CHARACTERIZATION OF PROTEINS AND TREHALOSE AS HEAT SHOCK REGULATORS IN INSECT SYSTEM

By

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(2006-11-107)

THESIS

Submitted in partial fulfillment of the
requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology

COLLEGE OF HORTICULTURE

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KERALA, INDIA

2008

DECLARATION

I hereby declare that the thesis entitled **"Characterization of proteins and trehalose as heat shock regulators in insect system"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that the thesis entitled entitled **"Characterization of proteins and trehalose as heat shock regulators in insect system"** is a record of research work done independently by Ms. Swetaleena Tripathy under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

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Acknowledgement

Words cannot express my deep sense of gratitude and indebtedness to ***Dr. Mani Chellappan,*** *Associate Professor, Department of Agricultural Entomology and chairperson of my Advisory Committee. I wish to place my heartfelt thanks to him for his inspiring guidance, untiring help, patience, encouragement, constructive criticism and valuable suggestions during the period of the investigation and preparation of the thesis. My sincere and heartfelt gratitude ever remains with him.*

I express my sincere gratitude to ***Dr. P.A. Nazeem,*** *Professor & Head, CPBMB and member of my advisory committee for her valuable suggestions and guidance rendered me for the completion of the research programme and preparation of the thesis.*

I am ever grateful to ***Dr. D. Girija****, Professor, CPBMB and member of my advisory committee for her invaluable help, guidance and critical assessment throughout the period of the work. I thank her for all the help and cooperation she has extended to me.*

I am deeply obliged to ***Dr. Jim Thomas****, Professor, Department of Agricultural Entomolgy and member of my advisory committee for his relevant suggestions and whole hearted cooperation throughout the period of investigation.*

I am extremely delightful to place on record my profound sense of gratitude to ***Dr. Anitha,*** *Department of Agronomy, for her suggestions and guidance in my statistical analysis during the conduct of this research work.*

I wish to extend my wholehearted gratitude to ***Dr. Pradeep G****., Senior scientist, Department of Molecular Endocrinology, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram and his team mates for their timely help during my research work.*

I also avail this opportunity to pay my sincere obligations and heartfelt thanks to ***Valsala*** *madam,* ***Kesavachandran*** *sir,* ***Rajendran*** *sir and* ***Sujatha*** *madam of CPBMB for their encouragement and kind help offered at different stages of the study.*

I thank ***Mr. P. K. Sreekumar****, Farm Assistant, CPBMB, for the help in photography.*

I wish to express my sincere thanks to all the non-teaching staff members and labourers of CPBMB for their whole hearted cooperation and timely assistance.

Special thanks go to ***Santhosh chettan****, Students Computer Club, COH, for rendering necessary help whenever needed.*

I express my deep sense of gratitude to Shailaja chechi, Shiba chechi, Manju chechi, Gini chechi, Firoz chettan, Soumya chechi, Sherin chechi, Alisha chechi, Reena chechi of CPBMB, who helped me in several ways for the completion of this venture.

I wish to express my thanks to my seniors Geena Chechi, Sameera Chechi, Neema Chechi, Likhitha Chechi, Anjali Chechi, Ramya Chechi, Liffey chettan and Fayas chettan for their timely help and encouragement.

Words fall short as I place on record my indebtedness to my class mates and friends, Niharika, Renu, Rashmi, Asha, Sindhu, Jusna, Regina, Sathish, Dinesh, Thiagarajan and Rahul for their prompt help and cooperation.

I am in dearth of words to thank my friends Malsawmkimi, Lamina, Hemalatha, Swapna, Divya, Thenmozhi, Sangeetha, Nisha, Julie, Sanju, Geetha, Jayashree, Suja, Sreevidhya, Saranya, Jagdish, Madhu, Mani, Harsha, Khelan, Devi, Natraj, Kiran, Sivaji, Vijith, Dinesh, Girish for their encouragement and unliving support.

With gratitude and affection, I recall the warm blessings and motivation from my parents and sisters without which this endeavour would never have become a reality.

Above all, I bow my head before the Great Truth, the ***Almighty****, for enlightening and making me confident and optimistic throughout my life.*

Swetaleena Tripathy


Dedicated to My
Loving family
and My Advisor

ABBREVIATIONS

APS	Ammonium persulphate
BSA	Bovine Serum Albumin
β	Beta
$CaCl_2$	Calcium chloride
cm	Centimeter
CPBMB	Centre for Plant Biotechnology and Molecular Biology
^0C	Degree Celsius
Da	Dalton
EDTA	Ethylene Diamine Tetra Acetic acid
γ	Gamma
g	Gram
h	Hour (s)
K	Kilo
KAU	Kerala Agricultural University
KDa	Kilodalton
Kg	Kilogram
m	Meter
M	Molar
mg	Milligram
ml	Millilitre
mm	Millimeter
mM	Millimolar
μg	Microgram
μl	Microlitre
μM	Micromole
NaCl	Sodium chloride
$(NH_4)_2SO_4$	Ammonium sulphate
ng	Nanogram
nm	Nanometer
PAGE	Poly Acrylamide Gel Electrophoresis
pH	Hydrogen ion concentration
ppm	Parts per million
%	Percentage
Rm	Relative mobility
rpm	Rotations per minute
SDS	Sodium Dodecyl Sulphate
TEMED	N, N, N, N- Tetramethylene ethylene diamine
UV	Ultra Violet
V	Volts
w/v	Weight by volume

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Introduction

1. INTRODUCTION

The capacity of individuals of the same species to survive short exposures to extreme temperatures is called thermo tolerance. It varies over a remarkable range, being contributed by the differences in growth condition and genetic background. Nutrient availability, oxygen tension, diurnal rhythms and an array of other variables exert highly reproducible effects on thermo tolerance.

A bright example for expression of thermo tolerance is in the red flour beetle *Tribolium castaneum* Herbst. (Tenebrioniidae: Coleoptera) also known as “bran bugs”. It is one of the serious household insect pest that originate from infested grain or from dry, stored food products, particularly, cereal products such as flour, cake mix, cornmeal, crackers, dry pet food and so forth. Other frequently-infested items are chocolates, nuts and seeds (*viz.*, bird seed). Both adults and small, off-white larvae are found in the infested food item. The adult beetles often wander away from the infested material and are found inside cupboards or anywhere in the house.

Insecticide sprays are not recommended for managing the insects in stored food cupboards. Washing shelves with detergent, bleach, ammonia or disinfectants will not have any effect on the pest. Moreover recent concern over ozone depleting ability of methyl bromide has renewed the interest in exploring heat treatment as a potential methyl bromide alternative (Makhijani and Gurney, 1995). Resistance to killing by extreme heat increases dramatically when the whole organisms or cultured cells are pretreated. This increased resistance is known as induced thermotolerance, which induces the synthesis of a set of proteins called heat shock proteins (Sanchez and Lindquist, 1990; Nover, 1991; Sanchez *et al*., 1992).

Historically, many observations have suggested that heat shock proteins play a vital role in inducing tolerance. It is striking that such proteins are induced at very different temperatures in different organisms but induction occurs at a temperature that constitutes a stress for that particular organism. A wide variety of regulatory mechanisms, acting at both the transcriptional and post transcriptional

levels, are employed to ensure that heat shock proteins are induced within minutes of exposure to high temperatures (Lindquist, 1986; Nover, 1991; Yost *et al*., 1991).

In general, heat shock proteins function by preventing accumulation of stress damaged proteins. Indeed, the artificial accumulation of 'abnormal' proteins is sufficient to induce heat shock protein synthesis (Pine, 1967; Goldberg, 1972; Goff and Goldberg, 1985; Ananthan *et al*., 1986; Parsell and Sauer, 1989). They prevent the accumulation of stress damaged polypeptides in two ways: some serve as 'molecular chaperones', preventing the aggregation of denatured proteins and promoting their proper refolding and others facilitate the degradation of abnormal protein.

Heat shock proteins are commonly reported to confer thermal tolerance in all living organisms and can indicate the level of tolerance to heat treatments. In both plants and animals, desiccation tolerance is associated with accumulation of both sugars and heat shock proteins, also referred to as LEA (Late Embryogenic Abundant) proteins (Wise, 2003). High concentration of a non-reducing disaccharide trehalose has also been reported to accumulate in large quantities during anhydrobiosis (Madin and Crowe, 1975).

The sugar 'trehalose' has been described as a 'chemical chaperone' since *in vitro* it is known to stabilize proteins and other biological structures against damage due to stress (Elbein *et al*., 2003 and Tunnacliffe and Lapinski, 2003). Trehalose has several properties of potential biological value. There is immense diversity of adaptation among different organisms and accordingly the virtues of trehalose are made use of in relation to the needs of the organism in several ways.

With loss of methyl bromide as a structural fumigant, heat treatments to disinfest mills of these pests are gaining popularity. In this context, it is important to understand the underlying mechanisms of insect heat tolerance to accurately develop the methods for reducing the expression of such proteins

produced under stress in order to manage this pest (Kabani, 2003). Hence the present study has been proposed with the following objectives:

1. To identify the life stages with anhydrobiosis in *Tribolium castaneum* Herbst. (Tenebrioniidae: Coleoptera)

2. To test the revival of anhydrobiosis at different stages with varying temperature

3. To study the heat shock regulators involved

4. To characterize the heat shock regulators *viz*., proteins and carbohydrates

Review of Literature

2. REVIEW OF LITERATURE

The red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae), is one of the serious insect pest associated with food processing facilities (Sinha and Walter, 1985). The red flour beetles are cosmopolitan pests of a wide range of grain and other food products, but they prefer milled grain, chocolates, nuts, seeds and spices (Mills and Pedersen, 1990). The red flour beetle is of Indo-Australian origin (Smith and Whitman, 2000) and is found in temperate areas, but will survive the winter in protected places, especially where there is central heat (Tripathi *et al*. 2001).

2.1 Biology of red flour beetle

The red flour beetle often referred as darkling beetles consists of a large and varied group of insects that contains more than 10,000 species of which about 100 are associated with stored products (Sallam, 2001). They are generally black or dark brown in color and are mainly phytophagous. The red flour beetle is found at an optimum temperature of 28^0C and requires a relative humidity of about 60 per cent for its growth and development. It is flat and oval in shape and usually ranges around 1/8 inches long. Their exoskeleton is reddish brown with a shiny and smooth texture (Linda and Gibb, 2008). The female beetle lays 2-3 eggs per day and the eggs usually tend to be white in color, or at times even colorless. They are very small in size and have a sticky outer covering that causes certain food particles to stick to it. The larvae are 3-4 mm long and have six legs, with two pointed projections toward the caudal end. Finally, the pupal stage (a cocoon-like form used to break itself down and emerge as a mature adult) is usually white or brownish color. The beetle life cycle lasts approximately 3 years or more, with the larval stage ranging anywhere from 20 to over 100 days, and the pupal stage around 8 days (Lyon, 2008).

Beetles usually breed in damaged grain, grain dust, high-moisture wheat kernels and flour. Females are highly fecund and able to lay 300 to 400 eggs with a maximum of 1000 eggs during a lifetime, at 40^0C and 22^0C set as upper and lower limits for development. Adults are characterized by the tarsi of the hind leg

with only four segments and cause serious damage by contaminating flour and flour products (Lewis, 1998).

2.2 Damage caused by red flour beetle

The red flour beetle is the most common secondary pest of all plant commodities in store throughout the world. Several other species of *Tribolium* are occasional minor pests and can be found in almost every store containing infested cereals or cereal products, especially in tropical and sub-tropical climates. Damage is done by both larvae and adults especially to broken or damaged grains in the tropical climate. Losses caused by these insects include not only the direct consumption of kernels, but also include accumulations of frass, exuviae, webbing, and insect cadavers and high levels of this insect detritus may result in grain that is unfit for human consumption. Insect-induced changes in the storage environment may cause warm, moist 'hotspots' that are suitable for the development of storage fungi that cause further losses (Sallam, 2001). In case of milled rice, the average mean loss due to red flour beetle was estimated at 21% for a period of thirteen months (Caliboso *et al.*, 1985).

Infestation by these beetles results in an unappealing smell due to the secretion of benzoquinones from the abdominal glands with an increase in age as a means of defense mechanism. Worldwide losses in stored products, caused by insects, have been estimated to be between five and ten per cent (Weaver and Petroff, 2005).

2.3 Heat treatment as an effective strategy

The use of elevated temperatures or heat treatments has long been recognized as an effective strategy for managing stored product insects associated with food processing facilities like flour mills (Dean, 1913). The ozone depleting ability of methyl bromide has given rise to the importance of heat treatment as a potential methyl bromide alternative (Makhijani and Gurney, 1995).

Heat treatment involves raising the ambient temperature of the whole or a portion of the facility to 50-60°C and holding these elevated temperatures for

a period till the insect gets killed (Dowdy and Fields, 2002; Wright *et al*., 2002). The rate of heating of different floors of a food processing facility during heat treatment can vary between 3 and 14°C (Roesli *et al*., 2003), and on some floors, heating rates can be as low as 0.3-0.9°C (Mahroof *et al*., 2003). Dowdy and Fields (2002) reported that the mortality of the red flour beetle adults was observed during steam heat treatment of a pilot flour mill at Kansas State University, Manhattan, Kansas. Heat treatment control insects in food processing facilities and also help in disinfesting fresh horticultural products, dried fruits/nuts, and other perishables to provide quarantine security (Waddell *et al*., 2000; Wang *et al*., 2002b). In heat treatments, high temperatures are used for short time periods to disinfest the products without adversely affecting product quality (Evans, 1986; Tang *et al*., 2000; Wang *et al*., 2002a).

Mahroof *et al*., (2003) selected temperatures below and above 50°C in the experiment while testing for the mortality of the red flour beetle because during heat treatment temperatures stratify both vertically and horizontally, resulting in non-uniform heating of a food processing facility, i.e., some areas may be under heated (< 50°C) while others may be overheated (> 50°C). At slow heating rates, most insects may have adequate time to adapt to the heat and increase thermal tolerance (Waddell *et al*., 2000). Temperatures more than 60°C generally are not recommended for heat treatments because of possible damage to heat sensitive equipment in the food processing facility (Dowdy, 1999a; Roesli *et al*., 2003). The differences in temperature tolerance of the insect species may be used to determine their natural geographical distribution limits (Huang and Kang, 2007). Heat tolerance in insects has been reported to vary within a developmental stage (Davidson, 1969; Barsell, 1973) and among stages (Oosthuizen, 1936; Evans, 1981) and it is due to the synthesis of a set of stress proteins called as heat shock proteins.

2.4 Heat shock proteins

Heat shock proteins can be induced in a wide range of organisms including bacteria, plants, insects and mammals (Lemaux *et al*., 1978; Yamamori

et al., 1978; Miller *et al*., 1979; Wilhelm *et al*., 1979; Mc Alister and Finkelstein, 1980; Welch, 1980; Jonston *et al*., 1980). They are of basic importance in many cells (Kelley and Schlesinger, 1982; Craig *et al*., 1982). Heat shock proteins have been discovered at both eukaryotic and prokaryotic level also. At eukaryotic level, heat shock proteins are studied extensively in *Drosophila melanogaster* (Tissieres *et al*., 1974). Heat shock proteins synthesis is associated with insect resistance also to thrive over stress (Mitchell *et al*., 1979; Loomis and Wheller, 1980; Li *et al*., 1982). They are expressed at low levels under normal physiological conditions but show dramatically increased expression in response to cellular stress (Slepenkov *et al*., 2003). Besides, heat shock proteins of hsp70 families of molecular chaperones assist in a number of molecular interactions that are essential under both normal and stress condition. Expression pattern of this hsp70 has been studied in red flour beetle, which is a major problem in flour and feed mills (Mahroof *et al*., 2005).

A variety of vertebrate cells show a heat shock response similar to *Drosophila* (Kelley and Schlesinger, 1978). Cells synthesize these proteins from different tissues at various stages of development (Tissieres *et al*., 1974 and Lewis *et al*., 1975). Synthesis of heat shock proteins is induced at high temperature where synthesis of cellular proteins is inhibited (Mitchell and Lipps, 1979; Buzin and Peterson, 1982). The temperature at which maximum synthesis occur is different for each heat shock proteins (Lindquist, 1980) that function in an ATP dependent manner (Feder and Hoffman, 1999). The hsp83 is the most ubiquitous of the heat shock proteins (Mirault *et al*., 1978; Storti *et al*., 1980). It shares homology with hsp83 of mice (Sanders *et al*., 1982). Similarly hsp70 shares homology with hsp68 (Holmgren *et al*., 1979). The amino acid sequences of the hsp70 gene have been deduced from the DNA sequences (Ingolia *et al*., 1980). It has been proved that hsp70 groups of proteins are characterized by multiple isoelectric variants (Kruger and Benecke, 1981). Small hsp21-28 is mainly referred to as minor proteins (Petersen *et al*., 1979; Wadsworth *et al*., 1980; Craig and Mc Carthy, 1980). Two proteins i.e., H2b (Sanders, 1981) and 26K ribonucleoprotein (Wieber and Pederson, 1982) have been identified as minor heat

shock proteins. Each of these heat shock proteins is distinct by peptide mapping (Corces *et al*., 1980; Voellmy *et al*., 1981).

2.5 Chaperoning functions of heat shock proteins and stress tolerance

Heat shock proteins underlie an important part of inducible thermo tolerance in many organisms (Feder and Hoffman, 1999). Cells that lack heat shock proteins tolerate less stress than those which express normal levels of the same (Solomon *et al*., 1991). At high temperatures, the physiology of the species changes due to production of hsp70 (Parsell and Lindquist, 1994; Feder *et al*., 1992). These conditions lead to the survival of *Drosophila melanogaster* (Feder and Krebs, 1998). Heat shock proteins function by facilitating the folding of other cellular proteins, preventing protein aggregation, or targeting improperly folded proteins to specific degradative pathways (Ohno, 2004). Specific heat shock proteins play a role in regulating apoptosis by interacting directly with key components of the apoptotic pathway (Feifel *et al*., 1998).

As molecular chaperones, heat shock proteins play diverse cellular roles, typically in minimizing dysfunction that may occur when other proteins are in non-native conformations (Feder, 2006). When normal protein synthesis is halted due to stress, then heat shock proteins work by stabilizing the proteins thereby preventing their aggregation, which promotes cell damage and death (Currie and Tuffs, 1997). Heat shock protein belongs to a highly conserved family of stress related proteins (Pierpaoli *et al*., 1998) and thus helps in protecting enzymes from desiccation damage (Anchordoquy and Carpenter, 1996).

2.6 Synthesis of heat shock proteins

The ability to respond to mild temperature shock by the synthesis of heat shock proteins within few hours of the shock is a general response and has been observed in microbes and animals as well as in plants. Most of the heat shock

proteins probably function as molecular chaperones that assist in protein folding and prevent protein denaturation (Lindquist, 1986). Some heat shock proteins are normally produced by the cell, while during stress more heat shock proteins are necessary because protein aggregation and denaturation are increased. Increased synthesis of heat shock proteins may help protect these proteins during the osmotic stress that follows cell dehydration (Feder, 2006).

Generally heat shock proteins are synthesized by tolerance inducing heat treatments (Li and Laszlo, 1985; Lindquist, 1986; Nagao *et al*., 1986; Subjeck and Shyy, 1986; Sanchez and Lindquist, 1990; Nover, 1991; Yost *et al*., 1991; Sanchez *et al*., 1992). The artificial accumulation of abnormal proteins is sufficient to induce heat shock proteins synthesis (Pine, 1967; Goldberg, 1972; Goff and Golelberg, 1985; Ananthan *et al*., 1986; Parsell and Sauer, 1989). Lewis *et al*. (1999) have proved that tolerance to high temperature could be used by the synthesis of stress proteins (heat shock proteins) and other key metabolites in many organisms. The impairment of cell structures need not always be irreversible. Low-intensity stressors have been found to increase the synthesis of heat-shock proteins that helps the organism to acquire tolerance to stress induced by the initial agents (Holubarova *et al*., 2000). Glycosylation is effective in mimicking the action of protein chaperones by preventing protein misfolding due to the production of chaperones like lectins and glycosides (Kern *et al*., 1992).

2.7 Classification of heat shock proteins

There are different groups of heat shock protein and they are classified mainly on the basis of their synthesis in an organism subjected to heat stress.

2.7.1 Heat shock protein hsp60

Heat shock protein 60 (hsp60) is a mitochondrial chaperonin that is typically held responsible for the transportation and refolding of proteins from the cytoplasm into the mitochondrial matrix. In addition to its role as a heat shock protein, hsp60 functions as a chaperonin to assist in folding linear amino acid chains into their respective three-dimensional structure (Johnson *et al*., 2003). This group of heat shock proteins is found in eukaryotes and cytosol of bacteria (Ellis and Vander Vies, 1991; Hartl *et al*., 1992; Lorimer, 1992; Horwich *et al*.,

1993) and it is strongly induced by heat (Neidhardt *et al*., 1992). It is also found in yeast mitochondria and promote protein refolding (Martin *et al*., 1992).

2.7.2 Heat shock protein hsp70

The 70KDa classes of heat shock proteins, which are true heat shock proteins, protect individuals from thermal stress (Pelham, 1986). The hsp70 group of proteins promotes translocation of proteins across mitochondrial and endoplasmic reticulum membranes (Velazquez and Lindquist, 1984; Welch and Feramisco, 1984; Cherico *et al*., 1988; Deshaies *et al*., 1988; Vogel *et al*., 1990; Larzlo, 1992). Members of 70KDa families of stress proteins which are discovered through rapid induction during exposure of cells to heat (Tissieres *et al*., 1974) got accumulated in the nucleoli of cells (Pelham, 1986). It mainly helps in the membrane translocation of proteins (Deshaies *et al*., 1988; Cherico *et al*., 1988; Murakami *et al*., 1988; Sheffield *et al*., 1990; Scherer *et al*., 1990; Langer, 1992). One of the major functions of this heat shock protein is its ability to renature the heat inactivated proteins both *in vitro* (Skowyra *et al*., 1990) and *in vivo* (Schroder *et al*., 1993).

They have a high affinity for ADP than ATP (Palleros *et al*., 1991) and they exert their function in conjunction with cycles of ATP binding and hydrolysis (Zimmer, 2001). This group of heat shock proteins is constitutively expressed (Lindquist and Craig, 1988) with an ATP binding domain (Flaherty *et al*., 1990; Mc Kay, 1991) and a substrate binding domain (Hightower *et al*., 1991). They bind to a variety of substrates (Gething and Sambrook, 1992) and hydrophobic surfaces (Pelham, 1986; Rothman, 1989). Different hsp70s are not functionally interchangeable (Brodsky *et al*., 1993) and they resemble the peptide binding site of MHC-1 molecules on the basis of computer modeling (Rippmann *et al*., 1991).

Other heat shock proteins potentiate the activities of hsp70. They reactivate the heat denatured enzymes (Skowyra *et al*., 1990; Schroder *et al*., 1993). Yeast cells express four cytosolic hsp70 proteins (Craig and Jacobsen, 1985; Nelson *et al*., 1992). The expression of hsp70 is generally more in the malphigian tubules of the insects (Feder and Krebs, 1998). They are induced more

than 1000-fold upon heat shock (Velazquez *et al*., 1983; Solomon *et al*., 1991; Welte *et al*., 1993) but are detrimental to growth as they replace some of the already synthesized proteins from the body (Feder *et al*., 1992). Transforming monkey cells and rat cells using human hsp70 genes helps in increasing thermal tolerance (Anderson *et al*. 1985; Riabowol *et al*., 1988; Angelidis *et al*., 1991; Li *et al*., 1991).

2.7.3 Heat shock protein hsp90

This group of heat shock proteins performs the same role in eukaryotes and prokaryotes (Borkovich *et al*., 1989) by interacting with many cellular proteins (Nishida *et al*., 1986; Redmond *et al*., 1989; Rose *et al*., 1989; Miyata and Yahara, 1991; Fostinis *et al*., 1992; Pratt *et al*., 1992; Minami *et al*., 1993). They function as a molecular chaperone under *in vitro* condition (Welch *et al*., 1992; Buchner, 1998; Miyata and Yahara, 1992).

The interaction between hsp90 and some target proteins are long lived (Pratt *et al*., 1992; Xu and Lindquist, 1993). Genetic analysis in yeast demonstrates that the amount of hsp90 required for growth increases as the temperature increases (Borkovich *et al*., 1989).

2.7.4 Heat shock protein hsp100

This group of heat shock proteins comprises a highly conserved family that includes proteins from bacteria, yeast and mammals (Gottesman *et al*., 1990; Parsell *et al*., 1991; Squires *et al*., 1991; Squires and Squires, 1992; Vierling, 1992) and they are reported to bind F-actin in a Ca^{2+}-calmodulin sensitive manner (Koyasu, 1989).

2.7.5 Small heat shock proteins

Some of small heat shock proteins are synthesized during normal development (Cheney and Shearn, 1981). Small heat shock proteins are encoded within 12 kb fragment of DNA (Petersen, 1979; Wadsworth *et al*., 1980; Craig and Mc Carthy, 1980; Voellmy *et al*., 1981 Corces *et al*., 1982). Minor puffs hybridize to cytoplasmic mRNA induced by heat (Mitchell, 1979; Lis *et al*., 1981;

Buzin and Petersen, 1982). Some of genes encoding smaller heat shock proteins are expressed during normal development (Zimmerman *et al*., 1982).

2.8 Late Embryogenesis Abundant (LEA) proteins

Heat shock proteins are first characterized in cotton and wheat as late embryogenesis abundant proteins or LEA proteins (Cuming, 1999). LEA proteins are induced by stress and are constitutively expressed in *Arabidopsis thaliana* (Welin *et al*., 1994). They protect the molecular structures from damaging effects due to stress and result in renaturation of unfolded proteins (Bray, 1993; Cuming, 1999). LEA proteins from tomato, barley and wheat confer increased resistance to stress when introduced into yeast (Zhang *et al*., 2000). They improve tolerance to stress in transgenic rice (Xu *et al*., 1996) and wheat (Senamani *et al*., 2000).

On the basis of expression pattern, there are three major categories of LEA proteins (Wise, 2003). Group I LEA proteins are unstructured in solution and contain a conserved 20 residue amino acid motif (Mc Cubbin *et al*., 1985). Group II LEA proteins are called “dehydrins” (Close *et al*., 1989) and are found in plants (Ceccardi *et al*., 1994; Close, 1996; Lissie *et al*., 1996). Group III LEA proteins have been discovered in nematodes and prokaryotes (Solomon *et al*., 2000; Dure, 2001; Browne *et al*., 2002). These proteins are natively unfolded in solution (Goyal *et al*., 2003) but become more structured on drying (Walkers *et al*., 2001) and help in desiccation tolerance (Battista *et al*., 2001). They also show molecular chaperone activity and anti aggregation activity thereby preventing protein inactivation during stress (Wise and Tunacliffe, 2004). Chaperones bind to non-native proteins and prevent irreversible aggregation (Welch and Brown, 1996; Browne *et al*., 2004).

LEA proteins suppress protein aggregation and inactivation under stress conditions (Bhattacharyya and Das, 1999; Goyal *et al.*, 2003; Sankar *et al*., 2004). Inactivation of enzyme activity is an early event during thermal stress (Dong *et al*., 1995; Buchner *et al*., 1998).

2.9 Heat shock gene expression

Expression pattern of three heat shock protein 70 (hsp70) genes have been studied in red flour beetle (Mahroof *et al*., 2005). Expression of hsp70 and related heat shock proteins is nearly universal, and these proteins are conserved across all organismal kingdoms (Lindquist, 1986). Five active gene copies encode hsp70, and these genes are distributed between two loci (Ish-Horowicz *et al*., 1979) that arises due to gene duplication (Leigh-Brown, 1981). Gene duplication changes the gene expression and regulation (Spotford, 1999).

The change in gene expression following heating is observed as change in the puffing pattern of polytene chromosomes (Ritossa, 1962), which is due to new transcription (Berendes, 1968; Ellgaard and Clever, 1971; Belyaeva and Zhimulev, 1976; Bonner and Pardue, 1976). Cytoplasm puffing occurs due to a heat stable inducer (Bonner, 1982; Parker and Hogness, 1983). Inhibition of protein synthesis leads to continued synthesis of heat shock mRNA and heat shock puffs (Ashburner and Bonuer, 1979). Heat shock mRNA hybridizes *in situ* to heat shock puffs (McKenzie *et al*., 1975; Spradling *et al*., 1975; Henikoff and Meselson, 1977; Greenleaf, 1978; Findley and Pederson, 1981). The regions of DNA encoding major heat shock proteins are specifically sensitive to nuclease (Levy and Noll, 1981). The distribution of non histone chromosomal proteins changes following heat shock (Silver and Elgin, 1977; Waideli *et al*., 1980; Jack *et al*., 1981). Induction of transcription of heat shock genes is studied by inducing expression of these genes *in vitro* by isolating salivary gland nuclei (Crompton and Bonner, 1978; Craine and Kornberg, 1981).

A strong inducible response to heat in *Drosophila* requires hsp70 (Solomon *et al*., 1991; Li and Duncan, 1999; Feder, 1996) but too much expression can reduce thermo tolerance (Krebs and Feder, 1998). *Drosophila* expresses hsp70 under near lethal stress conditions only when some hsp70 is present before they encounter extreme stress (Krebs and Loeschcke, 1994). The hsp70 expression covaries among different developmental stages. Where genetic variation occurs in a population, individuals of the same line are more alike for

thermo tolerance in *Drosophila* (Hosgood and Parsons, 1968) and in other insects (White *et al*., 1999). Divergence among groups reveals how genetic changes underlie variation in temperature tolerance (Levins, 1999; Huey and Bennett, 1990).

The first 60bp of *Drosophila* gene which has been transcribed in mammalian cell lines under heat shock condition are essential for expression of hsp70 in cells (Corces *et al*., 1981; Mirault *et al*., 1982; Pelham and Bienz, 1982; Voellmy and Rungger, 1982). The hsp70 gene is present in 5-6 copies distributed between two heat shock puff sites (Henikoff and Meselson, 1977; Ish-Horowicz *et al*., 1977; Mirault *et al*., 1978; Holmgren, 1989). The message for hsp70 is found to be abundant on large heat shocked polysomes (Moran *et al*., 1979) and it is used to isolate DNA clones which include the coding regions for hsp70 (Levak *et al*., 1978; Schedl *et al*., 1978; Artavanis-Tsakones *et al*., 1979, Moran *et al*., 1979; Goldschmidt-Clermont, 1980). All of the heat shock proteins messages in *Drosophila* have a long untranslated region at 5' end (Torok and Karch, 1980; Hackett and Lis, 1981; Ingolio and Craig, 1981; Mason *et al*., 1982).

The study of phenocopies offers insight into functions of heat shock proteins (Rappaport, 1939; Gloor, 1947; Sang and Mc Donald, 1954; Hadorn, 1955; Landaver, 1958 Goldschmidt, 1985). The best phenocopy prevention follows a heat treatment which fully induces heat shock proteins synthesis (Morato and Garcia-bellido, 1974; Santamaria, 1979; Seybold *et al*., 1979). Bettencourt *et al*. (1999) demonstrated that environmental trade offs can change hsp70 expression. Thermal tolerance and hsp70 expression varies in divergent lines due to the effects at multiple chromosomes (Cavicchi *et al*., 1999). Hoffmann and Parsons (1999) suggest that the natural selection on stress tolerance reduce the genetic variation. Conserved regions of the heat shock proteins are involved in the protein interaction (Arrigo *et al*., 1980).

2.10 Cellular localization of heat shock proteins

Heat shock proteins are not localized in mitochondria (Velazquez *et al*., 1980). But they are found preferentially in the nucleus (Mitchell and Lipps, 1975;

Vince and Tanquay, 1979). Nuclease digestion is required to release heat shock proteins from nucleus (Falkner *et al*., 1981; Sanders, 1982). Heat shock proteins function by stabilizing nuclei following the heat shock (Levinger and Varshavsky, 1981; Sinibaldi and Morris, 1981).

Small heat shock proteins are related evolutionarily even though not functionally (Sirotkin and Davidson, 1981). There is a lack of intervening sequences in all messages of heat shock proteins of *Drosophila* (Rubin and Hogness, 1977). A cell free protein synthesis system using *Drosophila* cells are found to be useful in examining the mechanism of selective translation in heat shocked cells (Scott *et al*., 1979; Storti *et al*., 1980; Kruger and Benecke, 1981; Scott and Pardue, 1981; Petersen and Mitchell, 1982). The effect of heat shock on protein synthesis is studied by shutting off protein synthesis with a short heat shock (Mitchell and Lips, 1979; Chomyn *et al*., 1979; Glover, 1982). Cellular localization of heat shock proteins in nucleus helps to prevent the developmental defects which are induced by heat. Heat shock proteins synthesis also improves the carbohydrate cell survival following heat shock (Li and Werb, 1981).

2.11 Mechanism of trehalose action

Trehalose is the major carbohydrate component of insect blood being a non reducing disaccharide (Wyatt and Kalf, 1956). During anhydrobiosis, trehalose is accumulated in larger quantities (Clegg, 1967; Madin and Crowe, 1975). Trehalose has been described as a ‘chemical chaperone’ (Welch and Brown, 1996; Ellis, 2004) since *in vitro*, it is known to stabilize proteins and other biological structures due to stress (Elbein *et al*., 2003; Tunacliffe and Lapinski, 2003), thus preventing heat induced aggregation (Viner and Clegg, 2001. *In vitro*, trehalose prevents irreversible inactivation of enzymes during desiccation (Colaco *et al*., 1992). Large amounts of trehalose have been reported in desiccatable eggs of mosquito (Clegg and Filosa, 1982) and in cysts of brine shrimp which is resistant to desiccation (Dutrieu, 1959).

Soluble sugars have also been reported to act as protectants during cell dehydration (Leprince et al., 1993). Trehalose is one of the most effective

osmoregulatory sugars in terms of the minimal concentration required. It has been reported in many organisms; however, there are only a few reports in higher plants. Trehalose seems to play many roles in increasing tolerance to dehydration (Potts, 1994). One role is to bind to the cell membrane and lower its melting temperature, thereby keeping the membrane in its liquid crystalline phase (Crowe et al., 1993). In addition, trehalose stabilizes enzymes (Carpenter et al., 1987) and vesicles (Crowe et al., 1993) during cell dehydration. Sucrose and maltose have also been shown to have protective effects over enzymes and membranes (Carpenter et al., 1987).

Disaccharides, like trehalose, appear to be one of the most effective stabilizers of dried enzymes and cell membranes *in vitro* and *in vivo*. Trehalose-6-phosphate synthase is involved in the production of trehalose, a disaccharide known to osmotically protect cell membranes during dehydration. (Potts, 1994). Generally, trehalose interacts directly with dry protein by hydrogen bonding -OH groups to polar residues in the protein (Carpenter, 1993). Studies of the interaction between trehalose and phospholipid bi-layers indicate that trehalose replaces water molecules around the polar head groups of the phospholipid in the dry state. This hypothesis has been discussed and direct evidence for trehalose binding to phospholipid membranes has been obtained for dry phospholipid vesicles (Potts, 1994). The interaction of trehalose with the membrane lowers the melting temperature of the membrane enabling it to keep its liquid crystalline phase during desiccation (Crowe et al., 1993). To give its protective effects, trehalose must be present on both sides of the membrane.

2.12 Heat shock proteins act synergistically with trehalose to prevent protein induced aggregation

The two previously known mechanisms for heat shock resistance are synergistic with each other, and together account for most of the heat shock resistance of stationary phase cells. One mechanism is provided by heat shock proteins that are known to provide thermotolerance to cells preconditioned to high temperatures (Sanchez and Lindquist 1990). The other mechanism is provided by

trehalose that has been increasingly implicated in the heat shock response. The synthesis of trehalose is well correlated with high temperatures (Hottiger *et al,*. 1987, 1989, 1992; Neves and Francois 1992), while *in vitro* and *in vivo* evidence suggests trehalose is a thermoprotectant (Colaco *et al.* 1992; Hottiger *et al.* 1994).

High temperature shock induces cells to accumulate the storage carbohydrates like glycogen and trehalose that provide a source of energy during starvation (Lillie and Pringle, 1980). High concentrations of trehalose are correlated with resistance to heat and desiccation in many organisms; *e.g.*, slime molds, insects, resurrection plants, yeast and other fungi (Killick and Wright 1972; Elbien 2003; Crowe *et al.* 1993; Hottiger *et al.*, 1987, 1989, 1992; Neves and Francois 1992). Trehalose may be partly able to replace water in forming a hydration shell around proteins, and it is now well established that many if not most enzymes become resistant to heat denaturation and to desiccation in the presence of trehalose (Colaco *et al.*, 1992; Hottiger *et al.*, 1994).

In addition, Tpsl (Trehalose phosphate synthetase) and Tps2 have been shown to be heat shock proteins (Bell *et al.* 1992; De Virgilio *et al.* 1993; Vuorio *et al.*, 1993). *In vitro* evidence suggests that trehalose acts as a thermoprotectant by stabilizing proteins and preventing heat inactivation (Colaco *et al.*, 1992; Hottiger *et al.*, 1994), whereas heat shock proteins repairs heat damaged proteins by resolubilizing the insoluble aggregate (Parsell and Lindquist, 1994). Thus the two functions appear to complement one another in the heat shock response, one putatively by protection, and the other by repair. If one of these two mechanisms is missing, then cells become sensitive to heat shocks. Trehalose and heat shock proteins together can account for most of the heat shock resistance of stationary phase cells.

2.13 Cellular consequences of stress

Lethal heat stress generates oxidative stress and cells become more resistant. Levels of sulfhydryl groups, dominated mainly by the high levels of the antioxidant glutathione (reduced form) and levels of vitamin E, decreases after heat stress and also leads to an increase in mitochondrial membrane disruption

(Davidson and Schiestl, 2001). Physical or chemical stressors induce direct or indirect structural changes in proteins, which results in protein aggregation and, subsequently, disturbed functioning of cell compartments (Holubarova *et al*., 2000). In higher eukaryotes, one of the most immediate effects of heat shock is extensive disruption of the cytoskeleton (Christiansen and Kramme, 1969; Falkner *et al*., 1981; Coss *et al*., 1982; Glass *et al*., 1985; Welch and Suhan, 1985; Iide *et al*., 1986). At high temperatures, the transcription of heat induced messages is at a maximum (Theodorakis and Morimoto, 1987; Peterson and Lindquist, 1988). Heat shock results as aggregation of intrinsic membrane proteins (Bass *et al*., 1978; Kruuv *et al*., 1983; Lepock *et al*., 1983).

The effects of exposure to sublethal temperatures on the subsequent molting under favourable conditions have been reported for some insects. Mellanby (1954) showed that exposure of larvae of yellow fever mosquito (*Aedes aegyptii* Reinert.) and yellow mealworm (*Tenebrio molitor* Kiihn.) to high temperature for brief period interrupts development, but after a delay of perhaps a month in *Aedes*, normal pupation occurs and adults are then produced after the usual interval. The life-cycle of the common house mosquito (*Culex pipiens* Galindo.) can be extended from 20-28 days under normal temperature conditions to 55-58 days by exposing larvae or pupae for different periods to 6° C. (Moulinier, 1959). Similar results are obtained when larvae of red flour beetle are exposed to low temperatures (Howe, 1962). Failure to cast larval or pupal skin in red flour beetle occurs after exposure to subthreshold low temperatures, and the proportion of individuals showing this abnormality tends to increase at lower temperatures and with longer exposure (Howe, 1962). In addition, high temperature causes defects in the formation of new plaques and bristles. This suppression is more pronounced in larvae exposed for longer periods. It was also noted that the bristles are scattered in disorder on the abdominal tergites. The bristles are thinner and shorter than normal, and the plaques are not developed as far as those of a normal insect (Okasha, 1968).

2.14 New role of heat shock proteins

It is already evident that chaperoning heat shock proteins have a role in the degradation of abnormal proteins (Baker *et al*., 1984; Kelley and Simon, 1988; Straus *et al*., 1988; Sherman and Goldberg, 1992). Newly synthesized proteins are shepherded to their correct native structure by molecular chaperones (Gething and Sambrook, 1992; Hendrick and Hartl, 1993). The molecular chaperones are initially described as nucleoplasmin (Laskey *et al*., 1978) and rubisco binding protein (Ellis, 1987). Compounds like glycerol, betaine and proline accumulate to a very high level under stressful condition (Yancy *et al*., 1982; Boon-Miermeijer *et al*., 1986; Hallberg, 1986; Laszlo, 1986; Lee and Dewey, 1988). Differences in thermo tolerance among cultivars are correlated with differences in heat shock proteins synthesis (Oogham and Stodalart, 1986; Feder and Connell, 1989; Howarth, 1989; Krishnan *et al*., 1989; Marmiroli *et al*., 1989). But the heat shock proteins are detrimental to the normal growth (Stone and Craig, 1990; Feder *et al*., 2006).

Recently it has been discovered that the heat shock proteins and other defense and immunity genes permit the blue green sharp shooter, a serious pest in grapevine plantation, to respond to various biotic and abiotic stresses, such as heat, cold, pathogens, and toxins and the identification of such heat shock genes helps in adopting biological control measures to reduce the blue green sharp shooter responsible for the spread of Pierces disease (Hunter, 2008).

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Characterization of proteins and trehalose as heat shock regulators in insect system" was undertaken in the laboratories of Department of Entomology and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University in Vellanikkara from 2007-2008 for the purpose of studying the effect of heat treatment and its significance on the red flour beetle, *Tribolium castaneum* Herbst. (Tenebrioniidae: Coleoptera).

3.1 CHEMICALS, GLASSWARE AND PLASTIC WARE

The chemicals used for the study were of good quality (AR grade) from various agencies including Merck India Ltd., Sisco Research Laboratories and Genei, Bangalore. The broad range protein marker was supplied by Bangalore Genei Ltd. All the plastic wares used were obtained from Axygen and Tarson India Ltd.

3.2 EQUIPMENT AND MACHINERY

The equipment items available at the Centre for Plant Biotechnology and Molecular Biology were used for the present study. Incubation of cultures was done in Incubator shaker, Dai Ki – S1010 (Dai Ki Scientific Co.). Centrifugation was done in KUBOTA centrifuge (Japan). The spectrophotometer readings were taken using the UV-VIS spectrophotometer (USA) and the Nanodrop ND 1000 spectrophotometer (USA). The gel electrophoresis system was from Bio Rad and the Millipore Centricon (Germany) was used for concentration purpose. Alpha Imager TM 1200 was used for imaging the protein gel.

3.3 EXPERIMENTAL ORGANISM

The red flour beetle, *Tribolium castaneum* (Tenebrioniidae: Coleoptera) mass culturing was done by the initial culture obtained from the provision store at Mannuthy, Thrissur. From the nucleus culture, mass culturing was done to obtain uniform aged and sized insects for various experiments.

3.4 MASS CULTURING OF TEST INSECT

The mass culturing of the test insects was done using plastic insect culture jars filled up with insect feed *viz*., wheat flour and semolina, procured from Mannuthy, Thrissur. The mass culturing of the test insects was done at 28°C with relative humidity of 70 % (Padin *et al*., 2002). A total of five pairs of insects were released per container.

3.4.1 Rearing the test insect

Food grade plastic insect culture jars (100ml capacity with wider mouth obtained from Mannuthy, Thrissur) were used for rearing the insect. Further the insect feeds were partially sterilized in hot air oven at 70°C for 15 minutes. The plastic insect culture jars were decontaminated with formaldehyde (2%) prior to their use in order to prevent the problem of contamination. The jars were filled up with insect feed *viz*., wheat flour, semolina and a combination of both. The mass culturing was done using wheat flour (about 25 g), semolina (about 25 g) and wheat flour and semolina (about 12.5 g each) in combination. Five pairs of test insect (red flour beetle) were released into the first five containers. Sub-culturing was done in an interval of seven days to one month depending upon the multiplication of the insects and monthly observation was recorded for twenty five insect rearing containers. The mean population build up of insect per month was determined.

3.4.2 Maintenance of the insect culture

Moults and faecal matters were removed from the culture jars at weekly intervals so as to avoid any sort of contamination (fungal contaminant). Sub-culturing was done to provide sufficient space for the insects to move about and multiply.

3.5 BIOLOGY OF TEST INSECT

As the biology of an insect gives accurate information on duration of different stages and its lifecycle, the biology of the test insect was studied under laboratory condition during March 2007 to March 2008.

3.5.1 Observations recorded

The following observations were taken with respect to the red flour beetle:

3.5.1.1 Oviposition period: The period up to which the female laid the eggs after pairing

3.5.1.2 Incubation period: Time taken from oviposition to the larval emergence

3.5.1.3 Larval period: The period starting from the day of hatching of the eggs to the day of final pupation. The total number of larval instars and the change of color in each moult were also observed

3.5.1.4 Pupation period: The number of days taken from the moulting of the final larval instars to pupa till the emergence of the adult beetle. The color of the pupa was also recorded

3.5.1.5 Emergence of the adults: The emergence of the adult's occurred from the pupa

3.6 BIOASSAY OF THE INSECT

The beetles were subjected to different temperature regimes varying from 35°C to 60°C at an incremental increase of 5°C for three different exposure time of 1 h, 2 h and 4 h. From the treatments, the most heat tolerant stage of the insect was found out. The temperature responsible for the mortality of the various stages of beetles was also determined.

3.7 BIOCHEMICAL ESTIMATION OF PROTEIN PRESENT IN INSECT HOMOGENATE

Total protein present in the heat stressed and unstressed insect homogenate was estimated by the method described by Lowry *et al*. (1951) with some modifications. The total protein content was also estimated using Nanodrop ND 1000 spectrophotometer for further confirmation.

3.7.1 Sample preparation

Samples for protein analysis were extracted before and after heat stress treatment. Four different stages of the test insect *viz*., neonate, V instar grub, pupa and adult were exposed to six different temperatures ranging from 35 to 60°C at an incremental increase of 5°C at a period of 1, 2 and 4h respectively. Protein content was estimated by taking known weight (0.1g) of insects. Crude homogenate of the sample was prepared using ice cold extraction buffer [(0.1g of test insect in 1 ml of 0.2M sodium phosphate buffer at pH 7.0 (Appendix II)] in a mortar following the procedure described by Sadasivam and Manickam (1996). The homogenate was spinned at 10,000 rpm for 10 minutes at 4°C to remove coarse materials. The supernatant was taken and the protein content estimated as per Lowry *et al*. (1951). The intensity of blue color developed was read in spectrophotometer at 660 nm absorbance and compared with the standard curve (Fig 1). The remaining sample was stored at -20°C for further analysis (SDS-PAGE).

3.7.2 Preparation of protein (BSA) standard

Bovine serum albumin (BSA) was used as standard. It (50 mg) was dissolved in the extraction buffer (50 mM sodium phosphate buffer ay pH 7.0) in a standard flask and kept as stock solution. From the 100 ml stock solution, 10 ml was drawn and made up to 50 ml with buffer in another standard flask. Different aliquots (200µl, 400 µl, 600 µl, 800 µl and 1000 µl) were pipetted out from the stock in different test tubes and the volume was made up to one ml with buffer. A test tube with extraction buffer (1 ml) alone served as blank. Reagent C (5 ml) (Appendix II) was added to each tube including blank and allowed to stand for 10 minutes. Further, Folin ciocalteau reagent (0.5 ml) was added and incubated for 30 minutes in dark at room temperature. The values are plotted on the graph to serve as standard graph.

3.7.3 Protein estimation using Nanodrop ND 1000 spectrophotometer

The test insect sample extracted with sodium phosphate buffer (1 µl) was pipetted out on to the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) was then brought in to contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap was enclosed to both 1 mm and 0.2 mm paths. A pulsed xenon flash lamp provided the light source and the spectrophotometer utilizing a linear CCD array to analyze the light after passing through the sample. The instrument was controlled by special software run from a computer, and the data was logged in an archive file. The following steps were followed to estimate the protein content.

1. The programme 'Protein A 280' was selected
2. With the sampling arm opened, the sample was pipetted on to the lower measurement pedestal.
3. The sampling arm was closed and initiated a spectral measurement using the operating software through the computer. The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement made.
4. When the measurement was complete, the sampling arm was opened and wiped the sample from both the upper and lower pedestals using a soft laboratory wipe. (Simple wiping prevents sample carryover in successive measurements and would give highly erroneous readings).
5. The reading was recorded at 280 nm

3.8 TREHALOSE ASSAY IN CRUDE INSECT HOMOGENATE

The trehalose content in the heat stressed and unstressed insect homogenate was estimated by the anthrone method as described by Dimler *et al*. (1952) with some modifications.

3.8.1 Sample preparation

Samples for trehalose estimation were extracted before and after heat stress treatment. The stress treatment at four different stages of the test insect *viz*., neonate, V instar grub, pupa and adults were given at six different temperatures ranging from 35 to 60°C at an incremental increase of 5°C at a period of 1, 2 and 4h respectively. Trehalose was determined by taking known quantities (0.05g) of insects. Crude homogenate of the sample was prepared with 1 ml ethanol using pestle and mortar. The homogenate was spinned at 13,000 rpm for 5 minutes at 4°C to remove coarse materials. The supernatant was collected, dried and estimated as per Dimler *et al*. (1952). The intensity of yellow color developed was read in spectrophotometer at 630 nm absorbance and compared with the standard curve (Fig 2). The sample was stored at room temperature.

3.8.2 Preparation of trehalose standard

Trehalose supplied by Merck India Ltd. was used as standard. Trehalose (100 mg) was dissolved in 100 ml distilled water in a standard flask and kept as stock solution. From the stock, 10 ml was drawn and made up to 100 ml with distilled water in another standard flask. Different aliquots (200µl, 400 µl, 600 µl, 800 µl, 1000 µl) were pipetted out in different test tubes and the volume was made up to one ml with distilled water. A test tube with distilled water (1 ml) alone served as blank. 200 µl of sodium hydroxide (2%) was added to all the test tubes including the blank and incubated at 100ºC for 10 minutes. Then cooling was done in ice cold water followed by the addition of 2 ml anthrone reagent prepared with sulfuric acid in 1:2 ratio and incubated for 10 minutes at room temperature. The values are plotted on the graph to serve as standard graph.

3.9 MOLECULAR WEIGHT DETERMINATION BY SDS-PAGE

The samples were subjected to electrophoresis in a vertical electrophoretic unit (BIO-RAD) according to the procedure described by Laemmli (1970) for sodium dodecyl sulphate polyacrylamide gel electrophoresis with some modifications

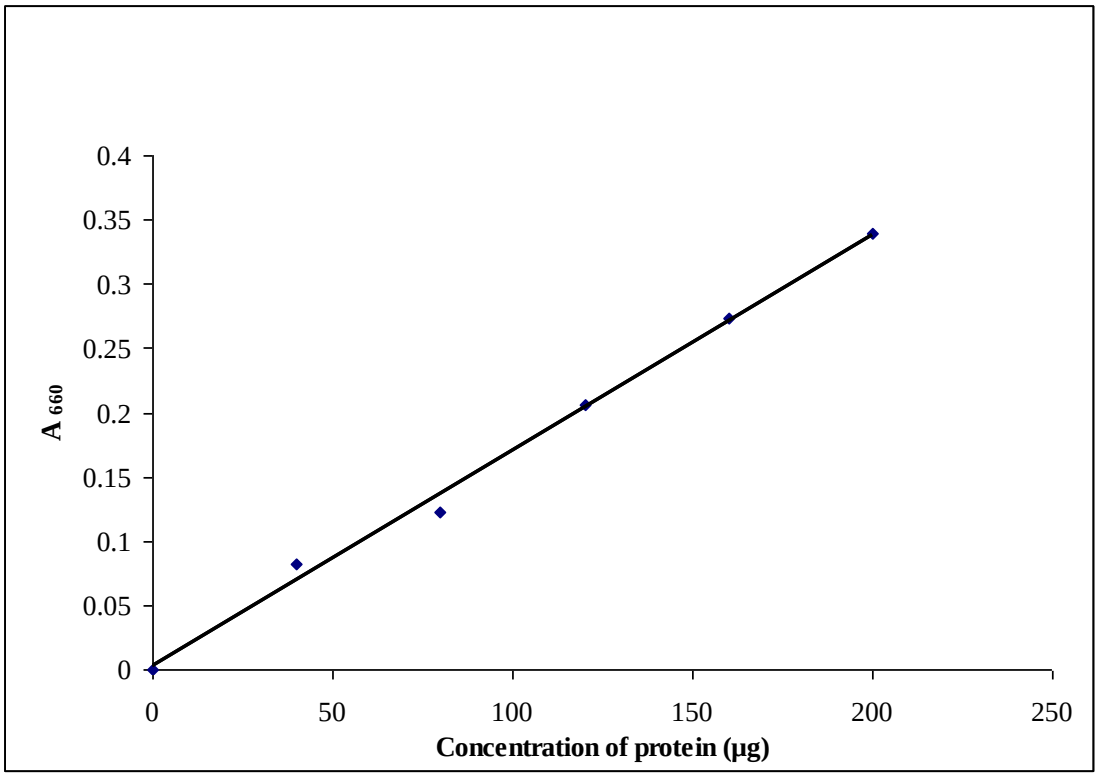


Fig 1. Protein standard curve

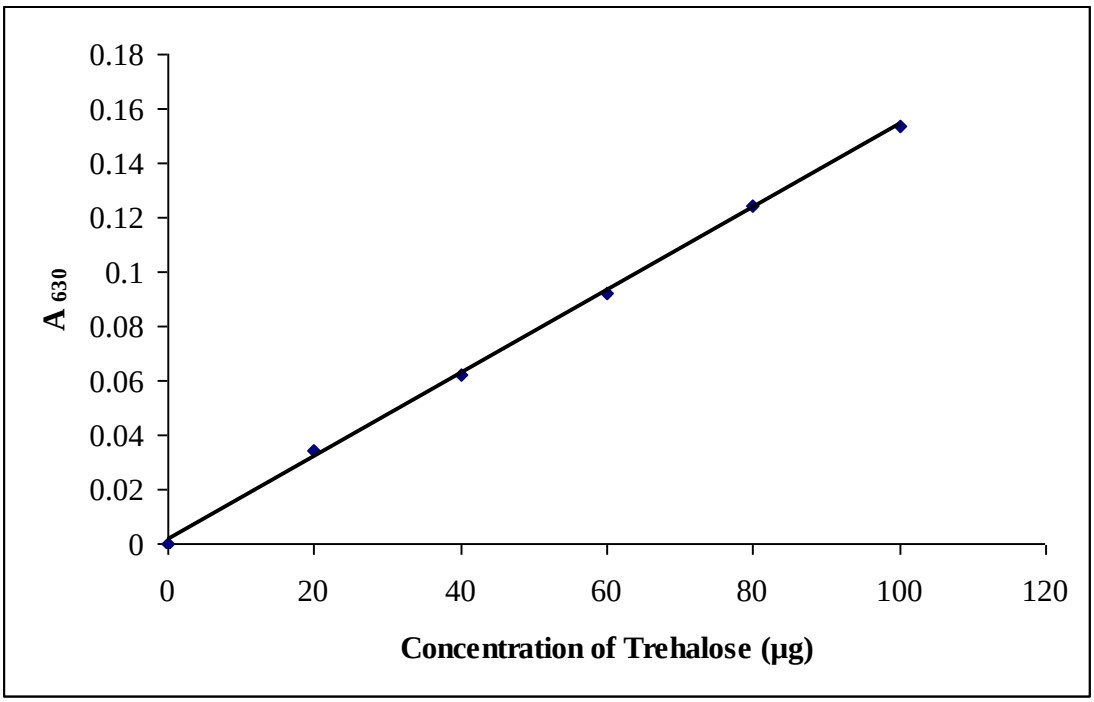


Fig 2. Trehalose standard curve

3.9.1 Reagents used

Detailed procedure is provided in Appendix III.

A. Monomer solution (30% Acrylamide and 2.7% Bisacylamide)

B. 4X resolving gel buffer (1.5 M Tris-Cl, pH 8.8)

C. 4X stacking gel buffer (0.5 M Tris-Cl, pH 6.8)

D. Electrode buffer (0.025 M Tris, pH 8.3, 0.192 M Glycine)

E. 2X Treatment buffer (0.125 M Tris Cl)

F. Initiator (10% APS)

G. SDS (10%)

3.9.2 Casting of the gel units

The glass plates and spacers (Hoefer Bio-Rad) were thoroughly wiped with acetone and assembled properly as per manufacture's instruction. The assembly was held together and the clips closed simultaneously and clamped in an upright position. The whole procedure was carried out wearing gloves.

3.9.3 Preparation of gel

1. Resolving gel (10%)

Sufficient volume of resolving gel (separating gel mixture) was prepared by mixing the components as given below.

1. Monomer : 3.33 ml
2. 4X resolving gel buffer : 2.5 ml
3. SDS (10%) : 0.1 ml
4. Distilled water : 4 ml
5. APS (10%) : 50 µl
6. TEMED : 5 µl

The ingredients were mixed gently and carefully. The gel solution was poured in the chamber between the glass plates with the help of micropipette up to the desired height (leaving 2 cm at top). Distilled water was layered on top of the gel and left to set for 60 minutes.

2. Stacking gel (6%)

Stacking gel was prepared by mixing the following solutions.

Monomer	: 0.67 ml
4x stacking gel buffer	: 1.25 ml
SDS (10%)	: 0.05 ml
Distilled water	: 3 ml
APS (10%)	: 25 µl
TEMED	: 30 µl

Water from the top of the gel was removed and the stacking gel mixture was poured over the resolving gel. Immediately the comb was placed in the stacking gel and the gel was then allowed to set for 30 minutes. After the stacking gel got polymerized, the comb was removed without distorting the shapes of the well. The gel was then carefully installed in the electrophoresis apparatus (filled with electrode buffer). Trapped air bubbles (if any) at the top of the gel was removed before the gel run.

3.9.4 Sample preparation and loading

Each 1g insect sample of *Tribolium castaneum* was ground with 1ml of 0.2 M phosphate buffer (pH 7.0). The extract was centrifuged at 10,000 rpm for 10 minutes at 4°C.

Ten microlitre of the extract was mixed with 10 µl 2X treatment buffer and heated at 100^0C for two minutes for denaturation of the protein and loaded in the wells. Broad range protein molecular weight marker (PMWB GENEI of 3000 to 2,05,000 kDa) of about 10 µl was also mixed separately with 10 µl of treatment

buffer (loading dye) and 30 µl of distilled water, heated at 100 ^{0}C for two minutes and loaded on other two separate lanes for the comparison of molecular weight.

3.9.5 Gel run

The electrophoretic unit after loading the samples was placed in a cold cell so that the heat generated during the run would be dissipated without harming the gel and resolution. The cathode and anode were connected to the power pack kept outside. Current was turned on to 15 mA for initial 15 to 20 minutes until the samples passed through the stacking gel. The gel run was then continued at 20 mA until the bromophenol blue (the tracking dye) reached the bottom of the gel.

3.9.6 Staining and destaining

Coomassie brilliant blue (Appendix IV) was used for staining the gel. After the electrophoresis, the gel was transferred to staining solution and kept overnight maintaining uniform shaking for the absorption of the stain (coomassie brilliant blue) by the protein. On the next day, the gel was transferred to a container with destaining solution and shaken gently and continuously. The destainer (acetic acid) was changed frequently until the background of the gel became colourless. The protein profile was viewed in transilluminator and documented using the Alpha Imager TM1200 (Alpha Innotech Corporation).

3.10 AMINO TERMINAL PROTEIN SEQUENCE ANALYSIS

3.10.1 Concentration of samples by Millipore centricon

The Millipore centricon was used for concentrating the test insect (adult beetles) samples extracted using sodium phosphate buffer from 1000 µl to 100 µl (Kumar *et al*., 2001). The sample reservoir was inserted into the filtrate vial. The solution required to be concentrated was added to sample reservoir (1 ml). Then the covered device was placed and filtrate vial was attached into the centrifuge rotor. Spinning was done at the rate of 4000 rpm at 4°C until desired concentration was achieved. Water was used prior to centrifugation for cleaning purpose. After centrifugation, the centrifugal filter assembly was removed from centrifuge and the filtrate vial was separated from membrane support base. For the

purpose of filtration the filtrate was reversed back and after the concentration process was completed, the filtrate was discarded. The retentate vial was placed over the sample reservoir and the unit was inverted to recover the retentate. Centrifugation was done at the same speed to transfer the concentrate into retentate vial.

3.10.2 Blotting

The protein samples were run on SDS-PAGE along with molecular weight markers. After completing the gel run, the gel was transferred into a Trans blot system (Bio Rad) using tank buffer for blotting. This was employed for transferring protein from the SDS gel to membrane (PVDF) in which the separated proteins were electroblotted onto PVDF (poly vinyl difluoride membrane) membrane (0.2 μm; Bio Rad, Hercules, CA) in presence of methanol (40 % v/v), Tris buffer, (25 mM at pH 8.2) and glycine (190 mM) at 200 mA for 1 h using Mini Trans Blot cell (Bio Rad, Hercules, CA) as described by Kumar *et al*., 2001. Blotting was done under constant current of 200 mA for 1 h at 4°C.

3.10.3 N-terminal sequencing of proteins

N-terminal sequencing was done in Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram. The test insect protein sample was run on a SDS-PAGE gel and the gel was then transferred onto a sequiblot PVDF membrane (0.2 μm) using the CAPS (3-[Cyclohexyl amino] – 1 propane sulfonic acid) transfer buffer (10 mM CAPS + 10 % methanol, pH 11.0) from Bio Rad at 200 mA for 1 h. After the transfer was completed, the membrane was stained with a staining agent, coomassie brilliant blue in methanol (50 %) and destained 1-3 times in acetic acid (5 %) followed by several washes in deionised water. The specific protein bands (70 KDa) were cut out from eight lanes and were processed for N-terminal protein sequence analysis which is based on Edman chemistry (cleaving reaction of peptides) followed by PTH (phenylthiohydantoin – a standard amino acid derivative) analysis using microbore HPLC (HP G-1000A equipped with a 1090 PTH analyzer) (Matsudaira, 1993).

3.11 SEQUENCE ANALYSIS

The sequence information obtained was named as ‘Trib hsp’ and further analyzed using various bioinformatics tools.

3.11.1 Homology search

The protein sequence ‘Trib hsp’ was compared with the sequence available in the database using BLAST tool offered by National Center for Biotechnology Information **(**NCBI**)**. Protein protein blast (blast p) was carried out for homology search. The BLAST progamme ‘blast p’ (Altschul, 1990) provided by NCBI (www.ncbi.nlm.nih.gov/Blast/Blast.cgi) was utilized for the purpose. The protein sequence of *Tribolium castaneum* was pasted in the blast web page and the programme was run at default settings. The blast sequence alignment of the search results were noted and saved.

3.11.2 Reverse translation

The protein sequence was translated to get the nucleotide sequence using the reverse translation tool. This programme is provided by the expasy server (http//www.expasy.org/tools). The protein sequence was pasted in the fasta format and the results obtained were saved.

3.11.3 Structural identity of protein

3.11.3.1 Primary structure prediction

The primary structure of the protein (its amino acid sequence) was predicted from ‘blast p’ programme. The further details about the primary structure of the protein were obtained by using the ‘protparam’ tool (http//www.expasy.ch/tools/protparam.html). The protein sequence was pasted excluding the fasta format and the results obtained were saved.

3.11.3.2 Secondary structure prediction

The secondary structure of the protein providing the details of alpha helices and beta strands was determined using the NN Predict tool (http://alexander.ucsf.edu/nomi/nnpredict.html). The per cent helices and strands

found in the secondary structure were obtained by using the ‘sopma’ tool (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html).

3.11.3.3 Tertiary structure prediction

The tertiary structure of the protein and the ramachandran plot displaying the psi and phi backbone conformational angles for each residue in a protein was designed using the rasmol tool, an offline tool. The details of ramachandran plot was obtained using the ‘savs’ (Structure Analysis and Verification Server) tool (http://nihserver.mbi.ucla.edu/SAVES).

3.11.4 Functional identity of protein

The functional domains present in the protein were identified using the ‘pfam’ tool (http://pfam.sanger.ac.uk) which described about the protein function.

3.12 STATISTICAL ANALYSIS

The data with respect to the total protein content, trehalose content and the recovered trehalose content under each treatment was tabulated and analyzed statistically in a two factor randomized complete block design as proposed by Mason *et al.* (2003).

Results

4. RESULTS

Results of the experiments conducted in the laboratory on the study entitled "Characterization of proteins and trehalose as heat shock regulators in insect system" are presented in this chapter.

4.1 MASS CULTURING OF TEST INSECTS

The test insect, red flour beetle, *Tribolium castaneum* Herbst (Tenebrioniidae: Coleoptera) was mass cultured from the nucleus culture using different insect feeds *viz*., wheat flour, semolina and wheat flour and semolina in combination (Plate 1). Five pairs of test insects were inoculated in the feed in February, 2007. The population build up of the insect in wheat flour (18.94±0.52), semolina (15.00±0.46) and in combination of both wheat flour and semolina (17.14±0.47) was observed in March 2007 (Table 1). There was an increase in the rate of multiplication in March 2007 starting since February 2007. The maximum population growth was seen in March 2008 in all the three feeds tested in the prevailing temperature (28.3°C) and relative humidity (64%) followed by February 2008. Comparatively, the population growth was less in July 2007 (21.80±0.48, 19.53±0.77, 21.70±0.24) and in August 2007 (22.23±0.36, 17.87±0.64, 21.56±0.40). The mortality was also relatively less in March 2008 in all the three feeds. Higher mortality per cent was recorded during July 2007 (20.85±0.09, 19.58±0.93, 19.13±0.08) and August 2007 (20.47±0.29, 20.58±0.07, 18.90±0.05).

As per the highest recorded population in March 2008, the multiplication of the insect was found to be faster (Fig. 3) in wheat flour (30.97±0.20) followed by the wheat flour and semolina combination (30.91±0.15) while it was less in semolina (30.29±0.13) alone. However, the per cent mortality of the insect was more in July 2007 in wheat flour (20.85±0.09) followed by the combination of both wheat flour and semolina (19.58±0.93) and the least in the semolina (19.13±0.08) (Table 1). The

a. Mass culturing of *Tribolium castaneum*

b. Growth stages of *T. castaneum* on wheat flour

c. Growth stages of *T. castaneum* on semolina

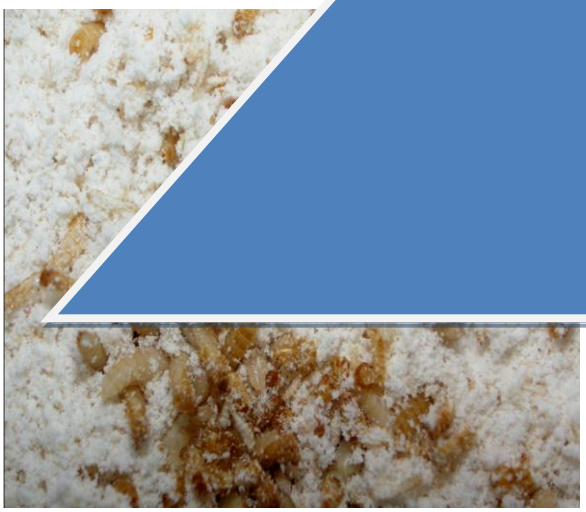
Plate 1. Culture of *Tribolium castaneum*

Table 1. Monthly variations in the population build up of *Tribolium castaneum* adults in different insect feed

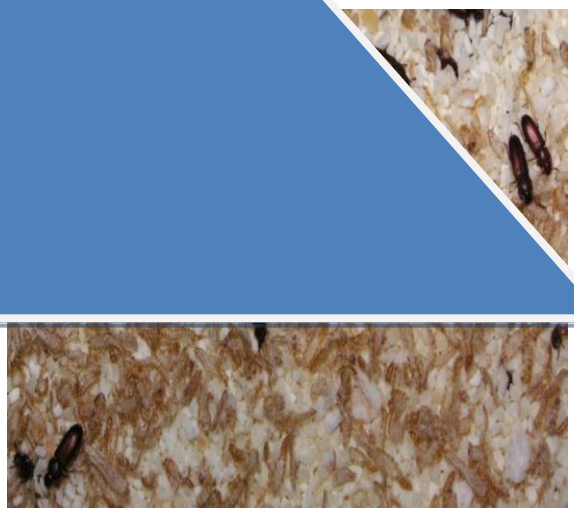
Period	Wheat flour		Semolina		Combination of wheat flour and semolina	
	Mean Population	Mortality (%)	Mean Population	Mortality (%)	Mean Population	Mortality (%)
Mar 07	18.94±0.52	18.29±0.24	15.00±0.46	16.23±0.05	17.14±0.47	14.52±0.08
Apr 07	23.12±0.45	14.05±0.19	22.25±0.66	12.54±0.08	21.90±0.58	12.71±0.08
May 07	23.05±0.23	14.82±0.13	20.00±0.32	11.44±0.07	22.09±0.39	12.59±0.04
Jun 07	22.77±0.41	19.56±0.13	21.00±0.52	17.89±0.08	22.70±0.50	17.77±0.15
Jul 07	21.80±0.48	20.85±0.09	19.53±0.77	19.58±0.93	21.70±0.24	19.13±0.08
Aug 07	22.23±0.36	20.47±0.29	17.87±0.64	20.58±0.07	21.56±0.40	19.06±0.06
Sep 07	22.60±0.50	19.67±0.14	18.73±0.34	21.78±0.06	21.37±0.42	18.90±0.05
Oct 07	24.06±0.33	14.56±0.11	20.73±0.31	12.96±0.06	23.73±0.33	12.96±0.06
Nov 07	25.35±0.31	11.47±0.10	23.94±0.26	8.54±0.03	24.28±0.27	9.22±0.03
Dec 07	28.00±0.26	6.12±0.16	27.72±0.19	3.98±0.04	27.75±0.26	5.32±0.03
Jan 08	28.10±0.20	4.25±0.08	27.20±0.27	3.65±0.03	27.21±0.28	4.05±0.05
Feb 08	29.43±0.26	4.54±0.08	28.10±0.12	3.24±0.03	28.70±0.18	4.08±0.05
Mar 08	30.97±0.20	3.66±0.07	30.29±0.13	2.28±0.04	30.91±0.15	2.57±0.03

SEm for three observations

a. Ma… …um



b. Growth stages of *T. castaneum* on wheat flour



c. Growth stages of *T. castaneum* on semolina

Plate 1. Culture of *Tribolium castaneum*

per cent mortality was found to be positively correlated (r = 0.84) with the relative humidity.

Insect feed was found contaminated by fungus during rainy season. The per cent contamination was highest in July 2007 (41.3±3.53) followed by August 2007 (34.7±1.33) due to the excessive relative humidity (88 and 84% respectively). However, comparatively lesser fungal contamination (Fig. 4) was observed during drier months viz., January 2008 (22.7±2.67) and February 2008 (20±2.31) when the relative humidity was 61 and 64 per cent respectively. There was very less contamination recorded in the feed in December 2007 (18.7±1.33) when the relative humidity was only 56 per cent (Table 2). A positive correlation was also found out between the relative humidity and per cent contamination (r = 0.91) which indicated the increase in per cent contamination with increase in relative humidity.

Table 2. Per cent contamination of insect feed

Period	Temperature	Relative humidity (%)	Contamination (%)
Mar 07	30.2	63	20.0±2.31
Apr 07	30.1	69	22.7±1.33
May 07	28.7	76	26.7±1.33
Jun 07	26.8	84	33.3±1.33
Jul 07	25.7	88	41.3±3.53
Aug 07	28.9	84	34.7±1.33
Sept 07	26.2	86	36.0±2.31
Oct 07	31.7	79	28.0±2.31
Nov 07	26.7	67	28.0±2.31
Dec 07	27.2	56	18.7±1.33
Jan 08	27	59	22.7±2.67
Feb 08	28.3	61	20.0±2.31
Mar 08	28.3	64	17.3±1.33

SEm for three observations

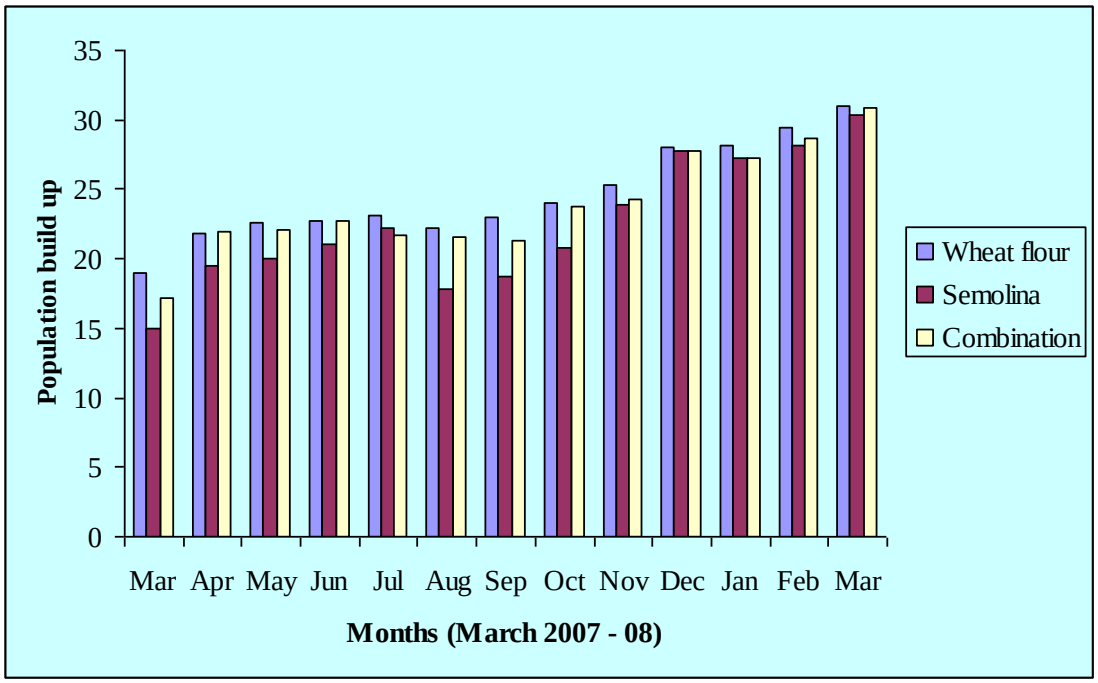


Fig 3. Populations build up of *Tribolium castaneum*

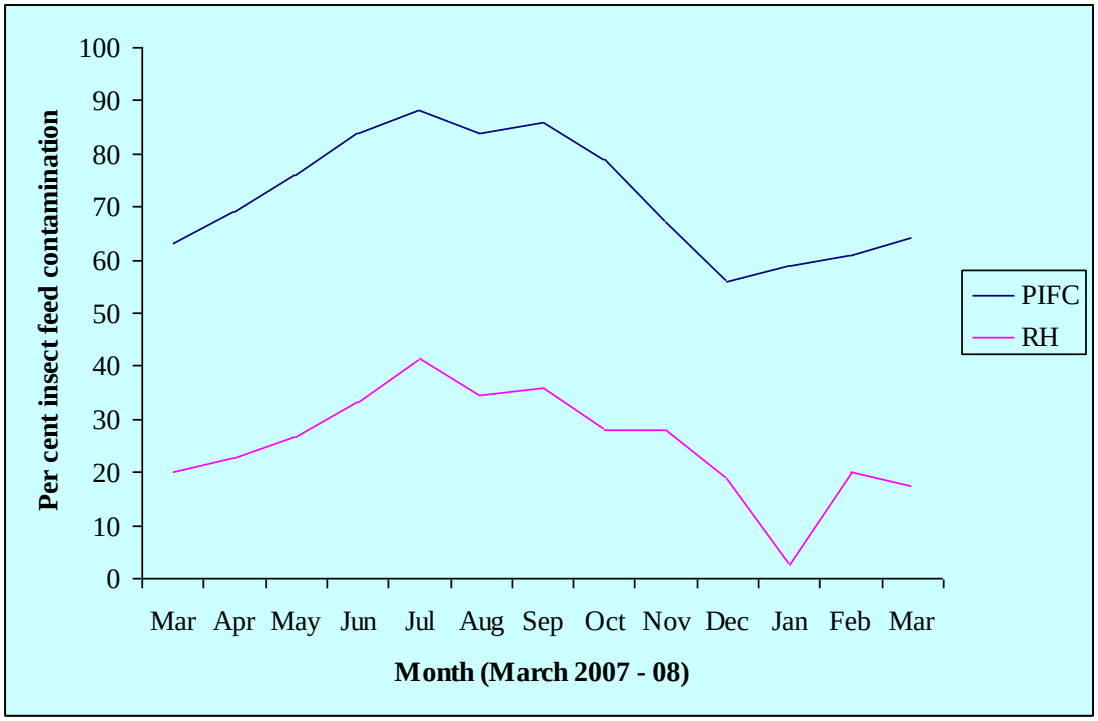


Fig 4. Insect feed contamination over a period of time

4.2 BIOLOGY OF THE TEST INSECT

The biology of Tribolium castaneum was studied during the period from March 2007 to March 2008. The data recorded gave an idea about the life cycle of the insect, duration of different life stages, per cent hatchability, mortality and per cent survival of the test insect.

4.2.1 Life stages of Tribolium castaneum

4.2.1.1 Egg

Eggs were very small, whitish with food particles adhering to the surface (Plate 2). The egg period varied from 3 to 5 days (Table 4).

4.2.1.2 Larva

Eggs hatch into slender, cylindrical, white larvae tinged with yellow. The brown headed larvae were cream to yellow, slender, and wiry, reaching a length of 1/4 inch. Larvae had six legs and two-pointed or forked projections at the last body segment. There were five larval instars and the length of the larval period varied from 16 to 18 days.

The first instar larva was cream colored (Plate 3) and the duration of the first larval instar varied from 7.0±0.58 to 10.0±0.58 days (Table 4). The duration of the second larval instar varied from 12±0.58 to 14.3±0.88 days. Similarly the duration of the third and fourth larval instars varied from 15±0.58 to 17.3±0.67 and 17.7±0.67 to 21±0.58 days respectively. Finally the last larval instar was yellow colored with a brown head and its duration varied from 23±0.58 to 25.3±0.33 days (Plate 4).

The increase in the number of neonate and the V instar grub was observed in March 2007 (12.54±0.58, 12.29±0.47). The highest rate of multiplication of the both the young and V instar grub was observed in February 2008 (18.67±0.26, 17.39±0.17) and March 2008 (18.31±0.20, 18.31±0.20) when the temperature was 28.3°C. It was comparatively lesser during July 2007 (13.92±0.42, 12.95±0.40) and

Plate 2. Eggs of *Tribolium castaneum* found adhering to the food particles

Plate 3. Neonate of *T. castaneum*

Plate 4. V instar grub of *T. castaneum*

Plate 5. Pupa of *T. castaneum*

Plate 6. Adult of *T. castaneum*

August 2007 (14.57±0.54, 13.76±0.42) due to the high relative humidity (more than 80%). The per cent mortality was also more during July 2007 (7.97±0.06, 8.00±0.05) and August 2007 (5.77±0.04, 5.69±0.04) owing to the 88 and 84 per cent relative humidity (Table 3).

4.2.1.3 Pupa

Fully grown larvae transformed into naked pupae. Pupae were white and immobile in nature (Plate 5) and the duration of the pupal instar varied from 7±0.58 to 7.4±0.58 days (Table 4). The highest rate of formation of the pupa was observed in the month of February 2008 (17.01±0.15) when the temperature was 28.3°C. Once again the pupal formation was less during the months of June 2007 (14.40±0.06), July 2007 (11.95±0.35) and August 2007 (12.48±0.21) due to a high relative humidity (Table 3). The per cent mortality was also more during July 2007 (7.85±0.03) and August 2007 (6.69±0.04).

4.2.1.4 Adult

Adults were reddish brown in color and duration lasted from 33±0.58 to 36±0.58 days (Plate 6). The life cycle required five to six weeks. The highest rate of emergence of the adults (fig.5) was observed in the month of October 2007 adults (Fig. 5) was observed in the month of October 2007 (17.63±0.10) when the temperature was 31.7°C. As in the larval and pupal stages, in June 2007 (14.00±0.00), July 2007 (14.25±0.18) and August 2007 (15.21±0.24), the adult emergence was less due to a high relative humidity (more than 80%). Similarly, the per cent mortality was also more during July 2007 (5.70±0.04) and August 2007 (6.62±0.04) due to high relative humidity (Table 3 & 4).

Table 3. Per cent monthly variation in mortality of the different life stages of *Tribolium castaneum*

Period	Neonate		V instar grub		Pupa		Adult		
	Total number	Per cent mortality	Total number	Per cent mortality	Total number	Per cent mortality	Total number	Per cent mortality	Mean adult population
Mar 07	12.54±0.58	2.25±0.12	12.29±0.47	2.65±0.12	12.20±0.31	1.14±0.18	12.06±0.18	2.28±0.10	11.78±0.08
Apr 07	17.33±0.33	5.81±0.13	16.09±0.24	4.67±0.10	15.32±0.33	3.53±0.13	12.58±0.22	3.36±0.13	11.88±0.09
May 07	17.00±0.26	4.70±0.13	16.52±0.29	4.74±0.07	15.90±0.27	4.59±0.04	11.89±0.33	2.25±0.15	11.19±0.18
Jun 07	16.10±0.27	4.70±0.15	15.40±0.12	5.80±0.06	14.40±0.06	4.60±0.06	14.00±0.00	3.38±0.02	13.62±0.02
Jul 07	13.92±0.42	7.97±0.06	12.95±0.40	8.00±0.05	11.95±0.21	7.85±0.03	14.25±0.18	5.70±0.04	13.63±0.14
Aug 07	14.57±0.54	5.77±0.04	13.76±0.42	5.69±0.04	12.48±0.35	6.69±0.04	15.21±0.24	6.62±0.04	14.78±0.20
Sep 07	16.82±0.29	3.47±0.08	16.35±0.33	2.91±0.08	15.95±0.41	3.33±0.04	15.62±0.43	3.85±0.05	15.27±0.38
Oct 07	17.83±0.17	2.36±0.06	17.72±0.18	2.39±0.06	17.01±0.17	2.39±0.06	17.63±0.10	2.39±0.06	17.24±0.04
Nov 07	18.00±0.21	2.36±0.05	17.63±0.26	2.43±0.04	17.21±0.29	2.29±0.05	16.98±0.34	2.40±0.08	16.58±0.26
Dec 07	18.17±0.20	2.32±0.04	17.85±0.24	2.35±0.05	17.50±0.28	1.24±0.01	17.32±0.31	2.36±0.05	16.96±0.26
Jan 08	17.67±0.20	2.35±0.04	17.32±0.22	1.23±0.04	17.09±0.26	1.21±0.03	16.92±0.28	2.32±0.04	16.60±0.24
Feb 08	18.67±0.26	2.45±0.08	17.39±0.17	2.38±0.02	17.01±0.15	3.45±0.03	16.71±0.16	2.36±0.06	16.35±0.10
Mar 08	18.04±0.16	2.33±0.04	18.31±0.20	2.28±0.01	17.43±0.20	1.24±0.02	17.25±0.22	2.33±0.04	16.92±0.18

SEm for five observations

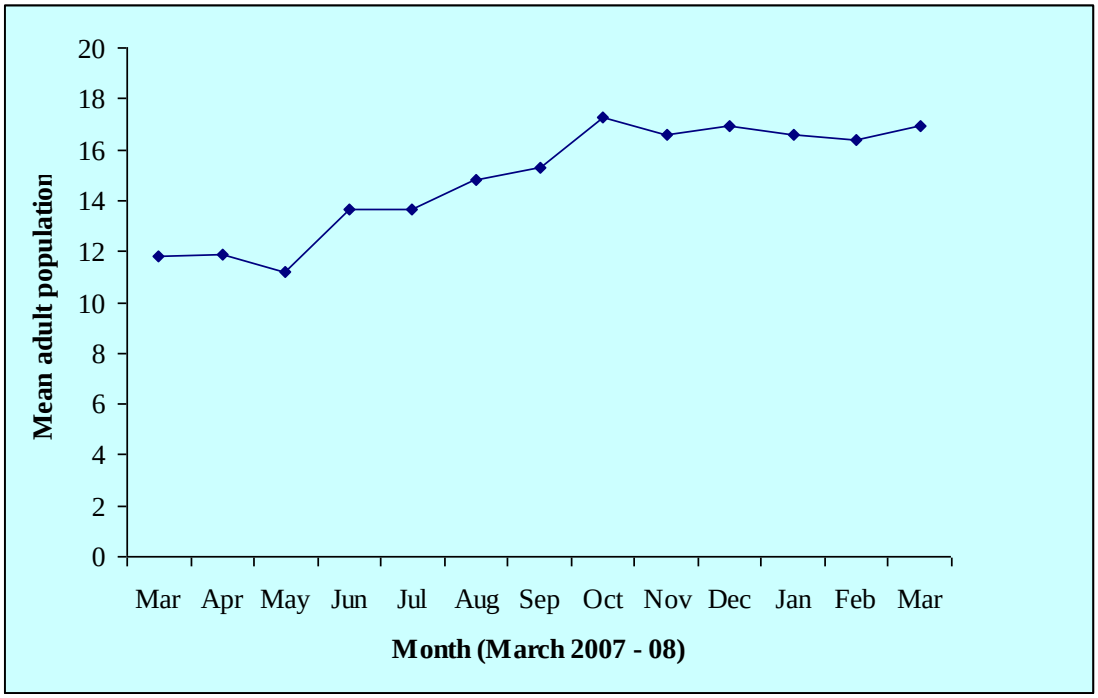


Fig 5. *Tribolium castaneum* adult emergence in different periods

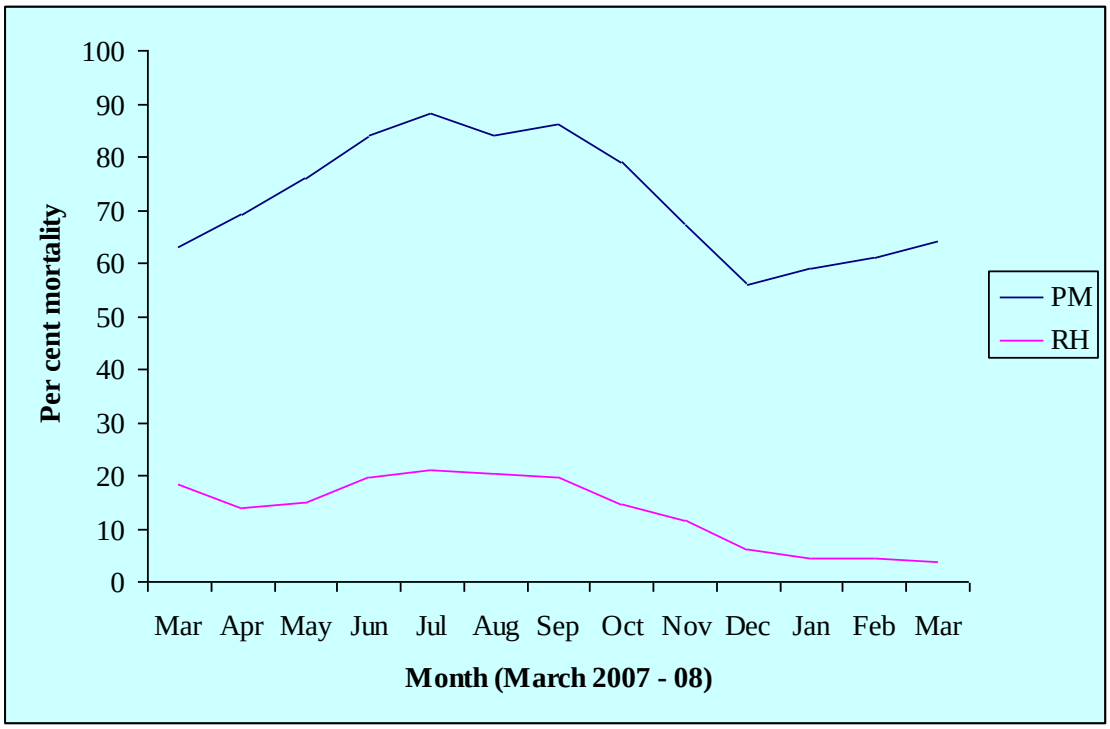


Fig 6. Per cent mortality of *Tribolium castaneum*

Table 4. Duration of different growth stages of *Tribolium castaneum*

	Duration (Days)	
Insect growth stage	**Minimum**	**Maximum**
Egg	3.3±0.33	5.3±0.33
1st instar	3.7±0.58	4.7±0.58
2nd instar	4.3±0.58	4.9±0.88
3rd instar	3.0±0.58	3.0±0.67
4th instar	2.7±0.67	3.7±0.58
5th instar	4.3±0.58	4.7±0.33
Pupa	7.0±0.58	7.4±0.58
Adult	8.0±0.58	8. 0±0.58

4.3 SURVIVAL OF THE TEST INSECTS UNDER DIFFERENT TEMPERATURE REGIMES AT DIFFERENT EXPOSURE PERIOD

The red flour beetles were subjected to six different temperature regimes ranging from 35°C to 60°C at three different exposure times *viz*., 1 h, 2 h and 4 h respectively. The temperature range was selected depending on the minimum horizontal and vertical limits designed for the flour mills. Initially, there was no mortality of the insects when they were exposed for 1h, 2h and 4h at 35°C to 60°C but when the exposure time was increased beyond 4h, the insects were killed.

It was observed that the mortality of the insects increased with increase in exposure time and temperature (Table 5). The mortality of the insect showed a significant increase at 35°C when exposed for 5 h (15.3±0.88), 6 h (24.3±0.88), 7 h

Table 5. Mortality of *Tribolium castaneum* adults exposed to different temperature and time

Time (h)	Temperature (°C)					
	35	**40**	**45**	**50**	**55**	**60**
5	15.3±0.88	21.0±1.15	27.7±0.88	53.7±0.88	81.3±1.15	100
6	24.3±0.88	34.7±0.88	39.0±1.15	66.0±1.00	90.7±1.20	100
7	28.7±0.20	44.7±1.20	52.3±0.67	84.3±0.88	100	100
8	53.7±0.88	71.7±1.45	84.0±1.15	98.7±0.33	100	100

SEm for five observations

(28.7±0.20) and 8 h (53.7±0.88). Similar trend was also found at 40°C, 45°C and 50°C. But higher mortality of the insects was observed at 50°C and 55°C when they were exposed for 8 h (98.7±0.33) and 6 h (90.7±1.20). Cent per cent mortality of the insects was observed at 55°C when the exposure time was increased to 7 h and 8 h respectively. At 60°C, when the insects were exposed for a time period of 5 h, 6 h, 7 h and 8 h, cent per cent mortality of the test insect was observed

4.4 BIOCHEMICAL ANALYSIS

4.4.1 Estimation of total protein present in the crude homogenate

The total protein content was estimated in the insects by the Lowry's method of protein assay and the readings were further confirmed by the Nanodrop spectrophotometer reading at a wave length of 280 nm and it was found to be approximately same.

The total protein content in the insect homogenate was found to decrease when they were subjected to stress at different temperatures for different time intervals. The total protein content was found to vary significantly from 35°C to 60°C for all the growth stages except for the V instar grub in which non significant difference was found out at 1 h (42.72 mg/g) and 2 h (41.70 mg/g) respectively (Table 6a). When the insects were subjected to heat stress at 35°C, the protein content was more for the neonate (51.53 mg/g) followed by V instar grub (49.23 mg/g), pupa (49.95 mg/g) and adult (50.74 mg/g). Similar result was observed up to 45°C but when the insects were exposed to 50°C, the corresponding protein content was in the order of neonate (36.81 mg/g) < pupa (38.16 mg/g) < adult (39.23 mg/g) < V instar grub (40.81 mg/g). Similar trend was also observed at 55°C and 60°C. The decrease in the total protein content was also significant with an increase in temperature and time period indicating the formation of increased heat shock protein in all the stages of the insect development (Table 6b).

Table 6a. Effect of exposure time and temperature on the total protein content (mg/g) of *Tribolium castaneum*

Exposure time (H)	Total protein content (mg/g)			
	Neonate	**V instar grub**	**Pupa**	**Adult**
1	44.19	42.72	45.49	47.02
2	41.64	41.70	43.29	44.55
4	40.48	39.76	40.86	42.42
CD (0.05)	0.88	1.18	0.35	0.42
Temperature (°C)				
Control (Unexposed insects at room temperature)	53.62	57.15	61.18	65.09
35	51.53	49.23	49.95	50.74
40	49.24	46.64	47.41	48.24
45	44.93	39.85	42.34	43.61
50	36.81	40.81	38.16	39.23
55	32.12	34.91	29.38	33.68
60	27.26	30.83	25.20	29.75
CD (0.05)	1.35	1.80	0.54	0.64

Table 6b. Effects of exposure time and temperature on the total protein content (mg/g) of different stages of *Tribolium castaneum*

Insect stage	Exposure time (H)	Temperature (°C)						
		Control (Unexposed insects at room temperature)	**35**	**40**	**45**	**50**	**55**	**60**
Neonate	1	53.62	53.28	50.79	48.34	38.69	30.48	27.81
	2	53.62	51.22	50.09	44.24	37.07	27.73	24.46
	4	53.62	50.09	46.85	42.23	34.67	29.93	23.32
	CD (0.05)	2.34						
V instar grub	1	57.60	52.05	48.91	40.39	44.98	33.58	29.78
	2	57.60	50.04	46.94	40.87	40.13	30.79	26.64
	4	56.24	47.68	44.06	38.29	37.33	31.98	25.37
	CD (0.05)	3.11						
Pupa	1	61.18	52.36	48.69	45.20	41.09	36.51	33.41
	2	61.18	50.00	44.30	42.05	38.47	33.45	28.56
	4	61.18	47.51	44.23	39.78	34.93	31.09	27.29
	CD (0.05)	0.93						
Adult	1	65.09	52.88	50.04	46.42	42.22	38.17	35.07
	2	65.09	50.48	49.26	43.32	39.21	34.28	29.43
	4	65.09	48.86	45.41	41.09	36.25	32.27	27.99
	CD (0.05)	1.11						

4.5 DETERMINATION OF MOLECULAR WEIGHT OF THE HEAT SHOCK PROTEIN BY SDS-PAGE

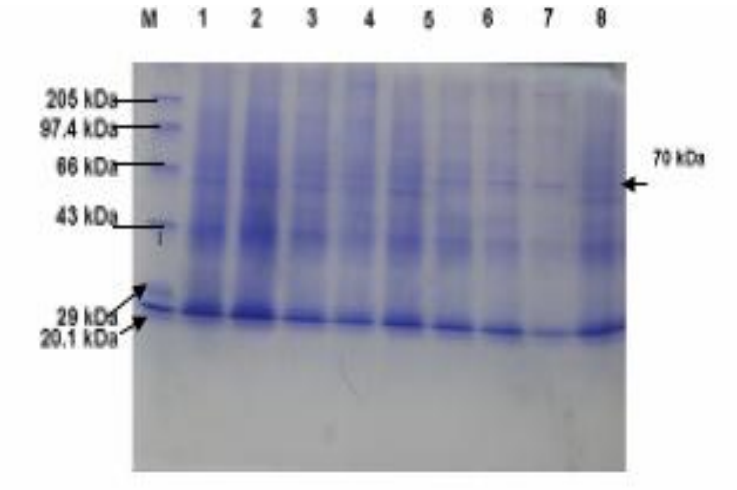
SDS-PAGE for proteins was carried out to characterize the heat shock protein. A very clear band was obtained in the insect samples (subjected to different temperature at different time intervals) as compared to the unexposed insect samples (Plate 7, 8, 9 and 10). The SDS-PAGE gel for proteins isolated from the neonate, V instar grub, pupa and adult under stress showed an extra band in all the lanes as compared to the control protein sample that was isolated at room temperature. The molecular weight of the band was determined using the alpha imager software and it was found out to be 70KDa. The 70KDa protein band got resolved clearly on the SDS-PAGE gel after staining with coomassie brilliant blue R 250 staining reagent. The Rf value was found to be 0.362 by point to point fit. The SDS-PAGE also confirmed 95 per cent purification of the hsp70 due to the appearance of a single band on the gel when 10 µl of sample containing 12 µg of protein was loaded into the wells.

4.6 N-TERMINAL SEQUENCING

The N-terminal sequence Y W P E A P W W W W (Tyr Trp Pro Glu Ala Pro Trp Trp Trp Trp) displayed all the ten amino acids present in the N- terminal region of the protein. The ‘Trib hsp’ was taken as the query sequence and it showed 80 per cent homology with the heat shock protein. The results of the BLAST output are furnished in accordance with the sequence data.

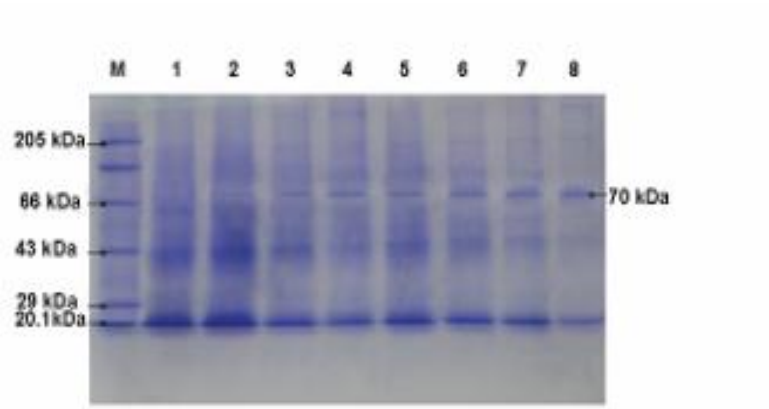
4.7 ANALYSIS OF SEQUENCE BY USING DIFFERENT BIOINFORMATICS TOOLS

Different computer algorithms were used to analyze the sequence data in a logical manner.



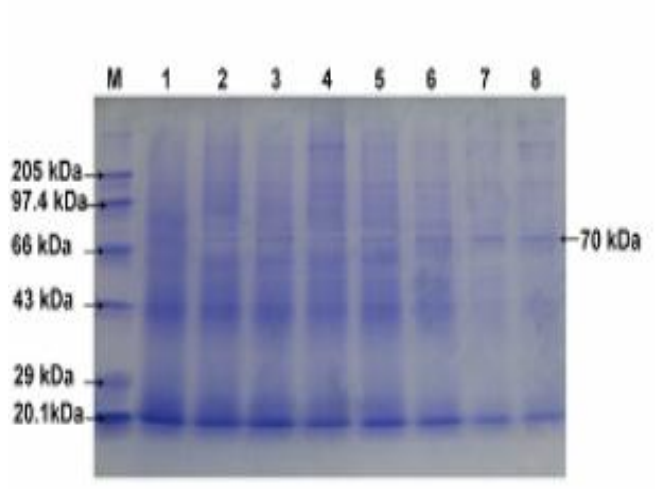
Lane M: Marker protein; Lane 1: Control; Lane 2 to 8: Protein isolated at different temperatures: 2 - 35ºC; 3 - 40ºC (10µl); 4 - 45ºC; 5 - 50ºC; 6 - 55ºC; 7 - 60ºC; 8 - 40ºC (15µl)

Plate 7. SDS-PAGE for proteins isolated from neonate under heat stress at different levels



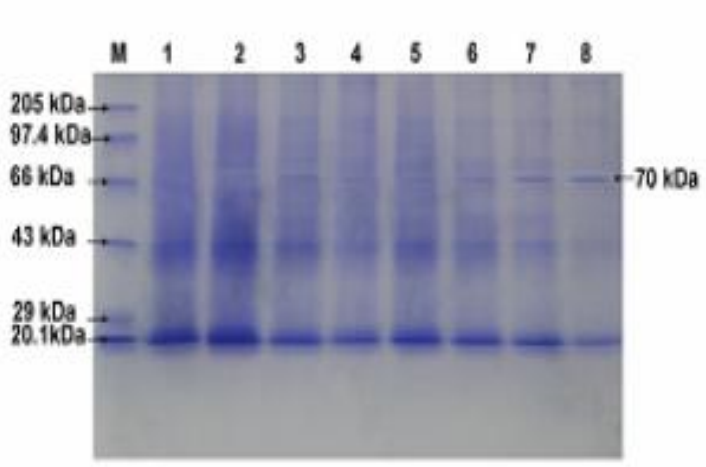
Lane M: Marker protein; Lane 1: Control; Lane 2 to 8: Protein isolated at different temperatures: 2 - 35ºC; 3 - 40ºC (10µl); 4 - 45ºC; 5 - 50ºC; 6 - 55ºC; 7 - 60ºC; 8 - 40ºC (15µl)

Plate 8. SDS-PAGE for proteins isolated from V instar grub under heat stress at different levels



Lane M: Marker protein; Lane 1: Control; Lane 2 to 8: Protein isolated at different temperatures: 2 - 35ºC; 3 - 40ºC (10µl); 4 - 45ºC; 5 - 50ºC; 6 - 55ºC; 7 - 60ºC; 8 - 40ºC (15µl)

Plate 9. SDS-PAGE for proteins isolated from pupa under heat stress at different levels



Lane M: Marker protein; Lane 1: Control; Lane 2 to 8: Protein isolated at different temperatures: 2 - 35ºC; 3 - 40ºC (10µl); 4 - 45ºC; 5 - 50ºC; 6 - 55ºC; 7 - 60ºC; 8 - 40ºC (15µl)

Plate 10. SDS-PAGE for proteins isolated from adult under heat stress at different levels

4.7.1 Homology search

Homology of the sequence ‘Trib hsp’ obtained from the protein product of *Tribolium castaneum* with the other reported sequence was analyzed. The sequence (Plate 11) showed significant homology to red flour beetle (*Tribolium castaneum*) protein sequence deposited in the public domain database using ‘blast p’ search tool. It had shown that 80 per cent identity to *Tribolium castaneum* heat shock protein (Plate 12).

4.7.2 Reverse translation

The nucleotide sequence determined from the protein sequence by the reverse translation tool showed that the gene corresponds to the thioredoxin (functions as heat shock protein) based heat shock gene. The nucleotide sequence showed the presence of a number of degenerate bases thus indicating degeneracy of codons.

4.7.3 Structural identity of protein

4.7.3.1 Primary structure prediction

The complete amino acid sequence of the protein and the physico-chemical properties of the protein giving the detailed information on the molecular weight, theoretical isoelectric point, amino acid composition (Table 7), instability index and grand average of hydropathicity (GRAVY) indicating the solubility of the proteins was determined by using the ‘protparam’ tool.

The molecular weight of the protein was found out to be 70KDa from the protparam results. Further the number of amino acids in the ‘Trib hsp’ was found out to be 620 with a theoretical isoelectric point of 8.62. The total number of negatively charged residues (Asp + Glu) was 80 while the total number of positively charged residues (Arg + Lys) was 89. Due to a low instability index (27.67), the protein was confirmed to be a stable protein. The negative GRAVY index (-0.36) suggested that the protein was hydrophilic.

N-TERMINAL SEQUENCE OUTPUT: YWPEAPWWWW

>gi|91094485|ref|XP_970942.1| PREDICTED: similar to CG9302-PA [*Tribolium castaneum*]
MKHFNTIFFLLLAFEINIYLTKDTKNAVVDNIYDIKEFKKLIRTKTNVLVCYTNSIKQASQVI
KVFREAADVIKGQGTMVVMDCSGEAKKVCKKLKVTPDPFIFKHYKNGEFNRDYDRKFTV
SSMVNFMRDPTGDLPWEEDASASDIVHVPDAETLAKFIRQESRPLMVMFYAPWCGFCKTL
KPEYVAAAKELKGHSVLAAIDVNKPENAVIRTLYNITGFPTLLYYKNGAMKFQYEGDNKR
QAIVNFMKNPSKPVKVKEQEWSEVDSEVVHLTTTNFDPVVKEEASLLVMFYAPWCGHCK
KIKPEYEKAAAKLKSDGIPGMMAAVDATKEVSIADRFSVKGYPTMKYFTYGEHKFDINLR
EATKIVEFMKNPKEPPPPPPPEKPWSEEESSVVHLNEENFKSFLKKKRHALVIFYAPWCGHC
KKAKPEFTKAAEFFKDDPKVEFAAVDCTTYQGVCSAHEVSGYPTIKYFSYLNKVVKAYNS
GRTADDFIAFMSDPEGNGSSQKTIVPQLTDANFEEEISSKSAVLVMFYAPWCKQCKEIKPE
YQKATNELKQDGFIQLASVDCSSNPVVTDKYDIGTFPTFKLFLNGKFAADFTGKSTKDDIK
SFVVDVKNRKNKEL

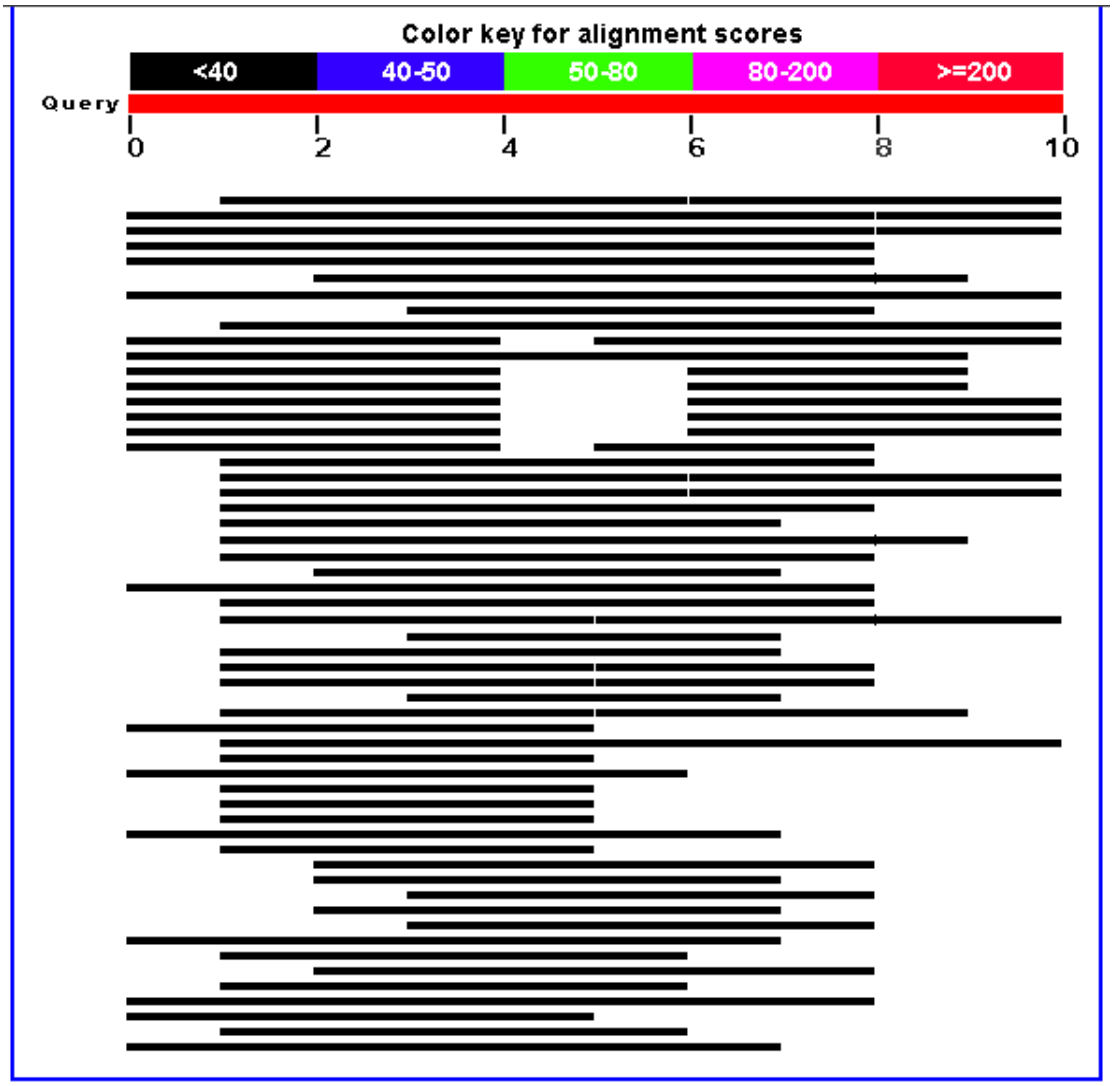
a. Protein sequence

>reverse translation of gi|91094485|ref|XP_970942.1| PREDICTED: similar to CG9302-PA [*Tribolium castaneum*] to a 1857 base sequence of consensus codons.
atgaarcayttyaayacnathttyttyytnytnytngcnttygarathaayathtayytnacnaargayacnaaraaygcngtngtngayaay
athtaygayathaargarttyaaraarytnathmgnacnaaracnaaygtnytngtntgytayacnywsnathaarcargcnwsncargtn
athaargtnttymgngargcngcngaygtnathaarggncarggnacnatggtngtnatggaytgywsnggngargcnaaraargtntg
yaaraarytnaargtnacnccngayccnttyathttyaarcay
tayaaraayggngarttyaaymgngaytaygaymgnaarttyacngtnwsnwsnatggtnaayttyatgmgngayccnacnggngay
ytnccntgggargargaygcnwsngcnwsngayathgtncaygtnccngaygcngaracnyt
ngcnaarttyathmgncargarwsnmgnccnytnatggtnatgttytaygcnccntggtgyggnttytgyaaracnytnaarccngartay
gtngcngcngcnaargarytnaarggncaywsngtnytngcngcnathgaygtnaayaarccn
garaaygcngtnathmgnacnytntayaayathacnggnttyccnacnytnytntaytayaaraayggngcnatgaa
rttycartaygarggngayaayaarmgncargcnathgtnaayttyatgaaraayccnwsnaarccngtnaargtnaargarcargartgg
wsngargtngaywsngargtngtncayytnacnacnacnaayttygayccngtngtnaargarga
rgcnwsnytnytngtnatgttytaygcnccntggtgyggncaytgyaaraarathaarccngartaygaraargcngcngcnaarytnaar
wsngayggnathccnggnatgatggcngcngtngaygcnacnaargargtnwsnathgcngaymgnttywsngtnaarggntaycc
nacnatgaartayttyacntayggngarcayaarttygayathaayytnmgnga
rgcnacnaarathgtngarttyatgaaraayccnaargarccnccnccnccnccnccnccngaraarccntggwsngargargarwsnws
ngtngtncayytnaaygargaraayttyaarwsnttyytnaaraaraarmgncaygcnytngtnathttytaygcnccntggtgyggncay
tgyaaraargcnaarccngarttyacnaargcngcngarttyttyaargaygay
ccnaargtngarttygcngcngtngaytgyacnacntaycarggngtntgywsngcncaygargtnwsnggntaycnacnathaartayt
tywsntayytnaayaargtngtnaargcntayaaywsnggnmgnacngcngaygayttyathcnttyatgwsngayccngarggnaa
yggnwsnwsncaraaracnathgtnccncarytnacngaygcnaayttygargargarathwsnwsnaarwsngcngtnytngtnatg
ttytaygcnccntggtgyaarcartgyaargarathaarccngartaycaraargcnacnaaygarytnaarcargayggnttyathcarytn
gcnwsngtngaytgywsnwsnaayccngtngtnacngayaartaygayathggnacnttyccnacnttyaarytnttyytnaayggnaa
rttygcngcngayttyacnggnaarwsnacnaargaygayathaarwsnttygtngtngaygtnaaraaymgnaaraayaargarytn

* r: Purine (AG), y: Pyrimidine (CT), k: Keto (GT), m: Amino (AC), s: Strong (GC), w: Weak (AT), b: (CGT), d: (AGT), h: (ACT), v: (ACG), n: Any nucleotide (AGCT)

b. Nucleotide sequence

Plate 11. SEQUENCE OUTPUT DATA



a. Graphical output

Plate 12. BLAST Output

Distance tree of results NEW

Sequences producing significant alignments:	Score (Bits)	E Value	
ref|XP_001811788.1| PREDICTED: similar to Sodium/potassium-tr...	25.2	1.4	G
ref|XP_966705.2| PREDICTED: similar to organic cation transpo...	23.1	6.0	G
ref|XP_973659.1| PREDICTED: similar to organic cation transpo...	23.1	6.0	UG
ref|XP_973694.1| PREDICTED: similar to organic cation transpo...	23.1	6.0	UG
ref|XP_973296.1| PREDICTED: similar to AGAP008335-PA [Triboli...	22.7	8.0	UG
ref|XP_968978.2| PREDICTED: similar to Calcium-independent ph...	21.4	19	G
ref|XP_001812825.1| PREDICTED: similar to CG32226 CG32226-PA ...	21.4	19	UG
ref|XP_970769.1| PREDICTED: similar to mitochondrial NADH-ubi...	21.0	26	UG
ref|XP_969670.2| PREDICTED: similar to sulfatase-modifying fa...	20.6	35	UG
ref|XP_967202.2| PREDICTED: similar to conserved hypothetical...	20.6	35	UG
ref|XP_001807536.1| PREDICTED: similar to AGAP009050-PA [Trib...	20.6	35	UG
ref|XP_971250.1| PREDICTED: similar to Valyl-tRNA synthetase ...	20.6	35	UG
ref|XP_974566.2| PREDICTED: similar to receptor-type tyrosine...	19.7	63	UG
ref|XP_970977.2| PREDICTED: similar to protein-tyrosine phosp...	19.7	63	G
ref|XP_001811209.1| PREDICTED: similar to brain RPTPmam4 [Tri...	19.7	63	G
ref|XP_969548.2| PREDICTED: similar to AGAP009919-PA [Triboli...	19.7	63	UG
ref|XP_001813960.1| PREDICTED: hypothetical protein [Triboliu...	19.7	63	G
ref|XP_970084.1| PREDICTED: similar to dynein heavy chain [Tr...	19.7	63	UG
ref|XP_969069.1| PREDICTED: similar to UDP-n-acteylglucosamin...	19.7	63	UG
ref|XP_968689.1| PREDICTED: similar to AGAP004533-PA [Triboli...	19.7	63	G
ref|XP_973912.1| PREDICTED: similar to synaptotagmin-14 [Trib...	19.7	63	UG
ref|XP_970523.1| PREDICTED: similar to GA15837-PA [Tribolium ...	19.7	63	G
ref|XP_968061.1| PREDICTED: similar to transmembrane 9 superf...	19.7	63	UG

b. Text output

Plate 12. Cont…

Table 7. Amino acid composition of the predicted heat shock protein sequence ('Trib hsp')

Amino acid			Molar percent of amino acids (Mol %)
			hsp
Non polar		Gly	4.0
		Ala	7.7
		Val	8.2
		Leu	5.2
		Ile	4.8
		Met	2.6
		Pro	5.8
		Phe	6.8
		Trp	1.1
Polar	Uncharged	Ser	5.6
		Thr	5.6
		Cys	2.3
		Tyr	4.0
		Asn	4.8
		Gln	2.3
	Basic	Lys	12.1
		Arg	2.3
		His	1.8
	Acidic	Asp	5.8
		Glu	7.1

4.7.3.2 Secondary structure prediction

The per cent alpha helices (34.25) and the beta turns (5.82) of the 'Trib hsp' sequence constitute the protein secondary structure (Table 8). Further the per cent of the alpha helices and beta turns was confirmed by the graphical representation using the 'sopma' tool (Plate 13).

Secondary structure prediction (SOPMA)

Secondary structure prediction *(H = helix, E = strand, - = no prediction)*:

```
-----HHHHHHHHHHHEEE-------------HHHHHHHHHHH---EEEE------HHH
HHHHHHHHHHHHHH----EEEE-----HHHHHH---------EE---------------
EE--HEE------------H------EEE----HHHHHHHHH----HHEEE---------
----HHHHHHHHHH---HEEE-------HHHHHEEEE------EEEH-----HEE-----
--HHHE-H----------------H-HHEEEE--------HHHHHHHHHEEE-------
-----HHHHHHHH---------EHHHH--HHHEEHHHE--------EEE-H------HHH
HHHHHHHHH-------------------HH-HEE-H-HHHHHHHHHHH-HHEEEE-----
--------HHHHHHHH------H-EE----------E---------EEEEEHHHHHHHHH
--------HHHE-------------EEE--------HHHHH---HEEEEE----------
---HHHH--HHH----HEEEE----------------------EHEHH------H-----
-----EEEE-H--------
```

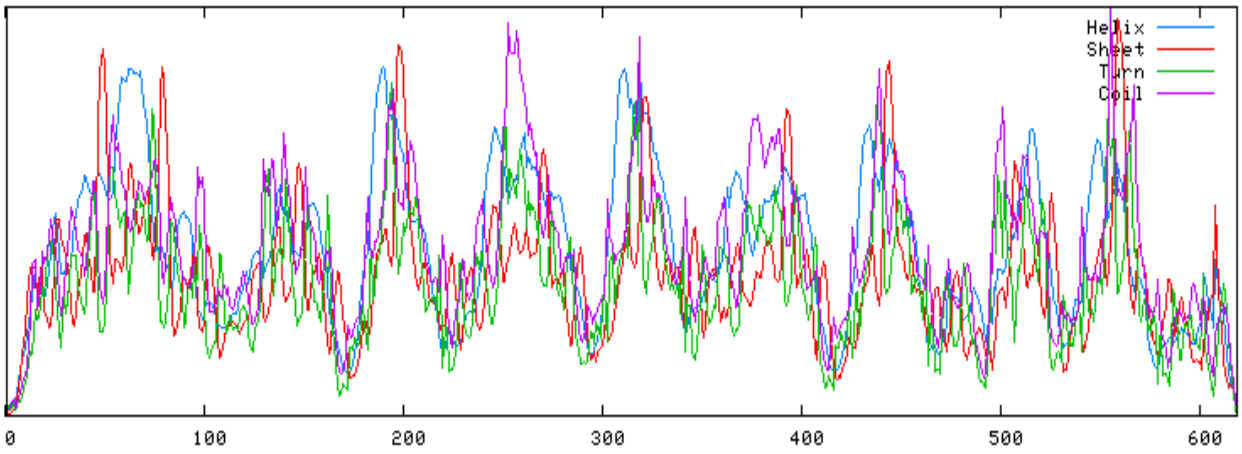


Plate 13. SOPMA result indicating the per cent alpha helices and beta turns

Table 8. Secondary structures present in the 'Trib hsp'

Secondary structures	Composition (%)
Alpha helix	34.25
3_{10} helix	0.00
Pi helix	0.00
Beta bridge	0.00
Extended strand	21.49
Beta turn	5.82
Bend region	0.00
Random coil	38.45
Ambiguous states	0.00
Other states	0.00

4.7.3.3 Tertiary structure prediction

The tertiary structure (Plate 14) of the protein was determined using the 'rasmol tool'. As per the tertiary structure prediction, the number of hydrogen bonds was found to be 90 followed by 5 helices, 5 strands and 8 turns respectively. The ramachandran plot was derived from the above structural data information. The ramachandran plot (Plate 15a and 15b) showed highest per cent of allowed (89.5) regions (Table 9) while none of the residues were found in the disallowed regions thereby justifying the reliability of the structure.

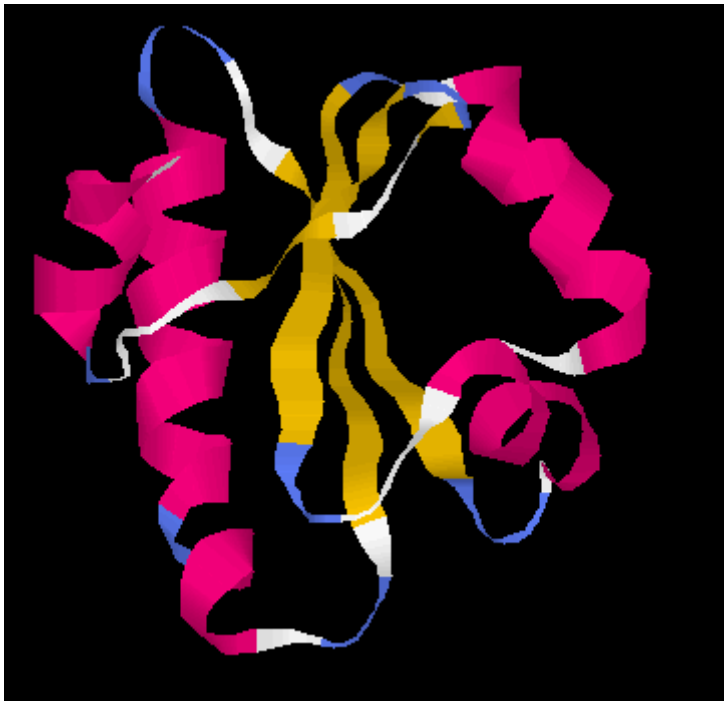


Plate 14. Tertiary structure prediction (Rasmol)

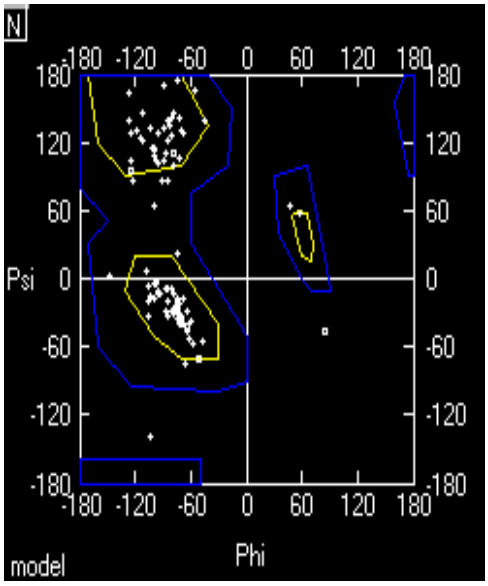


Plate 15a. Ramachandran plot

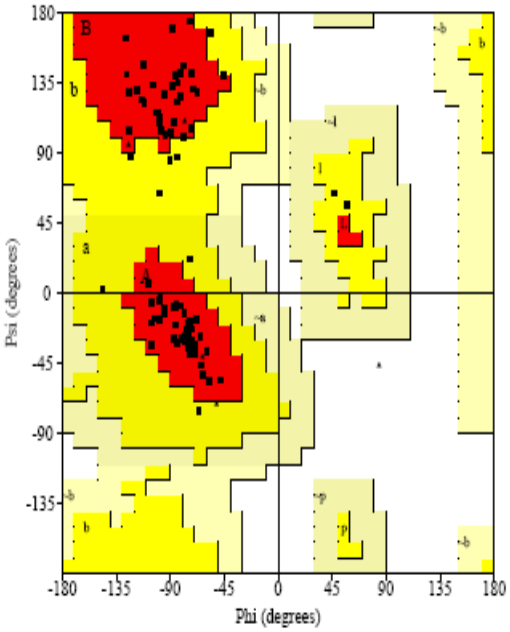


Plate 15b. Ramachandran plot model

Pfam-A	Description	Entry type	Sequence		HMM		Bits score	E-value	Alignment mode
			Start	End	From	To			
Thioredoxin	Thioredoxin	Domain	147	251	1	109	77.9	1.7e-21	fs
Thioredoxin	Thioredoxin	Domain	269	372	1	109	99.0	1.7e-27	fs
Thioredoxin	Thioredoxin	Domain	392	497	1	109	117.0	1.3e-32	fs
Thioredoxin	Thioredoxin	Domain	507	611	1	109	96.8	7.2e-27	fs

Pfam-B	Sequence Start	Sequence End	Score	E-value
Pfam-B_34895	22	134	357	7.6e-32
Pfam-B_101790	145	260	103	0.0013
Pfam-B_77902	148	234	99	0.00060
Pfam-B_9291	200	374	127	2.4e-06
Pfam-B_101790	278	355	89	0.16
Pfam-B_147832	287	349	67	0.80
Pfam-B_147832	412	471	122	2.0e-06
Pfam-B_9291	440	612	90	0.26
Pfam-B_101790	490	619	130	2.8e-07
Pfam-B_147832	527	586	67	0.80

Plate 16. Protein domains and functional description

Table 9. Allowed and disallowed regions in the tertiary structure of the 'Trib hsp'

Plot statistics	Composition (%)
Residues in most favored regions	89.5
Residues in additional allowed regions	10.5
Residues in generously allowed regions	0.0
Residues in disallowed regions	0.0

4.7.4 Functional identity of protein

The functional domains present in the 'Trib hsp' (Plate 16), defines the function of the protein and proves the nature of the protein as a heat shock protein. It was found out that four domains were present in the 'Trib hsp' and the domain with the minimum E-value (1.4) had the sequence length of 104 bp. This thioredoxin like protein to which hsp protein sequence showed 80 per cent similarity was found to possess chaperone properties, promoting protein folding and forming complexes with unfolded proteins, thereby playing the role of heat shock protein.

4.8 ESTIMATION OF THE TREHALOSE CONTENT IN INSECTS SUBJECTED TO STRESS AND AFTER STRESS

The trehalose content in insects was determined by the Anthrone method for trehalose determination using ice cold sulfuric acid. When the insects were subjected to heat stress at 35°C, the trehalose content in the neonate sample was (14.97 mg/g), V instar grub (16.49 mg/g), pupa (17.64 mg/g) and the adult (18.48 mg/g) (Table 10a). A similar increase in trend was also observed in the trehalose content of the insect sample exposed to still higher temperature. The trehalose content increased significantly from 35°C to 60°C (Table 10b) for all the stages of growth.

Gradually, when the insects were allowed a recovery period equal to the stress period, the trehalose content was reduced (Table 11a and 11b). When the

Table 10a. Effect of exposure time and temperature on the trehalose content (mg/g) in *Tribolium castaneum*

Exposure time (H)	Total trehalose content (mg/g)			
	Neonate	**V instar grub**	**Pupa**	**Adult**
1	17.46	18.90	20.31	22.08
2	18.33	20.04	21.84	23.15
4	19.79	21.22	22.76	23.99
CD (0.05)	0.12	0.16	0.14	0.19
Temperature (°C)				
Control (Unexposed insects at room temperature)	5.85	6.83	8.32	10.27
35	14.97	16.50	17.64	18.48
40	17.98	20.05	22.14	23.68
45	20.57	22.47	23.51	25.60
50	21.42	23.03	24.72	26.25
55	23.77	24.91	27.14	28.35
60	25.12	26.57	27.96	28.89
CD (0.05)	0.18	0.25	0.22	0.29

Table 10b. Effects of exposure time and temperature on the trehalose content (mg/g) of different stages of *Tribolium castaneum*

Insect stage	Exposure time (H)	Temperature (°C)						
		Control (Unexposed insects at room temperature)	**35**	**40**	**45**	**50**	**55**	**60**
Neonate	1	5.85	13.66	17.20	19.05	20.43	22.28	23.77
	2	5.85	14.83	17.25	20.59	21.15	23.61	25.05
	4	5.85	16.43	19.51	22.07	22.69	25.41	26.54
	CD (0.05)	0.30						
V instar grub	1	6.83	14.99	18.38	20.84	22.13	23.56	25.56
	2	6.83	16.58	20.33	22.28	22.79	25.10	26.38
	4	6.83	17.91	21.46	24.28	24.18	26.08	27.77
	CD (0.05)	0.44						
Pupa	1	8.32	15.71	20.43	21.82	23.36	25.46	27.05
	2	8.32	17.97	22.02	23.82	24.79	27.67	28.29
	4	8.32	19.25	23.97	24.90	26.03	28.28	28.54
	CD (0.05)	0.38						
Adult	1	10.27	16.48	22.69	23.87	24.95	27.67	28.64
	2	10.27	18.63	23.20	25.77	26.64	28.59	28.95
	4	10.27	20.33	25.15	27.15	27.15	28.80	29.07
	CD (0.05)	0.50						

Table 11a. Effect of exposure time and temperature on the trehalose content (mg/g) in *Tribolium castaneum* after stress recovery

Exposure time/Recovery period (H)	Total trehalose content (mg/g)			
	Neonate	**V instar grub**	**Pupa**	**Adult**
1	13.19	14.91	17.52	18.03
2	14.35	15.91	17.73	18.71
4	15.37	16.81	18.21	19.68
CD (0.05)	0.38	0.41	2.61	0.29
Temperature (°C)				
Control (Unexposed insects at room temperature)	5.85	6.83	8.32	10.27
35	10.25	11.69	12.44	13.38
40	13.02	15.18	17.03	18.60
45	15.96	18.16	22.34	20.84
50	16.27	17.97	19.64	21.60
55	18.74	19.83	22.06	23.24
60	20.04	21.49	22.89	23.72
CD (0.05)	0.58	0.63	3.98	0.45

Table 11b. Effect of exposure time and temperature on the trehalose content (mg/g) of different stages of *Tribolium castaneum* after stress recovery

Insect stage	Exposure time/Recovery period (H)	Temperature (°C)						
		Control (Unexposed insects at room temperature)	35	40	45	50	55	60
Neonate	1	5.85	8.21	12.17	14.73	15.35	17.35	18.68
	2	5.85	11.04	12.42	16.07	16.58	18.53	19.97
	4	5.85	11.50	14.48	17.09	16.89	20.33	21.46
	CD (0.05)	1.00						
V instar grub	1	6.83	10.47	13.71	17.25	17.15	18.48	20.48
	2	6.83	11.29	15.40	18.12	18.43	20.02	21.30
	4	6.83	13.29	16.43	19.10	18.33	20.99	22.69
	CD (0.05)	1.10						
Pupa	1	8.32	9.65	15.55	27.97	18.74	20.38	22.01
	2	8.32	13.40	16.94	19.46	20.17	22.59	23.20
	4	8.32	14.27	18.59	19.61	20.02	23.20	23.46
	CD (0.05)	6.90						
Adult	1	10.27	11.45	17.71	19.97	20.64	22.59	23.56
	2	10.27	13.29	18.12	20.58	21.56	23.46	23.72
	4	10.27	15.40	19.97	21.97	22.59	23.67	23.87
	CD (0.05)	0.77						

insects were recovered after being subjected to a heat stress of 35°C, the trehalose content was found to be reduced in the neonate (10.25 mg/g), V instar grub (11.69 mg/g), pupa (12.44 mg/g) and the adult (13.38 mg/g). Again a similar trend was seen at higher temperature too. The trehalose content when tested after stress recovery was observed to decrease significantly from 35°C to 60°C for neonate, V instar grub and pupa and adult. But in case of pupa, the trehalose content was seen to decrease non significantly at 1 h (17.52 mg/g) and 2 h (17.73 mg/g) respectively. A non significant decrease was also observed in the trehalose content of the insects at 45°C (22.34 mg/g), 50°C (19.64 mg/g), 55°C (22.06 mg/g) and 60°C (22.89 mg/g) respectively. But the decrease was seen to be significant at 35°C (12.44 mg/g) and 40°C (17.03 mg/g) respectively (Table 11a).

Discussion

5. DISCUSSION

Results obtained in the laboratory study on "Characterization of proteins and trehalose as heat shock regulators in insect system" with the red flour beetle, *Tribolium castaneum*, Herbst. (Tenebrioniidae: Coleoptera) are discussed under this chapter.

5.1 MASS CULTURING OF TEST INSECTS

The test insect was mass cultured from a nucleus culture using different insect feeds *viz*., wheat flour and semolina alone and in combination. The maximum population growth was seen in March 2008 (Table 1) in all the three feeds compared to the population build up in the other months of the year. The multiplication was found to be faster (Fig. 3) in the wheat flour (30.97±0.20) while it was less in semolina alone (30.29±0.13). The population build up was observed to be positively correlated with the temperature (28.3°C) and relative humidity (64%). At the same time, the per cent contamination was more in July 2007 (41.3±3.53) and August 2007 (34.7±1.33) due to the high relative humidity of 88 per cent and 84 per cent respectively and with comparatively lesser fungal contamination (Fig. 4) as was observed during January 2008 (22.7±2.67) and February 2008 (20±2.31) when the relative humidity was 61 per cent and 64 per cent (Table 2) respectively. The per cent fungal contamination (Fig. 4) was also positively correlated with the relative humidity. Similar studies were made by Yasmin (2001) on population fluctuation and mode of damage of red flour beetle in which the greater population build up of the insect was seen during the hot and humid season. But at the same time a high relative humidity of above 70 per cent was found responsible for the fungal infection and consequent mortality of the insects. However, in the present study, it was observed that the combination of both wheat flour and semolina was a better option for rearing and multiplication of the red flour beetle without any fungal contamination. According to Yasmin, the wheat flour was the most preferred food for the red flour

beetle (272.26 beetles\week) followed by semolina (65.08 beetles\week) and this data was obtained with an initial nucleus culture of 90 beetles. The insect population was well favored at a temperature range of 19-34°C under a relative humidity range of 49-68 per cent. Rustamani *et al*. (2001) also reported that the wheat flour was the best medium for the red flour beetle multiplication.

Higher per cent mortality of the insect was also observed during the months of July and August due to high relative humidity. The mortality per cent (Table 1) was positively correlated with relative humidity (Fig. 6). The mortality rate was high because of the severe green mold growth (*Aspergillus* spp) on the feed with the emission of a bad odour from the infected cultures during the rainy season coupled with high relative humidity. Studies by Buss and Fasulo (2006) showed that the high moisture content encouraged the growth of green mould (*Aspergillus flavus*) in flour cultures; a displeasing odour was also emanating and it was due to the secretion of a chemical, p-benzoquinone by the beetles. Both the factors were correlated with the mortality of the beetles, which, supports the present study.

5.2 BIOLOGY OF THE TEST INSECT

The biology of red flour beetle was studied under laboratory conditions over a period of thirteen months *i.e.*, during March 2007 to March 2008. Significant differences were observed in the biological parameters *viz*., total life cycle of the insect, duration of different stages, per cent hatchability, mortality and survival of the test insect.

5.2.1 Description of bio stages

The duration of the life stages was observed from March 2007 to March 2008. The mean number of days taken to complete the life cycle was 34±0.58 (Table 4) whereas Bousquet (1990) reported that it had taken about 36 to 40 days for the red flour beetle to complete one life cycle. The rate of multiplication of the insects was

faster in March 2008 with optimum temperature (28.3ºC) and relative humidity (64%). The highest population increase was observed in the month of February 2008 and March 2008 when the temperature was 28.3°C with a relative humidity of 61 and 64 per cent respectively (Table 3). Mewis and Ulrichs (2001) also reported that multiplication of red flour beetle was found to be highest at a mean temperature of 29 ± 2 °C and relative humidity of 70 ± 3 %.

5.3 SURVIVAL OF INSECTS UNDER DIFFERENT TEMPERATURE REGIMES AT DIFFERENT EXPOSURE PERIOD

The present laboratory study was designed to determine time mortality relationships for various life stages of *T. castaneum* at six different temperatures between 35 and 60°C. No mortality of the insects was observed within 4h but beyond that exposure time, the insects were found to be killed at 60ºC. The mortality per cent of the insect increased with increase in temperature and time (Table 5). There was a rise in the mortality per cent when the insects were exposed from 35 to 60°C. Moreover, a high mortality per cent was observed at 50°C for 8h (98.7±0.33) and 55°C for 6h (90.7±1.20). Cent per cent mortality was realized at 60°C when the insects were exposed for all the time interval of over 5h. Similar study was also done by Mahroof *et al*. (2003) and Arthur (2006) where a sharp increase in mortality of *Tribolium castaneum* was observed as the exposure temperature and time factor were increased. They also reported that latent mortality in *T. castaneum* (90.0±5.7) when exposed to 50 °C for a period of 5h.

5.4 PROTEIN CONTENT IN *Tribolium castaneum* HOMOGENATE

The total protein content was estimated in the insects by the Lowry's method of protein assay and further confirmed by the Nanodrop spectrophotometer for reproducible results. The total protein content in insects was found to decrease when they were subjected to a stress at different temperatures and different time intervals. It showed a significant decrease in its level when exposed at 35°C to 60°C for different

stages of insect (Table 6a). It was also observed that the protein content from 35ºC to 45°C was more for the neonate as compared to the other stages. Similar study was done by Gulen and Eris (2004) and they reported that in the plants, the total protein content decreased in the leaves by the heat stress when the strawberry seedlings were subjected to temperatures ranging from 30-45°C for 48 h. It was attributed to the synthesis of heat shock proteins when the other proteins got inhibited. When the insects were exposed to 50°C, the corresponding protein content was in the order of neonate (36.81 mg/g) < pupa (38.16 mg/g) < adult (39.23 mg/g) < V instar grub (40.81 mg/g). Thus, the decrease in the total protein content was observed to be more for neonate at 50°C when compared to the other stages. The sharp decrease in the total protein content in the neonate showed that it was the most heat tolerant stage at 50°C. This study was in accordance with Mahroof *et al*. (2003), observed 99 per cent mortality of all stages of insect when they were exposed at 55ºC for a period of over 6h.

The decrease in the total protein content was found to be significant among the different developmental stages and this decrease was observed with an increase in temperature and time period (Table 6b). The decrease in the protein content was as a result of replacement of already degraded proteins from the insect cell. Naito *et al*. (2000) reported that there was a decrease in the total muscle protein content of mice from (232.2±5.3) to (229.6±5.3) when subjected to a heat stress for 24 h at high temperature of 40°C; and the probable reason for the decrease in the protein content might be due to the increased synthesis of heat shock proteins in mice under stress.

5.5 DETERMINATION OF MOLECULAR WEIGHT OF THE HEAT SHOCK PROTEIN BY SDS-PAGE

The molecular weight of the heat shock protein in red flour beetle was determined by using a SDS-PAGE gel (10%). A very clear band was obtained in the insect samples (when tested at different temperature ranges at different time intervals) as compared to the control. The molecular weight of this band was 70 KDa with Rf

value of 0.362 by point to point fit (Plate 5, 6, 7 and 8). Tissieres *et al*. (1974) discovered that the members of the 70 KDa families of stress proteins were produced through the rapid induction during exposure of cells to heat while working in *Drosophila*. The SDS-PAGE also confirmed 95 per cent purification of the hsp70 due to the fine resolution of the bands on the gel and it was in conformation with the study done by Parsell and Lindquist (1994) in *Drosophila*.

5.6 N-TERMINAL SEQUENCING

The 'Trib hsp' was confirmed as the hsp70 produced in the red flour beetle under heat stress as per the sequencing data generated from the N-terminal sequencing for proteins followed by the bioinformatics analysis. Sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis, combined with electro-blotting and automated Edman degradation, was routinely used for protein purification and N-terminal amino acid sequence analysis. It was in accordance with Vandekerchove *et al*. (1985) and Kumar *et al.* (2001), a study done in egg avidin protein of chicken. Similarly, Mahroof *et al.* (2003) reported the presence of hsp70 through N-terminal sequencing in red flour beetle under conditions of heat stress.

5.7 THEORETICAL ANALYSIS OF SEQUENCE DATA

5.7.1 Homology search

The BLAST analysis determined the sequence and homology to predict the identity and function of the query sequence ('Trib hsp'). It was interesting to note that the sequence ('Trib hsp') obtained from the heat stressed red flour beetle displayed high homology about 80 per cent to the protein sequence of red flour beetle, *Tribolium castaneum* (Plate 12). The same homology per cent was found in accordance with the study of Mahroof *et al.* (2003) where the researchers deduced the sequence of hsp70 in red flour beetle.

5.7.2 Reverse translation

The ‘reverse translate’ tool accepted a protein sequence as input and used a codon usage table to generate a DNA sequence representing the most likely non-degenerate coding sequence. The nucleotide sequence determined from the protein sequence by the reverse translation tool showed that the gene corresponds to the thioredoxin based heat shock gene. The nucleotide sequence was found to be 1857 base pair long (Plate 11). This result was in confirmation with the study made by Thiesen and Bach (1990) and they found out that hsp70 gets expressed in the in *Drosophila* cells under heat stress.

5.7.3 Structural identity of protein

5.7.3.1 Primary structure prediction

The primary structure of the protein is its amino acid sequence which is predicted from the N-terminal generated sequence by using the ‘blast p’ tool. The primary structure of the given ‘hsp sequence’ showed that the protein was rich in amino acid lysine followed by alanine and glutamine (Table 7). The total number of negatively charged residues was made up of asparagine and glutamine while the total number of positively charged residues was made up of arginine and lysine. The instability index of the protein suggested that it was a stable protein and hydrophilic in nature. The primary structure of hsp70 was in confirmation with the study done by Horton and Nakai (1997) in *Drosophila* with respect to the stability of the protein. Similar studies were done by Heschl and Baillie (1990). They identified and characterized hsp70 multigene family in *Caenorhabditis elegans* which served as the basis for genetic characterization of a multicellular eukaryote.

5.7.3.2 Secondary structure prediction

The number of alpha helices and beta strands in the secondary structure of the protein was determined using the 'NN Predict' tool (Table 8). The secondary structure prediction as per the 'sopma' results (Plate 13) showed the presence of the alpha helices (34.25%) and beta turns (5.82%) in the heat shock protein. Horton and Nakai (1997) made a similar observation with respect to the secondary structure of the hsp70 in *Drosophila.*

5.7.3.3 Tertiary structure prediction

The tertiary structure was predicted by using the 'rasmol' tool and the ramachandran plot was drawn from the tertiary structure data (Ramachandran, 1968). The tertiary structure of the protein showed the coiling of the alpha helices and beta strands (Plate 14). The ramachandran plot (Plate 15a and 15b) indicated that only two amino acids lie in the disallowed region while rest of the amino acids lie in the allowed region. From the 'savs' result (ramachandran plot), it was further concluded that 89.5 per cent of the amino acid residues lies in the core region, 10.5 per cent in the allowed region and none in the disallowed region thus indicating the realibilty of the structure (Table 9). Similar results were obtained by Rassow *et al*. (1995) by identifying the sequence similarity between different types of heat shock proteins.

5.7.4 Functional identity of protein

The function of the protein as heat shock protein was identified from the functional domains using the 'pfam' tool. The functional domains present in the 'hsp protein sequence' (Plate 16), defined the function of the protein and proved the nature of the protein as a heat shock protein. It was found out that four domains were present in the 'hsp seq' and the domain with the minimum E-value (1.7e-21) had got a sequence length of 147 to 251 bp. This thioredoxin like protein to which hsp protein sequence showed 80 per cent similarity was found to possess chaperone properties. This data was also

in confirmation with Finn *et al*. (2006) who also reported the presence of four domains in *Drosophila*. Here the function of the 'hsp protein sequence' was similar to the role of heat shock protein (also functions as thioredoxin reductase) and played a role of molecular chaperone in promoting the protein folding during heat stress. The functional aspect of the hsp70 with respect to its role as thioredoxin reductase was also studied by Krebs and Holbrook (2001) in *Drosophila*.

5.8 TREHALOSE CONTENT IN *Tribolium castaneum* HOMOGENATE SUBJECTED TO STRESS AND AFTER STRESS

The trehalose content in insects was determined by the Anthrone method. When the insects were subjected to heat stress at 35°C, the trehalose content in the neonate sample was (14.97 mg/g) and it was significantly higher in V instar grub(16.49 mg/g), pupa (17.64 mg/g) and the adult (18.48 mg/g). Similarly, it was observed that the trehalose content at 40°C for the neonate, V instar grub, pupa and adult was relatively less compared to the trehalose content at 60°C in the respective stages. The trehalose content significantly increased from 35°C to 60°C (Table 8) irrespective of the development stages. Tunacliffe *et al.* (2005) also reported the accumulation of the non-reducing disaccharides *viz.,* trehalose occured in large quantities in insects undergoing anhydrobiosis.

So, the trehalose content showed a significant increase in its level from 35°C to 60°C (Table 10a and 10b). In the present study, the trehalose content was found to increase proportionately with increase in temperature and exposure time in insects subjected to heat stress. Trehalose was produced during stress in order to increase the activity of the heat shock proteins. Aurora *et al*. (2007) also reported the increase in trehalose content in cells of fungi when subjected to prolonged stress.

Gradually, when the insects were allowed a recovery period equal to the stress period, the trehalose content was reduced (Table 11a and 11b). When the insects were recovered after being subjected to a heat stress of 35°C, the trehalose content was found to be reduced in the neonate (10.25 mg/g), V instar grub (11.69 mg/g), pupa

(12.44 mg/g) and the adult (13.38 mg/g). Thus, the trehalose content was found to be more immediately after the stress imposition as compared to the same when estimated after a brief recovery period. These results are in confirmation with the reports of Berovic *et al*. (2007) in which trehalose content increased during heat shock at 37°C or 40°C and showed a declining trend upon temperature decrease from 40°C to 27°C.

There was also a significant reduction in the trehalose content after the recovery of the stress period (Table 11). Similar study was also done by Aurora *et al*. (2007) in yeast subjected to prolonged stress where it was reported that after the stress had ceased, trehalose returned to its basal concentrations, pointing to a role of neutral trehalase activity in heat shock recovery.

The data generated in the study gives an in depth knowledge on the biology of the red flour beetle, *T. castaneum*, the life stages which resist the heat stress and the factors responsible for the anhydrobiosis. Heat shock protein and the trehalose impart anhydrbiotic characters in the red flour beetle to resist the heat treatment. The characterization of such heat shock regulators may be utilized in the future for designing an effective anti heat shock regulator to manage the pest without affecting the quality of the food materials.

Summary

6. SUMMARY

The present study on "Characterization of proteins and trehalose as heat shock regulators in insect system" was carried out in the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 2007-2008.

The programme envisaged included identification of the life stages with anhydrobiosis characters in *Tribolium castaneum* Herbst. (Tenebrioniidae: Coleoptera), testing the survival of the test insects under different temperature regimes, identifying the heat shock regulators involved and characterizing the heat shock regulators like proteins and carbohydrates especially trehalose, the major insect sugar.

Different types of bioassays were carried out to test for the revival of the test insects under different temperature regimes at different incubation levels. Quantitative assays were carried out to determine the total protein and trehalose content in the insect under stressed condition.

Protein content of different stages of insect *viz*., neonate, V instar grub, pupa and adult at different levels of stress condition was analysed through SDS-PAGE assay. The N-terminal sequencing of the heat shock protein identified was done to characterize the protein. Bioinformatics tools were applied for the sequence analysis and 80 per cent homology of the 'Trib hsp' with the heat shock protein was obtained. The salient findings of the study are summarized below.

1. Mass culturing of insects in wheat flour showed high multiplication but also favoured more fungal contamination. Insect's multiplication in semolina medium was comparatively lesser with relatively less contamination

2. The combination of both wheat flour and semolina was found to be ideal for insect multiplication

3. Eggs were small, whitish with food particles adhering to the sticky surface. They hatch within 3-5 days

4. Brown-headed larvae were cream colored when young and turned yellow when a week old, slender and wiry, reaching a length of 1/4 inch. Larvae had six legs and two-pointed or forked projections at the last rear body segment. The total larval duration varied from 16 to 18 days

5. Fully grown larvae transformed to white colored naked pupa

6. The dark brown colored adult beetles emerged within a week

7. The life cycle required 5 to 6 weeks only

8. Maximum mortality of these insects was observed at a temperature of above 50°C when exposed for a time period of more than 6 hours

9. The mortality of the insects increased with increase in temperature and exposure time

10. Not even a single insect was able to revive after being exposed to a temperature of 60°C for a period of 5 h

11. The total protein content was analyzed by Lowry's method and it was observed that the total protein was reduced due to stress because of degradation of the native proteins

12. It was observed that the V instar grub showed a decrease in the total protein content when the insects were exposed to stress up to 45°C

13. At and beyond 50°C, it was the neonate that showed a decrease in the total protein content

14. The trehalose content obtained by Anthrone method was observed to increase positively with stress

15. When the insects were allowed a recovery period equal to the stress period; the trehalose content was found to decrease slowly

16. The molecular weight of the protein was found out to be 70 KDa on a 10% SDS-PAGE gel

17. The nature and function of the heat shock protein observed in the beetle during heat stress was confirmed to be hsp70 through blotting and N-terminal sequencing followed by the bioinformatics analysis

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Appendices

Appendix I
Meteorological parameters

Period	Temperature (°C)	Relative humidity (%)
Mar	30.2	63
Apr	30.1	69
May	28.7	76
Jun	26.8	84
Jul	25.7	88
Aug	28.9	84
Sep	26.2	86
Oct	31.7	79
Nov	26.7	67
Dec	27.2	56
Jan	27	59
Feb	28.3	61
Mar	28.3	64

Appendix II

Composition of buffer and reagent used for protein extraction

Phosphate buffer

A. 0.2M solution of monobasic sodium phosphate - 2.78g NaH_2PO_4/100ml

B. 0.2M solution of dibasic sodium phosphate

$Na_2HPO_4.7H_2O$ -5.365g/100ml or $Na_2HPO_4.12H_2O$- 7.17g/100ml

39ml of 'A' and 61ml of 'B' were mixed, pH was adjusted to 7.0 and volume was made up to a total of 200ml.

Alkaline Copper solution

A. 2% Na_2CO_3 in 0.1N NaOH

B. 0.5% $Cu_2SO_4.5H_2O$ in 1% Potassium sodium tartarate

C. Alkaline Copper solution: 50ml of 'A' and 1ml of 'B' were mixed and the solution had to be prepared afresh.

Appendix III

Composition of reagents used for SDS PAGE

A. Monomer solution

Acrylamide : 30g

Bis acrylamide : 0.8g

Distilled water to: 100ml

Stored at 4^{o}C in amber coloured bottle.

B. 4X resolving gel buffer (1.5MTris-Cl, pH 8.8)

Tris base : 18.5g

Distilled water to: 100ml

Tris base was dissolved in about 50 to 60 ml distilled water and pH was adjusted to 8.8 with concentrated HCl and stored at 4^{o}C.

C. 4X stacking gel buffer (0.5M Tris-Cl, pH 6.8)

Tris base : 6g

Distilled water to: 100ml

Dissolved the Tris base in about 50 to 60ml distilled water, adjusted the pH to 6.8 with concentrated HCl and stored at 4^{o}C.

D. Electrode buffer (0.025M Tris, pH 8.3, 0.192M Glycine)

Tris base : 1.525g

Glycine : 7.2g

SDS : 0.5g

Distilled water to : 500ml

E. 2X Treatment buffer (0.125M Tris Cl)

4X Tris Cl, pH 6.8 : 2.5ml

Glycerol : 2ml

2-Mercaptoethanol : 0.2ml

Bromophenol blue : 0.2g

SDS (10per cent) : 4ml

Distilled water : 10ml

F. Initiator (10% APS)

Ammonium persulphate: 0.1g

Distilled water : 1ml

This solution is prepared fresh, immediately before use.

G. SDS (10%)

Sodium dodecyl sulphate : 10g

Distilled water to : 100ml

Appendix IV

Composition of protein staining solution and destainer

1. Protein staining solution

Coomassive brilliant blue R 250	: 0.1g
Methanol	: 40ml
Acetic acid	: 10ml
Water	: 50ml

First dissolved the dye in methanol and then mixed acetic acid and water.

2. Destainer

Methanol	: 40ml
Acetic acid	: 10ml
Water	: 50ml

CHARACTERIZATION OF PROTEINS TREHALOSE AS HEAT SHOCK REGULATORS IN INSECT SYSTEM

By

SWETALEENA TRIPATHY
(2006-11-107)

ABSTRACT OF THESIS

Submitted in partial fulfillment of the

requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

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2008

ABSTRACT

The study entitled "Characterization of proteins and trehalose as heat shock regulators in insect system" was undertaken in the laboratory of Centre for Plant Biotechnology and Molecular Biology and Department of Entomology, College of Horticulture, Kerala Agricultural University in Vellanikkara to study the underlying mechanism of the red flour beetle, *Tribolium castaneum* Herbst. (Tenebrioniidae: Coleoptera) to resist stress. *Tribolium castaneum* had been seen as a serious pest of flour mills and food processing industries causing huge loss every year. The use of elevated temperatures had long been recognized as an effective strategy for managing the dreaded stored product insect.

The major objectives of the study were to find out the thermal death stage of the red flour beetle when exposed to a particular temperature and time period at which the insect was unable to combat stress further and to characterize the metabolites responsible for the survival of this insect during anhydrobiosis. The mass culturing of the insect was done at a temperature of 28°C with 70 per cent relative humidity. The population built up was faster on the wheat flour (30.97±0.20) followed by the combination of both wheat flour and semolina (30.91±0.15) while it was less in semolina alone (30.29±0.13). At the same time, the per cent contamination and per cent mortality were significantly more on wheat flour (41.3±3.53 and 19.58±0.93) when the relative humidity was higher (88%). The per cent contamination and per cent mortality were positively correlated with the temperature and relative humidity. The insect completed its life cycle within 34-38 days in the present study.

Different stages of insects (neonates, V instar grub, pupae and the adult beetles) were subjected to temperatures ranging from 35°C to 60°C at an incremental increase of 5°C for a period of 1 h, 2 h and 4 h respectively. The insects were not killed even at 4h exposure to the above temperatures. However, the mortality of each stage of the insects was increased with the exposure time and the incremental

temperature increase. A higher mortality per cent was observed at 50°C for 8 h (98.7±0.33) and 55°C for 6 h (90.7±0.67) respectively. Complete mortality of the insect was observed at 55°C and 60°C when the insect stages were exposed to the temperature for 7 h and 5 h respectively.

Subjecting the insects to stress showed accumulation of a heat shock protein (hsp) along with an insect sugar called trehalose. It was observed that the heat shock protein and trehalose content in insects increased significantly with the stress. When the insects were exposed to 50°C, the corresponding protein content was in the order of neonate (36.81 mg/g) < pupa (38.16 mg/g) < adult (39.23 mg/g) < V instar grub (40.81 mg/g). Thus, the decrease in the total protein content was observed to be more for the neonate at 50°C when compared to the other stages. Similarly, when the insects were subjected to heat stress at 35°C, the trehalose content in the neonate sample was (14.97 mg/g) and it was significantly higher in V instar grub (16.49 mg/g), pupa (17.64 mg/g) and the adult (18.48 mg/g). But there was significant decrease in the trehalose content of the insect after the recovery of the stress period.

The molecular weight (70KDa) of the 'Trib hsp' was determined from the SDS-PAGE analysis and its identity was further confirmed by the N-terminal sequencing. It showed 80 per cent homology with the heat shock protein. Further theoretical analysis of the protein sequence showed that the protein was stable and it was composed of four conserved domains.

The data generated so far could be useful in developing a heat control strategy to manage this insect. Further studies with respect to characterization of anti heat shock protein could also be carried out in order to see its effect on a heat shock cognitive. The characterization of the stress regulators would help to manage the public health pest's *viz*., mosquito. The genes coding for stress regulatory proteins could also be isolated, cloned and used for development of genetically modified insects.