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Minimum Structure of Diapause Hormone Required for Biological Activity

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Diapause hormone is a 24-amino acid peptide amide, and its C-terminal penta-peptide amide structure of FGPRL-NH_2 is believed to be essential for biological activity. The penta-peptide amide, the shorter peptide amides, and their derivatives and analogs were prepared to determine the minimal structure for biological activity. The C-terminal amide group of the penta-peptide amide was not replaced with the other functional groups, but Gly, the 4th amino acid from the C terminal, could be substituted with an other amino acid while maintaining the biological activity. The shorter peptide amide, PRL-NH_2, possessed low but significant activity, indicating the minimum structure of diapause hormone. By modifying its N-terminal, the aromatic ring of Phe is shown to enhance the activity of PRL-NH_2.

Key words: silkworm; diapause hormone; insect hormone; peptide hormone; biological activity

The egg diapause of the silkworm, *Bombyx mori*, is induced by a neuropeptide hormone, diapause hormone (DH), which is secreted from the pupal suboesophageal ganglion of this insect. The structure of DH has been determined to be a 24-amino acid peptide amide as shown below.[1,2]

Structure of diapause hormone

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
T	D	M	K	D	E	S	D	R	G	A	H	S	E	R	G	A	L	W	F	G	P	R	L-NH_2

Although there is no homology between the whole structure of DH and the other known proteins, DH shares the common C-terminal penta-peptide sequence of FXPR/KL-NH_2 (DH: X=G) with the other important insect neurohormones, myotropin,[3] pyrokinin[4] and PBAN.[5] Similar structures have also been found in the new peptides encoded in DH-PBAN m-RNA. These peptides have been named α-, β- and γ-SGNP (X=T, I and S, respectively) and were identified in an extract of pupal suboesophageal ganglions.[6] As all these peptides and the fragment peptide of DH bearing the C-terminal FXPRL-NH_2 structure have shown diapause egg-inducing activity to different degrees,[7-9] the penta-peptide amide moiety is believed to be a core structure for DH activity. In fact, synthetic FGPRL-NH_2 has shown low but significant DH activity.[7,9]

In the present work, we investigated the minimum structure of DH required to elucidate diapause egg induction. By comparing the biological activity of the penta-peptide amides, the shorter peptide amides, and their analogs and derivatives, PRL-NH_2 was concluded to be the minimum structure of DH. This is the first evidence to demonstrate the tri-peptide amide being an active center of peptide hormones.

Materials and Methods

All the protected amino acids were purchased from Peptide Institute Co., and the reagents were the purest ones available from Wako Chemicals Co. or Nacarai Tesque Co. and were used without further purification. Water was purified by Milli Q water purification apparatus (Millipore Co.) after being distilled.

Syntheses of the peptides and their analogs. A liquid-phase procedure was employed for the peptide syntheses starting from Leu-OMe according to the Boc strategy, so that the C-terminal ester moieties of the products could be easily converted into the other functional groups later. Four peptide methyl esters, FXPRL-OMe (X=G, S, V and I) were synthesized. The protected amino acids used in this work were Boc-Arg(Tos)-OH, Boc-Pro-OH, Boc-Gly-OH, Boc-Ser(O-Bzl)-OH, Boc-Ile-OH, Boc-Val-OH, and Boc-Phe-OH. The coupling reaction was facilitated by DCC-HOSu in DMF. Each intermediate BOC-peptide was treated with TFA to cleave the N-terminal Boc group, and the elongation reaction was then repeated. The protected penta-peptide esters thus obtained were treated with TFA, and then with TFMSA to cleave the Boc group and the side-chain protecting groups. The C-terminal ester moiety was finally converted into free carboxylic acid (entry 7 in Table 2), amides (entry 1, X=G; 2, X=S; 3, X=V; 4, X=I), or alkyl amides (entry 8, methylamide; 9, dimethylamide; 10, n-butylamide) by hydrolysis or aminolysis.

The di-, tri- and tetra-peptide amides (RL-NH_2, PRL-NH_2 and GPRL-NH_2) were derived from the corresponding synthetic intermediates, the protected peptide methyl esters, by treating with methanolic ammonia after cleaving the protecting groups.

The tri- and tetra-peptide amides modified with acyl groups bearing aromatic groups (Fig. 2) were next prepared. At first, the free N-terminal of the protected tri-peptide methyl ester was coupled with phenylhexanoic acid or 1-naphthaleneacetic acid, and that of the protected tetra-peptide methyl ester was modified with phenyl-

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propanoic acid or trans-cinnamic acid, according to the DCC-HOSu method in DMF. The ester groups of the products were then converted into amides after cleaving their side-chain Tos groups to afford phenylhexanoyl-PRL-NH_2, naphthaleneacetyl-PRL-NH_2, phenylpropanoyl-GPRL-NH_2, and trans-cinnamyl-GPRL-NH_2.

The intermediate product obtained from each reaction step was purified by silica gel column chromatography (BW-300, Fuji-Silysia Co.). The final products were isolated by reversed-phase HPLC (Develosil ODS-5, Nomura Chemicals Co., gradient elution between 0.1% TFA and CH_3CN). The structures of the purified products were confirmed by 500 MHz and/or 90 MHz ^{1}H-NMR (JNM-A500, Jeol Co., and R-90, Hitachi Co.) in CD_3OD, and by SIMS (M-80, Hitachi Co.).

Biological activity test. Each purified synthetic sample was dissolved in water and injected into five or seven female pupae of a polyvoltine, N4 strain of the silkworm, *Bombyx mori*. The injected insect was allowed to lay 200–250 eggs after copulation. In the present report, the diapause egg-inducing activity was indicated by the average number of diapause eggs laid by the injected insects.

No further bioassay with a larger amount of each chemical than that indicated in the figure was done, because an excessive dose could have caused death of the insect or a severe decrease in the number of eggs laid by the insect.

Results and Discussion

Confirmation of the structure and purity

The purity of each sample used for the biological activity test was analyzed by reversed-phase HPLC, detecting by the UV absorbance at 210 nm. Pure fractions, in which no contamination could be detected, were subjected to the biological activity tests. The SIMS data of each pure sample showed the correct $[M+H]^+$ ion (Table 1), and the result confirmed the structure. In each spectrum, only the $[M+H]^+$ peak was found, and no $[M+K]^+$ or $[M+Na]^+$ ion was detected. This result indi-

Table 1. $[M+H]^+$ Values Observed by SIMS and Characteristic Proton Signals in the 500 MHz ^{1}H-NMR Data of Synthetic DH Gragment Peptides and Their Analogs

	$[M+H]^+$	Characteristic proton signals in the 500 MHz 1H-NMR data
RL-NH_2	287	0.95, 3H, d, J=6.4 Hz & 0.97, 3H, d, J=6.7 Hz (Leu, CH_3), 3.21, 2H, t, J=7.0 Hz (Arg, N-CH_2), 3.9–4.0, 2H, m (α-H of AA)
PRL-NH_2	384	0.92, 3H, d, J=6.7 Hz & 0.95, 3H, d, J=6.7 Hz (Leu, CH_3), 3.20, 2H, t, J=7.0 Hz (Arg, N-CH_2), 3.3, 1H, m & 3.4, 1H, m (Pro, N-CH_2), 4.31, 1H, dd, J=6.4 & 8.5 Hz & 4.38–4.44, 2H, m (α-H of AA)
GPRL-NH_2	441	0.92, 3H, d, J=6.4 Hz & 0.95, 3H, d, J=6.7 Hz (Leu, CH_3), 3.19, 2H, t, J=7.0 Hz (Arg, N-CH_2), 3.5, 1H, m, & 3.6, 1H, m (Pro, N-CH_2), 3.89, 2H, s (Gly, CH_2), 4.36, 1H, dd, J=6.1 & 7.6 Hz, 4.41, 1H, dd, J=4.9 & 10.4 Hz & 4.48, 1H, dd, J=4.0 & 9.2 Hz (α-H of AA)
FGPRL-NH_2	588	0.91, 3H, d, J=6.4 Hz & 0.95, 3H, d, J=6.7 Hz (Leu, CH_3), 3.04, 1H, dd, J=8.5 & 14.3 & 3.28, 1H (Phe, benzyl-CH_2) 3.19, 2H, dd, J=6.7 & 11.9 Hz (Arg, N-CH_2), 3.57, 1H, m & 3.64, 1H, m (Pro, N-CH_2), 4.06, 1H, d, J=16.8 Hz & 4.10, 1H, d, J=16.8 Hz (Gly, CH_2), 4.13, 1H, dd, J=6.1 & 8.6 Hz, 4.36, 1H, dd, J=6.1 & 8.2 Hz, 4.39, 1H, dd, J=4.9 & 10.1 Hz & 4.45, 1H, dd, J=3.7 & 8.9 Hz (α-H of AA) 7.25–7.32, 3H, m & 7.32–7.40, 2H, m (Phe, arom-H)
FSPRL-NH_2	618	3.81, 2H, m (Ser, CH_2OH)
FVPRL-NH_2	630	0.83, 3H, d, J=6.4, 0.86, 3H, d, J=6.7 Hz, 0.91, 3H, d, J=6.7 & 0.94, 3H, d, J=6.7 Hz (Val, CH_3 & Leu, CH_3)
FIPRL-NH_2	644	0.92, 3H, t, J=7.5 Hz, 0.92, 3H, d, J=7.0, 0.95, 3H, d, J=6.5 Hz & 1.00, 3H, d, J=6.5 Hz (Ile, CH_3 & Leu, CH_3)
cin-GPRL-NH_2	571	7.35–7.45, 3H, m & 7.50–7.60, 2H, m (arom-H), 6.68, 1H, d, J=16.0 Hz & 7.53, 1H, d, J=16.0 Hz (viny I-H)
PhC3-GPRL-NH_2	573	7.2, 5H, bs (arom-H)*
PhC6-PRL-NH_2	558	7.1, 5H, bs (arom-H)*
NaphAc-PRL-NH_2	552	7.2–7.6, 4H, m & 7.6–8.0, 3H, m (arom-H)*
FGPRL-NHMe	602	2.71, 3H, s (N-CH_3)
FGPRL-NMe_2	616	2.93, 3H, s & 3.11, 3H, s (N-CH_3)
FGPRL-NH(n-Bu)	644	0.92, 3H, t, J=7.5 Hz (N-Bu-CH_3)
FGPRL-OCH_3	603	3.69, 3H, s (ester-CH_3)
FIPRL-OCH_3	659	3.70, 3H, s (ester-CH_3)

*: measured by 90 MHz NMR

AA: amino acid, cin: trans-cinnamyl-, PhC3-: henylpropanoyl-, PhC6-: phenylhexanoyl-, NaphAc-: 1-naphthaleneacetyl-

cates that the peptides used in the present work were not contaminated by the potassium ion which is known to induce egg diapause in the polyvoltine silkworm.

The structure of each synthetic sample was also confirmed by ^{1}H-NMR in CD_3OD. Table 1 shows the characteristic proton signals of the synthetic DH fragment peptides and their analogs obtained in this work. The NMR spectrum of FIPRL-COOH could not be measured because of the small amount of the sample, but the synthetic peptide showed the correct $[M+H]^+$ value (645) in the SIMS data.

Diapause egg-inducing activity of FGPRL-NH$_2$

The diapause egg-inducing activity of FGPRL-NH$_2$ was re-examined. The result is shown in Table 2 (entry 1) and in Fig. 1, and reconfirmed the previous observation that the peptide amide had diapause egg-inducing activity.[7,9] The threshold dose of the peptide amide was estimated to be 0.5 n mole (0.3 μg)/pupa, this being 30 times larger than that of intact DH. This lower activity may be attributable to lack of the remaining part of DH that contributes to enhanced activity.

Effect of substituting the C-terminal amide moiety on the diapause egg-inducing activity

The results of the previous research indicated that the 24-mer peptide with a free carboxylic acid C-terminal did not have any DH activity, even if its amino acid sequence was just the same as DH.[1,8] To examine whether the free carboxylic acid at the C-terminal completely extinguished the activity, the peptide methyl ester, FIPRL-OMe, was hydrolyzed and the activity of the product was measured. As can been seen in Table 2 (entry 7), no activity was apparent at a dose of 5 to 100 μg/pupa, although the corresponding peptide amide induced diapause in 20 eggs at a dose as low as 5 μg/pupa (entry 4). Although the reason why the peptide with the free carboxylic acid did not show any activity remains unknown, there is the possibility that an anionic charge at the free carboxylate moiety interfered with the interaction between the peptide ligand and the receptor.

To examine this possibility, the synthetic peptide methyl esters lacking an anionic charge at the C-terminal were subjected to a bioassay. The results are indicated as entries 5 and 6 in Table 2. Both of these penta-peptide methyl esters were completely inactive at doses of 5 and 100 μg. Consequently, the amide structure at the C-terminal of the peptide is concluded to have been essential for DH activity.

Table 2. Diapause-inducing Activity of Penta-peptide Analogs Expressed by the Average Number of Diapuse Eggs Layed by 5 Insects

	Dose (μg/pupa)	100	50	25	10	5	1	0.5
1	FGPRL-NH2	82.9	73.7	51.9	25.8	24.7	11.8	5.5
2	FSPRL-NH2	—	49.8	45.6	15.8	14.5	5.9	4.6
3	FVPRL-NH2	—	70.7	58.6	34.7	31	3.4	2.1
4	FIPRL-NH2	—	44.7	38.7	19.9	20	1.7	1.9
5	FGPRL-OMe	0	0	0	0	0	—	—
6	FIPRL-OMe	0	0	0	0	0	—	—
7	FIPRL-COOH	0	0	0	0	0	—	—
8	FGPRL-NHMe	0	0	0	0	0	—	—
9	FGPRL-N(Me)2	0	0	0	0	0	—	—
10	FGPRL-NH(n-Bu)	0	0	0	0	0	—	—

(—: not tested)

The next step was to discover whether modification of the amide nitrogen at the C-terminal of the peptide would affect the biological activity. To examine the effect of an alkyl substitution of the C-terminal amide hydrogen on the diapause-inducing activity, the three penta-peptide alkyl amides, FGPRL-N(R_1R_2) (entry 8: R_1=H, R_2=Me; 9: R_1=R_2=Me; 10: R_1=H, R_2=n-butyl), were prepared and their biological activities were tested. The results shown in Table 2 indicate that these analogs were completely inactive at doses of 5 and 100 μg/pupa, although they possessed the amide structure at the C-terminal. It was surprising that even the trivial change from a non-alkylated amide to a methyl amide completely extinguished the activity. The steric bulkiness of the substituted amide might have disturbed the interaction of the peptide with the DH receptor. Thus, we conclude that the non-substituted amide moiety at this position was very important for DH to exhibit its biological activity.

Effect of replacing amino acid X in FXPRL-NH$_2$ on the diapause egg-inducing activity

Some insect neuropeptides possess the common C-terminal penta-peptide sequence, FXPRL-NH$_2$, and amino acid X has no particular effect on the expression of different levels of diapause egg-inducing activity. To discover the real function of amino acid X, three peptide amides with different X components (entry 2, X=S; 3, X=V; 4, X=I in Table 2) were prepared to evaluate their biological activity. Their threshold doses were very close to each other and to that of 1 (about 0.5 n mole or 0.3 μg/pupa), and the activity of FXPRL-NH$_2$ was not affected to any large extent by substituting amino acid X from G to S, V or I. The hydrophilic nature, hydrophobicity or steric bulk of these amino acids was not important for the expression of biological activity. Instead, the various degrees of diapause egg-inducing activity of peptides with the FXPRL-NH$_2$ C-terminal were decided by the structure of the other parts. We thus propose the interesting hypothesis that the full structure of a penta-peptide amide is not essential for exhibiting biological activity and that the shorter peptide amide is responsible for diapause egg induction to a greater or lesser degree.

Diapause egg-inducing activities of the tetra-, tri- and di-peptide amides and their derivatives

To examine this hypothesis, three shorter peptides, GPRL-NH$_2$, PRL-NH$_2$ and RL-NH$_2$ were prepared and their biological activities were examined together with that of leucine amide. The results are shown as the dose response curves in Fig. 1, along with those of FGPRL-NH$_2$ and intact DH. The threshold dose for intact DH was about 0.015 n mole/pupa and that for the penta-peptide amide was about 0.5 n mole/pupa. Leucine amide and the di-peptide, RL-NH$_2$, were each complete-

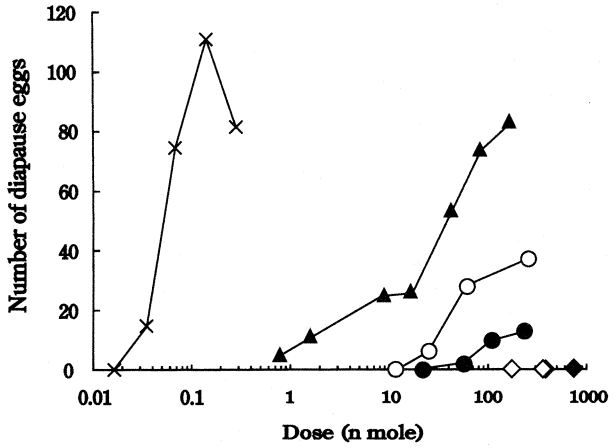

Fig. 1. Dose-response Curves for Diapause Hormone and Its Fragment Peptides.
× Bom DH-I [19-W], ▲ FGPRL-NH_2, ● GPRL-NH_2, ○ PRL-NH_2, ◇ RL-NH_2, ◆ L-NH_2

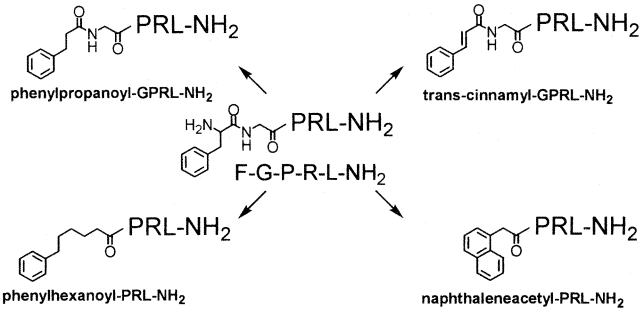

Fig. 2. Structures of FGPRL-NH_2 and the Derivatives of PRL-NH_2 and GPRL-NH_2.

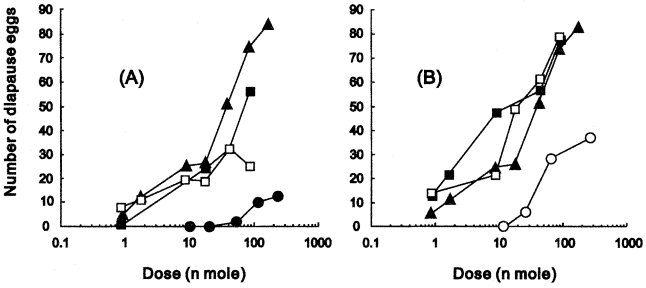

Fig. 3. Dose-response Curves for the Acylated Tri- and Tetra-peptide Amides.
(A), Acylated tetra-peptides ▲ FGPRL-NH_2, ● GPRL-NH_2, ■ Phenylpropanoyl-GPRL-NH_2, □ Cinnamyl-GPRL-NH_2
(B), Acylated tri-peptides ▲ FGPRL-NH_2, ○ PRL-NH_2, ■ Phenylhexanoyl-PRL-NH_2, □ 1-Naphthaleneacetyl-PRL-NH_2

ly inactive at doses of 40 and 800 n mole/pupa. These compounds might have been too-small molecules to exhibit any biological function and lack the essential partial structure(s) for the activity.

On the contrary, both the tetra- and tri-peptide amide showed weak but evident biological activity. Accordingly, we conclude that the tri-peptide amide, PRL-NH_2, was the smallest core structure with the ability to induce egg diapause in the polyvoltine silkworm, N4, and that the tri-peptide amide had the minimum characteristics to exhibit activity.

The threshold dose of the tri-peptide amide, PRL-NH_2, was estimated to be 10 n mole/pupa, being 700 and 20 times greater than those of DH and FGPRL-NH_2, respectively. The lower activity must have been due to a loss of the complementary parts. Comparing the structure of the tri-peptide amide and penta-peptide amide, it is evident that the former lacked Gly and Phe. The presence of Gly did not seem very important for enhancing activity, because the activity of GPRL-NH_2 was weaker still (the threshold dose was 20 n mole/pupa) than PRL-NH_2 and because the replacement of Gly in the penta-peptide amide with an other amino acid had no strong affect on the activity as shown in the previous section. Accordingly, Phe in the penta-peptide amide must have played a central role in the enhancement of activity. As the phenyl group of Phe seemed to strengthen the activity, the role of this group was examined by preparing aromatic fatty acyl derivatives of the shorter peptide amides. All the derivatives were designed to maintain the same distance through bonds between the phenyl group and the tri-peptide amide as that of Phe in FGPRL-NH_2 (Fig. 2), with the expectation that the aromatic moiety would act similarly to that of Phe and enhance the biological activity. The phenyl group is positioned seven bonds away from the nitrogen in Pro of the tri-peptide amide. The tetra-peptide amide was modified with a phenylpropanoyl and trans-cinnamyl group, and the tri-peptide amide was modified with a phenylhexanoyl and naphthaleneacetyl group. The results of biological activity tests are shown as dose-response curves in Figs. 3A and 3B. The dose response curves of FGPRL-NH_2, GPRL-NH_2 and PRL-NH_2 are also indicated for comparison. The threshold doses of phenylpropanoyl- and trans-cinnamyl-GPRL-NH_2 were around 0.7 n mole/pupa (Fig. 3A), and those of phenylhexanoyl- and 1-naphthaleneacetyl-PRL-NH_2 were about 0.5 n mole/pupa (Fig. 3B). These values are 30 and 20 times lower than those of the original non-modified tetra- and tri-peptide amides, respectively, and are very close to that of the penta-peptide amides.

These results suggest that the phenyl group of Phe in DH contributed to enhanced biological activity. Gly at the 4th position from the C-terminal of DH may be used as the spacer to keep the most suitable distance for biological activity between the phenyl group and the core structure of the tri-peptide amide. As all the derivatives tested in this work that lacked an amino nitrogen at the N-terminal position showed almost the same activity as that of the penta-peptide amide, the amino nitrogen in Phe is speculated to be used to connect the penta-peptide amide and the other part of DH, rather than to have any crucial role in the activity.

It was beyond our expectation that naphthaleneacetyl-PRL-NH_2 possessed similar activity to that of trans-cinnamyl-GPRL-NH_2. The benzene ring of the former is connected via a cis double bond in the naphthalene ring, while the latter is via a trans double bond, and these ben-

zene rings are expected to be located in different positions (Fig. 2). Although further experiments on the other derivatives are necessary to understand the reason why these two derivatives showed similar activities, the lipophilic environment around the modifying group may be important to enhance the biological activity of the shorter peptide amides. Similar enhancement of activity has recently been observed by Nachman *et al.*[10] in the case of a derivative of the C-terminal fragment of myotropin. They reported that a derivative of TPRL-NH_2, in which the phenyl group of Phe in the 5th position of the myotropin C-terminal was replaced with non-aromatic carborane, showed strong myotropic and pheromonotropic activity. Their result agrees with our present results, and both results show the importance of the lipophilic nature of the phenyl group of Phe at the position to enhance the activity. This lipophilicity may increase the affinity between the hormone molecule and the receptor.

Conclusions

This work has demonstrated that the smallest core structure of DH for exhibiting activity was the tri-peptide amide, PRL-NH_2, whose amide moiety was indispensable. The activity of this tri-peptide amide was enhanced to that of FGPRL-NH_2 by introducing the lipophilic benzene ring in a similar position to that of Phe in DH.

These findings have given us some hints to design new molecules with stronger diapause-inducing activity. A research program is in progress in our laboratories.

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