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Absorption of mulberry root urease to the hemolymph of the silkworm, *Bombyx mori*

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Abstract

Mulberry leaves are the sole diet of the silkworm, *Bombyx mori*. The host urease is incorporated into the larval hemolymph and involved in nitrogen metabolism in the insect. To investigate the selective absorption of the host urease to the larvae, crude urease was prepared from mulberry leaves and roots. Root urease was identical to leaf urease on the basis of electrophoretic analyses: (1) the urease activity appeared in the same migration position in a native gel; (2) There was no difference in molecular mass of the subunit. The root urease was orally injected to the fifth instar larvae of the silkworm. Just before spinning, the larvae absorbed intact urease from the midgut lumen to the hemolymph without the loss of activity. The capacity to absorb urease occurred only at the specific stage. Localization of host urease in midgut tissue was observed using confocal laser scanning microscopy and transmission electron microscopy. Based on spatial distribution of immunofluorescent signals and immunogold particles, host urease specifically attached to the surfaces of microvilli existing in the apical side of columnar cells and appeared in the cytoplasm of the cells for transport to the hemolymph. The incorporation efficiency of root urease into the hemolymph was significantly higher than for ureases from jack bean seeds and *Bacillus pasteurii*. The urease that was transported to the hemolymph was electrophoretically altered, compared with the host urease extracted.

Keywords: *Bombyx mori*; Midgut; Microvillus; Mulberry; Urease; Immunoelectron microscopy

1. Introduction

In the silkworm, *Bombyx mori*, a considerable amount of urea is detected in the hemolymph (Yamada et al., 1983; Sumida et al., 1990). The urea concentration in the hemolymph of the larvae is governed by the type of diet. When the larvae are reared on artificial diets, the urea concentration steeply increases from the beginning of spinning. In contrast, its concentration decreases rapidly when fresh mulberry leaves were used for rearing the larvae. These results led to the following speculation: (1) the urease activity which was detected in larvae reared on mulberry leaves originates from the mulberry leaves; (2) the leaf urease could pass across the midgut membrane without being attacked by digestive juice. Recently, Hirayama et al. (2000a,b) prepared two urease samples from host leaf tissues and the larval hemolymph, and then compared their enzymatic properties and N-terminal amino acid sequences. From this comparative study, it was revealed that the leaf urease, which was fed by oral administration, is definitely incorporated into the hemolymph of larvae at the specified age of the fifth instar. In addition, these results allow us to postulate that ammonia produced from urea by mulberry urease can be assimilated into amino acids

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via the glutamine synthetase–glutamate synthase pathway in the same way as plants and micro-organisms (Hirayama et al., 1999). Mulberry urease is most likely to play an important role in urea recycling in the silkworm larvae at older stages.

Leaves of the mulberry tree are the sole diet of silkworms. Therefore, it is rational to use mulberry leaf urease to examine various factors affecting the incorporation of host urease into the hemolymph. However, a large amount of mucilage polysaccharides contained in the leaves make it difficult to successfully separate urease with high activity. In the present study, we will describe the separation of crude urease from mulberry roots as an alternative source and satisfactory transportation of root urease from the midgut lumen to the hemolymph using silkworm larvae of a specified stage. We will also present here a possible route of urease transportation to the larval hemolymph by immunocytological detection and electrophoretic characterization of root urease which had been transported to the hemolymph.

2. Materials and methods

2.1. Preparation of urease from mulberry

Mulberry plants (*Morus alba* cv. 'Shin-Ichinose') were grown in a glass-house controlled at 25 ± 3 °C. Leaves and roots were separately harvested from young trees which were 50–80 cm tall, frozen in liquid nitrogen, and ground in a mortar into powder. The powder was homogenized in 10 mM phosphate buffer (pH 7.5) containing 1% (w/v) cross-linked polyvinylpyrrolidone, 10 mM EDTA and 10 mM DTT, and centrifuged at 20,000 *g* for 10 min at 4 °C. To the supernatant, cold acetone was added for removing 20% (v/v) acetone precipitate by centrifugation. From the resultant supernatant, 20–80% acetone precipitate was obtained by adding cold acetone to give a final concentration of 80% (v/v), and collected as a pellet by centifugation at 20,000 *g* for 15 min. The pellet was suspended in 10 mM phosphate buffer (pH 7.5), adding 10 mM EDTA and 10 mM DTT, which was treated as a crude urease.

2.2. Insects rearing, oral injection and collection of hemolymph

Larvae of the silkworm, *Bombyx mori*, 'Kinshu' × 'Shouwa' race, were reared on an artificial diet (Kyo-ya, Kyoto, Japan) from hatching through to the fifth instar in a room controlled at 25 °C. Under this condition, larval growth was fairly uniform, and the onset of spinning was 7 or 8 days after the fourth ecdysis. Only males were used for this study.

Urease solution (100 μl) was orally injected to the fifth instar larvae by using a micropipette. Preliminary experiments were carried out to check injection manipulation. The orally injected dye solution successfully penetrated the midgut lumen without leaking to the neighboring tissues. Larvae that regurgitated juicy contents from their mouth were omitted. After injection, the larvae were returned to the artificial diet and incubated for 24 h at 25 °C for further rearing. Hemolymph was collected by cutting the abdominal legs. To prevent melanogenesis, 25 μl of 100 mM DTT was added to 500 μl of the collected hemolymph, followed by centrifugation at 7000 *g* for 15 min. The supernatant was frozen in liquid nitrogen and kept at −25 °C prior to use.

2.3. Urease activity assay

The urease reaction mixture (150 μl) was composed of 50 μl hemolymph to be tested, 70 mM urea and 10 mM phosphate buffer (pH 7.5 for jack bean ureases and *Bacillus pasteurii*, pH 9.0 for mulberry urease). The reaction was carried out at 25 °C. After 30 min, 100 μl were pipetted out and diluted with 900 μl distilled water. The ammonium liberated during the incubation was assayed by the indophenol method reported by Witte and Medina-Escobar (2001). A standard curve was made by assaying a series of concentrations of ammonium chloride. Measurements were made at 636 nm in a spectrophometer (Shimadzu UV-1600). Unit definition of urease was as follows: one unit liberates 1.0 μmol of ammonium from urea per minute under the assay condition.

Urease activity was detected by in-gel staining (Witte and Medina-Escobar, 2001). Electrophoresis was performed at 4 °C under nondenaturing conditions. The acrylamide concentration of stacking and separating gels (1 mm thickness, 100 mm × 100 mm size) was 2.9% and 7.5%, respectively. A Tris–glycine buffer system (pH 8.3) consisting of 50 mM Tris and 384 mM glycine was applied and run at constant 20 mA until the tracking dye had just left the bottom of the gel. After electrophoresis, activity bands were visualized by 0.8% (v/v) *p*-nitrotetrazolium solution in the presence of 10 mM urea and 0.5 mM DTT.

2.4. Western blot analysis

Proteins in the crude urease preparation were separated by SDS-PAGE (12.5% gel, 0.1% SDS) according to Laemmli (1970), and transferred to PVDF membrane in 25 mM Tris–192 mM glycine (pH 8.3). Electroblotting was carried out at a constant 2 mA/cm^2 for 1 h. The membrane was then blocked overnight at 4 °C with 10% (w/v) skim milk in 10 mM Tris–HCl (pH 7.5) containing 0.9% (w/v) NaCl and 0.1% (v/v) Tween 20 (Tris-buffered saline incorporating Tween 20: TBST). After washing with TBST three times, the membrane was incubated with 1/2000 diluted anti-urease (jack