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JEB
Journal of Environmental Biology

ISSN: 0254-8704
CODEN: JEBIDP

Purification of *Colocasia esculenta* lectin and determination of its anti-insect potential towards *Bactrocera cucurbitae*

Kshema Thakur[1], Manpreet Kaur[2]*, Satwinder Kaur[3], Amritpal Kaur[3], Sukhdev Singh Kamboj[1] and Jatinder Singh[1]

[1]*Department of Molecular Biology and Biochemistry, Guru Nanak Dev University, Amritsar-143 005, India*
[2]*Department of Human Genetics, Guru Nanak Dev University, Amritsar-143 005, India*
[3]*Department of Zoology, Guru Nanak Dev University, Amritsar-143 005, India*
**Corresponding Author email : drdhunna@rediffmail.com*

Publication Info

Paper received:
19 August 2011

Revised received:
01 February 2012

Accepted:
22 March 2012

Abstract

The present study reports the purification of a lectin from *Colocasia esculenta* (L.) Schott corms and evaluation of its anti-insect potential towards *Bactrocera cucurbitae* (Coquilett). The lectin was found to be specific towards N-acetyl-D-lactosamine (LacNac), a disaccharide and asialofetuin, a desialylated serum glycoprotein in hemagglutination inhibition assay. Asialofetuin was used as a ligand to purify *Colocasia esculenta* agglutinin (CEA) by affinity chromatography. The purity of CEA was ascertained by the presence of a single band in reducing SDS-PAGE at pH 8.3. The affinity purified CEA was employed in artificial diet bioassay of second instar larvae (64-72 hr old) of the *B. cucurbitae* at concentrations ranging between 10-160 μg ml^{-1}. The lectin significantly ($p<0.01$) decreased the percent pupation and emergence with respect to control. Effect on various enzymes was studied by employing LC_{50} (51.6 μg ml^{-1}) CEA in the artificial diet bioassay of second instar larvae. All the enzymes tested namely esterases, phosphatases (acid and alkaline), superoxide dismutases, catalase and glutathione-S-transferase showed a significant ($p<0.01$, $p<0.05$) increase in their enzyme and specific activities. These results showed that CEA affected normal growth and development and presented stress to the larvae, activating their detoxification and anti-oxidant systems. Thus, the lectin seems to be a useful candidate for the control measures of *B. cucurbitae* under the integrated pest management (IPM) system.

Key words

Colocasia esculenta, Lectin, *Bactrocera cucurbitae*, Antioxidant enzymes, Hydrolytic enzymes, IPM

Introduction

Lectins are proteins or glycoproteins of non-immune origin with atleast one non-catalytic domain that bind specifically and reversibly to carbohydrates (Peumans and Van Damme, 1995). Lectins have been identified in all kingdoms of life (Lannoo and Van Damme, 2010). Since their discovery initially in the 19th century, much work has been done on the lectins to understand their properties and functions. Some lectins are mitogenic (Wong *et al.*, 2010; Ngai and Ng, 2007), some have anti-proliferative properties (Dhuna *et al.*, 2007; Kaur *et al.*, 2006a), while others are involved in nitrogen fixation (De Hoff *et al.*, 2009; Naeem *et al.*, 2001). One of the interesting role of lectins is in host defence against pathogens and predators (Fitches *et al.*, 2010: Hakim *et al.*, 2010; Kaur *et al.*, 2009, 2006a, b). As there is a need to replace conventional insect control measures which cause pollution and disturb the food chain, several alternative measures have been attempted including use of plant lectins. The anti-insect activity of plant lectins against a wide array of insect species have been well documented and represents a potential of using plant lectins as naturally occurring insecticidal agents against pests, which restrain increased crop production (Fitches *et al.*, 2010; Hogervorst *et al.*, 2006). *Bactrocera cucurbitae* is a major pest of cucurbitaceous vegetables and fruits in many parts of the world (Kumar *et al.*, 2006). The pest has so far defied almost all conventional control measures and the damage caused to the standing crop has been reported to be 100% in some cases (Singh *et al.*, 2009). The present study aimed at purification of a lectin from *Colocasia esculenta* corms and evaluation of its anti-insect potential towards this pest model. CEA was affinity purified and employed in the artificial diet bioassay of the pest. Effect of LC_{50} of CEA on various enzymes of this insect model was also studied.

Materials and Methods

Preparation of crude extract: The crude extract of *Colocasia* corms (obtained from the local market of Amritsar) was prepared in 0.01 M phosphate buffered saline, pH 7.2. The corms were washed, peeled and chopped off into small pieces. These were then mixed with 0.01 M PBS in 1:5 (w/v) ratio. The corms were blended in the mixer and then kept overnight at 4ºC. The extract was filtered with surgical gauze. The filtrate was then centrifuged at 20,000 × g for 35 min in refrigerated centrifuge (REMI - CPR 24) at 4ºC and the supernatant was collected. Unless mentioned, all the further experiments were performed with 0.01 M PBS.

Hemagglutination assay: Agglutination assay was performed in 96-well, U-bottom microtitre plate by using 2% suspension of rabbit erythrocytes (3.5×10^8 cells/ml) (Kaur *et al.*, 2002).

Hemagglutination inhibition assay: To find the carbohydrate specificity of *Colocasia* lectin, hemagglutination inhibition assay was performed (Kaur *et al.*, 2002). For this purpose, a series of 40 sugars/derivatives and glycoproteins were used which included 4 pentoses, 19 hexoses or their derivatives, viz; 7 disaccharides, 3 trisaccharides, 3 polysaccharides and 4 glycoproteins. Sugars or their derivatives were tested at a concentration of 100 mM while polysaccharides and glycoproteins at a concentration of 4 mg ml^{-1}. The lectin was used at twice the lowest concentration causing agglutination of rabbit erythrocytes as determined through serial double dilution technique. The minimum concentration of the sugar in the final mixture that completely inhibited the lectin-induced hemagglutination was taken as minimal inhibitory concentration (MIC).

Purification and purity check of lectin: The affinity matrix for the purification of lectin was prepared according to the method given by Shangary *et al.* (1995). The beads were packed in a polyprep column and stored in 2% sodium azide in PBS until further use. The crude extract containing 57.3 mg of protein (1.39 mg ml^{-1}) was loaded on the column. The sample was recirculated 2-3 times through the matrix to ensure complete adsorption of the lectin. The column was washed with 0.01 M PBS to remove any unbound molecules, until baseline absorbance was obtained. The bound lectin was eluted with the 0.1 M glycine-HCl buffer, pH 2.5 and 50 fractions of 1.6 ml each were collected. These were immediately neutralized with 2.0 M Tris-HCl buffer pH 8.8. The column was then equilibrated with 0.01 M PBS buffer, pH 7.2. The hemagglutination assay was performed to check lectin activity in all the affinity purified fractions. Protein estimation in the crude and affinity purified lectin sample was performed by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. To check the purity of affinity purified sample, the SDS-PAGE (pH 8.3) of the heat denatured lectin sample under reduced conditions was performed according to the method of Laemmeli (1970) using 11% (w/v) separating and 6% stacking gel.

Insect developmental parameters: Method described by Gupta *et al.* (1978) was used for the rearing of larvae of melon fruit fly under the artificial conditions. Cultures of *B. cucurbitae* were maintained at 25ºC±2ºC, photoperiod L10:D14 and 70-80% relative humidity. The initial culture was set on infested pumpkin fruits, *Cucurbita moschata* obtained from the local market. To ascertain the effect of CEA on developmental parameters of melon fruit fly, the second instar larvae (64-72 hr old) were reared on artificial diet prepared according to the method of Srivastava (1975). The affinity purified lectin at concentrations ranging between 10-160 μg ml^{-1} was incorporated in the diet. There were seven replicates for each concentration as well as control. The vials were kept in insect culture room at already mentioned conditions. The parameters studied were larval period, pupal period, total development period, percentage pupation and percentage emergence.

Biochemical assays: The effect of affinity purified CEA at LC_{50} (51.6 μg ml^{-1}) dose was ascertained on the activity of esterases, acid and alkaline phosphatases, superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST). The second instar larvae (64-72 hr old) were harvested from pumpkin pieces and transferred to the vials containing artificial diet with LC_{50} of lectin. The larvae were harvested from these vials after 0, 24, 48 and 72 hr intervals of treatment. Similar experiment was conducted with untreated medium (control). The enzyme extracts prepared at various stages were centrifuged and supernatants were stored at -20ºC till further use. Methodology given by Katzenellenbogen and Kafatos (1971) was followed for the estimation of esterases and by Mc Intyre (1971) with slight modifications for the measurement of phosphatase activity. For the estimation of SOD, methodology given by Kono (1978) was followed, for catalase estimation, method given by Bergmeyer (1974) and for GST estimation, methodology by Chein and Dauterman (1991) was followed.

Statistical analysis: The data obtained for insect bioassay as well as enzyme assay, were subjected to statistical analysis using MS Dos Prompt. Two statistical methods i.e. analysis of variance (ANOVA) and Student's 't'-test were employed depending on the type of data.

Results and Discussion

The lectin was found to agglutinate erythrocytes from rabbit blood with a minimal erythrocyte agglutinating protein concentration (MEAPC) of 20.63 μg ml^{-1} (Table 1). A very low value of MEAPC suggests easy availability and abundance of receptors for this lectin on the surface of rabbit erythrocytes. The hemagglutination inhibition assay showed the specificity of this lectin towards a disaccharide, N-acetyl-D-lactosamine (LacNAc) and a serum desialylated glycoprotein, asialofetuin, with a minimum inhibitory sugar concentration (MIC) of 25 mM and 125 μg ml^{-1} respectively. The results indicated that binding of CEA towards asialofetuin was due to LacNAc component while it failed to recognize all the other components of this complex glycoprotein. Virtually no interaction occurred with fetuin in which terminal T-disaccharide and LacNAc residues are masked by sialic acid. Based on these findings, asialofetuin, the inhibitory glycoprotein, was employed in affinity chromatography as a ligand to purify lectin.

The protein profile of the eluted fractions showed a single peak (Fig. 1). The log_2 hemagglutination titre correlated with the protein profile, that the fractions having highest protein had the highest lectin activity in comparison to the crude, raising the specific activity of purified sample to 489.72 HU mg^{-1} from 48.48 HU mg^{-1} of crude. Thus, the matrix was very efficient leading to 10 fold purification and a recovery of 293% (Table 1). The recovery of lectins after purification generally falls between a range of 20-100% (Bhat *et al.*, 2010; Dhuna *et al.*, 2007; Kaur *et al.*, 2006a, b). But in this case, the recovery was well above 100%. This may be the case when certain inhibitors are present in the crude extract amongst many other proteins that inhibit the lectin activity. But after purification, these inhibitors were removed and hence the lectin illustrates its full activity which is interpreted as enhanced percentages in recovery. The lectin represents 29% (w/v) of the total buffer extractable protein (Table 1).

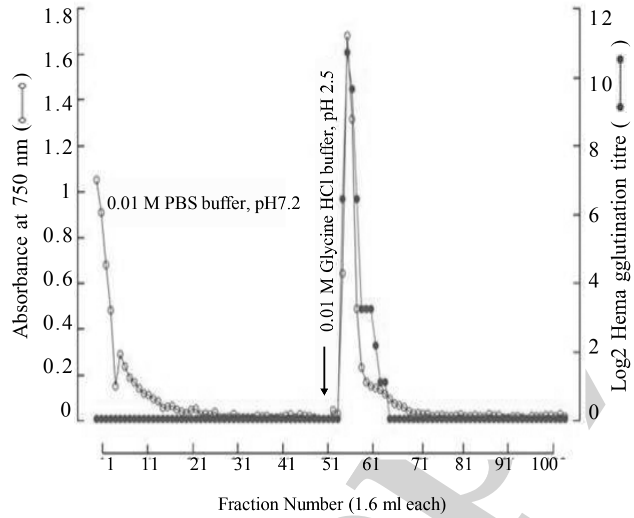

Fig.1 : Affinity purification of the *Colocasia esculenta* lectin

In SDS-PAGE, multiple bands were observed in case of crude sample while there was only one band of purified sample under similar conditions (Fig. 2). This is in consonance with the previous reports by Roy *et al.* (2002) and Van Damme *et al.* (1995).

Of the various developmental parameters investigated, two parameters were significantly ($p<0.01$) affected. There was a decline in emergence to 36.07% and in pupation to 37.25% for the highest concentration (160 μg/ml) employed in comparison to that of control (Fig. 3).

In the present study, there was a significant ($p<0.01$) increase in the esterases and phosphatases activity (Fig. 4 A, B, C) and specific activity in case of CEA treated larvae. This increase of enzyme activity in treated larvae in comparison to control might be supported by the hypothesis that the lectin introduced in the artificial diet of the pest exerts a stress to it in the form of a foreign toxin/anti-metabolic agent (Kaur *et al.*, 2009, 2006a, b; Singh *et al.*, 2009). As a consequence to this, the detoxification system of the insect might have been activated. Thus, it can be

Table 1: Affinity purification of lectin from the corms of *Colocasia esculenta*

Step	Protein (mg ml^{-1})	Activity (titre)	Total protein (mg)	Total activity (HU)[a]	Specific activity (HU mg^{-1})	Purification factor	Recovery (%)	MEAPC (μg ml^{-1})
Crude	1.32	64	660	32,000	48.48	1	100	20.63
Affinity purification								
PBS Fractions[b]	0.346	-	396.11	-	-	-	-	-
Glycine HCl fractions[c]	2.09	1,024	191.36	93757.44	489.95	10.10	292.99	2.04

Data given are for 100 g of corms, 500 ml of crude extract; [a] 2% rabbit erythrocytes were used for hemagglutination; [b] Pooled PBS fractions (50 fractions, 1.6 ml each), [c] Eluted lectin (compiled for active fractions 3, 4, 5 and 6, each 1.6 ml); MEAPC: Minimal Erythrocyte Agglutinating Protein Concentration

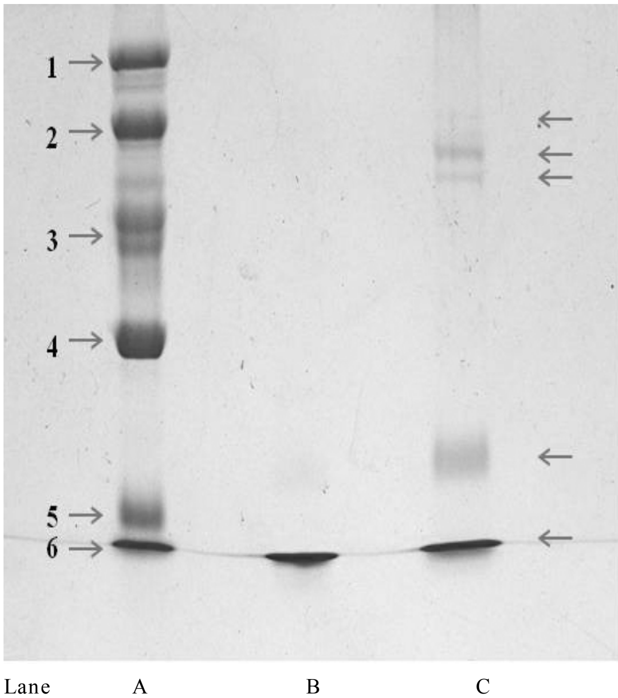

Fig. 2 : SDS-Page of affinity purified CEA with 11% separating gel, Lane (A): Standard protein molecular weight markers (1) Phosphorylase (97 kDa), (2) Albumin (66kDa), (3) Ovalbumin (45kDa), (4) Carbonic anhydrase (30 kDa), (5) Trypsin inhibitor (20.1kDa), (6) lactalbumin (14.4kDa); Lane (B): Affinity purified lectin sample; Lane (C): crude sample

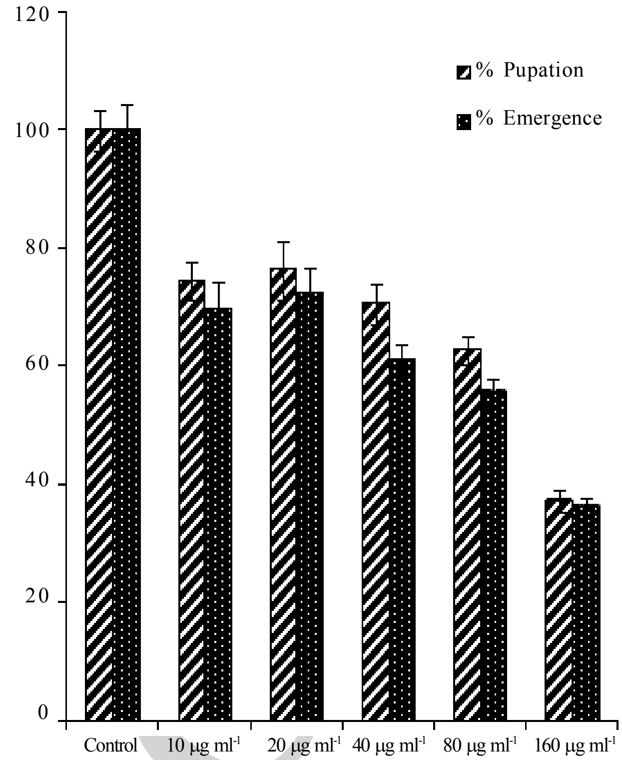

Fig. 3: The effect of *C. esculenta* lectin on pupation and percent emergence of *B. cucurbitae*. Values are mean of seven replicates ± SE

proposed that these hydrolytic enzymes are involved in the detoxification of CEA in this insect. The fact that the levels of the two hydrolytic enzyme systems, namely, Esterases and Phosphatases are raised at different stages of the treatment, it can be proposed that the two systems contribute to different detoxification systems or that the two systems contribute to different phases of the same detoxification system.

In the present study, CEA treated larvae showed significantly ($p<0.01$) higher values of SOD enzyme activity and specific activity as compared to the larvae treated on control diet after 24 hrs of treatment. Whereas, after 48 hours the treated larvae showed a significant ($p<0.01$) fall for both enzyme activity (Fig. 4D) and specific activity. That the levels of SOD activity showed a decline after 48 hrs of treatment indicated that either the presence of lectin did not further affect the production of ROS or if they were still produced, SOD were not involved in their scavenging.

The CAT activity and specific activity was significantly ($p<0.01$, $p<0.05$) raised in case of treated larvae as compared to the control larvae (Fig. 4E). This showed that the lectin presented oxidative stress to the insect which was indicated by the raised hydrogen peroxide production (Hernandez *et al.*, 2010). During the course of time, when the insect resists the stress, its catalase expression is induced and indicated in higher activities after treatment (Fig. 4E). Similar results about the increase in the levels of catalase activity were obtained by Tchankouo-Nguetchau *et al.* (2010) and Singh *et al.* (2009).

The *Bactrocera* larvae showed significantly ($p<0.01$) raised GST enzyme activity after treatment with a non-significant fall after 72 hrs of treatment as compared to control (Fig. 4F), whereas the treated larvae showed significantly ($p<0.01$) higher GST specific activity than control larvae for all the stages studied. This clearly indicated that the lectin induced stress to the insect and this stress was resisted by raising the GST activity levels. These results are supported by earlier studies (Zou *et al.*, 2011; Tchankouo-Nguetchau *et al.*, 2010; Singh *et al.*, 2009; Shengliang *et al.*, 2006).

In conclusion, *C. esutenta* lectin turns out to possess a significant anti-insect potential towards *B. cucurbitae*. The lectin gene presents a useful candidate for the integrated pest management. The value of this candidate gene is weighed by the fact that it expresses an edible protein and hence is not expected to pose any serious health threats on human health, if expressed in a transgenic plant.

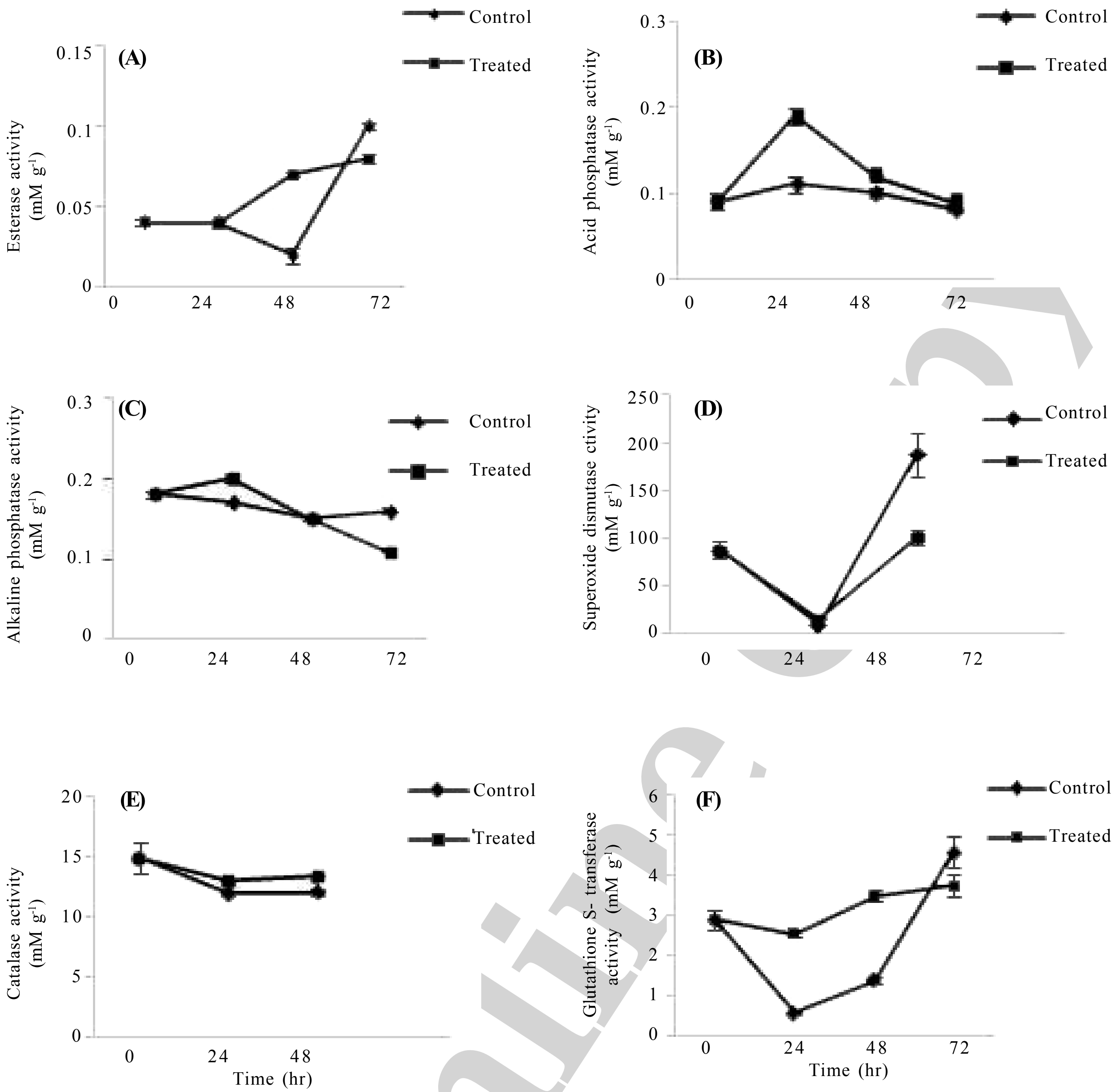

Fig. 4 : Influence of LC_{50} of affinity purified *C. esculenta* lectin on the enzyme activity of 2nd instar larvae of *Bactrocera cucurbitae* at various time intervals

Acknowledgment

The help provided for the statistical analysis of data by the Computer Centre of Guru Nanak Dev University is highly acknowledged.

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