Synthesis and resorption of a humoral chymotrypsin inhibitor, CI-8, by fat body of the silkworm, Bombyx mori

Koji Shirai 1,a, Hiroshi Fujii a,*, Hiroshi Doira a, Hisao Iwamoto b

a Institute of Genetic Resources, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
b Animal and Marine Bioresource Science, Division of Bioresource and Bioenvironmental Sciences, Kyushu University Graduate School, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

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Abstract

The Bombyx mori hemolymph contains up to 16 chymotrypsin inhibitors (CIs). The present in vitro culture of tissues in Grace’s medium indicated that CI-8, which belongs to the largest molecular-size group of CIs with sugar moiety, is synthesized in the fat body and secreted from it during the feeding period. When the fat body from other strain which synthesizes an allelic component (CI-7) instead of CI-8 was incubated in vitro in hemolymph from the strain which has CI-8, the fat body was found to receive CI-8. Thus it was concluded that CI-8, once secreted into the hemolymph, was again sequestered into the fat body after the onset of spinning. Protein granules isolated from the pupal fat body were shown to contain CI-8, indicating that the sequestered CI-8 is present in the protein granules. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Insect hemolymph has multiple sorts of proteinaceous protease inhibitors, which may play indispensable physiological roles, e.g. in blood clearance by entrapping protease leaked from histolytic tissues during metamorphosis and in the protection from invading microorganisms (Suzuki and Natori, 1985; Kannost and Wells, 1989; Kannost, 1990). In Bombyx mori, the expression of hemolymph chymotrypsin inhibitors (CIs) has been shown to be controlled by five genes, Ict-A, Ict-B, Ict-H, Ict-D and Ict-E, located on three separate chromosomes: the first to the third genes on chromosome 2, the fourth gene on chromosome 19, and the fifth gene on chromosome 22 (Fujii et al., 1996a,b). There are at least 16 isoforms of CIs, of which the allelic members for Ict-A (i.e. CI-13 or 13′), Ict-D (i.e. CI-6, 6′, 7, or 8), and Ict-E (i.e. CI-3 or 4) were found to consistently exist in all hemolymph specimens from B. mori strains examined so far (up to 400). We therefore suppose that the expression of these three genes is essential for the development and growth of the silkworm (Fujii et al., 1996a,b; Shirai et al., 1997). To date, CIs representing each of the three essential groups, i.e. CI-13 (Fujii et al., 1989; Shinohara et al., 1993), CI-8 (Shirai et al., 1997), and CI-3 (Shirai et al., unpublished data), as well as other Ict-A members CIs-1 and 2 (Deng et al., 1990), have been purified and characterized. Moreover, a systematic copurification method of CIs indicated that the above grouping on the basis of genetics is intimately related to the classification on the basis of molecular size and glycosylation (Shinohara et al., 1993, cf. also Shirai et al., 1997). CIs-13 and 13′, belonging to the smallest molecular-size group without sugar moiety, are detected in fertilized eggs, indicating that these CIs are sequestered by the ovary during oogenesis (Aratake et al., 1990). In this article, we report that CI-8, belonging to the largest molecular-size group with sugar moiety, is synthesized in the fat body and secreted from it, but again taken up into the fat body and stored in protein granules.
2. Materials and methods

2.1. Animals and the preparation of hemolymph and tissue specimens

The cz strain homozygous for Cls-3, 8, and 13’ was mainly used. Sometimes the r06 strain homozygous for Cls-4, 7, and 13 (allelic to Cls-3, 8, and 13’, respectively) was employed. Both strains are kept at the Institute of Genetic Resources, Faculty of Agriculture, Kyushu University. Larvae were raised on mulberry leaves. Hemolymph was collected from larvae or pupae, mixed with reduced glutathione at 0.6 mg/ml to avoid melanization, and centrifuged at 600 g for 5 min at 4°C to remove hemocytes. Tissues were dissected out of larvac or pupae, washed with sterilized physiological (0.75%) saline to remove hemolymph as far as possible, weighed, and used for experiments. If necessary, the tissues were homogenized in 3 vol (w/v) of the physiological saline with a Teflon-glass homogenizer, centrifuged at 12,000 g for 1 h at 4°C, and the supernatant was carried to subsequent steps.

2.2. Assay of CI activity and determination of CI-8 concentration

The inhibitory activity to protease was assayed using chymotrypsin and Hammarsten casein at pH 7.4, under the conditions specified previously (Fujii et al., 1989). To determine the CI-8 concentration, enzyme-linked immunoabsorbent assay (ELISA) was performed as described (Lanzavecchia, 1985) in flat-bottom Limbro EIA plates with 96 wells, using rabbit IgG raised against purified CI-8 (Shirai et al., 1997).

2.3. Electrophoresis and the detection of CI activity (zymogram)

Polyacrylamide gel electrophoresis under undenaturing conditions (native PAGE) was carried out, and the gels were stained for proteins with Coomassie brilliant blue or analyzed for CI activity (zymogram) as detailed previously using N-acetyl-D,L-phenylalanine-β-naphthylester as a protease substrate (Fujii et al., 1989).

2.4. Tissue culture in Grace’s medium or in the hemolymph

Different tissues were freshly prepared from 3 to 5 animals for each analysis and washed as described above and separately pre-incubated at 28°C for 1 h in 400 µl of methionine-deprived Grace’s medium containing 20 units of penicillin and 20 mg of streptomycin, and further incubated in 400 µl of the same medium with 10 µCi of [35S]methionine for 6 h. During incubation, the suspensions were covered with mineral oil to reduce oxidation. After brief centrifugation at 600 g for 5 min at 4°C the supernatants were directly subjected to immunoprecipitation using anti-CI-8 rabbit IgG, whereas the tissues were homogenized and centrifuged as described above and the supernatants were subjected to immunoprecipitation. Radioactivity was counted in an Aloka liquid scintillation spectrometer.

The fat body, dissected from 3 animals and washed, was incubated at 26°C for 12 h in the hemolymph collected from 3 animals. After incubation, the tissue was washed with physiological saline containing 0.05% Triton X-100, homogenized, and centrifuged as described above. The supernatant was analyzed for zymogram, whereas the pellet was homogenized in the physiological saline and the supernatant was subjected to zymogram analysis.

2.5. Isolation of protein granules from the fat body

Protein granules were isolated from the fat body by sucrose density gradient centrifugation, and the granules obtained were extracted for proteins by suspending in 0.05 M Na–phosphate buffer, pH 6.8 (Tojo et al., 1978).

3. Results

3.1. Developmental changes of the CI-8 concentration in the hemolymph

When the CI-8 concentration in the hemolymph was assessed daily by ELISA from the fourth molt to the late pupal stage of the cz strain, it increased after day 2 of the fifth instar, exhibiting a single and sharp peak at the onset of spinning, and fell rapidly thereafter (Fig. 1).
Substantially the same developmental changes were observed when the relative activity of whole hemolymph Cls was assayed using the same strain (patterns not shown). The overall changing patterns of relative Cl activity were roughly similar to those published previously using different strains (Eguchi et al., 1986; Aratake et al., 1990). In the present study, no large difference was observed between the female and male, although sexual difference has often been observed previously. The reason for this discrepancy remains to be elucidated.

3.2. Identification of the organ wherein Cl-8 is synthesized

Major tissues were dissected from the cz strain on day 4 of the fifth larval instar, when the Cl-8 concentration in hemolymph was increasing, and at the onset of spinning, when the Cl-8 concentration was at its peak. All were subjected to culture in vitro for 6 h in Grace’s medium containing [35S]methionine followed by immunoprecipitation of media and tissues using anti-Cl-8 IgG. As illustrated in Fig. 2, the fat body gave marked radioactivity in the medium. The value was larger for the feeding stage than for the spinning stage. Hardly any radioactivity was detected in the fat body tissue after incubation. The midgut, posterior silk glands, testes, and ovaries scarcely exhibited radioactivity both in media and tissues. Also hemocytes showed no signal of Cl synthesis in this experiment (data not shown). These results indicated that Cl-8 was synthesized in the fat body and secreted from it, and that the synthetic activity fell at the spinning period.

3.3. Development changes in content of fat body Cl-8

Dual peaks were observed when the developmental changes of Cl-8 content in the fat body extracts was determined by ELISA (Fig. 3): the first peak on day 4 (male) or 5 (female) of the fifth instar and the second one on day 3 after the larval–pupal ecdysis (both sexes). Similar conclusions could be obtained when the developmental changes in fat body Cl activity was determined by zymogram analysis after native PAGE (patterns not shown). The present zymogram exhibited a new Cl band appearing closely below the co-existing Cl-8 band during the pupal stadium. This component was shown to be immunologically cross-reactive with Cl-8 (Shirai, unpublished data), and thus it may be a derivative of Cl-8.

3.4. Sequestration of hemolymph Cl-8 into the fat body

The appearance of the second peak of the fat body Cl-8 content could not be explained by its synthetic activity, which was low at the pupal stadium (Shirai, unpublished data). Thus, there is a possibility that Cl-8, once secreted into the hemolymph, is resorbed into the fat body. To confirm this, the fat body of the ro6 strain, which has no Cl-8 but allelic component Cl-7, was dissected 1 day after hatching.
after the onset of spinning and incubated for 12 h in the hemolymph of the cz strain (having CI-8 instead of CI-7) prepared at the same age. Zymogram analysis after native PAGE (Fig. 4) showed that the r06 fat body after incubation (Lane 2) gave the band of CI-8 in addition to the intrinsic component CI-7, whereas the control r06 specimen before incubation (Lane 3) did not. Therefore, the CI-8 detected in the r06 fat body must be originated from the cz hemolymph, indicating that the fat body sequesters CI-8 from the hemolymph.

3.5. Detection of CI-8 in the protein granules of pupal fat body

Protein granules were isolated from the fat body using day 3 pupae of the cz strain (Fig. 5), and their extract was subjected to native PAGE followed by zymogram analysis (Fig. 6). The CI-8 band was seen (Lane 1) like the larval hemolymph specimen of the same strain (Lane 3, as Lane 2 before incubation (as a control). Note that the tissue of the r06 strain intrinsically without CI-8 had received it after incubation. When the fat body extract was analyzed, the bands for CIs-3 and 13' in the cz strain and those for CIs-4 and 13 in the r06 strain were often obscure although these are present.

Fig. 4. Sequestration of hemolymph CI-8 to the fat body in vitro. The fat body from the r06 strain with CI-7 was incubated in the hemolymph of the cz strain with CI-8 at 26°C for 12 h, extracted, and the supernatant was subjected to native PAGE (10%) followed by zymogram analysis. The fat body and the hemolymph were from males on day 1 after the onset of spinning. Lane 1, the fat body of the cz strain (as a reference); Lane 2, the fat body of the r06 strain after incubation; Lane 3, as Lane 2 before incubation (as a control). Note that the tissue of the r06 strain intrinsically without CI-8 had received it after incubation. When the fat body extract was analyzed, the bands for CIs-3 and 13' in the cz strain and those for CIs-4 and 13 in the r06 strain were often obscure although these are present.

Fig. 5. Micrograph of the protein granules from the fat body of pupae. The protein granules were isolated from the fat body of day 3 male pupae by the method described in Section 2.

Fig. 6. Detection of CIs in the protein granules of the pupal fat body prepared from day 3 male pupae of the cz strain. The extract of granules was subjected to native PAGE (10%) followed by zymogram analysis. Lane 1, the granule extract; Lane 2, the hemolymph at the same age (as a reference). All CIs-3, 8, and 13' were more markedly seen in the granule extract than in the fat body extract (cf. Fig. 4, Lane 1).
2). Immunoblotting of the gel with anti-CI-8 serum confirmed the identity of the band (patterns not shown). Also our preliminary immunohistochemical study made with cryostat sections of whole body indicated that the fat body had granules with positive signals for CI-8. These findings imply that CI-8 sequestered into the fat body is stored in protein granules.

4. Discussion

The increase of CI-8 concentration in the hemolymph during larval development of the cz strain may reflect the synthetic activity of CI-8 in the fat body, and the rapid decrease after the onset of spinning may be explained by its resorption into the fat body. This situation is reminiscent of those for the storage proteins as well as for some other humoral proteins of insects (Tojo et al. 1978, 1980; Fujii and Kawaguchi, 1983; Yoshiga et al., 1998). Unlike the storage proteins, which were almost sequestered by the fat body during pharate pupal stage, CI-8 exists in hemolymph throughout larval and pupal stage. Not only CI-8 but also CIs-3 and 13’ are expressed in the cz strain of B. mori and all were detected in the hemolymph during the whole developmental stages (details omitted but cf. Fig. 6, Lane 2). Moreover, the three CIs are present in the protein granules from the pupal fat body (Fig. 6, Lane 1). It is tempting to suppose that these CIs regulate the proteolytic activity in granules, although it is uncertain how CIs co-exist with other proteins.

During the present study, we found that the fat body tissues at the spinning stage was not uniform. The fat body tissues near the midgut (gFB) gave more marked signals for CI-8 than those near the epidermis (eFB) (details will be published elsewhere). We suppose that gFB is responsible for the sequestration of CI-8. Also the regional heterogeneity of fat body tissue has been pointed out in the corn earworm, Helicoverpa zea (Haunerland et al., 1990), wherein perivisceral fat body (=gFB?) but not peripheral fat body (=eFB?) turns blue due to the sequestration of blue-colored lipoprotein after the onset of spinning. It may be of interest to survey whether the differentiation of fat body tissues in terms of locality and function is ubiquitous or not.

B. mori CIs other than those studied in our laboratory have been well characterized mainly from the standpoint of molecular biology (Sasaki 1978, 1985 Sasaki, 1991; Sasaki and Kobayashi, 1984; Narumi et al., 1993; Eguchi and Shomoto 1984, 1985; Matsui and Eguchi, 1991; Eguchi, 1994). In a previous article (Shirai et al., 1994) we predicted that CI-8 is an allelic component of “inhibitor-d” and “sw-Achy” reported by Eguchi and Shomoto (1984, 1985) and Narumi et al., 1993, respectively. Some humoral CIs have been shown to inhibit the cleavage of prophenoloxidase to phenoloxidase (Aso et al., 1994; Ashida and Sasaki, 1994); thus CIs may be involved in the control of prophenoloxidase-activating cascade. Study along this line will provide a clue to the understanding about the implication of the complicated CI system of B. mori.

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