Biochemical Processes of Synthesis and Degradation of a Yolk Protein, Egg-Specific Protein, in the Silkworm, *Bombyx mori*

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Vitellin (Vitellogenin) is the major yolk protein of many arthropods which accounts for more than 80% of total yolk proteins (Engelmann, 1979; Yamashita and Indrasith, 1988). Whereas, in several lepidopteran insect eggs, non-vitellin yolk proteins are accumulated to various degrees along with vitellin. Egg-specific protein is a typical protein belonging to this group of yolk protein (Yamashita, 1986). This kind of yolk protein has recently been identified in several insects; paravitellin in *Hyalophora cecropia* (Telfer and Kulakosky, 1984), yolk polypeptides in *Prodia interpunctell* (Shirk *et al.*, 1984) and the related seven pyralid moths (Shirk, 1987). Compared to the advancement on biochemical and molecular studies on vitellogenin (Postlethwait and Giorgi, 1985), the limited attention has been paid on the non-vitellin yolk proteins. The present review describes the present state of biochemical studies on synthesis and degradation of egg-specific protein in the silkworm, *Bombyx mori*.

Synthesis of egg-specific protein during oogenesis

1. Characterization of egg-specific protein

The silkworm, *Bombyx mori*, undergoes oogenesis mainly during the pupal-adult development and at the time of adult emergence almost all oocytes become matured (Yamauchi and Yoshitake, 1984). The mature eggs were used for purification of yolk proteins. The non-denatured polyacrylamide gel electrophoresis (PAGE) of crude extracts gave 5

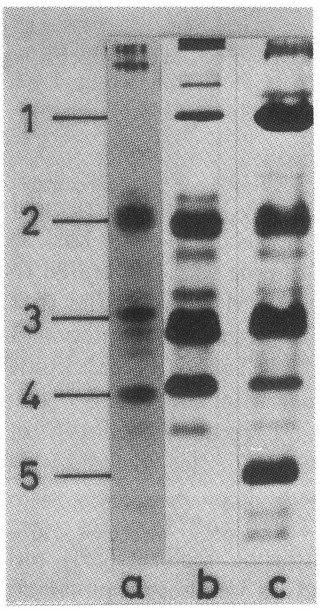

Fig. 1 Protein composition of eggs and hemolymph of silkworms. Egg extracts (c) and hemolymph from vitellogenic females (b) and males (a) were subjected to the non-denatured polyacrylamide gel electrophoresis. Band 1 corresponds to vitellin. Bands 2 to 4 are 30 kDa protein-1, -2 and -3, respectively. Band 5 is egg-specific protein (Modified from Zhu *et al.*, 1986).

major bands (Fig. 1). Hemolymph proteins from vitellogenic females and male pharate adults were coelectrophoresed to identify the yolk proteins. Band 1 was identified as vitellin, because of the deficiency in male hemolymph (Izumi *et al.*, 1980). Bands 2 to 4 were the non-sex limited serum proteins as referred to as 30 kDa proteins (Izumi *et al.*, 1981). Since band 5 protein was exclusively localized in eggs, it was referred as to egg-specific protein (ESP) (Irie and Yamashita, 1983; Yamashita, 1986). By monitoring mobilities of yolk proteins on non-denatured PAGE, vitellin, each 30 kDa protein and ESP were sequentially purified from the same starting materials by column chromatographies (Zhu *et al.*, 1986). Each purified protein was characterized to be the different protein in molecular properties (Table 1).
The molecular mass and subunit structure of vitellin and 30 kDa Proteins were in agreement with the results obtained different sources (Izumi *et al.*, 1980, 1981).

Table 1 Molecular properties of vitellin, egg-specific protein and 30 kDa proteins purified from silkworm eggs.

	Native molecular mass (kDa)	Subunit number	Subunit molecular mass (kDa)	Subunit molar ratio
Vitellin	420	2	178	1
			43	1.2
Egg-specific protein	225	2	72	2.0
			64	1
30 kDa protein−1	31.5	1	32.0	
−2	30.0	1	31.0	
−3	28.5	1	29.5	

(Modified from Zhu *et al.*, 1986)

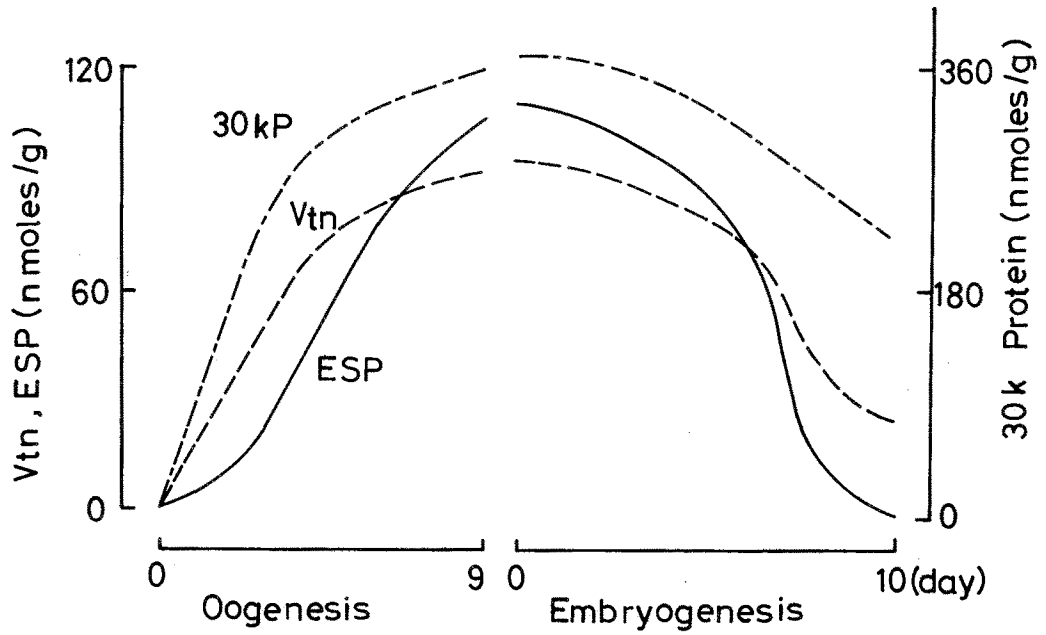

Fig. 2 Developmental changes in vitellin (Vth), 30 kDa proteins (30 K) and egg-specific protein (ESP) during oogenesis and embryogenesis of silkworms. (Modified from Yamashita and Indrasith, 1988).

ESP was shown to be a protein of 225 kDa composed with two 72 kDa peptides and one 64 kDa peptide (Zhu *et al.*, 1986).

Using the specific antiserum raised to each purified yolk protein, the titre changes of vitellin, 30 kDa proteins and ESP were followed throughout the oogenesis of silkworms (Fig. 2). Both vitellin and 30 kDa proteins exhibited a steep increase during the first half period, while ESP began to rise after a 2-day lag phase. In the mature eggs, vitellin accounted for about 40% of total yolk proteins, 30 kDa proteins for 35% and ESP for 23% (Table 2). In silkworm eggs, vitellin is the major yolk protein but remains less abundant compared to yolks of other insects (Engelmann, 1979; Kunkel and Nordin, 1985; Yamashita and Indrasith, 1988).

Table 2 Comparison of yolk proteins in silkworm eggs developed in female and male hosts.

Yolk proteins	Eggs developed in	
	Females	Males
	(μg/egg)	
Vitellin	19.0	1.0
Egg-specific protein	12.5	13.0
30 kDa proteins	17.0	17.4
Total proteins	52.1	33.2

Male eggs were obtained by transplanting ovarian discs into male fifth instar larvae (From Zhu *et al.*, 1986).

Due to the predominancy of vitellin as a yolk protein in many vertebrates as well as invertebrates (Engelmann, 1979; Wallace, 1985; Yamashita and Indrasith, 1988), it is believed that vitellin is an essential and indispensable reserve needed to the following embryogenesis. To examine the physiological role of vitellin in oogenesis and embryogenesis of silkworms, we have performed some biological experiments in which an ovary disk was transplanted to male larva so that oogenesis *undergoes* in the absence of vitellogenin. In the male hosts, oocytes grew up to mature eggs with chorion but the mature eggs were completely deficient in vitellin (Table 2). Whereas, eggs developed in male hosts ('male eggs') accumulated 30 kDa proteins and ESP at the comparable levels to those in normal eggs developed in females (Yamashita and Irie, 1980; Zhu *et al.*, 1986). The mature eggs were dissected out from male moths and subjected to an artificial parthenogenic activation to initiate embryogenesis. This treatment was enough to complete the embryogenesis of 'male eggs' as well as completion of their larval life (Yamashita and Irie, 1980). These results led us to conclude that vitellin is not essential for oogenesis and the following embryogenesis in silkworms.

By replacing the hemolymph with saline on the day of pupation by which time the synthesis of 30 kDa proteins has lasted, we were able to make the mature eggs which are deficient in 30 kDa proteins. However, ESP was accumulated at the normal levels in these eggs (unpublished data). Consequently, ESP seems to be more important yolk protein than vitellin and 30 kDa proteins for oogenesis and embryogenesis in silkworms. Therefore, our attentions have been focused on the biosynthesis and degradation of ESP.

2. Amino acid sequence of egg-specific protein deduced from cDNA

From developing ovaries, poly (A) $^{+}$RNAs were isolated and were used for construction of cDNA. The poly (A) $^{+}$RNAs contained mRNA which directs the synthesis of ESP when translated in a rabbit reticulocyte lysate system. The vector primer and tailed linker were prepared for construction of a cDNA expression vector (Inagaki *et al.*, 1987).

A 1.9 K bp (base pair) cDNA insert was excised and subcloned for sequencing of nucleotides (Inagaki and Yamashita, 1989). The cDNA was composed with 1889 bp including 21 bp in 5'-non-coding region, 1677 bp in coding frame, 115 bp in 3'-non-coding region and 76 poly (A) stretch (Fig. 3).

A first ATG codon (+1) was presumed to be the initiation site of translation, since this codon followed the consensus sequence (C/AAAC/A) for translation initiation. An inframe TAA stop codon appeared at 1678 and the G at 1792 was

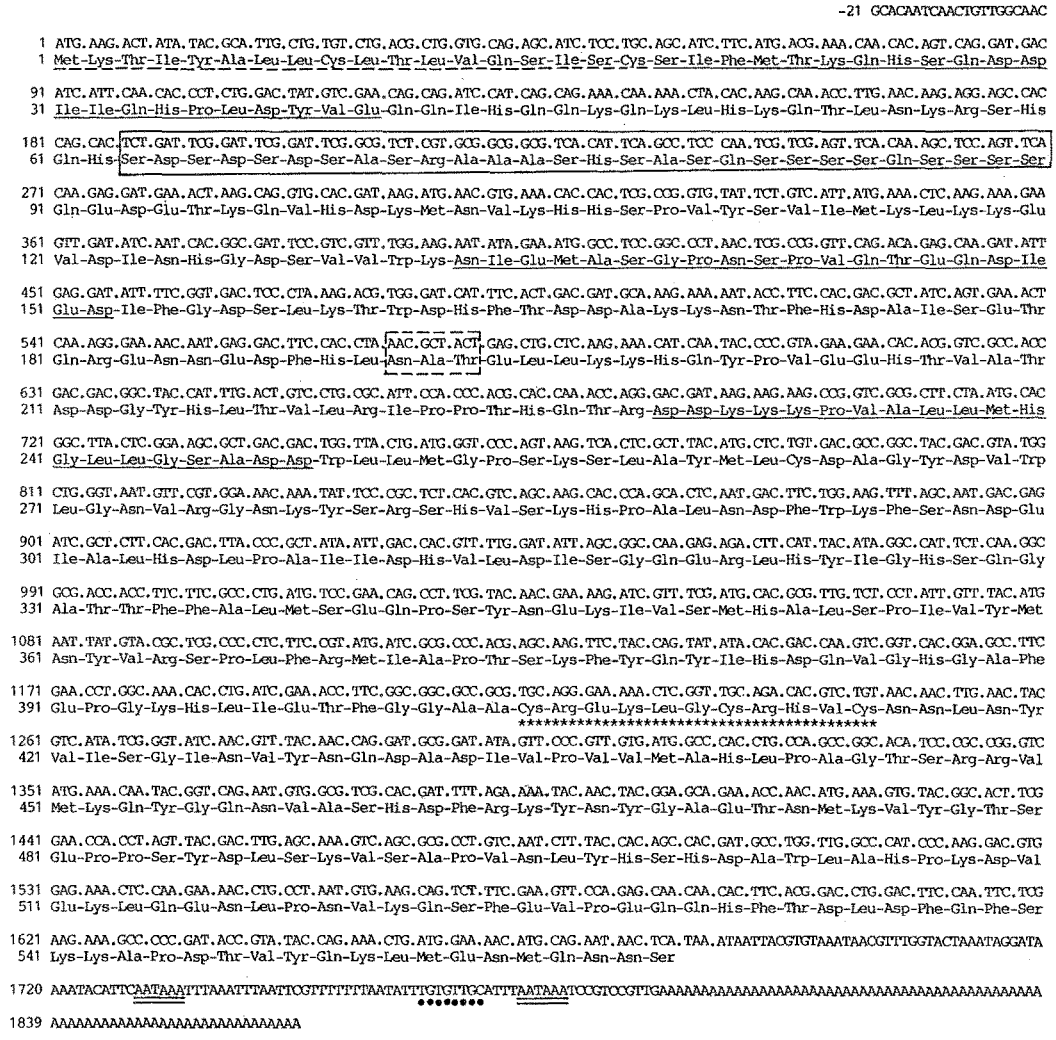

Fig. 3 Nucleotide sequence of a cDNA clone for egg-specific protein and the deduced amino acid sequence. Amino acids are numbered from the initial methionine. The broken lined amino acids are the signal peptide sequence. The underlined amino acids were confirmed by amino acid sequence determined chemically on the intact ESP and its degradation products. Serine rich domain was boxed. The possible N-linked glycosylation sequence is boxed with broken line. The possible metal binding sequence is marked with asterisks. The double lined sequences are presumed to be poly (A) signals. (Modified from Inagaki and Yamashita, 1988).

the poly adenylation site. Two hexamers, AATAAA, at 1730 and 1776 correspond to the polyadenylation signals (Inagaki and Yamashita, 1989).

The deduced amino acid sequence starting at the first ATG codon composed 559 amino acid residues (Fig. 3). The sequence from 19 to 40 was identical to that determined on the purified ESP by Edman method (Indrasith *et al.*, 1988b) . Thus, the first 18 sequence enriched in hydrophobic amino acids encodes the leader sequence which is known as the signal peptide. The signal peptide is to be released by the cleavage at Cys^{18} as the first step of post-translational processing of ESP (see below). A sequence, Asn-X-Thr, which is proposed to be a potential N-linked glycosylation site, was found at position of 191 to 193, indicating that ESP is glycosylated at Asn^{191}. The most striking feature of amino acid sequence of ESP was the existence of a serine-rich domain in which several serine residues showed a small clus-

ter. In vitellogenin of non-mammalian vertebrates, the serine clusters are the major sites of phosphorylation and considered to be a phosphate reserve needed for bone formation in embryos (Nardelli *et al.* , 1987) . No vitellins of invertebrates, *Drosophila* and nematodes contain such serine clusters in their sequences (Spieth *et al.*, 1985; Yan *et al.*, 1987). Therefore, the serine content is proposed to increase correlated with the evolution of oviporous animals. ESP and its analogues in stead of vitellin seem to denote the phosphate-carrying proteins in invertebrates (Inagaki and Yamashita, 1989).

3. Biosynthesis of egg-specific protein

In an *in vitro* translation experiment using a rabbit reticulocyte lysate, poly $(A)^+$RNAs from developing ovaries produced a 69 kDa peptide which was precipitated with the anti-ESP serum. The preliminary translation product was processed into a 67 kDa peptide by incubating with the dog pancreatic microsomal membranes. Translation of ESP was exclusively found in mRNAs prepared from developing ovaries (Kobayashi *et al.* , unpublished data). Thus, it is concluded that ESP mRNA is only transcribed in ovaries.

To analyze the post-translational modification in the course of ESP biosynthesis, we developed the *in vitro* incubation system of developing ovaries (Sato and Yamashita, 1989). The *in vitro* pulse and pulse-chase labeling of the selected follicles with ^{35}S-methionine showed that ESP was first synthesized as a 69 kDa peptide and then converted into 72 kDa peptide. By a long term incubation, some molecules of 72 kDa peptides were converted into 64 kDa peptides. ^{14}C-Mannose was incorporated into 69 kDa and 72 kDa peptides. Tunicamycin, a potent inhibitor of N-glycosylation, inhibited the synthesis of 69 kDa peptide and induced a new 67 kDa peptide which is not detected in the normal incubation system. Thus, 69 kDa peptide is concluded to be produced from 67 kDa peptide by a glycosylation. From the incorporation experiments using ^{32}P, 72 kDa peptide was shown to be phosphorylated. Digestion of 72 kDa peptide with alkaline phosphatase brought about 69 kDa peptide. These results led us to conclude that ESP is synthesized as follows: 67 kDa nascent peptide glycosylated 69 kDa peptide phosphorylated 72 kDa peptide (Fig. 4). However, it remains unknown on the process on conversion of 72 kDa peptide to 64 kDa peptide.

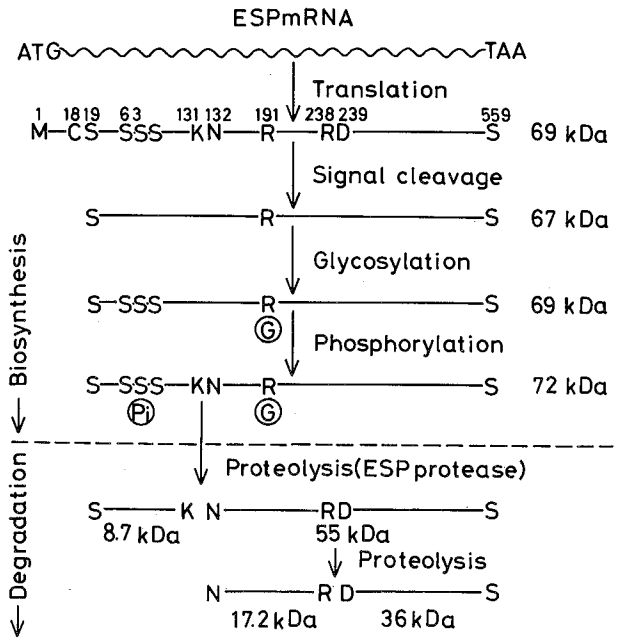

Fig. 4 The biochemical processes of synthesis and degradation of egg-sepecific protein in silkworms. Synthetic processes; (1) translation (69 kDa nascent peptide), (2) signal sequence cleavage (67 kDa peptide), (3) glycosylation (69 kDa peptide), (4) phosphorylation (72 kDa peptide). Degradation processes by ESP protease; (1) Cleavage between Lys^{114} and Asn^{115} of intact ESP giving rise to 8.7 kDa peptide and an intermediate 55 kDa peptide, (2) Cleavage between Arg^{210} and Asp^{221} of the intermediate to 17.2 kDa peptide and 36 kDa peptide.

Vitellogenin and 30 kDa proteins are synthesized in the extra ovarian tissue, fat body, and secreted into hemolymph to be taken up by the vitellogenic follicles in silkworms (Izumi *et al.*, 1981; Yamashita, 1986). ESP is synthesized by ovary itself but no information was available on the cells synthesizing ESP from the experiments using whole ovary. We isolated the follicle cells from oocytes / of developing ovaries and incubated *in vitro* them with ^{35}S-methionine as done on whole ovaries. The follicle cells clearly synthesized 69 kDa ESP peptide and release 72 kDa ESP peptide into the incubation medium (Sato and Yamashita, in preparation). An immunohistochemical observation has demonstrated that ESP is localized in follicle cells as well as oocytes (Irie and Yamashita, 1983). These results reveal that ESP is synthesized in follicle cells and released to be sequestered by developing oocytes.

Utilization of egg-specific protein during embryogenesis

1. Metabolic fate of egg-specific protein during embryogenesis

As shown in Fig. 2, the fates of yolk proteins during embryogenesis of silkworms are different each other. In the newly hatched larvae, an appreciable amount of 30 kDa proteins and vitellin remained unused. While ESP became utilized from the second half of embryogenesis and disappeared at larval hatching. Since yolk proteins are organized into a yolk granule, such a different utilization of each protein seems to be mediated by the different proteolytic mechanisms. A SDS-PAGE and the immunoblotting indicated that the intact ESP consisting of 72 kDa peptide and 64 kDa peptide breaks down into a 55 kDa peptide and then a 36 kDa peptide according to embryogenesis (Indrasith *et al.* , 1987). These facts imply that ESP is cleaved by a limited hydrolysis in eggs.

2. Protease responsible for selective degradation of egg-specific protein

To elucidate the enzymatic mechanisms responsible for the limited degradation of ESP occurring in embryonating eggs, the purified ESP was used as the substrate and incubated *in vitro* with the egg extracts of day 8 when the rapid degradation of ESP proceeded. As expected, the intact ESP was hydrolyzed into 55 kDa peptide and then 36 kDa peptide according to the incubation period of times, indicating that the sequential degradation is mediated by enzymatic reactions. The protease was purified to a homogeneous state from day 8 egg extracts using ion exchange column chromatography and HPLC. Molecular mass was estimated to be 30,500 kDa in native form and 29.6 kDa in subunit form. The inhibition experiment and action spectrum to the synthesized substrates showed that the protease is a trypsin-like seryl protease (Indrasith *et al.* , 1988c).

One of the interesting finding of this study is that the protease prefers the ESP as a potent substrate without attacking on the other yolk proteins as well as non-yolk proteins. ESP was clearly hydrolyzed at a ratio of substrate to enzyme of 5,000: 1 by weight. However, the protease becomes attackable to the denatured vitellin, suggesting the highly specified substrate for this protease (Table 3).

Table 3 Substrate specificity of the purified ESP protease.

Substrate	Relative activity
Egg-specific protein	1
Vitellin	3.8×10^{-4}
30 kDa proteins	1.3×10^{-4}
Storage protein-1	4.7×10^{-3}
Storage protein-2	1.7×10^{-4}
Ovalbumin	1.4×10^{-4}
Bovine serum albumin	2.2×10^{-4}
Casein	1.9×10^{-4}

(Modified from Indrasith *et al.*, 1988b)

A limited information has appeared on the specificity of protease toward the physiological proteins in stead of the synthesized or denatured peptides. To decide the substrate specificity, we further analyzed the degradation products of ESP after hydrolysis with the purified enzyme. In addition to 55 kDa peptide and 36 kDa peptide which were identified on SDS-PAGE, 8.7 kDa peptide and 17.2 kDa peptide were recovered by a HPLC equipped with gel permeation column (TSK 3000 SW) . The 55 kDa peptide was shown to be the intermediate form from which 36 kDa peptide and 17.2 kDa peptide were released. When the intact ESP was incubated for a longer time enough to complete hydrolysis, there was a stoichiometry between the disappearance of substrate and the appearance of the three peptides. Thus, it is concluded that the intact ESP is cleaved into 3 peptides; 8.7 kDa peptide, 17.2 kDa peptide and 36 kDa peptide through a 55 kDa intermediate peptide (Indrasith *et al.*, 1988c).

We sequenced several N-terminal amino acid of each product by Edman method. By relating these sequence to the amino acid sequence of the intact ESP (Fig. 3), the protease is shown to cleave first at Lyst-Asn site and secondly at Arg-Asp (Fig. 4).

3. Synthesis of ESP protease

The degradation rates of ESP in eggs increased from the midway of embryogenesis and attained the maximum level at the stage when larval differentiation completed (Fig. 2). The similar changing profile was found in ESP protease activity during embryogenesis (Indrasith *et al.*, 1988c). Thus, the utilization of ESP by developing embryos closely depends upon the appearance of the protease. In oviparous vertebrates, yolk protein breakdown has long been believed to be initiated by the disappearance of inactivation of protease inhibitors such as ovomucoid and ovoinhibitor which sometimes bind to the vitellin or yolk proteins (Salisbury *et al.* , 1980) . The mixing of homogenates from the different stages gave the additional activity found in both samples and there is no indication on the presence of inhibitors for ESP protease (Indrasith *et al.*, 1988c). Consequently, the developmental increase in ESP protease activity is due to the *de novo* synthesis of enzyme protein.

An *in vitro* translation of mRNAs extracted from the various stages of embryogenesis was carried out to detect the developmental changes in mRNA activity for ESP protease using the antiserum specific to ESP protease (Indrasith *et al.*, 1988a). The translation product with immunoreactivity to ESP protease had a molecular mass of 29 kDa and the activity increased at the late stage of embryogenesis. No translation occurred on mRNA from unfertilized eggs and young embryos. Upon the cotranslation with dog membrane fractions, the primary translation product was processed to 24.5 kDa peptide, indicating the signal peptide cleavage for intracellular translocation. These results confirm that ESP protease is synthesized in the developing eggs through the transcription of ESP protease gene. However, it remains unknown whether transcription and translation proceed in yolk cells or embryonic cells.

It is well conceived that many secretory proteases are synthesized as the enzymatically inactive forms, zymogens, and become activated after the chemical modification such as signal peptide cleavage and glycosylation. To estimate whether or not ESP protease becomes activated after the molecular modifications, we directly assayed proteolytic activity of the primary translation product. Since the reticulocyte lysate used in this experiment contains strong inhibitor(s) for ESP protease, the translation product was isolated from the reaction mixture by immunoprecipitation using the anti-ESP protease serum. The immuno-complexes retained activity to hydrolyze ESP and gave 55 kDa peptide and 36 kDa peptide on SDS-PAGE which were indentical to the hydrolysates produced by the purified ESP protease. No increase in activity was brought about in 24.5 kDa protease after the signal sequence was cleaved. Accordingly, the nascent peptide has been activated before the chemical modification. This is the first case in which a biologically active protease is directly translated as a nascent product throughout the organisms. Since this proteolytic action is limited to the specific physiological substrate, ESP, no potential hazard exists to other proteins consisting the cellular machinery for protein synthesis.

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