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Characterization of heat shock protein in red flour beetle *Tribolium castaneum* Herbst. (Coleoptera: Tenebrionidae)

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ABSTRACT: Red flour beetle, *Tribolium castaneum* Herbst. (Coleoptera: Tenebrionidae) tolerate the heat treatment in the food processing facilities and storage godowns. The anhydrobiotic character to tolerate the heat treatment of the insect is due to some special metabolites. Characterization of the insect homogenate showed that protein was one of the major constituent imparting the heat tolerance. Using SDS-PAGE (10%) analysis we could decipher the protein with molecular weight of the 70KDa (heat shock protein) act as molecular chaperone in protecting the normal protein in cells of the heat tolerant stages of the beetle. The identity of the heat shock protein (HSP) has been confirmed by the N-terminal sequencing. Further theoretical analysis of the protein sequence shows that the protein is stable and composed of four conserved domains.

KEYWORDS: Red flour beetle, *Tribolium castaneum*, heat shock protein (HSP), molecular chaperone, N-terminal sequencing.

INTRODUCTION

Red flour beetle (RFB), *Tribolium castaneum* Herbst. (Tenebrionidae:Coleoptera) also known as "bran bug" is one of the serious storage insect pests that originates from infested grains,

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or from dry stored food products, particularly, cereal products such as flours, cake mix, cornmeal's, crackers, dry pet foods and so forth. Other frequently infested items are chocolates, nuts and seeds. Both adult beetles and small white grubs are found in the infested food items. The adult beetles often wander away from the infested materials and are found inside cupboards, or anywhere in the house.

Insecticide sprays are not recommended for managing the RFB in stored food cup boards. 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 potential methyl bromide alternatives (Makhijani and Gurney, 1995). But resistance to killing by extreme heat increases dramatically when the whole insect or cultured cells are pre treated. This increased resistance also known as induced thermo tolerance, which induces synthesis of a set of proteins called heat shock proteins (HSP) (Sanchez and Lindquist, 1990; Nover, 1991; Sanchez *et al*.,1999). The ability of RFB to tolerate the temperature treatments prompted us to characterize the heat shock protein of their system.

Heat shock proteins commonly confer thermal tolerance in all living organisms and can indicate the level of tolerance to heat treatments (Feder and Hoffman,1999). The 70KDa classes of heat shock proteins, which are true heat shock proteins, protect individuals from thermal stress (Pelham, 1986; Lindquist, 1986). The expression of hsp70 gene of *T. castaneum* was heat inducible at various developmental stages (Mahroof *et al*., 2005a). HSP83 protein was involved in protection against thermal stress in newly hatched and matured beetles but in the ovary, HSP83 was only expressed in the follicle cells of mature beetles and not in newly hatched beetles, regardless if the beetles were subjected to heat shock or not (Xu *et al*., 2010). Another class of heat shock protein, HSP90 was expressed in all developmental stages of *T. castaneum* and highly expressed in early pupal and late adult stages (Zhang *et al*., 2013).

MATERIALS AND METHOD

Rearing of the test insect

Red flour beetles were reared on semolina. Food grade plastic insect culture jars sterilized with formalin (2%) were used for rearing the insect. The insect feed (semolina) was partially sterilized in hot air oven (at 70°C for 15 minutes). The culture jars were filled up with semolina (25 g) and five pairs of adult insects were released into each of the containers. Sub-culturing was done at regular intervals.

Level of heat tolerance in different stages of the test insect

The level of heat tolerance of the insect was studied by exposing the different stages of the insect *viz*., neonates, grub, pupa and the adult beetles of *T. castaneum* to different temperature regimes varying from 35°C to 60°C at an incremental increase of 5°C for 4 h. The temperature

ranges were selected on account of the prevailing temperature conditions of storage facilities in India. The time was selected to study the complete mortality of the insect exposed to a particular temperature.

Concentration of samples by Millipore centricon

Various growth stages *viz*., neonates (1 day old), fifth instar grub (19 days old), pupa (3 days old) and adult beetles (3 days old) of *Tribolium castaneum* were exposed to different temperature regimes varying from 35°C to 60°C at an incremental increase of 5°C for 4 h. A control treatment was maintained where the insects were maintained at ambience. From each treatment 1 g of RFB was sampled out and ground with 1 ml of 0.2 M phosphate buffer (pH 7.0). The extract was then concentrated to 100 µl using 0.22 µm millipore centricon (Kumar *et al*., 2001). The extract required to be concentrated was added to sample reservoir (1 ml) and then it was inserted into the filtrate vial. Spinning was done at the rate of 4000 rpm at 4°C until desired concentration was achieved. 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.

Blotting

Blotting was performed to excise the more concentrated and purified bands from the blot membrane for sequencing purpose. The protein samples were run on SDS-PAGE (10%) gel along with broad range molecular weight marker. 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 poly vinyl difluoride (PVDF) membrane in which the separated proteins were electroblotted onto PVDF 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 1h using Mini Trans Blot cell (Bio Rad, Hercules,CA) (Kumar *et al*., 2001). Blotting was done under constant current of 200 mA for 1 h at 4°C.

N-terminal sequencing of proteins

In order to excise the specific bands from the blot membrane with greater purity, 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 de-stained 1-3 times in acetic acid (5 %) followed by several washes in de-ionized water. The specific protein bands (70 KDa) were cut out from blotted membrane 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).

Sequence analysis

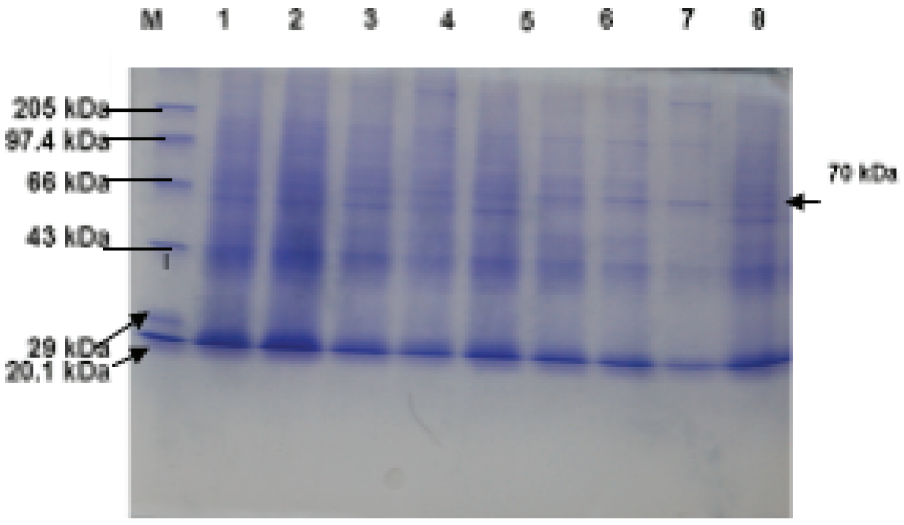
The protein sequence 'Trib hsp' was compared with the sequence available in the database using BLAST tool offered by **National Center for Biotechnology Information (NCBI)**. Protein blast (blast p) was carried out for homology search. The BLAST (Altschul *et al*., 1990) programme 'blast p' provided by NCBI (www.ncbi.nlm.nih.gov/Blast/Blast.cgi) was utilized for the purpose. The primary and the secondary structure of the protein were determined by using the protparam tool and sopma tool. Rasmol and Swiss PDB viewer were used to determine the tertiary structure of the protein.

RESULTS AND DISCUSSION

Determination of molecular weight of the heat shock protein by SDS-PAGE

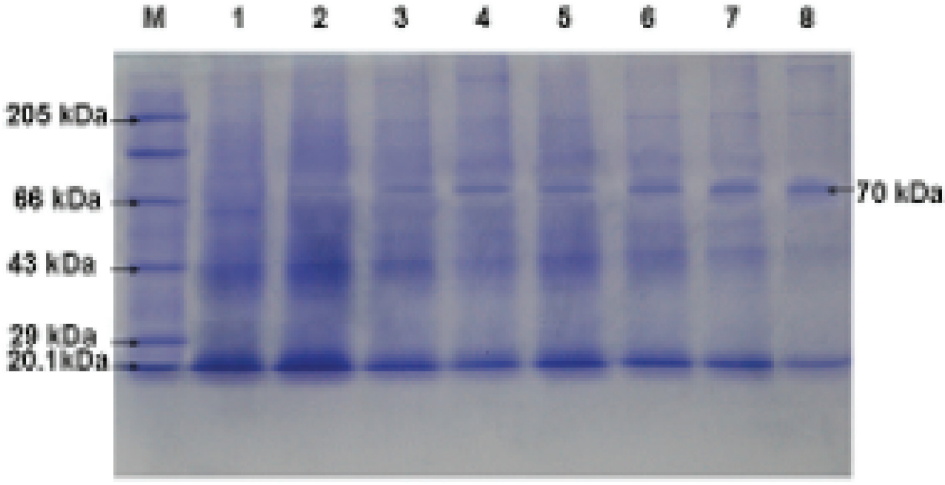
SDS-PAGE for proteins was carried out to characterize the heat shock protein. A very clear band was obtained in the insect samples (subjected to different temperature at 4 h exposure time) as compared to the unexposed insect samples. The SDS-PAGE gel for proteins isolated from the neonate, V instar grub, 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 (Fig 1, 2, 3 and 4). 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 Rf value was determined by the ratio of the distance moved by the solvent to the distance moved by the solution. 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. Blotting was performed to excise the band with greater purity for N-terminal sequencing. Although hsp70 antibody is commercially available, still specific HSP70 antibody for group of organisms belonging to class hexapoda is yet to be commercialized. Hence, the experiment was performed with the sequencing of the band excised from the blot. The members of the 70 KDa families of stress proteins were produced through the rapid induction during exposure of cells to heat in case of *Drosophila* (Tissieres *et al*. 1974). 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 in *Drosophila* (Parsell and Lindquist, 1994). Through this work, the difference in the level of expression of Hsp70 under seven different temperatures tested were studied.

N-terminal sequencing: The N-terminal sequence (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. Though it is a short sequence, it is enough to confirm the target protein Hsp 70. Total ten residues were obtained using N-terminal sequencing. The 'Trib hsp' was confirmed as the HSP70 produced



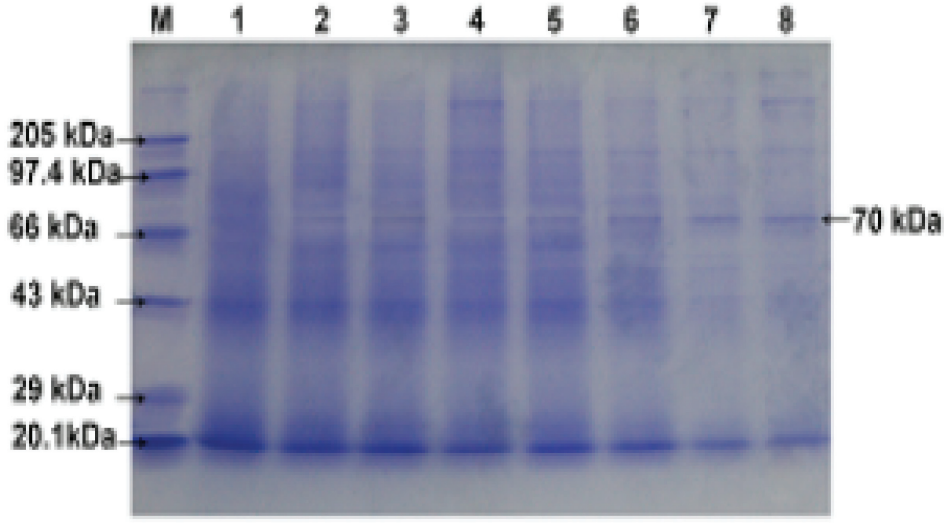
Lane M: Marker protein; Lane 1: Control; Lane 2 to 8: Protein isolated at different temperatures: 2 - 35ºC; 3 - 40ºC (10µl); 4 - 45ºC; 5 - 50ºC; 6 - 55ºC; 7 - 60ºC; 8 - 40ºC (15µl)

Fig 1. SDS-PAGE of *Tribolium castaneum* neonate homogenate under heat stress at different levels



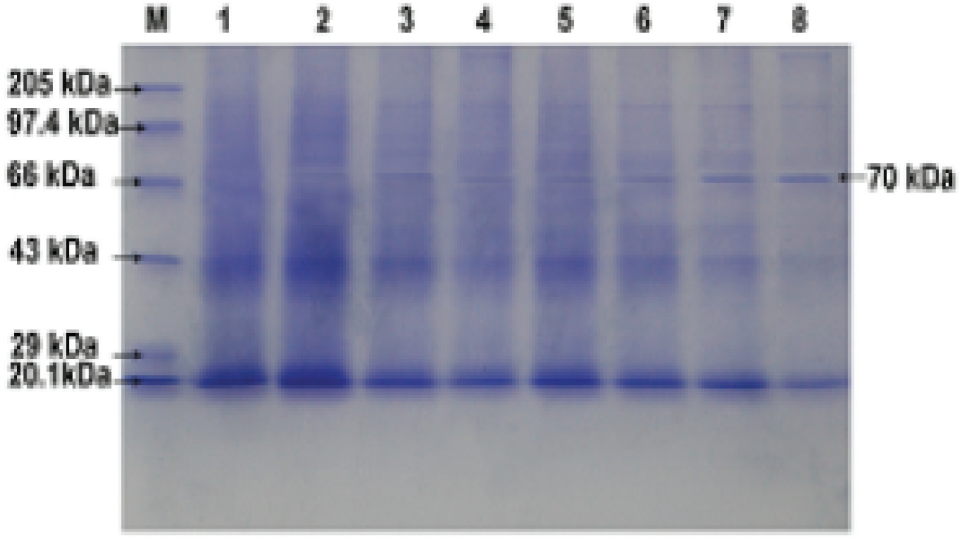
Lane M: Marker protein; Lane 1: Control; Lane 2 to 8: Protein isolated at different temperatures: 2 - 35ºC; 3 - 40ºC (10µl); 4 - 45ºC; 5 - 50ºC; 6 - 55ºC; 7 - 60ºC; 8 - 40ºC (15µl)

Fig 2. SDS-PAGE of *Tribolium castaneum* V-instar grub homogenate 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)

Fig 4. SDS-PAGE of *Tribolium castaneum* adult homogenate 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)

Fig 4. SDS-PAGE of *Tribolium castaneum* adult homogenate under heat stress at different levels

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 – poly acrylamide gel electrophoresis (SDS-PAGE), combined with electro-blotting and automated Edman degradation, was routinely used for protein purification and N-terminal amino acid sequence analysis (Vandekerckhove *et al*.,1985). It was in accordance with a study done in egg avidin protein of chicken (Kumar *et al*., 2001).

Theoretical analysis of sequence data

Homology of the sequence 'Trib hsp' obtained from the protein product of *T. castaneum* with the other reported sequence was analyzed. The sequence (Fig 5) showed significant homology to red flour beetle (*T. castaneum*) protein sequence deposited in the public domain database using 'blast p' search tool. The blast results showed 98 per cent query coverage and 80 per cent identity to *T. castaneum* heat shock protein.

Primary structure prediction

The physico-chemical properties of the protein with respect to the amino acid composition (Table 1), 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. The primary structure of hsp70 was in confirmation with the study done by in *Drosophila* (Horton and Nakai, 1997) with respect to the stability of the protein. Hsp70 multi gene family was also identified and characterized in *Caenorhabditis elegans* which served as the basis for genetic characterization of a multi cellular eukaryote (Heschel and Baillie, 1990).

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 2). Further the per cent of the alpha helices,beta turns and random coils has been depicted by the graphical representation using the 'SOPMA' tool (Fig 6). A similar observation with respect to the secondary structure of the hsp70 in *Drosophila* was also done (Horton and Nakai, 1997).

Tertiary structure prediction

The tertiary structure was visualized using the 'Rasmol' tool and the Ramachandran plot was drawn from the tertiary structure data. The tertiary structure of the HSP70 protein used for homology-based modeling showed the coiling of the alpha helices and beta strands (Fig 7).

Table 1. Amino acid composition of the predicted heat shock protein sequence ('Trib hsp') in *Tribolium castaneum* homogenate

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

Table 2. Secondary structures present in the 'Trib hsp' in *Tribolium castaneum*

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

The Ramachandran plot (Fig 8) indicated that only two amino acids lie in the disallowed region (red) while rest of the amino acids lie in the allowed region (yellow). 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 reliability of the structure (Table 3). Similar results were obtained by identifying the sequence similarity between different types of heat shock proteins (Rassov *et*

Table 3. Allowed and disallowed regions in the tertiary structure of the 'Trib hsp' in *Tribolium castaneum*

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

N-TERMINAL SEQUENCE OUTPUT YWPEAPWWWW:

>gi|91094485|ref|XP_970942.1| PREDICTED: similar to CG9302 -PA [*Tribolium castaneum*]
MKHFNTIFFLLLAFEINIYLTKDTKNAVVDNIYDIKEFKKLIRTKTNVLVCYTNSIKQASQVIKVFRE
AADVIKGQGTMVVMDCSGEAKKVCKKLKVTPDPFIFKHYKNGEFNRDYD RKFTVSSMVNFMRD
PTGDLPWEEDASASDIVHVPDAETLAKFIRQESRPLMVMFYAPWCGFCKTLKPEYVAAAKELKGH
SVLAAIDVNKPENAVIRTLYNITGFPTLLYYKNGAMKFQYEGDNKRQAIVNFMKNPSKPVKVKEQ
EWSEVDSEVVHLTTTNFDPVVKEEASLLVMFYAPWCGHCKKIKPEYEKAAAKLKSDGIPGMMAA
VDATKEVSIADRFSVKGYPTMKYFTYGEHKFDINLREATKIVEFMKNP KEPPPPPPPEKPWSEEESS
VVHLNEENFKSFLKKKRHALVIFYAPWCGHCKKAKPEFTKAAEFFKDDPKVEFAAVDCTTYQGV
CSAHEVSGYPTIKYFSYLNKVVKAYNSGRTADDFIAFMSDPEGNGSSQKTIVPQLTDANFEEEISSK
SAVLVMFYAPWCKQCKEIKPEYQKATNELKQDGFIQLASVDCSSNPVVTDKYDIGTFPTFKLFLN
GKFAADFTGKSTKDDIKSFVVDVKNRKNKEL

Fig 5. Protein sequence from NCBI database with which the hit was obtained

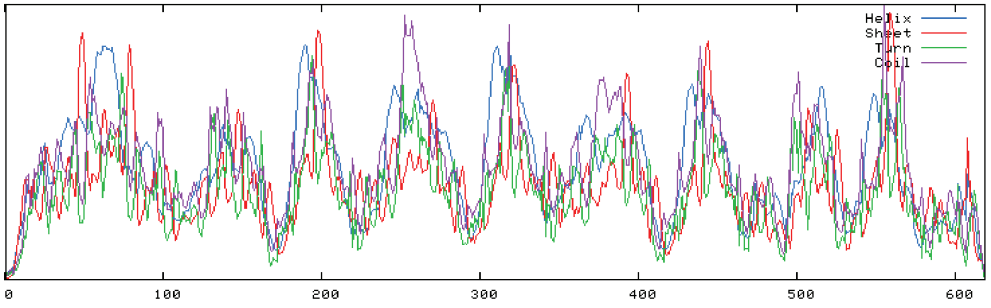


Fig 6. SOPMA result indicating the per cent alpha helices and beta turns

Fig 7. Tertiary structure prediction (Rasmol)

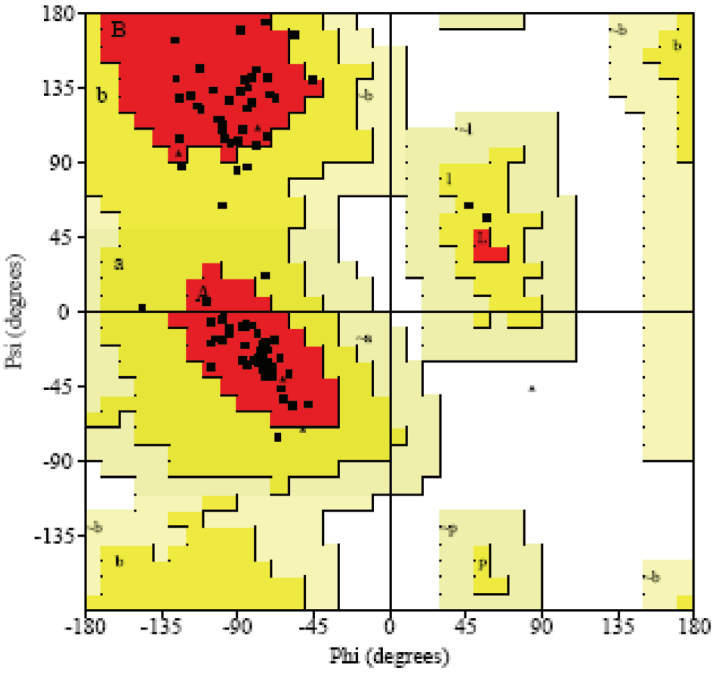


Fig 8. Ramachandran plot model

al., 1995).As temperature increases the pH and ion concentrations are altered, and there are also dramatic effects on macromolecules such as proteins, DNA, RNA, lipids, and carbohydrates, and on cellular structures such as cell and nuclear membranes, mitochondria and ribosomes. Though different classes of heat shock proteins are found in *T. castaneum* when they are subjected to conditions of heat stress, HSP70 is the most dominant heat shock protein found in the early instars that helps this pest to overcome unfavorable conditions (Mahroof *et al*., 2005b). However, HSP70 is found to synthesize at all stages of development in *T. castaneum* in our study. Newly synthesized proteins are shepherded to their correct native structure by these molecular chaperones during heat stress. The HSP70 helps the insect in preventing the aggregation of the already degraded proteins thereby protecting the beetle from progressive cell damage and death during stress.

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