Assignment of Disulfide Bond Location in Prothoracicotropic Hormone of the Silkworm, *Bombyx mori*: A Homodimeric Peptide†

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ABSTRACT: The disulfide bond location of a homodimeric peptide, prothoracicotropic hormone (PTTH) of the silkworm, *Bombyx mori*, was determined by a combination of partial reduction and sequence analysis of peptide fragments generated through a partial reduction of PTTH followed by alkylation and enzyme digestion. The partial reduction and *S*-alkylation broke the interchain disulfide bond but did not affect the intrachain disulfide bonds, generating monomeric PTTH whose intrachain disulfide bonds were kept intact. This monomeric PTTH has about one-half the biological activity of intact PTTH. Sequence analysis of the fragments generated by lysyl endopeptidase digestion of this monomeric PTTH after complete reduction and *S*-alkylation by another *S*-alkylating reagent showed that only the Cys15 residue was reduced and *S*-alkylated by the foregoing partial reduction, indicating that this residue formed the interchain disulfide bond. The other disulfide bonds which formed intrachain bridgings were determined by sequence and mass analyses of the fragments generated by two successive enzyme digestions of the monomeric PTTH. In conclusion, the disulfide bond location of PTTH was assigned to Cys15–Cys15′ as an interchain disulfide linkage and Cys17–Cys54, Cys40–Cys96, and Cys48–Cys98 as intrachain disulfide linkages.

Prothoracicotropic hormone (PTTH)[1] is a cerebral neuropeptide of insects that stimulates prothoracic glands to synthesize and release ecdysone, thereby playing a central role in molting and metamorphosis (Kopeć, 1922; Bollenbacher & Granger, 1985; Ishizaki & Suzuki, 1980). In 1987, PTTH was first isolated from heads of the adult silkworm, *Bombyx mori*, and its amino-terminal sequence was determined (Kataoka et al., 1987). Subsequently, the entire amino acid sequence and dimeric structure of PTTH were elucidated by a combination of peptide analysis (Kataoka et al., 1991) and molecular cloning of cDNA (Kawakami et al., 1990). PTTH is a homodimeric peptide composed of two identical chains which are held together by disulfide bond(s). Each chain is composed of 109 residues containing seven cysteine residues (Figure 1). Since reductive *S*-alkylation of disulfide bonds of PTTH completely abolished the biological activity, the tertiary structure maintained by disulfide bonds is essential to the biological function. Recombinant *Bombyx* PTTH possessing the biological activity has been obtained by using the *Escherichia coli* system (Kawakami et al., 1990).

Disulfide bond locations of peptides are conventionally determined by enzymatic and/or chemical cleavages to generate fragments which have a single disulfide bond, followed by sequence and/or mass analyses of these fragments. However, in the case of a homodimeric peptide like PTTH, it is necessary to distinguish between the interchain and intrachain disulfide bonds. In addition, the intact PTTH is relatively resistant to enzyme digestions because of the rigid structure made by many disulfide bonds. Therefore, we used a novel method which consists of a partial reduction of PTTH and enzyme digestions of the partially reduced PTTH. Here we report determination of the complete disulfide bond location of recombinant PTTH using this method.

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  1 GNIQVENQAIPDPPCTCKYKKEIEDLGENS  30
 31 VPRFIETRNCNKTQQPTCRPPYICKESLYS  60
 61 ITILKRRETKSQESLEIPNELKYRWVAESH  90
 91 PVSVACLCTRDYQLRYNNN            109
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FIGURE 1: Amino acid sequence of a PTTH subunit.

MATERIALS AND METHODS

Expression of Recombinant PTTH. Recombinant *Bombyx* PTTH was purified from cells of *Escherichia coli*, BL21 (DE3) strain, harboring the expression vector pETPTTH which was constructed as follows. The PTTH-subunit-coding DNA fragment was excised from another PTTH expression plasmid, pKKPTTH (Kawakami et al., 1990), by digestion with *Nco*I and *Hin*dIII and made blunt with the Klenow fragment of DNA polymerase. This DNA fragment was ligated to the blunted *Bam*HI site of the expression vector pET-3b (Rosenberg et al., 1987). The resultant plasmid was digested with *Nco*I and *Nde*I and self-circularized to remove the region of N-terminal 11 amino acid residues of the gene 10 protein.

The transformant was cultured in 1 L of LB medium containing 100 mg of ampicillin until an OD600 of 1.3. The PTTH expression was then induced by adding isopropyl

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[1] Abbreviations: PTTH, prothoracicotropic hormone; HPLC, high-performance liquid chromatography; LB medium, Luria–Bertani medium; TFA, trifluoroacetic acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)-methylamine; IPTG, isopropyl D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; *E. coli*, *Escherichia coli*; LSIMS, liquid secondary ion mass spectrometry.

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D-thiogalactopyranoside (IPTG) to give a final concentration of 0.5 mM. After 6 h of incubation, the cells were harvested by centrifugation and resuspended in 100 mL of 50 mM phosphate buffer (pH 7.0) containing 10 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride. The suspensions were allowed to stand on ice for 1 h with addition of 10 mg of lysozyme. The cells were frozen and thawed and disrupted by sonication three times for 100 pulses. After centrifugation, the inclusion bodies were solubilized in 100 mL of 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate buffer (pH 10.7) containing 8 M urea and 1 mM EDTA. The solubilized materials were dialyzed against 0.2 M sodium acetate buffer (pH 7.0) for 36 h at 4 °C. During dialysis, recombinant PTTH was refolded to a dimeric form and showed biological activity. The supernatant after centrifugation contained 10^6 *Bombyx* units (Ishizaki et al., 1983) of PTTH activity.

Purification of Recombinant PTTH. Purification of the recombinant PTTH was done using two steps of ion exchange chromatography followed by reverse-phase HPLC. The samples after each purification step were bioassayed using brainless pupae of *Bombyx mori* (Ishizaki et al., 1983).

The solution after the dialysis against 0.2 M sodium acetate (pH 7.0) was applied directly onto a DEAE-Sepharose CL-6B column (6 × 6 cm), and the unabsorbed fraction was purified by a CM-Sepharose CL-6B column (2 × 25 cm) as described previously (Kataoka et al., 1987). The active fractions after CM-Sepharose CL-6B purification were pooled and directly pumped onto a semipreparative reverse-phase HPLC column (Senshu pak VP-304-4253, 10 × 250 mm, Senshu Kagaku), equilibrated with 15% acetonitrile in 0.1% trifluoroacetic acid (TFA), and eluted with a 75-min linear gradient of 15–40% acetonitrile in 0.1% TFA at a flow rate of 5 mL/min using the L-6250 preparative HPLC system (Hitachi). About 1 mg of recombinant PTTH was obtained from the cell lysates of 1 L of culture. The specific activity of the recombinant PTTH was about one-half that of purified natural PTTH.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Samples (ca. 1 μg each) were analyzed without a reducing agent in a 1-mm thick, 15% acrylamide gel by the method of Laemmli (1970) using the AE 6450 system (ATTO). Each gel was run at a 25-mA constant current and stained with Coomassie brilliant blue.

Analytical High-Performance Liquid Chromatography (HPLC). Analytical HPLC was done using the HP1090 HPLC system (Hewlett-Packard) equipped with a photodiode array detector.

After a partial reduction, the samples were applied to a Hi-Pore RP-304 column (4.6 × 250 mm, Bio-Rad), equilibrated with 20% acetonitrile in 0.1% TFA, and eluted with a 40-min linear gradient of 20–30% acetonitrile in 0.1% TFA at a flow rate of 1 mL/min (HPLC method 1).

The reaction mixture after lysyl endopeptidase or thermolysin digestion was applied to a Senshu Pak VP-318-1151 column (4.6 × 150 mm, Shenshu Kagaku), equilibrated with 1% acetonitrile in 0.1% TFA, and eluted with 1% acetonitrile in 0.1% TFA isocratically at a flow rate of 1 mL/min in the first 10 min, followed by 1–40% linear gradient of acetonitrile in 0.1% TFA for 78 min at a flow rate of 0.5 mL/min (HPLC method 2).

Partial Reduction. Ten micrograms of PTTH was dissolved in 100 μL of 0.5 M Tris-HCl buffer (pH 8.5) containing 6 M urea, and then 1 μL of 4-vinylpridine and 2 μL of tributylphosphine were added. After incubation of the solution at 37 °C for 2 h under nitrogen, 100 μL of TFA was added, and the reaction mixture was analyzed by HPLC method 1. For the preparation of a large amount of partially reduced PTTH, 360 μg of PTTH was dissolved in 200 μL of the same buffer and reduced under the same conditions. About 60 μg of a partially reduced and *S*-pyridylethylated PTTH was obtained.

Full Reduction and S-Carboxymethylation. The partially reduced and *S*-pyridylethylated PTTH and intact PTTH (about 5 μg each) were lyophilized and redissolved in 10 μL of 0.5 M Tris-HCl buffer (pH 8.5) containing 6 M urea. They were reduced by addition of 55 μg of dithiothreitol (DTT) under nitrogen at 45 °C for 70 min, and then the reduced cysteine residues were *S*-carboxymethylated by addition of 145 μg of sodium iodoacetate at room temperature for 30 min in the dark.

Lysyl Endopeptidase Digestion of S-Carboxymethylated Peptides. The *S*-carboxymethylated peptides were digested with lysyl endopeptidase (Wako Chemical) by adding directly the enzyme solution (1.5 μg/10 μL 0.1 M Tris-HCl buffer, pH 8.5) to the reaction mixture for the reduction and *S*-carboxymethylation. After incubation at 37 °C for 2 h, 100 μL of 10% TFA was added and the resulting fragments were separated by HPLC method 2.

Lysyl Endopeptidase Digestion of Partially Reduced and S-Pyridylethylated PTTH. Sixty micrograms of partially reduced and *S*-pyridylethylated PTTH was lyophilized and redissolved in 100 μL of 0.5 M Tris-HCl buffer (pH 8.5) containing 1 mM sodium iodoacetate. The conditions of digestion with lysyl endopeptidase and HPLC for separation of fragment peptides were the same as mentioned above. One tenth of the digests was fully reduced and *S*-carboxymethylated and analyzed by HPLC using the same conditions to identify the disulfide-containing peptides.

Thermolysin Digestion of the Lysyl Endopeptidase-Digested Fragments. Two-fifths of the lysyl endopeptidase fragments were lyophilized and redissolved in 0.1 M *N*-morpholinoethanesulfonic acid buffer (pH 6.5) containing 1 mM $CaCl_2$. After addition of 1 μg of thermolysin (Boehringer) dissolved in 10 μL of the same buffer, the reaction mixture was incubated at 55 °C for 24 h. The resulting fragments were separated by HPLC method 2 after addition of 20 μL of 10% TFA. One fourth of the digest was reduced and *S*-carboxymethylated to identify the disulfide-containing peptides.

Digestion of Intact PTTH. One hundered and ninety micrograms of intact PTTH was dissolved in 100 μL of 0.5 M Tris-HCl (pH 8.5) containing 6 M urea and 1 mM sodium iodoacetate. Ten microliters of the lysyl endopeptidase solution (1 μg/10 μL of 0.1 M Tris-HCl buffer, pH 8.5) was added to the sample every day with incubation at 37 °C for a week. After separation of fragment peptides by HPLC method 2, each disulfide-containing peptide was lyophilized and then digested with thermolysin as mentioned above. The resulting fragments were separated by HPLC method 2, and the disulfide-containing peptide was identified by comparison of its HPLC elution pattern with that of a portion of the digests after reduction and *S*-carboxymethylation.

Amino Acid Sequence Analysis. Amino acid sequence analysis was performed using a gas-phase sequencer (Shimadzu PSQ-1) equipped with a phenylthiohydantoin amino acid analyzer.

Liquid Secondary Ion Mass Spectrometry (LSIMS). Approximately 1 μg of each sample was lyophilized and dissolved in 1 μL of 0.1% TFA. After being mixed with 1 μL

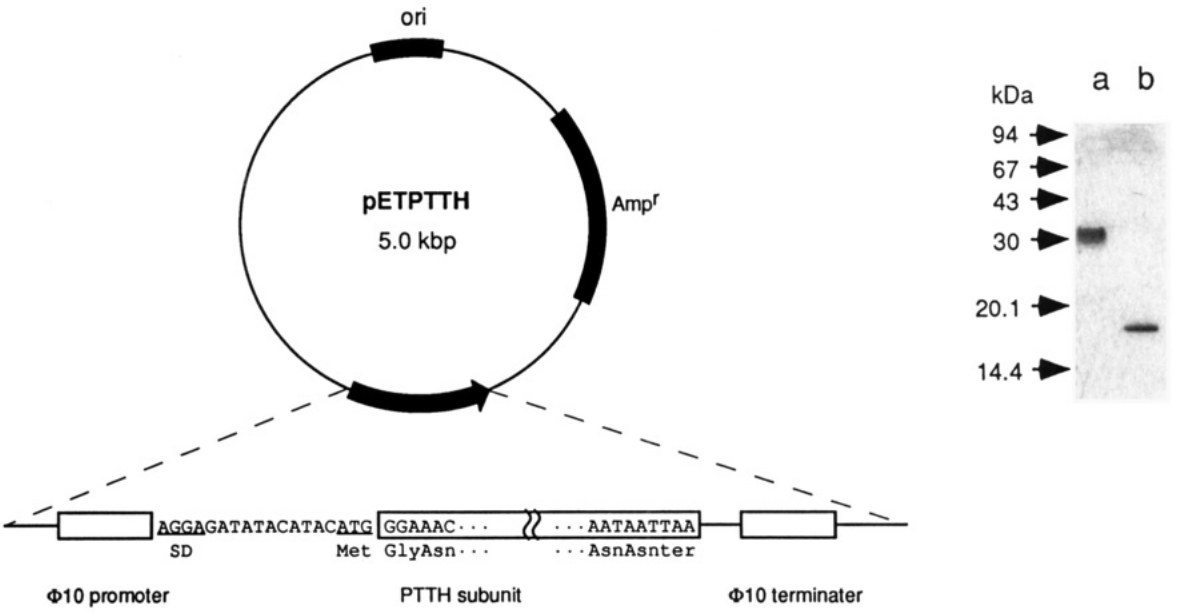


FIGURE 2: PTTH-expression plasmid and SDS–PAGE analysis of recombinant PTTH. (A, left) Construction of a PTTH-expression plasmid, pETPTTH. (B, right) SDS–PAGE analysis of recombinant PTTH. Samples (ca. 0.3 μg each) were analyzed by SDS–PAGE under a nonreductive condition. Lanes: a, intact PTTH; b, reduced and *S*-carboxymethylated PTTH.

of glycerol and 1 μL of thioglycerol as matrix, the sample was introduced into the ion source of the mass spectrometer using a stainless steel probe tip. Analysis was performed with a JEOL SX-102 mass spectrometer using Cs as the fast atom.

RESULTS

Expression of Recombinant PTTH in E. coli. We previously obtained the recombinant PTTH by using expression plasmid pKKPTTH (Kawakami et al., 1990). Since the expression yield was not sufficient, we constructed a new expression plasmid, pETPTTH, which carried the bacteriophage T7 gene 10 promoter and PTTH-subunit-coding DNA fragment (Figure 2A). *E. coli* strain BL21(DE3) was transformed with pETPTTH, and the PTTH expression was induced by adding IPTG to the culture. The recombinant PTTH was purified from the solubilized inclusion bodies by two steps of ion exchange chromatography followed by reverse-phase HPLC. In these steps, the recombinant PTTH was eluted in fractions similar to those of the natural PTTH. Finally, we obtained 1 mg of biologically active recombinant PTTH from 1 L of the culture. The recombinant PTTH possesses a specific activity comparable with that of the natural PTTH (recombinant PTTH, 0.6 ng/unit; natural PTTH, 0.3 ng/unit). The lower activity of recombinant PTTH compared with that of natural PTTH might be due to the lack of a carbohydrate chain bound to natural PTTH. Furthermore, SDS–PAGE analysis showed that the recombinant PTTH also had a dimeric structure linked by disulfide bond(s) (Figure 2B). Amino acid sequence analysis of recombinant PTTH and the fragment peptides generated by enzyme digestions of intact PTTH after reduction and *S*-carboxymethylation showed that the recombinant PTTH had an expected 109 residual sequence beginning with glycine residue and that a methionine residue added to the N-terminus of the PTTH subunit for recombinant expression had been deleted, presumably by methionine aminopeptidase in *E. coli*.

Identification of the Interchain Disulfide Bond. We first tried to determine the disulfide bond location of the recombinant PTTH by isolating disulfide-containing peptides after digestion of PTTH with lysyl endopeptidase or thermolysin. However, this attempt has been fruitless because PTTH was not sufficiently digested by these enzymes (data not shown). Therefore, we tried to reduce some of the disulfide bonds of the intact PTTH first and then digest a partially reduced PTTH with enzymes to isolate disulfide-containing peptides. We hypothesized that the interchain disulfide bonds may be more susceptible to reduction than the intrachain disulfide bonds and expected that reduction in appropriate conditions might produce the monomeric form whose intrachain disulfide bonds remain intact. We used as a reducing reagent tributylphosphine, a relatively moderate reducing reagent which can be used in the presence of the *S*-alkylating reagent, 4-vinylpyridine (Ruegg & Rudinger, 1977). The alkylation with 4-vinylpyridine could protect free sulfhydryl groups and prevent the disulfide bond exchange caused by free sulfhydryl groups during reduction.

In an appropriate condition of partial reduction, a peak (peak 3-2) appeared between the intact PTTH (peak 3-3) and the completely reduced and *S*-pyridylethylated form (peak 3-1) on HPLC (Figure 3A). The peak 3-1 was inferred from the sequence analysis of fragments after lysyl endopeptidase digestion as a completely reduced and *S*-pyridylethylated form. From the UV absorption at 255 nm for the *S*-pyridylethyl moiety, the peptide of the peak 3-2 was judged to contain no more than three *S*-(pyridylethyl)cysteine residues. The SDS–PAGE analysis under the nonreducing condition of the peptides from these fractions showed that their molecular masses were 15 kDa for peak 3-1, 16 kDa for peak 3-2, and 30 kDa for peak 3-3 (Figure 3B). These results suggest that the peak 3-2 material was a monomeric form produced by reduction and *S*-pyridylethylation of at least the interchain disulfide bond-(s) of PTTH. The difference of mobility between the materials of peaks 3-1 and 3-2 on SDS–PAGE is perhaps due to the intrachain disulfide bonds remaining intact in the peak 3-2 material.

In order to identify the location of the *S*-pyridylethylated cysteine residue(s), the peak 3-2 material was fully reduced by DTT, *S*-carboxymethylated by iodoacetic acid, and digested with lysyl endopeptidase. When the elution pattern of the lysyl endopeptidase digest of the peak 3-2 material was compared with that of PTTH after full reduction and *S*-carboxymethylation, only peak 4-1 had a different retention

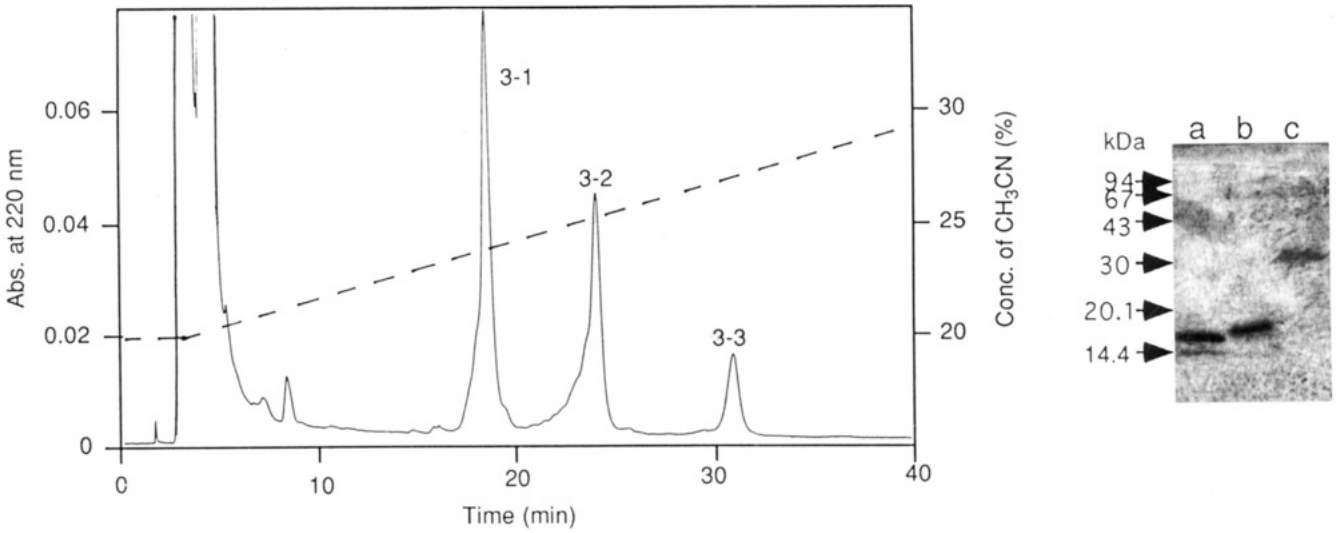


FIGURE 3: Partial reduction of PTTH. (A, left) HPLC separation of partially reduced and *S*-pyridylethylated PTTH. The reaction mixture after reduction and *S*-pyridylethylation of PTTH was separated with a Hi-Pore RP-304 column using HPLC method 1. Absorbance at 220 nm is shown by a solid line, and concentration of acetonitrile is given as a dotted line. (B, right) SDS–PAGE analysis of materials from peaks 3-1, 3-2, and 3-3. Samples (ca. 1 μg each) were analyzed by SDS–PAGE under nonreductive conditions. Lanes: a, peak 3-1; b, peak 3-2; c, peak 3-3.

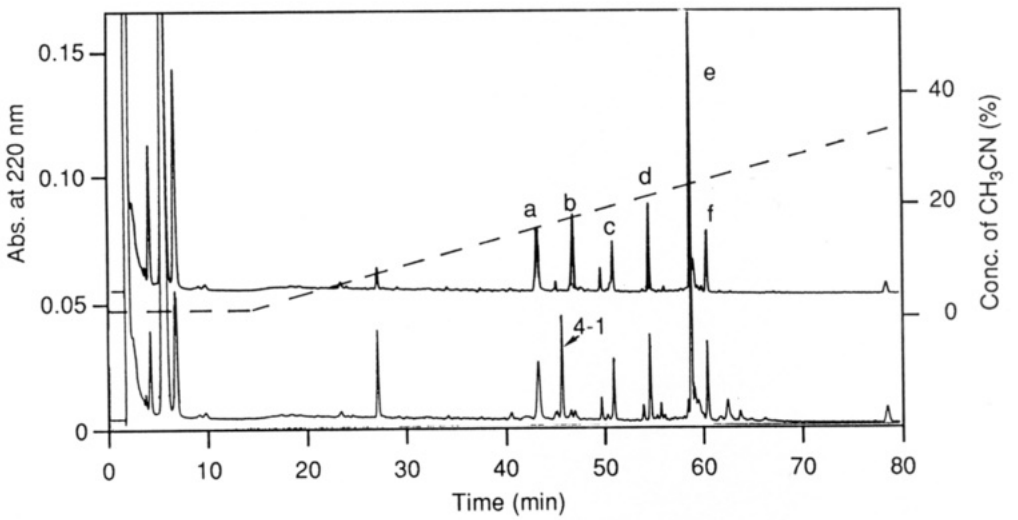


FIGURE 4: Comparative peptide maps of lysyl endopeptidase fragments after full reduction and *S*-carboxymethylation of intact PTTH (upper) and the partially reduced and *S*-pyridylethylated PTTH (lower). The fragment peptides were separated using HPLC method 2. Absorbance at 220 nm is shown by solid lines, and concentration of acetonitrile is given as a dotted line. Each fragment from intact PTTH was assigned as follows: a, position 43–55; b, position 1–18; c, position 71–82; d, position 22–42; e, position 83–109; f, position 56–65.

time, while other peaks had the same retention times between the two digests (Figure 4). Moreover, peak 4-1 possessed absorption at 255 nm for the pyridylethyl moiety, suggesting that the cysteine residue(s) of peak 4-1 was *S*-pyridylethylated and cysteine residues of other fragments were *S*-carboxymethylated. Amino acid sequence analysis of the peak 4-1 material showed that this peptide was the amino-terminal fragment of the PTTH subunit (position 1–18) containing two cysteine residues and that the Cys15 was *S*-pyridylethylated while the Cys17 was *S*-carboxymethylated. The other peptides shown in Figure 4 (lower trace) were sequenced, and it was identified that all cysteine residues were *S*-carboxymethylated. These results indicated that the partial reduction led to the dissociation of PTTH into two chains, in which only the Cys15 among seven cysteine residues in a chain was reduced and *S*-pyridylethylated. We conclude, therefore, that the Cys15 residue contributes to the formation of an interchain disulfide bond and that the other six cysteine residues form intrachain disulfide bonds.

Identification of Intrachain Disulfide Bonds. The locations of intrachain disulfide bonds were determined using the partially reduced and *S*-pyridylethylated PTTH by a strategy of two successive enzyme digestions with lysyl endopeptidase and thermolysin.

Digestion of this monomerized PTTH by lysyl endopeptidase produced a major peak and several minor peaks on a reverse-phase HPLC (HPLC method 2, Figure 5). To identify the fragments containing disulfide bond(s), a portion of the lysyl endopeptidase digest was reduced and *S*-carboxymethylated and then analyzed by the HPLC under the same conditions (data not shown). Only the major peak (peak 5-1) of Figure 5 disappeared after the reduction and *S*-carboxymethylation. Sequence analysis of the peptide in peak 5-1 revealed that this fragment consisted of four peptide chains, which were assigned to positions 1–18, 22–42, 43–55, and 83–109 by comparison with the sequence of PTTH. Since this fragment contained all intrachain disulfide bonds, this fragment was further digested with thermolysin, and the fragment peptides were separated on HPLC using method 2 (Figure 6). In the same manner as lysyl endopeptidase digestion, three fragment peptides 6-1, 6-2, and 6-3 were identified as disulfide-containing peptides. Sequence analysis showed that all

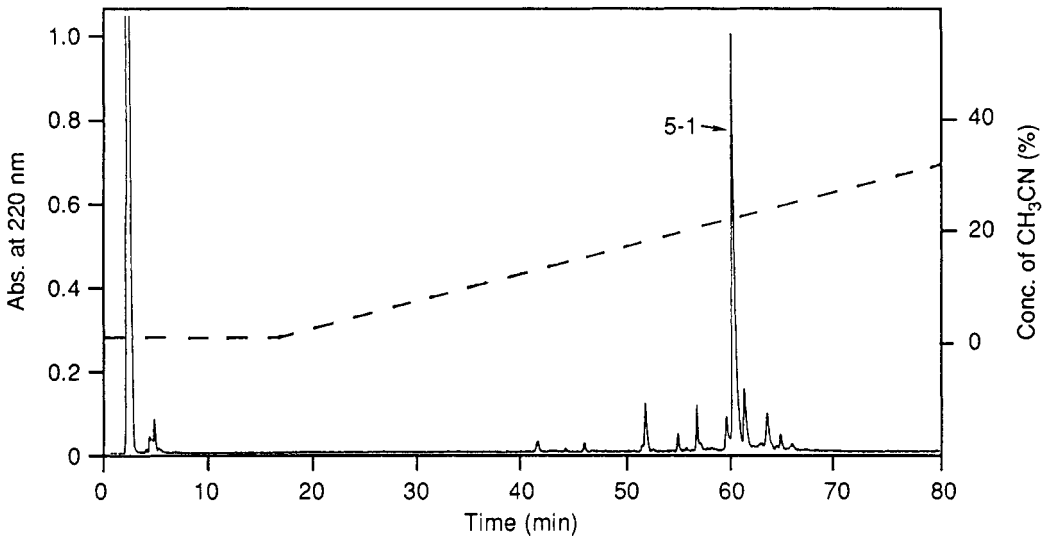
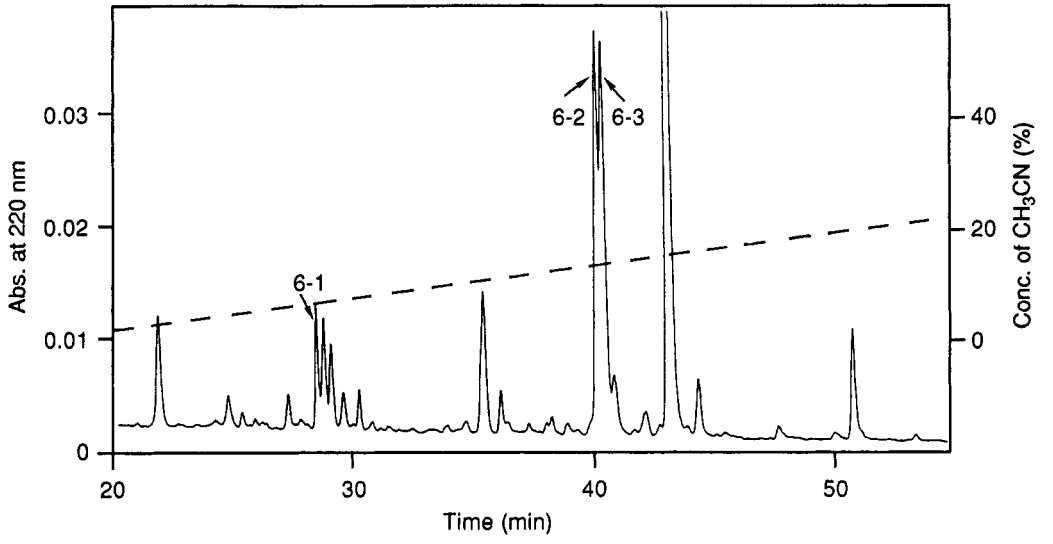


FIGURE 5: Separation of a lysyl endopeptidase digest of the partially reduced and *S*-pyridylethylated PTTH. The fragment peptides were separated using HPLC method 2. Absorbance at 220 nm is shown in a solid line, and concentration of acetonitrile is given as a dotted line. Peak 5-1 was identified as containing disulfide bonds by comparison with the spectrum of an aliquot which was reduced and *S*-carboxymethylated prior to the identical analysis.



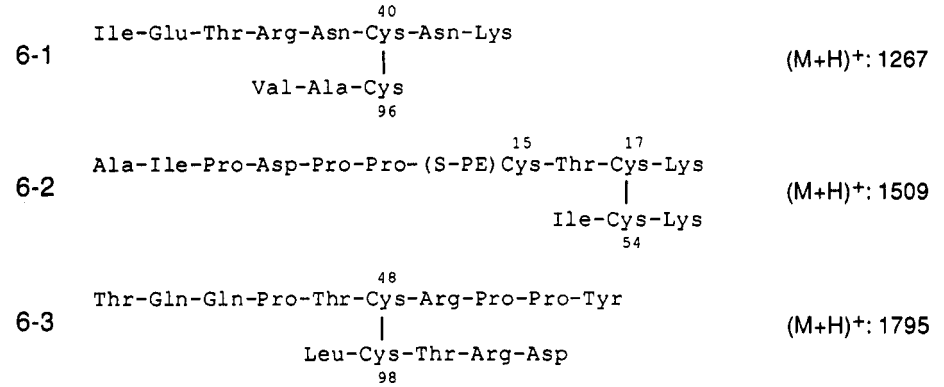


FIGURE 6: Separation of thermolysin fragments of the lysyl endopeptidase-digested fragment (peak 5-1 in Figure 5). The fragment peptides were separated using HPLC method 2. Absorbance at 220 nm is shown in a solid line, and concentration of acetonitrile is given as a dotted line. The peaks numbered were identified as containing disulfide bonds by comparison with the spectrum of an aliquot which was reduced and *S*-carboxymethylated prior to the identical analysis. The structures and molecular weights of peaks 6-1, 6-2, and 6-3 are presented.

peptides were composed of two chains, and the sequences were assigned to positions 35–42 and 94–96 for 6-1, 9–18 and 53–55 for 6-2, and 43–52 and 97–101 for 6-3. Although the cysteine residues could not be identified by sequencing, the LSIMS analysis of these fragments supported the conclusions of all these assignments. Thus, the three intrachain disulfide bonds were determined to be Cys17–Cys54, Cys40–Cys96, and Cys48–Cys98.

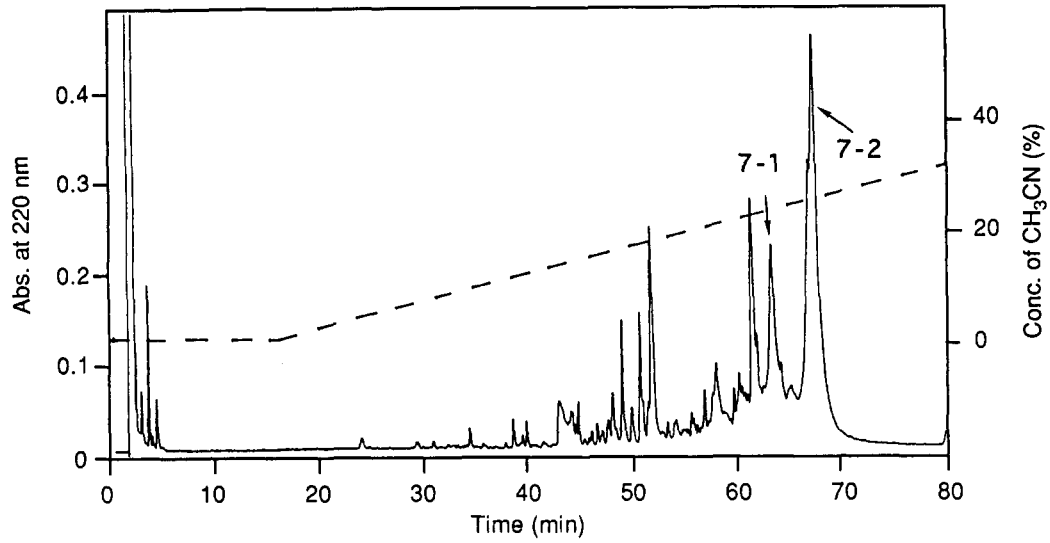
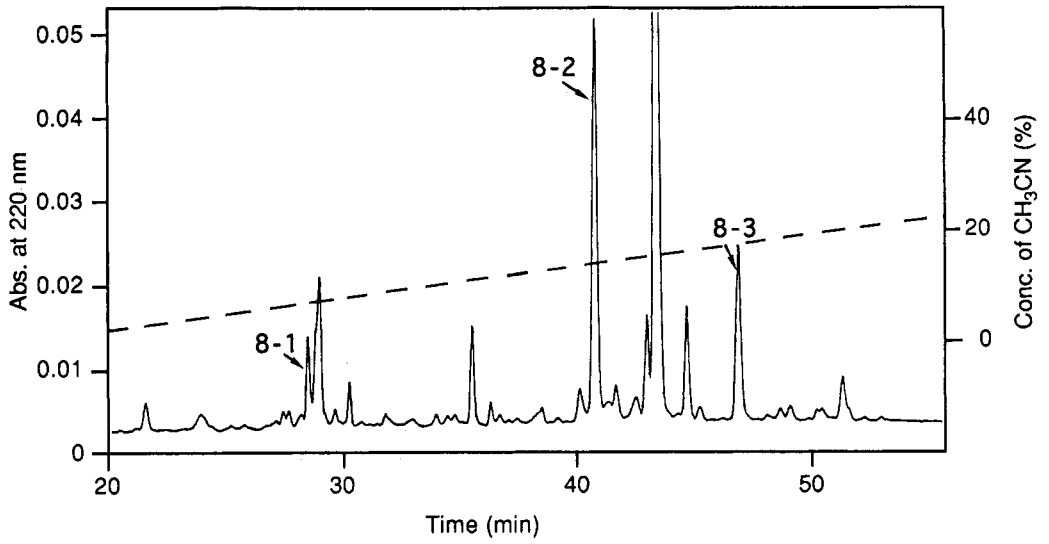


FIGURE 7: Separation of lysyl endopeptidase fragments of the recombinant PTTH without reduction. The fragment peptides were separated using HPLC method 2. Absorbance at 220 nm is shown in a solid line, and concentration of acetonitrile is given as a dotted line. The peaks numbered were identified as containing disulfide bonds by comparison with the spectrum of an aliquot which was reduced and *S*-carboxymethylated prior to the identical analysis.

Further Evidence for the Disulfide Bond Location. Since the intact PTTH failed to be digested effectively with the enzymes used under conventional conditions (data not shown), we tried to digest PTTH with lysyl endopeptidase in 6 M urea by prolonged exposure to a fresh enzyme which was renewed every day. After a week, we obtained two fragment peptides containing disulfide bonds when analyzed by HPLC method 2 (Figure 7, peaks 7-1 and 7-2). Sequence analysis of the peak 7-2 material showed a mixture of three amino acid sequences corresponding to positions 1–42, 43–55, and 83–109, while sequence analysis of the peak 7-1 material gave a mixture of four amino acid sequences corresponding to positions 1–18, 22–42, 43–55, and 83–109. The peak 7-1 material was further digested with thermolysin to generate fragment peptides. After the thermolysin digestion, three disulfide-containing fragments (peaks 8-1, 8-2, and 8-3 by HPLC method 2) were obtained (Figure 8). The peptides from peaks 8-1 and 8-2 had amino acid sequences identical with those of peak 6-1 and 6-3 of Figure 6, respectively. The LSIMS analysis of these peptides also gave the expected molecular weights. Although the fragment from peak 8-3 showed an amino acid sequence identical to that from peak 6-2 except for *S*-(pyridylethyl)cysteine, the molecular weight of this peptide was 2809, twice as large as that of the peak 6-2 fragment without the *S*-pyridylethyl moiety, suggesting that this peptide consisted of two chains of position 9–18 and two chains of position 53–55 and that these four chains were linked together with disulfide bonds. This result supports the idea that the homodimeric structure of PTTH is maintained by a disulfide bond between the Cys15 residues of two subunits. Figure 9 shows diagrammatically our conclusion regarding the interchain and intrachain disulfide bond locations in PTTH.

DISCUSSION

The disulfide bond location of PTTH was determined by partial reduction and *S*-alkylation of PTTH followed by enzymatic digestions and analysis of the derived disulfide-containing fragments. In the case of homodimeric peptides, it is impossible to determine whether the disulfide bond of the fragment generated by enzyme digestion originates from interchain or intrachain disulfide bonds. Fortunately, the partial reduction of PTTH in an appropriate condition generated a monomeric form whose intrachain disulfide bonds remained intact. This procedure made it possible to identify an interchain disulfide bond as well as to distinguish it from intrachain disulfide bonds. The method described in this article will be useful for determination of the disulfide bond locations of homodimeric peptides. In any condition of partial reduction using tributylphosphine and 4-vinylpyridine, we could not find any other partially reduced and S-pyridylethylated form besides the peak 3-2 material, suggesting that the interchain disulfide bond is more susceptible to reduction than the intrachain disulfide bonds and that the reduction of an intrachain is the rate-determining step of full reduction of PTTH. Andersson et al. (1992) recently reported the assignment of interchain disulfide bonds in platelet-derived growth factor (PDGF) using a similar partial reduction method. They used DTT as reducing reagent, followed by *S*-alkylation with iodoacetic acid. We first tested DTT as a reducing reagent, and it also gave a partially reduced form which was identical with that from the reduction using tributylphosphine (data not shown). But in the method using DTT, disulfide exchanges caused by free sulfhydryl groups may occur during the partial reduction. To reduce the disulfide exchange, we used as a reducing reagent tributylphosphine, which could be used in the presence of an *S*-alkylating reagent, 4-vinylpyridine. In our new method, free sulfhydryl groups could be *S*-alkylated immediately after being formed to prevent disulfide exchange. We could not find any disulfide bond isomers after partial reduction. Ruegg and Rudinger (1974) reported that partial reduction of insulin using tributylphosphine in the presence of ethylene imine gave a mixture of partially reduced and aminoethylated product attended by disulfide exchange. There is some possibility that disulfide bond exchange may occur during partial reduction, even in the presence of alkylating reagent, if disulfide exchange is much faster than alkylation by 4-vinylpyridine or ethylene imine. Furthermore, the partial reduction using tributylphosphine produced a relatively higher yield of the partially reduced form than the partial reduction using DTT. We digested disulfide bond-containing peptides with lysyl endopeptidase in the presence of sodium iodoacetic acid for the same purpose.



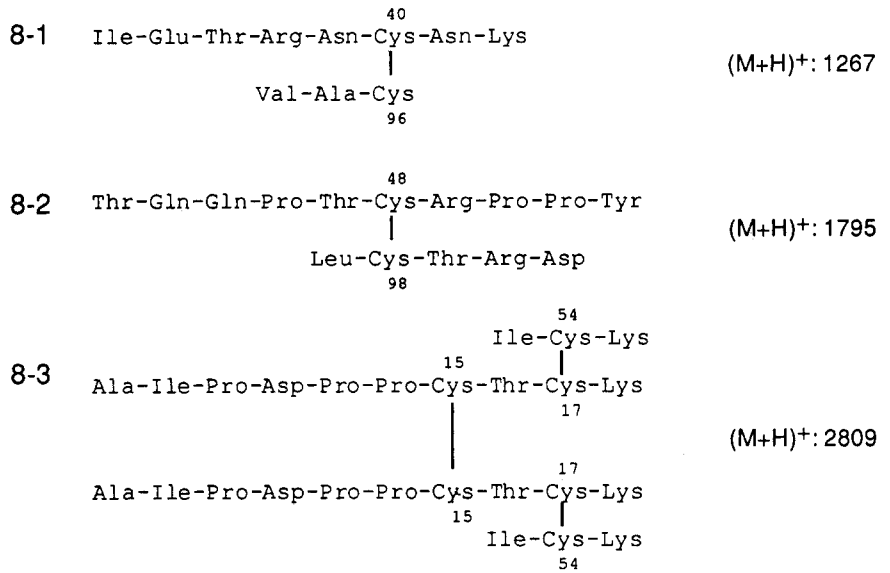


FIGURE 8: Separation of thermolysin fragments of the lysyl endopeptidase-digested fragment (peak 7-1 in Figure 7). The fragment peptides were separated using HPLC method 2. Absorbance at 220 nm is shown in a solid line and concentration of acetonitrile is given as a dotted line. The peaks numbered were identified as containing disulfide bonds by comparison with the spectrum of an aliquot which was reduced and *S*-carboxymethylated prior to the identical analysis. The structures and molecular weights of peaks 8-1, 8-2, and 8-3 are presented.

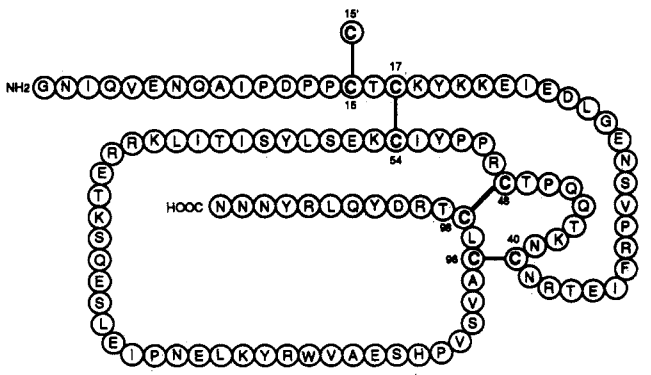


FIGURE 9: Schematic drawing of the covalent structure of PTTH.

Throughout the analysis of the enzyme-digested fragments, only a single disulfide bonding pattern was detected in any fragment, indicating that disulfide exchange did not occur during partial reduction and enzyme digestions and that the recombinant PTTH had a unique disulfide bond location. Since the recombinant PTTH was refolded by air oxidation during dialysis, this disulfide bond location of PTTH would be the most stable form thermodynamically. In fact, when the purified recombinant PTTH was reduced and reoxidized, the same disulfide bond formation occurred including the interchain disulfide bonds (data not shown).

The partial reduction gave another advantage for proteolytic cleavage to generate fragments containing disulfide bonds. The partially reduced and *S*-pyridylethylated PTTH was readily digested with lysyl endopeptidase, the reaction being completed in 2 h, whereas intact PTTH was not sufficiently digested with lysyl endopeptidase, even with incubation for a week with repeated addition of a fresh enzyme every day, suggesting that the dimeric structure of PTTH protects it from cleavage by proteolytic enzymes. In fact, when the intact PTTH was digested with lysyl endopeptidase, the cleavage at the Lys18, Lys20, and Lys21 residues near the Cys15, which makes an interchain disulfide bond, was slower than those at the other lysine residues (data not shown).

Since the content of PTTH in an insect brain is extremely low (1 μg of PTTH was obtained from 10 000 silkworm heads), the chemical and biological study has been limited. The

expression method using *E. coli* makes it possible to synthesize a large amount of recombinant PTTH and also the mutated PTTH. These recombinant PTTHs can be used for the determination of tertiary structure and the elucidation of structure–activity relationships of PTTH. In addition, the assignment of the disulfide bond location of PTTH gives important information for the determination of the tertiary structure of PTTH.

Interestingly, the partially reduced and *S*-pyridylethylated PTTH, in which all intrachain disulfide bonds remained intact, retained PTTH activity, although the specific activity of the partially reduced and *S*-pyridylethylated PTTH was about one-half that of intact PTTH. Since the fully reduced and *S*-alkylated PTTH monomer had no PTTH activity, the intrachain disulfide bonds are essential for making the conformation required for manifesting the biological activity, but the interchain disulfide bond is not essential for the activity. We are now developing the expression of mutated PTTH at each cysteine residue to confirm this notion. The monomeric PTTH whose interchain disulfide bond is lacking might be useful for the determination of teritary structure, especially using NMR, because each NMR signal of the monomeric PTTH is more simple than that of the intact PTTH.

We hope that the chemical and biochemical studies of PTTH will progress using the results described in this article.

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