC. Proton nuclear magnetic resonance studies on huwentoxin-I from the venom of the spider Selenocosima huwena. 2. Three-dimensional structure in solution. J Protein Chem 1997;16:565–74.
[13] Ryle AP, Sanger F. Disulphide interchange reactions. Biochem J 1955;60:535–40.
[14] Spiess C, Happersberger HP, Glocker MO, Spiess E, Rippe K, Ehrmann MJ. Biochemical characterization and mass spectrometric disulfide bond mapping of periplasmic α-amylase MalS of *Escherichia coli*. J Biol Chem 1997;272:22125–33.
[15] Yamashita H, Nakatsuka T, Hirose M. Structural and functional characteristics of partially disulfide-reduced intermediates of ovotransferrin N lobe. J Biol Chem 1995;270:29806–12.
[16] Wu J, Watson JT. A novel methodology for assignment of disulfide bond pairings in proteins. Protein Sci 1997;6:391–8.
[17] Zhang DY, Liang SP. Assignment of the three disulfide bridges of huwentoxin-I, a neurotoxin from the spider *Selenocosmia huwena*. J Protein Chem 1993;12:735–40.
[18] Zhou ZR, Smith DL. Assignment of disulfide bonds in proteins by partial acid hydrolysis and mass spectrometry. J Protein Chem 1990;9:523–32.