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Histochemistry of digestive organs and glands of *Deudorix isocrates* (Fab.) (Lepidoptera: Lycaenidae)

Ravindrakumar D Patil
Department of Zoology, Maratha Vidya Prasarak Samaj's K.T.H.M. College, Nashik, Maharashtra, India

Abstract
The pomegranate fruit borer, *Deudorix isocrates* is a serious pest of pomegranate causes remarkable economic loss of pomegranate growers. This pest was remained untouched to study its histology and histochemistry of the various organ systems which helps in understanding of the physiology of the pest. Knowledge of these fundamental aspects of pest studies is significant in pest management. Histochemistry is one of the important aspects studied by researchers to evaluate effect of insecticides to manage their population. The present research work was done to study histochemistry of midgut of larva and adult, rectal organ of adult and secretory glands of the larva and adult with respect to characterize the neutral polysaccharides, sulphated and non sulphated acid polysaccharides using PAS and Alcian Blue activities at pH 1.5 and pH 2.5.The results revealed that comparatively there was the highest accumulation of neutral polysaccharides in the apical cytoplasm and muscle coat of the larval midgut, muscle coat of the adult midgut, intima of the rectal caecum and luminal content of adult salivary glands. In the remaining tissues of the organs under study it was appeared very weak to moderate. The intima of rectal caecum and larval mandibular glands demonstrated the highest accumulation of sulphated acid polysaccharides while outer band of salivary secretion in larva, apical cytoplasm and muscle layer of adult midgut, rectal pad and luminal contents of adult salivary gland demonstrated the highest accumulation of non sulphated acid polysaccharides.

Keywords: *D. isocrates*, pest, histochemistry, PAS activity, Alcian blue activity, neutral polysaccharides, acid polysaccharides

Introduction

Deudorix is one of the lepidopteran genera (formerly known 'Virachola') includes some pest species which attack wide range of host plants, mostly the fruit plants. *D. isocrates* is one of the polyphagous pest species of this genus. It attacks wide range of host plants like guava, aonla, ber, citrus, tamarind, apple, sapota apart from the pomegranate (Singh and Singh, 2009; Balikai *et al*., 2011; Chhetry *et al*., 2015; Arya and Dubey,2017; Gundappa et al., 2017; Muthiah and Indragandhi, 2021) [22, 3, 5,1,8,16]. However, it appeared as the most destructive pest of Pomegranate, *Punica granatum* (Gupta and Dubey, 2005, Kumar, 2010) [9, 12] causing 65-70% yield loss worldwide (Kumar *et al*., 2017) [13]. It is distributed all over India and common in Maharashtra (Ritesh kumar, 2020) [18]. In India it causes 40-90% yield loss of pomegranate (Wadhi and Batra, 1969; Nair, 1978) [26,17] consequently results in to remarkable economic loss of the pomegranate growers. In Maharashtra and Karnataka, the incidence of *D. isocrates* on pomegranate has been reported throughout the year with varying degrees of intensity (Shevale and Khaire, 1999) [21]. The basic knowledge of internal systems of insect pest may prove to be beneficial in new trends of pest control or integrated pest management. The anatomical, histological and histochemical studies are very significant to provide background for understanding the future physiological aspects of the studies and its use in pest management. Recently, histochemical studies have been carried out to evaluate effect of botanicals on some tissues of the lepidopteran pest (Cruz *et al*., 2015) [6] and to understand development of the pest species (Vaca *et al*., 2019) [23]. Histochemical studies are useful to localize different chemical compounds or groups in the cells of internal systems. Including Lepidoptera, histochemical characterization of neutral, acidic mucopolysaccharides and different mucosubstances using PAS activity and Alcian blue (AB) at pH 1.0 or 1.5 and pH 2.5 in the tissue from various insect groups has been carried out (Verma and Sinha, 1980; Anwar, 1983; Zara and Caetano, 2004; Rivera et al., 2012; Lemos *et al*., 2018) [25, 2, 28, 20, 14]. The present work was undertaken to characterize neutral polysaccharides, sulphated and non sulphated acidic polysaccharides in the tissues of the midgut and salivary glands of the larva and adults, mandibular gland of larva and the rectum of adult using PAS, AB at pH1.5 and AB at pH2.5 activity. It is the first report on histology and histochemistry of the internal organs of *D. isocrates* larva and adult. The results offer histochemical understanding of the internal organs which will help to evaluate effect of various synthetic and herbal insecticides to control measures against this pest.

Materials and methods

The histochemistry of digestive organs of larva and adult of *D. isocrates* was studied to demonstrate mucopolysaccharides from the tissues of the midgut and salivary glands of the larva and adults, mandibular gland of larva and the rectum of adult. The live specimens which were obtained by collection from pomegranate field and laboratory reared stock were used to dissect and separate the organs under study. The organs were fixed in carnoy fixative, cleared in Benzene and infiltrated with paraffin

wax (M. P. 52ºC – 54ºC) for two and half hours. The cross sections were cut at 7 µ with a rotary microtome. Sections were spread on pre-egg albumin smeared slides. To understand general histological characteristics some sections were stained with Delafield's hematoxylin and 1% alcoholic eosine. The stained sections were mounted in DPX after clearing in xylene. The histological observations were made using light microscope. To characterize mucopolysaccharides a batch of various tissues was subjected to Periodic Acid Schiff's (PAS) reaction. The sections without periodic acid (without oxidation) were kept as control. One set of another batch of the tissues was subjected to Alcian blue (AB) at pH 1.5 while second set of another batch of the tissues was subjected to AB at pH 2.5. Permanent preparations were made of PAS subjected and AB (pH 1.5), AB (pH 2.5) subjected tissue sections. All the results obtained were tabulated according to colour intensity into different increasing grades ranging from -ve to 3+.

Results

In *D. isocrates* the wall of the larval midgut is composed with simple columnar epithelium with tall columnar epithelial cells, regenerative and goblet cells. The nuclei are situated misally or apically. The regenerative cells are distributed individually at the base of epithelium along the basement membrane. The circular muscle layer is thin. Bundles of longitudinal muscles are scattered around the circular muscle layer. In general the midgut cytoplasm was weakly (1+) PAS positive whereas apical boarder of epithelium, and muscle coat demonstrated intense (3+) PAS positive activity. The general epithelium was weakly (1+) AB positive at pH1.5 whereas the muscle layer was moderately (2+) AB (at pH 1.5) positive. The general epithelium of midgut and muscle layer both demonstrated moderate (2+) AB (at pH 2.5) positive activity (Table.1, Fig.1 a to c)

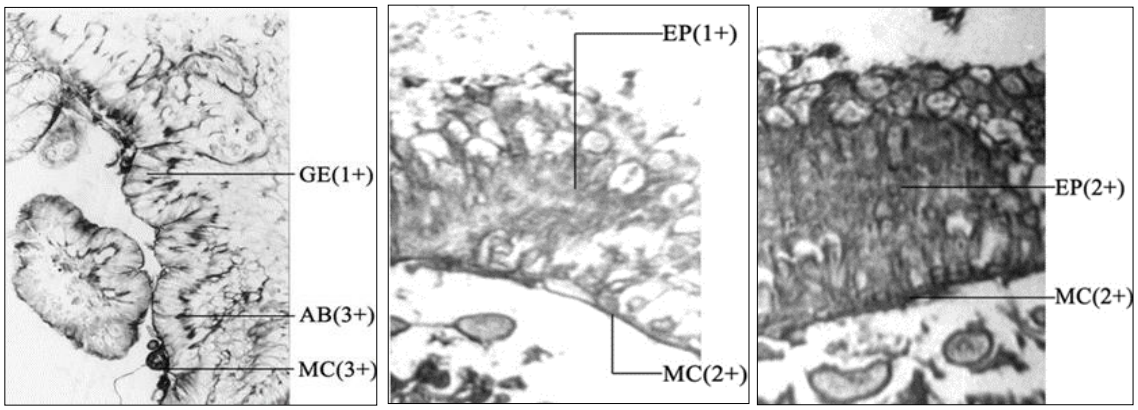

Fig 1a **Fig 1b** **Fig 1c**

Fig 1: T.S. of midgut, *D. isocrates* larva. a) PAS activity x115 b) AB activity at pH 1.5 x250 c) AB activity at pH 2.5 x250 AB: Apical border of midgut epithelium, EP: Midgut epithelium, GE: general cytoplasm of midgut epithelium, MC: Muscle coat

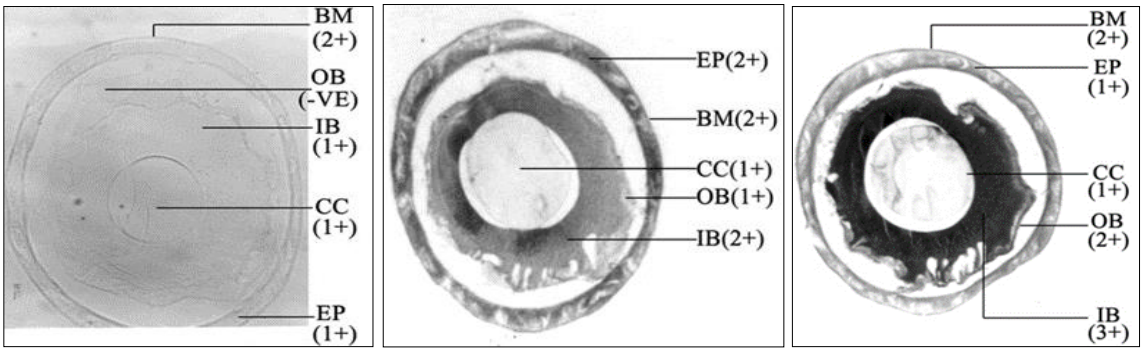

Fig 2a **Fig 2b** **Fig 2c**

Fig 2: T.S. of salivary gland reservoir, *D. isocrates* larva a) PAS activity x165 b) AB activity at pH 1.5 x159 c) AB activity at pH 2.5 x159 BM: Basement membrane, CC: Central core, EP: glandular epithelium, IB: Inner band, OB: Outer band.

The midgut wall of the adult shows absence of peritrophic membrane. The epithelium of midgut is lined with tall columnar cells with round to oval nucleus. The epithelium is thrown into little folds. The regenerative cells are distributed in the form of 'nidi'. The epithelium is encircled by a thin layer of circular muscles. The longitudinal muscle bundles run external to the circular muscle layer. The general epithelium of midgut was weakly (1+) PAS positive but its apical border was moderately (2+) PAS positive. The muscle layer of midgut demonstrated intense (3+) PAS positive activity. The general epithelium of midgut was weakly (1+) reactive to AB at pH 1.5. The muscle layer and apical border of epithelium were moderately (2+) reactive to AB at pH 1.5. The general epithelium of midgut was moderately (2+) reactive to AB at pH 2.5. The muscle layer and apical border of epithelium demonstrated intense (3+) AB at pH 2.5 activity (Table.1)

In the adult *D. isocrates*, the rectal wall is composed of intima, epithelium and muscle layer. The intima is thin which lines general epithelium as well as rectal pads. The

epithelium of rectum including the portion of rectal caecum is highly folded. It is composed of small cuboidal as well as columnar cells. The nuclei in both the type of cells are round. The rectal pads are present in the form of darkly stained disc like structures, which are distributed throughout the epithelium of the rectal caecum. In each rectal pad two cortical cells develops a single discoidal cytoplasmic mass. The membrane in between two cells is indistinct while the nuclei are distinctly visible. At the posterior most region of rectum, the rectal epithelium is remarkably different. The epithelium is composed with small cuboidal cells with ovoid nucleus. The epithelium is thrown into long and slender folds, which project in the lumen. Throughout the rectal region the circular muscles surround the epithelium and form thin layer. The bundles of longitudinal muscles lie outside to the circular muscle layer. The general epithelium of rectum was weakly (1+) PAS positive whereas rectal pad was moderately (2+) PAS positive. The rectal intima and muscle layer were intensely (3+) PAS positive. The epithelium and rectal pad (cytoplasm) were moderately (2+) AB (at 1.5) positive whereas the rectal intima and muscle layer were intensely (3+) AB (at 1.5) positive. The epithelium demonstrated weak (1+) AB (at pH 2.5) positive activity while intimal lining demonstrated moderate (2+) AB (at pH 2.5) positive activity. The cytoplasm of rectal pad and muscle layer were intense (3+) AB (at pH 2.5) positive (Table.1)

The salivary gland of the larva is distinguished into posterior secretory region, reservoir and salivary duct. The posterior secretory region composed of epithelial cells with the irregular shaped nucleus. The cytoplasm and nuclear sap appears granular. The epithelium is externally based on basement membrane. The lumen is completely filled with secretion, which forms a 'core' in the lumen of reservoir. The reservoir is composed of epithelium, which is based on basement membrane. The epithelial cells have nucleus with irregular shape. The luminal contents show three distinct bands around the central core. The duct of salivary gland is lined with well distinct intima. The epithelium is made up of large cuboidal cells. The oval nucleus is large and placed transversely at the center of cell. Cells rest on basement membrane. The paired duct anteriorly unites and forms a short common duct, which enters in the hypopharyngeal premental lobe and continued into the silk press. The dorsal wall of silk press invaginates deeply in to the lumen. The muscles from the hypopharyngeal premental lobe are inserted on the dorsal wall of silk press. A pair of Lyonet's glands is associated with salivary duct. Histologically it is composed of round cells arranged in a cluster. The cytoplasm of cell is characteristically vacuolated and granular. The nucleus is irregular shaped. All the gland cells rest on thin common basement membrane. For

histochemical study the sections were selected from the posterior region of reservoir where the luminal content shows core and two distinct bands; inner band and outer band. The glandular epithelium, central core and inner band of luminal content were weakly (1+) PAS positive. The basement membrane demonstrated moderate (2+) PAS positive activity. The outer band of luminal content was negative to PAS. The glandular epithelium, basement membrane and inner band of luminal content were moderately (2+) AB (at pH 1.5) positive whereas outer band and central core were weakly reactive (1+) to AB (at pH 1.5). The glandular epithelium and central core were weakly (1+) reactive to AB (at pH 2.5). The basement membrane and outer band were moderately (2+) reactive to AB (at pH 2.5). Inner band of luminal content demonstrated intense (3+) AB (at pH 2.5) activity (Table.1, Fig.2a to c).

The salivary gland of adult is composed of large cuboidal cells, which rest on basement membrane. The cells bear oval nucleus, which is placed centrally. The cytoplasmic content appears granular. The luminal space contains luminal contents. The glandular epithelium demonstrated very weak (1±) PAS positive activity whereas basement membrane was weakly (1+) PAS positive. The luminal content was intensely (3+) PAS positive. The glandular epithelium was weakly (1+) AB (at pH 1.5) positive whereas the basement membrane muscle coat was moderately (2+) AB (pH 1.5) positive. The basement membrane was weakly (1+) AB (at pH 2.5) positive. The glandular epithelium was moderately (2+) AB (at pH 2.5) positive while the luminal content was intensely (3+) AB (at pH 2.5) positive (Table.1).

D. isocrates larva has paired mandibular glands. The glandular intima is thin. The epithelium is composed with small cuboidal cells having basally situated, darkly stained round compact nucleus. The cells rest on basement membrane. The epithelium of mandibular gland was negative to PAS. The basement membrane and intima of the mandibular gland demonstrated weak (1+) PAS positive activity. The epithelium and basement membrane were weakly (1+) reactive to AB at pH 1.5 while the intimal lining was intensely (3+) reactive to AB at pH 1.5. The epithelium was weakly (1+) reactive to AB at pH 2.5 whereas the basement membrane and intimal lining demonstrated moderate (2+) AB (at pH 2.5) positive activity (Table.1).

Table 1: PAS and AB activity (at pH 1.5 and pH 2.5) in midgut and glands of *D. isocrates*

Organs	PAS	AB at p^H 1.5	AB at p^H 2.5
Midgut of larva			
Epithelium			
a. Apical border	3 +	1+	2+
b. General cytoplasm	1 +	1+	2+
Muscle coat	3 +	2 +	2+
Salivary gland of larva			
Luminal content			
a. Central core	1 +	1 +	1 +
b. Inner band	1 +	2 +	3 +
c. Outer band	-ve	1 +	2 +
Epithelium	1 +	2 +	1 +
Basement membrane	2 +	2 +	2 +

Mandibular gland of larva			
Intima	1+	3 +	2 +
Epithelium	-ve	1 +	1 +
Basement membrane	1 +	1 +	2 +
Midgut of adult			
Epithelium			
a. Apical border	2 +	2 +	3 +
b. General cytoplasm	1 +	1 +	2 +
Muscle layer	3 +	2 +	3 +
Rectal caecum			
Intima	3 +	3 +	2 +
Rectal pad (cortical cells)	2 +	2 +	3 +
Rectal epithelium	1 +	2 +	1 +
Muscle layer	3 +	3 +	3 +
Salivary gland			
Luminal content	3 +		3 +
Epithelium	1 ±	1 +	2 +
Basement membrane	1 +	2 +	1 +

Note: 1±: Very weak activity 1+: Weak activity
2 +: Moderate activity 3: Intense activity

Discussion

The present work reports the presence of neutral, sulphated and nonsulphated acid mucopolysaccharides in the various tissues of the larval and adult organs related with digestion, reabsorption and secretion activities in larval and adult *D. isocrates*. The neutral mucopolysaccharides were characterized by PAS activity while sulphated and nonsulphated acid mucopolysaccharides were characterized by Alcian Blue (AB) at pH 1.5 and pH2.5 activity.

The weak PAS positive activity in the secretion of midgut cells of the millipede *P. nigrolabiatus* (Joshi, 1972) [10] was demonstrated by the brush border and cell secretion which indicated the presence of neutral mucopolysaccharides in the respective sites of midgut. Similarly, the moderate to intense PAS positive activity at apical border of epithelium and the muscle coat in the larval midgut of *Musca domestica* (Mustafa and Kamat, 1973) [15] revealed the presence of neutral mucopolysaccharides. These reports supports the observations of the present work in which the presence of neutral mucopolysaccharides with the same intensity at the apical cytoplasm of the midgut epithelium and the muscle layer has been reported in the larval midgut. The AB reaction in midgut of housefly larva, *Musca domestica* (Mustafa and Kamat,1973) [15] revealed the presence of acidic mucosubstances, particularly, sulphomucins in the midgut region. In present study, the apical border of epithelium demonstrates AB positive activity with similar intensity to the general epithelium at both pH 1.5 and pH 2.5. These observations are indicative for presence of sulphated as well as non sulphated acid mucopolysaccharides in the midgut of *D. isocrates* larva.

PAS activity and AB at pH1.0 or 1.5 and AB at ph2.5 to demonstrate neutral, sulphated and nonsulphated acidic polysaccharides have been used in midgut of Heteroptera (Goverdhan *et al.*, 1981)[7], Diptera (Bruno *et al*., 2019) [4] and millipedes (Rost-Roszkowska et al., 2021) [19]. These studies indicated the presence of neutral polysaccharides and acidic polysaccharides in the midgut epithelium, except Heteroptera (Goverdhan *et a*l., 1981)[7]. In the present study the midgut and rectal caecum of adult *D.isocrates* were subjected to PAS reaction, AB at pH1.5 and AB pH2.5 activity. Intima of rectal caecum and muscle layer of both the organs showed intense PAS positive reaction. The rectal pads (cortical cells) and apical border of midgut epithelial cells demonstrate moderate PAS positivity whereas the epithelium in general of both midgut as well as the rectal caecum was weakly positive to PAS. All the above observations probably suggest that neutral mucopolysaccharides are present in various regions of adult midgut and the rectal caecum. However, their intensity varies from region to region. It was observed that apical border of midgut epithelial cells and muscle layer showed moderate activity towards AB at pH 1.5. The intima, general rectal epithelium and the rectal pads demonstrated moderate to intense activity towards AB at pH 1.5. These results probably suggest presence of sulphated acid mucopolysaccharides having differential intensities. The above said regions of adult midgut demonstrated moderate to intense activity towards AB at pH 2.5 also. However, the reactivity of parts of the rectal caecum was found to be weak to intense to AB at pH 2.5. These results also indicate probable presence of sulphated and non sulphated acid mucopolysaccharides in the above regions. The result of present study is supported by the earlier work (Bruno et al., 2019; Rost-Roszkowska *et al.*, 2021) [4, 19].

In Lepidoptera, the glands of mid fifth larva of the moth, *C. ethlius* demonstrate presence of PAS positive mucopolysaccharides (Wiley and Lai-Fook, 1974)[27]. The PAS positive reaction in cell membrane and cell secretion of the salivary glands of millipede, *P. nigrolabiatus* also revealed the presence of neutral mucosubstances in the salivary gland (Joshi and Vadgama, 1974) [11]. The present observations on PAS positive activity in salivary gland of larval *D. isocrates* also suggest the presence of neutral mucopolysaccharides with varying intensities at different tissues except the outer band of the luminal content (Silk thread). Alcian Blue (AB) activity has been employed to study the presence of sialic acids (acid mucopolysaccharides) in the salivary gland of some insect larvae (Vadgama and Kamat, 1969) [24], acid mucopolysaccharides in the cells of salivary glands of millipede, *P. nigrolabiatus* (Joshi, 1972) [10] and acidic mucosubstances in the luminal content of salivary (silk) gland of lepidopteran larvae *C. ethlius* (Wiley and Lai-Fook,1974)[27]. Their study revealed the presence of sialic acid in the salivary gland of the insect larvae and acidic mucosubstances in the luminal content of salivary (silk) gland using Alcian blue at pH 2.5 and pH 1.0. In the present

study on salivary gland of the larval *D. isocrates* it was observed that central core and outer band of luminal contents demonstrate weak AB Positive reaction at pH 1.5 whereas inner band of luminal content, epithelial cells and basement membrane show moderate activity to AB at pH 1.5. All these regions are positively reactive to AB at pH 2.5 also. These observations suggest the presence of sulphated as well as nonsulphated acid mucopolysaccharides in the salivary gland. Thus, the present observations are in concurrence with earlier studies (Vadgama and Kamat, 1969; Joshi, 1972; Wiley and Lai-Fook, 1974) [24,10,11].

The luminal content of the salivary gland of adult *D.isocrates* demonstrates intense PAS activity whereas the glandular epithelium is very weakly positive to PAS. The basement membrane is weakly positive to PAS. These observations suggest the presence of neutral mucopolysaccharides in the salivary gland with varying intensities at different sites. The glandular epithelium and basement membrane of salivary gland of adult demonstrated very weak to moderate activity towards AB at pH 1.5 and pH 2.5 whereas the luminal content was found to be intensely reactive to pH 2.5. The observations reveal probable presence of sulphated as well as non sulphated mucopolysaccharides in the salivary gland of adult *D. isocrates*.

The intima and basement membrane of the larval mandibular gland of *D. isocrates* demonstrate weak PAS reaction while general epithelium is negative to PAS reaction. These observations indicate that the neutral mucopolysaccharides are present in intima and basement membrane of the mandibular gland. In *D. isocrates*, under present study, the intima, epithelium and the basement membrane of the mandibular gland demonstrate weak to intense AB positive reactions at both pH 1.5 and pH 2.5. The observations probably indicate that these regions do contain sulphated and non sulphated acid mucopolysaccharides.

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