Molecular Heterogeneity of TIME-EA4, a Timer Protein in Silkworm Diapause Eggs*

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TIME-EA4 is an ATPase that measures time intervals as a diapause-duration clock found in diapause eggs of the silkworm, Bombyx mori. In the current studies, we report the molecular heterogeneity of TIME-EA4 protein regarding not only amino acid L62V, but also the numbers and linkage patterns of the sugar chain attached to the Asn22 residue. These sugar chain structures were determined in a picomolar amount of the protein by combining the methods of chemical modification (Smith degradation) and nano-HPLC-electrospray ionization-quadrupole-time-of-flight-mass spectrometry (ESI-Q-TOF-MS) and -MS/MS. The Japanese and bi-voltine Thai silkworm strains were compared to show the heterogeneity represented by four kinds of molecular species. Judicious choice of the combination methods led us to find the first example of a linkage position difference in the glycosidic bonds even in sugar moieties of the same molecular weight; thus, the Man(1-6)Man(1-4)GlcNAc(1-4)GlcNAc structure in the C108 pure strain and Man(1-3)Man(1-4)GlcNAc(1-4)GlcNAc structure in the Kinshu-Showa hybrid. A total of five kinds of molecular heterogeneity was determined, including the amino acids in TIME-EA4 protein. This paper describes the details for determining the sugar chain linkage in TIME-EA4 from the diapause eggs of various silkworm strains.

Key words: molecular heterogeneity; metallo-glycoprotein; glycoside bond heterogeneity; Smith degradation; timer protein

An ATPase, called TIME-EA4 (time interval measuring enzyme-esterase A4), was first found in diapause eggs of the silkworm, Bombyx mori, C108 pure strain. This timer protein is responsible for measuring the diapause duration as a key to embryonic diapause.3 The primary and higher structure of TIME-EA4 should be characterized in order to clarify the mechanism by which the protein measures the time intervals. In previous studies, the whole sequence of TIME-EA4 has been established as a metallo-glycoprotein by combined methods of peptide sequence analysis, nano-HPLC-ESI-Q-TOF-MS (electrospray ionization quadrupole time-of-flight mass spectrometry) and -MS/MS, and cDNA dictation.3) The TIME-EA4 protein showed a 46–55% identity from an amino acid sequence homology search (the BLAST algorithm) with Cu,Zn-SODs (superoxide dismutases); in particular the SOD active site (core domain) included metal-holding amino acid ligands and a disulfide bond, and these structures were completely identical in Bombyx SOD, bovine SOD and TIME-EA4 proteins.3) We have also proposed a computer-generated 3D structure on the basis of the fact that only one folding structure was found by overlaying all the main chains from the 25 reported X-ray crystal structures as Cu,Zn-SOD. This seems identical to the computer-generated structure3) and crystal structure forming the gene-overexpressed one reported recently by Hiraki et al., except for the sugar chain (not shown in the X-ray data).3) We found, however, that TIME-EA4 contained an additional copper atom (total of three metals; 2 × Cu and 1 × Zn) compared to other Cu,Zn-SODs that was proven under neutral non-denaturing conditions. As revealed through ESI mass spectrometric studies, the timer of TIME-EA4 was not in the SOD core domain. In addition, TIME-EA4 extends a sugar chain, which is indispensable to functioning as a timer protein, for the protein-folding element. We have also reported that the sugar chain of TIME-EA4 was essential for its binding with the time-regulating peptide PIN.3,5) It is therefore important to establish the structural background of the attaching sugar chain in relation to the

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Abbreviations: ESI-Q-TOF-MS, electrospray ionization-quadrupole-time of flight-mass spectrometry; BLAST, basic local alignment search tool; SOD, superoxide dismutase; PPG, preliminary packed gradient; ODS, octadecylsilane; ox/rd, oxidative/reductive
TIME-EA4 timer function. We have already reported that the N-linked sugar chain of TIME-EA4 was attached at Asn22.6 Furthermore, we have already confirmed that the TIME-EA4 sugar chain had an affinity to ConA lectin and had been cleaved with PNGase F.6 In the case of the silkworm C108 pure strain, digesting TIME-EA4 with trypsin produced 14 peptide fragments (respectively named T1 to T14 according to the amino-acid sequence) which was determined by a nano-HPLC-ESI-Q-TOF-MS analysis.7 Since the T5 and T14 peptides were observed as one T5–S–S–T14 fragment connected by a disulfide bond (Cys61–Cys150), this was reduced to each T5 and T14 by treating with dithiothreitol (DTT). Since T12 contained only one lysine residue, T12 was observed as T12→T13. On the other hand, we have observed the tetra-saccharide (Man1–6)[Man1–3]Man1–4GlcNAc1–4GlcNAc7) In that the penta-saccharide of TIME-EA4 was branched as another silkworm of the Showa strain, by using the same methodology.7) This methodology led us to conclude that the TIME-EA4 sugar chain had an N-linked tetra-saccharide (2Man+2GlcNAc) sugar chain linked to the T3-peptide (m/z=1019.44 [M+2H]2+), calculated as [M+H]+ = 2037.88; corresponding to the sum of peptide portion G2NITITQVQDGK at m/z = 1307.7 and tetra-sugar portion 730 = 2037.7) as reported previously.1,6 However, we only assumed that it had an N-linked tetra-saccharide (2Man+2GlcNAc) with no more details.1,6 The linkage-pattern was therefore unclear. We have subsequently established a new methodology for determining the sugar linkage on the basis of Smith degradation (NaIO4 and then NaBH4) combined with nano-HPLC-ESI-Q-TOF-MS and -MS/MS.6,8 We then examined the penta-saccharide-T3 peptide of TIME-EA4, which was also obtained from another silkworm of the Showa strain, by using the same methodology.7) This methodology led us to conclude that the T3 peptide of TIME-EA4 was branched as Man(1→6)[Man(1→3)]Man(1→4)GlcNAc(1→4)GlcNAc7). In that study, we determined the tetra-saccharide structure of TIME-EA4 by using our methodology of LC-MS and -MS/MS in a comparison of samples without and with the Smith degradation. In our current studies, we report that the sugar chain of TIME-EA4 had many varieties among different silkworm strains. An amino acid residue was also found to vary at the 62nd position located in the T5 peptide. In silkworm diapause eggs, these varieties might lead to a slight difference in measuring the synchronized time interval of the diapause duration on the TIME-EA4 timer. Since the time-regulating PIN peptide showed extremely strong binding affinity (Kd = 460 nm) with the sugar chain,7,10 this heterogeneity would also be related to the diversity of the limited number of hatching days of the diapause eggs, thus ending up by ensuring the copulation period.

Materials and Methods

Instrumentation. MS and MS/MS data were recorded with a Q-TOF (quadrupole time-of-flight) mass spectrometer (Micromass, Manchester, UK) fitted with a Z-spray ESI (electrospray ionization) source having a molecular weight resolution of about 8000. All experiments were performed in the positive ion mode. Data were acquired and processed by using MassLynx versions 3.4 and/or 4.0. LC-MS and MS/MS experiments were conducted by utilizing the appropriately adjusted nano-HPLC system (Jasco, Tokyo, Japan). The columns used were Develosil C30-UG-5 and ODS-HG-5 (Nomura, Seto, Aichi, Japan; 15 cm × 0.3 mm i.d.). The columns were equilibrated with water (260 μl) containing 0.025% trifluoroacetic acid at a flow rate of 10 μl/min and then developed with a linear gradient by the PPG method (preliminary packing gradient in 500 cm × 50 μm i.d. capillary tubing) from 0% to 100% of acetonitrile containing 0.025% trifluoroacetic acid for 40 min at a flow rate of 5 μl/min without flow splitting. The column effluent was monitored at 210 nm with a capillary UV detector and then introduced into the electrospray nebulizer for MS.

Materials. TIME-EA4 was from diapause eggs of the silkworms, Bombyx mori (synchronous egg batches of genetically pure strain C108), four Japanese strains (Sasamayu, Showa, Kinshu, and Showa-Kinshu) and five strains of Thai silkworm diapause eggs (TH-1, -2, -3, -4, and -5). Dithiothreitol (DTT), acetonitrile (HPLC grade), sodium metaperiodate (NaIO4), and trifluoroacetic acid were purchased from Nacalai Tesque (Kyoto, Japan). Trypsin (sequence grade) was purchased from Roche Diagnostics (Mannheim, Germany), and sodium borohydride (NaBH4) was purchased from Wako (Osaka, Japan).

Preparation of TIME-EA4. TIME-EA4 was prepared from each of the Bombyx diapause eggs according to the procedure established by Kai et al.9 For further purification, the crude TIME-EA4 precipitate with 80% saturated ammonium sulfate was treated in a HiTrap chelating column (5 ml; Amersham Pharmacia Biotech). The purification was performed according to the operation manual supplied by the manufacturer. TIME-EA4 was then eluted in a phosphate buffer at pH 4–5. Subsequently, the TIME-EA4 fraction was concentrated by ultrafiltration of the sample solution through Centricron YM-10 (Millipore, Billerica, MA, USA). After ultrafiltration, the TIME-EA4 solution (1 μl) was analyzed by means of nano-HPLC-ESI-Q-TOF-MS.

Trypsin-digestion and cleavage of the disulfide bond. The solution of TIME-EA4 (8 μg, 458 pmol) in a phosphate buffer (17 μl, 59 mM, pH 7.2) was heated at around 90 °C for 5 min to denature the protein. After cooling in an ice-water bath, a solution of trypsin (0.4 μg, 17 pmol) in water (3 μl) was added, and the mixture incubated at 37 °C for 18 h. After this incubation, the mixture was again heated at 90 °C for 5 min to deactivate trypsin. The solution of trypsin-digested TIME-EA4 (1 μl) was injected for analysis by nano-HPLC-ESI-Q-TOF-MS and -MS/MS. To cleave the disulfide bond, the solution of trypsin-digested TIME-EA4 (40 μl) was further treated with dithiothreitol (DTT; 1.2 μl, 175 mM in water). The mixture was incubated at 37 °C for 2 h. The MS/MS analysis of the trypsin-digested T3 peptide used 10 μl of the solution applied to nano-HPLC-ESI-Q-TOF-MS.

Smith degradation. We have already reported the established procedure of Smith degradation combined with LC-MS and tandem MS (MS/MS).7,8 In addition, the structure of the penta-saccharide in the glycoprotein from TIME-EA4 has been determined by utilizing our methodology.7 Therefore, in this study, we examined under similar conditions according to the method of Pitchayawasin and Isobe.4 A solution of TIME-EA4 in a phosphate buffer at pH 4–5 was mixed with 0.08 m sodium metaperiodate (NaIO4, to a final conc. of 0.02 m) in 0.1 m sodium acetate buffer at pH 4, and then the mixture was incubated to oxidize it for 3 d at 4 °C in the dark. After oxidation, in order to decompose the excess metaperiodate reagent, the solution was mixed with 3.2 m ethylene glycol (to a final conc. of 0.3 m; Nacalai Tesque), and then the mixture was incubated overnight at 4 °C. The solution of oxidized TIME-EA4 was brought to pH 7 with 0.1 m NaOH and then mixed with 0.1 m sodium borohydride (NaBH4; to a final conc. of 0.03 m) in borate buffer at pH 9. The mixture was incubated to reduce it overnight at 4 °C. After this reduction in the dark overnight at 4 °C, to decompose the borohydride, the solution was stirred with 4% trifluoro acetic acid and then applied to a mini-open column containing ODS (Cosmosil 75 C18 OPN; Nacalai Tesque) for desalting. The ox/rd-TIME-EA4 fraction was dried in a nitrogen gas flow.

Results

Molecular heterogeneity of TIME-EA4

In our previous studies, the total molecular weight of TIME-EA4, which had been obtained from Bombyx diapause eggs of the C108 pure strain, was 17336.79 Da.6 On the other hand, the ESI-MS results from other silkworm strains such as Showa and Kinshu indicated that TIME-EA4 had four different molecular masses,
corresponding to 17323 (or 17322), 17337 (or 17338), 17485, and 17499 Da (Fig. 1). According to the calculated mass difference between these proteins, differences of 14 and 162 Da could be found: 14 Da arose between 17323 and 17337, and between 17485 and 17499; 162 Da also arose between 17323 and 17485, and between 17337 and 17499. On the other hand, we have already confirmed that TIME-EA4 from the C108 strain had an attached tetra-saccharide,1,6) and TIME-EA4 from the Showa strain had not only a tetra-saccharide but also a penta-saccharide.7) It can therefore be assumed that the difference of 162 Da in Fig. 1 came from one hexose unit. By using the difference of 162 Da, the four kinds of molecular mass could be classified as a tetra-saccharide group (17323 and 17337) and a penta-saccharide group (17485 and 17499). Furthermore, the T3 glycopeptides of TIME-EA4 from both the C108 pure and Kinshu strains instead of the Showa strain, after trypsin-digestion, were analyzed by means of non-split flow LC-ESI-Q-TOF-MS/MS to find the T3 glycopeptides eluted at an earlier retention time from a column (15 cm × 0.3 mm i.d.; flow rate of 3–5 μl/min; gradient 0–100% CH3CN). Figure 2 shows that the T3-tetra-saccharide from the C108 pure strain was observed only at m/z 2038.3, and that the T3-tetra-saccharide from the Kinshu strain was observed at m/z 2200.3, having an additional hexose unit. This hexose is assumed to have been a mannose, but there is no evidence for this in the MS analysis. However, we have already assigned the penta-saccharide of Showa strain TIME-EA4 to branch as Man(1-6)[Man(1-3)]Man(1-4)GlcNAc(1-4)GlcNAc.7) It is therefore likely that the current position corresponded to a tri-mannosyl core unit in the N-linked sugar chain. Figure 1 shows that both the tetra-saccharide group and penta-saccharide group had a mass difference of 14 Da (between 17323 and 17337 Da). The T5-S-S-T14 fragment from the Kinshu strain after trypsin-digestion was observed at both m/z 928.8 ([M + 3H]3+; calculated [M + H]+ = 2784.34) and m/z 924.1 ([M + 3H]3+; calculated [M + H]+ = 2770.3).
differences of 14 and 162 Da would come from differences of 14 and 162 Da in the molecular weights. These strains indicated four kinds of molecular homologues makes it likely that the mother’s information is predominant.

**Thai silkworm diapause strain**

The Thai silkworm strains (TH-1, -2, -3, -4 and -5) were also examined on the basis of molecular heterogeneity of the TIME-EA4 protein. The native silkworm in Thailand is usually of the non-diapause (polyvoltine) race, but some bi-voltine races can be found by raising them under specific conditions to let them lay diapause eggs. We prepared diapause eggs with the foregoing methods. Four different molecular weights were observed by means of ESI-Q-TOF-MS as shown in Table 5. Based on the classification in Table 1, we assumed the following heterogeneity of the TH strains, although the sample amount prevented detailed experiments to be conducted. The TIME-EA4 protein from TH-5 predominantly showed the mass at 17338 Da. This value is the same as our originally reported value from the C108

![Fig. 4. ESI-Q-TOF-MS/MS Data for the Trypsin-digested T5 Peptides.](image)

A, the precursor ion at m/z 648.3, in the triply charged state, was obtained from the C108 pure strain; B, the precursor ion at m/z 643.6, in the triply charged state, was obtained from the Kinshu strain; and C, the precursor ion at m/z 643.6, in the triply charged state, was also obtained from the Kinshu strain. The T5 peptide was observed after reductive cleavage of the disulfide bond by a treatment with DTT. These MS/MS data were obtained at a cone voltage of 30 V and variable collision energy between 40 and 51 V.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Val62</th>
<th>Leu62</th>
<th>Val62</th>
<th>Leu62</th>
</tr>
</thead>
<tbody>
<tr>
<td>C108</td>
<td>17,323</td>
<td>17,337</td>
<td>17,485</td>
<td>17,499</td>
</tr>
<tr>
<td>Sasamayu</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Showa</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Showa-Kinshu</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Molecular mass gives rise to within a 2 mass difference, because the resolution of Q-TOF MS is ~8000.

![Table 1. Varieties of TIME-EA4 Proteins](image)

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In our previous study, the penta-saccharide of TIME-EA4 from the Showa strain was reported as being Man(1-6)[Man(1-3)]Man(1-4)GlcNAc(1-4)GlcNAc, consisting of a tri-mannosyl core structure.7) In this study, Bombyx diapause eggs of the Showa-Kinshu strain, which is an F1 hybrid between the Showa and Kinshu strains, showed both tetra- and penta-saccharides of TIME-EA4. We next examined whether or not the tetra-saccharide of the Showa-Kinshu strain was similar to that of the C108 pure strain with special reference to the 1-6 linkage between the terminal and internal Man residues. Although we obtained ESI-Q-TOF-MS data corresponding to the T3-tetra-saccharide from the Showa-Kinshu strain with/without SD, the fragment ions obtained from the T3-tetra-saccharide were complicated by including products from the T3-penta-saccharide fragment. It goes without saying that TIME-EA4 of the C108 pure strain did not have heterogeneity on the attached sugar chain, in contrast to TIME-EA4 of the Showa-Kinshu strain which had heterogeneity, having two kinds of sugar chain corresponding to tetra- and penta-saccharides. In order to determine the tetra-saccharide structure from the Showa-Kinshu strain, the two precursor ions of the T3-tetra-saccharide at m/z 1019.55 ([M + 2H]^{2+}) and 1005.50 ([M + 2H]^{2+}) obtained without and with SD, respectively, were analyzed by nano-HPLC-ESI-Q-TOF-MS/MS. Figure 8 illustrates the ESI-Q-TOF-MS/MS data for the T3-tetra-saccharide from the Showa-Kinshu strain which were respectively observed as the characteristic fragmentation pattern produced from the similarly prepared precursor ions at m/z 1019.55 ([M + 2H]^{2+}) and 1005.50 ([M + 2H]^{2+}) without/with SD. When comparing with Table 3, the terminal Man residue was found to be a 134-Da mass loss, instead of internal Man with the 162-Da mass loss, even after SD. The 162-Da mass loss after SD means that the internal Man residue had a substitution at the 3 position (a 1–3 linkage) or substitution at both the 3 and 6 positions (a 1–3,5 linkage).8) In this case, internal Man combined with terminal Man by a 1–3 linkage, because a tetra-saccharide never has a tri-mannosyl core structure. Thus, in Fig. 9, we show that the linkage pattern of the tetra-saccharide from the Showa-Kinshu strain was Man(1-3)Man(1-4)GlcNAc(1-4)GlcNAc.

### Discussion and Conclusion

The ESI-MS data for the C108 pure strain has shown TIME-EA4 at 17338 Da.1,6) In this study, we examined exactly how many kinds of heterogeneity could be observed among various silkworm strains. ESI-Q-TOF-MS data for TIME-EA4 from these strains indicated four kinds of molecular mass, corresponding to 17233, 17337, 17485, and 17499 Da (Fig. 1). These differences occur from variation of the attached sugar chain, corresponding to tetra- and penta-saccharides, and a lower homologue, corresponding to Leu and Val. We therefore concluded that TIME-EA4 had molecular heterogeneity among various kinds of silkworm strain. Thai silkworm diapause eggs also have heterogeneity of the TIME-EA4 protein, like the Sasamayu, Kinshu, Showa, and Showa-Kinshu strains. In addition, a lower homologue of the Thai silkworm’s TIME-EA4 protein

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**Figure 5.** Comparison of the ESI Mass Spectra of TIME-EA4 among Thai Silkworm Diapause Eggs. A, TH-5; B, TH-4; C, TH-3; D, TH-2; and E, TH-1. Mass spectra were produced by MaxEnt 1 processing of multi-charged molecular ions.
was also confirmed by using the in-source-fragmentation technique for the ESI-Q-TOF-MS analysis. This technique has turned out to be very useful for determining the Pro–Pro co-sequence in large molecules without enzymatic digestion.

In the current studies of the T3-tetra-saccharide, the terminal Man residue from the C108 pure strain was determined to have a 1–6 linkage with an internal Man residue, and the terminal Man residue from the Showa-Kinshu strain was determined to have a 1–3 linkage from the LC-MS and -MS/MS data without/with SD (Smith degradation). The C108 pure and Showa-Kinshu strains have different sugar chain structures, although having the same numbers of saccharides with the same molecular weight at $m/z = 1019.47$ ($\frac{1}{2}M + H^+ : 1019.47$) (Figs. 6 and 8). This result indicates the heterogeneity in TIME-EA4 of molecular weight 17337 Da. In addition, the sugar chain of TIME-EA4 also has a penta-saccharide, making the total pattern of the TIME-EA4 sugar chain to be of three types.

We have demonstrated in a previous study that the carbohydrate moiety of TIME-EA4 was indispensable for its molecular binding with the time-regulating peptide PIN. The region of the N-terminal must be significant in regard to the interaction between the PIN peptide and the carbohydrate moiety of the TIME-EA4 timer. In investigations of the interaction between TIME-EA4 and the PIN peptide, only TIME-EA4 from the C108 pure strain has been used as the test sample until now. The result of having heterogeneity of TIME-EA4 suggests that other sugar chains, for example Man(1–3)Man(1–4)GlcNAc(1–4)GlcNAc, could interact with the PIN peptide as well as C108 TIME-EA4. The binding constant of the TIME-EA4–PIN complex is 460 nM in the case of C108. This value might shift slightly higher or lower among the different sugar chains, so that interaction between TIME-EA4 and the PIN peptide might also change the time interval to the egg hatching day. In respect of the TIME-EA4–PIN complex, having heterogeneity is of great significance for investigating the time interval measurement mechanism of the

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**Table 2.** Assignment of MS Fragments of the Trypsin-digested T3-Tetra-saccharide from the C108 Pure Strain without and with Smith Degradation

<table>
<thead>
<tr>
<th>Fragment of T3-tetra-saccharide</th>
<th>Without Smith degradation precursor, $m/z$ 1019.47</th>
<th>With Smith degradation precursor, $m/z$ 991.49</th>
</tr>
</thead>
<tbody>
<tr>
<td>[M + H]$^{+\dagger}$</td>
<td>Obs. [M + 2H]$^{2+\dagger}$</td>
<td>Mass loss</td>
</tr>
<tr>
<td>T3 + 2GlcNAc + 2Man</td>
<td>(2037.94)</td>
<td>1019.47</td>
</tr>
<tr>
<td>T3 + 2GlcNAc + Man</td>
<td>(1875.92)</td>
<td>938.46</td>
</tr>
<tr>
<td>T3 + 2GlcNAc</td>
<td>(1713.82)</td>
<td>857.41</td>
</tr>
<tr>
<td>T3 + GlcNAc</td>
<td>(1510.76)</td>
<td>755.88</td>
</tr>
</tbody>
</table>

*aCalculated from the doubly charged ion.*

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**Fig. 6.** ESI-Q-TOF-MS Data for the Trypsin-digested T3-Tetra-saccharide, Obtained from the C108 Pure Strain, (A) with and (B) without Smith Degradation. The MS data were obtained at a cone voltage of 30 V.

**Fig. 7.** The Tetra-saccharide Structures, Attached with the Trypsin-digested T3 Peptide from the C108 Pure Strain, Consisting of Two Mannoses and Two N-Acetylglucosamines. A, without and B, with Smith degradation.
TIME-EA4 protein. It is a first step to measure the time for TIME-EA4 to dissociate from TIME-EA4–PIN complex with stimulation from a cold temperature. In general, Bombyx diapause eggs, after completing diapause development, synchronously hatch among those that were subjected to the same temperature. TIME-EA4 is a key protein for measuring the duration time for the diapause. With the same kind of silkworm strain, it is necessary to measure an identical time interval to complete the diapause development. In contrast, between different kinds of silkworm strain, we wondered whether an identical duration time for the diapause needed measuring or not. We consequently propose that the heterogeneity of TIME-EA4 requires measuring various interval times which would result in a longer copulation period for the short lifetime (adult < 1 week).

Hiraki et al. have recently reported the crystal structure of TIME-EA4 expressed from the gene originally obtained from diapause eggs of the silkworm hybrid between Shunrei and Shogetsu. This is similar to the 3D structure that we reported in 2006 by a computer-generated analysis, except for the monomeric or dimeric form. Therefore, the variety of the sugar chain structure might generate different interval times for interaction with the PIN peptide.

The heterogeneity on the attached sugar chain of TIME-EA4 involved three kinds of structure, corresponding to Man(1-6)Man(1-4)GlcNAc(1-4)GlcNAc, Man(1-3)Man(1-4)GlcNAc(1-4)GlcNAc, and Man(1-6)[Man(1-3)]Man(1-4)GlcNAc(1-4)GlcNAc. In addi-
tion, peptide heterogeneity having a lower homologue was found at the 62nd position of Leu in the TIME-EA4 protein. Although total of six types of molecular heterogeneity of TIME-EA4 could be calculated from 3 kinds of sugar chain in combination with 2 kinds of peptide ($3 \times 2 = 6$), the C108 pure strain having TIME-EA4 attached to Man(1-6)Man(1-4)GlcNAc(1-4)GlcNAc has not shown the lower homologue so far. Consequently, the heterogeneity of TIME-EA4 involves five kinds of molecular variety at present.

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**References**