

*Full Length Research Paper*

# Immuno-histochemical localization of cholesterol binding proteins in *Schistocerca gregaria* (Forsk.)

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**This manuscript aims to investigate immunocytochemical localization of cholesterol binding proteins (CBPs) in semi-thin sections of midgut of *Schistocerca gregaria* (Forsk.). For this purpose, polyclonal antibody specific to CBPs were raised in albino mice and used in immuno-fluorescence and immuno-blotting to determine the cellular location of CBPs. Midgut tissue sections were incubated with pAbs anti-cholesterol binding protein (primary antibody) and finally associate them with HRP conjugated anti-rabbit immunoglobulin (secondary antibody). Semi thin tissue sections of midgut portion were stained with hematoxylin and eosin for establishing general morphology of epithelial cells in control sections. Positive control tissue sections were stained with Sudan Black-B for microscopic visualization of cholesterol binding sites. Further, cholesterol association in tissue sections was confirmed by using tetramethylrhodamine isothiocyanate (TRITC) labeled florescent antibodies and immuno-blotting of CBPs. Finally, CBPs or cholesterol-carrying proteins were detected intracellularly in midgut epithelial/microvillus cells named as CBP+. Zymogene like dense granules localized were found scattered throughout the apical portion of microvillus cells. Further, presence of these CBPS was confirmed by SDS-PAGE gel electrophoresis and immuno-blotting. In treatments, dietary cholesterol was found to be internalized bound to complexed with CBPs before absorption. Further, same protein was also localized in other tissues like fat body, testis, and ovary of male and female insects of *S. gregaria*. However, present study done on immuno-cytochemical localization of cholesterol binding proteins in microvillus cells confirms that CBPS are main careers of cholesterol. These proteins also assist in transport of cholesterol to the various organs after its subsequent absorption.**

**Key words:** Cholesterol binding proteins (CBPs), cholesterol, lipoprotein, midgut, immunocytochemical localization, *S. gregaria* (Forsk.).

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## INTRODUCTION

Insects are unable to synthesize sterols de novo and rely on it exclusively on the exogenous source from diet (Laser and Clayton, 1966; Agarwal, 1970; Svoboda and Weirich, 1995) to fulfill their nutritional requirements (Lipke and Frankel, 1956). Sterols are indispensable components of animal cells, which conjugate with proteins to form lipoproteins. In many animals and plants, cholesterol is biosynthesized from smaller molecules or acetates but in insects, this pathway is totally absent

(Gilbert, 1967; Clayton et al., 1964). Mostly phytophagous insects' intake plant sterols in the natural diet and converts them into zoosterols and metabolizes them (Thomas and Gilbert, 1968). After the intake through diet, sterols are absorbed inside insect midgut (Joshi and Agrawal, 1977), in which some carrier proteins are involved (Gong et al., 2006). These proteins also serve as carrier vehicles of sterols (Nemeez and Schroeder, 1991; Mayer et al., 1985) and transport different types of lipids

mainly diacylglycerol (Chino et al., 1977) phospholipids (Klucken et al., 2000), cholesterol (Upadhyay et al., 2002) and hydrocarbons to various sites inside insect body (Abbey et al., 1985; Yun et al., 2002). These lipid carrier proteins are synthesized in fat body and play an important role in absorption and transport of different types of lipids such as diacylglycerol, cholesterol and phospholipids. These proteins also play very important role in transfer or exchange of lipids (Vahouny et al., 1985) across/between the membranes (Tam et al., 2006). After binding to surface receptors (Radhakrishnan et al., 2004; Wang et al., 2006) these protein assist in intracellular distribution of sterols mainly cholesterol (Upadhyay and Agarwal, 2007; Bass, 1988; Haunerland et al., 1992) and maintain cholesterol homeostasis inside cells (Brown and Goldstein, 1986). Similar few fatty acid binding proteins (M- FABPs) are purified also from different mammalian tissues (Ockner and Manning, 1982), which carry fatty acids to mitochondria for subsequent  $\beta$ -oxidation in flight muscles (Haunerland and Chisholm, 1990; Simons and Ikonen, 2000).

Besides lipoproteins, vitellogenins also transport lipids to developing insect oocytes (Atella et al., 2006). These proteins are major components of mature insect eggs and are considered as precursors of lipoproteins, and play a dual role in transport of lipids from fat body to ovaries. These are synthesized in the fat body and secreted into the hemolymph from where these are transferred to maturing follicles (Osir et al., 1986). These sterol carrier proteins have been identified and immunocytochemically localized in, liver cells (Pandak et al., 2006), rat brain (Horton and Shimomura 1999; Kim and Ong, 2009) and in mosquitoes (Kornick and Giesa, 1994; Lan and Massey, 2004; Schroeder et al., 2000). In addition, intracellular localization of insect sterol carrier protein-2 was successfully done (Seedorf et al., 2000). The sterol carrier proteins have been previously purified from insects but its cellular localization has not yet been established. Hence in the present investigation, immunocytochemical localization of CBPs was done in semi-thin sections of midgut of *S. gregaria*.

## MATERIALS AND METHODS

### Insects

Locusts also known as *S. gregaria* (Forsk.) were cultured in the laboratory by feeding on natural diets. Animal culture was maintained in the laboratory conditions at  $27 \pm 3^\circ\text{C}$ , 40 to 60% humidity

and a photoperiod of 12L: 12D. Experimental animals were made disease free by treating them with solvents and antibiotic substances (Streptomycin 20  $\mu\text{g/ml}$  and fungizone 20 IU/ml).

### Feeding the animals

Adult males and female of insects *S. gregaria* (in 10 to 15 in number) were placed in separate cages (18"×14"×16' unit) at room temperature. For treatment, insects were fed on cholesterol solution (300  $\mu\text{g/ml}$ , Merck India) coated on green leaves. Unfed insects were excluded from the observations.

### Preparation of homogenate

Midgut tissues (500 mg) were obtained from *S. gregaria* according to Thomas (1984) and were homogenized in a glass-glass homogenizer in 5 ml of Tris-HCl buffer (5 mM, pH7.8). Homogenate was centrifuged at 12 000 rpm for 30 min at  $4^\circ\text{C}$  in cold centrifuge (Remi) and supernatant was taken out for estimation of protein using Lowry's method (Lowry et al., 1951). From the supernatant, three aliquots of 10  $\mu\text{L}$  each were used for estimation of proteins.

### Purification and characterization of CBPs

CBPs were purified by gel filtration chromatography (column dimensions). For this, regular fractions of midgut homogenate of locusts were eluted (24 ml/h) and collected according to method of Spier (Spier, 1982). Flow rate was maintained between 20 to 24 ml per h by using a continuous buffer supply in a cold room. Eluted fractions were collected manually at a fixed interval of time at a constant flow rate. More than 130 fractions were eluted.

### Protein estimation in eluted fractions

Protein contents in the eluted fractions were estimated using Lowry's method (Lowry et al., 1951) and the values were plotted to show the presence of proteins on graph. Absorbance was taken at 640 nm in each fraction to get the elution pattern of CBPs. Besides these, eluted fractions were also evaluated at a wavelength of 260 and 280 nm.

### Molecular weight estimation of CBPs

Proteins of known molecular weights were passed through a gel filtration column at the same speed at which samples were eluted. The elution volumes of unknown proteins were compared with the log values on the x-axis for estimation of molecular weight.

### Production and purification of Polyclonal antibodies

Young albino mice (*Mus musculus*) weighing around  $30 \pm 5$  gm were used for immunization. Disease free animals were purchased from animal supplying agency and kept for one week in laboratory for acclimatization.

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**Abbreviations:** PBS, Phosphate buffer saline; **SDS-PAGE**, sodium dodecyl sulphate polyacrylamide gel electrophoresis; **pAbs**, polyclonal antibodies; **CBPs**, cholesterol binding proteins; **mv**, microvillus cells; **lm**, lumen; **ovf**, ovarian follicles; **tsf**, testicular follicles.

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### Immunization

Polyclonal antibodies (pAbs) directed against CBPs were raised in albino mice. Mice were injected with complete immunogen subcutaneously and intra-peritoneally. Only one injection of 0.30 mg of purified CBPs was given to each mouse and booster injection was given after 7<sup>th</sup> day of primary immunization. The first injection of immunogen was prepared by emulsifying purified CBPs with Complete Freund's adjuvant while the last two boosters contained incomplete adjuvant.

Anti-serum was separated by centrifugation. pAbs were purified from sera (10 ml) by precipitation with 50% of saturated ammonium sulphate following octanoic acid precipitation. In one volume of antiserum, two volume of sodium acetate buffer (60 mM, pH 4.0) were added at room temperature. Now 0.68 g n-octanoic acid were added drop wise per 10 ml of original antiserum, mixed thoroughly for 30 min and centrifuged at 1000 × g. After ammonium sulphate precipitation, pAbs were dialyzed against Tris-HCl buffer (Tris 100mM, NaCl 50 mM, pH 7.8). The serum was loaded on a Sep-Pak column (2.6 × 10 cm equilibrated with Tris HCl buffer (pH 7.8)).

For determination of relative concentration of antibodies and antigens, and to find confirmation of antigen and antibody interaction, Ouchterlony method was followed. For testing, four peripheral and one central well were cast in a molten agar gel glass slide (8 × 6 cm) by holes made. In the central well, 100 µL of polyclonal antibody (100 µg/ml) was filled up (140 µg/ml) while in peripheral wells, 100 µL of purified CBP (100 µg/ml) was used. It was kept inside a humidified box in cold (4°C) for double diffusion over night.

### Electrophoretic isolation of cholesterol binding proteins

The eluted fractions containing Caps were pooled and lyophilized to a desired concentration of protein. Proteins were separated on native and SDS polyacrylamide gel electrophoresis by using method of Laemmli (1970). Gels containing CBPs separated on SDS-PAGE were fixed in methanol: glacial acetic acid solution and stained with Comassie Brilliant Blue R 250. Gels were immersed in at least 5 volumes of the staining and placed on a slowly rotating platform for 4 to 8 h at room temperature. The over stained gels were de-stained in 30% methanol, 10% glacial acetic acid, and 60% water. The gels were photographed by a photo-densitometer camera and stored in water containing 20% glycerol in a glass tank. The specificity of pAbs anti-CBPs was estimated by protein blotting. For it, proteins were separated on SDS-PAGE and transferred to nitrocellulose membrane. Membranes were rinsed three times with PBS (phosphate buffer saline, 10 mM phosphate, NaCl 150 mM, pH 7.2) then saturated with 3% skimmed milk in PBS for 1 h at room temperature. Thereafter, membranes were incubated in pAbs anti-CBP diluted at 1:1000 with PBS containing 0.05% Tween-20. Membranes were further washed twice in fresh PBS/Tween-20 and incubated for 1 h at room temperature with a 1:20 000 dilution of alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Banglo-Genie). After washing as above, membranes were incubated with substrate solution of phosphatase containing 0.3 mg/ml of nitro-blue tetrazolium chloride (Banglo-Genie), 0.2 mg/ml of 5-bromo-4chloro-3indodylphosphate (Banglo-Genie) and 0.2 mg/ml of MgCl<sub>2</sub> to reveal the specific immuno-reactivity.

### Immuno-cytochemical localization of CBPs

Insects *S. gregaria* were kept for 24 h to feed on water swabs. These were provided pure crystalline cholesterol after dissolving it in absolute alcohol. Insects were fed after coating 50 µg cholesterol on small pieces of green grass. In controls, no cholesterol was given to insects. After 6 h, insects were dissected to collect midgut, ovary, and testis. Small segments of tissues (1 mm<sup>3</sup>) were fixed for

24 h at room temperature in PBS containing 2% w/v paraformaldehyde and 0.2% glutaraldehyde. The isolated tissues were cut into small pieces and preserved in formalin overnight. Fixed tissues were washed in de-ionized water and subjected to dehydration in different grades of alcohols and finally to xylene. The tissue pieces were embedded in molten wax. After embedding, tissue blocks were made and subjected to microtome sectioning.

### Positive control 1

In positive control, paraffin sections (3 to 4 µm) were cut using a microtome (Weswox Optik). These sections were stretched on microscopic glass slides, de-paraffinized and hydrated (100, 90, 70, 50%, water). Sections were put in hematoxylin, solution (0.3%), washed in water and dehydrated up to 90% alcohol and put in eosin for better differentiation. Sections were passed through 90, 100 and 100% alcohol + xylene and cleared in xylene and mounted in D.P.X.

### Positive control 2

Another positive control was set in which semi-thin sections were stained with Sudan black-B by using the method of Bayliss and Adams (1972).

### Negative control

Semi thin sections were de-paraffinized and passed through different grades of alcohols as used in previous controls. No treatment was given to these sections. These were simply dehydrated and mounted in DPX

### Test 1: primary and secondary antibody treatment

Semi-thin sections were incubated in PBS (10 mM sodium phosphate, 150 mM NaCl pH 7.4) with 0.1% BSA and 0.1 gelatin in cold water for 3 h. Sections were permeabilized with 0.2 of Triton X-100 in PBS for 30 min. Then these sections were rinsed in PBS and treated with 70, 90, 100, 90% ethanol for 2 min. These sections were incubated with 2.25% gelatin in 0.1 M PBS for 1 h. These were further incubated in primary antibody over night at 4°C. The next day, tissues were rinsed in PBS and fixed for 5 min in bromine water. Sections were rinsed in PBS and incubated for 4 h in HRP labeled secondary antibody (1:200 dilution). Sections were washed in PBS. After proper dehydration slides were cleared in xylene and mount in D.P.X.

### Test 2: treatment with primary antibody

Semi thin sections were incubated in PBS (10 mM sodium phosphate, 150 mM NaCl pH 7.4) with 0.1% BSA and 0.1% gelatin in cold water for 3 h. Small drops (35 µl sized) of PBS+ 4% BSA were put on each section and incubated at 4 °C for 30 minutes. Sections were washed twice with PBS and treated with 25 µL of purified primary antibody (1:500 dilutions in PBS) and incubated overnight at 4°C. Next day tissue sections were washed properly with PBS. It was then allowed to incubate for 4 h after treating them with few drops of (25 µL) diluted (1:200) HRP-labeled secondary antibody. It was washed in PBS and fixed in 4% glutaraldehyde for 5 min in 0.1 M Na<sub>2</sub>PO<sub>4</sub> phosphate buffer. Sections were rinsed in water, dehydrated in graded alcohol properly and mounted in DPX.

### Treatment with fetal bovine serum (normal serum)

Semi-thin sections were incubated in PBS (10 mM sodium phosphate, 150 mM NaCl pH 7.4) with 0.1% BSA and 0.1% gelatin in cold water for 3 h. Small drops (35  $\mu$ L sized) of PBS+4% BSA were put on each section and incubated at 4°C for 30 min. Sections were washed twice with PBS. After washing, 25  $\mu$ L sized drops of normal serum (Fetal Bovine Serum) were put on the sections and allowed to incubate overnight at 4°C. The next day sections were washed properly with PBS. Sections were fixed in 4% glutaraldehyde for 5 min in 0.1 M phosphate NaPO<sub>4</sub> and rinsed in water, dehydrated and mount.

### Immuno-florescent labeling

Tissue sections were saturated in PBS containing 1.5% BSA for 10 min at room temperature. Sections were washed for 10 min in fresh PBS. Tissue sections were incubated with pAb anti-CBP for 1 h at room temperature in 1:100 dilutions. These sections were washed in PBS and incubated with the TRITC labeled anti-rabbit immunoglobulin diluted 200 times in PBS containing 0.5% BSA for 30 min at room temperature. Tissue sections were further rinsed three times in PBS buffer and then mounted in DPX and photographed under 2-M florescent microscopes. Control experiments were carried out using pAbs anti-CBP as primary antibodies.

## RESULTS

### Purification and characterization of cholesterol binding proteins

Elution pattern of cholesterol binding proteins on gel filtration column gave two peaks at 260 and 280 nm, first soon after the void volume from fractions 31 to 50 and the second in fractions 61 to 71 (Figure 1a and b). Further, both peaks were compared with proteins obtained in each fraction. Interestingly, both peaks obtained coincided with the protein peak obtained at 640 nm absorbance. As a result the first peak contained large amount of cholesterol with less amount of protein but the second peak associated large amount of cholesterol with lesser amount of protein that represents specific binding (Figures 1c to e).

### Molecular weight determination

Molecular weight of CBPs was determined by gel filtration chromatography. The fractionation of midgut proteins presented associated large amount of cholesterol. These proteins showed molecular weight in a range of 34 to 70 kDa (Figure 1f). Further, proteins eluted in fractions 61 to 71 were pooled and their molecular weights were determined by electrophoresis.

### Production of polyclonal antibodies against cholesterol binding proteins

After purification, antibody recovery was obtained by 1.10 mg/ml in crude antiserum. It was partially purified by octa-

noic acid and consequently ammonium persulphate precipitation. % yield obtained in the precipitation was 70.90%. After diffusion of CBPs (antigen) and pAb anti-CBP in agar gel, and due to its close interaction, an immune complex precipitated in the gel and gave a thin visible white sickle shaped line of precipitation due to equivalence of both substances or formation of a concentration gradient (Figures 2a to d). It shows a visual signature of antigen recognition both in Ouchterlony test and precipitation ring test (Figures 2a to d).

### Electrophoretic separation of cholesterol binding proteins

When midgut and pooled fractions were subjected to 4 to 25% gel electrophoresis, it gave molecular weight of CBPs in a range of 68 kDa (Figure 3). Further, localization of proteins electrophoresis gels were stained with Sudan black-B. It showed specific binding to the CBPs and stained only cholesterol associating proteins.

### SDS-PAGE immuno blotting

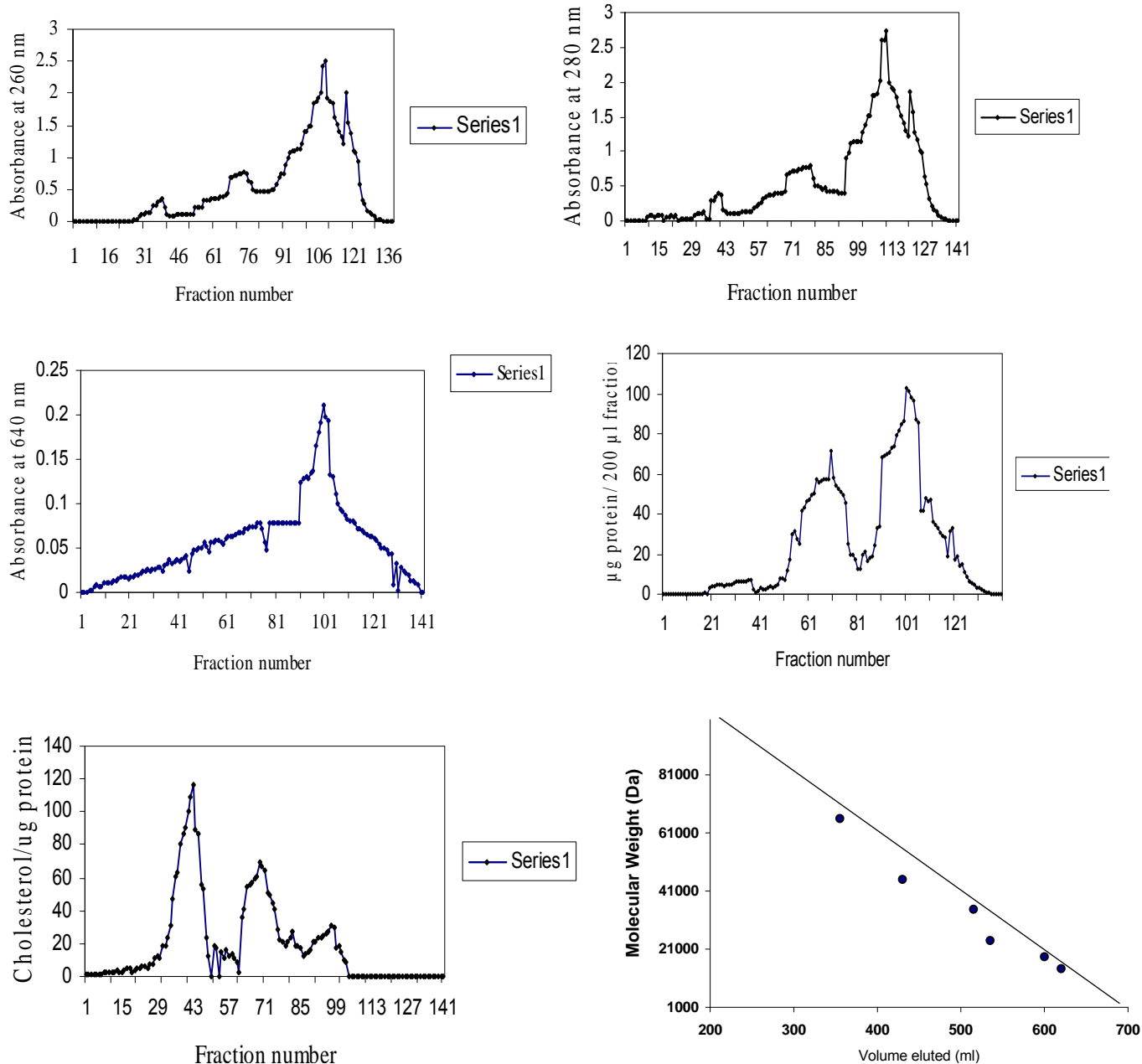
Immunoblotting technique confirmed CBP expression in midgut tissue of locusts. Immuno-blotting revealed similar band at about 68 kDa in both the SDS gel and immuno-blotting in insect midgut. In cholesterol unfed insects, these bands were totally absent, which was confirmed by the primary antibody interaction. The immuno-reactivity was found in the same band when allowed to incubate with primary and HRP labeled secondary antibody (Figure 4).

### Immunocytochemical localization of CBPs

Semi thin sections of insect midgut, ovary and testis were fixed in picric acid, 0.2% formaldehyde for 24 h. These tissue sections were allowed to be treated with primary and secondary antibody labeled with HRP. Semi-thin sections of midgut tissues of *S. gregaria* were incubated with pAbs anti-cholesterol binding proteins (CBPs) and in HRP conjugated goat anti-rabbit immunoglobulin. Following test and control experiments were conducted and results were obtained in tests and controls.

### Positive control 1

In positive control, semi thin sections of midgut, ovary and testis were processed using the same method and stained with Hematoxylin. For better differentiation, tissue sections were also stained with eosin and passed through various grades of alcohol starting from 90, 100 and 100% alcohol and xylene. These sections revealed the general morphology of the cells and localized presence of few stained granules (Figures 5e, 6a and 7e).



**Figure 1.** Elution pattern of PBS extractable proteins of *S. gregaria* midgut chromatographed on Sepharose CL-6B column. (a) absorbance at 260 nm; (b) absorbance at 280 nm; (c) absorbance at 640; (d)  $\mu\text{g protein}/200 \mu\text{l fraction}$ ; (e) specific activity; (f) Standard proteins chromatographed on Sepharose CL-6B 200 column for determining the molecular weights of cholesterol binding proteins isolated from *S. gregaria*. Proteins used were bovine albumin mol. wt 66,000, egg albumin mol. wt. 45,000, pepsin mol. wt. 34,700, trypsinogen mol. wt. 24,000, beta lactoglobulin mol. wt 18,400 and lysozyme mol. wt. 14,300. Elution volumes of unknown proteins were compared with log values on the X-axis for estimation of molecular weights.

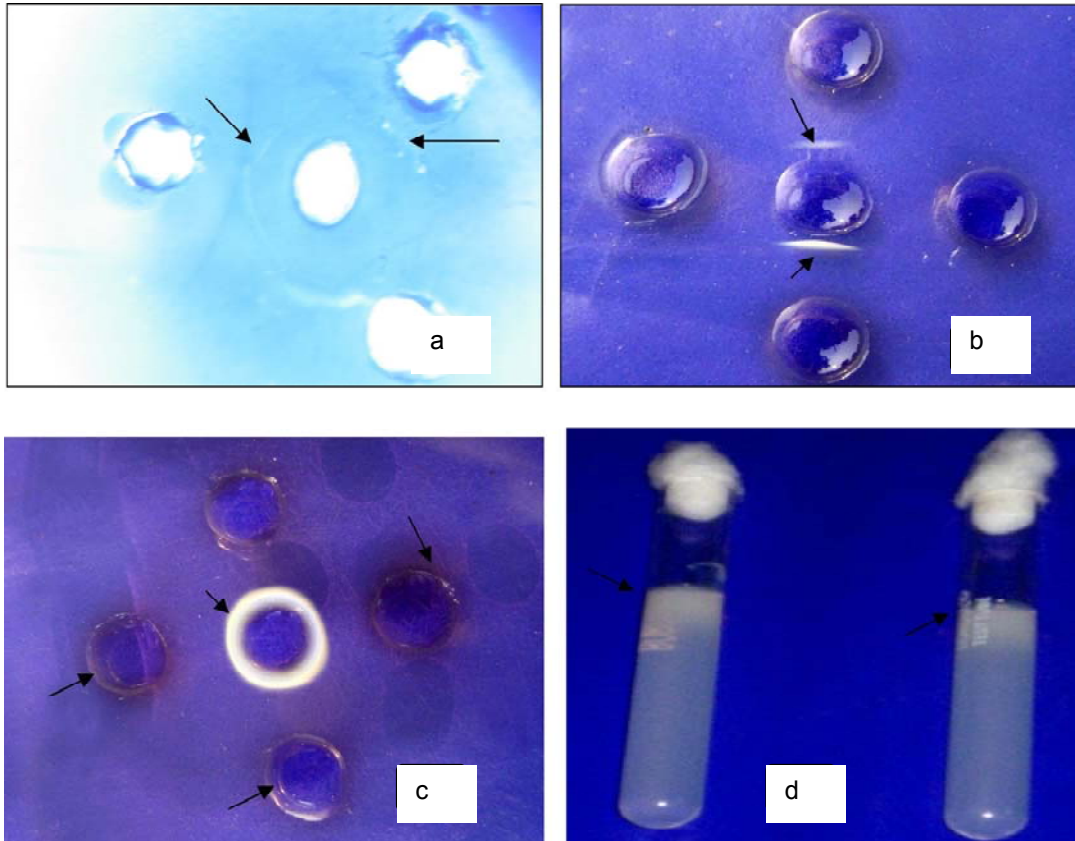
### Positive control 2

In positive control semi-thin midgut sections were deparaffinized and dehydrated up to 100% alcohol and stained with Sudan black-B dye in which midgut epithelium was found to be more visible which was composed of columnar microvillus cells. These showed cholesterol localized regions in form of droplets. Sudan black-B specially stained the cholesterol droplets in form of black

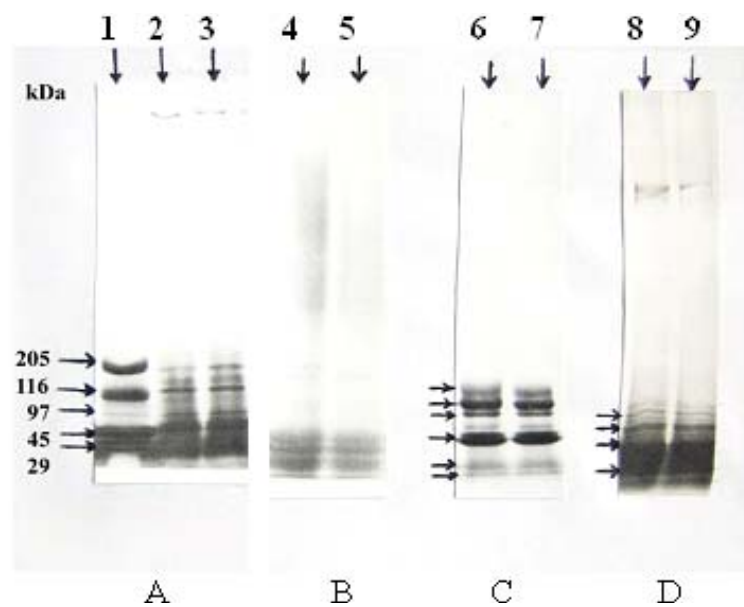
granules, which gave positive binding of cholesterol at appropriate binding sites (Figure 8).

### Negative control

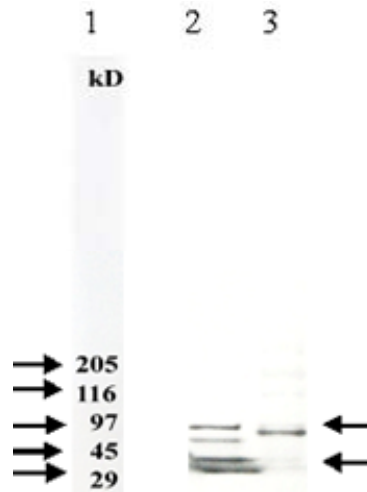
In the negative control, no reaction was done to localize the CBPs in midgut, ovary and testis tissue sections. It gave no reaction or stains because no treatment of any



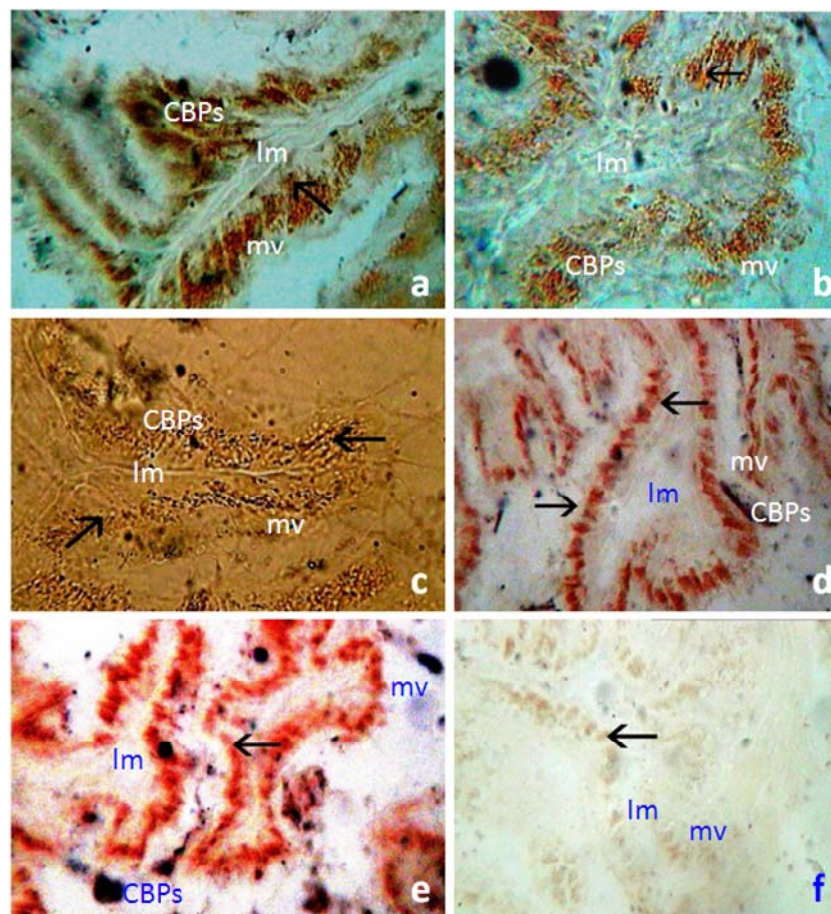
**Figure 2.** Confirmation of polyclonal antibodies generated against cholesterol binding proteins (CBPs) of locust. a and b showing results of immunodouble diffusion test; c and d precipitation ring test; d agglutination test respectively.



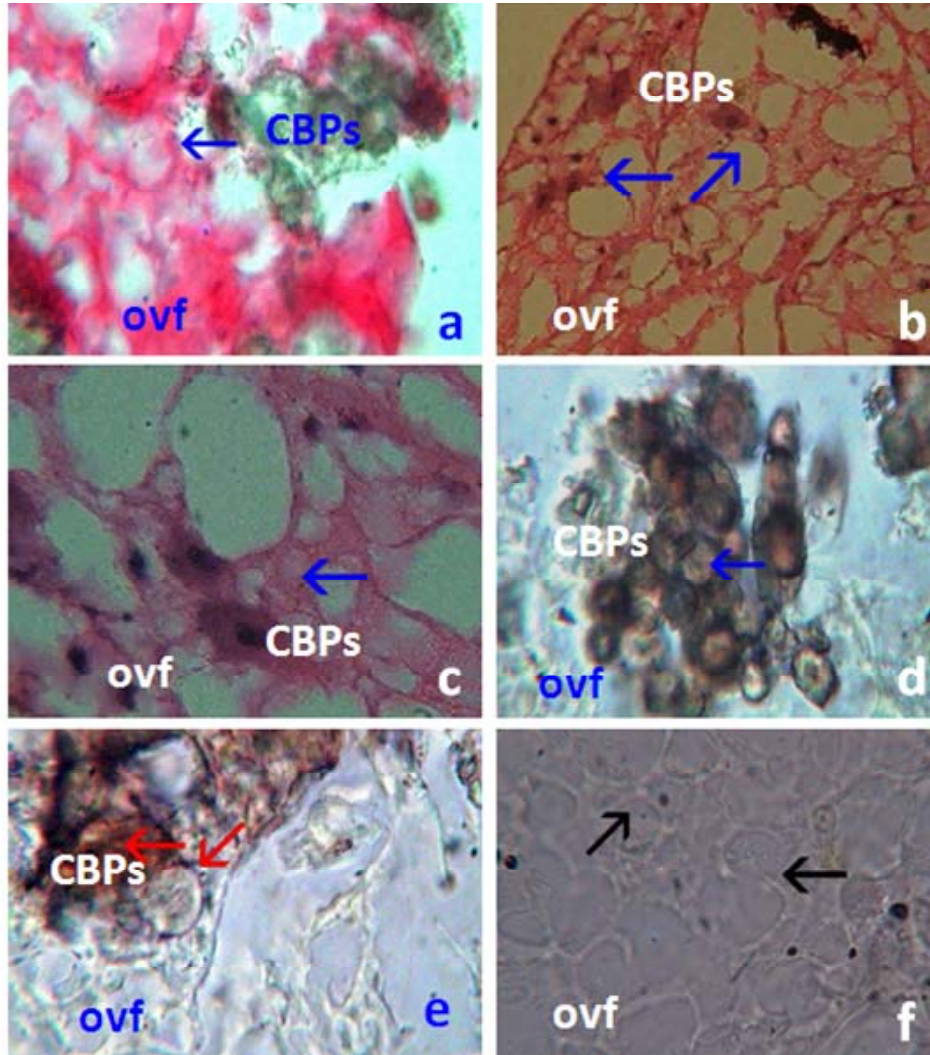
**Figure 3.** Electrophoretic separation of midgut proteins of male and female *S. gregaria* using 4-25% native gel electrophoresis (A). Lane 1 molecular weight marker; lanes 2 and 3 midgut protein from supernatant. (B) Lane 4 and 5 fat body proteins; (C) Lanes 6 and 7 ovary proteins. (D) Lane 8, 9 testis proteins. 70  $\mu$ l of sample was subjected to electrophoresis and gels were stained with Coomassie brilliant blue R-250.



**Figure 4.** Immunoblotting of CBP. Supernatant of midgut homogenate was subjected to SDS-PAGE (12%) analysis followed by immunoblotting. Lane 1 showing molecular mass markers (B-Genei); lane 2 pAbs and anti-CBP were found to react specifically with a 68 kDa protein band corresponding to that of that CBP.



**Figure 5.** Semi-thin sections of midgut epithelium of *S. gregaria* showing immunocytochemical localization of cholesterol binding proteins after ingestion of cholesterol. Immuno-reactivity is localized in microvillus cells in control and tests. (a) Treatments expressed the binding of primary and secondary antibody (1:200) with HRP labeling to cholesterol binding site. Proteins holding cholesterol gave color reaction; (b) Similar treatment showing higher binding; (c) treatments expressed only primary antibody binding; (d) positive control-tissue sections treated with normal serum; (e) Control sections stained with hematoxylin and eosin for general morphology and (f) Negative control-tissue sections without any staining and any treatment.



**Figure 6.** Semi-thin sections of ovarian tissue of *S. gregaria* showing immunocytochemical localization of cholesterol binding proteins after ingestion of cholesterol. Immuno-reactivity is localized in ovarian follicle cells in control and tests. (a) Stained with hematoxylin and eosin; (b) stained with hematoxylin for general physiology; (c) stained with eosin for general morphology higher magnification; (d) primary and secondary antibody HRP labeled; (e) positive control-tissue sections treated with normal serum; (f) negative control-tissue sections without any staining and any treatment.

substrate or staining material was given. It was proceeded only to pre fix the structural microscopic observations of midgut epithelium for better comparison or results (Figures 5f, 6f and 7f).

### Test 1

Tissue sections when treated with purified primary antibody (1:500 dilutions in PBS) and HRP labeled secondary antibody (1:200 dilutions in PBS) reaction and cholesterol binding sites were visualized clearly in form of granules. These were found deposited in apical areas of epithelial cells (mucosal cells) and mostly near the site of absorption of cholesterol (Figure 5a and b).

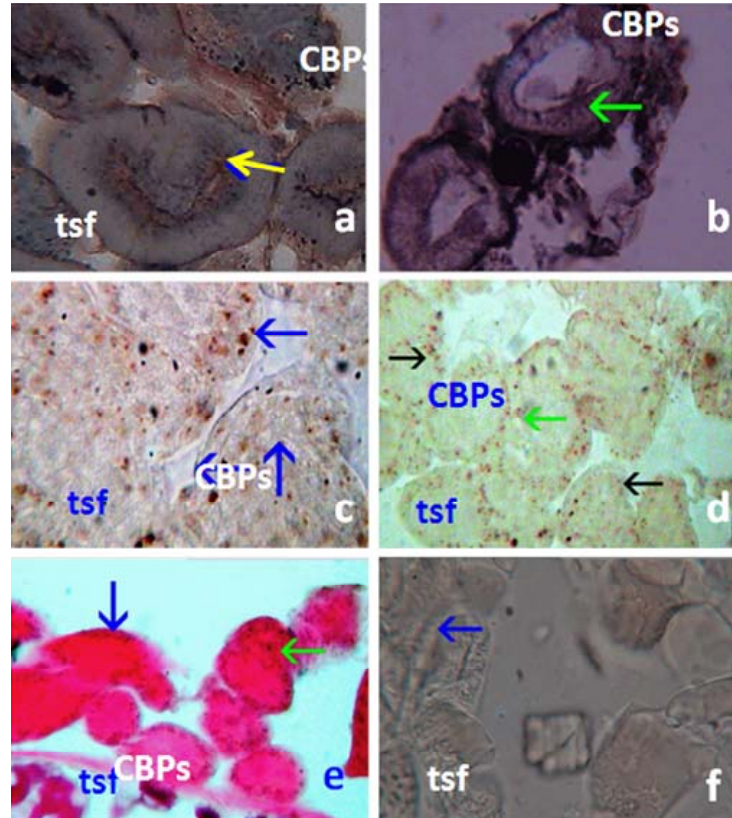
### Test 2

Tissue sections when incubated in PBS with 0.1% BSA and 0.1% gelatin in cold water for 3 h. Sections were permeabilized with 0.2% Triton-X 100 in PBS for 30 min. These sections were washed in PBS and passed through various grades of alcohols. Sections were treated with primary antibody, which showed positive binding to cholesterol (Figure 5c).

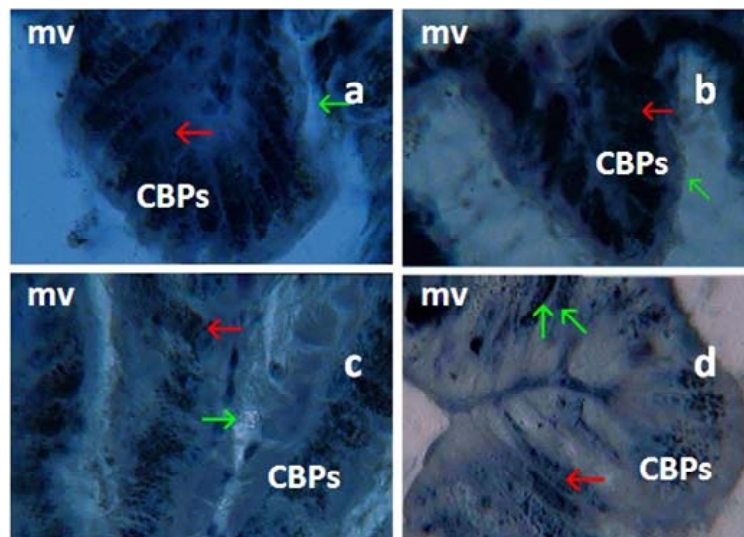
### Tests 3

Tissue sections were treated with normal serum. Serum components were found associating with cholesterol

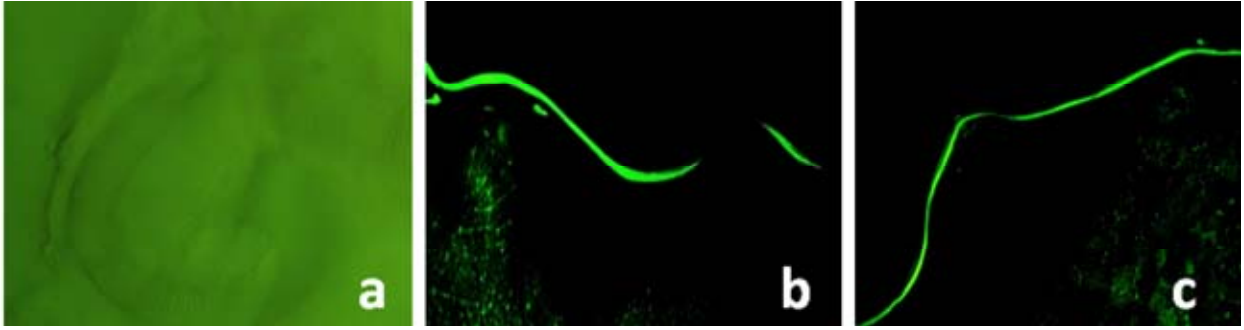




**Figure 7.** Semi-thin sections of testicular tissue of *S. gregaria* showing immunocytochemical localization of cholesterol binding proteins after ingestion of cholesterol binding proteins. Immuno-reactivity is localized in testicular follicle cells and vas deferens. (a) Tissue sections stained with Sudan black B; (b) Testicular follicle and vas deferens stained with Sudan black B; (c) tissue sections treated with primary antibody and secondary antibody labeled with HRP; (d) positive control- tissue sections treated with normal serum; (e) tissue sections stained with hematoxylin and eosin; (f) negative control-tissue sections without any staining and any treatment.



**Figure 8.** Semi-thin sections of midgut epithelium of *S. gregaria* showing localization of cholesterol binding proteins in microvillus cells. Tissue sections were stained with Sudan Black B method of Bayliss and Adams (1972).



**Figure 9.** Semi-thin cross sections of the midgut wall showing the basal region of the epithelium. Insects were provided cold cholesterol with diet. 8 h later, their midgut portions were isolated, thoroughly washed with insect culture medium and then incubated with primary antibody and then TRITC labeled secondary antibodies. (a) 15 min; (b) 60 min; (c) In control no treatment was given to the tissues.

binding proteins and showed positive binding in form of granules in the apical areas of micro-villous cells (Figure 5d).

### Immuno-fluorescent labeling

Immunocytochemical fluorescent labeling of midgut tissue sections exhibited strong fluorescence in side apical region on microvillus cells midgut epithelium. The fluorescence was found restricted to the pattern of an irregular lines partly connected to a network.

Semi-thin cross sections of midgut wall was stained with anti-CBPs and Sudan black-B resulted in an irregular staining of the midgut epithelial cells where CBPs were confirmed in the apical region of microvillus cells and in the lumen. However, the basal lamina exhibited stronger fluorescence in side of the midgut epithelium. The fluorescence was found restricted to the pattern of irregular lines partly connected to a network (Figure 9) which coincided with the out lines on the midgut epithelial cells and proved that the CBPs are localized along the baso-lateral region of microvillus cells.

The primary antibody generated for the immunocytochemical localization has shown specific binding to CBPs when it was revealed by Ouchterloy test and western blot technique. The western blotting had also revealed the presence of CBPs in the midgut epithelium showing specific binding with cholesterol.

### DISCUSSION

The results from the present investigation clearly demonstrate that cholesterol binding proteins are responsible for cholesterol absorption in insect midgut. These are histochemically localized in midgut microvillus cells of *S. gregaria* (Forsk.) after treatment. CBPs were isolated from midgut tissue of locusts and purified on a gel filtration column. Chromatogram obtained from gel filtration chromatography of locust midgut proteins resolved two major peaks, which associated a large amount of cho-

lesterol. First protein peak was obtained soon after the void volume in fraction number 31 to 50, while second peak was found between fractions 61 to 71 (Figure 1a and b). When eluted fractions were analyzed on UV-spectrophotometer at 260 and 280 nm absorbance; it also gave two peaks soon after the void volume. First peak was showing maximum association of cholesterol with protein in fractions 20 to 50, while second was associating a large amount of cholesterol between fractions 61 to 71 (Figures 1c to e).

Molecular weights of these proteins were determined after elution of standard proteins of known molecular weight at same flow rate. These proteins were showing molecular weight between 34 to 70 kDa (Figure 2f). It was probably HDLp, which carries the cholesterol to various organs (Ioannou, 2007). When pooled fractions 64 to 71 were applied on SDS gel electrophoresis, a protein band was obtained associating a large amount of cholesterol (Figure 3). It was further confirmed in immune-blot transfer analysis in which a CBPs and pAb anti-CBP interaction gave a positive while rests of the bands in the same lane remain undetected (Figure 4). Further, it was histochemically localized in treatments (tests) in apical zone of midgut epithelial cells which displayed positive labeling of CBPs in form of spherical protein bound cholesterol granules (photograph). More specifically, in tests where tissue sections were treated with primary antibody (pAbs anti-CBP) and HRP labeled secondary antibody, they have displayed specific binding to CBPs in apical areas, which appeared in zymogene-like granules. These granules could be cholesterol protein complexes, which were formed after immuno-reactivity (Photograph). It confirms presence of cholesterol binding proteins in midgut epithelial cells. However, cholesterol depositions were localized in form of large dense granules having irregular shape and size.

In another test, in which tissue sections were treated with normal serum also displayed few granules at the same site but lesser binding in comparison to HRP labeled antibodies. In another test, midgut tissue sections were incubated with primary antibody alone. For compa-

rative binding, tissue sections were stained with Sudan black-B, which also displayed dark color granules of cholesterol and CBPs complexes and demonstrate cellular localization of cholesterol (Figure 8). In all the cases, association of these proteins was found associated to some ligand at the place, where cholesterol binding protein and cholesterol complex granules were found. Further, cellular localization of CBPs and their homogeneous distribution in the apical portion of microvillous cells of midgut confirms the role of these proteins in cholesterol absorption. This study also inferred that midgut epithelial cells are major sites of cholesterol absorption because most of the binding sites were found in apical region of these cells. Further, these structural locations of granules in midgut epithelial cells of *S. gregaria* were examined under light microscope and photographed. In controls, cholesterol bound CBPs granules were totally absent because no antibody was used to confirm the specific binding. Furthermore, cholesterol immunohistochemical localization of CBPs was also done in ovary and testis, which also gave positive results after antigen and antibody interaction. It also confirms presence of CBPs in other tissues, and its important role in cholesterol transport in insects. (Figures 6 and 7). Similarly, few fatty acid and lipid carrier proteins were immunocytochemically localized in intestine, hemolymph and fat body of *Heliothis zea* by Kuthiala and Ritter, (1988), flight muscles of locusts (Li and Powanall, 2001; Xinemi and Haunerland, 1994) and midgut tissue of *Manduca sexta* (Stahl et al., 1999), Hamster cells (Bertolotti and Spady, 2001) and human intestine (Mayer et al., 1985).

HRP labeled rabbit anti-goat antibody specifically stained CBPs showed stronger reactivity in midgut epithelial cells in Western blot (Figure 4). Further, localization of CBPs was confirmed by using fluorescent labeling in epithelial cells, which also prove CBP might be an intracellular protein and involve in cholesterol absorption. Before absorption, cholesterol may bind to these proteins (Upadhyay and Agarwal, 2007) and release in to the hemolymph from where it transported to different organs of insects (Sakai and Rawson, 2001). These sterol carrier proteins are also characterized in different insects like cotton bollworm, *Helicoverpa armigera* (Du et al., 2012), caterpillar of *Helicoverpa zea*. (Jing et al., 2012), *Spodoptera litura* (Guo et al., 2009), and *Manduca sexta* (Jouni et al, 2002) and immunocytochemically localized in insect tissues (Dyer et al., 2003; Horton and Shimomura, 1999). It suggests that CBPs are very similar to SCPs and FABPs in function and might have belong to family of sterol carrier or lipid carrier proteins which are evolved in different insect groups during long evolution. These proteins also show structural diversity in higher invertebrates and vertebrates and perform lipid transport functions (Clarcke et al., 2000; Huang et al., 2002) and act as sterol regulatory element binding protein 1 in the rodent and primate brain (Ong et al., 2000) and perform cytoskeletal function in nerve cells (Caceres et al, 1986). These pro-

teins essentially take part in cholesterol absorption, trafficking and tissue distribution inside insect body (Zouari et al., 2006, Upadhyay and Agarwal, 2007). More exceptionally, few other proteins like lipase (Roberts et al., 1986; Dupont et al., 1992), and 3-beta-hydroxysteroid dehydrogenase (Wouters et al., 1995) were also immunocytochemically localized in animal tissues. These proteins also perform similar lipid transport functions. Therefore, it is important to note that transport functions of CBPs in present study are quite similar to sterol carrier proteins characterized in higher vertebrates previously. Hence, further progress is needed to explore major role of these proteins in different cell types for intracellular trafficking of cholesterol and other lipids as well as its absorption and transport in insects.

## Conclusion

On the basis of experiments conducted and results obtained, it can be concluded that CBPs are main carrier vehicles of cholesterol, which facilitate absorption in midgut epithelial cells. It also confirms that before absorption, cholesterol complexed with CBPs and internalized inside epithelial cells.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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