VISUALIZATION OF CHITIN-PROTEIN LAYER FORMATION IN ASCARIS LUMBRICOIDES EGG-SHELLS

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Abstract: The formation of chitin-protein layer in Ascaris lumbricoides egg-shells was studied using the method of chitin structures visualization by means of derivatives of stilbene-disulfonic acid (Blankophor, Bayer, FRG). Intensive formation of chitin structures in A. lumbricoides egg-shell occurred only in fertilized females in a very short portion of uterus from the site of connection of the oviduct with the uterus up to 25—30 mm distally, i.e. in the section forming about one fifth or less than one sixth of length of adult female uterus. Already 3 mm from the beginning of uterus there was a thin layer on the surface of the fertilized eggs which contained chitin detectable by Blankophor visualization, whereas the chitin-protein layer on the shells could not be demonstrated histologically. The thickness of this chitin-protein complex increased distally. At 25 mm from the beginning of uterus the chitin layer was already complete and its thickness did not increase any more.

It is known that only the zygote is involved in the formation of egg-shell chitin layer without participation of epithelial cells of uterus. The precursor of chitin is glycogen, whose amount and localization in relation with the formation of chitin-protein layer of nematode egg-shell has been studied by Lee (1960), Anya (1964), Poór (1968) and others. Dubinsky et al. (1979, 1980) demonstrated the increasing amount of glycogen in oocytes in the growth part of ovaries of both fertilized and unfertilized A. suum females. Immediately after the penetration of the sperm into the egg its glycogen reserves are mobilized and chitin synthesis begins. Chitin is a polysaccharide consisting of N-acetyl-D-glucosamine molecules which are synthesized from glucose released from glycogen reserves in the egg, from glutamine and other amino acids as nitrogen sources, and from acetate released from ascorbic esters (Fairbairn 1957).

The chitin synthesis in A. lumbricoides egg-shells was previously studied only indirectly, by following the level of enzymes catalyzing the metabolic ways of its synthesis (Dubinsky et al. 1988) of the difference in glycogen content in fertilized and unfertilized eggs. The glycogen content in unfertilized eggs decreases only by 22% during their shift through the uterus of unfertilized female. This value probably corresponds with the amount of energy necessary for keeping the basic metabolism of eggs. In fertilized eggs, however, gradually up to 80% of glycogen reserves are observed during this process (Dubinsky et al. 1979). However, no satisfactory specific histochemical test has been available for a direct chitin detection (Wharton 1980).

We utilized the reaction specificity of the stilbene-disulfonic acid derivative produced by Bayer (FRG) under the commercial name Blankophor for the fluorescence-microscopic visualization of chitin structures (Šromová and Hejtmánek 1987). This method is described in the present paper and it can help elucidate the process of chitin-protein layer formation in A. lumbricoides egg-shell and the localization of this process during the maturation of fertilized eggs and their passage through the uterus.
MATERIALS AND METHODS
A series of seminiferous sections was made of eggs obtained from adult fertilized females of *Ascarius lumbroides* recovered from fattened pigs in Provence, where the females were taken from natural ovulation and divided into short sections starting from the site of ovulation connection with uterus up to the distance of 50 mm distally. The uterus sections with eggs were fixed in 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2 and embedded into epoxy resin (Durcupan ACM, FLUKA). Thin sections were 100–120 nm thick. After removal of epoxy resin in saturated NaOH solution in absolute alcohol the slides with seminiferous sections were washed in absolute alcohol every 5 min (3×). Then they were put into phosphate buffer (pH 7.6) for 5 min, washed three times in distilled water and again placed in phosphate buffer (pH 4). After 5-min washing in running water the sections were stained in uranyl acetate in 100 mL of 0.25% NaOH for 3 min. After repeated thorough rinsing in distilled water the sections were transferred through an alcohol series and embedded in Canada balsam. The preparations were then examined in Fluovar (C. Zeiss, GDR) microscope.

Other seminiferous sections from the same portions of uterus were stained with a 1% solution of toluidine blue in 1% borax, pH 11, at 120 °C for 55 sec.

The results obtained by the two methods were compared.

RESULTS AND DISCUSSION
Figs. 1–5 show five studied sections of uterus measured from the site of ovulation connection: at 3–5 mm (PI. I, Fig. 1), at 5–10 mm (PI. I, Figs. 2a, b), at 10–15 mm (PI. I, Fig. 3), at 15–20 mm (PI. II, Fig. 1a, b), and at 20–25 mm (PI. II, Fig. 2a, b).

With regard to the demonstrated sensitivity of the described method for the detection of chitin structures we verified the gradual growth of the chitin-protein layer of egg-shells. The thickness of the chitin structures gradually increased in dependence upon the distance of eggs from the uterus beginning and, consequently, also from the time of fertilization. The radiation intensity also increases in this relation. The method enabled to detect even a very thin layer of chitin at the site where it could not be morphologically detected by other histological methods. This is evident particularly in the first section, where the starting formation of chitin layer is manifested by a visible fluorescence in the fertilized eggs. The chitin layer is here almost 0.5 μm thick in some eggs. However, the chitin-protein layer on the egg surface in these parts of uterus could not be demonstrated by histological methods (PI. I, Fig. 1).

A similar method can be observed also in the second section, where a thin but distinct morphological structure corresponding to the forming chitin-protein layer of egg-shells can be observed in the majority of eggs already in preparations stained by toluidine blue (see arrows in PI. I, Fig. 2b). The differences in the fluorescence intensity and thickness of growing chitin layer seem to be due to the asynchronous course of the fertilizing process in individual eggs. Consequently, the process of chitin formation is asynchronous as well. At the distance of 5–10 mm from the beginning of uterus, the chitin layer can be detected by Blankophor on the surface of all the eggs. A gradually increasing thickness of the chitin layer detectable by Blankophor and visible also in seminiferous sections stained by toluidine blue can be observed in further sections (PI. I, Figs. 3, PI. II, Fig. 1a, b). The thickness of these structures ceases to increase at the distance of about 20 mm distally from the beginning of uterus (PI. I, Fig. 2, a, b).

This thickness was up to 2.8 μm in our preparations. In further sections observed by us up to the distance of 50 mm, the thickness of chitin structures on the egg surface remained constant.

Due to the absence of a sensitive specific method for chitin detection, the majority of authors studying the formation of egg-shells in parasite nematodes have used only indirect methods for the study of its synthesis, which have been confronted with a morphologically visible formation of this shell layer. This way of chitin detection is imperfect and imexact. The Blankophor method described by us, on the contrary, is semiquantitative, very sensitive and enables the detection of even very small amounts of chitin structures in a relatively exact space during the passage of fertilized eggs through the uterus. However, individual data are variable in dependence on the female size, total length of uterus, and eventually on the time which elapsed since the moment of sperm penetration into the eggs.

Our study elucidates also the relation between the formation of chitin layer in egg-shells and ascaroside layer. It can be supposed that the ascaroside layer is formed only on the completion of a complete chitin-protein layer, since a part of refringent granule mass seems to participate in the formation of this layer (Four 1967, Tay et al. 1975). According to Four (1967), there forms first a space between the “shrinkaged” cytoplasm of the fertilized egg which appears in both light and electron microscopes as a homogeneous layer without any structure, surrounded by a superficial viteline layer arising from oolemma. This vitelline layer is therefore termed by some authors “zona pellucida” (Tay et al. 1975); it is filled by chitin structures rapidly but only secondarily. This would mean that the space under the vitelline layer is "empty" at the moment of its origin and is filled only secondarily. The sensitive chitin detection described by us enables us to express a serious presumption that the formation of the so-called free space by shrinking of the egg cytoplasm is a mere optical illusion. On the contrary, the rapid formation of the chitin structures of cytoplasm surface below the oolemma results in its "shift" caused by the intercellular increase in the thickness of the chitin-protein layer of egg-shells. Only after this layer has been completely formed, there begins the formation of the ascaroside layer by a "finger-like" sliding of the membranes of fat vacuoles and refringent granules (Bromová 1980) and a later larval structure of the ascaroside layer is formed (Lyck et al. 1965). All these processes are evidently very rapid and take their course within a few hours (Tay et al. 1975).

ВИДНОСТЬ ОБРАЗОВАНИЯ ХИТОННО-ПРОТЕИНОВОГО СЛОЯ ЯЙЦЕВОЙ ОБОЛОЧКИ ASCARIUS LUMBRICOIDES

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Резюме. Процесс образования хитино-протеинового слоя яйцовой оболочки *Ascarius lumbroides* исследовали методом видимости хитиновых структур с помощью левитации стабилизированных мазков (Blankophor, Vayer, FRP). Эти структуры и яйцевые оболочки *A. lumbroides* проникают только у оплодотворенных самок в очень короткий момент времени, начиная с места перехода яйцевода в матку в дистальном направлении до 25–30 мм, т. о. в участке, обладающем приблизительно одной циклической доминантой, чем одной скрытой. Уже на расстоянии 3 мм от начала матки на поверхности оплодотворенных яиц наблюдалось с помощью Blankophora тонкий слой, содержащий хитин, тогда как с помощью гистохимических методов хитино-протеиновый слой оболочки нельзя было показать. Толщина хитино-протеинового комплекса яйцевой оболочки увеличилась в дистальном направлении. На расстоянии 25 мм от начала матки тонкий слой был уже комбинированный и его толщина в следующих участках больше не увеличивалась.

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Figs. 1—3. Semithin uterus sections with eggs of *A. lumbricoides* taken at different distances from the site of oviduct connection with uterus. Fig. 1. At 3 mm; stained by toluidine blue (500×). Fig. 2a. At 5—10 mm (580×). Fig. 2b. Same as in Fig. 2a; stained by toluidine blue (500×). Fig. 3. At 10—15 mm (580×).

Figs. 1 and 2. Semithin uterus sections with eggs of *A. lumbricoides* taken at different distances from the site of oviduct connection with uterus. Fig. 1a. At 15—20 mm (580×). Fig. 1b. Same as in Fig. 1a; stained by toluidine blue (500×). Fig. 2a. At 20—25 mm (580×). Fig. 2b. Same as in Fig. 2a; stained by toluidine blue (500×).