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Induction of anhydrobiosis in fat body tissue from an insect

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Abstract

The larva of the African chironomid *Polypedilum vanderplanki* can withstand complete desiccation. Our previous reports revealed that even when the larva is dehydrated without a brain, it accumulated a great amount of trehalose and successfully went into anhydrobiosis. In this paper we determined the viability after rehydration in tissues from the larvae followed by complete dehydration. Only fat-body tissues that were the main producer of trehalose could be preserved in a dry state at room temperature for an extended period of more than 18 months in a viable form. Thus we have confirmed that the central nervous system is not involved in the induction of anhydrobiosis, even in this complex multicellular organism.

Keywords: *Polypedilum vanderplanki*; Anhydrobiosis; Cryptobiosis; Trehalose; Chironomid; In vitro culture; Fat body

1. Introduction

Life and death are mutually exclusive states, but some organisms without metabolic activity after complete desiccation are nevertheless able to resume active life after rehydration. Organisms in this peculiar biological state are highly resistant to dehydration in a condition referred to as "cryptobiosis" or "anhydrobiosis" (Keilin, 1959; Crowe et al., 1992; Wright et al., 1992; Danks, 2000; Clegg, 2001). Larvae of the African chironomid, *Polypedilum vanderplanki*, live in temporary rock pools in semi-arid areas on the African continent and are able to enter anhydrobiosis (Hinton, 1951, 1960a, b). When the rock pools dry up, the larvae become dehydrated but can resume activity within about an hour after water becomes available. This larva is the largest of all animals known to enter anhydrobiosis.

Many anhydrobiotic invertebrates accumulate large amounts of the disaccharide trehalose (Clegg, 1965; Madin and Crowe, 1975; Westh and Ramløv, 1991), which serves as a compatible solute thought to protect desiccating tissues by replacing the primary water of hydration and possibly through sugar glass formation, or vitrification (Burke, 1986; Green and Angell, 1989; Ingram and Bartels, 1996; Crowe et al., 1998; Clegg, 2001). Anhydrobiotic *P. vanderplanki* larvae also accumulate trehalose to levels of about 20% of their dry body weight (40 μg/individual), and we can measure the trehalose content of individual larvae (Watanabe et al., 2002).

Using a classical insect endocrine technique, we also demonstrated that even larvae without a brain, suboesophageal ganglion (SG) and thoracic ganglia (TG) could synthesize trehalose and enter anhydrobiosis successfully (Watanabe et al., 2002). Those observations indicated that induction of anhydrobiosis in *P. vanderplanki* is not under cerebral control (Watanabe et al., 2002), although in general the brain has significant roles in regulating both induction and termination of insect diapause (Denlinger, 1985).

It has been demonstrated, by introducing a recombinant adenovirus vector with trehalose synthase genes

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(Guo et al., 2000), human fibroblasts survived dehydration for 3 days if trehalose was synthesized in the cells. Because *P. vanderplanki* larvae can synthesize trehalose (Watanabe et al., 2002), we determined whether tissues could enter anhydrobiosis under in vitro conditions. We report here that indeed certain tissues from *P. vanderplanki* larvae could be successfully preserved in a dry state at room temperature for an extended period of 18 months in a viable form. Thus we confirm that the central nervous system is not involved in the induction of anhydrobiosis, even in a complex multicellular organism like *P. vanderplanki*.

2. Materials and methods

2.1. Insect rearing

Cryptobiotic larvae of *P. vanderplanki* were collected from rock pools in Nigeria in 2000, and a laboratory colony was established. Several egg masses were put into a glass container (diameter 105 mm, height 75 mm) containing milk agar (2% milk (v/v) and 1% agar (w/v), depth 8–12 mm) and water (depth, 50–70 mm). Last instar larvae were transferred to a plastic container (200 × 300 × 100 mm) containing autoclaved soil (depth 20–30 mm) and water (depth 20–30 mm). The container was covered with a nylon-mesh cage (200 × 300 × 250 or 300 mm). The rearing water was aerated continuously throughout the larval stage. Insects were reared for successive generations under 13 h light:11 h dark photoperiod and 27 °C.

2.2. Procedure for in vitro culture

Last instar larvae of a similar body mass (approximately 1 mg) were immersed individually into 70% ethanol solution for 10 s to sterilize their body surfaces, and washed twice in sterile distilled water. The end of the abdomen was severed from the larvae in a drop of sterile distilled water. Then, the head, including the brain and whole nerve cords, were pulled out together with the alimentary canal and Malpighian tubules. The remaining body parts, mainly fat body and larval skin, were rinsed to remove hemocytes with PBS (0.01 M, pH 7.2). We did not remove the cuticle completely to avoid physical damage to the fat body. The fat body with skin, or the alimentary canal with Malpighian tubules, was placed on a filter paper disc (diameter 6 mm) with 10 μl of various kinds of culture media in a plastic Petri dish (diameter 35 mm, height 10 mm). Seven kinds of media were used for the in vitro culture: supernatant of heat-treated hemolymph of *Bombyx mori* larvae (BmHem) (Nihon Nosan Kogyo, Yokohama, Japan), Carlson's solution (Carlson, 1946), fetal bovine serum (FBS) (GIBCO, New York), minimum essential medium (MEM Earle's, GIBCO, New York), 1% NaCl (w/v) solution, 1% glucose (w/v) solution and solution containing 1% NaCl and 1% glucose. The dish containing the tissues was transferred into a glass container (upper diameter 200 mm, bottom diameter 170 mm, height 250 mm) with 500 ml of saturated NaCl solution, where the relative humidity (r.h.) in the container was sustained at 76% at 25 °C (Winston and Bates, 1960). By exposing them to these conditions, the tissues in the dish were gradually dehydrated over 2 days. They were then completely dehydrated by transferring them to a desiccation box (200 × 250 × 300 mm) with 1 kg of silica gel, where the relative humidity was sustained at less than 5%. In quick desiccation (QD) treatments, the tissues were desiccated within 0.5 day by transferring the dish containing the tissues without the cover into the desiccation box with silica gel. The dried tissues were kept for 1 week, 3 months, or 18 months in the box before the viability check after rehydration was done.

2.3. Trehalose measurements

Each body part desiccated over 2 or 0.5 days was homogenized separately with 0.1 mg of sorbitol as an internal standard in 0.5 ml of 90% ethanol. Trehalose present in the homogenate was measured by the method described in Watanabe et al. (2002). As BmHem contains trehalose at about 0.5% by volume, trehalose de novo synthesized by the various body parts in BmHem was calculated by deducting the endogenous trehalose.

2.4. Assessment of tissue survival

Each desiccated tissue sample on a filter disc was rehydrated with a drop (10 μl) of the medium used for desiccation and incubated for 1–3 h at 100% r.h. The samples were then examined by a double staining method using carboxyfluorecein diacetate succinimidyl ester (CFSE) and propidium iodide (PI) to check the viability of cells in these tissues. The tissues in intact living larvae and larvae frozen overnight at −23 °C were also stained as standards of live and dead cells. Living cells fluoresce green due to esterase activity with the substrate CFSE and nuclei of dead cells fluoresce red by the non-selective penetration of PI. A 0.1 mM stock solution of CFSE was prepared in dimethyl sulphoxide and 1 mg/ml stock solution of PI was prepared in PBS (0.01 M, pH 7.2). Rehydrated tissues were stained for at least 1 min in 0.1 ml of working solution (5 μl of CFSE stock solution and 10 μl of PI stock solution in 1 ml PBS), rinsed twice in PBS and observed under epifluorescence microscopy.