THE INITIATOR AMINO ACID IN SILK FIBROIN BIOSYNTHESIS

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1. Introduction

At the fifth larval instar of *Bombyx mori*, L., silk fibroin is synthesized exclusively in the posterior silk glands which may provide a good system for studies of the initiation of protein biosynthesis [1]. In a previous work [2] it was demonstrated that one of the two methionine tRNAs, which occurs in the cytoplasm of the posterior silk gland of silkworm, was formylated with transformylase [EC 2.1.2.9] of *Esherichia coli* in the presence of formyl donor. The suggestion has been presented that methionine is a possible initiator amino acid in the fibroin synthesizing system as in eukaryotic systems [3–7].

The present paper deals with N-terminal amino acid analysis of nascent peptides from pulse-labeled peptidyl–tRNA in the posterior silk glands. These revealed the occurrence of methionine in the N-terminal residue.

2. Materials and methods

Larvae of silkworm, *Bombyx mori*, L., hybrid from Japanese and Chinese strains (Taihei × Choan), on the fourth day of the fifth instar were dissected, and the posterior silk glands were collected as described previously [8]. L-[methyl-^{14}C]methionine (56 mCi/mmole) and L-[U-^{14}C]cysteine hydrochloride (336 mCi/mmole) were obtained from the Radiochemical Centre, Amersham, England. L-[U-^{14}C]amino acid mixture (Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Tyr, Val, 100 mCi/mmole) was purchased from ICN Chemical and Radioisotope Division, USA.

Five hundred mg (wet weight) of the posterior silk glands were preincubated for depletion of endogenous amino acids in a medium (4 ml) containing 20 mM phosphate buffer (pH 7.4), 86 mM NaCl, 6.8 mM $CaCl_2$, 0.4 mM $MgCl_2$, and 0.25 mM α-ketoglutaric acid. The amount of free amino acids in the glands decreased rapidly, and reached a nearly minimum level in the first 30 min, and stayed at a similar level for the next 30 min (unpublished data). A suitable preincubation time therefore is 30 min. Incubation was then performed in the presence of labeled amino acids (1 μCi of each of the following; Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val) at 30°C for 2 min. The glands were disrupted with 4% sodium dodecyl sulfate and shaken with an equal volume of phenol saturated with 20 mM acetate buffer (pH 5.4) for 2 days at room temperature. The aqueous phase was separated, and NaCl was added to it to a final concentration of 1.0 M. After treatment overnight at 0°C, the supernatant was separated from the insoluble materials and mixed with two volumes of cold ethanol. The precipitate formed (peptidyl–tRNA fraction) was then dissolved in a small volume of 10 mM acetate buffer (pH 5.4).

From the solution, peptidyl–tRNA was prepared by a slight modification of the method of Slabaugh and Morris [5]. The peptidyl–tRNA fraction was applied to a Sephadex G-100 column (1.5 × 140 cm) previously equilibrated with 10 mM acetate buffer (pH 5.4) and

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chromatographed. The flow rate was 10 ml/hr. Each fraction contained 3.0 ml of eluate. Peptidyl–tRNA fraction (Fraction No. 44–58 in fig. 1a) was rechromatographed on the same column. The peptidyl–tRNA (Fraction No. 44–56 in fig. 1b) thus prepared was stripped of peptides by incubation for 3 hr at 30°C after adjustment of pH to 10.0–10.5 with 1N NaOH. The solution was adjusted to pH 3 with formic acid, and placed on a Sephadex G-25 (superfine) column (1.5 × 90 cm). Elution was carried out with 0.5% formic acid at a flow rate of 4.0 ml/hr. Each fraction contained 2.0 ml of eluate.

Amino acid residues (N-terminal and non-N-terminal amino acids) were separated as the 2,4-dinitrophenyl

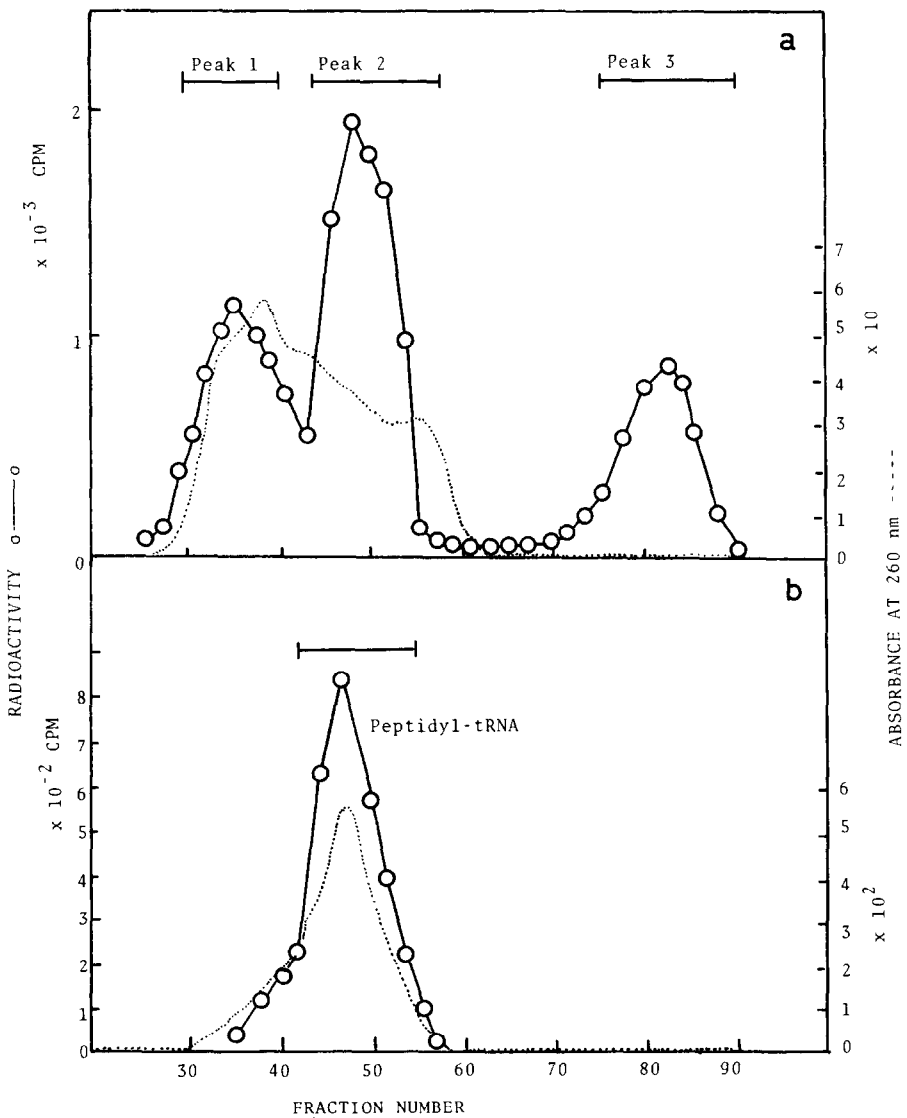

Fig. 1. a) Sephadex G-100 column chromatography of peptidyl–tRNA fraction was performed as described in 'Materials and methods'. The fractions (Fraction No. 44–58; peak 2) were pooled and rechromatographed. b) The peptidyl–tRNA fraction indicated above were pooled, dialyzed against water, and then chromatographed on Sephadex G-100 column. (o——o) radioactivity; (×——×) absorbance at 260 nm.

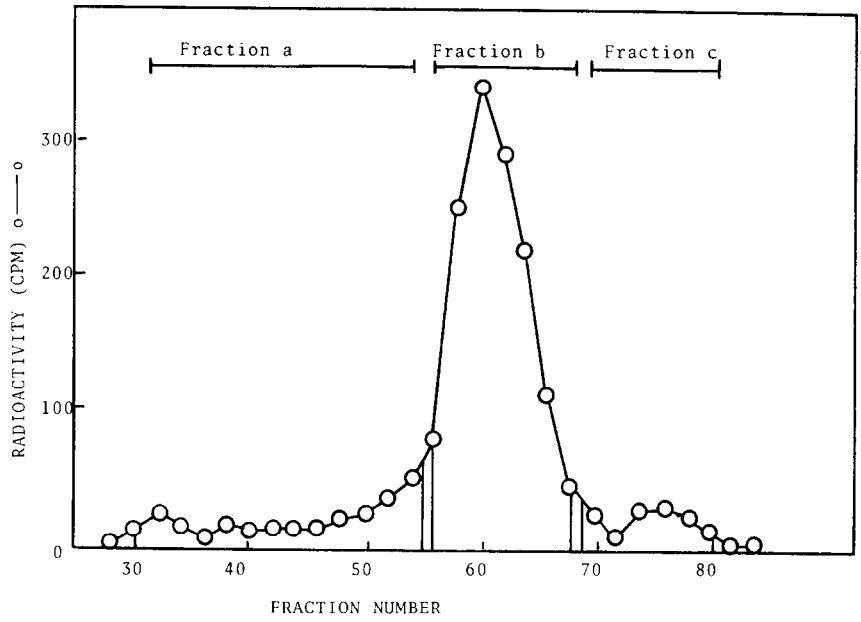

Fig. 2. Elution profile of nascent peptides from Sephadex G-25 column. Nascent peptides (the deacylation products of charged tRNA fractions) were placed on a Sephadex G-25 column (1.5 × 90 cm) and eluted with 0.5% formic acid as described in 'Materials and methods'. (o——o) radioactivity.

derivatives by thin layer chromatography according to the Sanger's DNP-method [9]. Each of the spots was scraped off and extracted with 1.5 ml of 1% $NaHCO_3$. The DNP-amino acids were counted for radioactivity in a liquid scintillation counter.

3. Results

After incubation at 30°C for 2 min of posterior silk glands in the presence of 17 labeled amino acids, the peptidyl–tRNA fraction was isolated and loaded onto Sephadex column. Peak 2 (Fraction No. 44–58 in fig. 1) corresponded to the elution position of glycyl–tRNA or peptidyl–tRNA. Radioactive components liberated from peak 2 were shifted to the elution position of peak 3 after alkaline treatment. Peak 1 might consist of alkaline stable long peptidyl–tRNA, since the radioactivity was not shifted from peak 1 position after alkaline treatment (unpublished data). Peak 2 was rechromatographed (fig. 1b). The peptidyl–tRNA fraction (Fraction No. 44–56 in fig. 1b) thus prepared was stripped of peptides as described in 'Materials and methods'.

The elution profile of the nascent peptides from Sephadex G-25 column is shown in fig. 2. There was one major peak. Each fraction (Fraction a; Fraction No. 30–55, Fraction b; Fraction No. 56–68 and Fraction c; Fraction No. 69–80) was pooled and analyzed for N-terminal and non-N-terminal amino acids by DNP-method. The N-terminal amino acid of Fraction b was rich in methionine and methionine sulfoxide (table 1). From the elution profile of nascent peptide and several standard peptides on the column, a mol. wt. of 1150 was estimated for the peptide (Fraction b) as shown in fig. 3.

4. Discussion

More than 90% of the protein synthesized in the posterior silk glands of *Bombyx mori*, L., after the fourth day of the fifth instar stage is fibroin [1]. It is, therefore, reasonable to assume that the nascent

Table 1
N-terminal and non-N-terminal amino acids of nascent peptides fractionated from posterior silk glands

DNP-amino acid	Fraction a		Fraction b		Fraction c	
	N-terminal	Non-N-terminal	N-terminal ($\times 10^{13}$ moles)	Non-N-terminal	N-terminal	Non-N-terminal
Aspartic acid	1.0	6.3	1.9	108.0	1.5	9.3
Glutamic acid	0.8	12.0	0.5	254.1	–	4.6
Cysteine	–	1.6	–	21.2	–	0.8
Threonine	–	10.5	–	191.4	–	6.3
Serine	1.7	21.8	–	331.5	–	19.1
Glycine	3.8	52.3	1.3	697.4	–	45.1
Alanine	–	22.5	–	292.9	–	10.6
Proline	–	13.8	–	140.9	–	4.0
Phenylalanine	–	8.9	–	67.1	0.1	5.4
Methionine	0.3	4.5	33.1	22.6	33.1	2.8
Valine	0.5	5.7	0.4	139.3	0.6	5.8
Leucine	0.2	2.9	–	27.5	0.2	2.9
Isoleucine	–	4.0	–	35.7	–	4.3
Lysine	–	0.5	–	11.7	–	0.3
Tyrosine	–	–	–	–	–	–
Histidine	–	–	–	–	–	–
Arginine	–	–	–	–	–	–
Methionine sulfoxide	–	–	47.6	–	27.5	–

–: undetectable

N-terminal and non-N-terminal amino acid analysis of peptides were performed by DNP-method as described in 'Materials and methods'. The values were corrected for losses caused by decomposition during hydrolysis and by thin layer chromatography. The values were calculated based on the specific activity on the free amino acids which existed in the posterior silk gland after the incubation.

peptides from peptidyl–tRNA in this stage are mostly of fibroin. On this basis, nascent peptides from the pulse-labeled peptidyl–tRNA were analyzed for N-terminal and non-N-terminal amino acids. There was one major peptide (mol. wt.: 1150) detected by Sephadex chromatography (fig. 2). The N-terminal amino acid of the peptide was methionine or methionine sulfoxide exclusively. Since methionine sulfoxide can be considered as methionine oxidized during acid hydrolysis and alkaline treatment, methionine seems to be the N-terminal amino acid. Non-N-terminal amino acids were rich in glycine, serine, alanine, and glutamic acid. At the present stage, the purity of the peptide is unknown. The peptide, however, should consist of 10–15 amino acid residues from the molecular weight.

The N-terminal amino acids of a peptide (Fraction a in fig. 2) longer than the main component were rich in glycine, serine, aspartic acid, and glutamic acid. In hemoglobin biosynthesis, it has been reported that the N-terminal amino acid of the initial peptides (smaller than 16 amino acids) is mainly methionine [6]. Yoshida has proposed the hypothesis that the N-terminal methionyl peptides of peptidyl–tRNA is buried in the ribosome matrix and that further chain elongation is required to expose the N-terminal to aminopeptidase. The same idea might be applied to the initiation in fibroin biosynthesis.

Lucas [10] suggests that there are at least four chains in fibroin molecule, since the N-terminal residues are glycine, serine, aspartic acid, and alanine. In view of the above facts, two conclusions to be drawn from available data may considered: 1) There is one kind of fibroin mRNA containing the methionine codon

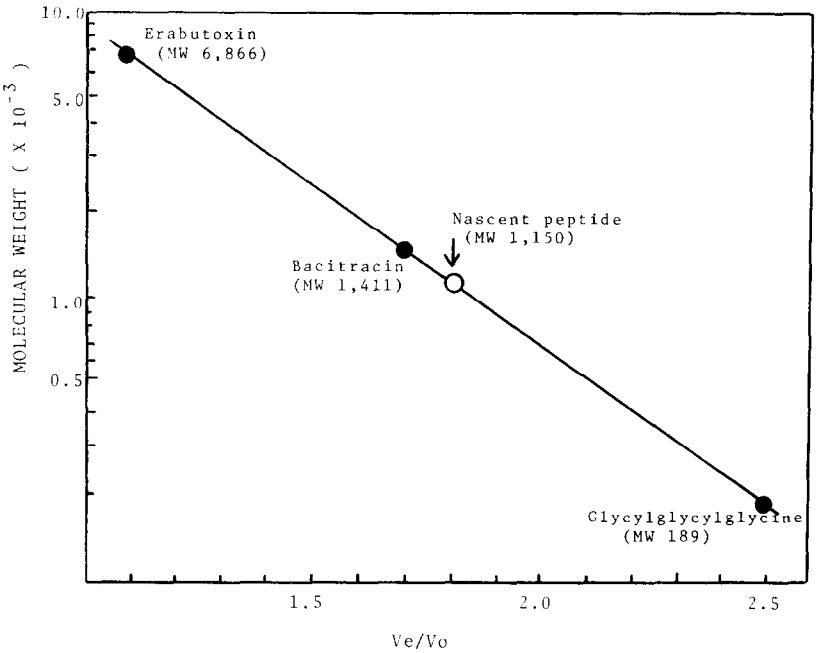

Fig. 3. Molecular weight estimation of nascent peptide by Sephadex G-25. The solid circles indicate the position of peak midpoints for peptides of known molecular weights. The arrow indicates the position of nascent peptide.

as an initiator codon. After 16 steps of chain elongation, the methionyl residue is removed with an aminopeptidase. Since the aminopeptidase has ambiguous properties in amino acid specificity, various amino acids are detected in the N-terminal residue, and 2) There are four kinds of fibroin mRNA containing the methionine codon as an initiator codon, and corresponding to four N-terminal amino acids. After 16 steps of chain elongation, the initial methionyl residue is removed. The resulting N-terminal amino acid are four kinds of amino acid. Whether or not one of these explanations is true or whether another one is required cannot be known until further study.

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