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Amino acid sequence of PIN peptides conducting TIME (Time-Interval-Measuring-Esterase) activation for resumption of embryonic development in the silkworm, *Bombyx mori*

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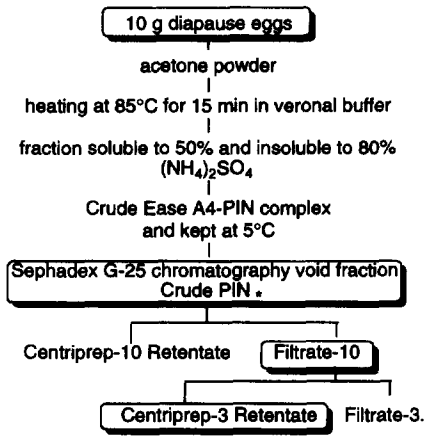
Abstract: Resumption of arrested development in the silkworm at the embryonic stage is parallel to the activation of TIME (Time-Interval-Measuring-Esterase), which initially exists as complex form with PIN peptide. TIME-PIN complex dissociates by exposing to low temperatures, then PIN was separated by ultra-filtration and chromatography. Amino acid sequences of 6 peptides, as PIN components varying from 28 to 38 residues, were determined by amino acid sequencer and by HPLC-ESI/MS. Their sequences are the parts of a common and the longest sequence of PIN, which is SIFMT KQHSQ DDIIQ HPLDY VEQQI HQQKQ KLQKQ TLN.

Diapause is a phenomenon for over-wintering, and in the silkworm, *Bombyx mori* the diapause is induced by diapause hormone (DH) which is secreted from suboesophageal ganglion. DH has been studied for long time[1] to conclude its structure to be peptide comprising 24 amino acids by Imai *et al.*[2] Necessity of the C-amide terminal was also studied, and several structure activity relationships have been known to have a sequence including FXPRL-NH_2.[3] After induction of the embryonic diapause, the embryonic cells carry diapause-specific metabolism, which is called as diapause development. A fixed period of exposure to cold temperature is essential for completion of diapause development. Recently Kai *et al.* reported one of esterases (Ease A4) in the diapause eggs exhibited transitory burst activation in a coincidental behavior with the completion of diapause development; namely, time-interval activation of Ease A4 by low temperatures, which is necessary for diapause termination.[4] Such change in Ease A4 activity was observed only in chilled diapause eggs and was not observed in diapause eggs without chilling nor in non-diapause eggs. In fact, the establishment of hatch ability in chilled eggs was observed after Ease A activity had reached the maximum level. Thus, the increase of Ease A4 activity could be regarded as associated with the termination of diapause *per se* but not with the subsequent process of post-diapause development.[4] The time interval activation of Ease A4 occurred suddenly at about two weeks of the egg chilling, and such activation also occurred during chilling of purified Ease A4 in the test tubes. The sudden elevation of Ease A4 activity *in vitro* was equivalent to that observed *in vivo* and was coincident with the chilling period which was indispensable for diapause termination.[5]

Ease A4 may exist in the eggs with a small peptide (named as PIN) to form a complex of 25 kDalton, which is heat stable even at 85°C for 15 min. The complex collapses into Ease A4 and PIN *in vitro* by keeping in cold for a few hours, so that these components become separable according to these thermal characters. Kai suggested that the Ease A4 may undergo conformational changes by an identical mechanism both *in vivo* and *in vitro*.[5c] Ease A4 was indicated to be a DNA-dependent ATPase that possessed some sort of time-measuring ability inherent in the molecule and so Kai has given TIME (Time Interval Measuring Esterase) for Ease A4.[5c] In the mixing of PIN with Ease A4, the clock-run of Ease A4 was delayed equally to the period of PIN inclusion.[5d] Removal of PIN was required for the initiation of the timer activation of Ease A4. The function

of PIN was to hold the timer probably by forming Ease A4-PIN complexed substructure. The interaction between Ease A4 and PIN differed greatly in temperature, and only in cold PIN and Ease A4 may dissociate from each other.[5d] This paper deals with the elucidation of amino acid sequence of PIN peptides.

Those diapause eggs laid by diapause egg producers, C108 strain of the silkworm, *Bombyx mori*, were used as material source for PIN and Ease A4 according to the method described by Kai *et al.*[5c] The acetone powder from the diapause eggs was purified by heat treatment at 85°C, precipitation from 80% saturated ammonium sulfate and gel filtration through Sephadex G-25 to provide Crude PIN.[5a] Molecular cut-off membrane (Centriprep-10 or C.-3, Amicon, U.S.A.) was used to separate crude PIN from high molecular weight components in the void volume. Standard procedure is summarized in **Scheme 1**. PIN activity was routinely assayed as the relative inhibition of Ease A4 activity as previously described.[6,7] An example is shown in **Fig. 1**.



Scheme 1. Preparation of crude PIN

* According to Kai *et al.*[5a]

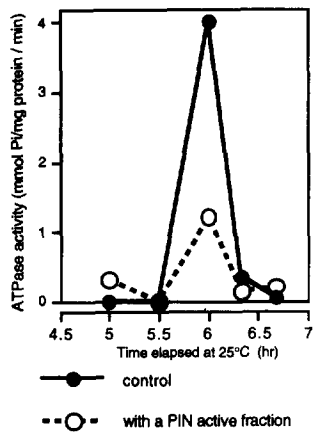


Fig. 1. Example of Assay[6]

Curves depict changes in Ease A4 activities without (solid circle) and with PIN fraction (open circle); showing inhibitory effect by PIN fraction in ca. 70% decrease.

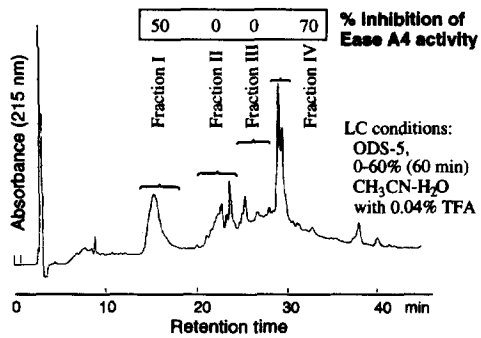


Fig. 2 Chromatogram of Filtrate-10

ODS-column (4.6 x 250 mm), a linear gradient (0-60%) CH_3CN-water (0.04% TFA) for 60 min, monitored at 215 nm.

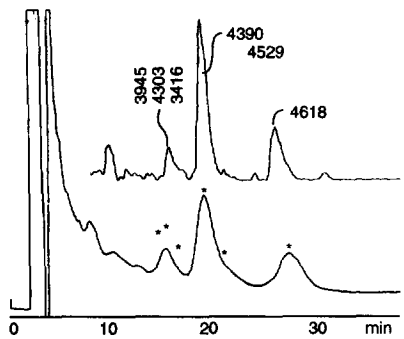


Fig. 3 HPLC and LC-ESI/MS[7]

ODS-column (4.6 x 250 mm), isocratic (20.2 %) CH_3CN-water. Monitor: lower, 215 nm; upper, total ion mass.[7b,8]

Filtrate-10 or Retentate-3 of Crude PIN was firstly fractionated on a Develosil ODS-5 column (4.6 x 250 mm).[8] A typical chromatogram showing fractionation of peaks of Filtrate-10 is shown in Fig. 2. The sample was fractionated into 4 fractions (Fractions I-IV) as indicated by "{". Fraction IV showed the highest

inhibition activity (70%) whereas Fraction I showed only half activity. But Fractions II and III were non-active.[6] Further analysis of Fraction I did not give sharp peak, which was no-further purified. The electrophoretic mobility in Tricine-SDS-PAGE of Fraction IV was equal to the major band of crude PIN (data not shown). Hence, Fraction IV should contain PIN peptide(s).

Fraction IV (either from Filtrate-10 or Retentate-3) was further separated with HPLC on an ODS column.[7a,8] A typical chromatogram (**Fig. 3**, lower chromatogram) shows fractions with asterisks that indicate those with amino acid sequence analyses.[7c] The resulting sequences 1 to ca. 30 were classified into 3 groups; namely, starting from (1) IFMTK..., (2) SIFMTK... and (3) SQDDI... as summarized in **Table 1**. In **Table 1**, the amino acid dictation of PIN was clear in the beginning 1-30, but not so clear toward the end. Since these peptides seemed to have common portion among fractions, we employed HPLC-ESI/MS (Electrospray Ionization Mass Spectrometry) instrument to ensure the sequences toward the C-termini.[7d,9]

Table 1 Correlation of amino acid sequences and observed MWs of Fraction IV components

group	Amino acid sequence matching with MW 1234567890 1234567890 1234567890 12345678	Observed MW (M ± S.D.)	Calc. MW[9] average mass
I	IFMTKQHSQ DDIIQHPLDY VEQQIHQQKQ KLQKQTLN	4530.04 ± 0.12	4531.13
	IFMTKQHSQ DDIIQHPLDY VEQQIHQQKQ KLQKQT	4302.90 ± 0.08	4303.87
	IFMTKQHSQ DDIIQHPLDY VEQQIHQQKQ KLQ	3946.19 ± 0.00	3946.46
II	SIFMTKQHSQ DDIIQHPLDY VEQQIHQQKQ KLQKQT	4390.00 ± 0.00	4390.95
	SIFMTKQHSQ DDIIQHPLDY VEQQIHQQKQ KLQKQTLN	4617.84 ± 0.40[7e]	4618.21
III	SQ DDIIQHPLDY VEQQIHQQKQ KLQKQT	3416.47 ± 0.50	3417.78

Sequence analysis of components of Fraction IV disclosed that they have only one common sequence. These peptides differed only in their chain length of both N- and C-termini. The whole sequence of each peptide correlating to its observed molecular weight is summarized in **Table 1**. These data suggest that the longest sequence of PIN consisted of 38 amino acids as: **SIFMT KQHSQ DDIIQ HPLDY VEQQI HQQKQ KLQKQ TLN**. Shorter chain lengths than 38 amino acids were found such as 37, 36, 35, 32, and 28 residues. The N-terminus of these peptides started either from Ser at sequence no. 1, Ile at sequence no. 2 or Ser at no. 9. The C-terminus mostly ended at no. 36, but also at no. 33 or 38. The 38 residual PIN was always not the major constituents.

Table 2 Homology of AA sequence between PIN and Egg-Specific Protein (ESP) in *Bombyx mori*

Gene /Peptide	Amino acid sequence
	1 40
ESP	MKTIYALLCL TLVQSISCSI FMTKQHSQDD IIQHPLDYVE QQIHQQKQKL QKQTLNKRSH QHSDSDSDSA .....
PIN	SI FMTKQHSQDD IIQHPLDYVE QQIHQQKQKL QKQTLN

It is of great interest that the full sequence of PIN is 100% identical to a portion of the deduced amino acid sequence of Egg-Specific Protein (ESP) of the silkworm, *Bombyx mori*, which had been deduced from ESP gene by Sato.[10] The beginning 70 sequential part of ESP is referred in **Table 2**. The sequence of PIN relates to the one of ESP from the 19th to the 56th amino acid residues in the whole deduced 559 residues, in which the 1st-18th residues are likely to be a signal peptide and the 57th-58th residues (K-R) are possibly a cleavage site sequence. Thus, it is the most possible that the resulted peptide after processing from this precursor protein would be PIN. Although PIN has its amino acid sequence identical to a portion of ESP, it has never been reported before to be a component of the ESP molecule[11] nor any known peptides. PIN has its own regulatory function on Ease A4 activity which is different from those of egg-specific protein.[12]

Consequently, PIN plays a quite important role through the association-dissociation with TIME-protein upon long time-cold treatment, that is essential for the resumption of embryonic development in the silkworm, *Bombyx mori*. The study on PIN should help further elucidation of the sustaining mechanism of diapause in the silkworm. Thus, this paper has focused on the chemical structure of PIN. We have obtained a preliminary result that synthetic peptides having identical partial sequences to the PIN did inhibit Ease A4 activity. Further studies on structure activity relationship are in progress, and will be reported in a near future.

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6. Assay methods: Each tested peptide or PIN sample was dissolved in a phosphate assay buffer pH 7.4 before being added to the Ease A4 solution. Then, the mixture was transferred to 25°C and measured the changes in ATPase activity of Ease A4 during keeping at 25°C. Amount of each fraction tested per amount of Ease A4 was equal to their amounts obtained from the same starting quantity of eggs. See also: Carter, S. G; Karl, D. W. *J. Biochem. Biophys. Methods.*, **1982**, *7*, 7-13; for inorganic phosphate assay with malachite green.
7. Equipment: (a) HPLC; a Gulliver HPLC chromatograph equipped with an ODS column (Develosil) two PU-980 HPLC pumps, a UV-970 UV-VIS wavelength detector and an 807-IT integrator (JASCO, Tokyo). (b) under an isocratic condition of 20.2 % (or 19.3 %) acetonitrile-water containing 0.04% TFA at a flow rate of 0.7 (or 0.8) ml/min. (c) Protein Sequencer; an ABI model either 477/120A or 476A with 610A Data Analysis System, (Applied Biosystems Inc., U.S.A.). (d) LC-ESI/MS; a model VG Platform (FISONS Ins., U.K.), quadrupole mass spectrometer having MassLynx software for operation and data analysis; a model VG QUATTRO-SQ in some experiments. (e) Analyses were carried out with other lot of sample due to very trace amount of sample being available.
8. The similar mobile phase 19.3 % CH_3CN-water (0.04% TFA) at a flow rate of 0.8 ml/min were also applied to Retentate-3 and Fraction IV of Retentate-3.
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