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LECTIN GENE EXPRESSION IN DIFFERENT TISSUES IN THE MATURE L ARVAL INSTAR OF SILKWORM, *Bombyx mori* L.

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ABSTRACT

Tissues of *Bombyx mori* mature larval instar can be classified into two major groups according to the presence or absence of lectin mRNA; *i. e*. hybridization of the mRNA with the 50 KDa lectin probe: The first group contains fat bodies, midgut, fore and hind wings discs, malpighian tubes, prothoracic gland, anterior silk gland and ovary.These tissues had active lectin gene which was transcribed into lectin mRNA. Therefore, expression of lectin gene was detected in these tissues. The second group of tissues includes testis, trachea, suboesophageal ganglion and thoracic ganglia. They did not give any indication for the presence of lectin gene. Thus, these tissues may have a silent lectin gene or lack lectin mRNA which is responsible for encoding lectin.

INTRODUCTION

The silkworm, *Bombyx mori* L. genes may produce female and male mRNAs by sex-specific alternative splicing, so that expression of one gene can give rise to a family of related protein. Nucleic acids are growth factors for insects where as RNA accelerate the growth rate and the capacity of the call to synthesize protein (Alonso, 1973). Due to the identity of immunochemical properties of the 50 KDa protein lectin (about 600 bp of cDNA clone of the haemocyte 50 KDa lectin) and the haemolymph 350 KDa protein lectin, Amanai *et al*. (1991) clearly mentioned that these are two homologous lectins (hepameric). Since the purification and characterization of the hemagglutinin in the haemolymph of *B. mori* by Amanai *et al.*(1990) and the discovery of the site of haemolymph lectin production and its activation *in vitro* by 20-hydroxyecdysone (Amanai *et al*., 1991), it was possible to employ the reverse transcriptase polymerase chain reaction (RT-PCR) and *in situ* hybridization action as tool to investigate the expression of this gene in different tissues. The present work aims to detect the total RNA from different tissues sites of mature instar larvae of *B. mori* to obtain more direct insight into the localization of the hem agglutinating sugar specific-binding protein (lectin) production *in situ* hybridization using non-radioactive probe, which would hybridize directly with the intact mRNA of the investigated tissue which were fat bodies, midgut, fore and hind wings discs, malpighian tubules, prothoracic gland, anterior silk gland, ovary, testis, trachea, suboesophageal ganglion and thoracic ganglia.

MATERIALS AND METHODS

A standard culture of the silkworm Bombyx mori larvae was reared under laboratory condition (25 ± 2 ºC, 60-75% RH). Larvae were fed on fresh leaves of Morus alba var kukuso, 27, four meals per day were offered to larvae except during the molting periods. To obtain more direct insight into the localization of lectin (Haemagglutinating sugar specific-binding-protein) production, in situe hybridization and control (without probe). Serial sections were carried out on fore and hind wing discs, testis, ovary, interior silk gland, midgut, Malpighain tubes, prothoracic gland, fat body, trachea, suboaesophageal ganglion and thoracic ganglia, tissues of the mature instar larvae.

Detection of total RNA from different tissues of mature larval instar of *B. mori* was carried out according to Chmezynski and Sacchi (1987) in the following steps:

1- Fifth instar larvae which possessed the pigmented spinnerets on day 6 were anaesthetized with diethyl ether for 1 – 2 min. Midgut, fore and hind wing discs, testis, malpighian tubes, prothoracic gland, anterior silk gland and ovary were fixed on ice as quick as possible in a solution of absolute ethyl alcohol (8.36 vol.): formaldehyde (1.14 vol.): glacial acetic acid (0.5 vol.) for 30 – 40 min.

2- Fixed tissues were soaked in 1 X PBS pH 7.4 constraining 15% sucrose and incubated for overnight at 4 ºC increased accessibility by digesting the proteins that surround the target nucleic acid.

3- Tissues were dehydrated in an ascending series of ethyl alcohol, then immersed for overnight in absolute N-butanol, transfused to 1 : 1 N-butanol paraffin (Paraplast plus, Oxford, UK) for 20 min. at 50 – 60 ºC, followed by three changes of pure paraffin within an hour.

4- The microtome knife was sterilized with absolute ethyl alcohol. Sectioning was carried out at 7µm. Sections were left for overnight at 42 ºC and another overnight at 37 ºC and make sure that the section stick firmly on the glass slides.

5- Sections were dewaxed through 2 changes of xylene, hydrated and treated with 0.2 N HCL for 20 min. to improve the ratio of signal to noise level.

6- Sections were rinsed in water for 5 min. and incubated with 1 ng/ml protinase K in 20 m M Tris-HCL (pH 7.4) contained 2 m M $CaCl_2$ for 25 min. at 37 ºC. This treatment increased accessibility by digesting the proteins that surround the target nucleic acid.

7- Sections were treated with 0.2% glycine in 1X PBS, pH 7.4 followed by 1X PBS, pH 7.4 for 2 min. each.

8- Section were refixed again using 4% paraformaldhyde in PBS, pH 7.0 for 20 min. with 3X PBS, followed by 10 min washing with 1X PBS.

9- Prehybridyzation was carried out by using prehybridyzation buffer which containd 50% deionized formamide, 5X SSPE, 5% dextran sulfate, 500 µg/ml denaturated SSS DNA, 250 µg/ml yeast tRNA and 1X Denhardt's solution for overnight at 50 ºC.

10- Hybridyzation process was done by adding 300 µg/ml of the labeled probe (labeled insert DNA) to the prehybridyzation solution and 250 µl of the resulting solution (per slide) was added and incubated at 37 ºC for overnight.

11- Sections were washed three times within 2-3 hours with a solution containing 50% deionized formamide and 5X SSPE. The second washing was carried out through 3-5 changes of PBT within 2 hours.

12- Sections were treated with 5% sheep serum (Cappel, USA) in PBT for overnight at 4 ºC, followed by incubation with anti-digoxigenin-AP Fab fragments for overnight at 20 ºC.

13- After washing sections several times with PBT within 2 hours, they were incubated with the coloring buffer for an hour at room temperature.

14- Sections were developed using coloring solution for 10-25 min. The reaction was stopped by PBT and sections were photographed immediately.

The non radioactive probe (about 600 bp of cDNA clone of the haemocyte 50 KDa lectin, labeled with Digoxigenin) was kindly provided by Dr. Masafum Iwami, Biology Department, Faculty of Science, Kanazawa University, Japan. This probe would hybridize directly with the intact mRNA of the investigated tissues. Control serial sections were treated with the prehybridization solution without the labeled probe.

RESULTS AND DISCUSSION

1. Midgut:

Control serial sections did not show any signal of positivity (Fig. 1 d). Treated sections show a moderate positive signal represented by a very fine purple dots corresponding to the hybridization between the probe and the mRNA in some midgut columnar cells and some regenerative cells (Fig. 1 b&c). Amanai *et al*. (1990) recorded that *Bombyx* lectin has glucuronic and galacturonic acid binding specificity.

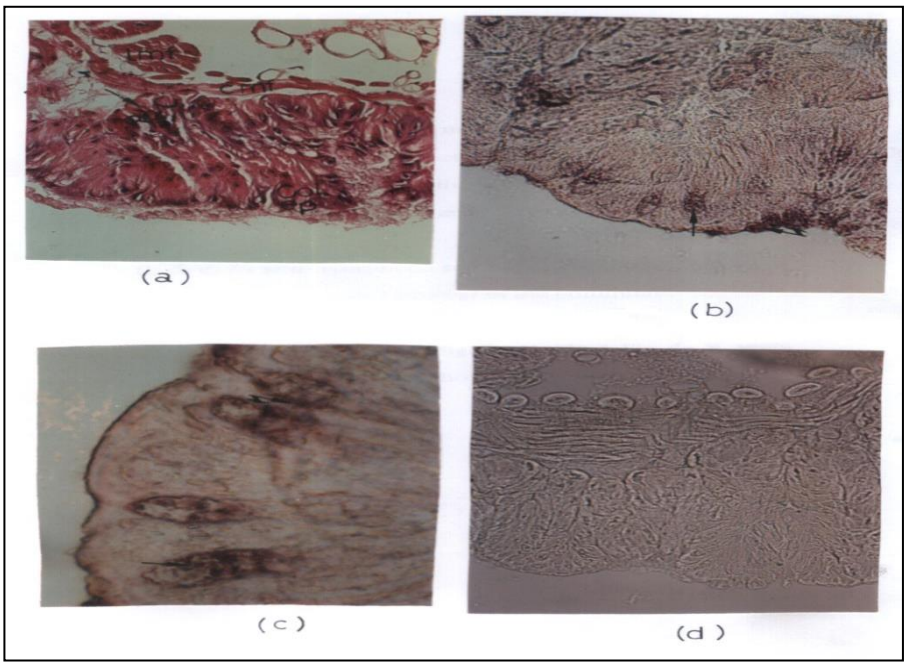

Fig. 1. Transverse sections of the midgut:(a) H & E-stained transverse section showing, the columnar epithelium (Col. ep.), regenerative cells (Reg. c.), thin layer of circular muscle fibers (c.m.f.), and longitudinal muscle fibers (l.m.f.). X. 200. (b & c) *in situ*-hybridized sections showing weak hybridization signals (arrows) in the columnar epithelium and some regenerative cells .(Fig. 1b X. 400 & Fig. 1c X. 1000). (d) In situ control section showing no hybridization signals. (Fig. 1d X. 200).

2. Silk gland:

Control serial sections in the anterior silk gland (Fig. 2 d) did not show any positive signal neither in the call cytoplasm nor in the nucleus comparing with figure (2a). Treated sections show a slightly high positive signals, homogeneously distributed throughout the cytoplasm specially in the lower twothird of the cells (Fig. 2 b&c). This means that the hybridization between the probe and the mRNA has a specifically binding to the anterior silk gland cells which have hemocytes 50 KDa lectin mRNA. This finding is coincide with Amanai *et al.* (1991) whose reported the presence of this lectin in the cuticular intima. This result can be suggested that *Bombyx* lectin gene is transcribed to the corresponding mRNA. This mRNA could be translated into 50 KDa lectin which secreted into the cuticular intima. On the other hand, Couble *et al.* (1983) detected during the course of the last instar quantitative changes in mRNAs for ribosomal proteins, enzymes and other peptides involved in the synthesis and secretion of silk proteins.

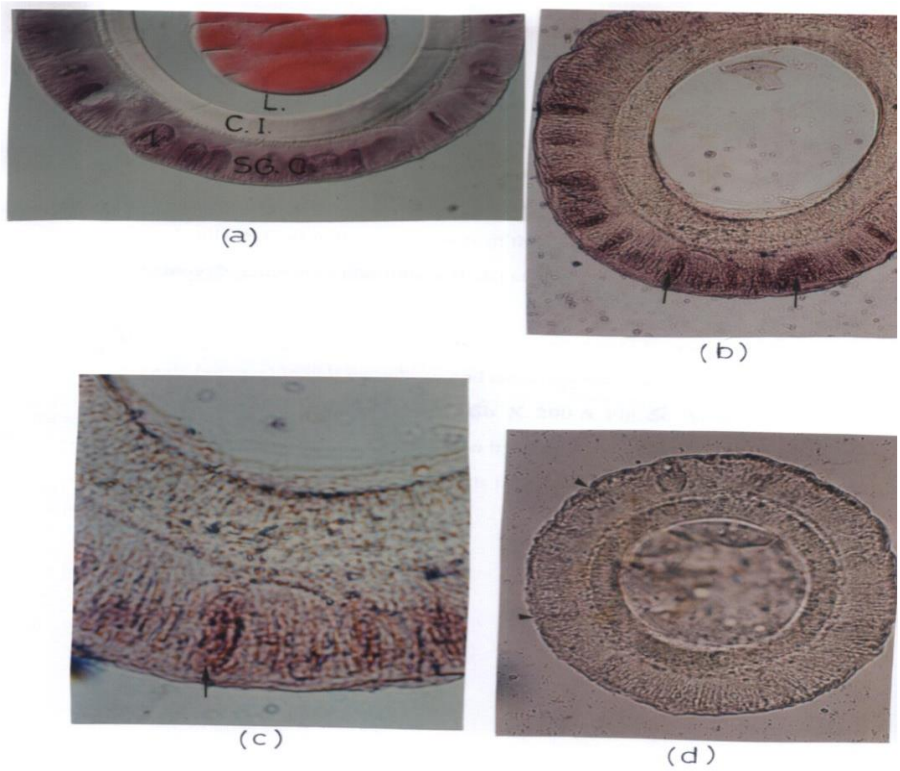

Fig.2. Transverse sections of the anterior silk gland: (a) H & E-stained transverse section showing, the anterior silk gland cells (S.G.c) with several parts of the highly ramified nucleus (N.), the cuticular intima (C.I.) enclosing the large lumen. X. 400. (b & c) *In situ*-hybridized sections showing moderated hybridization signals (arrows) in cell cytoplasm and some nuclear parts. (Fig.2 b X.200 & Fig.2 c X.1000).(d) *In situ* control section showing no hybridization signals. The distance between two arrow heads is one cell

3. Malpighian tubes

The cells of malpighian tubes have a large nuclei and are standing on a tough basement membrane (Fig. 3a). In situ hybridization experiment (Fig. 3 d) did not show any positive signal in control serial sections. Treated sections show the transcribed mRNA of the *Bombyx* 50KDa lectin gene in the malpighian tube cells. Highly positive signals are observed in the apical cytoplasm part of the cells and near the absorption areas of the tubules. The same signals were noticed in the nucleus (Fig. 3 b& c).

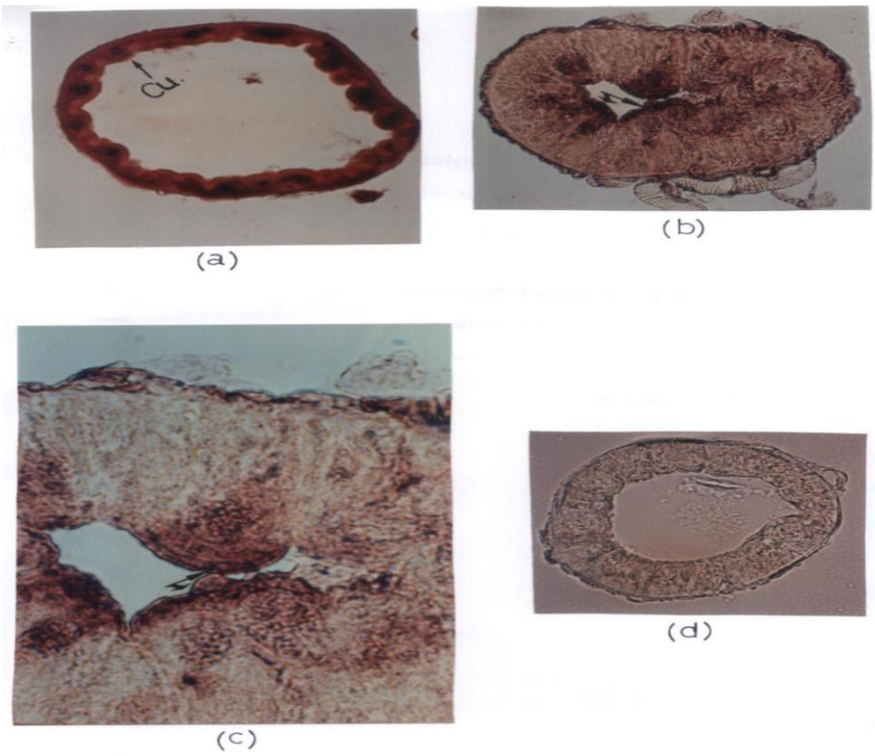

Fig.3. Transverse sections of the Malpighian tubes.(a) H & E-stained transverse section showing, the tube wall is one cell-layer with very thin cuticle (Cu). X. 200. (b & c) *In situ*-hybridized stained sections showing the slightly high signal of hybridization (arrows) at the apical cytoplasm of the cells. (Fig3b X. 400 & Fig. 3c X. 1000).(d) In situ control section does not show hybridization signals at any site.X. 200.

4. Prothoracic gland:

The cells nuclei of prothoracic gland are small and oval when the secretory activity is low, but in the active gland they become enlarged and lobulated and the cells has more extensive and deeply staining cytoplasm (Fig. 4). Control serial sections did not show any positive results (Fig. 4 a). Treated sections show high intense signals in the cells (Fig. 4 b). These results revealed that the Bombyx 50KDa lectin is synthesized in the prothoracic gland of insects. Amanai *et al*. (1990) recorded that the hemagglutinating activity in the larval haemolymph of *B. mori* fluctuates during the 4th instar inconcert with the changes in ecdysteriod secretory activity of the prothoracic glands. However, relationship between the lectin titer and the ecdysteriod titer in the haemolymph was not clear during the 5th larval instar.

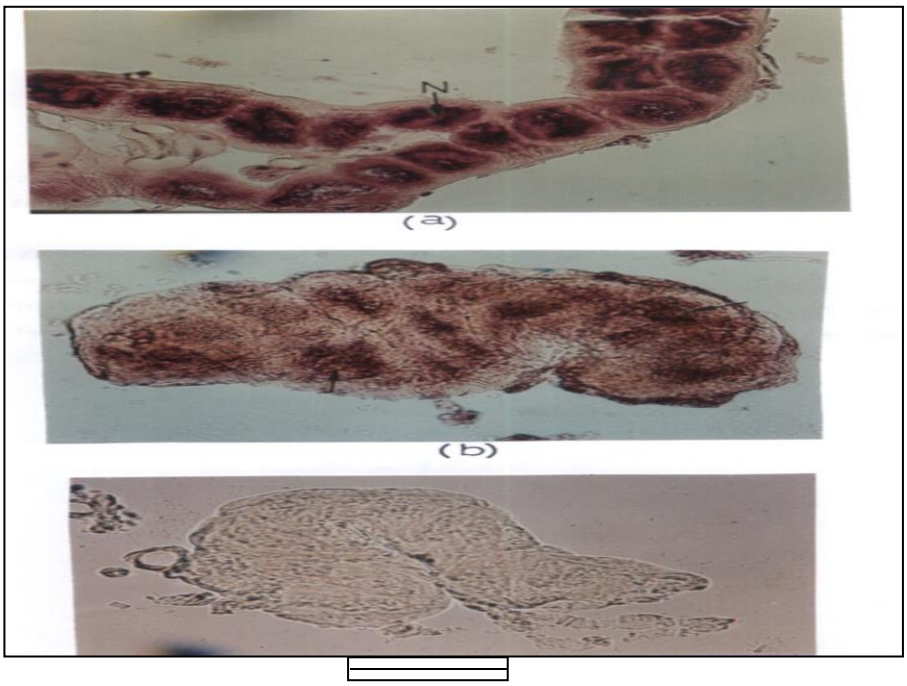

Fig.4. Transverse sections of the prothoracic gland.a) H & E-stained transverse section showing, the large active gland cells with lobulated nuclei. (N). X 400. (b) *In situ*-hybridized sections slightly high positive hybridization signals (arrows) distributed throughout the cytoplasm. X.400. (c) *In situ* control section showing no hybridization signals. X. 400.

5. Ovary:

Ovaries of mature larval instar of *B. mori* is shown in Fig. 6 a&d. *Bombyx* larval ovary shows strong positive signal in the nurse cells, follicular cells and in the inter-ovatiole connective tissue cells (fig. 5 b & c) which bind the ovarioles to each other while no signal was observed in the oocytes (fig. 5 e). Consequently, we can concluded that the *Bombyx* 50KDa lectin gene is transcribed into lectin mRNA in the nurse cells, and will release later into oocytes. Our results are coincide with the result of Amanai *et al*. (1994) about the participation of this lectin in early embryonic development. They found that lectin mRNA was transcribed mainly in the nurse cells, transferred via the ring canal into the ooplasm and finally deposited in the oocyte durin the course of oogenesis. In the present work, Bombyx 50KDa lecti gene is active and transcribed to lectin mRNA which will translated to the 50KDa lectin protein. On the other hand, Amanai *et al.* (1991) observed that the ovaries release *in vitro* significant amount of proteins with hemagglutinating activity (lectins) into the culture medium.

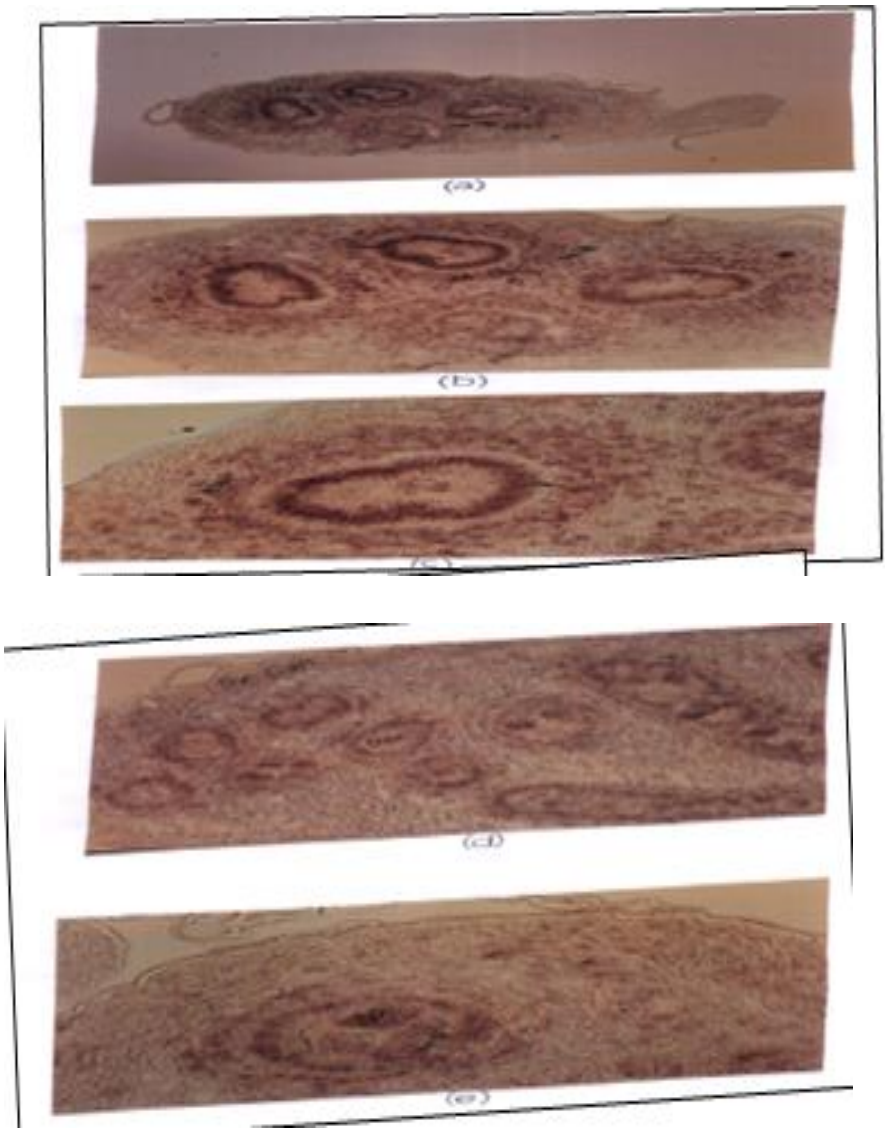

Fig.5. Transverse sections of the Ovary. (a) *In situ*-hybridized sections showing the four ovarioles (biheaded arrows).X. 100. (b, c) *In situ*- hybridized sections showing highly positive hybridization signals (arrow) in the follicular cells (F.c.), and (biheaded arrow) in the inter-ovariole connective tissue (int.ovl. C.T.). (Fig. 5b X. 200 & Fig. 5c X. 400). (d) *In situ*-hybridized sections showing no hybridization singles neither in the ovarian capsule (Ov.Cap.) nor in the oocytes(Oo). X.200. (C) *In situ*-hybridized section showing highly positive Hybridization signals (biheaded arrow) in the nurse cells(Nc). X.400.

6. Imaginal wing discs and hemopoietic organ:

In the 4th and 5th larval instars, the wing initiates to develop as an evagination within the prepodial cavity and finally everted at the larval-pupal moult. The wing discs cosist of single folded layer of epithelial cells. The disc is surrounded by a basement membrane of the larval epidemis. The most common cell type is the columnar cells (Figs. 6a & 7 a). Treated serial sections of both fore and hind wing discs show a very high intensity of hybridization signal in the columnar cell of the fore wing discs (Figs. 6b & c) while the intensity is slightly lower in the hind wing discs (Figs. 7b & c).

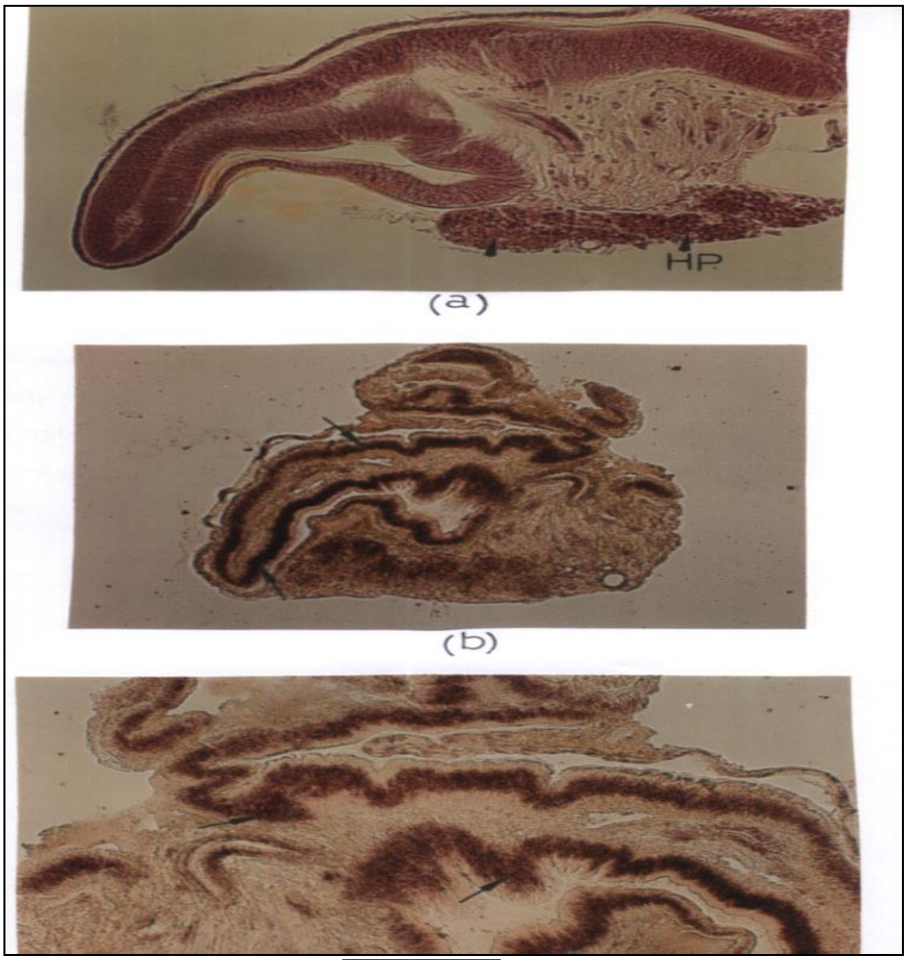

Fig. 6. Transverse sections of the fore wing disc:(a) H & E-stained transverse section showing, the single cell layer of the columnar epithelium , and the hemopoeitic organ. (H.P. & arrow heads X. 200 .(b,c) *In situ*-hybridized stained sections showing very strong hybridization signal (arrows) in the columnar cell layer of the fore wing disc. (Fig. 6b X. 100 & Fig. 6c X. 400).

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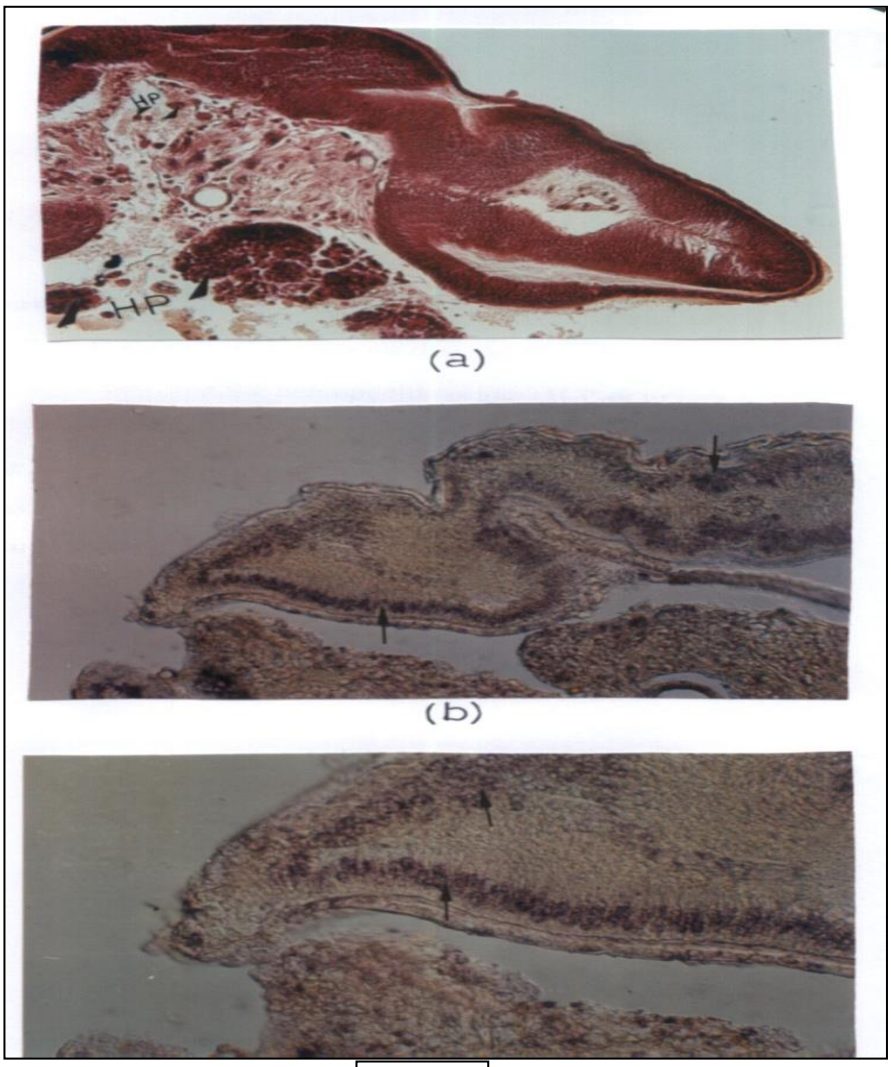

Fig. 7. Transverse sections of the fore wing disc.(a) H & E-stained transverse section showing, the single layer of columnar cell , and the hemophilic organ. (H.P. & arrowheads) X. 200. (b,c) *In situ*- hybridized stained sections showing highly positive hybridization singnals in the hind wing disc columnar cells (arrows) (Fig. 7b X. 200 & Fig. 7c X. 400)

This indicates that both of the fore and hind wing discs have 50 KDa *Bombyx* lectin gene expression. Okuda *et al.*(1985) mentioned that ecdysteroid control the expression of many genes involved in metamorphosis when larvae were dissected *i. e.* at gut purge, the haemolymph ecdysteroids

titer was high. This high ecdysteroid titer may be responsible for triggering 50 KDa protein gene transcription and mRNA translation into the *Bombyx* 50 KDa protein by the fore, hind wing discs and by the haemopoietic organ. Figure (8a & b) shows the most highly intense signals in the hemopoietic organ at the site of the hemocyte differentiation. This result was expected because the used probe is a cDNA clone of the 50 KDa lectin from hemocyte. It is difficult to carry out the *in situ* hybridization experiment for hemocytes in haemolymph, thus the experiment was done for the organ (hemopoietic organ) which produce the hemocytes.

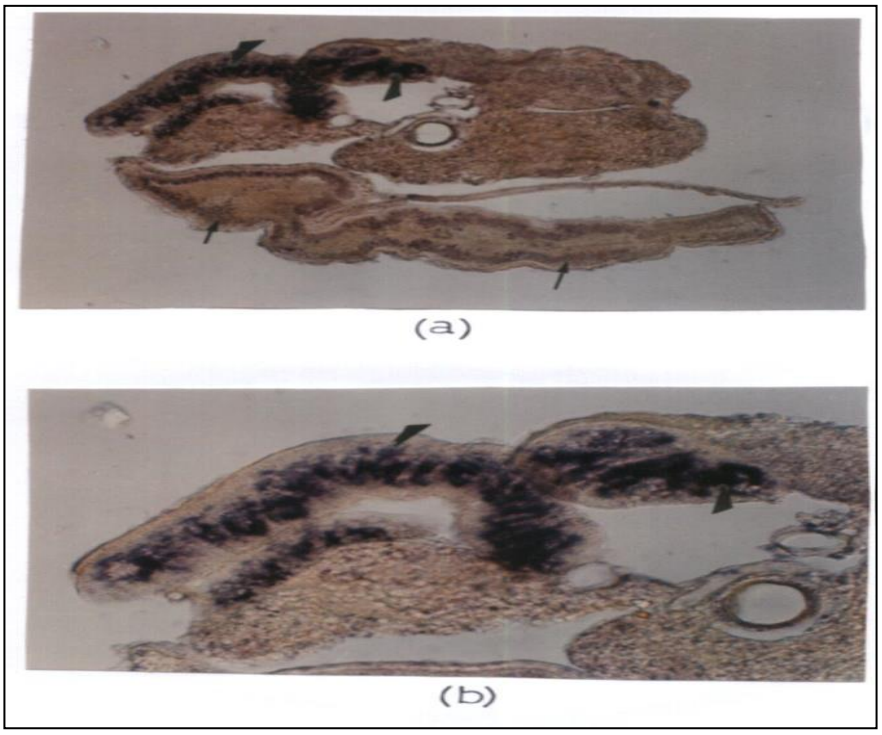

Fig.8. Transverse sections of the hind wing disc with the hemopoeitic organ. (a,b) *In situ*–hybridized sections showing the most highly hybridization signals (arrow heads) in early hemocytes differentiation stages in the hemopoeitic organ. (Fig. 8a X. 100 & Fig. 8b X. 200)

7. The fat bodies:

RNA extracted from different locations of the fat body, followed by RT-PCR for the mRNA and southern hybridization clearly demonstrated that fat body tissue contains an active 50 KDa protein gene. This gene was transcribed into detectable mRNA, which in turn could be translated to lectin (Abou el ela *et al.*, 2001). Our result indicate that the fat body contains 50 KDa protein, but this result may not emphasis that this protein is secreted by the fat body or it selectively removed from the haemolymph and stored intracellular granules in the fat body for use at metamorphosis.

8. Testis, trachea, suboesophageal ganglion and thoracic ganglia:

The testis, trachea, suboesophageal ganglion and thoracic ganglia did not give any indication for the presence of lectin gene, thus these tissues may have lectin gene switched off. This finding is in agreement with the data of haemolymph electrophoresis which shows that the band with molecular weight of 49.634 KDa was not found in males while it was found in females.

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جين اللاكتين في الأنسجة المختلفة لليرقات تامة النمو لدودة الحرير التوتية *Bombyx mori* L.

محمد أحمد عيد ، سعد بن عايض العتيبي ، صلاح عبد الله صالح المعصراوي و محمد رفعت أبو العلا

قسم الحشرات الاقتصادية والمبيدات – كلية الزراعة – جامعة القاهرة – الجيزة – مصر .

يمكن تصنيف الأنسجة في اليرقات تامة النمو لدودة الحرير التوتية بناء على وجود أو غياب لاكتين الحامض النووي الريبوزي الرسول mRNA إلى مجموعتين . المجموعة الأولى تشمل أنسجة الأجسام الدهنية ، والمعدة ، وبراعم الأجنحة الأمامية والخلفية ، وأنابيب ملبيجى ، وغدة الصدر الأمامي ، وغدة الحرير ، والمبايض . هذه الأنسجة تحتوي على جين اللاكتين النشط الذي تم الكشف عنه فيها . المجموعة الثانية تشمل الخصية ، والقصبات الهوائية وغدة تحت المرئ والغدة الصدرية ، وهذه المجموعة لم تظهر أي مؤشرات لوجود جين اللاكتين بها ، وقد يكون الجين موجود بها في صورة صامتة (غير نشطة).

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